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I. Baldani, José; L.D. Baldani, Vera

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## History on the biological nitrogen fixation research in graminaceous plants: special emphasis on the Brazilian experience

JOSÉ I. BALDANI and VERA L.D. BALDANI

Embrapa Agrobiologia, BR465, Km 07, 23851-970 Seropédica, Rio de Janeiro, Brasil

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### ABSTRACT

This review covers the history on Biological Nitrogen Fixation (BNF) in Graminaceous plants grown in Brazil, and describes research progress made over the last 40 years, most of which was coordinated by Johanna Döbereiner. One notable accomplishment during this period was the discovery of several nitrogen-fixing bacteria such as the rhizospheric (*Beijerinckia fluminensis* and *Azotobacter paspali*), associative (*Azospirillum lipoferum*, *A. brasilense*, *A. amazonense*) and the endophytic (*Herbaspirillum seropedicae*, *H. rubrisubalbicans*, *Gluconacetobacter diazotrophicus*, *Burkholderia brasilensis* and *B. tropica*). The role of these diazotrophs in association with grasses, mainly with cereal plants, has been studied and a lot of progress has been achieved in the ecological, physiological, biochemical, and genetic aspects. The mechanisms of colonization and infection of the plant tissues are better understood, and the BNF contribution to the soil/plant system has been determined. Inoculation studies with diazotrophs showed that endophytic bacteria have a much higher BNF contribution potential than associative diazotrophs. In addition, it was found that the plant genotype influences the plant/bacteria association. Recent data suggest that more studies should be conducted on the endophytic association to strengthen the BNF potential. The ongoing genome sequencing programs: RIOGENE (*Gluconacetobacter diazotrophicus*) and GENOPAR (*Herbaspirillum seropedicae*) reflect the commitment to the BNF study in Brazil and should allow the country to continue in the forefront of research related to the BNF process in Graminaceous plants.

**Key words:** bacterial endophytes, diazotrophs, semi-solid N-free medium, inoculation, cereals, grass plants.

### INTRODUCTION

Research on BNF with grasses in Brazil was initiated by Johanna Döbereiner when she joined the research team at the National Center of Education and Agricultural Research of the Ministry of Agriculture, located at Km 47 in the fifties. The first studies were

on the occurrence of *Azotobacter* in acid soils of the “Baixada fluminense” (Döbereiner 1953). These studies gained visibility with the discovery of two new nitrogen-fixing bacteria associated with the rhizosphere of some graminaceous plants: *Beijerinckia fluminensis* with sugarcane (Döbereiner and Ruschel 1958) and *Azotobacter paspali* with *Paspalum notatum* cv. batatais (Döbereiner 1966). In the seventies, a significant advance in the area of BNF in grasses was the introduction of the acetylene reduction method. Almost at the same time, the semi-solid NFb medium that replicated the oxygen level

Dedicated to the memory of Dr. Johanna Döbereiner by two of her disciples who learned through working with her that research could be done with simplicity, perseverance, honesty, ethics and sagacity.

Correspondence to: José Ivo Baldani  
E-mail: ibaldani@cnpab.embrapa.br

found in soil niches was developed to isolate microaerophilic nitrogen-fixing bacteria associated with plant roots. This medium allowed the isolation of two new species of *Azospirillum*: *A. lipoferum* and *A. brasilense*. This marked the beginning of BNF research in grasses in Brazil as well in other countries. The research concentrated on several areas of the interaction between plants and bacteria including the microorganisms themselves. A new species of *Azospirillum* named *A. amazonense* (Magalhães et al. 1983) was isolated and identified using semi-solid LGI medium, derived from modification of the pH and carbon source of NFb medium (Baldani et al. 1984).

Several lines of research focusing on agricultural applications were developed. Hormonal effects, nitrogen assimilation, biological nitrogen fixation and even negative responses were frequently observed (Boddey and Döbereiner 1982). During this period, other groups working on BNF with non-leguminous plants were established in Brazil. Currently, besides the Embrapa Agrobiologia (Km 47) team there is groups in the States of Paraná, Rio Grande do Sul, Rio de Janeiro, Minas Gerais, Goiás, Ceará and Distrito Federal.

Research on the colonization of plant tissues by diazotrophic bacteria received a lot of attention from 1985 to 1990 and consequently some aspects of the plant-bacteria interaction began to be elucidated. Two new nitrogen-fixing bacteria able to colonize the interior of plant tissues were found: *Herbaspirillum seropedicae*, was isolated from plants of maize, sorghum and rice (Baldani et al. 1986a) and *Gluconacetobacter diazotrophicus* (synon. *Acetobacter diazotrophicus*) was isolated from sugarcane plants (Cavalcante and Döbereiner 1988).

This intimate interaction between macro and micro symbionts modified the concept known as associative and Döbereiner (1992a) introduced the endophyte concept to the field of BNF. From this point on, a new area within BNF was established which led to great advances in the understanding of physiology, ecology and genetics as well as in the interaction of the bacteria with the plant (Baldani

et al. 1997a). New nitrogen-fixing bacteria were identified: *Herbaspirillum rubrisubalbicans* (Baldani et al. 1996), *Herbaspirillum frisingense* (Kirchhof et al. 2001), *Azospirillum doebereineriae* (Eckert et al. 2001), *Gluconacetobacter johannae* and *Gluconacetobacter azotocaptans* (Fuentes-Ramírez et al. 2001) and *Burkholderia tropica* (Reis et al. 2004).

This review aims to rescue the history of BNF studies with grasses in Brazil. These studies were mainly coordinated by Johanna Döbereiner and through her efforts this line of investigation was established in several parts of the World. In addition, it presents recent advances in the area.

#### FREE-LIVING AND RHIZOSPHERIC NITROGEN-FIXING BACTERIA

##### *Azotobacter chroococcum*

These studies were mostly done between 1950 and 1970 when very little was known about the occurrence of these bacteria in tropical soils. In the first paper on the subject, Döbereiner (1953) observed a very frequent occurrence of *Azotobacter chroococcum* in 22 out of 27 acid soil samples collected in the "Baixada fluminense".

##### *Beijerinckia fluminensis*

The occurrence of nitrogen-fixing bacteria of the genus *Beijerinckia* was mentioned for the first time in Brazil and it was demonstrated that the size of the populations was related to vegetation, physical, and chemical characteristics of the soil (Döbereiner and Castro 1955). Additional studies on the occurrence of this genus in soil of several Brazilian States (Rio de Janeiro, São Paulo, Pernambuco and Paraná) led to the description of a new species of *Beijerinckia* named *B. fluminensis* (Döbereiner and Ruschel 1958). Analysis of 158 samples collected in different regions of Brazil showed that this species occurred predominantly in soils where sugarcane was cultivated (Döbereiner 1959a) and a direct influence of the plant on the development of the bacteria was suggested (Döbereiner 1959b). Addi-

tional studies showed that roots as well as leaves and stems had a positive influence on *Beijerinckia* populations. This was influenced by the exudation of substances into the soil by the roots during rainfall (Döbereiner and Alvahydo 1959). Other studies involving the roots showed that the population of *Beijerinckia* was much more pronounced in the rhizoplane region (refers to the soil adherent to the root surface) than in the rhizosphere. In addition, it was shown that removal of the aerial part of the plant significantly reduced the population of bacteria in both the rhizoplane and rhizosphere regions (Döbereiner 1961). Rice plants grown in greenhouse and inoculated with *Beijerinckia*, showed the establishment of the bacteria as well as an increase in the yield (Döbereiner and Ruschel 1961). However, these types of experiments were not continued. Studies on the occurrence of *Beijerinckia* and *Azotobacter* were extended to other forage grasses and the results showed that *Beijerinckia* appears mainly in rhizoplane soil and *Azotobacter* in the rhizosphere (Ruschel and Döbereiner 1965).

In the 1970's, the introduction of acetylene reduction methodology stimulated further studies involving *Beijerinckia* and sugarcane. The measurement of the nitrogenase activity in roots of sugarcane showed that it was much higher than that observed in the rhizosphere and in soil between the plant rows. *Beijerinckia indica* was the most abundant bacterial species in both roots and soil samples (Döbereiner et al. 1972a). Quantification of BNF in sugarcane based on the extrapolation of the nitrogenase activity data indicated a contribution of 50kg N/ha/year to the soil/plant system (Döbereiner et al. 1973).

#### *Azotobacter paspali*

A gramineous plant that caught the attention of Johanna Döbereiner in the 1960's was *Paspalum notatum*, also known as forquilha or batatais grass. Today this grass still covers the campus of Km 47 and lends the landscape a green color in the absence of added nitrogen fertilizer. A preliminary analysis on the occurrence of nitrogen-fixing bacteria with

this grass showed that *Beijerinckia* and *Azotobacter* were the predominant bacteria in the rhizosphere, even with plants grown in acid soils (Ruschel and Döbereiner 1965). The use of silica gel plates, containing Winogradsky salts and calcium citrate as a carbon source, inoculated with rhizoplane or rhizosphere soil of *Paspalum notatum*, led to the isolation of a new species of *Azotobacter*, named *Azotobacter paspali* (Döbereiner 1966). This name was later changed to *Azorhizophilus paspali* (Thompson and Skerman 1981). The high frequency of this bacterium in the rhizoplane of *P. notatum* (75 out of 76 samples) and in two of three samples of *P. plicatum*, as well as its complete absence in 81 rhizoplane samples of other plants including 5 species of *Paspalum*, led to the suggestion that the observed specificity could represent an intermediate stage between the legume symbiosis and the non-symbiotic nitrogen fixation (Döbereiner 1970). Additional studies concerning the dependency of the bacteria on the plant showed that the bacteria developed much better on the rhizoplane (Machado and Döbereiner 1969). These authors also showed that root exudates stimulated growth of the bacterial population and that active substances present in these exudates tolerated high temperatures. One of the first evaluations on the BNF contribution of *A. paspali* to the plant was carried out by Donald Kass in 1970, and he detected a gain of 18 kg N/ha/year (Kass et al. 1971). At this time, the acetylene reduction activity (ARA) technique was being used and one addendum in the paper mentioned above indicated nitrogenase activities up to 25 nmols C<sub>2</sub>H<sub>4</sub>/h/g dry root weight of batatais grass harvest in the field (Döbereiner and Campêlo 1971). Other studies confirmed the ARA observed for the cultivar batatais and also showed that it was almost zero for the pensacola cultivar (Döbereiner et al. 1972b). The highest activity was observed in the roots and rhizomes, located lower in the soil and was zero in the aerial parts. In addition, the authors observed that the bacteria formed microcolonies adjacent to roots of cv. batatais but not in the cv. pensacola. Extrapolation of the ARA data to BNF suggested a contribution of up to 50 kg

N/ha/year (Döbereiner et al. 1973). BNF in cv. batatais was confirmed by the  $^{15}\text{N}_2$  technique with the incorporation of  $^{15}\text{N}$  into the plant tissues (De-Polli et al. 1977). Later, Boddey et al. (1983) used the  $^{15}\text{N}$  dilution technique and demonstrated a BNF contribution of 20 kg N/ha/year in plants grown under field conditions.

#### *Derxia spp*

Other nitrogen-fixing bacteria studied by Dr. Johanna Döbereiner's group in the 1960's belonged to the genus *Derxia*, represented by the species *D. gummosa* and *D. indica*. A preliminary study on the occurrence of *D. gummosa* in soils of Rio de Janeiro State, led to the isolation of this bacterium from 20 rhizosphere soil samples collected from different forage grasses (Döbereiner 1968). A more complete evaluation on the occurrence of *Derxia* in 100 soil samples from 4 Brazilian States (SP, RJ, PA and PE), cultivated mainly with grasses, showed its occurrence in 36% of root samples collected in RJ and PA, but not from other States represented mostly by samples from very dry regions (Campêlo and Döbereiner 1970). A unique experiment on the inoculation of *Azotobacter vinelandii*, *Azotobacter paspali*, *Derxia* sp. and *Beijerinckia indica* in *Pennisetum purpureum* plants, grown in greenhouse, was conducted by Souto and Döbereiner (1967). They observed a small but significant increase due to inoculation of the first three bacteria, however these bacteria did not colonize the rhizosphere. On the other hand, *B. indica* significantly increased the dry weight and total N of the plant and also colonized the rhizosphere. Unfortunately, these grass inoculation studies were not continued.

#### *Paenebacillus azotofixans*

Dr. Johanna Döbereiner also made an initial contribution to the study of the nitrogen-fixing bacterium *Paenebacillus azotofixans* (formerly *Bacillus azotofixans*) (Seldin et al. 1984), characterized by Dr. Elisa Gastão da Cunha Penido's group in the Microbial Genetic Laboratory of the Microbiology

Institute, Universidade Federal do Rio de Janeiro. Nowadays, Dr. Lucy Seldin's group is continuing the study of this Gram positive diazotroph. It has been demonstrated that *P. azotofixans* is genetically diverse and that there are a predominance of certain genotypes of *P. azotofixans* in the rhizosphere of grasses such as wheat and sugarcane (Rosado et al. 1998). A "rhizosphere effect" promoted by the wheat rhizosphere was also demonstrated (Rosado et al. 1996). A significant difference among the populations of *P. azotofixans* isolated from rhizoplane, rhizosphere and soil of maize variety commonly cultivated in Brazil has been observed. In addition, it has been shown that the soil type was responsible for this diversity (Seldin et al. 1998). These studies led to the isolation and description of a new species of *Paenibacillus* named *P. brasiliensis* (von der Weid et al. 2002).

### ASSOCIATIVE NITROGEN-FIXING BACTERIA

#### SEMI-SOLID MEDIUM AND DISCOVERY OF *Spirillum*

The studies on associative bacteria began with the use of the acetylene reduction method to measure the capacity of gramineous plants to fix nitrogen in association with diazotrophs. The results showed high nitrogenase activity rates (ARA), however there was no direct relation to the nitrogen-fixing bacteria known at that time. During a talk at the International Conference on the Global Impact of the Applied Microbiology, held in São Paulo, 1973, nitrogen-free semi-solid medium was mentioned for the first time. This medium, containing starch or glycerol as a carbon source and calcium carbonate, when inoculated with small pieces of washed roots from gramineous plants gave rise to the development of an abundant pellicle with high nitrogenase activity. However, it was not possible to isolate and identify the predominant bacteria due to the difficulties in growing the organisms on plates containing nitrogen-free medium, even under low oxygen levels (Döbereiner 1973). During the first International Congress on BNF, held in Pullman, WA, USA, 1974, it was demonstrated that *Spirillum lipoferum*

was the main nitrogen-fixing microorganism associated with the roots of the forage grass *Digitaria decumbens* (Döbereiner and Day 1975). In addition, the authors suggested that this bacterium was responsible for the high ARA rates detected. Changing the carbon source of the above-mentioned semi-solid medium to sodium malate led to the isolation of this microaerophilic bacterium from roots of several gramineous plants. Later studies on the physiology of *S. lipoferum* showed that growth was pH dependent (best at pH 6.8 to 7.8). The optimum growth temperature under nitrogen-fixing conditions was between 32 and 40°C. Although growth was aerobic, this organism was sensitive to oxygen and the best carbon sources were organic acids (Day and Döbereiner 1976). Studies on the localization of *S. lipoferum* in roots of *D. decumbens*, using the tetrazolium reduction technique, showed that the bacteria were mostly found in the inner cortex layer. This led to the suggestion that *S. lipoferum* was an intermediate between bacteria involved in the rhizospheric association and the legume symbiosis (Döbereiner and Day 1975).

#### BNF GRASS POTENTIAL MEASUREMENTS BY ARA

After the discovery mentioned above, studies were carried out to evaluate the BNF potential of several forage grasses. In addition, it was observed that there were a few problems in applying the acetylene reduction technique to samples harvested directly from the field. A lag phase was detected during the measurement of the nitrogenase activity when extracted roots were used but not when the intact soil/plant system was evaluated. The strategy used to solve this problem was to maintain the root samples in flasks containing water and then substitute it with nitrogen gas in the laboratory. The flasks were incubated overnight at a low oxygen level before the injection of 10% of acetylene (Abrantes et al. 1976a). The use of this technique to evaluate the BNF potential of several forage grasses (*Panicum maximum*, *Pennisetum purpureum*, *Brachiaria mutica*, *Digitaria decumbens*, *Cynodon dactylon* and *Melinis minutiflora*) gave rates of 239 to 750 nmols

C<sub>2</sub>H<sub>4</sub>/h/g of roots, which varied with the season and stage of plant development (Day et al. 1975). Soil temperature (Abrantes et al. 1976b) and the level of ammonia in the soil (Neves et al. 1976) also interfered with the nitrogenase activity. Due to the potential of BNF in grasses, the studies were directed towards plants of higher agricultural and economical importance such as cereals. Values of ARA around 10,000 nmols C<sub>2</sub>H<sub>4</sub>/h/g dry roots were detected in maize plants grown in pots containing very wet soils and under high light intensity (Dommergues et al. 1973). At that time, the authors suggested that anaerobic nitrogen-fixing bacteria were the responsible for the activity. In 1975, von Bülow and Döbereiner published a study on the BNF potential of 276 S1 lines of maize. After an ARA pre-screening, 17 lines were tested under field conditions. The best lines continued to show high ARA values (between 2,000 and 7,000 nmols C<sub>2</sub>H<sub>4</sub>/h/g roots) compared to 313 nmols C<sub>2</sub>H<sub>4</sub>/h/g roots in the original cultivar. The authors verified the higher ARA activity during the flowering stage of maize and were able to isolate *S. lipoferum* even after surface sterilization of the roots with different agents (alcohol, chlorinated water, and hydrogen peroxide). They suggested that the association between *S. lipoferum* and the roots was located within the root tissues, since the very high ARA could not be explained by a simple causal association at the rhizosphere level. Based on the differences among genotypes, it was suggested that genetic studies on cereals that are able to associate with diazotrophs should be initiated on a species other than maize since maize breeding programs were mostly directed toward an N fertilizer response. Therefore, the characteristics that favored the association with nitrogen fixing bacteria were eliminated in this plant (Döbereiner 1976).

#### NFb MEDIUM AND DESCRIPTION OF *Azospirillum* GENUS

At the end of 1975, the semi-solid medium developed for the isolation of *Spirillum* species was officially named NFb (N stands for new and Fb for Fábio Pedrosa). This medium was used to study the

occurrence of *S. lipoferum* in several plants grown in tropical and temperate regions of Brazil and the USA (Döbereiner et al. 1976). The results showed that the *S. lipoferum* was very common in soil and in the roots of plants grown in tropical regions (Brazil and several African countries). Forage grasses and cereals always had a large bacterial population. Despite its lower occurrence, *S. lipoferum* was also isolated from plants grown under non-tropical conditions in the USA and Southern Brazil. A soil pH, between 5.5 and 7.0, favored the occurrence of the bacteria (Döbereiner et al. 1976). Preliminary field inoculation experiments carried out in Madison, Wisconsin, USA showed the establishment of the bacteria in the roots of the plants. However, at that time the *S. lipoferum* inoculation practice in tropical regions did not offer great prospects because of the wide distribution of the bacteria in tropical soils.

Advances in physiology and biochemistry suggested that other aspects should be studied. It was suggested that inoculation should only be done in the presence of low doses of nitrogen fertilizers to prevent inhibition/repression of the BNF process and to also lessen the environmental risks caused by the nitrogen fertilizer excess (Döbereiner 1977a). During an International symposium dealing with the application of genetic engineering to the field of nitrogen fixation, several aspects of the plant/bacteria interaction were discussed concerning the possibility of increasing the BNF association in grass plants (Döbereiner 1977b). At the meeting an interesting comment was made by Dr. Döbereiner. She said that the international scientific community did not carefully read the papers published on BNF in grasses therefore leading to a race to find *S. lipoferum* strains and maize lines to be used in agriculture. Although the potential of BNF in grasses was already demonstrated, the studies indicated that the best strategy to maximize BNF was through plant breeding programs (Döbereiner 1977c). Several studies carried out at that time showed that the plant genotype played an important role in the BNF process since there were highly significant differences in the nitrogenase activity observed among maize (von Bülow

and Döbereiner 1975) and wheat cultivars (Nery et al. 1977). A highly significant negative correlation was observed between nitrogenase ( $N_2$ -ase) in the roots and nitrate reductase (NR) in the leaves with maize lines UR-I selected for high and low BNF. The results suggested that breeding programs focusing on BNF should also include studies on NR to select genotypes that gain from both N sources (Baldani et al. 1979).

#### THE ARA METHOD AND ITS ROLE IN THE BNF IN GRASS

Due to its high impact on the study of BNF in grasses, the ARA method was the subject of discussion by the scientific community. The main topic referred to the use of isolated roots to measure the potential of BNF in different cereal genotypes. Multiplication of bacteria during the incubation of roots at low oxygen levels was the main criticism. In contrast to legumes, nitrogenase activity was detected only after incubation of the roots for 10 to 16 hours at low oxygen tension. This lag phase could, however, be reduced when carbonate was added to the roots (Baldani et al. 1978). A positive correlation between the nitrogenase activity produced by pieces of maize and *Digitaria* roots pressed into semi-solid NFb medium and by isolated roots of the same plants was demonstrated by Döbereiner and collaborators (Döbereiner 1978). This suggested that *Spirillum* was the main microorganism responsible for the ARA in these plants. Studies on BNF in 5 tropical forage grasses using isolated roots showed that the methodology underestimated the ARA values in comparison to those detected using the intact soil/plant system, although both techniques showed a significant correlation (Souto and Döbereiner 1984).

#### PHYSIOLOGICAL AND BIOCHEMISTRY STUDIES

Several physiological and biochemistry studies were conducted to gain a better understanding of the role played by *Spirillum lipoferum* in the association with grass plants. These studies confirmed observations that the bacteria were sensitive to high levels of oxy-

gen and that the optimal pH for growth was between 6.8 and 7.0 and the optimal incubation temperature was between 32 and 36°C. In addition, it was found that organic acids were the preferred carbon sources. Therefore, malic acid was the carbon source incorporated in the semi-solid NFB medium used to grow and isolate *Spirillum lipoferum* (Neyra and Döbereiner 1977). Another characteristic of the bacteria discovered at that time was the ability of some strains to participate in several steps of the nitrogen cycle in nature. The most surprising aspect was the ability to carry out denitrification since nitrogen-fixing bacteria were not known to carry out this process at that time (Neyra et al. 1977). Based on physiological and biochemical characteristics, three groups of bacteria were identified: Group I – no biotin requirement and the ability to use glucose as carbon source for growth and nitrogen fixation; Group II – a biotin requirement and glucose utilization; Group III – similar to Group I, except that it was able to reduce  $\text{NO}_2^-$  to  $\text{N}_2$  (Sampaio et al. 1978).

*A. lipoferum* and *A. brasilense* species – Later studies, based on DNA:DNA homology, led to the creation of a new genus called *Azospirillum* with two species: *Azospirillum lipoferum* (synon. of *Spirillum lipoferum*) and *Azospirillum brasilense* (Tarrand et al. 1978). Serological studies showed that fluorescent antibodies could be used to differentiate the two species, however 3 subgroups were formed within *A. brasilense* (De-Polli et al. 1980).

*A. amazonense* species – A new species of *Azospirillum*, named *A. amazonense*, was later described (Magalhães et al. 1983). The species was isolated from forage grasses grown in the Amazon and in the State of Rio de Janeiro and later from rice, maize and sorghum plants grown in Seropédica, Rio de Janeiro (Baldani 1984). Its broad ecological distribution was later confirmed through the detection of large numbers of the bacteria in other grasses such as sugarcane (Baldani et al. 1999). The main characteristics that differentiate it from other species are the ability to use sucrose as carbon source, a smaller cell diameter and an inability to tolerate alkaline pHs (Magalhães et al. 1983).

Other *Azospirillum* species – During the last two decades, other species of *Azospirillum* have been described: *A. halopraeferens* associated with Kallar grass grown in saline soils of Pakistan (Reinhold et al. 1987), *A. irakense* associated with rice plants grown in Iraq (Khammas et al. 1989), *A. largimobile* (the original name *largomobile* was orthographically incorrect) isolated from a water sample collected from a lake in Australia (Dekhil et al. 1997) and *A. doebereineriae* associated with *Pennisetum* plants grown in Germany (Eckert et al. 2001). Very few studies have been carried out with these *Azospirillum* species, except for *A. irakense* where pectinolytic activity was observed (Vande Broek and Vanderleyden 1995).

#### PHYSIOLOGICAL AND BIOCHEMICAL STUDIES

Evaluation of the nitrogen fixation mechanisms in these *Azospirillum* species showed that inhibition of nitrogenase activity by nitrate was dependent on the reduction of nitrate (Magalhães et al. 1978). In addition, the authors showed that under aerobic conditions, where nitrogenase is inhibited by oxygen, nitrate could be used as a nitrogen source for growth. An elegant schema presented by Döbereiner (1979) illustrates how oxygen and mineral nitrogen sources interfere with the BNF process in *Azospirillum*. It was observed that when the oxygen supply to the bacterial site exceeds its consumption,  $\text{NH}_3$ ,  $\text{NO}_3$  and  $\text{NO}_2$  are assimilated at maximal rates, but there is no BNF. In contrast, when the consumption of oxygen corresponds exactly to the amount transported to the site, the conditions are optimum for nitrogenase synthesis by the bacteria and in the case where mineral N sources ( $\text{NH}_3$ ,  $\text{NO}_3$  and  $\text{NO}_2$ ) are not available, atmospheric  $\text{N}_2$  is used as nitrogen source. If oxygen is removed, respiration is interrupted, and ATP is not generated. This, in turn, results in the cessation of nitrogen fixation. On the other hand, nitrate can be substituted by oxygen. In this case, the product of respiration (nitrite) is excluded from the cells. Nitrogen fixation is not inhibited and the process becomes dependent on nitrate as demonstrated by Scott et al. (1979). A



study on the inorganic N transformation processes in presence of *Azospirillum*, showed that the bacteria participate in all steps except in the nitrification process (Bothe et al. 1981). The authors verified that nitrogenase activity dependent on nitrate occurs only during 3 to 4 hours until the assimilatory enzymes involved in the reduction of nitrate are synthesized. During this period, nitrite accumulates and the nitrogenase is inhibited when the concentration reaches about 1 mM. Additional studies on the tolerance of *Azospirillum* to oxygen carried out in a fermenter, showed that the level of tolerance is dependent on the age of the culture, optical density and rate of shaking (Volpon et al. 1981). Oxygen tolerance is greater in *A. lipoferum* and occurred when the concentration of lactate and glucose in the medium decreases to less than 0.5% (Stephan et al. 1981, Volpon et al. 1981). More information on the physiology and biochemistry of these 3 species of *Azospirillum* can be found in a book written by Döbereiner and Pedrosa (1987).

#### ECOLOGICAL AND COLONIZATION STUDIES

Several studies were carried out on the colonization process and the establishment of *Azospirillum* in different grasses. One of the first studies made use of the tetrazolium reduction method (Patriquin and Döbereiner 1978). The authors observed bacterial colonization of the cortex tissues and the inner central region of maize roots and other forage grasses. Further studies, using maize plants grown in the field confirmed the endorhizospheric nature of the association with bacteria present in the central cylinder of the roots as well as in the xylem vessels in colm nodal regions (Magalhães et al. 1979). The highest frequency of colonization occurred during the grain filling stage ( $10^5$  to  $10^7$  cells/g root tissue) when the nitrogenase activity is usually much higher. Although this methodology and the results have been subjected to criticism, the intercellular colonization of maize and wheat plants by *Azospirillum* has been confirmed more recently by molecular techniques (Assmus et al. 1995).

A survey on the occurrence of the *Azospirillum*

species known up to 1980, showed that there was a certain host plant specificity in the colonization of  $C_3$  and  $C_4$  plants by *Azospirillum* (Baldani and Döbereiner 1980). The authors observed that maize plants were preferentially colonized by *A. lipoferum* while wheat and rice were colonized by *A. brasilense*. These results were later confirmed for other forage grasses with  $C_3$  and  $C_4$  photosynthetic pathways and in addition showed a higher occurrence of denitrifying strains colonizing the interior of roots (Baldani et al. 1981). Another interesting characteristic of the *Azospirillum* that colonized the interior of roots of cereals was the high frequency of isolates with a tolerance for up to 20 ppm of streptomycin as compared to strains isolated from the soil or rhizosphere (Döbereiner and Baldani 1979). This discovery led to a suggestion that the plants developed a new mechanism to select root-colonizing bacteria (Döbereiner 1979). Further studies showed that liming stimulated the production of streptomycin in the rhizoplane of several gramineous plants (Baldani et al. 1982).

In the International Workshop on Associative  $N_2$ -Fixation, held in 1979, in Piracicaba, SP, Brazil a new term "diazotrophic biocoenosis" was introduced to describe the association of plants of the family *Gramineae* (renamed *Poaceae*) with nitrogen-fixing bacteria (De-Polli and Döbereiner 1980). Three more specific terms were also considered; "rhizocoenosis" (roots), "caulocoenosis" (stems) and "phylocoenosis" (leaves). However, these new terms were not used by the scientists working in this area, therefore the term "associative" continued to be used to describe the association of diazotrophic bacteria, mainly *Azospirillum*, with non-leguminous plants. Later 1992a, Döbereiner introduced (as discussed below) the term endophyte, to define bacteria able to colonize internal plant tissues. A group coordinated by Dr. Fátima Moreira (Universidade Federal de Lavras, UFLA, Minas Gerais) detected a large population of this species in plants of the Orchidaceae family as well as in species of other plants (Lange and Moreira 2002). Further studies carried out by her group on the ecology of *Azospirillum* in

heavy metal contaminated area as well as in bauxite mining reclamation area showed that the population of *Azospirillum* was similar to the one detected in non-contaminated agricultural ecosystems, but was drastically reduced in the bauxite mining area. On the other hand, the use of several gramineous species in this area promoted a qualitative and quantitative increase in diazotrophic bacteria populations with values higher than those observed for reference areas (Melloni et al. 2004). The analysis of the nitrogen-fixing bacteria present in these areas showed a high diversity of the diazotrophs including *Azospirillum* and *Herbaspirillum* species as well as other unidentified ones (Nóbrega et al. 2004).

#### INOCULATION RESPONSES

The advances generated in the 70's led several researchers to evaluate the effect of *Azospirillum* inoculation on gramineous plants. The results although inconsistent, indicated a potential contribution by the BNF process of around 40% of the nitrogen requirement based on observations by groups in Brazil and Israel (Boddey and Döbereiner 1982). However, the debate about the principal role played by bacteria in association with plants still remained. Several authors as summarized by Patriquin et al. (1983) demonstrated hormonal effects, biological nitrogen fixation and interference in other processes of the nitrogen assimilation.

Field wheat inoculation experiments with *Azospirillum* strains isolated from sterilized roots of wheat (Sp 245, Sp 107 st), showed a consistent increase in total plant N however, this was not the case for the heterologous strain Sp 7 (Baldani et al. 1983). High correlation ( $r=0.92$ ) was observed between N accumulation and the number of *Azospirillum* present in sterilized roots but there was not a significant correlation with the number of *Azospirillum* detected in washed roots. A positive inoculation effect on maize plants was observed when homologous strains of *Azospirillum* were compared with strains isolated from other plants (Freitas et al. 1982). At this time, studies were initiated to evaluate the location of different strains of *Azospirillum* (ho-

mologous and heterologous) in wheat and sorghum plants grown in the field (Baldani et al. 1986b). The authors observed that homologous strains were preferentially located in the interior of roots of wheat plants (Sp245 and Sp107) and sorghum (Sp S82) while the heterologous strains (Sp 7 and Cd) were found on the root surface.

Other studies confirmed the inoculation effect of *Azospirillum* strains on wheat plants grown in the field. It was demonstrated that the observed effect, mainly with homologous strain Sp 245, was not due to BNF but to an increase in the nitrate reductase (NR) activity of the bacteria in the roots (Boddey et al. 1986). The role of bacterial NR in the plant N metabolism was confirmed by inoculating wheat plants grown under gnotobiotic conditions with nitrate reductase negative mutants (Ferreira et al. 1987). Besides BNF and its role in the plant metabolism, inoculation with *Azospirillum* can stimulate plant growth through production of auxins, gibberellins and cytokinins (Hartmann and Zimmer 1994). Some countries, not including Brazil, are commercially producing inoculants based on *Azospirillum* (Baldani et al. 1999). However, practical application still represents an incognito due to inconsistent results.

#### GENETICS STUDIES

Reviews published in the last decade have made a lot of advances in the physiology, biochemistry and genetics of the *Azospirillum* species. In addition, the role of these bacteria in the interaction with gramineae plants and other non-legume plants has been confirmed (Vande Broek and Vanderleyden 1995, Bashan and Holguin 1997, Steenhoudt and Vanderleyden 2000). In Brazil, studies on the organization and regulation of the *nif* genes in the BNF process of the genus *Azospirillum* have been conducted by groups led by Dr. Fábio de Oliveira Pedrosa, Universidade Federal do Paraná (UFPR) and Dr. Irene Schrank, Universidade Federal do Rio Grande do Sul (UFRGS).

Dr. Pedrosa's group played an important role in research involving the regulation of BNF in

*Azospirillum brasilense*. They isolated the first mutants with mutations in the regulatory genes *nifA* and *ntrC* that code for the transcription activating proteins NifA and NtrC (Pedrosa and Yates 1984). These mutants aided the isolation of the *ntrBntrC* operon of *A. brasilense* by genetic complementation of *nifA*, *nifB* genes (Knopik et al. 1991, Machado et al. 1995). This work led to an intensified effort at the molecular level in order to understand the structural organization and regulation of the *nif* genes of this endophytic diazotrophic bacterium. The work on *Azospirillum brasilense* was initially centered on the isolation and characterization of regulatory mutants able to constitutively fix nitrogen in presence of  $\text{NH}_4^+$  (Machado et al. 1991). These mutants were also able to excrete ammonia, the product of the nitrogen fixation process (Machado et al. 1991, Victorino et al. 2001). The group has also dedicated considerable effort on studying the regulation of the nitrogen fixation process in *A. brasilense*. This regulation did not depend on the transcription activating proteins NtrC, NifA and the Sigma RNA polymerize factor,  $\sigma^N$ . The *nifA* promoter, which does not show typical structural motifs of the promoters of promoter dependent proteins, was characterized as a typical  $\sigma^{70}$  promoter. Studies on chromosomal *nifA::lacZ* fusions and plasmid borne fusions showed that *nifA* expression of *A. brasilense* is repressed by ammonium, the principal effector. Low oxygen tension activates while high oxygen tension represses. Other results showed that the repression of *nifA* expression reaches its maximum level as a result of a synergistic effect between ammonium and oxygen (Fadel-Picheth et al. 1999).

Dr. Fabio's group recently described NtrX NtrY as a potential regulator involved in nitrate metabolism in *A. brasilense*, however it does not participate in regulation of nitrogen fixation (Ishida et al. 2002). The role of GlnZ in the reactivation of dinitrogenase reductase under ammonium-limiting conditions as well as the requirement of the PII protein to turn the enzyme off were also important to understanding the control of nitrogenase activity in *A. brasilense* (Klassen et al. 2001). In

addition, the group determined the genomic structure using pulse-field eletrophoresis of the *Azospirillum* genus (Martin-Didonet et al. 2000) and also constructed gene fusions using *gfp* and *gusA* reporter genes to monitor the plant/bacteria interaction (Ramos et al. 2002).

The studies coordinated by Dr. Schrank's group, Universidade Federal do Rio Grande do Sul/UFRGS, initially concentrated on the genes that encode the nitrogenase subunits in the *A. brasilense* species (Schrank et al. 1987). The nitrogenase structural gene operon was completely sequenced and the gene organization was found to be *nifHD-Korf1Y* (Passaglia et al. 1991). Furthermore, the operons *orf2nifusvorf4* (Frazzon and Schrank 1998), *nifENXorf3orf5fdxAnifQ* (Potrich et al. 2001a) and *fixABCX* (Irene Schrank, personal communication) were characterized. The group also studied the regulation of the *nif* operon in *A. brasilense*. The sequence of the structural gene operon showed the presence of two overlapping UASs, a unique characteristic among all of the *nif* operons so far analyzed. *In vivo* assays showed that one UAS (UAS2) has higher activity than UAS1 and that NifA binds to the two muted promoters (Passaglia et al. 1995). Antibodies specific for *A. brasilense* NifA were also produced (Passaglia et al. 1998). It was demonstrated that the binding activity of the purified NifA protein is not altered even when it is inactivated (presence of oxygen) and it is also able to bind to the *nifH* gene promoter when the UASs are mutated (Passaglia et al. 1995). Two mutants with higher nitrogen fixation activity than the wild type were obtained during selection of the *nif* genes (Araújo et al. 1988). One mutant was characterized and it was shown that the gene (ORF280) containing the mutation encodes a predicted gene product that is homologous to proteins involved in stress (Revers et al. 2000). More recently Dr. Schrank's group initiated studies with *A. amazonense* to determine similarities and difference between this species and *A. brasilense*. Using PCR primers designed for *nif* or *fix* gene sequences of *A. brasilense*, products from *A. amazonense* ge-

nomic DNA were isolated and sequenced from the *nifHDK*, *nifUSV*, *nifENX* and *fixABC* operons. Southern blot hybridization was also carried out using different operon probes against the genome of *A. amazonense*. These results suggest that the *nif/fix* operons are present in *A. amazonense*, however they exhibited different patterns of restriction enzyme cleavage. The nitrogenase structural genes, which are conserved among different bacterial genera, are also different in *A. brasilense* and *A. amazonense*. A conserved region was found in the *nifD* gene, but the intergenic region of *nifH-D* and the promoter region of *nifH* were quite different. Also, genes involved in the regulation of nitrogen fixation such as those that encode NifA and PII protein were isolated and partially sequenced (Potrich et al. 2001b).

#### ENDOPHYTIC BACTERIA

##### ENDOPHYTIC DEFINITION

The term endophyte was first introduced to the area of nitrogen fixation research associated with Graminaceous plants by Döbereiner (1992a, b). The term was defined by De Bary (1866 cited by Stone 1986) and refers to mycotic flora that inhabits the interior of plant tissues. The term was then applied to bacteria and was the subject of several conceptual definitions. In general, the term includes all microorganisms that are able to colonize, during some portion of their life cycle, the inner tissues of plants without causing any apparent damage to the host (Petrini 1991). *Herbaspirillum*, *Glucanacetobacter* and *Burkholderia* are the three endophytic nitrogen-fixing bacteria studied by the Brazilian groups presented below.

##### *Herbaspirillum seropedicae*

The first nitrogen-fixing bacterium with endophytic characteristics was isolated in 1984 from the rhizosphere, washed roots and surface sterilized roots of maize, sorghum and rice plants and was first named *Azospirillum seropedicae* (Baldani et al. 1984). Although this group of bacteria showed several mor-

phological and physiological characteristics similar to the genus *Azospirillum*, DNA: DNA homology studies showed that they formed a new genus named *Herbaspirillum*, thus *Azospirillum seropedicae* was renamed *Herbaspirillum seropedicae* (Baldani et al. 1986a).

##### GENETICS STUDIES

Not much progress was made until the beginning of the 90's when Dr. Fábio Pedrosa's group focused their attention on the organization and regulation of the *nif* genes in this species. They isolated the *nifA*, and *nifB* genes along with the *ntrBntrC* operon of *H. seropedicae* by genetic complementation of *A. brasilense* Nif minus mutants (Souza et al. 1991a, b, Pedrosa et al. 1997). The nitrogenase structural genes, other *nif* genes, and genes involved in the regulation of the nitrogen fixation of *H. seropedicae* were sequenced. The *nif* genes were found in two regions; region I contains the genes contiguous with *nifA* and *nifB*, while region II contains the *nifHDKENXorf1orf2orf3* operon (Machado et al. 1996, Klassen et al. 1999, Pedrosa et al. 2001). The *nifQmodABC* and *fixXC* genes are located 3 kbp upstream (Klassen et al. 1999). The group studied the motif functions present in the promoter region of *nifA* in detail. Structurally, this region shows a very high complexity. It has two potential NtrC-binding sites, three NifA sites and one IHF (integration host factor) – binding site localized upstream of an  $\sigma^N$  type promoter (–24/–12) (Souza et al. 1991b, Wasseem et al. 2000, 2002). In summarizing, the studies showed that NtrC and RpoN are essential for expression from the *nifA* promoter and that IHF positively modulates activation by NtrC and acts negatively for activation by NifA (Souza et al. 1991a, b, 1999, 2000, Wasseem et al. 2000). *In vivo* and *in vitro* studies of the function and structure of the *H. seropedicae* NifA protein showed that this transcription activator responds to external signals such as  $\text{NH}_4^+$  and  $\text{O}_2$ . The N-terminal region of NifA is involved in auto regulation in response to the negative regulator  $\text{NH}_4^+$  in concert with PII which is essential for activation of NifA in

absence of  $\text{NH}_4^+$  (Monteiro et al. 1999a, b). Dr. Pedrosa's group also demonstrated that the NifA protein of *H. seropedicae*, similar to those from the  $\alpha$ -*Proteobacteria* (including *A. brasilense*), is sensitive to oxygen. Besides, it requires Fe for *in vivo* activity and the interdomain region between the C-terminal domain and the central domain is involved in oxygen sensitivity (Monteiro et al. 1999a, Souza et al. 1999). In addition, Dr. Pedrosa's group isolated and characterized the *glnB* gene of *H. seropedicae* and determined the three-dimensional structure of the purified protein product, a transducer protein of the PII signal. This structure is similar to that of the GlnK of enterobacteriaceae and it has a role in the control of NifA activity (Benelli et al. 1997, 2001, 2002). The genes *glnAntrBntrC* were also sequenced by the group (GenBank accession number AF0828730) and constitutes a unique operon where transcription is initiated from two promoter sequences. One promoter is sN dependent and the other is  $\sigma^{70}$  dependent and, both promoters are located upstream of *glnA* (Persuhn et al. 2000). Two genes involved in the SOS repair system, *recA* and *recX* of *H. seropedicae* were isolated, sequenced and their functions are being determined (Steffens et al. 1993).

#### GENOMIC STUDIES

The sequence and annotation of the *H. seropedicae* genome has almost been completed by the GENOPAR Consortium of Parana State, coordinated by Dr. Pedrosa. The consortium involves a net of 16 laboratories from Paraná, three from Santa Catarina, two from Rio de Janeiro and one from Rio Grande do Sul. The objective of the project is to gain an understanding of the overall metabolic pathways of *H. seropedicae* with a view towards increasing the efficiency of the plant/bacteria association.

#### *H. rubrisubalbicans*

Also in the 90's, a bacterium responsible for the mottled stripe disease in sugarcane was isolated. This strain belongs to the species *Pseudomonas*

*rubrisubalbicans* and shows many characteristics similar to those of the genus *Herbaspirillum* (Döbereiner et al. 1990, Gillis et al. 1991). It was shown that it has the ability to fix atmospheric nitrogen and that it could also be reisolated from sugarcane leaves 60 days after artificial inoculation of the leaves (Pimentel et al. 1991). Additional physiological studies showed that this bacterium has some characteristics that are different from those of *H. seropedicae* (Baldani et al. 1992a). Based on this information, a complete study on this group of bacteria was carried out and culminated with the description of a new species named *H. rubrisubalbicans* (Baldani et al. 1996). The genus has recently been expanded with the description of a new species named *H. frisingense*, isolated from roots and leaves of forage grasses grown in Germany and Brazil (Kirchhof et al. 2001). A survey on the occurrence of this genus showed that the species *H. seropedicae* is found associated with several non-leguminous plants but not in soil in the absence of plants (Olivares et al. 1996). *H. rubrisubalbicans* has been isolated from many Brazilian sugarcane varieties with no symptoms of the mottled stripe disease (Döbereiner 1992b), however its ecological distribution is still unknown. Bacteria belonging to the genus *Herbaspirillum* were detected in wild and commercial rice varieties cultivated in Japan, however no relation with known species of *Herbaspirillum* could be found (Elbeltagy et al. 2001).

One aspect that was a strong consideration for inclusion of this genus in the endophytic group is its lower survival in soil (Baldani et al. 1992a). Olivares et al. (1996) verified the observation that the recovery of *H. seropedicae* and *H. rubrisubalbicans* strains inoculated into soil only occurred in the presence of the host plant. This was probably due to the release of growth promoter substances in the rhizosphere by the plant. However, Arcanjo et al. (2000) were unable to reisolate strains of *Herbaspirillum* from soil samples collected within sugarcane fields even when sugarcane micropropagated plants were used as the trapping host. Similarly, no *Herbaspirillum* strains could be isolated and analyzed us-

ing the Immunocapture technique from soil samples collected between rows of sugarcane in the field (Santos et al., unpublished data).

#### COLONIZATION AND INFECTION STUDIES

The interaction of *H. seropedicae* and *H. rubrisubalbicans* strains with plants has been extensively studied through the inoculation of sugarcane and rice plants grown under sterile conditions followed by microscopic analysis. In rice plants, *H. seropedicae* has been found to colonize the intercellular spaces near the tip of young roots. Apparently, the bacteria move intercellularly to the cortex region, since the cortical cells are where large numbers of bacteria have been detected (Baldani et al. 1992b). More recent results, from studies with aluminum-tolerant rice cultivars grown under axenic and field conditions, confirmed observations that *H. seropedicae* colonizes the interior of roots and the aerial part of the plant (Gyaneshwar et al. 2002). Colm of sorghum plants artificially inoculated with *H. rubrisubalbicans* showed slight symptoms of the "red stripe" disease in the leaves suggesting an active translocation of the bacteria (Döbereiner et al. 1994). In contrast, no symptoms were observed with the inoculation of *H. seropedicae* strains. Further studies showed a very high rate of infection of the xylem vessels and the formation of a structure originating from the plant involving grumes of bacteria (James and Olivares 1998). In the case of sugarcane, it was observed that the bacteria enter the plant near the region of root emergence and invade the vascular tissues by colonizing the parenchymatic cells and metaxylem vessels (Olivares et al. 1995).

Most of the microscopic researches carried out in Brazil with these endophytic diazotrophic bacteria are now coordinated by Dr. Fábio Lopes Olivares (UENF University, Rio de Janeiro State). Besides the studies involving endophytic establishment in sugarcane by *Herbaspirillum* spp. using conventional microscopy (James et al. 1997, Olivares et al. 1997), cryotechnique approaches have opening insights for new ultrastructural aspects of the bacteria in free state and under endophytic interac-

tion (Silva et al. 2003). Using cryofracture technique it was possible to conclude that the amount and the polymeric composition of the PHB granules changes depending on the physiological condition of the bacteria growing in the medium, based mainly on the status of Nitrogen and Carbon. Under endophytic interaction viewed by TEM after high pressure freezing followed by freezing substitution, *Herbaspirillum* spp. infected leaves as microcolonies into the lumen of the metaxylem vessel and as single cell/micro-colony at apoplast. In roots, *H. seropedicae* colonized cortex apoplast, adhered to plant cell wall (PCW), such as xylem vessels and inside apparently dead vascular parenchyma cells. Plant roots inoculated with the bacteria showed the bacteria associated with the intercellular root cortex. Cryotechniques were able to reveal altered aspects of all bacteria and PCW at adhesion sites and bacterial protrusions could be observed too. With great frequency, the bacteria were observed involved by abundant unknowing electron-dense material and many of vesicular structures coming from plant cell wall were also observed. All substances produced by PCW are clearly stimulated in response to bacterial presence. These bacterial compounds could stimulate the plant cell not only in relation to N<sub>2</sub>-fixing process but also in process involved other plant-growth promotion process (Silva et al. 2004).

#### INOCULATION RESPONSE

The potential of cereal plants to respond to inoculation with *H. seropedicae* strains has been evaluated by many experiments in the last 15 years. The initial results showed no positive response for sorghum (Pereira et al. 1988) and maize (Pereira and Baldani 1995). In the case of rice, inoculation promoted a yield increase equivalent to the treatment with 40 kg of N/ha (Pereira and Baldani 1995). However, large advances have been made in the last few years through a highly detailed study based on selection of *Herbaspirillum* strains grown in association with rice plants grown under gnotobiotic conditions (Baldani et al. 2000). It was observed that the response of the rice variety to inoculation

was dependent on the strain used. In some cases, highly significant increases in phytomass accumulation were observed with the inoculation of some strains, whilst the opposite was observed for others (Baldani et al. 2000). Evaluation of these strains under greenhouse and field conditions also showed significant increases in yield, however this increase depended on the rice cultivar tested (Guimarães et al. 2000).

#### *Gluconacetobacter diazotrophicus*

The other diazotrophic bacterium with endophytic characteristics and object of intense research is *Gluconacetobacter diazotrophicus* (synon. *Acetobacter diazotrophicus*). The search for this bacterium was initiated with the observation that some sugarcane varieties could obtain around 60% of their nitrogen through biological nitrogen fixation (Lima et al. 1987). Initially, different semi-solid media were used. However, the medium containing ¼ of LGI salt supplemented with 250 mL of sugarcane juice and 25 g of crystal sugar was chosen because *G. diazotrophicus* formed a very thick yellow pellicle on the surface of the medium. This medium was suitable for the detection of bacteria in root and colm samples up to 10<sup>7</sup> dilution (Cavalcante and Döbereiner 1988). Through knowledge acquired on the physiology of this organism, the authors modified the semi-solid medium, now named LGIP, and established as a rule the use of 10% crystal sugar with the pH adjusted to 5.5 using acetic acid. The medium was again modified by Reis et al. (1994) with the addition of sugarcane juice in the concentration of 0.5%.

Based on morphological, physiological and biochemical characteristics of this group of bacteria, a new genus named *Saccharobacter* was created with a new species called *S. nitrocaptans* (Cavalcante and Döbereiner 1988). However, DNA:DNA and rRNA hybridization analysis showed that this group of bacteria belonged to an rRNA branch of *Acetobacter* with many characteristics similar to *A. liquefaciens*. Therefore, it was included in the

genus *Acetobacter* with the creation of the species *Acetobacter diazotrophicus*, a unique nitrogen-fixing bacterium of this genus (Gillis et al. 1989). Recently, this species was renamed *Gluconacetobacter diazotrophicus* based on the 16S rDNA sequence and the predominant type of ubiquinone produced (Yamada et al. 1997, 1998). Recently, two new nitrogen-fixing species of this genus were described: *G. azotocaptans* and *G. johannae*, with the last one in honor of Johanna Döbereiner (Fuentes-Ramírez et al. 2001).

#### ECOLOGICAL STUDIES

*G. diazotrophicus* was initially isolated from roots and colms of sugarcane varieties grown in the Northeast (PE and AL) and Southeast (SP, MG) of Brazil (Cavalcante and Döbereiner 1988). It could not be detected in soil samples collected between sugarcane rows or in roots of 12 weed plants grown in sugarcane fields nor in grain of saccharine sorghum. However, it could be isolated from washed roots and aerial tissues of *Pennisetum purpureum* cv. cameroon plants (Döbereiner et al. 1988). Its occurrence was confirmed in other Brazilian sugarcane areas (Döbereiner et al. 1990) as well as in other countries that produce sugarcane such as Australia and USA (Baldani et al. 2002a).

Until quite recently, it was thought that *G. diazotrophicus* could only occur in plants that propagate vegetatively such as sugarcane, sweet potato (Paula et al. 1990, Döbereiner et al. 1993) and pineapple (Tapia-Hernandez et al. 2000). However, it was detected in plants propagated by seeds such as the grass *Eleusine coracana* (Loganathan et al. 1999) and in coffee (Fuentes-Ramírez et al. 2001), although these observations refer to the new species mentioned above, *G. johannae* and *G. azotocaptans*. *G. diazotrophicus* has already been detected in mealybugs that inhabit sugarcane fields (Ashbolt and Inkerman 1990) and in mycorrhiza spore fungi collected from sweet potato roots (Paula et al. 1991). Genetic diversity studies conducted on *G. diazotrophicus* strains isolated from sugarcane plants cultivated in Brazil and Mexico showed that diversity

is very limited (Caballero-Mellado and Martinez-Romero 1994). Studies from our laboratory with 45 strains isolated from sugarcane varieties, maintained for 30 years in a germoplasma bank and analyzed by the ITS/RFLP technique, also confirmed this low genetic diversity (Santos et al. 1999).

Other studies using ELISA, Immunocapture and PCR techniques have been conducted in an effort to detect *G. diazotrophicus* in soil samples and plant tissues. Using the ELISA technique, large populations of *G. diazotrophicus* were detected in different plant tissues of sugarcane varieties cultivated in Brazil and Australia (Boddey et al. 2000). In the case of the Immunocapture technique it was possible to detect *G. diazotrophicus* in plant tissues but not in soil collected between rows of sugarcane plants grown in the field (Santos et al., unpublished data). With the PCR technique, fragments of the same size as those from *G. diazotrophicus* genomic DNA were detected in soil samples collected in a sugarcane field. However, *G. diazotrophicus* could not be reisolated from micropropagated sugarcane plants used as a trapping host (Arcanjo et al. 2000). These results suggest that the bacteria could be in a viable but non cultivable stage or that the number of the bacteria in the soil was too low to colonize the roots of the plants.

#### PHYSIOLOGICAL AND BIOCHEMICAL STUDIES

The physiological and biochemical studies carried out during the last 15 years on *G. diazotrophicus* showed that this bacterium is able to grow diazotrophically using different carbon sources in a culture medium with a pH much lower than that used for most of the diazotrophic bacteria (Cavalcante and Döbereiner 1988). *G. diazotrophicus* is able to grow at pH 3.0 and fixes nitrogen in culture medium at pH 2.5 (Stephan et al. 1991). This bacterium does not use sucrose directly – it has an extracellular enzyme (invertase) with saccharolytic activity. This carbon source is a key compound during the isolation of the bacteria since the LGIP semi-solid medium contains 10% sucrose (crystal sugar) which is known to inhibit the growth of other diazotrophs.

The optimum pH for its growth is around 5.5 and one interesting characteristic is the absence of the nitrate reductase enzyme (Cavalcante and Döbereiner 1988). Nitrogenase activity is not inhibited or suppressed by high concentrations (25 mM) of nitrate (Stephan et al. 1991), but is partially inhibited by  $\text{NH}_4^+$  (Teixeira et al. 1987). Reis and Döbereiner (1998) demonstrated that the nitrogenase activity is less inhibited by  $\text{NH}_4\text{Cl}$  (5 mM) when the bacteria are grown in media containing 10% sucrose as compared to 1%. Studies involving a mixed culture of *G. diazotrophicus* and amylolytic yeast showed that *G. diazotrophicus* is able to support growth of the yeast with about 40% of the fixed nitrogen being used by this organism (Cojho et al. 1993). The form of nitrogen excreted by *G. diazotrophicus* during the biological nitrogen fixation process is currently unknown (Baldani et al. 1997a).

#### PLANT DEPENDENCE

The dependence of the *G. diazotrophicus* on plants, the majority of which propagate vegetatively (setts-sugarcane and *Pennisetum*, stem cutting-sweet potato), emphasizes its endophytic life style and suggests that this is the major way of dissemination of this organism. Paula et al. (1991) demonstrated that micropropagated sugarcane and sweet potato showed high colonization of the aerial part by *G. diazotrophicus* when co-inoculated with mycorrhizal fungi. Thermal treatment of sugarcane setts (30 minutes at 52°C), applied to eliminate phytopathogenic bacteria responsible for the ratoon standing disease, did not eliminate the endophyte (Reis et al. 1994), confirming that the setts are one way of dissemination of the bacteria. In addition, it has been observed that the bacteria have a very low rate of survival in soil (Baldani et al. 1997a), although it has been demonstrated that the rate of cell death depends on the humidity at the time of inoculation into the soil (Oliveira et al. 2004). It is already known that the bacterial population is also influenced by the nutritional stage of the sugarcane plants (Reis-Jr et al. 2000) and that nitrogen fertilization also decreases the population of *G. diazotrophicus*.



associated with the sugarcane (Fuentes-Ramírez et al. 1999).

#### GENETICS STUDIES

In the 90's, many genes involved in nitrogen fixation by *G. diazotrophicus* were cloned and sequenced (Sevilla et al. 1997, Teixeira et al. 1999). The organization of the *nif*, *fix* and *mop* genes is known, however the mechanisms involved in their regulation still needs further study (Lee et al. 2000). Because of its endophytic life style, this bacterium has been used as a vector to express heterologous genes of interest. One example is the expression of *cry3A* and *cry1Ab* genes isolated from *Bacillus thuringiensis* that are responsible for the entomopathogenic activity against coleopteran and lepidopteran insects that cause damage to sugarcane plants (Salles et al. 2000, Baldani et al. 2002b).

#### INOCULATION RESPONSE

One way to introduce *G. diazotrophicus* into sugarcane plants is during the micropropagation process since the setts are naturally colonized by this bacterium. The micropropagation process eliminates both the phytopathogenic and the nitrogen-fixing bacteria. Therefore, a method to introduce selected endophytic diazotrophic strains during the acclimatization process was developed (Reis et al. 1999). The bacteria are able to infect and colonize the root tissues and aerial parts by penetrating root tip and lateral roots formed during the rooting process (James et al. 1994). At the colm base, where the tissues are heavily colonized, the bacteria move to the aerial part using the xylematic vessels (James et al. 1994, 2001). Relatively large populations of the bacteria are found in these tissues, suggesting that this is one of the main sites for nitrogen fixation due to the low oxygen level and the energy available in form of sucrose for nitrogen fixation (James et al. 1994).

Few inoculation studies have been done to evaluate the potential of *G. diazotrophicus* strains to fix nitrogen in association with sugarcane with contributions to the plant's nitrogen metabolism, because sugarcane plants are mostly propagated by

setts. Nevertheless, inoculation of micropropagated sugarcane plants with the *G. diazotrophicus* strain PAL5 increased the aerial fresh weight of the plant by 28% (Baldani et al. 1999). Similar results were obtained when the inoculation of the strain PAL5 was done in the presence of low amounts of nitrogen fertilizer (Moraes and Taub-Tornisielo 1997). Proof that *G. diazotrophicus* fixes nitrogen was demonstrated by inoculation of micropropagated sugarcane plants with wild-type strain PAL5 and its *nif* minus mutant followed by measurement of  $^{15}\text{N}_2$  gas incorporated into the plant tissues (Sevilla et al. 2001). Recent results have shown that sugarcane derives benefit from inoculation with a mixture of diazotrophic bacteria (Oliveira et al. 2002). This result was also obtained when the inoculation process is associated with mycorrhizae (Muthukumarasamy et al. 1999). Other effects of *G. diazotrophicus* inoculation are related to phyto-hormone production (indol-acetic acid) which acts on initial root development which in turn benefits the whole plant as demonstrated by Fuentes-Ramírez et al. (1993). Other studies of co-inoculation involving *G. diazotrophicus* and mycorrhizal fungi showed an increment in the root system of sweet potato plants as well as the uptake of some nutrients (Paula et al. 1992). The BNF contribution to sugarcane plants grown in the field for 18 months after inoculation at the micropropagated stage with a mixture of endophytic diazotrophic bacteria, was in the range of 20 to 30% of the total N accumulated in the plant tissues (Oliveira et al. 2003). Other measurement studies using the delta 15 technique showed that the average BNF contribution to the sugarcane varieties harvested in sugarcane producing areas in Brazil is around 30% (Boddey et al. 2001). However, these values varied from 0 to 70% depending on the level of the molybdenum available in the soil (Polidoro 2001).

#### ANATOMICAL AND PHYSIOLOGICAL ROOT RESPONSE TO INOCULATION

The group coordinated by Dr. Olivares has carried out studies on the inoculation effect of this

bacterium on physiological changes of the plant metabolism. The authors have demonstrated that the inoculation of different micropropagated sugarcane genotypes (mainly RB cultivars) with selected strains of *G. diazotrophicus* render a sort of anatomical and physiological changes over the plant host. These effects included an increase of lateral root mitotic sites as well as emerged lateral roots with changes at the root geometry by increasing the fine root portion and the overall root system (Olivares et al. 2002). Furthermore, these anatomical changes have been accomplished by an increase on the  $H^+$ -ATPase activity of the root cell microsomal fraction and protein contents. Besides that, studies were carried out to link the endophytic inoculation and photosynthetic processes. The results showed that the net photosynthesis, stomatal conductance, transpiration rates as well as the relative quantum dependence of photosystem II are not affected by the endophytic establishment of *Herbaspirillum/G. diazotrophicus*, indicating no photoinhibitor effect and no altered leaf gas exchange. The physiological changes that takes place in sugarcane during the endophytic interaction could be, in part, related to the plant growth promoting effects such as it has been observed in many experiments that includes increase of nutrient accumulation and biomass.

#### MOLECULAR MECHANISM OF THE PLANT-BACTERIA INTERACTION

An important feature of the plant interaction with these endophytes is that bacteria colonize most plant organs, promoting plant growth without causing any disease symptoms. It raises the question if there is an active role of the plant in the process, or if it is just a niche for bacterial growth. Since 1994, Dr. Hemery's group from the UFRJ is addressing this question by investigating sugarcane gene expression during the association with *Gluconacetobacter diazotrophicus* using different approaches: (i) cDNA-AFLP fingerprinting, (ii) transcriptional profiles generated from the SUCEST (Sugarcane EST Sequencing Project) database and (iii) *microarray*. The novelty of this studies was to show for the

first time that sugarcane might be actively involved in the association, because several genes involved in different plant physiological processes were identified as candidates to be differentially expressed during the association (Nogueira et al. 2001). The group focus now the studies on the characterization of signaling pathways by which sugarcane plants can decipher bacterial signals and respond properly for a successful association (Vargas et al. 2003); and the molecular mechanisms that promote plant growth by association with the endophytes. By using functional genomic tools, a group of genes related to nitrogen metabolism and plant development are being characterized, in order to understand how plants benefit by this association. In addition, receptors involved in signaling plant/bacteria interactions are also being studied. The data observed for most of the studied genes indicate that the modulated gene expression during association is not a general stress response against microorganisms, but seems to be specific for benefic associations. Interestingly, the data showed that expression of several genes is not altered in sugarcane genotypes with low contributions of BNF, indicating that the plant genotype has an important role on the efficiency of the association.

#### GENOMIC AND PROTEOMIC STUDIES

The economical potential for the use of *G. diazotrophicus* for the inoculation of sugarcane cultivated in the Rio de Janeiro State and Brazil stimulated the creation of a network to sequence the genome of this bacterium. The collaborative network called RIOGENE is supported by FAPERJ and CNPq and has the participation of Embrapa Agrobiologia, four universities (UFRJ, UFRRJ, UENF and UERJ) and Laboratório Nacional de Computação Científica (LNCC). The aim of the project is to obtain the genome sequence by the end of this year with a goal to understand gene functions and consequently their manipulation to increase the efficiency of the plant/bacteria interaction and nitrogen fixation. A proteomic network was created to support the sequencing program of *G. diazotrophicus*.

### *Burkholderia* spp

The last group of diazotrophic bacteria showing endophytic characteristics, but not as well studied as those described above, was renamed in the last decade (Yabuuchi et al. 1992). This new genus called *Burkholderia* consists of 47 species, however only three are known to fix nitrogen (Gillis et al. 1995, Zhang et al. 2000, Reis et al. 2004). The first, named *B. vietnamiensis*, was isolated from the rhizosphere of rice roots cultivated in the Vietnam (Gillis et al. 1995). This nitrogen-fixing species also included two strains isolated from human materials belonging to the older *Pseudomonas cepacea* species, demonstrating that the new genus is not restricted to plant materials. The other species called *B. kururiensis* was isolated in Japan from an aquifer area contaminated with trichloroethylene (TCE) (Zhang et al. 2000). The ability of this species, represented only by one isolate, to fix nitrogen was demonstrated by Santos et al. (2001) in a study that compared different *Burkholderia* strains isolated from plants grown in Mexico and from other countries including Brazil.

In the mid 90's, a large number of nitrogen-fixing bacteria, with characteristics similar to those of the *Burkholderia* genus, were isolated from rice, sugarcane and sweet potato plants using semi-solid JMV medium containing mannitol as a carbon source with the pH adjusted to near 4.5 (Baldani 1996). The partial sequence of the 23S and 16S rDNA region of two representative strains (M130 and Ppe8) indicated that these isolates belong to the genus *Burkholderia*, but they were not *B. vietnamiensis* species. Later, studies using probes derived from the sequenced regions from M130 and Ppe8 showed the presence of two distinct groups, one formed by the rice, manhiot and sweet potato isolates and the other by the isolates from sugarcane (Hartmann et al. 1995).

*Burkholderia tropica* – Based on the morphological, physiological and genetic characteristics, two new species were proposed: *B. brasiliensis* (Baldani et al. 1997b) and *B. tropicalis* (Kirchhof et

al. 1997, Reis et al. 2000). Recent studies showed that the *B. brasiliensis* strain M130 and the *B. kururiensis* strain KP23 showed the same ARDRA pattern (Santos et al. 2001) with a similarity of 99.9% between the 16S rDNA subunits, suggesting that these two strains belong to the same species. Partial sequencing of the *nifH* and *glnB* genes also showed that these strains (M130 and KP23) were similar but distinct from the reference PPe8 strain belonging to the *B. tropicalis* species (Marin et al. 2003). DNA: DNA experiments are been carried out to define either the *B. brasiliensis* isolates constitute a new species or belongs to the *B. kururiensis* species. On the other hand, the proposed name of *B. tropicalis* was officially accepted as *B. tropica* (Reis et al. 2004).

### MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES

Among the morphological and physiological characteristics that distinguish the two species are: pH tolerance, use of carbon sources, colony type grown in JMV medium containing nalidixic acid, and osmotic tolerance. The “*B. brasiliensis*” species grows and fixes nitrogen in semi-solid JMV medium with a pH range of 4.0 to 6.0 with mannitol or +D,L-carnitine as carbon sources. This strain, however, does not grow in semi-solid LGIP medium with 10% sucrose. The colonies in semi-solid JMV medium are brownish and become irregular, smooth and transparent in the presence of nalidixic acid. “*B. brasiliensis*” strains are able to oxidize the following carbon sources (Biolog test): maltose and xylitol but not D-lactose and L-raffinose. On the other hand, the *B. tropica* species grows and fixes nitrogen in semi-solid JMV medium with a pH higher than 5.0 with one of the following carbon sources: mannitol, +arginine, +adipate or +ribose. It is able to fix nitrogen in semi-solid JMV medium with 10% of sucrose. Colonies grown on Potato medium with 10% crystal sugar are light brown and when grown in LGIP medium the colonies are orange with brownish borders. Colonies in JMV medium (pH 5.5) containing 2.5 µg/L of nalidixic acid are rounded, and orange with yellow borders.

*B. tropica* strains are able to oxidize the following carbon sources (Biolog test): D-lactose and L- raffinose, but not maltose and xylitol. Strains from both species can be identified through the hybridization with probes based on the sequences of the 16S ribosomal subunits. The “*B. brasiliensis*” species can be identified using the Bbra62 and Bbra636 probes while the *B. tropica* species can be identified using the Btrop636 and Btrop463 probes (Boddey 2003).

#### ECOLOGICAL STUDIES

Studies on the ecological distribution of these species demonstrated their high frequency of occurrence (especially “*B. brasiliensis*”) in sugarcane plants grown in different regions of Brazil and in Australia (Boddey 2003). “*B. brasiliensis*” has also been isolated from different rice varieties grown in soil from Rio de Janeiro State and the Cerrado soil from Goiás (Rodrigues et al. 2001). Strains from these two species were also isolated from banana and pineapple (Weber et al. 1999) and their identity was confirmed by the partial sequencing of the 16S rDNA subunit (Cruz et al. 2001). The infection and colonization process of rice by “*Burkholderia brasiliensis*” strain M209 showed that the bacteria first colonize the root surface and then penetrate the cells via the intercellular spaces of the damaged membrane (Baldani et al. 1997a). The bacteria can also penetrate through wounds in the epidermal cell region and points of emergence in the secondary roots (Baldani et al. 1995). Additional studies showed that these species also colonize the stomata of rice seedlings (Silva et al. 2000). The infection and colonization process of micropropagated sugarcane roots inoculated with the strain Ppe8 of *Burkholderia tropica* was very similar to that observed for other endophytic bacteria (Boddey et al. 1999). Large populations of strain Ppe8 are mainly found on the surface of micropropagated sugarcane roots, when this strain is inoculated together other endophytic diazotrophic bacteria (Oliveira et al. 2002).

#### INOCULATION RESPONSES

Several inoculation experiments have been carried out to determine the BNF contribution to graminaceous plants by these *Burkholderia* species. Rice varieties grown in low fertility acid soil in Vietnam inoculated with the *Burkholderia vietnamiensis* TVV75 strain gave yield increases of 13 to 22% (Tran Van et al. 2000). In Brazil, a detailed study has been conducted involving strains of “*Burkholderia brasiliensis*” and different rice varieties with the goal to select those strains that are more efficient in the association leading to a greater contribution to the development of the plant (Baldani 1996). The results show that an interaction exists between the strain and rice cultivar (Baldani et al. 2000). A BNF contribution on the order of 20 and 30%, as determined by the <sup>15</sup>N isotopic dilution technique was observed in rice plants grown under gnotobiotic and greenhouse conditions (Baldani et al. 2000). The yield response of the same rice cultivars grown in the field and inoculated with these strains was very variable (Guimarães et al. 2000). A yield increase of 54% was observed for the IAC4440 rice variety inoculated with “*Burkholderia brasiliensis*” strain M209, however the increase was very low for the IR42 variety inoculated with this strain (Guimarães et al. 2002). The results suggest that BNF research on rice should be increased considering the progress that has been made. One aspect that should be exploited should be the plant/bacteria interaction as well as interactions between bacteria as has been observed for sugarcane inoculated with a mixture of bacteria (Oliveira et al. 2002).

#### PERSPECTIVES

A historical analysis of studies on BNF in Graminaceous plants demonstrates significant advances in several aspects of plant/bacteria interactions. However, the expectation that the nitrogen fixation efficiency might be equivalent to the rhizobia/legume symbiosis did not turn out to be true, although the endophytic diazotrophic bacteria/plant association

shows some characteristics that are similar to the legume symbiosis. A biotechnological program devoted to defining the functionality of genes present in most of the nitrogen-fixing bacteria as well as knowledge generated by genome sequences of several plants of agronomic interest, should contribute to a better understanding of these associations, particularly the endophytic ones. Consequently, it may be feasible to convert the potential of this association into a standard inoculation practice in agriculture. However, responses similar to those observed for the Brazilian soybean should not be expected for cereals and other grasses inoculated with endophytic diazotrophic bacteria. As has been emphasized in most of Dr Johanna Döbereiner's papers, breeding programs with Gramineous plants should always take the interaction of endophytic diazotrophic bacteria and plant genotype into account so that the biological nitrogen fixation process can be optimized.

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#### RESUMO

A presente revisão aborda a história da Fixação Biológica de Nitrogênio (FBN) em Gramíneas no Brasil, procurando mostrar a evolução da pesquisa na área iniciada a mais de 40 anos sob a liderança da pesquisadora Johanna Döbereiner. Um aspecto marcante deste período foi a descoberta de diversas bactérias fixadoras de nitrogênio atmosférico tais com as rizosféricas (*Beijerinckia fluminensis* e *Azotobacter paspali*), associativas (*Azospirillum lipoferum*, *A. brasilense*, *A. amazonense*) e as endofíticas

(*Herbaspirillum seropedicae*, *H. rubrisubalbicans*, *Gluconacetobacter diazotrophicus*, *Burkholderia brasilensis* e *B. tropica*). O papel destas bactérias diazotróficas em associação com as gramíneas, especialmente os cereais, tem sido estudado e muito se avançou sobre os aspectos ecológicos, fisiológicos, bioquímicos e genéticos. Os mecanismos de colonização e infecção dos tecidos das plantas foram melhor entendidos e a contribuição da FBN para o sistema solo-planta foi determinado. Estudos de inoculação de cereais com bactérias diazotróficas, têm mostrado que as endofíticas têm um maior potencial de contribuição da FBN e que o genótipo da planta influencia na associação da planta/bactéria. Os avanços alcançados apontam para uma maior exploração e entendimento desta associação endofítica. Os programas de sequenciamento do genoma: RIOGENE (*Gluconacetobacter diazotrophicus*) e GENOPAR (*Herbaspirillum seropedicae*) mostram a importância da FBN no Brasil e devem permitir que o país continue na fronteira do conhecimento em relação ao processo de FBN em gramíneas.

**Palavras-chave:** bactéria endofítica, diazotrófica, meio semi-sólido, inoculação, cereais, planta forrageira.

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