Nitrogen-fixing Actinorhizal Symbioses

Nitrogen Fixation: Origins, Applications, and Research Progress

VOLUME 6

The titles published in this series are listed at the end of this volume.

Nitrogen-fixing Actinorhizal Symbioses

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"Scanning electron micrograph of a sporulating *Frankia* Cc13 culture grown on agar (upper left triangle; courtesy of David R. Benson, University of Connecticut, USA, and reproduced with permission) and a light microscopy picture of the infection zone near the tip of the lobe of a *Casuarina glauca* nodule, showing fully infected cells at bottom left and a line of three cells undergoing hyphal invasion (lower right triangle; courtesy of Kirill Demchenko, Komarov Botanical Institute, Russian Academy of Sciences, St. Petersburg, Russia, and reproduced with permission)."

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PREFACE TO THE SERIES

Nitrogen Fixation: Origins, Applications, and Research Progress

Nitrogen fixation, along with photosynthesis as the energy supplier, is the basis of all life on Earth (and maybe elsewhere too!). Nitrogen fixation provides the basic component, fixed nitrogen as ammonia, of two major groups of macromolecules, namely nucleic acids and proteins. Fixed nitrogen is required for the N-containing heterocycles (or bases) that constitute the essential coding entities of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA), which are responsible for the high-fidelity storage and transfer of genetic information, respectively. It is also required for the amino-acid residues of the proteins, which are encoded by the DNA and that actually do the work in living cells. At the turn of the millennium, it seemed to me that now was as good a time as any (and maybe better than most) to look back, particularly over the last 100 years or so, and ponder just what had been achieved. What is the state of our knowledge of nitrogen fixation, both biological and abiological? How has this knowledge been used and what are its impacts on humanity?

In an attempt to answer these questions and to capture the essence of our current knowledge, I devised a seven-volume series, which was designed to cover all aspects of nitrogen-fixation research. I then approached my long-time contact at Kluwer Academic Publishers, Ad Plaizier, with the idea. I had worked with Ad for many years on the publication of the Proceedings of most of the International Congresses on Nitrogen Fixation. My personal belief is that congresses, symposia, and workshops must not be closed shops and that those of us unable to attend should have access to the material presented. My solution is to capture the material in print in the form of proceedings. So it was quite natural for me to turn to the printed word for this detailed review of nitrogen fixation. Ad's immediate affirmation of the project encouraged me to share my initial design with many of my current co-editors and, with their assistance, to develop the detailed contents of each of the seven volumes and to enlist prospective authors for each chapter.

There are many ways in which the subject matter could be divided. Our decision was to break it down as follows: nitrogenases, commercial processes, and relevant chemical models; genetics and regulation; genomes and genomics; associative, endophytic, and cyanobacterial systems; actinorhizal associations; leguminous symbioses; and agriculture, forestry, ecology, and the environment. I feel very fortunate to have been able to recruit some outstanding researchers as co-editors for this project. My co-editors were Mike Dilworth, Claudine Elmerich, John Gallon, Euan James, Werner Klipp, Bernd Masepohl, Rafael Palacios, Katharina Pawlowski, Ray Richards, Barry Smith, Janet Sprent, and Dietrich Werner. They worked very hard and ably and were most willing to keep the volumes moving along reasonably close to our initial timetable. All have been a pleasure to work with and I thank them all for their support and unflagging interest.

Nitrogen-fixation research and its application to agriculture have been ongoing for many centuries – from even before it was recognized as nitrogen fixation. The Romans developed the crop-rotation system over 2000 years ago for maintaining and improving soil fertility with nitrogen-fixing legumes as an integral component. Even though crop rotation and the use of legumes was practiced widely but intermittently since then, it wasn't until 1800 years later that insight came as to how legumes produced their beneficial effect. Now, we know that bacteria are harbored within nodules on the legumes' roots and that they are responsible for fixing N_2 and providing these plants with much of the fixed nitrogen required for healthy growth. Because some of the fixed nitrogen remains in the unharvested parts of the crop, its release to the soil by mineralization of the residue explains the follow-up beneficial impact of legumes. With this realization, and over the next 100 years or so, commercial inoculants, which ensured successful bacterial nodulation of legume crops, became available. Then, in the early 1900's, abiological sources of fixed nitrogen were developed, most notable of these was the Haber-Bosch process. Because fixed nitrogen is almost always the limiting nutrient in agriculture, the resulting massive increase in synthetic fixed-nitrogen available for fertilizer has enabled the enormous increase in food production over the second half of the 20^{th} century, particularly when coupled with the new "green revolution" crop varieties. Never before in human history has the global population enjoyed such a substantial supply of food.

Unfortunately, this bright shiny coin has a slightly tarnished side! The abundance of nitrogen fertilizer has removed the necessity to plant forage legumes and to return animal manures to fields to replenish their fertility. The result is a continuing loss of soil organic matter, which decreases the soil's tilth, its water-holding capacity, and its ability to support microbial populations. Nowadays, farms do not operate as self-contained recycling units for crop nutrients; fertilizers are trucked in and meat and food crops are trucked out. And if it's not recycled, how do we dispose of all of the animal waste, which is rich in fixed nitrogen, coming from feedlots, broiler houses, and pig farms? And what is the environmental impact of its disposal? This problem is compounded by inappropriate agricultural practice in many countries, where the plentiful supply of cheap commercial nitrogen fertilizer, plus farm subsidies, has encouraged high (and increasing) application rates. In these circumstances, only about half (at best) of the applied nitrogen reaches the crop plant for which it was intended; the rest leaches and "runs off" into streams, rivers, lakes, and finally into coastal waters. The resulting eutrophication can be detrimental to marine life. If it encroaches on drinking-water supplies, a human health hazard is possible. Furthermore, oxidation of urea and ammonium fertilizers to nitrate progressively acidifies the soil – a major problem in many agricultural areas of the world. A related problem is the emission of nitrogen oxides (NO_x) from the soil by the action of microorganisms on the applied fertilizer and, if fertilizer is surface broadcast, a large proportion may be volatilized and lost as ammonia. For urea in rice paddies, an extreme example, as much as 50% is volatilized and lost to the atmosphere. And what goes up must come down; in the case of fertilizer nitrogen, it returns to Earth in the rain, often acidic in nature. This uncontrolled

deposition has unpredictable environmental effects, especially in pristine environments like forests, and may also affect biodiversity.

Some of these problems may be overcome by more efficient use of the applied fertilizer nitrogen. A tried and tested approach (that should be used more often) is to ensure that a balanced supply of nutrients (and not simply applying more and more) is applied at the right time (maybe in several separate applications) and in the correct place (under the soil surface and not broadcast). An entirely different approach that could slow the loss of fertilizer nitrogen is through the use of nitrification inhibitors, which would slow the rate of conversion of the applied ammonia into nitrate, and so decrease its loss through leaching. A third approach to ameliorating the problems outlined above is through the expanded use of biological nitrogen fixation. It's not likely that we shall soon have plants, which are capable of fixing N_2 without associated microbes, available for agricultural use. But the discovery of N_2-fixing endophytes within the tissues of our major crops, like rice, maize, and sugarcane, and their obvious benefit to the crop, shows that real progress is being made. Moreover, with new techniques and experimental approaches, such as those provided by the advent of genomics, we have reasons to renew our belief that both bacteria and plants may be engineered to improve biological nitrogen fixation, possibly through developing new symbiotic systems involving the major cereal and tuber crops.

In the meantime, the major impact might be through agricultural sustainability involving the wider use of legumes, reintroduction of crop-rotation cycles, and incorporation of crop residues into the soil. But even these practices will have to be performed judiciously because, if legumes are used only as cover crops and are not used for grazing, their growth could impact the amount of cultivatable land available for food crops. Even so, the dietary preferences of developed countries (who eats beans when steak is available?) and current agricultural practices make it unlikely that the fixed-nitrogen input by rhizobia in agricultural soils will change much in the near-term future. A significant positive input could accrue, however, from matching rhizobial strains more judiciously with their host legumes and from introducing "new" legume species, particularly into currently marginal land. In the longer term, it may be possible to engineer crops in general, but cereals in particular, to use the applied fertilizer more efficiently. That would be a giant step the right direction. We shall have to wait and see what the ingenuity of mankind can do when "the chips are down" as they will be sometime in the future as food security becomes a priority for many nations. At the moment, there is no doubt that commercially synthesized fertilizer nitrogen will continue to provide the key component for the protein required by the next generation or two.

So, even as we continue the discussion about the benefits, drawbacks, and likely outcomes of each of these approaches, including our hopes and fears for the future, the time has arrived to close this effort to delineate what we know about nitrogen fixation and what we have achieved with that knowledge. It now remains for me to thank personally all the authors for their interest and commitment to this project. Their efforts, massaged gently by the editorial team, have produced an indispensable reference work. The content is my responsibility and I apologize

upfront for any omissions and oversights. Even so, I remain confident that these volumes will serve well the many scientists researching nitrogen fixation and related fields, students considering the nitrogen-fixation challenge, and administrators wanting to either become acquainted with or remain current in this field. I also acknowledge the many scientists who were not direct contributors to this series of books, but whose contributions to the field are documented in their pages. It would be remiss of me not to acknowledge also the patience and assistance of the several members of the Kluwer staff who have assisted me along the way. Since my initial dealings with Ad Plaizier, I have had the pleasure of working with Arno Flier, Jacco Flipsen, Frans van Dunne, and Claire van Heukelom; all of whom provided encouragement and good advice – and there were times when I needed both!

It took more years than I care to remember from the first planning discussions with Ad Plaizier to the completion of the first volumes in this series. Although the editorial team shared some fun times and a sense of achievement as volumes were completed, we also had our darker moments. Two members of our editorial team died during this period. Both Werner Klipp (1953-2002) and John Gallon (1944-2003) had been working on Volume II of the series, *Genetics and Regulation of Nitrogen-Fixing Bacteria*, and that volume is dedicated to their memory. Other major contributors to the field were also lost in this time period: Barbara Burgess, whose influence reached beyond the nitrogenase arena into the field of iron-sulfur cluster biochemistry; Johanna Döbereiner, who was the discoverer and acknowledged leader in nitrogen-fixing associations with grasses; Lu Jiaxi, whose "string bag" model of the FeMo-cofactor prosthetic group of Mo-nitrogenase might well describe its mode of action; Nikolai L'vov, who was involved with the early studies of molybdenum-containing cofactors; Dick Miller, whose work produced new insights into MgATP binding to nitrogenase; Richard Pau, who influenced our understanding of alternative nitrogenases and how molybdenum is taken up and transported; and Dieter Sellmann, who was a synthetic inorganic chemistry with a deep interest in how N_2 is activated on metal sites. I hope these volumes will in some way help both preserve their scientific contributions and reflect their enthusiasm for science. I remember them all fondly.

Only the reactions and interest of you, the reader, will determine if we have been successful in capturing the essence and excitement of the many sterling achievements and exciting discoveries in the research and application efforts of our predecessors and current colleagues over the past 150 years or so. I sincerely hope you enjoy reading these volumes as much as I've enjoyed producing them.

William E. Newton
Blacksburg, February 2004

PREFACE

Nitrogen-fixing Actinorhizal Symbioses

This book is part of a seven-volume series that was launched in 2004 and covers all aspects of nitrogen fixation from the biological systems to the industrial processes. Volume 6 covers nitrogen-fixing actinorhizal symbioses, which occur between soil actinomycetes of the genus *Frankia* and a diverse group of dicotyledonous plants, collectively called actinorhizal plants. These symbioses play vital roles in native ecosystems as well as important components in both forestry and land reclamation.

The volume is divided into 11 chapters, all authored by well-known scientists in the field. As in previous volumes of this series, the first chapter presents an historical perspective and describes the development of actinorhizal research with its focus on the period after the first reproducible isolation of the responsible microorganism by John Torrey's group in 1978.

Very early on, the initial attempts to characterize the bacterium taxonomically had considered this endosymbiont as an obligate symbiotic bacterium and used its ability to form root nodules and its morphological characteristics within root-nodule cells as discriminative criteria to distinguish it from other actinomycetes. These efforts led to the emendation of the family *Frankiaceae* with the type genus *Frankia* and also to the definition of host-specificity groups based on inoculation experiments using crushed nodules. However, after *Frankia* strains were isolated from nodules and pure cultures became available, many of these early results had to be discarded. Chapter 2 describes the techniques used to obtain phenotypic, genotypic and phylogenetic information on the members of the genus *Frankia*.

In contrast to most rhizobia (see Volume 7, *Nitrogen-fixing Leguminous Symbioses*), *Frankia* strains can fix N_2 in the free-living state which improves their ability to survive in soil and *Frankia* strains have been found in soils devoid of actinorhizal plants. Chapter 3 covers the recent advances in knowledge of *Frankia* strains as soil microorganisms and their relationship to other soil microorganisms.

Actinorhizal symbioses occur with dicotyledonous plants from eight different families, *i.e.*, in quite a diverse group of plants. Chapter 4 deals with the phylogeny of both the host plants and the *Frankia* endosymbionts. Host and endosymbiont phylogeny are then compared in an effort to address the question of whether the partners are an example of co-evolution.

Aerobic organisms, like *Frankia*, suffer from the so-called 'oxygen (O_2) dilemma of nitrogen fixation'. Nitrogenase (see Volume 1, *Catalysts for Nitrogen Fixation*) is highly sensitive to O_2 and can only function in an O_2-free environment, so a high respiratory O_2 flux has to exist adjacent to a vanishingly low O_2 tension at the sites of nitrogen fixation. Generally, strategies combine external O_2 barriers with high O_2 utilization at the nitrogenase site to maintain a steep O_2 gradient. Because, in contrast to the rhizobia, *Frankia* can provide its own O_2-protection system by forming specialized vesicles with restricted O_2 access, O_2-protection mechanisms in actinorhizal symbioses involve more than modification of nodule structure and physiology. Chapter 5 covers the diverse forms in which the O_2 dilemma is solved in different actinorhizal symbioses.

The different ways by which *Frankia* strains can enter the plant root and induce organogenesis, as well as mechanisms for autoregulation of nodule formation, are reviewed in Chapter 6. The N_2 fixed by the resulting actinorhizal plants is often the major nitrogen input to many terrestrial ecosystems and Chapter 7 deals with the interplay of carbon and fixed-nitrogen metabolism in actinorhizal nodules of different plant species.

Although actinorhizal plants play important functional roles in native uncultivated ecosystems and are usually the first species to colonize devastated land, relatively little is known about ecological constraints on their capacity for nitrogen fixation because most studies of actinorhizal associations have been conducted in laboratories, growth chambers, and greenhouses. The impact of ecological effects is addressed in Chapter 8, which reviews studies of actinorhizal symbioses in a variety of natural situations.

The molecular-level analysis of actinorhizal symbioses has lagged behind similar studies of legume symbioses for several good reasons and not for the lack of effort! Actinorhizal plants are, with one exception, woody plants, trees or shrubs, and have a long generation time, which together renders them recalcitrant to molecular-genetic analysis. In the last decade, however, molecular-level studies have been initiated with several actinorhizal species, aided by the development of transformation procedures for actinorhizal trees of the Casuarinaceae family. Chapter 9 describes the contribution of plant molecular-biology approaches to our understanding of actinorhizal symbioses.

We've known, since 1995, that all plant species, which are able to enter into root-nodule symbiosis with nitrogen-fixing soil bacteria, *i.e.*, legumes, *Parasponia* sp., and actinorhizal plants, belong to a single clade that also contains many non-symbiotic plant species. It appears that the common ancestor of this clade (called Rosid I) had acquired a property based upon which a root-nodule symbiosis could and, in some cases did, develop. If this property could be identified, it would offer the possibility of transferring the capacity to form nitrogen-fixing root-nodule symbioses to plant species outside the Rosid I clade. As a preliminary in the search for such a property, comparative analysis of different symbiotic systems should allow the identification of common features *versus* system-specific adaptations. Hence, a comparison of actinorhizal and legume symbioses is presented in Chapter 10. Finally, Chapter 11 discusses the prospects for the future of actinorhizal research.

It's been nearly five years from its inception to the completion of this volume and we would like to sincerely thank all of the contributors for their efforts and patience. We dedicate this volume to Antoon Akkermans, a long-time proponent of this area of research, who died suddenly in 2006. We remember him fondly.

Katharina Pawlowski
Stockholm, April, 2007

William E. Newton
Blacksburg, April, 2007

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Antoon D. L. Akkermans
(1940-2006)

This book is dedicated to the memory of Antoon Akkermans, who was Associate Professor at Wageningen University from 1987 to 2003. His early research concerned symbiotic nitrogen fixation between *Frankia* and actinorhizal plants, particularly the symbiosis of black elder. Later, he focused more on the molecular ecology of bacterial communities, examining bacterial interactions in the mammalian (human, pig and mouse) gastrointestinal tract, in grassland soil, and in anaerobic sludge. *Akkermansia muciniphila,* a human intestinal mucin-degrading bacterium, was named for him to honor his contributions to microbial ecology. Actinorhizal symbioses were Antoon's first love and he maintained his interest in them even after his switch to microbial ecology. He co-authored the first chapter in this volume, a review of the history of actinorhizal research, a history in which he played an important role.

Antoon Akkermans had many interests besides science, most particularly music and art. In fact, after his retirement, he turned to painting with the proceeds of the sale of his oil and acryl paintings of bacterial communities going to the Akkermansia Foundation, which supports young scientists (< 65 year) working in the field of microbial ecology. Antoon's experience, wisdom and sense of humor will be missed by all who knew him.

Chapter 1

FRANKIA AND ACTINORHIZAL PLANTS: A HISTORICAL PERSPECTIVE

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1. INTRODUCTION

> *"…when in the wide estuaries the mangroves have in due time reclaimed the swampy land from the water, the Casuarina tree plants itself and in its turn settles, solidifies and fertilizes the soil till it is ripe for a more varied and luxuriant growth"*
> *W. Somerset Maugham, 1926.*

The origins of the two organismal names in the title of this chapter are of particular historical interest. First, the generic name *Frankia* was proposed initially by Brunchorst (1886-1888), in honor of Professor Frank, to describe the endophyte of root-nodulated non-legumes. The name was adopted by Becking (1970) in his early taxonomic study and is now universally accepted. *Frankia* is at present the only confirmed member of the family Frankiaceae. Prior to 1979, the term "non-leguminous plant symbioses" was in common usage to describe not only what are now known as "actinorhizal plants" but all nitrogen-fixing associations between microorganisms and plants that do not belong to the Leguminosae (Fabaceae). In an excellent review in 1965, Allen and Allen considered the history of research not only of root-nodulated non-leguminous members of the Angiospermae but also of the Cycadaceae colonized by cyanobacteria. The term "actinorhizal" was proposed

K. Pawlowski and W. E. Newton (eds.), Nitrogen-fixing Actinorhizal Symbioses, 1–24.

at the first international meeting on "Symbiotic Nitrogen Fixation in Actinomycete-nodulated Plants", held at Harvard Forest, to provide a convenient and more positive designation for the field than the term "non-legume" (Torrey and Tjepkema, 1979).

Following the first reproducible isolation of the microorganism by the John Torrey's group (Callaham *et al.,* 1978), which was a watershed event in the development of *Frankia*-actinorhizal research, there was a rapid increase internationally in the number of scientists interested in *Frankia* and an exponential increase in the number of isolates in culture. This focused attention on the taxonomy of *Frankia* and necessitated the establishment of a classification system. At the international conference on the "Biology of *Frankia*", held in Wisconsin in 1982, it was agreed that criteria were lacking for a system based on species names, and so a system for numbering *Frankia* strains was proposed in the first "Catalog of *Frankia* strains" (Lechevalier, 1983; 1986), which is still in use. This system employs three letters to designate the collection and up to ten numbers to reflect the origins of the strain. Thus CpI1, the first *Frankia* strain to be isolated at Harvard Forest from *Comptonia peregrina*, is designated HFP070101. The first two numbers encode the host genus from which the strain was isolated and the third and fourth number the species. Thus, one hundred and fifty years after Meyen (1829) first published a description of alder root nodules, the designation and acceptance by the scientific community of a unique and distinguishing nomenclature accorded proper and full recognition of the scientific and economic importance of these symbiotic relationships. By definition, the term "actinorhizal" excludes *Parasponia* of the Ulmaceae, the only non-legume shown definitively to bear rhizobial nodules.

Actinorhizal plants are perennial, woody species, with the exception of *Datisca* spp., which are herbaceous perennials. Most genera fruit heavily or spread vegetatively and show relative shade intolerance, which are characteristics of species of early- to mid-succession in plant community development (Dawson, 1990). The primary colonization of deglaciated areas in Alaska, first by *Dryas drummondii* and then by *Alnus sinuata*, both actinorhizal species, provided a benchmark chronosequence that has served as a paradigm for the role of actinorhizal plants as pioneer species and facilitators of fixed-nitrogen accretion in nutrient-poor habitats (Crocker and Major, 1955; Lawrence *et al.,* 1967; Bormann and Sidle, 1990; Chapin *et al.,* 1994). The invasion of young volcanic fields by *Myrica faya*, after its introduction in the Hawaiian Islands, illustrates the successful domination of this ecosystem (to the exclusion of native trees) due to its characteristics of nitrogen fixation, wind pollination, prolific fruiting, and ready seed dispersal by birds (Vitousek, 1989).

In this chapter, key advances made in the period prior to 1978 will be discussed briefly because excellent accounts, including historical aspects, are available in the literature (McKee, 1962; Allen and Allen, 1965; Bond, 1973; Akkermans and van Dijk, 1981; Quispel, 1990; Quispel *et al.,* 1993). Further, because the pace of research has increased rapidly since 1978, not all of the major advances can be considered in this description of the historical development of the area. The authors extend their apologies to any who feel that their contributions to the history of the area have been neglected. However, due accord is given to the work of many other

major contributors in the specialist chapters in this volume. Earlier scientific progress in this area is covered in "The Biology of *Frankia* and Actinorhizal Plants" edited by Schwintzer and Tjepkema (1990) and has also been the subject of many reviews in recent years, e.g., Normand and Lalonde, 1986; Tjepkema *et al.,* 1986; Benson and Silvester, 1993; Berry, 1994; Mullin and Dobritsa, 1996; Pawloski and Bisseling, 1996; Dommergues, 1997; Huss-Danell, 1997; Franche *et al.,* 1998; Benson and Clawson, 2000; Laplaze *et al.,* 2000; Wall 2000; Schwencke and Caru, 2001. The history of research on casuarinas is admirably documented in a specialist volume (Subbarao and Rodriguez-Barrueco, 1995).

2. THE EARLY YEARS

This section draws primarily on the review of Quispel (1990), who divided the efforts of scientists prior to 1978 into three periods. By the end of the nineteenth century, good experimental evidence had been obtained for the utilisation of atmospheric N_2 by nodulated *Alnus glutinosa* plants (Hiltner, 1895). Anatomical studies showed the presence of intercellular hyphae in nodules (Woronin, 1866), but the nature of the microorganism was in much dispute, being described variously as either a parasitic fungus or a myxomycete. The next 50 years were characterised by research that consolidated and extended these earlier findings. Notably, careful cytological studies provided strong evidence that the organisms in nodules of several genera of plants, such as *Alnus*, *Myrica*, *Casuarina*, and *Elaeagnus*, were actinomycetes (reviewed in Schaede, 1962), although definitive proof was not obtained due to failure to isolate the organism reproducibly. Cross-inoculation experiments with crushed nodules indicated that there were specific differences between the microorganisms that nodulated different plant genera.

In the period from 1950 to 1978, interest in actinorhizal root nodules greatly increased, promoted by the efforts of several influential scientists. Notable among these were George Bond and Anton Quispel. Bond and his co-workers conducted many classical experiments to demonstrate an increase in total nitrogen in nodulated actinorhizal plants. Their experiments showed how environmental factors, such as pH, combined nitrogen, O_2, and light periodicity, affected nodulation and N_2 fixation. Importantly, in experiments that paralleled those of Virtanen *et al.* (1954), ^{15}N methodology was used to prove unequivocally that actinorhizal nodules fix atmospheric N_2 (Bond, 1955; 1956). Biochemical studies confirmed the importance of citrulline as the main product of N_2 fixed in alder and its role in nitrogen transport from root nodules (Leaf *et al.,* 1958). One of Bond's other important contributions was as coordinator of that part of the International Biological Programme of the International Council of Scientific Unions concerning the biological importance of actinorhizal plants; this activity focused on a world-wide survey of root nodulation of these plants involving some 50 collaborators from 30 countries (Bond, 1976). The ubiquitous nodulation of *Alnus* species and the irregularity of nodulation of some other genera, notably *Casuarina* and *Dryas*, were noted. A further 36 species in genera known to be nodulated were recorded and *Colletia* was described as a new nodulated genus in the Rhamnaceae. In addition to

the important scientific contributions, the IBP programme served to arouse interest in actinorhizal-plant biology and to provide added stimulus to all aspects of nitrogen-fixation research. Bond died in 1988 and a fascinating summary of his life and achievements is found in the Memoirs of the Royal Society of London, to which he was elected as Fellow in 1972 (Nutman, 1990).

Quispel also had significant interests in the host plant, but particularly with respect to the processes of infection and nodulation (Quispel, 1974). He had a long-standing personal interest in the endophyte and its cultivation *in vitro*. Early publications by others of their attempts to grow *Frankia* in pure culture were highly controversial because the experiments either did not fulfill Koch's postulates or were not reproduced. In 1959, Pommer published his study on the isolation of the micro-symbiont of alder, the characteristics of which suggest that his attempt was almost certainly successful (Pommer, 1959). Unfortunately, his cultures were lost. In the early nineteen fifties, Quispel designed a series of experiments to help lay the foundations for the isolation and culture of the endophyte. He demonstrated that surface-sterilized root-nodule tissue of *Alnus glutinosa* remained infective during storage on nutrient agar and that infectivity increased in media to which an alcoholic extract of alder nodules had been added (Quispel, 1954; 1955). Actinomycete hyphae were evident at the border of the infective nodule pieces (Quispel, 1960). Following his appointment as Professor in Experimental Botany at the University of Leiden, it was another eight years before Quispel and Teun Tak returned to research on actinorhizal plants and demonstrated that there was a close correlation between *Frankia* hyphal biomass and infectivity (Quispel and Tak, 1968). A further twenty years were to elapse before dipterocarpol, the compound in alcoholic extracts that enhanced the infectivity of nodule fragments, was isolated and identified (Quispel *et al.,* 1989). Quispel supervised many doctoral students, several of whom have contributed with distinction to our knowledge of actinorhizal plants. Quispel's involvement in the early work of his students is often not apparent for he encouraged their publication of doctoral work as the sole author. Quispel's contributions are highlighted further in Akkermans *et al.* (1983).

The availability of the electron microscope facilitated the resolution of important questions concerning the pleoiomorphic nature of the endophyte. The bacteria-like cells seen in sections of many actinorhizal nodules were identified as spores contained within sporangia (van Dijk and Merkus, 1976). The ultrastructure of the vesicles of several genera was described and histochemical techniques coupled with light microscopy identified them as the locus for nitrogenase (Akkermans, 1971), observations since confirmed using immunocytological methods (Huss-Danell and Bergman, 1990). Vesicles are absent from nodules of *Casuarina* and here nitrogenase is located in the hyphal tips (Berg and McDowell, 1987).

The discovery by Dilworth, Burris and co-workers in 1966 (see an historical account by Turner and Gibson, 1980; and volume 1 of this series, *Catalysts for Nitrogen Fixation: Nitrogenases, Relevant Chemical Models and Commercial Processes*) that nitrogenase reduces acetylene to ethylene was soon utilised as an assay for nitrogenase activity in field studies of nitrogen fixation by actinorhizal plants (Stewart *et al.,* 1967). The technique has been used frequently in both field and laboratory assays of nitrogenase activity since, but many problems arise in the

quantitative extrapolation of acetylene-reduction data to nitrogen-fixation rates. These include an acetylene-induced decline in nitrogenase activity, inactivation of hydrogenase in some actinorhizal nodules, and effects of water stress on the acetylene-to-$^{15}N_2$ conversion ratio (Winship *et al.,* 1987; Schwintzer and Tjepkema, 1994; Johnson *et al.,* 1997). Although nitrogenase has not been purified from actinorhizal nodules, studies with nodule homogenates and cell-free extracts show that actinorhizal nitrogenase is similar to that of rhizobia (Benson *et al.,* 1979; Roelofsen and Akkermans, 1979). Further, molecular-genetic techniques show that *nifHDK,* the structural genes for the Fe and MoFe proteins, of the actinorhizal nitrogenase have extensive sequence similarity with those of other nitrogen-fixing organisms (Ruvkun and Ausubel, 1980) and, in several *Frankia* strains, are clustered on the chromosome as occurs in other bacteria (Simonet *et al.,* 1990).

3. TWO DECADES TO THE NEW MILLENNIUM

By 1978, a battery of new techniques was becoming available to study actinorhizal symbioses and, as indicated above, the advent and application of molecular techniques was providing new ways to tackle problems previously thought to be impossible to resolve. However, the number of scientists involved and the funding available was much less than for research on legumes, so that progress with actinorhizal plants has been slower. Although several actinorhizal genera contain species that are economically important in forestry and land regeneration and all are of ecological importance, only a few e.g. *Hippophae* berries, are used for food (Wheeler and Miller, 1990). Because of their slower growth and generation time, and their high phenolic content, woody plants in general are less amenable to physiological and molecular analysis than herbaceous plants. Nevertheless, micropropagation techniques offer a partial solution to these last problems and have been devised for species such as *Alnus* (Perinet and Lalonde, 1983; Tremblay and Lalonde, 1984); *Myrica* (Tavares *et al.,* 1998), *Hippophae* (Yao, 1995); *Datisca* (Wang and Berry, 1996), and casuarinas (Duhoux *et al.,* 1996).

The availability of such methodology has facilitated efforts to genetically transform casuarinas by the group of Emile Duhoux, Claudine Franche and Didier Bogusz. Following inoculation with *Frankia,* active nitrogen-fixing nodules were formed on a high proportion of transformed plants of *Allocasuarina verticillata*, which had been regenerated from calli induced on wounded embryos co-cultivated with *Agrobacterium tumefaciens*. Integration of the transgenes into the *Allocasuarina* genome did not interfere with the nodulation process (Franche *et al.,* 1999a; 1999b).

Although the actinomycetous micro-symbiont is slow-growing and more difficult to culture and use than the single-celled rhizobia, there have been striking advances in recent years in our knowledge of *Frankia* and its symbioses. In the next section, we have attempted to place into a historical context some of the most important research findings or “milestones” that have influenced the direction of actinorhizal research in the last quarter century.

3.1. Isolation, Culture and Taxonomy of Frankia

The repeated early failures to isolate the endophyte and to obtain proof of re-infectivity (see Baker and Torrey, 1979) led many to suggest that either an obligate association with the host or a synergistic interaction among one or more different microorganisms was necessary for endophyte growth and host-plant infection. As late as 1970, there were suggestions that the endophyte forms associations akin to that of an obligate parasite (Becking, 1970). Such a possibility was effectively eliminated when *Frankia* strain CpI1 was isolated in 1978 (Callaham *et al.,* 1978). The medium used was of a relatively complex composition and a range of media of differing composition have been used subsequently (Lechevalier and Lechevalier, 1990). Because of the relatively slow growth rate of the organism in culture, successful isolation from nodules required careful application of procedures both to surface-sterilise nodules and to remove as far as possible contaminating organisms and inhibitory plant compounds, such as phenolics (Lechevalier and Lechevalier, 1990). However, even today and despite numerous attempts since 1978, *Frankia* strains, which have been identified as a single clade by 16S-rDNA amplification from actinorhizal root nodules and infective only on rosaceous hosts, *Ceanothus* spp., *Datisca* and *Coriaria* spp. (Benson and Clawson, 2000), have still not been isolated successfully in pure culture.

In the mid–1970s, Torrey branched out into actinorhizal-plant research from his major and internationally distinguished contributions to root biology, root tissue culture, and legume-nodule physiology. Initially, his special interest was *Casuarina* because of its importance in the Tropics. He developed his interests further during a visit with Bond, which encouraged him to begin studies on the initiation and development of *Casuarina* root nodules (Torrey, 1976). It is remarkable that Torrey's group achieved the first reproducible isolation of the endophyte, the objective of many researchers previously, so soon after establishing his research program on actinorhizal plants. This important advance attracted many researchers to his laboratory and many of these scientists have gone on to distinguished careers and to make major contributions to the subject. John Torrey retired from active research in 1992, when he, along with Yvon Dommergues and Mary Lechevalier, were honoured at the International Conference on *Frankia* and Actinorhizal Plants, held in Lyon, France in September 1991 (Normand *et al.,* 1992). Sadly, John Torrey died only a few months later. The meeting on *Frankia* and actinorhizal plants, held in Ohakune, New Zealand in 1993, was dedicated to his memory and contains a tribute to his life and work (Baker and Berry, 1994).

Although actinorhizal-plant research had been included since 1976 in both the International Symposia on Nitrogen Fixation and the Symposia on Nitrogen Fixation with Non-Legumes, these symposia were largely focused on legume and on non-symbiotic fixation, respectively. The increased interest and activity in actinorhizal symbioses that was stimulated by the work of the Harvard group led to the organisation of a series of meetings devoted to *Frankia* and actinorhizal symbioses, which have helped to coordinate scientific activities (see Table 1). In addition, progress has been reviewed at specialist meetings, for example on *Casuarina* in Canberra (1981), Cairo (1990), and Da Nang, Vietnam (Pinyopusarerk

Table 1. Proceedings of International Conferences on Frankia *and Actinorhizal Plants.*

Number	Year	Place	Proceedings
1	1978	Petersham, MA, USA	Torrey and Tjepkema (1979)
2	1979	Corvallis, OR, USA	Gordon *et al.* (1979)
3	1982	Madison, WI, USA	Torrey and Tjepkema (1983)
4	1983	Wageningen, The Netherlands	Akkermans *et al.* (1984)
5	1984	Québec, Canada	Lalonde *et al.* (1985)
6	1986	Umeå, Sweden	Huss-Danell and Wheeler (1987)
7	1989	Storrs, CT, USA	Winship and Benson (1989)
8	1991	Lyon, France	Normand *et al.* (1992)
9	1993	Okahune, New Zealand	Harris and Silvester (1994)
10	1995	Davis, CA, USA	Berry and Myrold (1997)
11	1998	Champaign, IL, USA	Dawson *et al.* (1999)
12	2001	Carry-le-Rouet, France	Normand *et al.* (2003)

et al., 1996), and at many local meetings, where some presentations were published in journals such as *Acta Botanica Gallica* (Duhoux and Diem, 1996).

Biochemical and physiological techniques have continued to play an important role in unravelling the taxonomy of both *Frankia* and the host plant. Research in the Lalonde laboratory showed early on that *Frankia* is characterised by the presence of 2-*O*-methyl–D-mannose, a sugar not found in other actinomycetes (Mort *et al.,* 1983). Biochemical and serological study of different strains initially suggested a preliminary division of the genus into two groups: one group that nodulates *Alnus* and *Myrica*, and a second, more heterogeneous group (Baker *et al.,* 1981; Lechevalier, 1984). However, cross-inoculation experiments suggested that strains fell into at least three or four host-compatibility groups (Normand and Lalonde, 1986; Baker, 1987).

A more definitive analysis of *Frankia* taxonomy became available as molecular techniques for analysing *Frankia* DNA were developed. Analyses of DNA–DNA relatedness and of the DNA, which encodes the universal, slowly evolving and functionally conserved 16S rRNA sequence, have been particularly important. The groups of Normand and Fernandez and Dobritsa (Akimov and Dobritsa, 1992) played significant roles in the early application of these techniques. Fernandez *et al.* (1989) applied DNA-hybridisation techniques to 43 isolates of *Frankia* and differentiated nine genomic species, including three among strains compatible with *Alnus* species, five among strains compatible with Elaeagnaceae, and one among strains compatible with Casuarinaceae. Nazaret *et al.* (1991) determined phylogenetic relationships among eight of the genomic species by amplification and sequencing of 16S rDNA. They first showed that strains in the *Alnus* and *Casuarina* infectivity groups were closely related, but well separated from those in the *Elaeagnus* infectivity group, which also included atypical strains isolated from *Casuarina*. Three cohesive *Frankia* clades with distinct host-specificity ranges have now been defined by DNA analysis, as well as a fourth clade of non-nodulating, non-nitrogen-fixing *Frankia* relatives (Benson and Clawson, 2000).

Reverse-transcriptase sequencing of 16S rRNA led Hahn *et al.* (1989) to suggest a close phylogenetic relationship between *Frankia* and *Geodermatophilus*. Both organisms have multilocular sporangia. However, Maréchal *et al.* (2000) re-sequenced the *rrs* gene and the *recA* gene of *Acidothermus cellulolyticus* to show an even closer proximity of this actinomycete to *Frankia*, compared to the morphologically more similar *Geodermatophilus.* Further, both *Acidothermus* and *Frankia* contained high levels of hopanoid lipids, which had been found earlier to be abundant in *Frankia* cells and in nodules (Berry *et al.,* 1991).

3.2. Taxonomy and Evolution of the Host Plant and New Nodulating Genera

There are eight Angiosperm families known to be nodulated by *Frankia*. Until 1979, only seven families were commonly known to be actinorhizal hosts, when Chaudhary (1979) reported that *Datisca* (Datiscaceae) also forms *Frankia* symbioses. Interestingly, *Datisca* was first described as a nodulated plant by Severini (1922), but this report had gone relatively unnoticed until Chaudhary's rediscovery. Three new genera of the Casuarinaceae were defined by dividing the former genus *Casuarina* into *Casuarina, Allocasuarina, Gymnostoma* and *Ceuthostoma* (Johnson, 1980; 1982; 1988). Nodulation of species in the first three of these genera has been observed regularly.

New nodulated genera in the Rhamnaceae (*Colletia*, *Trevoa*, *Talguenea and Retanilla),* which are native to South America, have also been discovered and *Frankia* strains from these shrubs characterised (Caru, 1993), whereas nodulation of *Rubus* has now been discounted (Stowers, 1985). Nodulation of genera in new families, e.g. *Atriplex* of the Chenopodiaceae (Caucas and Abril, 1996), always requires careful, independent confirmation. "Nodules", often called tubercles in older literature, which are produced by mycorrhizal or other forms of microbial infection, may easily be confused with *Frankia* nodulation. Arbuscular mycorrhizal nodules, which like actinorhizas are modified lateral roots, have been reported recently for *Gymnostoma* (Duhoux *et al.,* 2001).

Classical taxonomy, based on morphology and floristics, suggested that the distribution of actinorhizal plants through eight families was characterised by taxonomic unrelatedness (Bond, 1983), thus introducing the possibility that actinorhizal and legume-nodule symbioses have arisen in taxonomically unrelated plant groups. This concept has been effectively eliminated by the findings of Swensen and Mullin, who used molecular techniques rather than morphological criteria to study the phylogeny of actinorhizal plants. Working with collaborators in the USA and Australia, they used chloroplast-gene sequence data (*rbcL*) to show that representatives of all eight actinorhizal-plant families, together with representatives of the rhizobia-nodulated families, occurred in a single "nitrogen-fixing clade", interspersed with non-nodulating genera (Soltis *et al.,* 1995). Additional molecular data, together with phylogenetic trees constructed from *Frankia nifH*-gene and 16S-rDNA sequences, have given insights into the co-evolution of actinorhizal symbioses and suggests that actinorhizal symbioses originated three times after the divergence of the large plant clade (Swensen, 1996;

Swensen and Mullin, 1997a; 1997b; Jeong *et al.,* 1999, Benson and Clawson, 2000). Here, as in virtually all areas of biology, the "molecular revolution" has transformed our ability to answer questions that previously either could not be tackled or could be studied only with the expenditure of much time and effort. The impact on the ecology of actinorhizal plants is considered below.

3.3. Infection and Nodule Development

The application of electron microscopy facilitated further detailed observations of infection pathways and nodule development (Berry and Sunell, 1990). One of the most important developments was the recognition of two different infection pathways used by *Frankia* hyphae. The "traditional" pathway, which occurs in genera such as *Alnus*, *Myrica* and *Casuarina*, involved penetration of deformed root hairs, followed by intracellular penetration of cells of the root cortex. The "alternative" pathway, which was first recognised in *Elaeagnus* (Miller and Baker, 1985), involved epidermal penetration, followed by intercellular colonisation of the cortex, before intracellular penetration of mature cortical cells and ultimately the host cells of the developing nodule. Furthermore, whereas hyphae in intracellular infections are encapsulated in a host-derived matrix of polysaccharides, cellulose, hemicellulose and pectin (Berg, 1990), during intercellular colonisation, the hyphae are not encapsulated until they penetrate the host cells.

The molecular signals that initiate infection remain unknown. No convincing evidence of either *nod* genes or Nod-factor homologs has been demonstrated in *Frankia* (Cérémonie *et al.,* 1998a; 1998b), although a root hair-deforming factor (or Had factor) is produced constitutively by some *Frankia* strains (van Ghelue *et al.,* 1997; Cérémonie *et al.,* 1998b). Because of their role as signal molecules in legume symbioses, flavonoids excreted by the host plant have been examined, but clear evidence for their involvement in nodulation has not been obtained (Benoit and Berry, 1997; Hughes *et al.,* 1999).

The possibility that plant hormones may regulate nodule development has led to the observation of elevated levels of cytokinins, auxins and gibberellins in nodules (Wheeler *et al.,* 1979) and changes in abscisic acid and polyamines in relation to dormancy and the supply of mineral nitrogen, respectively, have been reported (Watts *et al.,* 1987; Wheeler *et al.,* 1994). Auxins and cytokinins are known to be secreted by *Frankia* both *in vitro* (Wheeler *et al.,* 1984; Stevens and Berry, 1988; Berry *et al.,* 1989) and in nodules (Hammad *et al.,* 2003), but direct evidence of their involvement in nodule development is lacking. It has been suggested that the failure of most transgenic plants of *Allocasuarina verticillata* to nodulate could be due to effects of the auxin genes of the transforming organism, *Agrobacterium rhizogenes,* on host-plant hormone balance (Franche *et al.,* 1999a). The availability of genetic transformation systems will undoubtedly facilitate resolution of the role of hormones in the nodulation process and the nature of the molecular signalling systems that must regulate interactions between *Frankia* and the host plant.

Progress is being made in identifying and determining the expression of nodule-specific genes – actinorhizal nodulin genes – and their gene products. Séguin and

Lalonde (1993) used two-dimensional polyacrylamide gel electrophoresis to detect several nodule-specific polypeptides in developing actinorhizal nodules. This research area has been developed further, particularly by Katharina Pawlowski, together with Ton Bisseling and Antoon Akkermans, and also independently by Beth Mullin, Didier Bogusz and Claudine Franche, Alison Berry, Ann Hirsch, and Chung Sun An. Screens of cDNA libraries from nodules of *Alnus glutinosa* and *Datisca glomerata* revealed a number of genes that are expressed during early nodulation and the products of some are known, *e.g*., *ag12* encodes a subtilisin-type protease (Ribeiro *et al.,* 1995), whereas *Dg93* shares sequence homology with an early nodulin gene from legumes (Okubara *et al.,* 2000). After expression *in vitro*, proteins, which are encoded by two nodule-specific cDNAs isolated from *A. glutinosa* nodules, have been purified and characterised (Gupta *et al.,* 2002). These proteins represent a new class of plant metal-binding proteins, which have potential use in bioremediation. Cell-specific expression of chitinase genes in nodules of *Elaeagnus umbellate* has also been described (Kim and An, 2002). Urgent challenges for future research include matching gene structure and protein function with the processes involved in nodule developmental and metabolism.

3.4. Life with Oxygen (O_2)

Nitrogen fixation by *Frankia* in both the free-living and symbiotic state is supported by aerobic metabolism and is maximal at about atmospheric O_2 partial pressures, so special mechanisms must be in place to protect nitrogenase from inactivation by O_2. Early identification of vesicles as the probable site of nitrogen fixation in cultured *Frankia* was confirmed by immunogold labelling of nitrogenase (Tjepkema *et al.,* 1981; Meesters *et al.,* 1987). The individual research programs of Tjepkema, Silvester, and Huss-Danell and Berry have been instrumental in resolving the complexity and diversity of protective mechanisms that operate in free-living and symbiotic *Frankia*. Actinorhizal nodules are well aerated with large numbers of air spaces and do not have the diffusion-resistant "nodule endodermis" that restricts O_2 diffusion in legume nodules. In the cultured organism, the layered walls of the vesicles, which contain large amounts of hopanoid lipids (Berry *et al.,* 1993), vary in thickness in response to changes in pO_2, thus regulating O_2 diffusion (Parsons *et al.,* 1987). Similarly, at least in the *Alnus* symbiosis, where nitrogenase is located in vesicles, increased O_2 concentration results in both an increase in vesicle envelope thickness and changes in the relative proportions of hopanoid lipids present (Silvester *et al.,* 1988; Huss-Danell, 1990; Kleemann *et al.,* 1994).

The production of vesicles in actinorhizal nodules of different species is highly variable. At one end of the spectrum, vesicles are not produced in *Casuarina* nodules, most probably because they are only ever exposed to low pO_2. Experimental proof (Tjepkema, 1979) of the earlier observation of O_2-transporting hemoglobins in *Casuarina* nodules (Davenport, 1960) provided the basis of this explanation because low pO_2 in the infected cell areas is consistent with the presence of a functional O_2 transporter. Later, Murry *et al.* (1985) showed that, under conditions of very low pO_2, vesicles do not form in cultured *Frankia* but the

hyphae still develop nitrogenase activity. In contrast, nodules of *Alnus* and some other genera form well-defined vesicles, but contain only a low haemoglobin concentration (Suharjo and Tjepkema, 1995).

For *Casuarina glauca*, hemoglobin is synthesised early in young infected nodule cells to prepare the environment for functional nitrogenase. Hemoglobin cDNA has been cloned and induction of the hemoglobin gene prior to the detection of *Frankia nifH* mRNA has been demonstrated by *in situ* hybridisation (Gherbi *et al.,* 1997). Interestingly, cultured *Frankia* produces haemoglobin, raising important questions concerning the source and regulation of hemoglobins *in vivo* (Beckwith *et al.,* 2002). An additional method of O_2 protection was found in nodules of *Coriaria*, which has thin vesicles that radiate inwards towards a central cell vacuole. Here, mitochondria are distributed around the vesicles and also around the intercellular spaces that penetrate the nodule, indicating that pO_2 could be kept low by respiratory scavenging of O_2 (Silvester *et al.*, 1999). It should be noted that the shape of the *Frankia* vesicle *in vivo* is host-plant dependent (Tjepkema *et al.,* 1986).

Nitrogenase in actinorhizal nodules seems also to be protected by "oxygen transients" in which nitrogenase switches off rapidly in response to an increase in pO_2, possibly by conformational protection, and then recovers when O_2 levels return to normal (Silvester and Winship, 1990). Free radical scavenging systems, such as superoxide dismutase, have also been detected in cultured and symbiotic *Frankia* and may complement the battery of defenses that help prevent O_2 inactivation (Steele and Stowers, 1986; Puppo *et al.,* 1989; Alskog and Huss-Danell, 1997). Interestingly, expression of the *Frankia* gene for Fe superoxide dismutase, *sodF*, is induced by host-root exudates (Hammad *et al.,* 2001), suggesting it plays an additional earlier role in the symbiosis (Maréchal *et al.,* 2003).

3.5. Metabolism, Nitrogen Cycling, and the Regulation of Metabolism

Frankia in most actinorhizal nodules has a particularly active H_2-uptake system, catalysed by uptake hydrogenase (Hup), which metabolises H_2 evolved by nitrogenase during nitrogen fixation (Schubert and Evans, 1976; Roelofsen and Akkermans, 1979). Respiratory activity eventually leads to donates of the electrons from H_2 oxidation to O_2 and may thus contribute to both energy conservation through ATP generation and help prevent O_2 inactivation of nitrogenase. Support for these suggestions came from studies of *Alnus incana* in symbiosis with a Hup(–) *Frankia* strain, which showed lower nitrogen fixation than plants inoculated with Hup(+) *Frankia* (Sellstedt *et al.,* 1986). Further, nitrogenase activity of *Frankia* increased when cultured in a gas mix with elevated pO_2 and pH_2 (Murry and Lopez, 1989). Immunological studies by Anita Sellstedt have shown that the hydrogenases of *Frankia* are located primarily in vesicles and to a lesser extent in hyphae (Lindblad and Sellstedt, 1989; Sellstedt and Lindblad, 1990) and are similar to membrane-bound [NiFe] hydrogenases (Mattson *et al.,* 2001).

The composition of different media used to culture *Frankia* shows that the organism can use a wide range of carbon substrates *in vitro*, such as amino acids, pyruvate, propionate, and glucose. However, the nature of the carbon substrate that

is transported into symbiotic *Frankia* to support endophyte growth and nitrogen fixation is still unknown despite comprehensive physiological studies by Kerstin Huss-Danell's group, who developed assay techniques for symbiotic *Frankia* cells (Lundquist and Huss-Danell 1992) that continue to be used today.

The likely first step in the assimilation of the ammonia produced by nitrogenase activity is the formation of glutamine. Extensive biochemical studies by David Benson's laboratory showed that this is true for cultured *Frankia*, which possesses two glutamine synthetases. GS-I is present during growth on either NH_4^+ or N_2 and is similar to the classical bacterial glutamine synthetase, being regulated by adenylation. GS-II is derepressed when cultures are starved of combined nitrogen and accounts for most of the glutamine-synthtase activity in such cultures (Edmands *et al.,* 1987). However, glutamine synthetase is thought not to be present in *Frankia* in symbiosis (Lundquist and Huss-Danell, 1992). The NH_4^+ from fixation is exported to the plant-cell cytosol for assimilation into an organic form and exported to the host plant as either citrulline (alders and casuarinas) or amides (most other actinorhizal genera). The cells of the nodule pericycle of *Alnus* show high levels of expression of genes that code for enzymes, such as sucrose synthase and glutamine synthase, and of several other nodulin genes, different from those of the root pericycle. These observations suggest that the *Alnus* nodule pericycle may play a special role in the exchange of metabolites between the stele and the nodule cortex (Pawlowski and Bisseling, 1996).

Photosynthesis is the main source of carbon and provides translocated sucrose to drive nodule metabolism, with lesser amounts coming from CO_2 fixation through the action of phosphenol pyruvate carboxylase and, in alders, ornithine carbamyl phosphate synthase (McClure *et al.,* 1983). It was thought originally that either the supply of carbohydrates or nitrite inhibition following uptake of nitrate were primary regulators of nitrogenase activity. However, Parsons, Raven, Sprent and co-workers proposed that the concentration of nitrogen-containing compounds (amino acids, amides, ureides) in phloem sap inversely regulates the rates of both nitrogen fixation and nodule growth (Parsons *et al.,* 1993). This mechanism has gained favour as the primary metabolic signal that regulates nodule growth and activity in actinorhizal nodules (Baker *et al.,* 1997a; 1997b; Parsons and Sunley, 2001) and further work is in progress to identify the sensor mechanisms that detect fluctuations in supply of combined nitrogen to nodules.

3.6. Ecology and Applications

The utilisation of actinorhizal plants in land reclamation and forestry continues to be researched (Dawson, 1983; 1986; Dommergues, 1997; Gordon and Wheeler, 1983; Schwenke and Caru, 2001; Tjepkema and Schwintzer, 1990). Traditional techniques of ecological physiology were employed in the Himalayas to determine the contributions of *Alnus nepalensis* to nutrient cycling and primary production in agroforestry, both in different aged plantations and in naturally regenerated landslip sites (Sharma *et al.,* 1998). These studies showed clearly how uptake of recycled mineral nitrogen replaced the high-energy processes of both nitrogen fixation and

nodule production as soil-nitrogen concentration increased with stand age (Sharma and Ambasht, 1988).

New molecular techniques have facilitated investigation of questions such as the persistence and competitiveness of introduced *Frankia* strains in managed environments and provided approaches to determine the contributions of indigenous and introduced actinorhizal plants to the nitrogen economy of particular ecosystems. The groups of Antoon Akkermans, Philippe Normand, Dittmar Hahn, David Myrold, and David Benson have been particularly successful in developing molecular techniques to study the ecology of *Frankia* populations. These techniques include both utilising probes that will hybridise specifically with *Frankia* 16S or 23S rRNA (Hahn *et al.,* 1990; Akkermans *et al.,* 1994; Hönerlage *et al.,* 1994) and sequence analysis of PCR-amplified ribosomal DNA, *nif* genes or intergenic spacers (Hahn *et al.,* 1999; see chapter by Hahn in this volume). These last authors note that, whereas studies to date have focused on the analysis of *Frankia* populations in nodules and to a lesser extent in soil and the rhizosphere, the establishment of more sophisticated methods should allow detailed studies of the environmental dynamics of *Frankia* populations.

3.6.1. Sporulation in Frankia

The capacity of molecular techniques to provide unequivocal answers to previously insoluble questions is well illustrated by research on the sporulation of symbiotic *Frankia* in natural plant communities. In the mid-seventies, microscopic studies of the endophyte of *Alnus glutinosa* nodules led van Dijk and Merkus (1976) to propose that the term "spore" should be used to replace "bacteroids", which was in common use to describe the "granulated bodies" seen in some infected cells. Further analysis of the root nodules of different *Alnus glutinosa* populations showed two types of nodules, one containing spores and the other from which spores were absent (van Dijk, 1978). The distribution of these two types showed considerable clustering and, in due course, led to the terminology "spore(+)" and "spore(–)" to describe the two nodule types. These nodules types are not confined to alders and, in general, the spore(–) nodule type seems to be less infective but more effective than spore(+) nodules (VandenBosch and Torrey, 1984; Holman and Schwintzer, 1987). Cross-inoculation experiments suggested that the ability to form sporangia in the symbiotic state is controlled by the endophyte (van Dijk, 1978; Vanden Bosch and Torrey, 1985). Proof of this was finally obtained by molecular characterisation through PCR amplification and sequencing of 16S-rRNA sequences, which showed specific differences in the DNA of endophytic *Frankia* from spore(+) and spore(–) nodules (Simonet *et al.,* 1994).

3.6.2. Irregular Nodulation

As mentioned earlier, there are many reports of irregular nodulation among different host species. Most frequent causes are growth in either unfavourable environmental/soil conditions or the occurrence in soil of non-infective, poorly effective or ineffective strains of *Frankia*. A study of alders growing in a wet soil in the Netherlands showed that these strains can form a significant proportion of the