PROGRESS REPORT

U.S. PARTICIPATION IN AN INTERNATIONAL PROGRAM OF COOPERATION IN TRAINING AND RESEARCH IN NITROGEN FIXATION IN THE TROPICS

AUGUST 1979 - JUNE 1981

Report of activities undertaken by the Board on Science and Technology for International Development, National Academy of Sciences, in cooperation with the Brazilian National Research Council and Empresa Brasileira de Pesquisa Agropecuaria, with grant funds provided by the Office of Agriculture, Technical Assistance Bureau, Agency for International Development, Grant No. AID/ta-G-1329
CONTENTS

I. Introduction and Background

II. Highlights of the Program, August 1979 - June 1981

III. Summary of Research, August 1979 - June 1981

IV. Summary of Consulting Visits by Dr. David Pramer and Dr. Carlos Neyra

V. List of Publications, 1981

VI. Financial Summary
I. INTRODUCTION AND BACKGROUND

The International Program of Cooperation in Training and Research in Nitrogen Fixation in the Tropics was conceived at a meeting in November 1974 between a group of Brazilian scientists, headed by Dra. Johanna Dobereiner, and its counterparts from the United States, United Kingdom, Canada, and Australia. The purpose of the meeting was to review and discuss the research and training program at the Soils Section, Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), at km 47 on the old highway from Rio de Janeiro to Sao Paulo, and the Federal Rural University of Rio de Janeiro (UFRRJ) at the same location. The Brazilian National Research Council (CNPq) and the U.S. National Academy of Sciences (NAS) were also represented. These two organizations have a long-standing program of cooperation in scientific endeavor linked to Brazilian development.

Recent advances in knowledge of nitrogen-fixing symbiotic associations in Brazil were discussed at the meeting, in particular, the recent discovery by Dra. Dobereiner's group of bacteria (Spirillum lipoferum) that fix atmospheric nitrogen in the root cells of nonleguminous plants--tropical grasses and cereal crops grown on tropical soils in Brazil.
It was concluded at the meeting that this area of research and training was especially significant because the shortage of nitrogen in tropical soils, coupled with rapidly rising costs of nitrogenous fertilizer as a result of higher energy costs, places an increasingly important constraint on the worldwide improvement of agricultural production from tropical soils. The prospect of supplying even a small part of this requirement from symbiotic fixation of atmospheric nitrogen led the group to recommend that the research and training at km 47 be strengthened, and to agree to support it through an International Program of Cooperation.

The International Program provides a framework through which research on nitrogen fixation in both legumes and tropical cereal and forage crops can be supported at km 47 through (1) periodic exchanges of scientists, including postdoctoral research fellows from the United States and elsewhere to work at km 47 for periods of up to 2 years; (2) assistance in purchasing essential equipment not readily available in Brazil; and (3) periodic meetings of the International Advisory Committee to discuss progress in research and training.

In 1976, the Office of Agriculture of AID's Technical Assistance Bureau provided the Academy with a grant for support of 3 years of U.S. participation in the program. Following an evaluation in the third year, this grant was extended to April 1980 to cover activities taking place during the period of this report.
II. HIGHLIGHTS OF THE PROGRAM, AUGUST 1979 - JUNE 1981

During the final year of the grant, two senior scientists from the United States, Dr. David Pramer and Dr. Carlos Neyra, both of Rutgers University, visited Brazil for consultations with Brazilian colleagues concerning experiments on key aspects of nitrogen fixation—streptomycin resistance and plant physiology, respectively. Arrangements were made for two Brazilian scientists to visit Rutgers during the summer of 1980; however, they were unable to visit.

Dra. Johanna Dobereiner, Brazilian coordinator of the program, visited the National Academy of Sciences in March 1981, and participated in a review of nitrogen fixation research priorities. This was undertaken both to bring American scientists up to date on the program, and to identify promising areas of future research that might be undertaken under a new research grants program administered by the Academy. The meeting indicated that nitrogen-fixing trees, associative fixation (particularly physiological aspects), symbiotic mechanisms of fixation in sugarcane and wheat, and azolla offer particular opportunities for research. Future NAS support of nitrogen fixation research in developing country institutions will be possible under the terms of this AID-funded grant; however, the precise areas remain to be identified by an expert committee in early 1982. The activities indicated in this report exhausted the original grant funds, and this phase of cooperation with the International Program in Brazil was concluded.
III. SUMMARY OF RESEARCH, AUGUST 1979 - JUNE 1981*

In accordance with the plan of activities of the Biological Nitrogen Fixation Program, research concentrated on problems related to nitrogen fixation in legumes and grasses and the solubilization of natural phosphates. In the area of legumes, it was possible to explain the occurrence of highly streptomycin-resistant varieties of rhizobium in cerrado soils in terms of the increase in the number of actinomycetes on the surface of the roots, and the percentage of streptomycin-resistant bacteria other than rhizobia, principally after fertilization and liming of the acid soils, indicating the production of antibiotics in the rhizosphere and assimilation by the plant. A similar case to the cerrados was encountered in the Amazon where, after 5 years, natural selection and multiplication of rhizobia resistant to streptomycin and chloramphenicol resulted in abundant nodulation without requiring inoculation. The use of inoculants of this type of rhizobium should permit increased nodulation in the first year.

The sensitivity of rhizobium to elevated temperatures in the nodulation of beans was the other basic problem studied. The bacteria show a genetic constitution of the rhizobium species in temperate climates which easily lose the genetic information responsible for nitrogen fixation.

*Translated from original Portuguese version prepared by Dra. Johanna Dobereiner.
The plant-bacterium interaction permitting tolerance to aluminum and phosphate deficiency was confirmed in pot experiments, with both LVA and LVE soils, indicating the importance of selecting plant-bacteria combinations suitable for difficult soil conditions. In these experiments, an unexpected effect was observed in the rhizobium family in the translocation of fixed nitrogen to the seed (in soybeans); however, there were insufficient measurements of total fixed nitrogen to evaluate the efficiency of the rhizobia. Analysis of the composition of the sap of the soy plants and the beans inoculated and fertilized with mineral nitrogen revealed differences in the content of allantoin, indicating a much more efficient method in the plant for assimilating fixed nitrogen than by the traditional route through asparagine.

In the grasses research, the physiological studies of *Azospirillum* revealed surprising characteristics of this bacterium important in their association with cereals. Its capacity to grow and fix nitrogen was demonstrated autotrophically, i.e., without organic sources of energy, as a result of the oxidation of H₂ or methane. Since H₂ is principally produced with methane in deep soil layers, it is possible that this characteristic of *Azospirillum* increases the efficiency of grass systems, complementing the supply of photosynthates by the plant from exogenous substrate. Experiments with $^{15}$NO₃ using *Paspalum notatum* and *Brachyaria* spp. under field conditions (lysimeters) confirmed nitrogen fixation on the order of 20 percent of the total nitrogen incorporated, i.e., 50 kg of nitrogen per hectare.
fixed per year. The first results were also obtained under field conditions from the inoculation of corn with specific strains of *Azospirillum* which contributed from 25 to 60 percent of the total nitrogen incorporated corresponding to 20-40 kg per hectare per year. Multiplication of *Azospirillum* in soils treated with vinaca (alcohol stillage residues) and appreciable nitrogen fixation were also verified.

In the phosphate research, a pronounced effect of the application of organic matter (manure) on the nodulation of beans was demonstrated even when the phosphate was not solubilized. Incubation of these phosphates with vinaca transformed part of the soluble phosphate into organic phosphate; however, this was not observed to lead to greater phosphorus availability to the plant. In various centers in the country, a crop rotation experiment of cultivars, which almost exclusively based their biological fixation of nitrogen on natural phosphate, was established in the hope of obtaining subsidies for the highly productive agricultural system in each region.

IV. SUMMARY OF VISITS BY DR. DAVID PRAMER AND DR. CARLOS A. NEYRA

Dr. David Pramer, Rutgers University

Dra. Johanna Dobereiner requested that I visit her laboratory to advise on her research program on nitrogen fixation.

A series of studies performed there have demonstrated a high correlation of resistance to streptomycin with a capacity of bacteria
to establish intimate associations with plant roots. The relationship was displayed by bacteria that establish nitrogen-fixing, symbiotic associations with plant roots, as well as by nonsymbiotic nitrogen-fixing bacteria. Specifically, populations of rhizobia isolated from the nodules of legumes growing on cerrado and Amazon soils, as well as azospirilla and other bacteria isolated from maize roots, were demonstrated to be predominantly streptomycin-resistant. The correlation has been demonstrated to hold in a number of different cases; however, the studies are not exhaustive and one should anticipate that exceptions can and will be reported.

Dra. Dobereiner is interested in an explanation for the observed relationship. There is the obvious Darwinian possibility: if streptomycin and other antibiotics are produced in a root zone, the only organisms fit to survive in the root environment would be those that have developed resistance to antibiotics. However, there is a paucity of evidence to support that hypothesis. When Dra. Dobereiner sought evidence in the literature, she encountered my work on the production, fate, and biological effects of antibiotics in soil, which led to my visit to Brazil.

An explanation of the observed relation of streptomycin resistance to a capacity of bacteria to establish an intimate association with plant roots is of academic interest, but it may also provide an understanding of how we might promote bacterial/plant root interactions that are beneficial to soil fertility and crop production.
During my trip I was able to meet and talk at length with Dra. Dobereiner as well as with various members of her research group. Of particular interest was Dr. Robert Boddy, a relatively young and energetic associate who appears to be directing a good deal of the laboratory work, and two graduate students, Scotti and Sa, who are working at the Federal University in Minas Gerais on cerrado and Amazon soils.

In private discussions with various individuals and in a seminar conducted specifically for Dra. Dobereiner's group, I traced the history of efforts to demonstrate antibiotic production in soil. Emphasis was placed on the fact that if antibiotics are produced at all, their occurrence will be limited to soil microenvironments that are discontinuous in place and time. Moreover, the amounts produced will not be great. No one has demonstrated streptomycin production in soil unless samples have been sterilized, supplemented with high levels of readily available organic matter, and inoculated with a streptomycin-producing microorganism. What is true for streptomycin applies to other antibiotics as well, with the exception that low levels of some substances (gliotoxin, fusaric acid, actinomycin) have reportedly been produced in unsterilized soils that are amended with organic material such as wheat straw or soy bean meal.

I assisted in the design of experiments to demonstrate by direct and indirect methods whether or not streptomycin is produced in soils typical of those Dra. Dobereiner has employed in her studies. For
this purpose I brought with me a streptomycin-dependent strain of
*Escherichia coli* to be used as an assay organism, as well as various
other items to facilitate the work. I am not at all optimistic about
Dra. Dobereiner's position, and I expressed doubt that evidence would
be obtained to support her conviction that the production of
antibiotics in the rhizosphere is responsible for the appearance of
streptomycin-resistant bacteria in association with root plants.

In the course of our discussions I tended to be more discouraging
than encouraging. It was repeatedly necessary to make the point that
antibiotic resistance is a complex genetic phenomenon. It can be the
result of a chromosomal mutation, manifested by a permeability change
that excludes the antibiotic from entering the cell, or it can be the
result of plasmid-induced synthesis of an enzyme, such as
phosphotransferase, which is able to inactivate streptomycin. A number
of other possibilities were discussed. Moreover, I reminded
Dr. Dobereiner and her associates that antibiotic resistance can
develop in cells which have never been exposed to antibiotics, and that
multiple resistance is the rule rather than the exception.

My primary message was that antibiotic production is but one of a
number of possible explanations for the observed occurrence of
streptomycin-resistant bacteria in association with plant roots. Among
a number of alternatives, it is less rather than more probable, and it
will not be accepted without experimental support.

During the visit, I presented a university-wide seminar on
nematode-trapping fungi which was well attended. A great deal of
interest was expressed in the subject, and I left copies of our
published papers in the area, as well as a time-lapse
cinephotomicrographic study of the growth and predaceous activity of
nematode-trapping fungi, with those working in plant protection and
biological control.

I am convinced that Dra. Dobereiner benefited from my visit, which
I found both interesting and informative. I have since sent some
cultures and some antibiotics to Dra. Dobereiner, and no doubt we will
continue to communicate for some time in the future.

I am indebted to the Academy and to AID for making the trip
possible.

Dr. Carlos A. Neyra, Rutgers University

This visit included the following activities:

- Discussions of projects under way with a number of
  investigators associated with the program.

- Participation in the design of new experiments to include the
  physiological approach and methods in the following areas:
    -- Relationships of nitrogen fixation to nitrate assimilation
       in grain crops
    -- Enhancement of nitrogen fixation under limiting
       environmental conditions
Consulting trip of 3 days to EMBRAPA at Goiânia, north of Brasilia.

- Collaboration in the preparation of a cooperative research program between the International Program for Nitrogen Fixation, Rutgers University, and the University of Hawaii. The principal investigators are Dra. J. Dobereiner (Brazil), Dr. Carlos A. Neyra (Rutgers), and Dr. B.B. Bohlool (University of Hawaii).

- Completion of two manuscripts submitted to the *Canadian Journal of Botany* as part of our collaborative research:
  -- Nitrogen assimilation and dissimilation in five genotypes of *Brachiaria* spp. (Bot. 80-205).
  -- Nitrate reduction and nitrogenase activity in excised corn roots (Bot. 80-206).

In addition, the preparation of two other manuscripts was initiated, and they will be submitted for publication shortly.

V. LIST OF PUBLICATIONS, 1981


International Workshop on Associative N₂-Fixation, Cali, Colômbia.


LEE, K.K. and DOBEREINER, J. (1981) Effect of excessive temperatures on growth of cowpea type rhizobia in vitro, on nodulation and nitrogen fixing activity in symbiosis with Siratro, Enviado para a PAB.


VI. FINANCIAL SUMMARY

U.S. Participation in an International Program of Cooperation in Training and Research in Nitrogen Fixation in the Tropics, Grant No. AID/ta-G-1329

<table>
<thead>
<tr>
<th>Description</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salaries</td>
<td>$15,820</td>
</tr>
<tr>
<td>Fringe Benefits</td>
<td>3,091</td>
</tr>
<tr>
<td>Travel - international</td>
<td>32,075</td>
</tr>
<tr>
<td>Communications &amp; Shipping</td>
<td>1,264</td>
</tr>
<tr>
<td>Materials, Services &amp; Equipment</td>
<td>18,334</td>
</tr>
<tr>
<td>Indirect costs</td>
<td>16,609</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>$87,193</strong></td>
</tr>
</tbody>
</table>