Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations

Nitrogen Fixation: Origins, Applications, and Research Progress

VOLUME 5

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

Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations

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"Colonization of a wheat root hair by *Azospirillum*. Photograph courtesy of Claudine Elmerich, Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, and Institut Pasteur, Paris, France, 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

Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations

This book is part of the seven-volume series that was launched a few years ago with the ambitious objectives of reviewing the field of nitrogen fixation from its earliest beginnings through the millennium change and of consolidating the relevant information - from fundamental to agricultural and environmental aspects – all in one place. Volume 5 covers the biology of bacteria that associate with non-leguminous plants. The subject matter includes a wide range of associations; it covers the bacterial species that associate either with the surface or within the tissues of grasses (often referred as plant growth-promoting rhizobacteria) and also the symbiotic associations that cyanobacteria form with fungi, algae, and both lower and higher plants. This volume does not deal with the *Frankia*-actinorhizal plant associations, which is the topic of Volume 6.

The book is divided in 13 chapters, each of which is the work of well-known scientists in the field. Just like in the other volumes of this series, the first chapter is an historical perspective. It describes how, as early as the end of the 19th century, it was shown that plant exudation favoured the proliferation of soil bacteria in the rhizosphere, and how the first nitrogen-fixing bacteria, including cyanobacteria were isolated. The chapter covers the landmarks and scientific concepts that arose from more than one century of research in this area.

Recently, implementation of the techniques of molecular phylogeny has led to the identification of an increasing number of N_2-fixing genera and species associated with grasses. The taxonomic status of both old and recently discovered species of the α- and β-subgroups of the Proteobacteria is the topic of the second chapter. Chapter 2 also outlines the ecology of these genera and then describes both tools and molecular probes that can be used for *in situ* localization of associated bacteria, in particular, to distinguish the bacteria located on the root surface from the endophytes resident within the plant tissues.

The genetics and regulation of nitrogen fixation in free-living bacteria is dissected in detail in Volume 2, however, it is of such importance that selected coverage of this subject is provided here in Volume 5, especially as it relates to the current understanding of the *nif* genetics of the most important grass-associated species; *Azospirillum brasilense*, *Herbaspirillum seropedicae*, *Gluconacetobacter diazotrophicus, Azoarcus sp.,* and *Pseudomonas stutzeri.* Indeed, Chapter 3 uses the established knowledge of *Klebsiella* and *Azotobacter nif* genetics as a basic framework on which to provide a comprehensive and comparative view of the grass-associated bacterial systems, while simultaneously emphasizing the unique features of each system and their regulatory networks.

Five chapters of Volume 5 focus on the molecular bases of the plant growth-promotion effect and the plant response to inoculation. Chapters 4 and 5 review more specifically the physiological and molecular bases of the root colonization. The molecular mechanisms of chemotaxis and the role of the chemotactic response

in adaptation to the soil and plant rhizosphere are reviewed in Chapter 4. Chapter 5 continues the colonization process, from attachment through to root-surface colonization, with a detailed review of the involvement of flagella, pili, and surface polysaccharide components. This chapter also presents a comprehensive analysis of the factors required for rhizosphere competence both at the physiological and genetic levels. Next, the idea that plants benefit from associated bacteria as a consequence of microbial phytohormone production was launched more than 50 years ago and this is the subject of Chapter 6. It is apparent that soil bacteria produce a wide range of plant hormones and that there is a multiplicity of biosynthetic pathways. For example, the routes for indole-3-acetic acid biosynthesis differ in plants, in pathogenic bacteria, and in plant-associated N_2-fixing bacteria. Chapter 6 describes this multiplicity of pathways and discusses the role(s) of these compounds in the association.

Chapter 7 reviews the overall plant response to inoculation, including the changes in root morphology, root metabolism, and effect on plant productivity. It also includes a review of the effect of *Azospirillum* and other bacterial inoculation on legume nodulation. To complete the presentation of plant-growth promotion by inoculation, Chapter 8 deals with the role of the N_2-fixing bacteria associated with grasses as biocontrol agents, even though the amount of information in the particular case of nitrogen fixers is still limited. Biocontrol is the property of beneficial bacteria to compete with pathogens through, for example, antibiosis, iron sequestration, or aggressive root colonization. The chapter also describes the mechanisms of activation of plant defences.

Although Chapters 4 to 8 include information on the colonization ability of a range of microorganisms, the main emphasis is on *Azospirillum* as the paradigm for root-surface colonization. With the discovery some 15 years ago of endophytic associations involving N_2-fixing bacteria that did not cause disease symptoms, a new research era arrived. The example of *Azoarcus* is treated in Chapter 9, which reviews the phylogeny and physiology of *Azoarcus* and related bacteria. It describes the cytology and the molecular biology of the interaction of *Azoarcus* with rice and Kallar grass. Chapter 10 deals with sugarcane-cropping systems and focuses on the diversity of N_2-fixing bacteria associated with sugarcane. It emphasizes the modes of endophytic colonization and the molecular biology of both *G. diazotrophicus* and *H. seropedicae*.

Cyanobacteria coverage is limited to two chapters, but additional information on the physiology, genetics, and genomics of cyanobacteria is given in Volume 2, *Genetics and Regulation of Nitrogen Fixation in Free-living Bacteria*, and Volume 3, *Genomes and Genomics of Nitrogen-fixing Organisms*. Because differentiation of the non-N_2-fixing vegetative cells into N_2-fixing heterocysts is crucial for a successful cyanobacterial symbiosis, Chapter 11 summarizes current knowledge of the physiology and genetics of filamentous cyanobacteria, emphasizing the differentiation process. This chapter is followed by a comprehensive and extensive review of the various plant associations involving filamentous cyanobacteria. Chapter 12 describes the biology of the different symbioses of cyanobacteria with diatoms, *Geosiphon*, lichens, liverworts, hornworts, mosses, *Azolla*, Cycads, and *Gunnera*. Volume 5 then concludes with a chapter describing the potential of

endophytic nitrogen fixers for the future and discusses the ideal model of a diazotrophic endophyte.

It took several years to compile the contents of this volume and to finalize the chapters. We give special recognition to all the authors, who shared their knowledge and ideas in this fascinating field, and we hope that their invaluable contributions will promote nitrogen-fixation and related research efforts and drive us onward to more spectacular discoveries in the future.

We give a special thought to Johanna Döbereiner, a leading figure in this field, who passed away in 2000. This volume is dedicated to her memory. Many researchers learnt from her and are proud to have done so; they continue to work in her spirit. We also remember Jean-Paul Aubert, deceased in 1997, for his support of nitrogen-fixation research for more than 20 years. Finally, we thank our families, friends, and colleagues for their interest and continual support during the time spent editing this volume.

Claudine Elmerich
Gif-sur-Yvette, April 2005

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Blacksburg, April 2005

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Johanna Döbereiner (1924 – 2000)

This volume is dedicated to the memory of Johanna Döbereiner in recognition of her forty-nine years of research in soil microbiology. Johanna Döbereiner was born in Czechoslovakia in 1924, she studied agronomy at the University of Munich and, in 1951, emigrated with her family to Brazil. She started work in the "soil microbiology laboratory" in the National Department for Agricultural Research of the Ministry of Agriculture in Seropédica, which later became the EMBRAPA. Johanna was at the centre of biological nitrogen-fixation research from the early discovery of *Azotobacter paspali* associated with the roots of *Paspalum notatum* until the "endophytic" associations of N_2-fixing bacteria within the tissues of forage grasses, cereals, and sugarcane. She published more than 500 scientific papers and she was ranked seventh among Brazilian scientists in the citations of her publications and the first amongst female scientists. But above all, those of us who understood her strong personality prized her friendship, her encouragement, and her capacity to face work as happy and enthusiastic as a person going on holiday. Johanna was more than a leader, she was a mother to many scientists (and a grandmother to the youngest), and she was a great friend and a source of pride for all of us. Johanna was awarded the degrees of Doctor *Honoris Causa* by both the University of Florida, USA, and the Universidade Federal Rural do Rio de Janeiro, plus the National Frederico de Menezes Viega Prize, the Bernard Houssay Prize, the UNESCO Science Prize, the Science and Technology Prize of Mexico, the Order of Rio Branco, the Order of Merit of the National Judiciary, and the Order of Merit of the Federal Republic of Germany. She was a member of the Academy of Sciences of the Vatican, the Brazilian Academy of Sciences, and the Third World Academies of Sciences. We thank V. Massena Reis, A. A. Franco, J. I. Baldani, M. C. Prata Neves, R. M. Boddey, V. L. Divan Baldani, and F. Pedrosa for supplying this dedication.

Chapter 1

HISTORICAL PERSPECTIVE: FROM BACTERIZATION TO ENDOPHYTES

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1. THE NITROGEN CYCLE: HERITAGE FROM THE 19TH CENTURY

The various steps of the nitrogen cycle and the major groups of microorganisms involved were discovered during the 19th Century (Figure 1; Table 1). Reyset, in 1856, was the first to describe the decomposition of organic matter in the soil that resulted in the release of nitrogen gas into the atmosphere, so providing the basis of the nitrogen cycle (see Payne, 1990). Schlossing and Müntz discovered the nitrification process in 1877 and Winogradsky obtained the first culture of *Nitrosomonas* by 1890. Gayon and Dupetit discovered denitrification in 1886 (Payne, 1990; Aubert, 1995). According to Wilson (1957), the notion of biological nitrogen fixation was born around 1837, although "gestation had been under way for many years". This idea, therefore, preceded the historical discovery of Hellriegel and Wilfarth, who established in 1886 that legumes, bearing root nodules induced by bacteria, could use gaseous nitrogen (Wilson, 1957; Nutman, 1987).

Even earlier, by 1771, Priestley was already convinced that plants could absorb nitrogen gas and this view was later adopted by many others (reviewed by Payne 1990). But scientists, including de Saussure and Liebig, challenged this view and declared that the fixed nitrogen originated only from the ammonia present in water, air, and fertilizers. Jean-Baptiste Boussingault performed the first set of experiments in 1838 that showed nitrogen fixation with clover and pea. Between 1851 and 1855, he implemented a new set of experiments that were unsuccessful. The experiments carried out by Georges Ville at the same time showed a positive gain not only with legumes, but also with wheat, rye, and watercress. To kill the controversy, Ville performed new experiments under the control of a committee mandated by the French Academy (Dumas *et al*., 1855). Although this committee

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confirmed that Ville's observations were consistent with the conclusions drawn from his previous work, a number of questions were raised in the committee's report (Dumas *et al*., 1855) and Cloez (1855) highlighted a number of experimental difficulties casting doubt on the conclusions drawn. At about the same time, Gilbert, Lawes and Plugh conducted similar experiments at Rothamsted in England. The conclusions of these scientists, like those of Boussingault and Ville, were also censured by their contemporaries, in particular, the German scientist Liebig (Nutman, 1987).

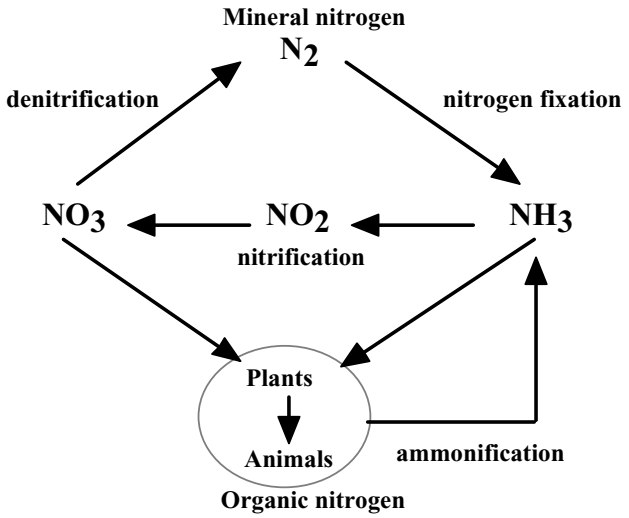

Figure 1. Schematic representation of the nitrogen cycle

Jodin, at the French Academy, reported the first observation of N_2 fixation by unknown microorganisms in a nutrient solution incubated under controlled conditions (Jodin, 1862). This observation was followed, 26 years later, by the isolation of a strain from root nodules by Beijerinck (1888). The strain, initially designated *Bacillus radicicola*, was later renamed *Bacterium radicicola*, and then as *Rhizobium leguminosarum* by Frank in 1890 (reviewed in Virtanen and Miettinen, 1963). A few years later, Winogradsky (1893) isolated the first anaerobic nitrogen fixer, *Clostridium pastorianum* (now *pasteurianum*) and, in 1901 and 1903, *Azotobacter* spp. were isolated by Beijerinck and Lipman (Table 1). Nitrogen fixation with blue-green algae (now classified as cyanobacteria) was also discovered during the 19th Century (see Chapter 12). However, as these algae were always associated with bacteria, it was only much later that their ability to fix nitrogen was confirmed (Drewes, 1928).

In 1883, the Danish scientist, Johann Kjeldahl, introduced an analytical method for the determination of total nitrogen and, one year later, the first digestion and

distillation equipment became available (produced by the C. Gerhardt Company). Berthelot (1885) first demonstrated chemical nitrogen fixation, by lightning for example, before turning his attention on nitrogen fixation by microscopic organisms in the soil, which he estimated would account for 15-to-30 kg of fixed N per ha.

By the end of the 19th Century, it was widely accepted that plants encourage the proliferation of microorganisms in the root zone. This led Lorenz Hiltner to define the rhizosphere as the soil immediately surrounding the roots under the influence of the plant (Starkey, 1958; Rovira, 1991).

2. NUTRITIONAL INTERACTIONS BETWEEN BACTERIA AND PLANTS

Following the initial discovery of *Clostridium* and *Azotobacter* an increasing number of nitrogen-fixing organisms were isolated (reviewed by Virtanen and Miettinen, 1963; Wilson, 1969; Postgate, 1982; Balandreau, 1983; Döbereiner and Pedrosa, 1987; Table 1). A dozen of genera had been discovered by 1969, including *Aerobacter* (*Klebsiella*), *Azotobacter*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Derxia*, *Spirillum*, and various photosynthetic bacteria and cyanobacteria (Stewart, 1969). Interestingly, *Spirillum* (*Azospirillum*) received little attention until the early 1970's (Döbereiner and Day, 1976). In fact, for more than 50 years after their initial discovery, *Azotobacter* and *Clostridium* were regarded as the only genera of bacteria capable of fixing nitrogen in the free-living state and *Nostoc* as the only nitrogen-fixing blue-green alga (Stewart, 1969; Wilson, 1969). But many soil bacteria were known to produce plant growth substances and to proliferate in the rhizosphere. Soon, "bacterization" was considered as a mean to benefit non-leguminous crops (Brown, 1974).

2.1. Azotobacter *and the Nitrogen-Fixation Potential of Soils*

In his volume on soil microbiology, which consists essentially of a compilation of his publications plus comments, Winogradsky (1949) expressed the view that *Azotobacter* was the only aerobic non-symbiotic bacterium able to fix nitrogen, with nitrogen fixation by other genera remaining doubtful. For Winogradsky, a key question was that of the role of *Azotobacter* in its natural environment. He differentiated "sugar *Azotobacter*" (grown in laboratories) from "soil *Azotobacter*" and considered that physiological experiments with pure cultures overfed with sugars provided the agrobiologist with no real insight into the role of *Azotobacter* in the soil. He developed several methods both for isolating *Azotobacter* and for estimating the density of this bacterium in soil, based on the use of either silica gel plates devoid of combined nitrogen or sifted soil to which mannitol or other carbon sources were added (see Pochon and Tchan, 1948). He proposed that the number of *Azotobacter* colonies was correlated with the nitrogen-fixation potential of the soil.

Table 1. Landmarks in nitrogen-fixation research with special reference to free-living, associative and endophytic nitrogen-fixing bacteria

Year	Event	Reference or citation
1838-1880	Experiments of Boussingault, Ville, Lawes and Gilbert, and others; controversy in the demonstration of nitrogen fixation by plants	Dumas et al, 1855[(a)]; Wilson, 1957; Nutman, 1987; Payne, 1990
1862	Jodin demonstrated nitrogen fixation by microorganisms in culture under controlled conditions	Jodin, 1862; Wilson, 1957
1856-1868	Reyset established the principle of the nitrogen cycle	Payne, 1990; Aubert, 1995
1877	Schlossing and Müntz discovered nitrification	Payne, 1990; Aubert, 1995
1883	Kjeldahl's method of total nitrogen determination	
1885	Berthelot observed nitrogen fixation in soil	Berthelot, 1885
1886	Gayon and Dupetit isolated the first pure culture of bacteria capable of denitrification	Payne, 1990; Aubert, 1995
1886-1888	Hellriegel and Wilfarth established nitrogen fixation by root nodules of Legumes	Wilson, 1957; Nutman, 1987
1888	Isolation of *Bacillus radicicola* [(b)]	Beijerinck, 1888
1890	Isolation of *Nitrosomonas* by Winogradsky, initially referred to as the "ferment nitrique"	Winogradsky, 1949; Payne, 1990
1893	Isolation of *Clostridium pasteurianum* [(c)]	Winogradsky, 1893
1901-1903	Isolation of *Azotobacter* spp. by Beijerinck and by Lipman	Virtanen and Miettinen, 1963
1904	Definition of the "rhizosphere" by Hiltner	Rovira, 1991
1925	Isolation of *Spirillum lipoferum* by Beijerinck	Becking, 1963; 1982
1927	First bacterization experiments in Soviet Union	Macura, 1966 [(d)]
1928	Isolation of *Aerobacter aerogenes* by Skinner	Virtanen and Miettinen, 1963
1928	Isolation of *Nostoc* and *Anabaena* by Drewes	Drewes, 1928; Chapter 12
1931	Discovery of production of phytohormones by bacteria	Boysen Jensen, 1931
1939	Isolation of *Beijerinckia* spp. by Starkey and De	Döbereiner and Pedrosa, 1987
1941	Application of N^{15} to Nitrogen fixation research	Burris and Miller, 1941
1949	Clark proposed the term of "rhizoplane" for the microbiology of root surface	Starkey, 1958; Rovira, 1991
1958	Isolation of *Bacillus polymyxa* by Hino and Wilson	Balandreau, 1983
1960	Nitrogenase activity is obtained in cell free extract of *C. pasteurianum* by Carnahan, Mortenson, Mower and Castle	Wilson, 1969
1961	Production of growth regulators by *Azotobacter*	Vancura, 1961
1966	Association *Azotobacter paspali -Paspalum notatum*	Döbereiner, 1974
1966	Acetylene reduction technique to assay nitrogenase activity by Schollhorn and Burris and by Dilworth	Hardy *et al*. 1968
1974	§ First international congress on nitrogen fixation, Pullman, Washington, USA, with the Döbereiner and Day paper on "Associative symbiosis in tropical grasses"	Newton and Nyman, 1976
1974-1978	Clarification of the taxonomic status of *Azospirillum* spp.	Tarrand *et al*., 1978

1979	‡ International workshop on associative N_2 fixation, São Paulo, Brazil	Vose and Ruschel, 1981
1980	Ausubel's and Haselkorn's groups used the high degree of conservation among *nif* genes for their detection in heterologous hosts by Southern hybridization	Elmerich, 1991
1981	¶ First "*Azospirillum* workshop", Bayreuth, Germany	Klingmüller, 1982
1986	Isolation of *Herbaspirillum seropedicae*	Döbereiner, 1992
1986	Isolation of nitrogen-fixing rod from Kallar grass (later identified as *Azoarcus* spp.)	Reinhold *et al*., 1986
1987	Nitrogen fixation in *Pseudomonas stutzeri*	Krotzky and Werner, 1987
1988	Isolation of (*Glucon-)Acetobacter diazotrophicus*	Döbereiner, 1992
1992	Development of the N_2-fixing endophytes concept	Döbereiner, 1992
1994	Discovery of nitrogen fixation in *Burkholderia* associated with rice	Tran *et al*., 1994; Chapter 2
1995	International Symposium on sustainable agriculture, Rio de Janeiro, Brazil, organized by Franco and Boddey in honour of the 71st birthday of Johanna Döbereiner	Special issue of Soil Biol. Biochem., 1997, 29, N°5/6
2001	Non-culturable *Burkholderia* endophyte	Minerdi *et al*. 2001
2001	Development of the β-rhizobia concept: some β-Proteobacteria can nodulate legumes[(e)]	Moulin *et al*. 2001; see Chapter 2
2002	Genome projects: *Azotobacter*, *Herbaspirillum*	Kennedy, Pedrosa *et al*

(a) *This ref. corresponds to the report presented to the French Academy by the committee members who evaluated the experiments performed by Ville;* ***(b)*** Rhizobium leguminosarum*;* ***(c)*** *In his initial publication of 1893, Winogradsky isolated a mixture of 3 bacilli; in 1894, he successfully isolated the nitrogen-fixing agent in pure culture in anaerobic conditions; and the name* Clostridium pastorianum *appeared only in 1895;* ***(d)*** *J. Macura presented his general report at the Soil Microbiology Colloquium, devoted essentially to "bacterization", organized by J. Pochon in Feb. 1966.* ***(e)*** *The ability to establish a symbiosis with legumes is found outside the α Proteobacteria and among β Proteobacteria in the Burkholderiales:* Burkholderia *and* Ralstonia. ***§‡ ¶****- The numerous International Congresses covering different aspect of nitrogen fixation cannot be cited here, but these three series have been of particular importance for the field.* ***§*** *1974 saw the first of a series of international congresses set up by W. E. Newton, covering chemistry, biochemistry, genetics, ecology and agricultural aspects of nitrogen fixation; the following were in Salamanca, Spain (1976), Madison, USA (1978), Canberra, Australia (1980), Noordwijkerhout, The Netherlands (1983), Corvallis, USA (1985), Cologne, Germany (1987), Knoxville, USA (1990), Cancun, Mexico (1992), St Petersburg, Russia (1995), Paris, France (1997), Foz do Iguaçu, Brazil (1999), Hamilton, Canada (2001) and Beijing, China (2004).* ***‡*** *After the 1979 workshop in Brazil, the symposia on "Nitrogen Fixation with Non legumes" were held successively in Canada (1982), Finland (1984), Brazil (1987), Italy (1990), Egypt (1993), Pakistan (1996), Australia (1999) and Belgium (2002).* ***¶*** *The first four workshops were organized by W. Klingmüller in Bayreuth, Germany in 1981, 1983, 1985 and 1987, with I. Fendrik and associates continuing the tradition in Hanover, Germany (1991) and Hungary (1994). The* Azospirillum *workshops are now a part of the "Nitrogen Fixation with Non legumes" series. Congresses on "photosynthetic prokaryotes", not mentioned here, are also regularly held and are of importance for the research on cyanobacteria.*

2.2. Contribution of Free-Living Nitrogen Fixers to Soil Fertility

With the emergence of ^{15}N tracer techniques (Burris and Miller, 1941), the relative contribution to soil fertility by free-living nitrogen-fixing organisms continued to stimulate considerable interest (Delwiche and Wijler, 1956; Chang and Knowles, 1965; Stewart, 1969). *Azotobacter* decreased in importance in the eyes of researchers (Starkey, 1958) as new nitrogen-fixing species were confirmed by the isotopic technique (Wilson, 1969).

Beijerinckia was demonstrated to be of importance in soils following the discovery that *Azotobacter* distribution is limited to neutral soils, whereas *Beijerinckia* predominates in tropical acid soils (Dommergues and Mutaftschiev, 1965; Döbereiner, 1974; Döbereiner and Pedrosa, 1987). Similarly, anaerobic nitrogen fixation by *C. pasteurianum* in soils and aerobic nitrogen fixation by genera other than *Azotobacter* was observed in the soils of Quebec (Chang and Knowles, 1965). The addition of soluble organic substrates to soils greatly increased the rate of nitrogen fixation. In natural conditions, free-living heterotrophs in the soils have been shown to fix insignificant quantities of nitrogen unless organic substrates, such as grass cuttings, straw or other plant residues, are available (Delwiche and Wijler, 1956).

2.3. Bacterization

Plants were first inoculated with bacterial preparations in 1895, when Nobbe and Hiltner reported the benefit of inoculating legume seeds with rhizobia (Subba Rao, 1982). This development constituted the birth of the commercial inoculant industry, with the establishment in 1898 of Nitragin, a company that still produces rhizobial inoculants.

Inoculation was later extended to non-leguminous crops, such as cereals and vegetables. Shortly after the discovery of *Azotobacter*, the effects of inoculating the soil with this bacterium were investigated with a view to improving soil nitrogen balance and plant growth. Further experiments followed in which seeds or roots were directly inoculated with *Azotobacter*. The term "bacterization" was coined in 1926 and field inoculations with "azotobacterin" began in the Soviet Union shortly afterwards (see Macura, 1966). Russia was very active in this field of research, because Russian soils contained large numbers of *Azotobacter*. By 1958, about 10 million ha in Russia were treated with preparations of either *Azotobacter chroococcum* or *Bacillus megaterium* (Brown, 1974; Rovira, 1991) with *Bacillus* used for organic phosphate mineralization (phosphobacterin).

The reported results of bacterization in Russia generated strong controversy due to claims of high yield increases that were not reproduced in other parts of the world. However, further experiments confirmed some increase in yield for various crops. Moreover, changes were also reported in the general growth of the plants, for example, early flowering in tomato and wheat (Michoustine, 1966; Dénarié and Blachère, 1966; Brown 1974; Rovira, 1991). Also, other bacterial species, which may have beneficial effects, were detected in the plant rhizosphere (Rivière, 1963; Dénarié and Blachère, 1966; Brown 1974; Döbereiner and Day, 1976).

2.4. Role of the Bacterial Inoculant in the Bacterization Process

A prerequisite for successful bacterization is the survival of the inoculum and its multiplication in the rhizosphere. However, *Azotobacter* is often described as a fragile organism, highly sensitive to pH variations, with a low survival rate in the soil (Pochon and Tchan, 1948; Postgate, 1981; Döbereiner and Pedrosa, 1987). Many researchers have reported rapid declines in the number of *Azotobacter* after inoculation and a lack of rhizoplane (root surface) colonization (Jackson and Brown, 1966; Michoustine, 1966; Subba Rao, 1982; Kloepper, 1994).

The means by which the plants benefit from the inoculation was unclear. Different hypothesis were proposed to explain the benefit observed. These included: (i) changes in the rhizosphere microbial population; (ii) production of growth regulators (phytohormones) that stimulated the plant development; and (iii) disease suppression (Brown, 1974; Subba Rao, 1982; Rovira, 1991). Bacteria are known to produce growth regulators (Boysen Jensen, 1931; Vancura, 1961). *Azotobacter* cultures produce gibberellic acid and indole-3-acetic acid (Vancura, 1961; Brown, 1974). Rivière (1963) noted that a high percentage of bacterial strains isolated from the rhizosphere of wheat produced phytohormones. The list of phytohormone producers among soil bacteria and plant pathogens and the variety of the compounds synthesized is very large (see Chapter 6, this volume). Furthermore, nitrogen fixation and phosphorus mineralization were not considered to play a major role in the efficient application of "azotobacterin" and "phosphobacterin" (Brown, 1974; Subba Rao, 1982; Rovira, 1991).

2.5. The Rhizosphere Effect

The work of Hiltner in particular increased our understanding of the differences between the bulk soil and the rhizosphere soil. The microbial population is dense around plant roots. Plants affect microbe development and, in turn, the plant is affected by the activity of the microbes in the rhizosphere (Starkey, 1958). From 1950 onwards, studies of the composition of root exudates increased (reviewed by Starkey, 1958; Rovira, 1962). These analyses demonstrated that root exudates provide a source of nutrients for the soil microflora, favouring the proliferation of certain microorganisms in the rhizosphere and preventing others (Rovira, 1962). Clark defined the rhizoplane in 1949 (Starkey, 1958; Rovira, 1991) and the term "rhizodeposition" was later coined to account for the carbon loss by the plant that generates the rhizosphere effect (Lynch and Whipps, 1991). An important feature of the root exudates is their high C/N ratio, which may promote enrichment in nitrogen-fixing bacteria in the rhizosphere (Döbereiner, 1974).

The term "rhizobacteria" is now currently used for the bacteria that colonize the rhizosphere. Rhizobacteria with beneficial effects on plant development (involving growth stimulation or disease prevention/suppression) are referred to as plant growth-promoting rhizobacteria or PGPR (Kloepper and Beauchamp, 1992; Kloepper, 1994).

3. ASSOCIATIVE NITROGEN-FIXING BACTERIA

The terms "associative symbiosis", "rhizocoenoses", and "associative nitrogen fixation" have all been used to describe the interaction between *Azospirillum* and other rhizosphere bacteria and their host plants. None of these is fully satisfactory as a generic term. The process and the degree of interaction between the bacteria and the plant may differ between species; there are no differentiated structures on the roots induced by the bacteria; the extent of rhizoplane colonization is not always well defined; and the benefit of the association has often been challenged. Thus, soil nitrogen-fixing bacteria that can be found in close association with the root of grasses are usually designated as "associative nitrogen-fixing bacteria". Due to their growth-promoting effect, they are also referred to as nitrogen-fixing PGPR.

3.1. Evidence for Nitrogen Fixation in non-Legume Cropping Systems

Early evidence for non-symbiotic nitrogen fixation (reviewed by van Berkum and Bohlool, 1980) was provided by studies of the N balance in various ecosystems, such as salt marshes, fallow fields, and pasture fields. Crops, such as sugarcane in the tropics, and wetland rice in Asia, together with certain cereal fields in Canada and fallow fields in UK, shared the common characteristic of receiving no fertilizers over several centuries, and were all thought to benefit from nitrogen fixation by some means (van Berkum and Bohlool, 1980; Boddey and Döbereiner, 1982).

The advent of new techniques, based on acetylene reduction by nitrogenase, made it easier to estimate nitrogen fixation in natural ecosystems (Hardy *et al*., 1968; 1973). A critical review of the various techniques available for use with bacterial cultures, legumes, and non-leguminous plants can be found in the manual edited by Bergersen (1980). The determination of acetylene reduction either on excised roots or on plant soil cores was a source of some controversy because it did not reflect the actual rate of nitrogen fixation in intact plants in their natural environments (Hirota *et al*., 1978; van Berkum and Bohlool, 1980). In most cases, there was a considerable time lag before nitrogenase activity became detectable, and large variations between samples were observed. Balandreau *et al.* (1974) determined *in situ* acetylene reduction for a grass (*Panicum maximun*), rice, and peanut and not only confirmed the existence of non-symbiotic nitrogen fixation, but also demonstrated plant-specific diurnal variations in nitrogen-fixation rates.

In the preface of the book devoted to "Nitrogen Fixing Bacteria in Non-leguminous Plants" (Döbereiner and Pedrosa, 1987), Johanna Döbereiner describes her sabbatical leave at Rothamsted (UK) in 1970-1971. It was during her stay that she, together with other members of the "Grass-N_2-Fixation-Club", found acetylene reduction with roots of sugarcane and several tropical grasses, including *Paspalum notatum* (Döbereiner *et al*., 1972a). The discovery of *Azotobacter paspali* was an important step into associative nitrogen fixation. This species of *Azotobacter* is specific for *Paspalum notatum* cv. batatais and estimates of N_2 fixation with different soil cores ranged from 15-90 kg N/ha/year (Döbereiner *et al.*, 1972b). The ^{15}N isotope-dilution method showed that 10% of the total-N accumulated in *Paspalum notatum* originated from biological N_2 fixation (Boddey *et al*., 1983).

However, Brown (1976) observed that acetylene reduction was not always associated with the presence of *A. paspali* on the roots and she claimed that *A. paspali* improved the growth of *P. notatum* primarily by producing phytohormones rather than by N_2 fixation. This nitrogen fixation *versus* phytohormones production appears as a recurrent theme in the history of associative nitrogen-fixation research.

Boddey and Döbereiner (1982) reviewed early reports of N_2 fixation in rice, which showed up to 20-30 % of total-N in rice plants originates from biological N_2 fixation (Ventura and Watanabe, 1982; Watanabe and Roger, 1984). However, as the wetland rice microflora is highly complex, it was difficult to estimate the contribution of the heterotrophic bacterial population to N_2 fixation. Sano *et al*. (1981) measured the acetylene-reduction rates of various rice cultivars *in situ* and showed seasonal and diurnal variation as well as cultivar-dependent N_2 fixation.

3.2. Microbiology of the Association

3.2.1. Identification of the Nitrogen-Fixing Bacteria

In a review paper, Johanna Döbereiner listed her "ten recommendations" for the identification of root-associated diazotrophs (Döbereiner, 1989). In addition, the development of both numerical and molecular-taxonomy techniques, plus phylogenetic analyses, has greatly advanced the identification of the putative nitrogen-fixing isolates (Rennie, 1980; Balandreau, 1983; Hartmann *et al*., 2000; Roselló-Mora and Amann, 2001). Indeed, with time, many isolates have been renamed and reclassified in new genera (Young, 1992). Tools and strategies for the identification of bacterial isolates and for the *in situ* localization of these bacteria in the rhizosphere or within the plant tissues are detailed in Chapter 2 of this volume.

Determination of the nitrogen-fixation capacity of the bacterial isolates is often a critical step. In most cases, growth in N-free solid, semi-solid or liquid media is insufficient proof of nitrogen-fixing activity. Physiological conditions compatible with nitrogenase activity and the detection of this activity by the acetylene-reduction test may also be ineffectual or inconclusive (Postgate, 1981). Therefore, a molecular demonstration of the presence of the *nif* genes in the genome of the putative nitrogen fixer is generally considered as the reliable indicator. After the identification and cloning of the nitrogenase structural genes, *nifHDK*, and related genes, Southern hybridization experiments were performed to identify *nif* genes in a large number of Eubacteria and Archaea (Table 1, reviewed by Elmerich, 1991). Nowadays, the polymerase chain reaction (PCR) amplification with *nif* specific oligonucleotides probes, such as "*nifH* universal primers", is preferred (Zehr and McReynolds, 1989). This technique can be applied to the DNA of pure bacterial cultures, but it can also be used to follow the fate and distribution of nitrogen fixers by amplifying *nif* DNA fragments from DNA extracts from environmental samples (Rosado *et al.*, 1998; Hamelin *et al*, 2002; see Chapter 2).

3.2.2. Old and New Nitrogen-Fixing Bacteria

By the end of the 1980's, the presence of various species of *Azotobacter, Bacillus*, *Beijerinckia, Derxia, Enterobacteriaceae* (*Klebsiella, Enterobacter, Pantoae*),

Pseudomonas, and *Pseudomonas*-like bacteria was well established in the rhizosphere of cereal crops, weeds, and sugarcane (Rennie, 1980; Balandreau; 1983; Bally *et al.*, 1983; Haahtela *et al*, 1983b; Ladha *et al.*, 1983; Seldin *et al.*, 1984; Young, 1992). For nitrogen-fixing *Pseudomonas,* most of the initial reports dealt with bacteria that have since been reclassified to other genera (see Chapter 3), however, Vermeiren *et al*. (1999) confirmed the identity of the isolates classified as *Pseudomonas* by Haahtela *et al*. (1983a) and Krotzky and Werner (1987) (see Chapter 3). Johanna Döbereiner was responsible for discovering most of the new nitrogen-fixing species that were isolated from close association with the roots of various forage grasses, sugarcane, and maize (Table 1). In 1961, she found *Beijerinckia* associated with sugarcane roots (Döbereiner, 1961); in 1966, she isolated *Azotobacter paspali* from the grass growing in front of her laboratory (Döbereiner, 1974); and she then isolated several species of *Azospirillum* (Chapter 2), followed by *Herbaspirillum* in 1986, and of *Gluconacetobacter* in 1988 (Table 1; see Section 4.2).

Little attention was paid to the spirillum-like bacteria isolated by Beijerinck in 1923 and rediscovered by Becking (1963; 1982) until Döbereiner and Day (1976) described the association of these bacteria with grasses and many cereal crops. These bacteria were eventually assigned to a new genus, called *Azospirillum* (Tarrand *et al*., 1978), and the number of reports dealing with these bacteria increased rapidly thereafter. Although this volume extends well beyond *Azospirillum*, much of the information published on this genus can be found in Chapters 2-8. *Azospirillum* species display an extremely wide ecological distribution and are associated with a large diversity of plants (van Berkum and Bohlool, 1980). Seven species are known (Chapter 2) and many aspects of their physiology and genetics have been reviewed (Eskew *et al*, 1977; van Berkum and Bohlool, 1980; Patriquin *et al*., 1983; Döbereiner and Pedrosa, 1987; Elmerich *et al*., 1992; 1997; Okon, 1994; 1985; Bashan and Levanony, 1990; Costacurta and Vanderleyden, 1995; Steenhoudt and Vanderleyden, 2000), including *nif* genetics (Chapter 3), colonization of the root system (Chapters 4 and 5), phytohormone production (Chapter 6), and the plant response to inoculation (Chapter 7). These bacteria are also known to produce siderophores and bacteriocins, which may serve as biocontrol agents in the competition with other members of the soil microflora (see Chapter 8).

The microbiology of rice, maize, coffee, sugarcane, pineapple, sorghum, and Kallar grass also resulted in the characterization of new species of *Alcaligenes*, *Azoarcus*, *Burkholderia*, *Campylobacter*, *Gluconacetobacter*, *Herbaspirillum,* and *Paenibacillus*, many of which are probably endophytes (see Section 4.2 and Chapters 2, 9, 10, and 13).

Flooded rice fields are an important source of methane emissions into the atmosphere (Liesack *et al*., 2000). Methane is produced as a result of a complex interaction between bacteria in anoxic soil, the oxic interface, and the rice rhizosphere (Watanabe and Roger, 1984). The bulk soil is considered as an anoxic compartment that favours the proliferation of fermentative bacteria (*Clostridium* spp.), methanogenic Archaea, and sulfate-reducing bacteria (*Desulfovibrio*),

whereas methane-oxidizing bacteria are present at the aerobic interface. Members of all of these groups are known to fix N_2 (Postgate, 1981; Young, 1992).

3.3. Plant-Growth Promotion

Efficient nitrogen-fixing associations require an adequate supply of substrates from the host plant, appropriate environmental conditions to support nitrogen fixation by the associated bacteria, and transfer of the fixed nitrogen to the host plant (Klucas, 1991). In 1975, von Bülow and Döbereiner reported high rates of nitrogen fixation by *Azospirillum* in association with maize. These findings generated a considerable long-standing controversy, even though experiments with the ^{15}N isotope dilution technique confirmed that, in some cases, biological nitrogen fixation could account for several percent of the total nitrogen in the plant (Boddey and Döbereiner, 1982; Klucas, 1991). The controversy remained until 1994, when Okon and Labandera-Gonzales published a compilation analysis of field trials. This survey of 20 years of field inoculation worldwide concluded that significant (5-30%) increases in yields could be achieved by inoculation with *Azospirillum*. These crop-yield increases were more marked when the use of chemical fertilizer was low. Yield increases result from better development of the root system, which correlates with an increase in the rate of water and mineral uptake by roots. *Azospirillum* possesses several pathways for IAA synthesis and also produces gibberellins. The plant growth-promoting effect of this genus is currently attributed to production of indole-3-acetic acid (IAA) and other phytohormones (see Chapters 6 and 7). The nitrogen-fixing capacity of these bacteria is thought to contribute little to plant growth.

4. DISCOVERY OF NITROGEN-FIXING ENDOPHYTES

The presence of bacteria resident within the tissues of healthy plants was first reported as early as 1926 (Starkey 1958; Hallmann *et al*, 1997). The names "endorhizospheric" and "endophyte" are used to describe this particular type of bacteria-plant association that does not induce disease symptoms (You and Zhou, 1988; Döbereiner, 1992; Reinhold-Hurek and Hurek, 1998). The systematic isolation of nitrogen-fixing bacteria, which belonged to bacterial species that did not survive in the soil, from externally sterilized root and stem samples led Döbereiner and co-workers to define a novel type of nitrogen-fixing bacterium-plant interaction involving nitrogen-fixing endophytes (Döbereiner, 1992; Döbereiner *et al.*, 1993).

4.1. Azospirillum*: a Root Surface Colonizer or a Facultative Endophyte?*

Azospirillum species are indigenous soil bacteria and common root-associated diazotrophs, essentially located on the surface of the root. They are connected to the root surface by fibrillar material and are sometimes found in the superficial layers of the root cortex (Bashan and Levanony, 1990). Indeed, most of the *Azospirillum* isolates have been obtained from surface-sterilized root samples, suggesting that a proportion of these cells are protected from sterilizing agents and