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FINAL REPORT

IDENTIFICATION OF SUPERIOR BEAN-RHIZOBIA COMBINATIONS FOR UTILIZATION IN CROPPING SYSTEMS SUITABLE TO SMALL FARMS IN BRAZIL

1981 - 1989

BRAZIL/UNIVERSITY OF WISCONSIN/BLISS PROJECT

August 1990

A Collaborative Effort Among:

CENTRO NACIONAL DE PESQUISA DE ARROZ E FEIJAO (CNPAF)/
EMPRESA BRASILEIRA PESQUISA AGROPECUARIA (EMBRAPA),
THE UNIVERSITY OF WISCONSIN-MADISON and
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BEAN/COWPEA COLLABORATIVE RESEARCH SUPPORT PROGRAM (CRSP)

EXECUTIVE SUMMARY

The Bean/Cowpea CRSP Project entitled "Identification of Superior Bean-Rhizobia Combinations for Utilization in Cropping Systems Suitable to Small Farms in Brazil" was the result of a collaborative effort among the CNPAF/EMBRAPA, The University of Wisconsin-Madison and Michigan State University, with The U.W.-Madison as the lead University. The overall goal of this project was to develop superior common bean cultivars capable of enhanced biological nitrogen fixation that, in association with superior strains of Rhizobium leguminosarum biovar phasioli, produce high yields under bean monoculture and multiple cropping systems without supplemental nitrogen fertilizer. Research supporting this project was conducted at all three institutions, with most of the research judged to be site specific done in the field in Brazil. The training components included two completed and one soon-to-be completed Ph.D. recipients, and a BNF workshop at U.W.-Madison; short-term non-degree recipient training at M.S.U.; and short- and medium-term non-degree training of students and professionals at CNPAF/EMBRAPA.

The research accomplishments included the following:

Development of breeding methodology for the incorporation of enhanced N_2 fixation into bean cultivars suitable for Brazil.

Release and distribution of high-N_2 fixing black bean germplasm for testing and development in Brazil and elsewhere.

Participation in Network Experiments for testing rhizobial strains and bean lines at multiple sites in Brazil.

Observation of plant, microbe and environmental factors that either enhance or limit N_2 fixation by beans in monoculture or in relay cropping with maize.

Identification and use of rhizobial strains that provide high performance on either existing or improved bean lines in the field.

Determination of timing of N fertilizer application to optimize bean productivity while encouraging enhanced levels of N_2 fixation.

Detection of bacterial symbiont influence on branching and growth of bean roots apart from effects of greater N availability.

Characterization of important events that occur in conjunction with nodule senescence.

Dissemination of new information through presentations and printed articles to groups ranging from local to international audiences.

Another important accomplishment was the institutionalization of the concept of breeding for increased N_2 fixation at CNPAF/EMBRAPA. At the beginning of this project it was not evident whether bean lines, especially early, type I and type II cultivars, could be bred for increased N_2 fixation. It is now clear that it is possible to improve genetically the ability of most bean cultivars to fix more atmospheric nitrogen. Under favorable field conditions in Wisconsin and using the ^{15}N-isotope methods, the improved black bean lines have been shown to fix routinely in excess of 50 kg N ha^{-1} and up to 100 kg N ha^{-1} in some trials. Under less optimal field conditions in Brazil, these lines have been shown to fix up to 30 kg N ha^{-1}. Numerous breeding lines within different market classes are currently being tested in Brazil to determine whether they merit release.

The five black bean lines released as improved germplasm has been undergoing rigorous field testing in various states of Brazil. Some show promise and may be released as named cultivars. Others lack the necessary combinations of traits and may be used as breeding lines. Application of rhizobial inoculant for bean production in Brazil is not always done routinely. The results of the network experiments and other studies of the microsymbiont provide the basis for deciding which strains to use in commercial inoculant production.

Several recommendations were made as follows.

CNPAF/EMBRAPA should seek continuing support for a project of this nature in the form of 1) a staff scientist trained in agronomy/physiology to work in collaboration with a rhizobiologist and plant breeder, 2) technical staff to assist the scientists, and 3) staff assigned to development of technology packages that will allow the potential of BNF in beans to be fully utilized in appropriate production systems for small farms able to utilize limited purchased inputs. It is recommended that CNPAF/EMBRAPA seek additional support for a broad program to deliver this applied technology. Such a program should emphasize practicality and on-farm testing.

The Bean/Cowpea CRSP has provided an effective vehicle for linking U.S. research expertise with host country institutions that are in need of collaboration to address critical problems. These efforts should be continued.

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BEAN/COWPEA CRSP FINAL REPORT

1.0 INTRODUCTION

One of the constraints to the availability of beans and cowpeas, as identified during the planning process in 1978 and 1979 (Adams, 1984) was low grain yields resulting from inadequate nitrogen for the developing plants. Many soils on which beans are grown by small farmers are inherently low in N; and for those producers, nitrogen fertilizer often is unavailable or too costly to be applied as a routine production input.

In addition to the limitations of low soil N and costly fertilizer, common bean is often regarded as a poor nitrogen-fixer which is unresponsive to inoculation with rhizobia. Furthermore, the addition of N fertilizer may be in some cases "self-defeating", since the presence of large amounts of combined N is considered to have an adverse effect on N_2 fixation. The availability of improved bean cultivars, responsive to either effective native rhizobia or added inoculum, and to N fertilizer without experiencing a depressing effect on N_2 fixation would benefit small farmers who grow beans with limited purchased inputs.

The University of Wisconsin-Madison was chosen as the U.S. Lead Institution because, in 1978, the P.I. was one of the few breeders in the U.S. who was conducting genetic research on improving N_2 fixation of common bean. That research was being supported through funding from a USAID/CSRS Special Grant. The U.S. co-investigator, Frank Dazzo, was chosen because of his expertise in rhizobiology and the need to study the micro-symbiont concurrently with host plant improvement. The choice of the host

country, Brazil, reflected the fact that, even though they are the largest producer and consumer of beans, productivity there is quite low especially on small, limited-input farms. Several other factors were considered also. Historically, considerable research on N_2 fixation has been done in Brazil, and largely because of the leadership of Dr. J. Dobereiner, a number of outstanding rhizobiologists have been trained there and have active research programs in Brazilian institutions. However, in initially assessing constraints to increased bean production, Brazilian scientists expressed the need for more fundemantal research, particularly on breeding for improved fixation. Because of not only a demonstrated need but also a good existing research infrastructure relating to N_2 fixation, the National Rice and Bean Center (CNPAF) of EMBRAPA, located near Goiania, Goias State was chosen as the Host Country Institution. Also, because of the wide diversity of production conditions and of bean types grown in Brazil, it was felt that much of the research would be applicable to bean production systems elsewhere.

The Centro International de Agricultura Tropical (CIAT) had initiated a program on improving N_2 fixation in beans prior to the CRSP project. Much of the initial information utilized by the U.S. P.I. was based on the work done earlier at CIAT. Studies at that institution are continuing, and throughout the CRSP project, there has been exchange of information, plant materials and rhizobial strains.

2.0 GOAL AND OBJECTIVES

The overall goal of this project was to develop superior common bean cultivars capable of enhanced biological dinitrogen fixation that, in association with superior strains of *Rhizobium leguminosarum* biovar *phaseoli*, produce high yields under bean monoculture and multiple cropping systems without supplemental nitrogen fertilizer.

More specific objectives were:

1. Plant improvement through selection of superior genotypes
2. Rhizobial improvement through collection and evaluation
3. Study of factors affecting N_2 fixation in cropping systems
4. Dissemination of plant materials, rhizobia and information
5. Provision of degree and non-degree training to support research
6. Institutionalization of the concept of breeding for improved N_2 fixation at CNPAF/EMBRAPA in Brazil

3.0 RELEVANCE AND IMPACT OF THE PROJECT

Global Concerns of the Bean/Cowpea CRSP

The low yields of beans grown on small farms in areas where they are an important dietary component are a result jointly of limitations of the physical environment (i.e., inadequate N from N-poor soils) and plant response limitations (i.e., the inability of the bean plant to support adequate N_2 fixation with the bacterial symbiont, *R. phaseoli*). By improving the capability of the bean plant to fix large amounts of atmospheric N_2 efficiently, the limitation imposed by the physical environment can be reduced as well.

This genetic approach to the problem is also cost effective, since use of an improved, high fixing cultivar rather than a low N_2-fixing standard cultivar does not require large recurring costs such as fertilizer purchases. It is relatively easy for the small farmer to realize the benefits of this improved technology. If the breeder has been successful, there are only a few production practices that must be changed to use a new cultivar. These include; 1) obtaining new seed periodically (a good practice anyway), 2) adding rhizobial inoculant if native rhizobia are scarce or ineffective, and 3) possibly altering harvest, storing and marketing practices.

Perhaps most important, there is in Brazil, an organization in place to test new cultivars and assist farmers in deciding when and how to use the new materials. The success of this project in showing that breeding for increased N_2 fixation can be done, will

assist greatly in reducing one of the major constraints to increased bean production, i.e., inadequate N to support high yields even when other constraints are minimized.

Brazil

According to Vieira (1988), about 5 million hectares of beans and cowpeas are planted annually in Brazil, with about 80% being common bean and 20% cowpea. Although total production oscillates over years, some 2.2 million metric tons of dry beans are produced annually. This relatively steady production coupled with a population increase of some 2.5% per year means that per capita bean consumption is decreasing, while consumption of other staples, i.e., wheat, has increased. It is imperative that productivity per unit area be increased or per capita consumption of beans will decrease further, and therefore impact on the diets of especially those people dependent on beans as a source of protein.

Consumer preference for beans in Brazil varies greatly among regions (Vieira, 1988), with seed color, size, taste and cooking qualities being important. Indeed, this varying preference is found world-wide. It is important to understand that if a trait such as improved N_2 fixation is to make a wide and substantial impact, breeders must be able to incorporate it into a wide array of different cultivars, e.g., red, black, carioca types etc. easily and quickly. Thus, the results of this project are important not only for improving black beans but also because the breeding methods, analytical techniques, testing etc. can be applied to the improvement of other types of grain legumes as well.

Never-the-less, black beans are the most popular class in the states of Rio Grande do Sul, Santa Catarina, southern and eastern Parana, Rio de Janeiro, southeastern Minas Gerais and southern Espirito Santo (Viera, 1988). In addition, on small farms and local markets, black beans often are a predominant type in cultivar mixtures. The combined total common bean production for these six states in 1984 was approx. 1,177,000 metric tons according to FIBGE, 1984 (cited by Vieira, 1988). If only one-half (a conservative estimate) of the total production of these states is of black beans, their importance is readily apparent. The national mean yield has slipped from 600-700 kg/ha in the 1960's to about 450 kg/ha (Vieira, 1988). Thus, the newly released cultivars and germplasm (Bliss et al., 1989) with potential for increased N_2 fixation can contribute substantially to raising productivity of beans in Brazil and elsewhere.

A list of recommended bean cultivars is maintained for each state in Brazil (CNPAF/EMBRAPA, 1983). Although the recommended cultivars for each are different, two standard black-seeded cultivars, Rico 23 and Rio Tibagi, are expected to be prominent among those which the newly-released materials will replace if successful. Data collected in regional trials in Brazil provided an indication of the superiority of promising lines in trials grown with modest to low input (CIAT, 1986; 1987). In the Preliminary Yield Trial (EPR) 1986/88 at Ponta Grossa, Parana, the grain yield of WBR 22-24 was 2107 kg/ha vs. 1234 kg/ha for Tio Tibagi. In Rio Grande do Sul, the yields of WBR 22-24 vs. Rio Tibagi in three trials were: 1017 vs. 1050, 2050 vs 0, and 1783 vs. 1304 (CIAT,

1986 pp 274-275). At two trials in Goias state, Honduras 35 produced 1689 kg/ha vs. 774 and 1456 vs. 841 for Rio Tibagi.

The experiments that compared N_2 fixation in beans grown in monoculture and in relay with maize were not fully conclusive, but it is evident that factors interact in a complex manner to affect N_2 fixation. Future experimentation on intercropping with grain legumes should consider the effects of the production system on N_2 fixation, since available N is often an important constraint to the productivity of the system.

The potential for the various bean lines identified by the Bean/Cowpea CRSP as having enhanced ability to fix N_2 effectively appears to be quite good. In addition, they possess adequate levels of diseaase resistance and good adaptability for different states. The rigorous testing by independent Brazilian agencies in addition to trials at CNPAF/EMBRAPA, give reason to be optimistic.

United States

In addition to the black bean germplasm released, two snapbean germplasm lines have been released to breeders, primarily in the U.S. The application of N fertilizer in the U.S. is still very cost effective, never-the-less the development of cultivars able to fix much of their required N would be desirable. For example, Wisconsin is the leading producer of snapbeans for processing and the majority of production is on sandy soils in Central Wisconsin. Because of the intensive agriculture and this soil type, high levels of nitrate in the groundwater pose a potentially serious

problem. The ability to reduce the amount of N applied would be both cost effective and environmentally sound in that area, realizing that it is not uncommon to recover less than 1/3 of the N applied to a crop of snapbeans.

It is likely that the future release of white dry bean germplasm will be useful to the U.S. dry bean industry as parents for crosses to incorporate improved N_2 fixation. These genetically well-defined lines that differ in their ability to fix N_2 offer unique materials with which to study the genetic control of physiological traits associated with uptake and use of N by grain legumes.

4.0 INSTITUTIONAL AND HUMAN DEVELOPMENT

Two objectives of this project were; 1)to develop well-trained scientific personnel who can continue breeding beans for improved N_2 fixation in their H.C. institutions (e.g., CNPAF/EMBRAPA and Sokoine University, Tanzania), and will serve as a continuing resource in their respective countries and regions for future bean breeding expertise, and 2) to institutionalize, especially at CNPAF/EMBRAPA, the concept of breeding beans for increased N_2 fixation. Since this was the only Bean/Cowpea CRSP Project that emphasized solely N_2 fixation, the results were expected to have an important effect on efforts to alleviate production constraints due to inadequate N. Therefore, if it has been shown to be possible to develop superior bean cultivars able to fix substantial atmospheric N_2, the appropriate methodology, information and plant materials should be made available to other bean improvement programs.

Previous research had shown that, while addition of rhizobial inoculant sometimes increased bean yields, results were often eratic and the returns were uneconomical. Clearly, research to improve the host plant genotype should not follow the example set by previous attempts to improve only one component (the bacteria) of the symbiotic system. Breeding for high-fixing bean cultivars should include selection and testing in the presence of known inoculant rhizobial populations as well as native strains.

Institutionalization of the Concept of Breeding for Increased Nitrogen Fixation at CNPAF/EMBRAPA

There has been continuing discussion among breeders and agronomists about whether selection and testing of potentially superior bean lines should be done either without the benefit of added N or in the presence of higher fertility (i.e., on N-fertile soils and/or with fertilizer N being added). The information gathered to date suggests that effective selection and evaluation for high N_2 fixation should be practiced on low N soils and without adding fertilizer N. If selection is done in the presence of high combined N, it is unlikely that the selected lines will fix large amounts of N_2 when grown on N-poor soils.

At the beginning of this project it was not evident whether bean lines, especially early, type I and type II cultivars, could be bred for increased N_2 fixation. Before the concept of breeding beans for increased fixation could become institutionalized as part of an ongoing breeding strategy at CNPAF/EMBRAPA, other research institutes in Brazil, and elsewhere, it was necessary to obtain data relevant to this question. It is clear, after some 10-15 years of research, that it is possible to improve genetically the ability of most bean culitvars to symbiotically fix atmospheric N_2. The earlier observation that determinate and bush beans generally fixed less N_2 than indeterminate and climbing beans is largely true, but that conclusion should not be interpreted to mean that fixation in determinate and bush beans cannot be increased.

The amount of actual N_2 fixed by various bean lines (and

legumes in general) is difficult to quantify even under the best conditions using isotopic ^{15}N techniques and suitable non-fixing test plants. It is even more difficult to obtain precise estimates for beans growing under less-than-optimal small farm conditions, realizing that no well-defined, adapted non-nodulating bean genotype is abailable. Under favorable field conditions in Wisconsin and using ^{15}N-isotope techniques, the improved blackbean lines have been shown to fix routinely in excess of 50 kg N/ha and up to 100 kg N/ha in some trials. Under less optimal field conditions in Brazil, these lines have been shown to fix up to 30 kg N/ha. Clearly, the cultivars have the potential, but numerous other limiting factors, i.e., presence of effective and efficient rhizobia, soil acidity, low P, high Al, insects, diseases etc. are influencing yields.

Never-the-less, fixation of 20-30 kg N/ha is not inconsequential. It is important to note that usually not more that 30-50% of the actual N applied as fertilizer is recovered in a bean plant under field conditions. To recover 20-30 kg N/ha from applied fertilizer, two to three times that amount (40-90 kg actual N/ha) must be applied, that being at considerable expense to the farmer.

The concept of breeding beans for increased N_2 fixation is now recognized by breeders at CNPAF/EMBRAPA as being feasible and desirable, just as is selection for high yield, disease resistance etc. Trials have shown that lines bred for high N_2 fixation can perform well for other traits as well, and that yields of improved lines can exceed those of standard cultivars over a range of

conditions (see the various tables in the results section).

The presence of effective rhizobia, either native or applied as inoculant, is essential for efficient N_2 fixation to occur. Although this is well recognized in Brazil from previous work, these studies produced some data to suggest that there are useful differences in efficiency among rhizobia. More important, the concept of using a high-fixing bean genotype to distinguish efficiency among strains was shown. The support of the Bean/Cowpea CRSP project for collaborative trials to compare both rhizobial strains over a set of standard bean cultivars and new bean lines over a set of standard rhizobial strains has been important in institutionalizing the concept of optimizing N_2 fixation during breeding and in the production field.

Continuity of Research and Development. After CRSP support for the project at CNPAF is phased-out in May, 1989, the project will be continued under the direction of Dr. Pedro A.A. Pereira. Because of financial constraints in Brazil, it is unlikely that another full-time scientist will be added to replace Dr. Henson as we have suggested. Contributing further to reduced scientist involvement in the near future, is the fact that Mr. Ricardo Silva Araujo is on leave to complete the Ph.D. degree at the Univ. of Wisconsin-Madison. Never-the-less it is important that Mr. Araujo completes the degree to provide greater future scientific capability upon his return.

Even with a reduced scientist component, the project should be able to continue to be productive in the development of superior, high-fixing cultivars under the leadership of Dr.

Pereira. Many promising breeding lines of different types, i.e., Carioca, Goiania Precoce, Mulatinho, and Rosinha are in various stages of testing. New populations are being created by intercrossing superior selections (Table 4.1). Wild bean accessions are being evaluated for unique new characteristics that may not be available in cultivated materials, and there is a rich collection of landrace cultivars from Brazil that are being evaluated by CNPAF. Because much of the methodology required for effective breeding and evaluation of both plant materials and rhizobia has been developed, efforts can be concentrated on developing improved cultivars and identifying optimum bean/rhizobia combinations.

Students from various universities in Brazil often contact scientists at CNPAF to arrange for work experiences or studies for advanced degrees. The N_2 project can continue to provide research opportunities and in this way, involve them in meaningful mission-oriented research.

Adoption of Concepts by Other Programs. During the course of this project, many bean lines from the CNPAF National Genotype Evaluation Program and other research programs were evaluated for N_2 fixation; and selections for N_2 fixation were evaluated within the national program for other important traits (i.e., disease resistance, yield and adaptability). Although the current emphasis in evaluation for N_2 fixation has shifted, from bean lines developed for other traits by other projects to developing breeding lines for improved fixation, lines selected for N_2 fixation potential are not only accepted but also are requested by other CNPAF projects for

Table 4-1. Crosses by other CNPAF breeding programs using lines selected for N_2 fixation.

PEDIGREE	DATE	PROGRAM[1]	CURRENT GENERATION
Honduras 35 x AN512879	07/87	B,An	F_3
Honduras 35 x Mexico 168	07/87	B,An	F_3
Honduras 35 x Mexico 92	07/87	B,An	F_3
Honduras 35 x AN512519	07/87	B,An	F_3
WBR 22-55 x CB511687-1	07/87	An	F_3
WBR 22-55 x A 252	07/87	An	F_3
Mexico 29 x WBR 22-55	07/87	B	F_3
WBR 22-55 x A 248	07/87	Ar	F_3
WBR 22-55 x AN3484-1	07/87	Ar	F_3
WBR 22-8 x LM20952	07/87	Ar	F_3
(AN512575 x XAN30)F_1 x (Honduras 35 x AN512519)F_1	07/87	An	F_3
(Honduras 35 x Mexico 29)F_1x (AN512575 x AN512586)F_1	07/87	An	F_3
(WBR 22-55 x CB511687-1)F_1 x 733002	07/87	An	
84VAN18 x Honduras 35	1988	Y	F_2
AN512567 x WBR 22-52	1988	Y	F_2
WBR 22-34 x 84VAN163	1988	Y	F_2
(WBR 22-55 x A 248)F_1 x Milionario 1732 x LA721477)F_1	1988	Ar	F_1
(WBR 22-55 x A 248)F_1 x 84VAN196	1988	Ar	F_1
(LA721493 x ICA COL 10102)F_1 x (WBR 22-55 x A 248)F_1	1988	Ar	F_1
(ICA Pijao x AN3484-1)F_1 x (WBR 22-8 x LM20952)F_1	1988	Ar	F_1
(ICA Pijao x AN3484-1)F_1 x (WBR 22-55 x AN3484-1)F_1	1988	Ar	F_1
(WBR 22-8 x LM20952)F_1 x (WBR 22-55 x AN 3484-1)F_1	1988	Ar	F_1
(WBR 22-8 x LM20952)F_1 x (Milionario 1732 x LA721477)F_1	1988	Ar	F_1
(WBR 22-8 x LM20952)F_1 x (LA721493 x ICA COL 10103)F_1	1988	Ar	F_1
(WBR 22-55 x AN3484-1)F_1 x (Milionario 1732 x LA721477)F_1	1988	Ar	F_1

Table 4.1 cont'd

PEDIGREE	DATE	PROGRAM[1]	CURRENT GENERATION
(WBR 22-55 x AN3484-1)F_1 x (LA721493 x ICA COL 10103)F_1	1988	Ar	F_1
(Milionario 1732 x AN512879)F_1 (WBR 22-55 x AN3484-1)F_1	1988	Ar	F_1
(WBR 22-55 x A 248)F_1 x LA721419	1988	Ar	F_1
(WBR 22-55 x A 248)F_1 x BGMV 005	1988	Ar	F_1

[1]An = Anthracnose, Ar = Architecture, B = Common Bacterial Blight, Y = Yield.

evaluation and use in their programs. Several lines selected by the CRSP project have already entered breeding programs at CNPAF (Table 4.1).

The project research up to this time has dealt largely with improving the components of the system. The full benefits of growing improved cultivars and applying inoculant when needed will be realized only if the practices are adopted and found to be economically viable by the farmers and the seeds acceptable to consumers. The presence of an "on-farm" research unit at CNPAF/EMBRAPA should allow for continuation of the necessary testing under a variety of real-world conditions. This will be a crucial component to realization of the full potential of N_2 fixation to alleviate physical/environmental constraints imposed by low N fertility.

Linkages With Other Institutions. Increased contact between CNPAF/EMBRAPA and Brazilian state programs is needed to integrate the concept of selection of beans for N_2 fixation into the EPR and state research philosophies and trials. Cooperators and potential cooperators exist in several locations in Brazil, but the level of collaboration should be increased to further integrate N_2 fixation improvement into the Brazilian system of genotype evaluation and selection. Dr. Walter Ribeiro, Jr. of EMGOPA (the Goias state agricultural research organization), Goiania, was hired to select bean genotypes for improved N_2 fixation. Headquartered at CNPAF, his presence offers an ideal opportunity to begin integration of selection for N_2 fixation at the state level. Walter has participated in some aspects of the N_2 fixation project at CNPAF,

but the level of collaboration between the CNPAFand EMGOPA projects should be increased, especially while Ricardo Araujo is on study leave.

There is a small project supported by the IAEA located at CNPAF, with Bob Henson initially, and now Pedro Pereira, as the P.I. This is an important link to other institutions in Brazil (i.e., CENA at Piracicaba) and other countries (i.e., Mexico, Peru, Chile, Costra Rica). There have been important linkages developed by the U.S.P.I. (Bliss) and Co.-P.I. (Dazzo) through their direction of USAID/CSRS Special Grants for N_2 fixation in Honduras and Mexico, respectively.

There is interaction between CNPAF/EMBRAPA and CIAT on several levels. Promising lines from this project are entered routinely in regional trials conducted by Dr. M. Thung of CIAT. There is interaction among bean breeders and rhizobiologists at the two institutions regarding improved N_2 fixation.

Equipment Purchased for CNPAF/EMBRAPA with Project Funds. An important part of enhancing the capability of CNPAF/EMBRAPA to continue research on N_2 fixation has been the purchase of equipment and specialized supplies (Table 4.2). These purchases were not extensive since the Center is well established and has considerable resources.

Professional Degree and Non-Degree Training

Dr. Pedro Pereira earned the M.Sc. and Ph.D. degrees in Plant Breeding and Plant Genetics at U.W.-Madison during this project with Fred Bliss as his major professor. Ricardo Araujo is

currently enrolled in a Ph.D. program in the Plant Pathology Dept. at U.W.-Madison. Both students were funded by EMBRAPA.

Dr. Susan Nchimbi, funded by the W.S.U.-Tanzania Bean/Cowpea CRSP project, earned the M.Sc. and Ph.D. degrees in Plant Breedin and Plant Genetics at U.W.-Madison, with Fred Bliss as her major professor.

Dr. Bob Henson, stationed at CNPAF/EMBRAPA, received post-doctoral training in N_2 fixation research on beans and project management under Brazilian conditions as a research associate on this project.

All CRSP-funded support staff at CNPAF were trained in conducting field, greenhouse, and laboratory research on N_2 fixation in beans.

Each year, CNPAF hosted a 3-week training course for university agronomy students from around Brazil. Researchers in the various disciplines presented background data and results from their research programs. During each training course, a 2-hour to 1/2 day presentation was given on CNPAF/EMBRAPA N_2 fixation research. Laboratory and field visits were included whenever possible. In August 1984, a similar half-day session on N_2 fixation was held at CNPAF as part of a 3-week EMBRAPA training course for extensionists. In 1986 and 1987, presentations of our work on N_2 fixation in beans were given during Agronomic Week at the Federal University of Goias. The audience was primarily agronomy students.

After completion of a bachelor's degree, Brazilian students often enter a non-degree program of additional training as bolsistas at an appropriate research institution. Five people have

Table 4-2. Equipment and reagents purchased with Bean/Cowpea CRSP funds, then donated to CNPAF/EMBRAPA.

Equipment

Air Blast seed cleaner and screens

Blue M gravity convection incubator

Serological H_2O bath

Variable speed, explosion proof shaker with flask holders

Mettler top-loading balance

Heavy duty type 2200 hot plate

Multifuge

Nuova 7 hotplate

Hygrothermograph with replacement charts and pens

Mettler electronic analytical balance

Mighty Small II gel electrophorosis unit and power supply

Udy cyclone grinding mill

Two Isotemp gravity convection drying ovens

Near Infrared N analyzer

Plot thresher

Reagents

^{15}N-enriched ammonium sulphate fertilizer

Miscellaneous reagents for Kjeldahl total N and gel electrophoresis analyses

contributed to the N_2 fixation project at CNPAF as bolsistas (Table 4.3 Project Roster). Each one gainee hands-on experience in the design, installation, management, sampling, sample processing, and data collection and analysis of field and greenhouse experiments, as well as standard laboratory procedures used in N_2 fixation research at CNPAF. In addition to participating in project experiments, each bolsista was responsible for a specific research problem.

At M.S.U., the following people received training in rhizobiology of the Rhizobium phaseoli - Phaseolus vulgaris symbiosis: Ricardo Aruajo, Jaime Maya-Flores, Subash Thaker, Deborah Barnes, Charles Yokoyama, Glenn Harris, Henry Mlozabanda, Jorge Acosta-Gallegos, Catalina Samper, Kathryn Smith, Margaret Welsch, Shiquing Wang, Thomas Olen, and David Baker (Table 4.3). Work on this project by Jaime Maya-Flores, a Visiting Research Scholar in F. Dazzo's lab at M.S.U. will be included in his doctoral dissertation for the Ph.D. degree at the National Polytechnic Institute in Mexico City, Mexico.

Workshop on Nitrogen Fixation at U.W.-Madison

A biological Nitrogen Fixation Workshop sponsored by the Brazil/U.W. project was held July 17-20, 1983 on the campus at Madison and at the Hancock Agricultural Research Station in central Wisconsin. Eighteen students participated: Four from the U.S. and 14 from developing countries. Of the participants, five were women and 13 were men. Presentations were made by Dr. Ken Shapiro, Assoc. Dean International Agric. Programs, CALS, U.W.-Madison; Dr.

Table 4-3. List of Contributors and Participants in the Project

CNPAF/EMBRAPA AND U.W.-Madison

Name	Sex	Position	Dates of Service	Funding Agency
Frederick A. Bliss	M	US PI	Start-9/88	UW
Herbert J. Hopen	M	US PI	9/88-End	UW
Pedro Antonio Arraes Pereira	M	HC PI	Start-7/83	EMBRAPA
		Graduate Student (UW)	8/83-8/87	
		HC PI and Plant Breeder	8/87-End	
Susan F. Nchimbi	F	Graduate Student (UW)	8/82-8/88	CRSP
Ricardo Silva Araujo	M	Rhizobiologist	8/82-12/87	EMBRAPA
		HC PI	8/83-8/87	
		Graduate Student (UW)	1/88-End	
Robert A. Henson	M	Research Associate	3/83-End	CRSP
Carlos Alberto Cavalcante	M	Laboratory Technician	Start-3/84	EMBRAPA
Alfonso Celso da Costa	M	Laboratory Technician	6/84-9/85	CRSP
Amadeu Francisco de Jesus	M	Field Technician	1/83-7/83	CRSP
Abdala Fernandes Borges	M	Field Technician	8/83-1/87	CRSP
Paulo Tadeu Lobo	M	Field Technician	2/87-End	CRSP
Dalva de Fatima Bastos	F	Laboratory Auxilliary	6/85-End	CRSP
Elsa	F	Laboratory Auxilliary	5/88-End	EPAMIG/EMBRAPA
Neyre de Sousa Lobo	F	Laboratory Auxilliary	4/88-10/88	CRSP
Aldimar Ferreira dos Santos	M	Laboratory Auxilliary	Start-2/87	EMBRAPA
Helio Dionizio de Rezende	M	Field and Lab. Staff	Start-12/87	EMBRAPA
Jose Simiao de Rezende	M	Field and Lab. Staff	6/85-End	CRSP
Tiago Monteiro Damasceno	M	Field Staff	Start-End	EMBRAPA
Jose Aparecido Damasceno	M	Field Staff	6/86-8/86	CRSP
Sandro Vaz da Silva	M	Field Staff	2/88-End	EMBRAPA
Lilian Ferro da Cunha	F	Bolsista	8/84-7/85	CNPq
Eliane Lopes	F	Bolsista	2/87-11/88	CRSP
Ester de Moura Rios	F	Bolsista	2/87-7/87	CRSP
			8/87-1/88	CNPq
Jose Rodrigues de Moura	M	Bolsista	2/88-7/88	CNPq
			8/88-11/88	CRSP
Claudia Regina de Souza	F	Bolsista	8/88-3/89	None
Jesuino Andriollo	M	Graduate Student (UFG)	4/88-End	None
Paulo Marra	M	Graduate Student (UFG)	4/88-End	None
Susan Nchimbi	F	Graduate Student (UW)	5/85-3/88	CRSP (WSU)

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Table 4-3 cont'd

Michigan State University

Name	Sex	Position	Year	Location
Frank Dazzo	M	Professor, Co-PI	1981-9	MSU
Pedro Pereira	M	Visitor	1982	EMBRAPA, Goiania
Subash Thaker	M	Lab Aide	1982	MSU
Deborah Barnes	F	Lab Aide	1983	MSU
Charles Yokoyama	M	Lab Aide	1983-4	MSU
Ricardo Araujo	M	Visiting Scholar	1983	MSU
Henry Mlozabanda	M	Graduate Student	1983	MSU/Malawi
Jaime Maya-Flores	M	Visiting Scholar	1984-9	ITC/MSU
Alvin Smucker	M	Professor	1985	MSU
Maxwell Mortland	M	Professor	1985	MSU
Gene Safir	M	Professor	1985	MSU
Kathryn Smith	F	Lab Technician	1986	MSU
Margaret Welsch	F	Lab Technician	1986	MSU
Jorge Acosta-Gallegos	M	Graduate Student	1986	MSU/Mexico
Thomas Olen	M	Undergrad. Student	1986	MSU
Maria Valdes	F	Visiting Scholar	1986	IPN/Mexico
Rawle Hollingsworth	M	Research Associate	1986	MSU
Chris Cheng	M	Professor	1986	MSU
Thomas Olen	M	Lab Technician	1987-8	MSU
Eldor Paul	M	Professor	1988	MSU
David Harris	M	Research Associate	1988	MSU
Siquing Wang	F	Visiting Scholar	1988	Peoples Rep. China
Maureen Peterson	F	Visiting Scholar	1988	Univ. Florida
David Baker	M	Lab Technician	1989	MSU

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Frank Dazzo, U.S. Co-P.I., M.S.U.; Dr. Joe Burton, NifTAL Research Project, Univ. of Hawaii; Dr. Peter Graham, Univ. of Minnesota; Dr. Fred Bliss, U.S. P.I.; Mr. Juan Carlos Rosas, Research Assistant, U.W.; and Ms. Dina St. Clair, Research Assistant, U.W. A handbook of workshop materials was prepared and distributed to participants and other interested persons.

5.0 DISSEMINATION OF BIOLOGICAL MATERIALS AND INFORMATION

Bean Cultivars and Improved Plant Germplasm

Release of Improved Black Bean Germplasm Lines. Five common bean (*Phaseolus vulgaris* L.) germplasm lines of the black bean class of dry beans, WBR22-3, WBR22-8, WBR22-34, WBR22-50, and WBR22-55 (Reg. no. GP-71-PI520603, GP-72-PI520604, GP-73-PI520605, GP-74-PI520606, GP-75-PI520607, respectively) were developed, tested cooperatively and released in 1988 by personnel of the College of Agricultural and Life Sciences, University of Wisconson-Madison and the Centro Nacional de Pesquisa de Arroz e Feijao-Empresa Brasileira de Pesquisa Agropecuaria (CNPAF-EMBRAPA),. Goiania, Goias, Brazil (Bliss et al., 1989) (See p 5-11, this report). Seed of these lines is available from either of the two participating institutions.

Designation of Improved Black Bean Lines as Recommended Cultivars in Brazil. As of July, 1989, it is anticipated that three black bean lines selected by the Project as being good nitrogen fixers, will be released in Brazil as recommended cultivars WBR22-24 in Rio Grande do Sul; WBR22-50 in Minas Gerais and Honduras 35 in Rio de Janeiro states. Prior to release as recommended cultivars, the experimental lines were grown extensively by official organizations for observation and comparison to standard cultivars. We believe that this positive evaluation by independent groups provides a good indication that these cultivars will make a valuable contribution to improved bean productivity in Brazil and perhaps elsewhere.

Distribution of Improved Snapbean Germplasm. The seed of two snapbean germplasm lines, SFX11-048 and SFX11-073, has been distributed in 1988, primarily to commercial breeders. Others may request seed from the Department of Horticulture, University of Wisconsin-Madison. These breeding lines possess desirable snapbean traits, fix high levels of nitrogen, and have high levels of field resistance to root rot inciting pathogens. A formal description for the release of these lines will be published in HortScience.

Other Improved Germplasm. The release of germplasm lines of the small white and white navy classes of dry beans is anticipated in the future. Inbred backcross lines resulting from the initial crosses of Sanilac x Puebla 152 and Ex Rico 23 x Puebla 152 have undergone field testing in Wisconsin and have shown increased levels of N_2 fixation, higher yields and other desirable traits (e.g., St. Clair et al., 1988).

It is anticipated that plant germplasm resulting from this project will be released through CNPAF/EMBRAPA for several years to come, since there are many promising lines in various stages of evaluation. Currently, the most promising of these are in the Goiania Precoce, Rosinha, and Carioca classes.

Rhizobial Strains

Peat based inoculants of the most effective nitrogen fixing strains of *R. leguminosarium* biovar *phaseoli* identified in this project on bean line U.W.21-58 were prepared and disseminated to investigators for use in field trials. The recipients included:

R. Araujo and R. Henson, this project; Jaime Maya-Flores, Instituto Tecnologico de Celaya, Celaya, Mexico; M.W. Adams, Michigan State University for use in the Bean/Cowpea CRSP projects in Malawi and Mexico.

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REGISTRATION OF FIVE HIGH NITROGEN FIXING COMMON BEAN GERMPLASM LINES

FIVE common bean (*Phaseolus vulgaris* L.) germplasm lines of the black bean class of dry beans, WBR22-3, WBR22-8, WBR22-34, WBR22-50, and WBR22-55 (Reg. no. GP-71-PI520603, GP-72-PI520604, GP-73-PI520605, GP-74-PI520606, and GP-75-PI520607, respectively) were developed, tested cooperatively, and released in 1988 by personnel of the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and the Centro National de Pesquisa de Arroz e Feijao—Empresa Brasileira de Pesquisa Agropecuaria (CNPAF-EMBRAPA), Goiania, Brazil. The lines, tested as numbers 22-3, 22-8, 22-34, 22-50, and 22-55, respectively, were selected from a population of inbred backcross (BC_2S_3) lines resulting from the cross of 'ICA Pijao' (recurrent parent) × 'Puebla 152' (black donor parent) and produced following the procedure described by McFerson et al. (1).

The five lines have an erect, Type II plant habit with a medium guide stem developing. Seeds are dull (opaque) black similar to ICA Pijao, the recurrent parent, but slightly larger than ICA Pijao. In a 1985 trial at CNPAF-EMBRAPA, the 50-seed weights of the five lines ranged from 8.5 g (WBR22-50) to 10.0 g (WBR22-3 and WBR22-8) compared to 7.4 and 8.2 g for 'Rio Tibagi' (a standard Brazilian cultivar) and ICA Pijao, respectively. Although these breeding lines were not selected specifically for disease resistance, field observations at CNPAF-EMBRAPA in Brazil have indicated that they possess at least moderate levels of resistance to bean rust, caused by *Uromyces phaseoli* (Reben) Wint., and angular leaf spot, caused by *Isariopsis griseola* Sacc. Each of the five lines carries the single dominant I-gene form of resistance to bean common mosaic virus, based on the occurrence of the total necrosis reaction when inoculated with the NL 3 strain in greenhouse tests at CNPAF-EMBRAPA (de Faria, J., Personal communication, 1987).

Field tests in Brazil and Wisconsin have shown that the lines fix more atmospheric N_2 than Rio Tibagi, based on comparisons using total accumulated shoot N, ^{15}N isotope dilution, acetylene reduction activity; and visual evaluation of nodulated roots, nodule mass, and grain yield of plants grown on soils where N is limiting. Although actual levels of N_2 fixation will vary, often depending on environmental factors such as soil characteristics, native rhizobia, moisture, etc.; the relative superiority of these lines compared to Rio Tibagi are consistent. In one field trial in Wisconsin and three field trials in Brazil, the five breeding lines accumulated from 3 to 42% more total N in the shoots than Rio Tibagi. In Brazil, when ^{15}N isotope dilution was used to estimate fixation, WBR22-34 had 29% more fixed N_2 in the shoot an 109% more fixed N_2 in the seeds than Rio Tibagi. In WBR2 34, 44% of the seed N was derived from fixation, compare to 35% in Rio Tibagi (Saito, S.M.T., personal communica tion, 1986). Indirect measures of fixation, such as total shoc N, which are positively correlated with the amount of N fixed by plants grown on low-N soils, indicate that the amoun of N_2 fixed by the other four breeding lines are comparabl to WBR22-34.

The grain yields of the five breeding lines usually hav been higher than that of Rio Tibagi, whether fixed atmos pheric N_2 or fertilizer N is the primary N source. In a 198 trial at CNPAF-EMBRAPA, where fertilizer N was not ap plied, the yields of the breeding lines exceeded that of Ri Tibagi by 10 to 25%. In the National Preliminary Yield Trial at 16 sites in Brazil during the 1984–1985 and 1985–198 growing seasons, the mean yield of each breeding line ex ceeded the standard Rio Tibagi by the following amounts WBR22-8, + 22%; WBR22-55, + 18%; WBR22-3, + 18% WBR22-50, + 13%; and WBR22-34 + 10%. Based on mear yields over 16 sites, WBR22-8 ranked 8th; WBR22-3, 12th WBR22-55, 16th; WBR22-50, 25th; and WBR22-34, 34th among 81 entries, which included four local cultivar checks at each of the 16 sites.

Small amounts of seeds can be obtained by writing to F.A. Bliss at Dep. of Horticulture, Univ. of Wisconsin, Madison, WI 53706, or to P.A.A. Pereira at CNPAF-EMBRAPA, Caixa Postal 179, 74000 Goiania, Goias, Brazil.

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References and Notes

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2. Dep. of Horticulture, Univ. of Wisconsin, Madison 53706; and P.A.A. Pereira, R.S. Araujo, R.A. Henson, M.G. Teixeira, and C.C. da Silva, CNPAF-EMBRAPA, Caixa Postal 179, 74000 Goiania, Goias, Brazil. Registration by CSSA. Accepted 30 June 1988. *Corresponding author.

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6.0 RESEARCH RESULTS

Introduction

Initial estimates of N_2 fixation based on indirect measures of fixation or fixation potential, such as nodulation characteristics (e.g., mass, number, visual ratings), estimates of composition of xylem sap (e.g., ureide content), supported the general conclusion that most common bean cultivars fixed small amounts of atmospheric N_2. Therefore, beans grown on N-poor soils without the benefit of fertilizer N were likely to be low yielding, in part because of inadequate N for support of vigorous plant growth, development of pods and seed maturation.

Although these indirect measures of N_2 fixation have not proved to be precise enough for good quantitative measures of N_2 fixation, the presence of relatively large differences among cultivars based on nodule traits and acetylene reduction activity, led to the conclusion that there was genotypic variability among bean lines for greater N_2 fixation ability. When better methods of estimating N_2 fixation based on application of ^{15}N-labelled fertilizers were used, some lines such as 'Puebla 152' and 'Cargamento' which had been identified as high-fixing based on indirect measures, were verified as being superior. Puebla 152 has been used extensively as a high-fixing parent in crosses made to study the inheritance of variability for N_2 fixation and to develop variable populations in which to select for improved fixation. The identification of the genetically superior parent, Puebla 152, by Graham and co-workers at CIAT proved to extremely important to the success of this CRSP project.

Prior to initiation of the Bean/Cowpea Project, collaborative research on improving N_2 fixation was sopported through a CSRS/AID Special Research Grant to the U.W.-Madison and the Escuela Agricola Panamericana (E.A.P.) in Honduras. Crosses were made between Puebla 152 and the cultivars Porrillo Sintetico, Jamapa, ICA Pijao, Samilac and Ex Rico 23 to develop five populations of inbred backcross lines in which to study the imheritance of differences in N_2 fixation and to select for improved small white and black-seeded breeding lines. Evaluation of four populations, one each of Porrillo Sintetico, Jamapa, Sanilac and Ex Rico 23 x Puebla 152, was conducted and reported by McFerson (1983) and Rosas (1983). Their results, based on acetlyn reduction and yield (McFerson, 1983) and visual evaluation (Rosas and Bliss, 1986) to estimate fixation potential provided evidence that increased fixation was a heritable trait and that selection of improved breeding lines with suitable agronomic traits was feasible. Evaluation of the population of inbred backcross lines resulting from ICA Pijao x Puebla 152 was made as a part of this Bean/Cowpea project and will be reported on in detail.

Prior to initiation of this project, one of the major concerns expressed was that there seemed to be "premature nodule senescence on many field-grown beans. The specific cause(s) of this general phenomenon were not known. Presumably, factors associated with the plant, rhizobia, some interaction of the symbionts and physical and environmental factors could be responsible. Although considerable progress has been made toward understanding nodule senescence, still the precise causes and the magnitude of this problem are not

understood fully. Studies on the microbiology, physiology, cytology and biochemistry of premature nodule senescence conducted during this Bean/Cowpea CRSP Project are summarized later in this report. Regardless, progress has been made in breeding bean lines and identifying rhizobia that together result in fixation of substantial atmospheric nitrogen.

Improved Plant Materials

Breeding Methods. Choice of an efficient breeding method is essential to the success of each breeding program. Factors to be taken into account include; type of cultivar used commercially (e.g., pure line or mixtures of near-homozygous plants), method of reproduction (i.e., self-fertilization), inheritance patterns and heritability of the trait(s) being improved, methods used to discriminate between superior and inferior phenotypes, unit of selection (e.g., single plant or family selection), adaptedness of the source of enhanced variability (e.g., parents which are cultivars or poorly-adapted accessions), and ease of recovery of agronomically-acceptable breeding lines which are potential new cultivars.

Based on these considerations, the inbred backcross line method (Fig. 6-1) was used to develop populations of segregating families. The lines were grown in replicated field trials on low-N soils, with evaluation based on family rather than single plant performance. Usually, estimates of fixation were made on five plants in a plot with the remaining ten plants being used for evaluation of traits such as days to maturity, plant lodging, seed

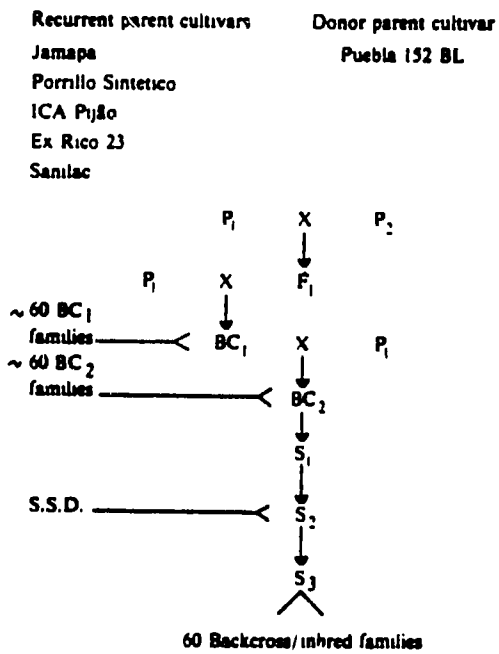


Figure 6-1. Schematic diagram of the method used to develop inbred backcross lines of common bean selected for increased BNF in the Bean/Cowpea CRSP Project (Source: McFerson et al., 1982).

yield and seed characteristics.

Methods for Assessing Fixation. Although used widely, estimates of N_2 fixation based on acetylene reduction activity are not sufficiently accurate for quantitative and comparative purposes. Therefore, methods based on the addition of either ^{15}N-enriched or ^{15}N-depleted ammonium sulphate applied to growing bean plants were utilized to obtain more precise estimates of N_2 fixation. Such estimates are expensive and require use of a mass spectrometer, thus limiting their application to analysis of relatively few experimental units.

To develop a simple, cheap, and accurate method of estimation, comparisons were made with other parameters. A high positive correlation was found between N_2 fixed based on isotope dilution methods and total accumulated N when plants were grown on low-N soils (St. Clair et al., 1988). A suitable non-fixing check plant is desirable to facilitate estimation, and in the absence of a non-nodulating bean, a non-nod. soybean line was used effectively in most situations.

Selection Within Breeding Populations. Selection within Population 22, the population of inbred backcross lines resulting from the cross of ICA Pijao x Puebla 152, was carried-out in the low-N field at the Hancock Agricultural Research Station in Wisconsin, at CNPAF/EMBRAPA in Brazil and in a controlled environment room at the U.W.-Madison. The results of these experimants have been described by Pereira (1987) and Pereira and Bliss (1989). Based on extensive field trials, five promising black-seeded lines have been released and registered as high N_2-

fixing common bean germplasm lines by the U.W.-Madison and CNPAF/EMBRAPA (Bliss et al., 1989).

Pereira (1987) compared the breeding lines in Pop. 22 with the parents ICA Pijao and Puebla 152, for ability to fix N_2 under different levels of phosphorus in controlled growth room conditions. The relative performance of lines in the growth room was compared to N_2 fixation under low-N field conditions in Wisconsin. Two lines, 22-28 and 22-34, were efficient fixers under controlled phosphorus levels and also fixed high levels of N_2 in the field (Table 6-1). Line 22-27 which was a high-fixing line on the low-N soil conditions in Wisconsin (Table 6-2), has been among the promising black-seeded lines in 1986/88 EPR field tests conducted by CIAT in Brazil (Table 6-3) (CIAT Annual Report, 1987).

Field evaluations of the lines in Pop. 22 at CNPAF/EMBRAPA for fixation based on acetylene reduction, total shoot N, and seed yield were used to identify lines with greatest potential as cultivars. Five lines, WBR22-3, WBR22-8, WBR22-34, WBR22-50, and WBR22-55, have shown consistently good performance in Brazilian field trials and have been released as "High Nitrogen Fixing Common Bean Germplasm Lines" (Bliss et al., 1989). Depending on the location, these five lines have been consistently among the best 10 black-seeded entries in field tests conducted by CIAT in Brazil (Table 6-4). These lines are now in Brazilian state trials (Table 6-5) to determine whether they will be released as commercial cultivars for wide-spread production.

<u>Continuation of Breeding for N_2 Fixation at CNPAF/EMBRAPA</u>. With the return of Dr. Pedro Pereira to CNPAF/EMBRAPA after

Table 6-1. Mean shoot N content and shoot dry weight of four - inbred backcross lines compared to the recurrent parent, ICA Pijao, the donor parent, Puebla 152 and the Brazilian black bean cultivar, Rio Tibagi (Source: Pereira, 1987).

Line	Shoot N content[a] (mg/2plants)	Shoot dry weight (g/2 plants) 100 mM P	400 mM P
22-43	115.1	2.62	4.90
22-28	109.9	2.95	4.22
22-52	106.2	2.24	3.90
22-16	105.9	2.37	4.01
ICA Pijao	85.2	2.24	3.71
Puebla 152	109.3	2.22	4.66
Rio Tibagi	71.8	1.62	2.12
LSD (0.05)	16.0	0.97	

[a] averaged over two P levels.

Table 6-2. Mean shoot N content, shoot dry weight, seed yield and N_2-ase activity of 54 inbred backcross bean lines compared to the recurrent parent ICA Pijao, donor parent Puebla 152, and Brazilian black bean cultivar, Rio Tibagi grown in a low-N soil at Hancock, WI 1984 (Source: Pereira, 1987).

Line	Shoot N content (mg/plant)	Shoot dwt. (g/plant)	Seed yield (kg/ha)	N_2-ase activity (umol C_2H_2/h/pl)
22-27	331	10.3	2082	23
22-06	302	9.6	1857	21
22-43	279	9.1	1705	23
22-49	254	8.0	1405	12
22-28	252	8.1	1635	18
ICA Pijao	205	6.7	1257	15
Puebla 152	283	9.0	3337	30
Rio Tibagi	160	5.9	1047	6
LSD (0.05)	67	1.9	495	10

Table 6-3. The ten best black bean lines from EPR trials in Brazil, 1986/88 (Source: CIAT Annual Report, 1987).

	IPAGRO/RS		CNPFT 1/RS		CNPFT 2/RS		CNPFT 3/RS	
	ADVANCED BREEDING LINES							
01	AN 512573-0	1500	PRETO CARIRI	1050	AN 512638-0	2640	AN 512574-0	3247
02	BZ 1719-2	1238	AN 512575-0	1050	HONDURAS 35	2283	AN 512575-0	2765
03	84 VAN -163	1150	W 22-24	1017	AN 512573-0	2240	AN 512567-0	2174
04	W 22-52	1150	BZ 1719-2	950	BZ 1719-2	2090	W 22-52	1994
05	PRETO CARIRI	1113	W 21-58	833	84 VAN - 196	2070	AN 512573-0	1943
06	W 22-27	1100	AN 512646-0	783	84 VAN - 163	2050	84 VAN - 18	1941
07	AN 512572-0	1088	W 22-52	750	W 22-24	2050	AN 512572-0	1834
08	84 VAN - 18	1088	AN 512630-0	700	AN 512646-0	2040	W 22-24	1783
09	AN 512567-0	1075	AN 512572-0	700	84 VAN - 18	1990	HONDURAS 35	1720
10	AN 512575-0	1000	84 VAN-196	700	AN 512631-0	1980	84 VAN - 163	1644
X10		1150		853		2143		2105
	ELITE CHECKS							
01	LM 30074-0	775	LM 30074-0	517	LM 30074-0	2130	LM 30074-0	1694
02	LM 21135-0	375	LM 21135-0	550	LM 21135-0	1770	LM 21135-0	1570
03	CARIOCA	350	CARIOCA	1017	CARIOCA	1870	CARIOCA	1801
04	RIO TIBAGI	775	RIO TIBAGI	1050	RIO TIBAGI	0	RIO TIBAGI	1304
X4		569		784		1442		1592
	LOCAL CHECKS							
01	GUATEIAN 6662	1138	GUATEIAN 6662	433	RAI 76	1750	FT 83-120	2481
02	RIO TIBAGI	638	RIO TIBAGI	433	RIO NEGRO-EMPASC	1280	RAI 76	1213
03	MAQUINE	563	MAQUINE	583	FT 83-120	1570	TURRIALBA 4	941
04	TURRIALBA 4	788	TURRIALBA 4	400	TURRIALBA 4	1360	RIO NEGRO-EMPASC	1034
X4		782		462		1490		1417
EXP. MEAN X36		871		589		1814		1495
LSD (5%)		408		456		676		733
CV (%)		22.5		39.4		17.9		23.8

	CNPAF/GO		ANAPOLIS/GO		BARREIRAS/BA	
	ADVANCED BREEDING LINES					
01	HONDURAS 35	1689	AN 512646-0	1479	PRETO CARIRI	1657
02	AN 3508	1426	HONDURAS 35	1456	AN 512575-0	1447
03	SPB 5	1337	SPB 5	1449	W 22-24	1334
04	W 22-14	1273	AN 3508	1434	BZ 1719-2	1293
05	AN 512573-0	1204	AN 512574-0	1351	W 21-58	1281
06	W 21-58	1177	AN 512637-0	1306	AN 512646-0	1263
07	W 22-27	1133	AN 512575-0	1299	W 22-52	1246
08	AN 512637	1117	W 22-27	1254	AN 512630-0	1181
09	W 22-24	1104	AN 512567-0	1231	AN 512572-0	1179
10	SPB 1	1096	VAGEM ROXA.EV BR.	1224	84 VAN-196	1143
X10		1256		1348		1302
	ELITE CHECKS					
01	LM 30074-0	666	CARIOCA	938	LM 30074-0	1210
02	LM 21135-0	1073	RIO TIBAGI	841	LM 21135-0	999
03	CARIOCA	1006	LM 30074-0	1351	CARIOCA	1445
04	RIO TIBAGI	774	LM 21135-0	578	RIO TIBAGI	773
X4		880		927		1107
	LOCAL CHECKS					
01	BAT 1647	998	SPB 1	1351	CARIOCA	1761
02	BAT 451	946	PRETO CARUARU	1014	IPA 74-19	1218
03	BAT 67	1358	RIO NEGRO	983	EPABA 1	947
04	ICA COL 10103	1061	RICO 23	908	MIL.VAGEM.ROXA	679
X4		1091		1064		1151
EXP. MEAN X36		1003		1099		1068
LSD (5%)		443		584		528
CV (%)		22.5		27.1		24.0

Table 6-4. The 10 best black advanced lines and their common and local checks tested in 1984-1986 EPR over 4 locations in the southeast of Brazil (Source: CIAT Annual Report, 1986).

	CAMPOS84*		EMCAP8586*		PESAG8586*		PESAG8586*		EMGO8586*	
Advanced breeding lines:										
01	NAG 24	1948	82 B VAN 38	954	W 22-8	1380	BAT 1037	2257	NEGRITO 897	1432
02	W 22- 3	1899	LM 30063	904	LM 21132	1287	LM 30036	2179	W 22- 3	1293
03	W 22-55	1826	NAG 25	899	IPA 74-19	1280	NAG 15	2067	LM 21124	1280
04	NAG 87	1692	LM 30074	858	NAG 24	1259	LM 10360	2048	RIOO 1735	1186
05	LM 30063	1664	NAG 26	848	LM 30063	1225	NAG 24	1982	BAT 871	1140
06	82 B VAN 38	1644	LM 21132	834	LM 10364	1223	LM 10364	1959	LM 10360	1137
07	LM 20816	1605	82 B VAN 39	813	LM 21135	1186	82 B VAN 39	1955	82 B VAN 74	1119
08	NAG 26	1554	LM 10360	743	BAT 871	1173	LM 10377	1940	LM 21135	1104
09	LM 20720	1545	W 22-50	742	CNF 0376	1156	NAG 26	1910	LM 10364	1076
10	82 B VAN 39	1505	CNF 295	738	LM 30074	1146	CNF 295	1899	NAG 40	1075
$\bar{X}_{10}$		1853		833		1232		2020		1184
Common Checks:										
01	JALO EEP 558	1131	JALO EEP 558	549	JALO EEP 558	1005	JALO EEP 558	1388	JALO EEP 558	761
02	CORNELL 49242	1550	CORNELL 49242	470	CORNELL 49242	898	CORNELL 49242	1652	CORNELL 49242	529
03	CARIOCA	1479	CARIOCA	606	CARIOCA	1022	CARIOCA	1580	CARIOCA	724
04	RIO TIBAGI	811	RIO TIBAGI	327	RIO TIBAGI	651	RIO TIBAGI	1203	RIO TIBAGI	872
$\bar{X}_4$		1243		488		894		1456		722
Local Checks:										
01	T.LOCAL 1	1391	CAP.PRECOCE	1053	T.LOCAL 1	753	BR1 XODO	2033	EMGOPA OURO 1	819
02	T.LOCAL 2	1470	COSTA RICA 1031	89	T.LOCAL 2	691	BR2 GDE RIO	1350	EMGOPA OURO 2	908
03	T.LOCAL 3	1505	RIOO 1735	526	T.LOCAL 3	561	BR3 IPANEMA	1755	EMGOPA OURO 3	844
04	T.LOCAL 4	993	ESAL 1	720	T.LOCAL 4	881	C.PRECOCE	2054	EMGOPA OURO 4	831
$\bar{X}_4$		1340		597		722		1798		851
MEAN(81)		1218		535		908		1595		849
LSD(5%)		524		338		372		609		499
CV(%)		21.9		32.2		20.9		19.5		29.9

* CAMPOS84 = PESAGRO - Campos - Rio de Janeiro; EMCAP8586 = EMCAPA - Venda Nova - Espirito Santo
PESAG8586 = PESAGRO - Campos - Rio de Janeiro; PESAG8586 = PESAGRO - Campos - Rio de Janeiro; EMGO8586 = EMGOPA - Goiania - Goias

Table 6-5. Brazilian state trials conducted by PESAGRO during 1984/86 and 1985/86 (Source: CIAT Annual Report, 1986).

Identification	Wet Season 1985/86			Dry Season 1986		
	Campos	Itaocara	Natividad	Campos	Bom Jesus do Itabapoana	Mean
	kg/ha					
1. PV 99 N	1826	473	668	1143	2040	1230
2. LM 30063	1633	520	799	850	2260	1212
3. A 222	1640	433	784	1023	1766	1129
4. EMP 84	1700	433	527	890	1900	1090
5. LM 21124	1603	420	735	763	1860	1076
6. NAG 24	1600	386	464	910	1873	1047
7. LM 30036	1446	340	345	833	2013	995
8. LM 10363	1600	333	299	890	1786	982
9. W 22-3	1366	466	297	903	1873	981
10. LM 20816	1396	366	326	686	1966	948
11. W 22-8	1286	313	426	796	1733	911
12. LM 00574	1413	200	92	913	1833	890
13. 82 B VAN 74	1093	366	378	-	-	612
14. NAG 87	953	500	219	-	-	557
15. W 22-55	980	333	340	-	-	551
CHECKS						
1. BR 2-Grande Rio	1906	433	567	1220	1926	1210
2. BR 1-Xodo	1786	453	446	1200	1873	1152
3. BR 3-Ipanema	1406	666	469	930	1826	1059
4. Capixaba	1953	526	680	-	-	1053
5. Porrillo Sintetico	1706	566	498	-	-	923

IDENT.	Wet season 84/85	Dry season 85	Wet season 85/86	Dry season 86	Mean
	kg/ha				
1. NAG 24	1946	2038	2033	992	1752
2. LM 30036	1237	2073	2283	1066	1664
3. NAG 15	1473	1885	2033	1066	1614
4. LM 30063	1661	1825	1800	1125	1602
5. LM 10360	956	1921	2100	1316	1573
6. W 22-55	1826	1861	1425	1083	1548
7. A 231	1486	1833	1558	1283	1540
8. LM 21124	1297	2088	1600	1150	1533
9. 82 B VAN 39	1505	1581	1866	1158	1527
10. W 22-3	1898	1725	1633	466	1511
11. DOR 218	1393	1720	1466	1458	1509
12. W 22-8	1452	2080	1816	683	1507
13. LM 10363	1384	2006	1966	656	1503

completion of the Ph.D. degree in Plant Breeding and Plant Genetics at the U.W.-Madison and the departure of Dr. Fred Bliss from U.W.-Madison, breeding responsibilities for increased N_2 fixation have entirely to CNPAF. The following is the current status of the breeding program within each seed type or group.

Carioca. From the cross between Carioca and BAT 336, three lines were selected at U.W. with high yield potential and excellent N_2 fixation capacity, but with a non-erect plant type similar to Carioca. The BC_1S_1 families derived from one backcross to Carioca were planted in the field in Wisconsin and individual plants with improved architecture were selected. One seed of each selected plant was adnvanced by SSD. Field evaluation of these 129 lines was begun at CNPAF in 1989.

Black. Six crosses were made within population 21, previously developed from a cross between Porrillo Sintetico x Puebla 152 (McFerson et al., 1982):

W21-16 x W21-38

W21-16 x W21-43

W21-16 x W21-58

W21-38 x W21-43

W21-38 x W21-58

W21-43 x W21-58

Forty lines from each cross were advanced by SSD and were planted in a field evaluation in 1989.

The F_1 of Cargamento x Preto Cariri was crossed with ICA Pijao and Porrillo 70, thus developing two populations of 68 and 45 families, respectively. In 1989, the BC_2S_2 generation was planted

as single rows in the field at CNPAF for early selection for yield potential and N_2 fixation.

Rosinha. From the cross Rosinha G2 x W24-21, 28 BC_1S_2 lines were selected for seed type at U.W.-Madison. Field evaluation of these lines for N_2 fixation was begun at CNPAF in 1989.

Mulatinho. The F_1 of Cargamento x Preto Cariri was crossed with Riz 29. The 62 BC_2S_2 lines developed from this cross were planted in single-row plots at CNPAF in 1989 for early selection for yield potential and N_2 fixation.

Short-season. With advances in irrigation technology for use in Brazil, interest in early-maturing beans for winter (dry season) planting has increased. From the cross W24-21 x Goiano Precoce, 60 BC_2S_2 lines were developed. These materials were planted in a field evaluation at CNPAF in 1989.

Wild beans. After two cycles of selection in the greenhouse at CNPAF, six wild bean lines were chosen for use as parents in the breeding program for N_2 fixation. Each of these lines was crossed with lines A 247(Carioca), Rubi(Rosinha), and AN 350910(anthracnose resistant). In early 1989, the first backcross was begun on these populations.

IAEA REGIONAL PROGRAM

The CNPAF Microbiology Project has participated since 1986, in an International Atomic Energy Agency (IAEA) program to enhance symbiotic N_2-fixation by Phaseolus vulgaris L. in Latin America. The objectives of this research were to quantify N_2 fixed by elite bean lines selected for N_2-fixation at CNPAF, to evaluate potential non-N_2-fixing control crops for use with the isotope dilution technique (IDT), and to compare applying all the ^{15}N label with sugar before planting with adding the ^{15}N in split applications without sugar.

In the first experiment, 16 bean lines and four grasses (non-fixing checks) were evaluated in a split-pilot design with three replicates. The main plot factor was method of ^{15}N application: either before planting addition with sugar or split applications. The plot area not labeled with ^{15}N was fertilized with the same amount (10 kg N ha^{-1}) of natural-abundance N and at the same times as the isotope-treated area. The subplot factor was different bean lines/grass control crop. Samples were taken twice during the vegetative growth phase and twice during the reproductive phase to determine shoot, root, and nodule dry matter accumulation and shoot total N concentration and content. The sample at the second reproductive phase was taken from the ^{15}N-labeled area, but a drying oven caught fire and most of the ^{15}N samples were lost. At

maturity, seed and stover (stems + roots + pod walls, without senesced leaves) dry matter and total N were measured.

The experiment was repeated in 1987 with 17 bean lines and three grass treatments. Samples were taken twice during the vegetative growth phase and once during the mid-reproductive phase to determine shoot, root, and nodule dry matter accumulation and shoot total N concentration and content. At approximately physiological maturity (66 DAE), when the bean leaves were beginning to senesce, a sample was collected for determining the ^{15}N concentration and content. On that date, bean samples were divided into pods and stems + leaves for separate analyses. Weighted data from these subsamples were combined for whole shoot analyses. Grain yield, seed moisture content, and seed weight were determined at maturity.

Total N analyses were performed at CNPAF. The 1987 ^{15}N samples were analyzed at the IAEA Seibersdorf Laboratory. Data were analyzed using the MSTAT program. The 1987 results are discussed in detail, since they were more complete and are typical of results from both experiments.

Split application of N fertilizer during the growing season was complicated by difficulties in transporting water to the experimental site and synchronizing fertilizer with irrigations and samples. Although the same mass of N and ^{15}N was applied in both treatments, disproportionately larger applications of N were added during the reproductive phase which may have influenced data collected at 66 DAE.

Nodule dry weight during vegetative growth was reduced by applying all of the N prior to planting, but there were no other significant differences due to method of N application through the 51 DAE sample. Although the application of all N prior to planting produced higher dry matter in all plant parts studied at 66 DAE, the split N treatment appeared to alter the mass distribution of both N species to the pods rather than other organs (Table 6-6). Concentrations were unaffected.

The difference method (DM) estimates of N_2 fixation for the first three samples and IDT estimates at 66 DAE were higher with the split N application than with one application prior to planting. Although the mass of N added in the one-application treatment was small (10 kg N ha^{-1}), apparently the bean plants were sensitive to this level, as shown by reduced nodule mass, quantification estimates, and data from other experiments (R.A. Henson, unpublished data). Thus, N_2 fixation probably was higher when the N fertilizer was added in smaller amounts throughout the growing season. Harvest parameters measured were not significantly affected by method of N application (Table 6-7). The application of all N prior to planting produced a higher grain yield, but this may be a reflection of applying the final split N applications relatively late in the growing season, when N fertilizer is less effective.

Nodulation was relatively sparce, and by the mid-reproductive phase (51 DAE), most nodules had senesced. Among lines, Negro Argel, Carioca, WBR 22-24, and Honduras 35 produced the best early-

Table 6-6. Effect of applying N fertilizer in 1 application or in 6 split applications on mean dry matter and N contents of beans at 66 DAE (R7) in the IAEA ^{15}N Genotype Evaluation, CNPAF, August-November 1987. Values are means of 3 replicates and are on a per plant basis.

		Mass				Concentration		
Measurement	N Applications	Whole Shoot	Pods	Stems & Leaves	Pods: Whole Shoot	Whole Shoot	Pods	Stems & Leaves
		---- g plant $^{-1}$ --------				-------- % ----------		
Dry Matter	1	12.20	6.30	5.90	0.512	--	--	--
	6	11.80	6.17	5.63	0.517	--	--	--
		---- mg plant $^{-1}$ -------				------- % ----------		
Total N	1	221	125	96	0.559	1.83	2.02	1.64
	6	216	127	89	0.586	1.85	2.09	1.56
		---- mg plant $^{-1}$ -------				--- at. % excess -----		
^{15}N	1	0.601	0.336	0.265**	0.558	0.276	0.277	0.284
	6	0.595	0.352	0.243**	0.594	0.275	0.271	0.298

** Denotes significant difference ($P < 0.01$) between N application treatments by Tukey's HSD.

Table 6-7. Effect of applying N fertilizer in 1 application or in 6 split applications on maturity parameters of beans in the IAEA ^{15}N Genotype Evaluation, CNPAF, August-November 1987. Values are means of 3 replicates.

N Applications	Grain Yield (oven dry)	Seed Moisture at Harvest	Seed Test Weight (oven dry)
	- Kg ha $^{-1}$ -	----- % -----	-- g (50 seeds) $^{-1}$--
1	1197	8.06	8.38
6	1015	8.40	8.12

season nodulation; WBR 22-3, Puebla 152, and WBR 22-24 were best during the late vegetative stage; and Puebla 152, Negro Argel, WBR 22-24 and WBR 22-14 showed the best duration of nodulation into the reproductive phase. Rio Tibagi, included among the bean entries as a poor-fixing control, was a consistently poor nodulator.

Puebla 152 contained a high shoot dry matter content on all sampling dates. Honduras 35 grew best in the early season, while WBR 22-34 showed the best late season growth. Among the grasses, wheat grew too fast during the vegetative phase, but was not significantly different from the beans during the reproductive phase. Solid-seeded sorghum consistently produced more dry matter (on a per area basis) than the beans, while hilled sorghum started out very slowly but surpassed the beans in dry matter production by the reproductive stage. At 66 DAE, Honduras 35, Carioca, and Negro Argel were the most efficient in converting whole shoot dry matter into pod dry matter. Preto Cariri and Puebla 152 were the least efficient, which may be due to poor adaptation.

Root dry matter did not vary significantly among bean lines tested, but Carioca and Honduras 35 showed characteristically low values, while WBR 22-34 had the largest root system during reproductive growth. Honduras 35, Carioca, Negro Argel, and Puebla 152 showed the highest shoot:root dry matter ratio due to both relatively small root masses and relatively high shoot growth. Root growth of wheat and sorghum paralleled shoot growth. The grasses consistently had lower shoot:root ratios than the beans, due to relatively high root masses.

Shoot N concentration did not vary appreciably among beans and was, in general, slightly lower in grasses than in beans. Among beans, the mass of shoot N generally reflected the shoot dry matter content. Honduras 35 accumulated the most N during early growth, while Puebla 152 was consistently high throughout the season, and WBR 22-34 and Preto Cariri accumulated large amounts of N during the reproductive period. ICA Pijao accumulated a relatively small amount of shoot N throughout the season. Wheat accumulated N faster than the beans during the vegetative phase, but was surpassed by almost all bean entries during reproductive growth. Solid-seeded sorghum accumulated more, but not significantly different, N than the beans during vegetative growth, and far surpassed the beans by 66 DAE. Hilled sorghum accumulated N very slowly during the early season, but also surpassed the beans by 66 DAE.

The distribution of total N between pods and stems + leaves at 66 DAE reflected dry matter distribution. Honduras 35, Negro Argel, and Carioca were the most efficient lines in translocating N to the pods, while Preto Cariri and Puebla 152 were the least efficient (Table 6-8).

DM estimates of N_2 fixed using the three control crop grasses at each sampling date were inconsistent. Due to higher N accumulation than the beans during vegetative growth, wheat resulted in negative values for the first 2 samples (Table 6-9). Solid-seeded sorghum gave negative values at 66 DAE, and hilled sorghum gave positive, but very low values. ICA Pijao contained the lowest N

Table 6-8. N content of shoots and shoot components and pod: whole shoot N content ratio at 66 DAE in the IAEA ^{15}N Genotype Evaluation, CNPAF, August-November 1987. Values are means of 6 replicates.

Genotype	Whole Shoot		Pods		Stems & Leaves		Pods: Whole Shoot	
	mg plant $^{-1}$							
Rio Tibagi	200	ABC[1]	103	DEF	96	BCD	0.510	DE
Carioca	234	ABC	159	ABCD	75	CD	0.680	ABC
Negro Argel	238	ABC	165	AB	73	CD	0.688	AB
Preto Cariri	261	A	82	F	179	A	0.303	F
Puebla 152	249	AB	111	BCDEF	138	AB	0.444	E
ICA Pijao	166	C	94	EF	71	CD	0.572	BCD
Honduras 35	228	ABC	178	A	50	D	0.778	A
WBR 22-3	207	ABC	126	ABCDEF	81	CD	0.600	BCD
WBR 22-8	203	ABC	109	BCDEF	94	BCD	0.544	DE
WBR 22-14	233	ABC	133	ABCDEF	99	BC	0.567	CD
WBR 22-24	192	ABC	116	BCDEF	76	CD	0.593	BCD
WBR 22-34	259	AB	151	ABCDE	108	BC	0.579	BCD
WBR 22-50	178	BC	106	CDEF	72	CD	0.595	BCD
WBR 22-52	195	ABC	104	CDEF	91	BCD	0.525	DE
WBR 22-55	210	ABC	124	ABCDEF	86	CD	0.589	BCD
Ven. 350 PS	257	AB	160	ABC	97	BCD	0.598	BCD
WBR 22-27	212	ABC	122	ABCDEF	90	CD	0.568	CD
	---		---		---		---	
$\bar{X}$	219		126		93		0.572	

[1] Means within a column followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-9. Wheat control crop Difference Method estimates of N_2 fixed in the IAEA 15N Genotype Evaluation, CNPAF, August-November 1987. Values are means of 6 replicates.

	DAE							
Genotype	19	33	51	66	19	33	51	66
	%				kg ha^{-1}			
Rio Tibagi	0 A[1]	0 A	5 AB	23 AB	0 A	0 A	2 A	10 AB
Carioca	0 A	0 A	9 AB	29 A	0 A	0 A	5 A	15 AB
Negro Argel	0 A	0 A	23 AB	31 A	0 A	0 A	15 A	15 AB
Preto Cariri	0 A	0 A	23 AB	31 A	0 A	0 A	18 A	17 AB
Puebla 152	0 A	0 A	24 AB	32 A	0 A	0 A	15 A	17 AB
ICA Pijao	0 A	0 A	4 AB	12 B	0 A	0 A	2 A	4 B
Honduras 35	1 A	0 A	26 AB	26 AB	0 A	0 A	17 A	12 AB
WBR 22-3	0 A	0 A	6 AB	24 AB	0 A	0 A	3 A	11 AB
WBR 22-8	0 A	0 A	4 AB	23 AB	0 A	0 A	2 A	12 AB
WBR 22-14	0 A	0 A	3 B	28 AB	0 A	0 A	2 A	13 AB
WBR 22-24	0 A	0 A	8 AB	20 AB	0 A	0 A	4 A	8 AB
WBR 22-34	0 A	0 A	13 AB	35 A	0 A	0 A	8 A	19 A
WBR 22-50	0 A	0 A	10 AB	18 AB	0 A	0 A	10 A	7 AB
WBR 22-52	0 A	0 A	7 AB	21 AB	0 A	0 A	5 A	9 AB
WBR 22-55	0 A	0 A	8 AB	23 AB	0 A	0 A	6 A	11 AB
Ven. 350 PS	0 A	0 A	12 AB	29 AB	0 A	0 A	7 A	18 AB
WBR 22-27	0 A	0 A	7 AB	25 AB	0 A	0 A	4 A	12 AB
$\bar{X}$	0	0	11	25	0	0	7	12

[1] Means within a column followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-10. ICA Pijao control crop Difference Method estimates of N_2 fixed in the IAEA 15N Genotype Evaluation, CNPAF, August-November 1987. Values are means of 6 replicates.

Genotype	DAE 19	33	51	66	19	33	51	66
	%				kg ha^{-1}			
Rio Tibagi	1 B[1]	15 AB	13 AB	20 ABC	0 B	2 AB	5 AB	9 AB
Carioca	21 AB	21 AB	19 AB	29 AB	1 AB	3 AB	9 AB	14 AB
Negro Argel	19 AB	28 A	30 A	29 AB	1 AB	5 AB	18 AB	14 AB
Preto Cariri	19 AB	19 AB	33 A	36 A	1 AB	3 AB	23 A	19 A
Puebla 152	19 AB	34 A	34 A	33 AB	1 AB	7 A	20 AB	17 A
ICA Pijao	0 B	0 B	0 B	0 C	0 B	0 B	0 B	0 B
Honduras 35	35 A	27 A	34 A	28 AB	3 A	5 AB	22 A	13 AB
WBR 22-3	16 AB	26 A	12 AB	22 AB	1 AB	5 AB	5 AB	9 AB
WBR 22-8	25 AB	21 AB	12 AB	20 ABC	2 AB	3 AB	5 AB	9 AB
WBR 22-14	5 B	10 AB	9 AB	29 AB	0 B	1 AB	4 AB	13 AB
WBR 22-24	14 AB	19 AB	17 AB	16 ABC	1 AB	3 AB	8 AB	6 AB
WBR 22-34	8 AB	14 AB	25 AB	36 A	0 B	2 AB	14 AB	19 A
WBR 22-50	10 AB	12 AB	16 AB	14 BC	1 B	2 AB	11 AB	5 AB
WBR 22-52	15 AB	22 AB	8 AB	17 ABC	1 AB	4 AB	5 AB	7 AB
WBR 22-55	14 AB	20 AB	13 AB	22 AB	1 AB	4 AB	7 AB	9 AB
Ven. 30 PS	6 B	22 AB	14 AB	31 AB	0 B	5 AB	8 AB	18 A
WBR 22-27	10 AB	25 AB	13 AB	21 ABC	1 B	5 AB	6 AB	11 AB
$\bar{X}$	14	20	18	24	1	4	10	11

[1] Means within a column followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-11. Isotope Dilution Technique estimates of N_2 fixed at 66 DAE (R7) in the IAEA 15N Genotype Evaluation, CNPAF, August-November 1987. Values are means of 6 replicates.

	Control Crop					
		Sorghum			Sorghum	
Genotype	Wheat	Solid	Hills	Wheat	Solid	Hills
	%			kg ha^{-1}		
Rio Tibagi	30 A[1]	22 A	25 A	12 A	8 A	10 AB
Carioca	33 A	24 A	29 A	16 A	11 A	13 AB
Negro Argel	36 A	24 A	32 A	17 A	12 A	15 A
Preto Cariri	27 A	15 A	23 A	15 A	8 A	12 AB
Puebla 152	34 A	23 A	30 A	17 A	11 A	15 AB
ICA Pijao	31 A	22 A	26 A	11 A	8 A	9 AB
Honduras 35	36 A	25 A	32 A	17 A	12 A	15 AB
WBR 22-3	33 A	20 A	28 A	14 A	9 A	12 AB
WBR 22-8	33 A	21 A	28 A	14 A	9 A	11 AB
WBR 22-14	31 A	22 A	27 A	14 A	10 A	12 AB
WBR 22-24	31 A	22 A	27 A	12 A	8 A	10 AB
WBR 22-34	34 A	22 A	29 A	18 A	11 A	15 A
WBR 22-50	29 A	19 A	24 A	10 A	6 A	8 AB
WBR 22-52	21 A	14 A	16 A	8 A	6 A	6 AB
WBR 22-55	33 A	21 A	29 A	14 A	9 A	12 AB
VEN. 350 PS	32 A	20 A	28 A	17 A	9 A	13 AB
WBR 22-27	20 A	12 A	15 A	8 A	4 A	5 B
$\bar{x}$	31	20	26	14	9	11

[1] Means within a column followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

mass among bean genotypes on three of the four sampling dates; and therefore was also used as a control crop in DM calculations to establish a minimum value underestimate of N_2 fixed by the other entries (Table 6-10). Using this method, the beans fixed an average of 11 kg N_2 ha^{-1} (24% of total shoot N) across genotypes during the growing season. Preto Cariri, WBR 22-34, Venezuela 350 PS, and Puebla 152 were the best N_2-fixing lines.

The concentration of ^{15}N as a percentage of total N in the shoot at 66 DAE was higher in grasses than in beans. Among the beans, Honduras 35, Negro Argel, WBR 22-34, and Puebla 152 contained the lowest concentrations of ^{15}N. The concentrations of ^{15}N in pods and in stems + leaves were similar.

Sorghum shoots accumulated a much higher mass of ^{15}N than the beans, while the value for wheat was similar to those of beans. Among beans, no statistically significant differences were observed in whole shoot ^{15}N content. Distribution differences between pods and stems + leaves were similar to differences in dry matter and total N (Table 6-8).

IDT estimates of N_2 fixed showed almost no statistically significant variation among bean lines (Table 6-11). Honduras 35, Negro Argel, Puebla 152, and WBR 22-34 were the best N_2-fixing entries based on this method, but the apparent lack of an appropriate control crop made the accuracy of these quantification estimates questionable.

Grain yield values generally paralleled N accumulation and IDT values, with Honduras 35, Negro Argel, Carioca, and WBR 22-34

being the most productive entries (Table 6-12). Yield of Puebla 152 was undoubtably affected by poor within-plant distribution.

Summary. Although adding all the N fertilizer prior to planting or in split applications had a minimal effect on plant dry matter and N accumulation, N_2 fixation estimates were affected significantly. Among bean genotypes, Honduras 35, Negro Argel, Puebla 152, and WBR 22-34 emerged as the best N_2-fixing lines tested.

Under the conditions of this experiment, none of the grass treatments appeared to be a satisfactory control crop for either DM or IDT estimates of N_2 fixation. The growth cycle of dwarf sorghum was considerably longer than that of the beans tested, the plant was too large, and the pattern of seasonal N uptake was different from that of the beans. The growth cycle of the wheat cultivar tested was similar to that of the beans, but the N uptake pattern was different. A non-nodulating bean line is needed desperately for quantification of N_2 fixation by *P. vulgaris* L. This trait has been reported (Davis et al., 1988), but the non-nodulating genes must be transferred to adapted germplasm of various seed types. Until a non-nodulating plant is available, relative accumulation of total shoot N is probably the best parameter for selecting genotypes for N_2 fixation.

Network Experiments

The Brazilian National Program on Symbiotic Nitrogen Fixation in Beans was formed in 1980, with funding from the FINEP.

Table 6-12. Maturity parameters in the IAEA ^{15}N Genotype Evaluation, CNPAF, August-November 1987. Values are means of 6 replicates.

Genotype	Grain Yield (oven dry)	Seed Moisture at Harvest	Seed Test Weight (oven dry)
	- kg ha $^{-1}$ -	----- % -----	- g (50 seeds)$^{-1}$ -
Rio Tibagi	934 BC[1]	9.62 AB	6.27 G
Carioca	1404 AB	6.63 AB	9.70 BC
Negro Argel	1433 AB	8.54 AB	8.75 CD
Preto Cariri	928 BC	12.74 AB	7.48 DEFG
Puebla 152	1163 ABC	13.92 A	11.40 A
ICA Pijao	842 C	8.51 AB	7.34 EFG
Honduras 35	1609 A	6.16 B	11.04 AB
WBR 22-3	1173 ABC	7.32 AB	7.75 DEF
WBR 22-8	859 C	6.90 AB	8.35 CDEF
WBR 22-14	1058 BC	8.13 AB	7.76 DEF
WBR 22-24	1069 BC	7.07 AB	7.73 DEF
WBR 22-34	1301 ABC	5.66 B	8.46 CDE
WBR 22-50	921 BC	5.69 B	7.31 EFG
WBR 22-52	966 BC	9.53 AB	7.70 DEF
WBR 22-55	1022 BC	8.39 AB	8.15 DEF
Ven. 350 PS	1166 ABD	7.53 AB	7.02 FG
WBR 22-27	961 BC	7.62 AB	8.10 DEF
	---	---	---
$\bar{x}$	1106	8.23	8.25

[1] Means within a column followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Participants include researchers from Brazilian institutions working on N_2 fixation. Objectives of the program are defined according to constraint areas, with each participant working on the constraint areas appropriate to their research program.

In conjunction with this program, a network experiment was conducted in agricultural years 1984/85 and 1985/86 to test the performance of selected bean lines. At each location, split plots were assigned in a randomized complete block design. The main plot factor was N treatment: inoculation with R. phaseoli, fertilization with a split application of N, and a control with neither inoculum nor N. Subplots were planted to four standard bean lines: Rio Tibagi and Carioca, standard cultivars, and Negro Argel and WBR 22-34, lines identified at CNBAP as good N_2-fixers. In the CNPAF trial, which also included line WBR 22-3, selected for improved N_2-fixation, the following data were recorded: shoot, root, and nodule dry matter early in the vegetative phase, at flowering, and during podfilling; number of nodules at 13 DAE; acetylene reduction activity at flowering and during podfilling; shoot total N content during podfilling; and pod and grain yield at maturity.

In the two experiments at CNPAF, WBR 22-34 and WBR 22-3 were clearly superior to the other entries for nodulation (Table 6-13), shoot N accumulation (Table 6-14), and grain yield (Table 6-15). These two lines, bred and selected for N_2-fixation under this CRSP project, produced comparable or higher yields without N fertilizer than the two standard production cultivars receiving added N. Therefore, by planting a line with improved N_2 fixation capability,

Table 6-13. Nodule dry weight in the Network Experiment at CNPAF. Values are means of 2 experiments and 4 replicates.

N Treatment	Genotype	Days After Emergence 12	39	59	$\bar{X}$
		------- mg. plant^{-1} -------			
Control	Rio Tibagi	2	11	20	11
	Carioca	8	40	48	32
	Negro Argel	12	60	32	34
	22-34	5	47	73	41
	22-3	6	41	31	26
	$\bar{X}$	6B[1]	40A	41A	29B
Innoculum	Rio Tibagi	7	22	31	20
	Carioca	15	45	46	35
	Negro Argel	16	54	51	40
	22-34	7	105	75	62
	22-3	7	47	62	39
	$\bar{X}$	10A	54A	53A	39A
N Fertilizer	Rio Tibagi	0	2	2	2
	Carioca	1	6	9	5
	Negro Argel	1	9	16	8
	22-34	0	27	13	14
	22-3	1	25	6	10
	$\bar{X}$	1C	14B	9B	8C
HSD (0.05)[2]		6.1	40.2	31.9	16.9

[1] Means within a column followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

[2] Applies to comparisons within N Treatments.

Table 6-14. Shoot N content at mid-podfill in the 2-year Network Experiment at CNPAF.

Genotype	Control	Innoculum	N Fertilizer	$\bar{X}$
	---------- mg. plant^{-1} ----------			
Rio Tibagi	188 b[1]	230a	309 b	242 c
Carioca	244ab	255a	329 b	276 bc
Negro Argel	308ab	314a	341 b	321ab
22-34	328a	258a	485a	357a
22-3	261ab	314a	427ab	334ab
$\bar{X}$	266B[1]	274B	378A	

[1] Values within a column followed by the same small letter and means within a row followed by the same capital letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-15. Grain Yield in the 1984 and 1985 CNPAF Network Experiment. Values are means of 4 replicates in each year.

Genotype	Control	Innoculum	N Fertilizer	$\bar{X}$
	kg. ha^{-1}			
Rio Tibagi	826 c[1]	960 d	1066 c	951 c
Carioca	984 b	1063 c	1282 b	1109 b
Negro Argel	847 c	886 d	1054 c	929 c
22-34	1204a	1176 b	1663a	1348a
22-3	1136a	1286a	1609a	1344a
$\bar{X}$	999 b	1074 b	1335a	1136

[1] Values within a column followed by the same small letter and within a row followed by the same capital letter do not differ significantly ($P<0.05$) by Tukey's HSD.

bean yields equivalent to those of unimproved lines which receive N fertilizer, can be obtained. Lines WBR 22-34 and WBR 22-3 also showed the best yield response to N fertilization, which is desirable for those producers who do apply N.

Network experiment results at other participating institutions (Table 6-16) showed that, across locations and years, grain yield of WBR 22-34 without N was similar to that of Rio Tibagi with added N. Line WBR 22-34 with N fertilizer also produced higher yields than the other entries. However, yield levels and results varied greatly among trials, with none of the genotypes being the best in all trials and with all treatments. Response to inoculation also varied among sites, but a positive yield response to N fertilization was observed in almost all line x site combinations. These results reinforce the importance of multiple-location and multiple-year trials.

Brazilian Regional Trials

CNPAF Regional Trials for N_2 fixation. Most materials evaluated in the two initial screenings for N_2 fixation at CNPAF in 1981 and 1982 were Brazilian landraces from the CNPAF germplasm collection unit. Lines bred specifically for improved N_2-fixation at the University of Wisconsin-Madison entered evaluation at CNPAF in 1983.

In 1984, after emphasizing evaluation of the existing cultivated germplasm and materials from three populations of breeding lines initiated at Wisconsin, there was greater emphasis

Table 6-16. Grain yield (kg ha^{-1}) in the 1983/84 and 1984/85 Network Experiment conduc[illegible] by the Brazilian National Program on N_2 fixation in Beans.[1] Values are means of 4 replicates.

LINE AND TREATMENT	ENCAPA 1984	UAPNABS 1984	CENA 1984	IAPAR 1983	EMPASC 1983	CNPAF 1984	CNPAF 1985	~~CNPAF~~ 1984	~~CNPAF~~ 1985	$\bar{X}$[2]
Rio Tibagi										
Control	-	608	480	436	1980	1352	300	622	560	792
Inoculum	734	908	576	706	1662	1462	458	675	554	875
N Fertilizer	807	815	1121	895	1813	1661	471	814	765	1044
$\bar{X}$	770	777	726	679	1818	1492	410	704	626	904
Carioca										
Control	-	868	1290	590	1697	1465	502	697	567	960
Inoculum	773	990	1565	597	1543	1606	520	897	781	1062
N Fertilizer	847	1318	1992	852	1840	1806	758	1116	940	1328
$\bar{X}$	810	1059	1616	680	1693	1626	593	903	763	1117
Negro Argeli										
Control	-	1007	870	740	1947	1139	554	523	539	91[illegible]
Inoculum	780	1012	876	694	1802	1214	558	695	860	9[illegible]
N Fertilizer	860	1340	1590	1281	1958	1348	761	857	859	1249
$\bar{X}$	820	1120	1112	893	1902	1234	624	692	759	1042
WRR 22-34										
Control	-	776	1193	698	1882	1517	891	814	474	1031
Inoculum	1057	680	1260	665	1188	1562	790	928	644	965
N Fertilizer	1247	1148	2490	1377	1777	2169	1158	1009	898	1503
$\bar{X}$	1152	864	1648	913	1616	1749	946	917	672	1166
$\bar{X}$										
Control	-	812	958	607	1876	1368	562	664	540	923
Inoculum	836	898	1069	666	1549	1461	582	799	710	967
N Fertilizer	940	1155	1798	1101	1847	1746	787	949	866	1281
$\bar{X}$	888	955	1276	791	1757	1525	643	804	705	1057

[1]Data courtesy of: A. Athayde, EMCAPA, Cariacica, Espírito Santo; Duque et al., 1985, EMBRAPA/UAPNPBS, Seropédica, Rio de Janeiro; S. M. T. Saito, CENA/USP, Piracicaba, São Paulo; M. Voss, IAPAR, Londrina, Paraná; F. Andriollo, EMPASC, Chapecó, Santa Catarina; R. A. Henson and R. S. Araujo, EMBRAPA/CNPAF, Goiânia, Goiás; and R. Perez, EMBRAPA/CPAC, Planatina, Distrito Federal.

[2]Means across locations do not include EMCAPA data.

on evaluation for N_2 fixation of lines from regional yield performance trials (Ensaio Preliminar de Rendimento or EPR). Prepared by CNPAF, the EPR trials at that time consisted of bean lines grouped by color: black, mulatinho (including carioca types), and rosinha, which were submitted directly to the trials by various state, national, and international breeding and selection programs. Then and now, the state research organizations which plant and manage the trials request only the color group(s) grown in their respective areas. After initial state tests, the state programs decide whether lines will be discarded or tested further in advanced trials, usually for a minimum of two additional years at various locations, for possible release as approved cultivars. Results of EPR and other advanced trials form the basis for selecting groups of recommended and tolerated cultivars for each state within the region. Farmers who do not plant recommended or tolerated cultivars are not eligible for government financing to cover bean production costs.

1982/83 and 1983/84 EPR Trials. At the time selection for N_2 fixation began on EPR materials, these groups were nearing the end of a 2-year evaluation cycle (agricultural years 1982/83 and 1983/84), and seeds of all entries in those regional trials were not available. Most of the materials screened for N_2 fixation were from CIAT, but a number of lines from CNPAF and state breeding programs were included also (data not shown). The single-observation plots of the two treatments, with added inoculum and with N fertilizer, were evaluated for: shoot N content at flowering, shoot

and root dry matter content and visual nodulation at flowering and at mid-podfilling, and seed and pod yield at maturity.

The N-fertilized plants as a group were consistently higher for all shoot, root, and yield parameters but showed poorer nodulation (visual observation). Differences were evident in early growth and persisted throughout the season.

Eleven lines (5 black and 6 mulatinho) were noted to have good relative nodulation in the N-fertilized plots. Comparisons of N_2 fixation and dry matter parameters were made to begin compiling baseline data on the relative performance of classes with various seed colors and growth types.

Black trials. Eighteen of the 55 black lines tested for N_2-fixation in the 1st evaluation of 1982/83 and 1983/84 EPR lines were selected for a 2nd-stage evaluation in 1986. These materials, with WBR 22-34 and Rio Tibagi as good-fixing and poor-fixing controls, respectively, were planted in a split-plot design with three replicates. The standard CNPAF inoculum mixture and N fertilizer were the main plot treatments, and lines was the subplot factor. Samples were taken at the R3 and R7 stages for shoot, root, and nodule dry matter content and at maturity for pod and seed yield and seed test weight.

Although the N fertilizer treatment was again superior to inoculum, yields of five entries without N fertilizer were higher than Rio Tibagi with N (data not shown). The best entries in this trial were advanced to a 3rd evaluation in 1988.

1984/85 and 1985/86 Black Bean EPR. All entries in this trial were evaluated for N_2 fixation in 1986 by Walter Q. Ribeiro, Jr., at the EMGOPA (Goias state agricultural research organization) experiment station near Goiania. The 20 entries which placed among the 10 highest yielders in more than one of the nine trials were selected for evaluation at CNPAF. The five lines which our project submitted to this EPR (each placing in the top 10 in one trial) were also included, with Rio Tibagi as a control.

One replicate of each entry received the standard CNPAF inoculum mixture and one replicate received N fertilizer. Samples were taken at R2 and R7 for shoot, root and nodule dry matter content. The R7 shoots were analyzed for total N content. At maturity, grain and pod yield and seed test weight were measured. Although selected for traits other than N_2-fixation, several entries were outstanding without N fertilizer and were re-evaluated in 1988.

Carioca/Mulatinho Trials. When the entries for the 1984/85 and 1985/86 EPR trials were assembled at CNPAF, a set of superior black lines had been selected for N_2 fixation. However, breeding work had been concentrated on black beans, and previous evaluations for N_2 fixation had emphasized performance relative to all entries, with little attention paid to individual color groups and seed types. Therefore, advanced selections were predominantly by black lines, since this group usually performed better than lines of other seed types. At the time of this EPR, almost no non-black lines had been selected for high N_2 fixation, so there were none to

recommend for the EPR or other research programs or producers interested in materials to evaluate.

Selection programs were initiated within the carioca and mulatinho types. From the 1984 evaluation of materials from the 1982/83 and 1983/84 EPR trials, 20 carioca and 17 mulatinho lines were selected for a second evaluation in 1985. In three replicates, inoculum and N fertilizer were the main plot factors, with lines as the subplot factor. To spread the labor requirements, carioca and mulatinho lines were planted in separate evaluations. Shoot, root, and nodule dry matter, ARA, and shoot N content were measured at 14 DAE and at flowering. At maturity, pod and seed yield and seed test weight were determined. Notes on disease incidence and plant architecture were collected also.

Based upon results from the 1985 evaluations, six carioca and three mulatinho lines were planted, with the variety Carioca as a check, in a 3rd-stage evaluation in 1986. Main plots consisted of the standard CNPAF inoculum mixture and N fertilizer, with lines as the subplot factor, and combination replicated from times. Shoot, root, and nodule dry matter and shoot N content were determined at flowering and at R7. Grain and pod yield and seed test weight were recorded at maturity.

Over the 3-stage evaluation, lines CENA 164, A246, A255 and A285 produced the highest yields both with inoculum and with N fertilizer (Table 6-17). These lines and others found to have high nodule production, N accumulation, etc., are available for use as

Table 6-17. Fields of Mulatinho and Carioca bean lines receiving inoculant (In) or nitrogen fertilizer (N) in Brazilian Regional Trials.

Evaluation	Carioca							Mulatinho			$\bar{X}^2$
	A241	A246	A255	A285	A2883	Car.	CENA 164	A353	A357	Bat 160	
1984											
In	1623	2098	1585	1821	1526	1068	1617	963	1170	1655	1513
N	1172	1220	1822	1756	1320	1724	1708	2322	1306	139	1494
$\bar{X}$	1398	1659	1704	1788	1423	1396	1662	1642	1238	897	1481
1985											
In	2069	1935	1945	2399	2154	2113	2033	1836	1887	1452	1982
N	2530	2374	2523	2720	2294	2528	2802	2335	2260	2054	2442
$\bar{X}$	2299	2154	2234	2559	2224	2320	2418	2085	2074	1753	2212
1986											
In	1960	2005	2040	2350	2055	1894	2030	1904	1896	2112	2025
N	1976	2215	2138	2483	2145	1782	2046	2020	1887	2140	2083
$\bar{X}$	1968	2110	2089	2416	2100	1838	2038	1962	1891	2126	2054
$\bar{X}$											
In	1884	2013	1857	2190	1912	1692	1893	1568	1651	1740	1840
N	1893	1936	1261	2320	1920	2011	2185	2226	1818	1444	1991
$\bar{X}$	1888	1974	2009	2255	1916	1852	2039	1897	1734	1592	1916

parents in crosses to improve these characteristics or N_2 fixation in general.

1984/85 and 1985/86 Carioca/Mulatinho EPR. Entries from this trial and lines selected previously as controls were evaluated for N_2 fixation at CNPAF in 1986, one replicate of each line with the standard CNPAF inoculum mixture and one with N fertilizer. Grain yield was the only trait recorded.

In the carioca group, none of the lines greatly surpassed the controls, but several mulatinho lines were promising, especially compared to the best carioca lines. These materials were re-evaluated in a final evaluation in 1988.

Roxo Trials. Within the EPR classification, the Roxo group is a combination of roxo (dull, dark reddish-purplish), rosinha (dull, pinkish-beige, pink hilum), and chumbinho (dull, brown to grayish). Although not grown as widely as black or carioca lines, there are strong local preferences for these types, which often command a higher market value. With few exceptions, the existing germplasm of these groups is notorious for weak plants, disease susceptibility, and low yields. Some entries from the 1984/85 and 1985/86 Roxo EPR were evaluated for N_2 fixation in 1986, and the best materials were re-evaluated in 1988. It is expected that the best lines from this evaluation will be used as parents in breeding for improved N_2 fixation within this group.

Bean/Cowpea CRSP Project Lines in CNPAF Regional Trials. The black bean EPR for agricultural years 1984/85 and 1985/86 was sent to 22 locations in Brazil and one site in Bolivia. The trial

consisted of four local controls and 77 lines sent from CNPAF, including five entries bred and selected for N_2 fixation as part of this project: WBR 22-3, WBR 22-8, WBR 22-34, WBR 22-50, and WBR 22-55. Fertilizer was applied according to local recommendations, and most trials received approximately 15-20 kg N ha^{-1} at planting or were planted on fertile soil. In the 16 trials for which CNPAF received results, mean yield rankings of the N_2 fixation lines were good compared to the widely-grown varieties Carioca and Rio Tibagi (Table 6-18). In the state of Minas Gerais, line WBR 22-50 placed 1st and 2nd for yield in the two test years, and was advanced to their state trials, the last evaluation before becoming a recommended cultivar.

The EPR trials for the 1986/87 agricultural year were again composed of lines submitted directly by breeding and selection programs and consisted of four groups: black, carioca, mulatinho, and roxo. The 36 entries in the black bean trial included eight lines selected for N_2 fixation under this project: 21-58, 22-2, 22-14, 22-24, 22-27, 22-52, Honduras 35, and Preto Cariri. The former six lines were bred for high N_2 fixation, while the latter two are from the CNPAF germplasm collection and preservation program.

In the West-Central Region, Honduras 35 produced the top yield in the CNPAF trial and was 2nd best in the EMGOPA trial at Anapolis (Table 6-19). Five of our lines were among the top 10 yielders at CNPAF. Reports from the Southern Region (Rio Grande do Sul, Santa Catarina, and Parana) show inconsistent results for some lines and good yield stability for others (Table 6-20). In 1988, six lines

Table 6-18. Performance of selected entries in the 1984/85 and 1985/86 Black Bean preliminary yield trial. Values are means of 16 trials.

Genotype	Yield (Kg Ha^{-1})	S.E.[1]	Rank
Best Entry	1582	156	1
WBR 22-8	1468	128	8
WBR 22-3	1444	122	12
WBR 22-55	1425	138	16
WBR 22-50	1364	144	25
WBR 22-34	1323	147	34
Overall Mean	1323	123	39
Carioca	1302	158	41
Rio Tibagi	1210	134	65
Worst Entry	1072	123	77

[1] Calculated using the means of the 2 replicates in each trial.

Table 6-19. Grain Yield (kg ha^{-1}) of the 1986/87 black bean EPR at two sites in the Central-West region of Brazil. Values are means of four replicates.

Entry	CNPAF[1]	EMGOPA[1]	$\bar{X}$
Honduras 35	1689 a	1456 a	1572
AN 3508	1426 ab	1434 a	1430
BAT 67	1358 ab	--	--
SPB-5	1337 ab	1449 a	1393
ICA COL 10103	1061 ab	--	--
AN 512642-0	968 ab	1479 a	1224
AN 512637-0	1117 ab	1306 a	1212
22-27	1133 ab	1254 a	1194
22-14	1273 ab	1029 a	1151
AN 512574-0	949 ab	1351 a	1150
22-24	1104 ab	1186 a	1145
AN 512573-0	1204 ab	1081 a	1142
AN 512630-0	1078 ab	1171 a	1124
22-52	1027 ab	1186 a	1106
BAT 1647	998 ab	--	--
BAT 451	946 ab	--	--
AN 512575-0	860 ab	1299 a	1080
AN 3484	967 ab	1141 a	1054
84 VAN 18	927 ab	1156 a	1042
CF 840627	857 ab	1224 a	1040
21-58	1177 ab	893 a	1035
AN 511619-0	863 ab	1201 a	1032
SPB-1	1096 ab	923 a	1010
LM 30074-0	666 b	1351 a	1008
AN 512567-0	767 ab	1231 a	999
BZ 1719-2	853 ab	1119 a	986
Carioca	1006 ab	938 a	972
84 VAN 196	1052 ab	833 a	942
84 VAN 163	790 ab	1066 a	928
AN 512631-0	717 b	1059 a	888
AN 512638-0	1016 ab	713 a	864
LM 21135-0	1073 ab	578 a	826
AN 512572-0	707 b	938 a	822
Rio Tibagi	776 ab	841 a	808
Preto Cariri	722 b	623 a	672
22-2	544 b	796 a	670
Local Controls			
SPB-1	--	1351 a	--
Preto Caruaru	--	1014 a	--
Rio Negro	--	983 a	--
Rico 23	--	908 a	--

[1] Data courtesy of C.C. da Silva, J.G.C. da Costa, and G.E. de S. Carneiro ((CNPAF) and M.J. Peloso (EMGOPA).

Table 6-20. Grain yield (kg ha^{-1}) of the 1986/87 black bean EPR at two sites in the Southern region of Brazil. Values are means of four replicates.

Entry	Cascata 1	Cascata 2	UEPEL	Osorio	Chapeco	Ponta Grossa
Honduras 35	2283	1720	700	835	1484	2000
AN 3508	1690	1311	483	940	1400	1700
SPB-5	1650	763	617	508	855	1617
22-14	1630	505	433	416	1305	2083
AN 512573-0	2240	1943	1050	1586	1951	1950
21-58	1520	931	833	762	1092	1567
22-27	1770	1523	633	935	784	1700
AN 512637-0	--	860	250	752	956	1500
22-24	2050	1783	950	928	778	2117
SPB-1	1450	608	517	--	392	1550
AN 512630-0	--	1040	533	823	1060	1542
LM 21135-0	1770	1570	550	404	1610	1950
84 VAN-196	2070	1471	633	445	1455	1967
22-52	--	1944	700	960	1132	2117
AN 512638-0	2640	1007	483	557	1047	1867
Carioca	1870	1801	1017	--	1742	1667
AN 512642-0	2040	1351	333	--	1215	2183
AN 3484	1540	864	633	954	1265	1667
AN 512574-0	1800	3248	750	914	1596	2467
84 VAN-18	1990	1941	700	935	1956	2550
AN 511619-0	1880	1601	700	--	1582	2250
AN 512575-0	1810	2765	467	--	1646	2517
CR 840627	1500	863	133	556	600	1333
BZ 1719-2	2090	1154	550	1339	1705	2150
84 VAN-163	2050	1644	417	1132	1752	2367
Rio Tibagi	--	1304	1050	726	946	1450
AN 512567-0	--	2174	783	1182	1680	2267
Preto Cariri	1400	1463	533	1176	1075	1217
AN 512631-0	1980	1543	633	905	1108	1783
AN 512572-0	1470	1834	600	1292	1565	2000
LM 30074-0	2130	1694	517	778	1590	2250
22-2	1840	874	183	1023	863	1433
Local Controls						
RAI 76	1750	1213	--	--	--	--
FT 83-120	1570	2481	--	--	--	--
Turrialba 4	1360	1034	400	732	--	--
Rio Negro-EMPASC	1280	941	433	--	--	--
Maquine	--	--	583	407	--	--
Guateian 6662	--	--	433	1321	--	--
Rio Tibagi RS	--	--	--	626	--	--
1	--	--	--	--	1445	1517
2	--	--	--	--	966	1917
3	--	--	--	--	1634	1067
4	--	--	--	--	1130	1667

[1] Data courtesy of I. Antunes (EMBRAPA/CNPFT Ext. Stn. Cascata and EMBRAPA/UEPEL); R.L. De Moura (Osorio); R. Flech (EMPASC, Chapeco); and A.S. Takeda (COTIA Agric. Coop., Ponta Grossa).

selected for N_2 fixation by this project had been advanced to the level of state trials in Brazil (Table 6-21).

Future Directions. During the period of this project, the National Genotype Evaluation Program at CNPAF has gone through several modifications in quest of a more efficient and effective procedure for testing the many lines that should be evaluated in a manner that best meets the needs and expectations of everyone involved. In the current program, candidate lines for the EPR will be evaluated first in a Preliminary Line Evaluation (EPL). Breeding and selection programs may submit as many EPL entries as they wish. Although details for EPL testing are still being defined, multiple-season and multiple-location trials are anticipated. Based upon the results of those trials, a subsequent EPR will be formed.

Previously, as many EPR lines as possible have been evaluated for N_2 fixation and the entire 1st EPL was evaluated in 1987. However, with the development of additional populations bred for improved N_2 fixation and the increased size of the EPL, the large number of materials to evaluate has become prohibitive. Therefore, the N_2 fixation project at CNPAF will concentrate on developing and selecting breeding lines for submission to the EPL and evaluate only occasionally other EPL/EPR entries which show exceptional promise for use as parents in further crosses for improved N_2 fixation.

Table 6-21. N_2-fixation lines included in 1988 Brazilian state trials.

Line	State	Locations
Honduras 35	Goias	Goiania, Brasilia, Itapuranga, Pirenopolis, Morrinhos, Anapolis
	Minas Gerais	Vicosa, Ponte Nova, Leopoldina
	Rio Grande do Sul	Osorio, Sobradinho
	Espirito Santo	Venda Nova
WBR 22-24	Goias	Goiania, Brasilia, Itapuranga, Pirenopolis, Mossamedes, Anapolis
	Mato Grosso do Sul	Dourados, Sindrolandia, Maracaju, Indapolis, Campo Grande
	Rio Grande do Sul	Osorio, Sobradinho, Erexim, Cascata, Irai, Frederico Whestphalen
WBR 22-52	Goias	Goiania, Brasilia, Itapuranga, Pirenopolis, Mossamedes, Anapolis
	Rio Grande do Sul	Osorio, Sobradinho, Erexim, Cascata, Canguru, Frederico Whestphalen
WBR 22-27	Goias	Goiania, Brasilia, Itapuranga, Pirenopolis, Mossamedes, Anapolis
WBR 22-50	Minas Gerais	Vicosa, Ponte Nova, Leopoldina
WBR 21-58	Rio Grande do Sul	Osorio, Sobradinho

Project Lines in Other Trials

Stress Tolerance. Elite lines selected for N_2 fixation were included in an anthracnose resistance evaluation at CNPAF. Honduras 35, CNF 1896, and 20-9 showed good resistance. A similar group of materials was evaluated at CNPAF for reaction to Bean Common Mosaic Virus. Several lines were shown to carry the desired I gene for virus resistance (Table 6-22). A set of lines was evaluated by CNPAF for resistance to Brazilian Bean Golden Masaic Virus, but no resistance or tolerance was found. Line WBR 22-34 was entered in a CNPAF screening for resistance to rust and advanced to other tests, but has not yet been used as a parent in the rust breeding program.

Rio Tibagi, Carioca, Negro Argel, and WBR 22-34 were entered into a CIAT screening trial for tolerance to low soil phosphorus at CNPAF, and thirty-seven lines selected for high N_2-fixation capacity were evaluated at CNPAF for drought tolerance, with the results yet to be forthcoming.

On-farm Trials. Negro Argel, WBR 22-34, WBR 22-8, and WBR 22-55 were included in on-farm trials conducted by the CNPAF extension unit. In 1983/84 trials, the yield levels of Negro Argel were good for on-farm conditions in the state of Goias and clearly superior to the farmer's variety in most instances where that comparison was made (Table 6-23). Part of this difference in performance was undoubtedly due to planting higher-quality seed in the demonstration plots, but in light of the relatively small amounts of fertilizer used, a large contribution of N from fixation was needed

Table 6-22. Evaluation of bean lines for common bean mosaic reaction at CNPAF, 1987.

Line	Cultivar	Reaction
41	Rio Tibagi	10/10 nt
42	Negro Argel	10/3 nt + 7 ms
43	Carioca	10/10 nt
44	20-9	10/10 nt
45	480	10/10 ms
	Rico 23	9/9 ms
	Top crop	2/2 nt
46	21-58	10/10 nt
47	22-2	9/9 nt
48	22-3	10/10 nt
49	22-8	9/9 nt
50	22-14	9/9 nt
51	22-24	9/9 nt
52	22-27	10/10 nt
53	22-34	9/8 nt + 1 ms
54	22-50	10/10 nt
55	22-52	10/10 nt
56	22-55	9/9 nt
57	Preto Cariri	5/5 nt
58	LM 30074	10/10 nt
59	ICA Pijao	9/9 nt
60	Puebla 152	8/8 nt
61	A 241	10/10 nt
62	A 246	10/10 nt
63	A 255	10/10 nt
64	A 285	11/11 nt
65	A 288	10/10 nt
66	ICA Quetzal	10/10 nt
67	PV 99	11/11 nt
68	BAT 165	9/9/ nt
69	BAT 429	10/10 nt
70	BAT 451	10/10 nt
71	EF 33	10/4 nt + 6 ms
72	BAT 431	10/10 nt
73	EMP 60	10/10 nt
74	EMP 84	10/10 nt
	9 C	6/6 ms
	Rico 23	9/9 ms
	Top crop	3/3 nt
	18 R	10/10 ms
	46 M	8/8 nt
	47 M	7/7 ms
	Rico 23	10/10 ms
	Top Crop	2/2 nt

Obs: nt = Necrose total (gene I)
ms = Mosaico sistemico

Planting date: 28/09/87
Innoculation date: 05/10/87
Evaluation date: 09/10/87 and 13/10/87
Strain: NL-3

Table 6-23. Data collected from on-farm demonstration plots installed in the state of Goias by the CNPAF extension team, 1983/84.[1]

City	Bean Variety/ Line	Soil Preparation	Planting	Fertilization ($kg.ha^{-1}$) and Formulation	Dates		Grain Yield (kg/ha)		Cropping System[2]	Observations
					Planting	Harvest	Demo Plot	Variety		
Anicuns	Carioca	No	Manual	120, 4-14-8	29/02/84	15/05/84	790	372	M	
Anicuns	Negro Argel	Yes	Manual	120, 4-14-8	21/02/84	21/05/84	800	390	M	
Rubiataba	Negro Argel	No	Manual	200, 4-30-10	17/02/84	27/07/84	750	300	M	Excess rain at flowering
Rubiataba	Carioca	Yes	Mechanized	120, 4-30-10 +70 Ammonium Sulfate	28/02/84	20/05/84	1,020	300	P	
N. Gloria	Negro Argel	Yes	Manual		12/02/84	07/05/84	600	50	P	Lack of rain
C.R. Verde	Negro Argel	Yes	Mechanized	140, 4-30-10	09/02/84	10/05/84	1,080	630	P	
C.R. Verde	WBR 22-34	Yes	Mechanized	140, 4-30-10	09/02/84	10/05/84	860	630	P	
Itapuranga	Negro Argel	Yes	Manual	300, 3-20-10	28/02/84	17/05/84	987	297	P	
Itapuranga	Carioca	Yes	Manual	140, 3-20-10	29/02/84	17/05/84	770	297	P	Lack of rain
Ceres	CNF-10	Yes	Manual	None	21/02/84	11/05/84	1,250	660	M	
Ceres	Negro Argel	Yes	Manual	None	21/02/84	11/05/84	1,784	660	M	
Ceres	CNF-10	Yes	Manual	None	13/02/84	11/05/84	1,500	660	M	
Ceres	Carioca	No	Manual	150, 4-30-10	15/02/84	10/05/84	2,500		P	Floodplain
Ceres	Carioca	No	Manual	150, 4-30-16	03/02/84	27/04/84	270	248	P	Excess & lack of rain, high incidence of anthracnose and rust

P. Bernardo	Negro Argel	No	Manual	None	16/02/84	30/05/84	424		P	Excess rain, web blight
Inhumas	Costa Rica	Yes	Mechanized	300, 4-14-8	15/02/84	25/05/84	911		P	Deep fertilizer placement
Inhumas	Negro Argel	Yes	Mechanized	175, 4-30-10	28/02/84	late May	635			
Itaguaru	CNF-178	Yes	Manual	200, 4-14-8	14/02/84	04/05/84	400		P	Lack of rain, rootworm damage
Itaguaru	Negro Argel	Yes	Manual	200, 4-30-10	09/02/84	25/04/84	1,640		M	Stemborer & rootworm damage, excellent cooking quality & palatability
Itaguaru	CNF-10	Yes	Manual	150, 4-30-10	03/03/84	25/05/84	1,250		P	Rootworm damage, rust
Itaberai	Negro Argel	Yes	Animal	140, 4-30-10	24/02/84	21/05/84	1,150	600	M	Lack of rain at flowering, 2 tons lime applied
Ipora	Negro Argel	Yes	Manual	150, 4-30-10 +100 Amonium Sulfate	09/02/84	07/05/84	640	324	P	Stemborer damage, lack of rain
Ipora	CNF-10	No	Manual	None	23/02/84	25/05/84	116	47	M	Lack of rain
S.L.M.B.	Negro Argel	No	Manual	15 kg P_2O_5 24 kg K_2O	22/02/84	15/05/84	1,410	1,340		
S.L.M.B.	CNF-178	Yes	Manual	None	22/02/84	20/05/84	3,450	1,114	P	
B. Vista	Negro Argel	Yes	Manual	250, 4-14-8	15/02/84	20/05/84	270	220	M	Lack of rain

[1] Data courtesy of E. T. de Oliveira, CNPAF.
[2] M = multiple-cropped, P = bean pureculture.

to produce yields of over 1000 kg ha^{-1}. Despite these promising results, testing of Negro Argel in on-farm trials was discontinued because of a severe incidence of anthracnose in some trials. Results of WBR 22-8 and WBR 22-55 in 1988 trials have not yet been received.

Trials at Other Institutions. Elite lines selected for N_2 fixation at CNPAF were sent to Uruguay, Argentina, and Colombia (CIAT) for evaluation, but no results were received.

Two sets of black bean lines selected for N_2 fixation were entered in CNPAF regional trials (see section on Brazilian Regional Trials). Eight lines selected for N_2 fixation at CNPAF (WBR 22-3, WBR 22-34, WBR 22-50, WBR 22-55, WBR 21-58, Negro Argel, and Honduras 35) were among those tested at the Center for Nuclear Energy in Agriculture (CENA), University of Sao Paulo, Piracicaba, Sao Paulo, Brazil, under an NRC/BOSTID Research Program grant to study mycorrhizae and N_2 fixation in maize/bean relay cropping.

Each year, CNPAF supplies seeds for the network experiment conducted by participants in the Brazilian National Program on Symbiotic Nitrogen Fixation in Beans. Upon request, lines selected for N_2 fixation at CNPAF were sent to other institutions, most notably EMBRAPA/UAPNPBS and CENA, for use in experiments on limiting factors to N_2 fixation.

Since 1986, CNPAF has participated in an International Atomic Energy Agency program to improve N_2 fixation by *P. vulgaris* in Latin America, with emphasis on selecting host plants. Based upon results of this CRSP project at CNPAF, Honduras 35 will be tested

in 1988-89 trials in Chillan, Chile; Cali, Colombia; Guatemala City, Guatemala; Irapuato and Colima, Mexico; and Piracicaba, Brazil. Line WBR 22-34 will also be included at Piracicaba.

Rhizobial Strain Evaluations

Strain Evaluations at MSU. *Rhizobium leguminosarum* biovar *phaseoli* strains evaluated for nodulation and N_2 fixation potential at MSU were obtained from two main sources: a) 323 isolates taken directly from within root nodules of 25 cultivars/lines of beans collected at field growing sites in several countries (Table 6-24); and b) 19 recommended, effective strains obtained from established *Rhizobia* culture collections (Table 6-25). This collection of isolates enabled us to examine many diverse stains adapted to different geographical locations and to compare their symbiotic potential relative to recommended inoculant stains, so that superior combinations of improved bean lines and rhizobia could be identified. The large difference in nodule number and mass per plant root system among bean lines growing at the same site (MSU Montcalm Experiment Station) is illustrated in Fig. 6-2a-d. The isolates from root nodules on uninoculated plants at CNPAF provided a representative sample of the indigenous population of bean rhizobia which would compete with our inoculant strains in field tests. Most of the indigenous strains induced small, white nodules which were ineffective for nitrogen fixation.

Throughout the 8-year project, isolates were evaluated for induction of nodules and nitrogen fixation potential on the

Table 6-24. Rhizobium phaseoli isolated from root nodules of various uninnoculated bean cultivars/lines collected from different geographical regions.

No. of Isolates	Field Location & Collector	Bean Cultivar/Line Sampled
11	MSU Campus Farm S. Thacker, F. Dazzo	Domino, Neptune
24	UW Experiment Station F. Dazzo, F. Bliss	Sanilac, Ex-Rico, P21-16, 23
45	MSU Experiment Station Montcalm, MI F. Dazzo, W. Adams, C. Semper	Jamapa, Domino, Neptune, Puebla 152, Fleetwood, Seafarer, Porillo Sintetico, I 2119, I 82072, 80B06596, I 82055, I82063, I82065, N81037
215	CNPAF, Goiania Brazil EMBRAPA, Km 47, Seropedica R. Araujo, D. Isleib, A. Franco, F. Dazzo	21-45, 22-34, Mexico 307, Puebla 152, IPA 7419, Black Turtle Soup
25	Instituto Tecnologico de Celaya Celaya, Mexico J. Maya-Flores	Flor de Mayo, Carioca
3	Malawi H. Mlozabanda, W. Adams	Unknown

Nodules were washed, surface-sterilized, squashed, and their contents streaked on BIII and YEM agar plates. After 5 days growth, cultures producing uniform colonies which were large, pearl-white, mucoid, raised, and with entire edges were restreaked on BIII agar. Cultures consisting of Gram negative rods were verified as R. phaseoli by root nodulation tests using bean line 21-58 under microbiologically controlled conditions, and were maintained in sub-culture on BIII agar slants.

Shown below are photographs of an isolated colony of R. phaseoli on BIII agar (left) and bacteroid cells from within bean nodules (right).

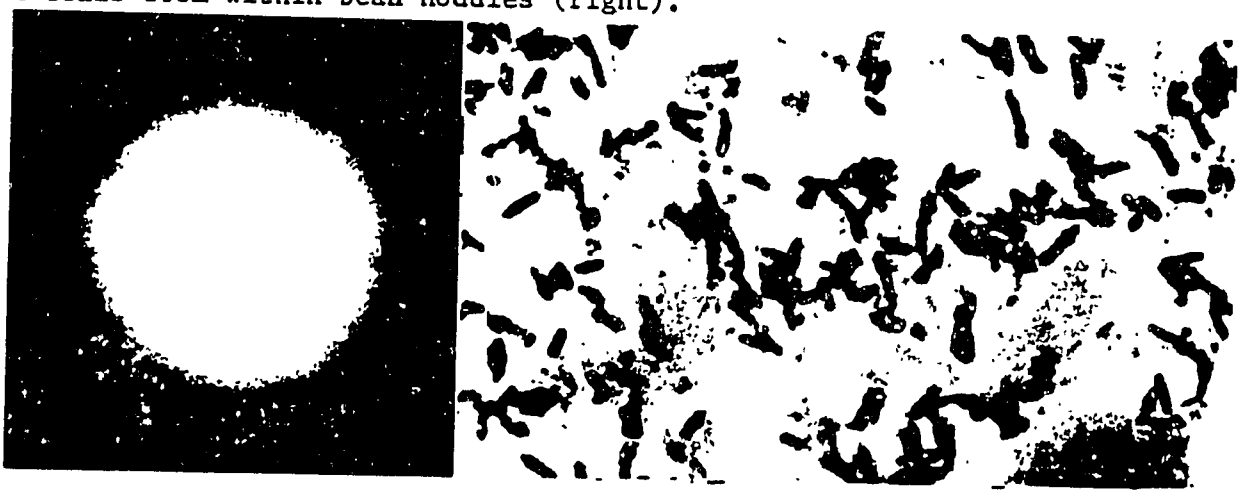


Table 6-25. Recommended effective *Rhizobium phaseoli* strains obtained from established culture collections and evaluated in this study.

Strain #	Origin	Culture Collection
CIAT 899, CIAT 632, CIAT 640	Columbia	P. Graham, Univ. Minn.
Viking 1, UMR 1024, UMR 1026	Brazil	P. Graham, Univ. Minn.
CNPAF 150, CO 5	Brazil	R. Araujo, CNPAF Goiania
KIM-5	Washington, USA	D. Bezdicek, Washington State University
TAL 182	Maui, Hawaii	J. Halliday, Niftal
127K81	Columbia	S. Smith, Nitragin Co.
RCR 3603, RCR 3644, RCR 3622	England	J. Jebb, Rothamsted Experiment Station, England
ALN-1	Unknown	T. Wacek, North American Plant Breeders (Kalo)
F. Mayo, CIAS, ITC-49, ITC-50	Mexico	J. Maya-Flores, Instituto Technologico de Celaya, Mexico

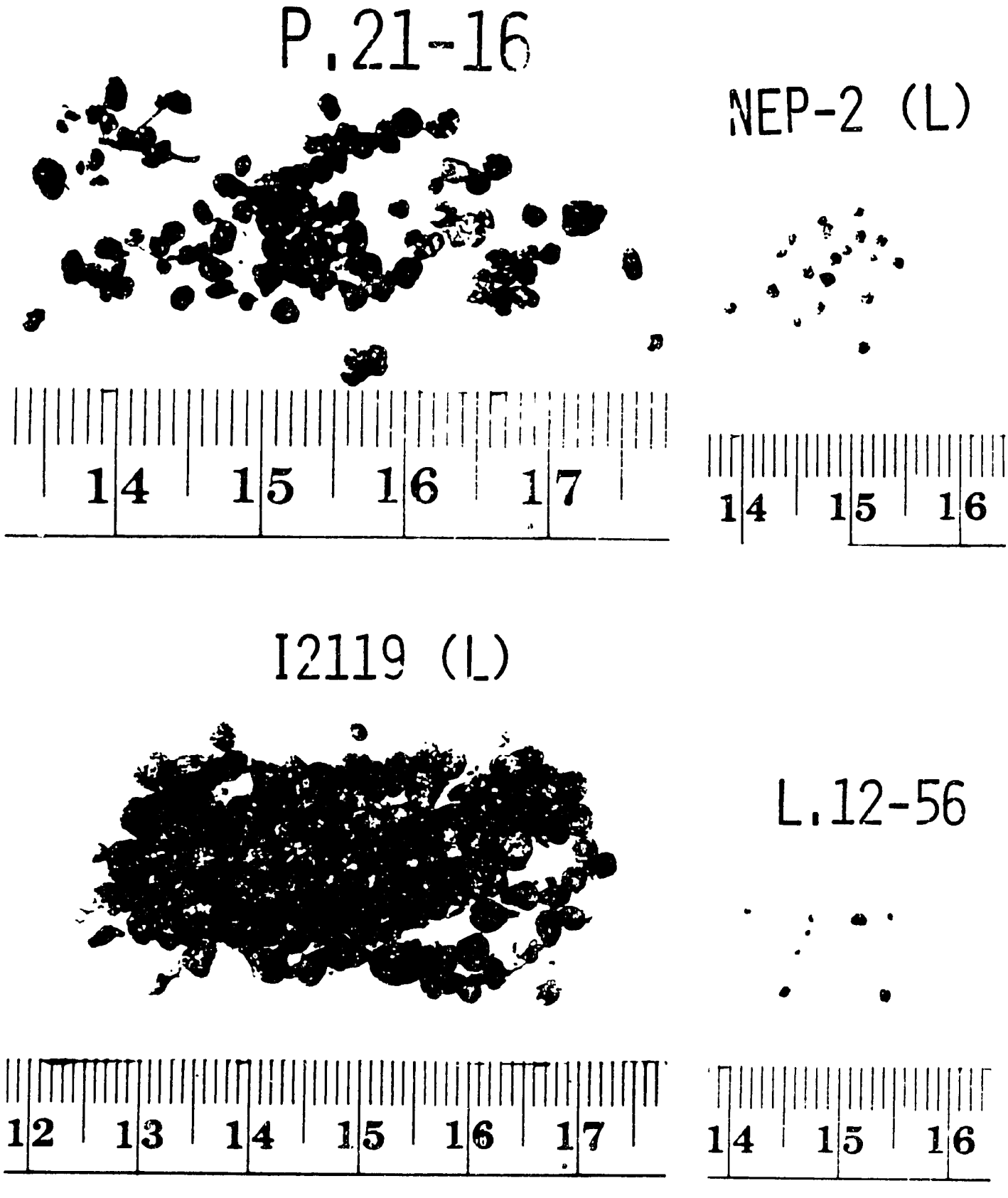


Figure 6-2. Representative nodules sampled from the bean lines; A) 21-16, B) NEP-2, C) 82055, and D) L12-56 grown at the Montcalm Michigan Experiment Station.

superior bean line 21-58, developed and recommended by F. Bliss. All screening was performed in growth chamber environments (20/22 C night/day cycle, 16 hr photoperiod of mixed incandescent/fluorescent lighting) using microbiologically controlled plant cultures containing a nitrogen-free, mineral salts medium (Araujo et al., 1986). Seeds were surface-sterilized with diluted chlorox, washed, and germinated on sterile moistened filter paper in the dark at 22°C. In studies from 1982 through 1985, seedlings were transferred to enclosed 250-ml Erlenmeyer flasks containing 50-ml of sterile medium solidified with agar. After 1985, acid-washed quartz sand which had been washed with water and then autoclaved was substituted for agar in the flask cultures. Each seedling root was inoculated with 1 ml of medium containing 10^8 bacteria that had been grown for 5 d at 30 C on B III plates (Aruajo et al., 1986). The flasks were covered with aluminum foil throughout the growth period so that roots would remain in the dark. When the stems had grown to a sufficient height, they were withdrawn aseptically from the flask opening and the foam plug closure was replaced so the aerial part of the plants could grow unrestricted.

The acetylene reduction assay (Dazzo, 1982) was used to measure dinitrogen fixation potential of the entire nodulated root system after one month of growth, while the plants were in the vegetative phase. At this age, effective vs. ineffective symbioses could be distinguished also by the green or yellow color of the bean leaves (Fig. 6-3a). The acetylene reducing activity of the

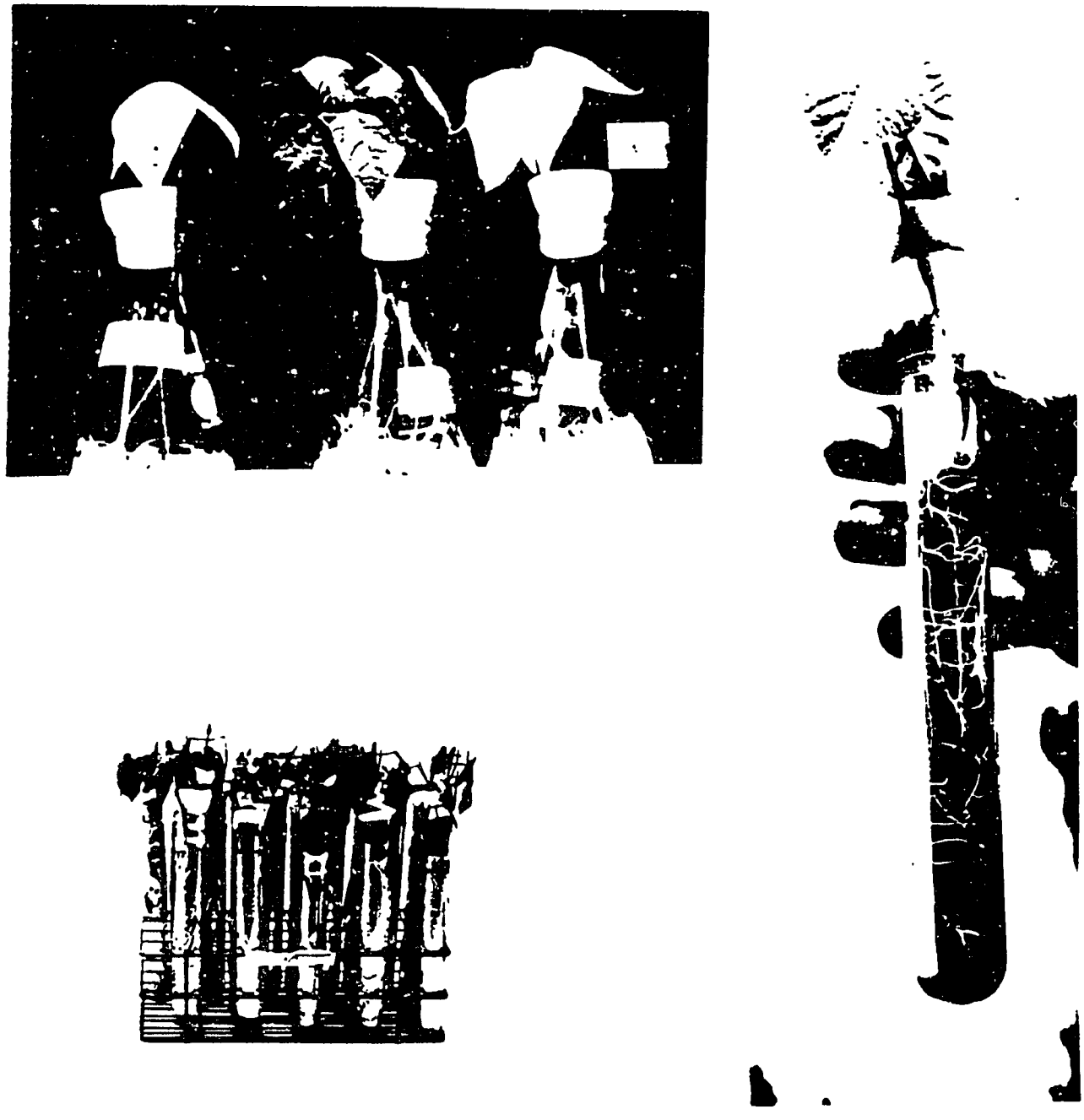


Figure 6-3. Bean plants grown in flask and tube cultures inoculated with R. leguminosarum bv. phaseoli.

most effective strains in this project is presented in Table 6-26.

Inoculant Preparation at MSU. Although peat inoculants are traditionally prepared from diluted batch, broth cultures of the *Rhizobium* species (Somasegaran and Hoben, 1985), we used plate-grown cells instead. We know from other research at MSU that rhizobia produce more extensive capsules when grown on agar plates rather than in shaken broth culture. We believe that encapsulation helps the bacteria survive better under desiccation stress. To prepare peat inoculants, cells from five 5-day old YEM agar plate cultures were aseptically harvested into YEM broth, centrifuged at 12,000 x G for 30 min, resuspended into YEM broth, and inoculated into gamma-irradiated sterile peat purchased from NifTAL. The fresh YEM broth supplement was added to provide nutrient for continued growth of the bacteria in the peat. The inoculated peat bags were sealed, cured for 2 weeks at 30°C and stored at 4°C until shipment to CNPAF or other sites. This procedure for preparing inoculants produced inoculants having very high viable counts of bean rhizobia. The population sizes of bean inoculants containing the most effective strains identified for this project are presented in Table 6-26.

Rhizobial Field Evaluations at CNPAF. *Rhizobium leguminosarum* biovar *phaseoli* strain selections from various research programs were evaluated on bean line 21-58 at MSU. The best 12 strains were planted in a field experiment at CNPAF in 1985 in a randomized complete block design with a split-plot layout and 4 replicates.

Table 6-26. Effective Inoculant Strains of *R. phaseoli*.

Strain	Acetylene Reduction Activity[a] (μmole ethylene/plant/hr)	Population in Peat Innoculant[b] (log-10 cells/gram)
CIAT 899	1.85	10.38
CIAT 640	1.61	8.41
CIAT 632	2.03	9.18
F. Mayo	1.19	10.60
CIAS	1.12	10.92
CNPAF 150	1.34	8.53
Kim 5	1.24	8.88
127K81-3	1.63	9.23
Viking 1	1.27	8.46
ALN-1	1.73	ND
ITC-49	2.39	ND
J025	0.41	Representative indigenous strains
J029	0.35	isolated from nodules of uninnoculated beans grown at CNPAF Goinia, Brazil

[a] Assayed by acetylene reduction in 1 month-old flask cultures grown in a growth chamber. 5-6 replicate cultures/strain.

[b] Peat innoculants were cured for 2 weeks and the population enumerated by viable plate counts after 1 week incubation on YEM agar at 30° C.

The main plot factor was bean genotype: Rio Tibagi, normally a poor nodulator, and WBR 22-34, one of our elite selections. The subplot factor was rhizobial strain, with each strain being seed-inoculated individually. An N-fertilized treatment and a control which received neither inoculum nor N fertilizer were included also.

A plant sample was taken at the bud stage to measure shoot, root, and nodule dry matter production, acetylene reduction activity (ARA), and shoot N content. Samples of nodules from roots in approximately a 5-cm radius from the seed were taken to MSU to test for the presence of inoculated strains. At maturity, pod and seed yields, seed N concentration and content, and seed test weight were determined. Stems, roots, and pod walls, without senesced leaves, were collected to measure dry matter and N content. From these data, dry matter and N harvest indices were calculated.

Based upon their performance in 1985, strains CNPAF 150, CIAT 899, Cias, and Viking 1 were selected for a 2nd evaluation in 1986. An N fertilizer treatment and a control treatment with neither inoculum nor N fertilizer were included, as well as the following inoculi: a mixture of the above four strains, the rhizobial mixture recommended by Nitragin, the rhizobial mixture used at CNPAF, and the Brazilian commercial inoculum. A sample was taken at flowering (R1) for shoot, root, and nodule dry matter and shoot N content. At R7, shoots were collected for dry matter and N content. Pod, seed, and stover yields were determined at maturity. The 50-seed weight was recorded and HI values were calculated on the basis of stover and R7 shoot dry matter.

Table 6-27. Dry matter content at flowering in the Rizobium Strain Evaluation, CNPAF, 1985, 1986 and 1987. Values are means of 4 replicates.

Genotype	CIAT 899	VIKING 1	CNPAF 150	Fertilizer N	Control	$\bar{x}$
	Shoot Dry Matter (g plant^{-1})					
Rio Tibagi	3.10	2.97	3.13	4.84	2.75	3.36
WBR 22-34	3.06	3.21	3.13	4.68	3.33	3.48
$\bar{x}$	3.08 B[1]	3.09 B	3.13 B	4.76 A	3.04 B	3.42
	Root Dry Matter (g plant^{-1})					
Rio Tibagi	0.59	0.60	0.63	0.86	0.57	0.65*
WBR 22-34	0.67	0.69	0.68	0.87	0.72	0.73*
$\bar{x}$	0.63 B	0.65 B	0.66 B	0.86 A	0.65 B	0.69
	Shoot: Root Ratio					
Rio Tibagi	5.43	5.12	5.12	5.85	4.89	5.28*
WBR 22-34	4.67	4.70	4.71	5.36	4.66	4.82*
$\bar{x}$	5.05 B	4.91 B	4.92 B	5.61 A	4.78 B	5.05

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

* denotes a significant difference between genotype means at $P<0.05$.

Table 6-28. Maturity parameters in the Rhizobium Strain Evaluation, CNPAF, 1985, 1986 and 1987. Values are means of 4 replicates.

Genotype	CIAT 899	VIKING 1	CNPAF 150	Fertilizer N	Control	$\bar{x}$
	Grain Yield (kg ha^{-1})					
Rio Tibagi	1181	1208	1230	1551	1222	1278***
WBR 22-34	1593	1573	1781	2048	1530	1705***
$\bar{x}$	1387 B[1]	1391 B	1505 B	1800 A	1376 B	1492
	Pod Yield (m^{-2})					
Rio Tibagi	200	205	202	255	202	213***
WBR 22-34	164	168	188	204	160	177***
$\bar{x}$	182 B	187 B	195 B	229 A	181 B	195
	50-seed Field Test Weight (g)					
Rio Tibagi	6.87	6.67	6.77	6.84	6.97	6.84***
WBR 22-34	9.69	9.61	9.77	9.74	9.60	9.68***
$\bar{x}$	8.28 A	8.18 A	8.27 A	8.29 A	8.28 A	8.26

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

*** denotes a significant difference between genotype means at $P<0.001$.

Table 6-29. Dry matter and N data at flowering in the Rhizobium Strain Evaluation, CNPAF, 1985 and 1986. Values are means of 4 replicates.

Genotype	CIAT 899	VIKING 1	CNPAF 150	Cias	Fertil-izer N	Control	$\bar{x}$
	Shoot Dry Matter (g plant^{-1})						
Rio Tibagi	3.41	3.07	3.42	3.00	5.04	2.83	3.46
WBR 22-34	3.33	3.37	3.28	3.68	5.10	3.41	3.70
$\bar{x}$	3.37 B[1]	3.22 B	3.35 B	3.34 B	5.07 A	3.12 B	3.58
	Root Dry Matter (g plant^{-1})						
Rio Tibagi	0.69	0.68	0.74	0.68	0.96	0.62	0.73
WBR 22-34	0.75	0.77	0.78	0.78	0.94	0.81	0.80
$\bar{x}$	0.72 B	0.73 B	0.76 B	0.73 B	0.95 A	0.72 B	0.77
	Shoot: Root Dry Matter Ratio						
Rio Tibagi	4.97	4.54	4.74	4.50	5.33	4.61	4.78
WBR 22-34	4.41	4.41	4,23	4.82	5.33	4.10	4.56
$\bar{x}$	4.69 B	4.47 B	4.49 B	4.66 B	5.35 A	4.35 B	4.67
	Shoot N Concentration (%)						
Rio Tibagi	3.25	3.26	3.21	3.26	3.59	3.24	3.30
WBR 22-34	3.18	3.16	3.00	3.08	3.56	3.27	3.21
$\bar{x}$	3.21 B	3.21 B	3.11 B	3.17 B	3.57 A	3.25 B	3.25
	Shoot N Content (mg plant^{-1})						
Rio Tibagi	111	100	110	98	181	92	115
WBR 22-34	106	107	99	114	180	113	120
$\bar{x}$	109 B	104 B	104 B	106 B	181 A	102 B	118

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-30. Maturity parameters in the Rhizobium Strain Evaluation, CNPAF, 1985 and 1986. Values are means of 4 replicates.

Genotype	CIAT 899	VIKING 1	CNPAF 150	Cias	Fertil-izer N	Control	$\bar{x}$
			Pod Yield (m^{-2})				
Rio Tibagi	212	208	213	223	285	206	225**
WBR 22-34	174	186	201	188	220	167	189**
$\bar{x}$	193 B[1]	197 B	207 B	206 B	252 A	186 B	207
			Grain Yield (kg ha^{-1})				
Rio Tibagi	1110	1097	1102	1215	1466	1140	1188***
WBR 22-34	1631	1633	1844	1665	2104	1486	1724***
$\bar{x}$	1370 B	1365 B	1473 AB	1440 B	1785 A	1304 B	1456
			Stover Yield (kg ha^{-1})				
Rio Tibagi	1056	991	1027	1075	1427	1018	1099***
WBR 22-34	1396	1399	1586	1542	1693	1221	1473***
$\bar{x}$	1226 B	1195 B	1307 B	1308 B	1560 A	1120 B	1286
			Harvest Index				
Rio Tibagi	0.512	0.522	0.517	0.525	0.509	0.527	0.519
WBR 22-34	0.534	0.537	0.538	0.519	0.555	0.538	0.537
$\bar{x}$	0.523A	0.529A	0.528A	0.522A	0.523A	0.532A	0.528
			50-seed Field Test Weight				
Rio Tibagi	6.11	5.94	6.05	6.08	5.98	6.22	6.06***
WBR 22-34	9.08	8.96	9.22	9.19	9.12	8.88	9.07***
$\bar{x}$	7.59 A	7.45 A	7.64 A	7.64 A	7.55 A	7.55 A	7.57

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

** and *** denote significant differences between genotype means at $P<0.01$ and $P<0.001$, respectively.

In 1987, a 3rd-stage evaluation of these strains at CNPAF was combined with the network experiment of the Brazilian National Program on Symbiotic N_2 fixation in Beans. Strains CNPAF 150, CIAT 899, Viking 1, the Nitrogen mixture, and the Brazilian commercial mixture were again compared to N fertilizer and a control with neither N nor inoculum. Strains ITC 53 (Celaya, Mexico, via MSU), IAPAR 115 (Londrina, Parana, Brazil), UMR 1135 (University of Minnesota), and CNPAF 146 (Ribeira do Pombal, Bahia, Brazil, via CNPAF) were included for the first time. Each treatment was tested with three host plant lines: Rio Tibagi, WBR 22-34, and Carioca.

Samples were taken during the vegetative stage, at flowering, and at the end of podfilling to determine shoot, root, and nodule dry matter content. The shoot samples taken at pod-filling were analyzed for N content. At maturity, pod and grain yield and seed test weight were measured. Data were grouped for analysis to maximize year, inoculation treatment, and genotype combinations and parameters measured.

Analysis of the treatment combinations included in all three experiments showed N fertilizer application to be significantly superior to the other treatments at flowering and at maturity (Tables 6-27 and 6-28). The only parameter not affected was seed test weight. Inoculation treatments did not differ significantly from the uninoculated control even though the mean values for the inoculated treatments were often higher, particularly for grain yield (Tables 6-28 and 6-30). Line WBR 22-34 was generally superior to Rio Tibagi.

Combined analyses of treatment combinations and parameters from years 1985 and 1986 and from 1986 and 1987 also showed N fertilizer to be superior to the non-fertilized treatments and failed to show a significant response to inoculation (Tables 6-29 thru 6-33). Grain yield of WBR 22-34 inoculated with CNPAF 150 was highest of the non-N-fertilized treatments across three years (Table 6-28), but was lower than the uninoculated control when data from only 1986 and 1987 were combined (Table 6-34). Based upon these results, the program of field evaluation of seed-inoculated strains in the CNPAF bean growing area was discontinued.

The observation that some strains excel under controlled conditions but not in the field is not a new revelation, and is not surprising in light of the competition with other microorganisms for survival under environmental stresses encountered in a field situation. Research emphasis at CNPAF has been shifted to studying inoculation methodology, applying inoculum as a side-dress in an attempt to provide fresh inoculum at the optimum time during the growing season and in the optimum location (near the secondary roots, where nodulation is most abundant) to derive the most benefit.

Tube Cultures for the Most Probable Number Technique. The most common way to enumerate bean rhizobia in soil samples is the plant infection count using the Most Probable Number (MPN) technique in Leonard jars. However, since this technique is open to microbial contamination, it will overestimate the true MPN when cross-contaminated with bean rhizobia. This is a frequent and

Table 6-31. Maturity parameters in the Rhizobium Strain Evaluation, CNPAF, 1985 and 1986. Values are means of 4 replicates.

Genotype	CIAT 899	VIKING 1	CNPAF 150	Cias	Fertil-izer N	Control	$\bar{x}$
	Grain N Concentration (%)						
Rio Tibagi	3.30	3.18	3.24	3.27	3.70	2.99	3.28
WBR 22-34	3.27	3.25	3.17	3.33	3.33	3.31	3.28
$\bar{x}$	3.29AB[1]	3.22AB	3.20AB	3.30AB	3.51A	3.15 B	3.28
	Grain N Content (kg ha^{-1})						
Rio Tibagi	37	35	36	39	55	34	39***
WBR 22-34	54	55	59	55	71	47	57***
$\bar{x}$	46 B	45 B	47 B	47 B	63 A	40 B	48

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

*** denotes a significant difference between genotype means at $P<0.001$.

Table 6-32. Dry matter content at flowering in the Rhizobium Strain Evaluation, CNPAF, 1986 and 1987. Values are means of 4 replicates.

Genotype	CNPAF 150	CIAT 899	Nitra-gen	VIKING 1	Braz. Commer-cial	Fertil-izer N	Control	$\bar{x}$
	Shoot (g plant^{-1})							
Rio Tibagi	2.95	3.06	2.86	3.04	3.09	4.56	2.77	3.19
WBR 22-34	3.25	2.88	3.11	3.16	3.40	4.62	3.75	3.45
$\bar{x}$	3.10B[1]	2.97B	2.99B	3.10B	3.24B	4.59A	3.26B	3.32
	Root (g plant^{-1})							
Rio Tibagi	0.53	0.52	0.51	0.55	0.54	0.75	0.52	0.56***
WBR 22-34	0.65	0.58	0.58	0.63	0.64	0.82	0.72	0.66***
$\bar{x}$	0.59B	0.55B	0.55B	0.59B	0.59B	0.79A	0.62B	0.61
	Shoot: Root Ratio							
Rio Tibagi	5.62	5.99	5.77	5.69	5.85	6.26	5.37	5.80*
WBR 22-34	5.15	4.99	5.42	5.07	5.46	5.57	5.34	5.28*
$\bar{x}$	5.38A	5.49A	5.59A	5.38A	5.66A	5.92A	5.36A	5.54
	Nodule (mg plant^{-1})							
Rio Tibagi	19	15	12	16	17	7	12	14*
WBR 22-34	29	36	27	14	36	8	20	24*
$\bar{x}$	24 AB	25 AB	19 AB	15 AB	26 A	8 B	16 AB	19

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

* and *** denote significant differences between genotype means at $P<0.05$ and $P<0.001$, respectively.

Table 6-33. Shoot Dry matter and N content at R7 in the Rhizobium Strain Evaluation, CNPAF, 1986 and 1987. Values are means of 4 replicates.

Genotype	CNPAF 150	CIAT 899	Nitra-gen	VIKING 1	Braz. Commer-cial	Fertil-izer N	Control	$\bar{x}$
	Shoot Dry Matter (g plant^{-1})							
Rio Tibagi	11.10	12.38	11.17	10.89	10.44	12.99	11.22	11.46***
WBR 22-34	14.66	13.66	14.26	13.66	13.28	16.92	16.31	14.68***
$\bar{x}$	12.88AB[1]	13.02AB	12.72AB	12.28AB	11.86 B	14.95A	13.77AB	13.07
	Shoot N Concentration (%)							
Rio Tibagi	1.90	1.80	2.21	2.03	2.11	1.99	1.95	2.00*
WBR 22-34	1.91	1.80	1.97	1.81	1.92	1.75	1.80	1.85*
$\bar{x}$	1.90 A	1.80 A	2.09 A	1.92 A	2.02 A	1.87 A	1.88 A	1.94
	Shoot N Content (mg plant^{-1})							
Rio Tibagi	212	212	243	215	224	250	214	224**
WBR 22-34	267	234	270	235	251	284	270	259**
$\bar{x}$	240 A	223 A	257 A	255 A	237 A	267 A	242 A	241

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

*, ** and *** denote significant differences between genotype means at $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Table 6-34. Maturity parameters in the Rhizobium Strain Evaluation, CNPAF, 1986 and 1987. Values are means of 4 replicates.

Genotype	CNPAF 150	CIAT 899	Nitra-gen	VIKING 1	Braz. Commer-cial	Fertil-izer N	Control	$\bar{x}$
	Pod Yield (m^{-2})							
Rio Tibagi	201	194	216	208	214	234	206	211**
WBR 22-34	175	149	161	149	155	181	162	162**
$\bar{x}$	188 AB[1]	172 B	189 AB	179 AB	185 AB	207 A	184 AB	186
	Grain Yield (kg ha^{-1})							
Rio Tibagi	1293	1177	1284	1225	1253	1512	1304	1293**
WBR 22-34	1678	1425	1418	1403	1546	1875	1693	1577**
$\bar{x}$	1485AB	1301 B	1351 B	1314 B	1399 B	1693A	1498AB	1435
	50-seed Field Test Weight (g)							
Rio Tibagi	7.09	7.23	7.07	7.00	6.86	7.25	7.35	7.12**
WBR 22-34	9.90	9.76	10.06	9.51	9.78	9.89	9.77	9.81
$\bar{x}$	8.49 A	8.50 A	8.56 A	8.25 A	8.32 A	8.57 A	8.56 A	8.47

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

*** denotes a significant difference between genotype means at $P<0.001$.

major problem in greenhouse studies of growing bean plants. Thus, an MPN method using a modification of the Gibson semi-enclosed tube culture technique was developed which could be adapted for bean rhizobia growing under microbiologically controlled conditions (Araujo et al., 1986). Advantages of the tube culture over Leonard jars to estimate the MPN include: 1) less frequent need to water; 2) less space required; and 3) less chance of cross-contamination. The MPN using the tube cultures reached a plateau by 21 days after inoculation and was in close agreement with the viable plate count of pure cultures of the R. leguminosarum biovar phaseoli strains on YEM plates. The appearances of the tube culture at 21 days is illustrated in Figs. 6-3b and c. The accuracy of this tube culture method was verified with aqueous suspensions of rhizobia, peat-based inoculants, and soil. From 1 to 3 viable cells of R. leguminosarum biovar phaseoli were sufficient inoculum to induce nodule formation. This probably reflects the superiority of bean line 21-58 to serve as an efficient "trap" capable of forming root nodules from a low concentration of bacterial cells. A more comprehensive test of this hypothesis is described under the section "Early-season Nodulation Studies at MSU" in this report. The indigenous population of bean rhizobia at the CNPAF field sites was measured using this technique.

Evaluation of Methods to Identify Inoculant Strains in Nodules on Field-Grown Plants. In order to measure inter-strain competitiveness at MSU, we evaluated several methods to identify our inoculant strains against the diverse, indigenous strains in the

field, including serology, intrinsic antibiotic resistance, and protein profiles in SDS-polyacrylamide gel electrophoresis (PAGE). Several strains immunologically cross-reacted. Only one strain (CIAT 899) carried sufficient intrinsic antibiotic resistnce (spectinomycin) to be useful in its identification. On the other hand, each of the bean rhizobial strains used in this research for peat-based inoculants produced a protein profile in SDS-PAGE which can distinguish that strain. The major advantage of strain identification based on protein patterns in SDS-PAGE is that the total genetic background of the cells is compared rather than a few selected traits.

We have improved on the protein profile method to identify strains by significantly shortening the period required to culture sufficient cells of the nodule occupant strain for analysis by SDS-PAGE. This is accomplished with the use of BIII broth which provides some selectivity for rhizobia and cycloheximide to inhibit fungi, enabling us to inoculate cells from a squashed, surface-sterilized nodule directly into the medium. Compared to 3-4 days incubation on conventional YEM agar plates, sufficient cells for SDS-PAGE are obtained by overnight incubation. To evaluate this modified method, we compared the nodule occupants from 50 field-grown bean nodules (uninoculated border rows from J. Acosta and W. Adams plot, MSU) grown in BIII broth vs. streak plating on YEM agar. Approximately 92% of the nodule occupants obtained by streak plating were accurately typed after 1-day growth in BIII broth. The remaining 8% of the nodules had multiple strain occupants. In

summary, we have improved on the methods to enumerate and identify strains of bean rhizobia.

Nodules on Field-Grown Plants

Early-season Nodulation Studies at MSU. Much interaction occurs between the bacterial and plant symbionts before either partner benefits from nitrogen fixation. Since genetic variability for both the plant and the bacteria impacts centrally on this Bean/Cowpea CRSP project, we decided to examine how different bean and *R. phaseoli* genotypes respond when cultured together before nitrogen fixation commences.

The first set of experiments was designed to determine the nodulation efficiency of various bean lines/cultivars to a low bacterial inoculum under microbiologically controlled conditions. In one study, we found that bean line 21-58 was superior to Puebla 152, Sanilac, and Rio Tibagi in formation of root nodules following inoculation with one cell of CIAT 899 (Fig. 6-4). This result indicates that bean line 21-58 is extremely efficient in all the processes of the host plant involved in nodule formation. In a second study, the ability of 10 bean lines/cultivars to serve as an efficient "trap" for *R. phaseoli* was evaluated to optimize the MPN plant infection technique (see section on Tube Cultures for the MPN Technique). Puebla 152 and line 21-58 were the most efficient trap hosts, requiring the least number of bacterial cells in the inoculum to develop nodulated roots (Table 6-35). These data indicate that the accuracy of the plant infection technique will

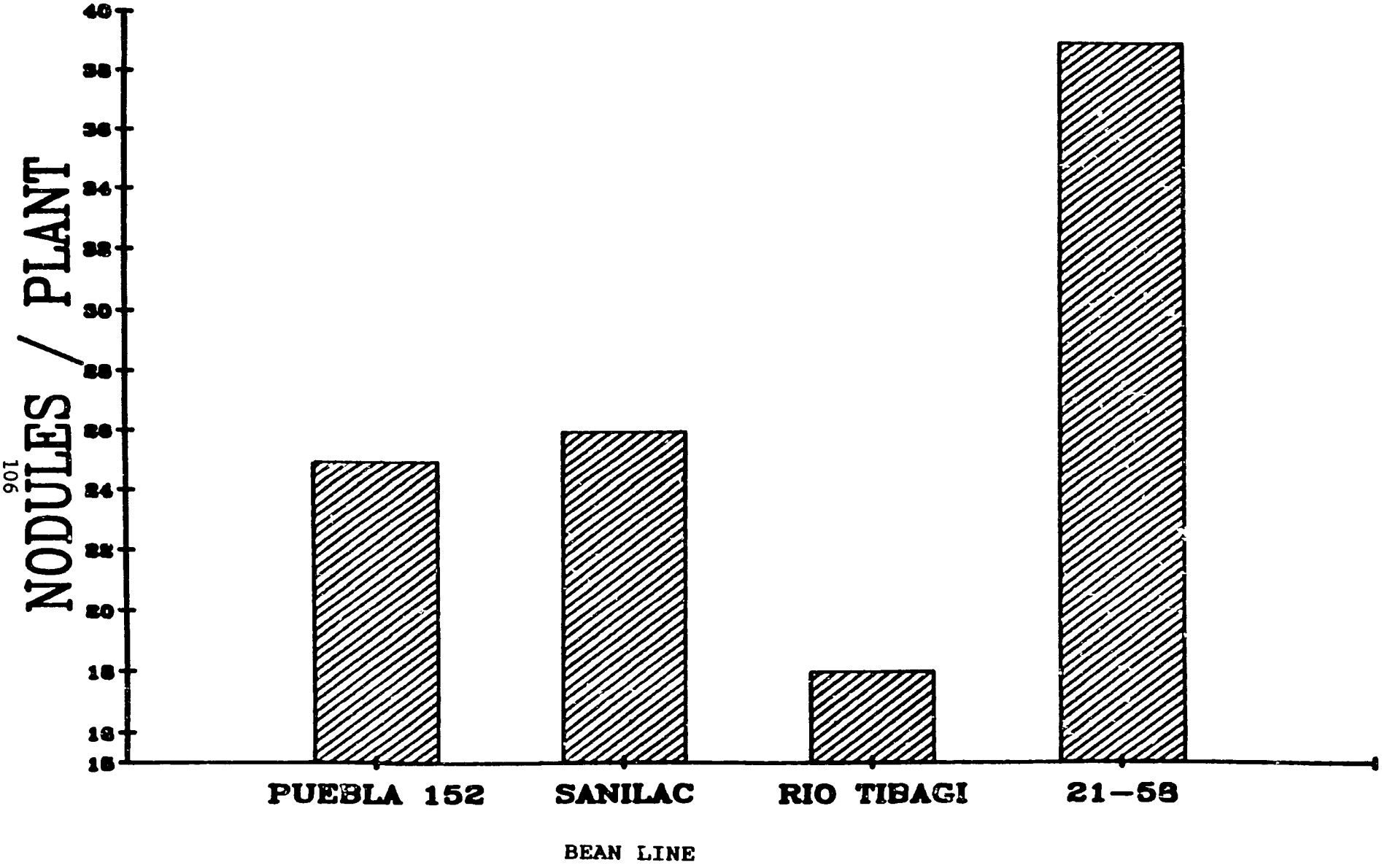


Figure 6-4. Nodule induction efficiency of four bean lines grown in semi-enclosed tube cultures using one cell inoculum of R. phaseoli.

Table 6-35. Nodulation of Different Bean Lines/Varieties by Low Inoculum Sizes of *Rhizobium phaseoli* CIAT 899[a]

Bean Line	CFU/MPN[b]	Cell innoculum[c] 8 plants^{-1}	Nodules 8 plants^{-1}	Nodules cell innoculum^{-1}
Rio Tibagi	4.5	64	78	1.2
Puebla 152	1.4	64	119	1.9
Negro Argel	2.4	64	76	1.2
Sanilac	2.4	64	109	1.7
21-58	1.4	64	185	2.9
22-17	6.5	84	191	2.3
22-27	11.8	84	175	2.1
22-37	11.8	84	210	2.5
22-34	3.5	84	218	2.6
ICA Pijao	6.5	84	112	1.3

[a] Using tube cultures as described in 1986 Appl. Environ. Microbiol. 52:954-956.

[b] Colony forming units (plate counts)/most probable number using plant infection test.

[c] Based on CFU on YEM agar.

vary with the bean line serving as trap host, and we recommend these two bean lines to enumerate the native population of bean rhizobia in soils where field trials are being conducted. The high nodulation response of line 21-58 at low rhizobial inoculum dose may contribute to its superior nodulation performance and be related to the fixation potential of the host plant.

The second set of experiments was designed to examine the impact of the bacteria on bean development prior to symbiotic nitrogen fixation by evaluating the early growth response of various bean lines to inoculation with R. phaseoli. These experiments also measured how well different bean lines stimulate the bacterial population in the rhizosphere and form root nodules in response to their presence.

The following bean lines were evaluated in sand culture: Negro Argel, Rio Tibagi, Puebla 152, ICA Pijao, Sanilac, Sanilac L12-50, Porrillio Sintetico, 21-19, 21-58, 22-03, 22-17, 22-27, 22-34, and 22-37 (all seed provided by F. Bliss). Acid-washed quartz sand was washed with deionized water, dried at 160 C , cooled, and mixed with peat-based inoculant containing either R. phaseoli CIAT 899 or CNPAF 150 (two strains which perform well under field conditions) to a final density of 10^4 cells/g of sand. Trays of sand were irrigated with sterile Smucker's nitrogen-free medium, planted with seeds of each bean line to a 1-2 cm depth, covered in Al foil, and incubated in a growth chamber. Beginning with the third day, each row was irrigated daily with 5 ml of sterile water. The foil cover was removed on the 4th day and plants emerged after

5-6 days. At 10 days, plants were examined for plant height. Top height varied with bean line and was influenced by the bacterial inoculant, with most plant tops developing slightly better with CIAT 899 (Fig. 6-5).

The development of the bacterial inoculant strain in the sand, the rhizospheres, and the bean root was examined. Roots were placed in tared tubes containing sterile water, rocked for ca. 1 min, and removed. The tared difference represented the rhizosphere and sand dislodged from the root. Due to the intrinsic antibiotic resistance of CIAT 899, it was possible to enumerate the rhizosphere and non-rhizosphere populations of this strain by viable plate counts. Diluted samples were plated on BIII agar containing 200 ug/ml cycloheximide and 500 ug/ml spectinomycin. R. phaseoli strain CIAT 899 developed high populations in both the non-rhizosphere sand and in the bean rhizospheres (Fig. 6-6), with little difference in stimulation among the various lines (Fig. 6-7). The high productivity of this bacterial strain both inside and outside of the bean rhizosphere illustrates its success as an adaptable microorganism and leads to a marginal R:S ratio. Attempts were made to enumerate CNPAF 150 on B III plus cycloheximide and congo red, but viable counts of this strain could not be reliably recognized among other bacteria producing the same colony morphology. The roots were placed on agar plates containing BIII medium plus cycloheximide and spectinomycin to examine the distribution of CIAT 899 on the root system. After imprinting the root to deposit the bacteria, the plate was marked on the back to

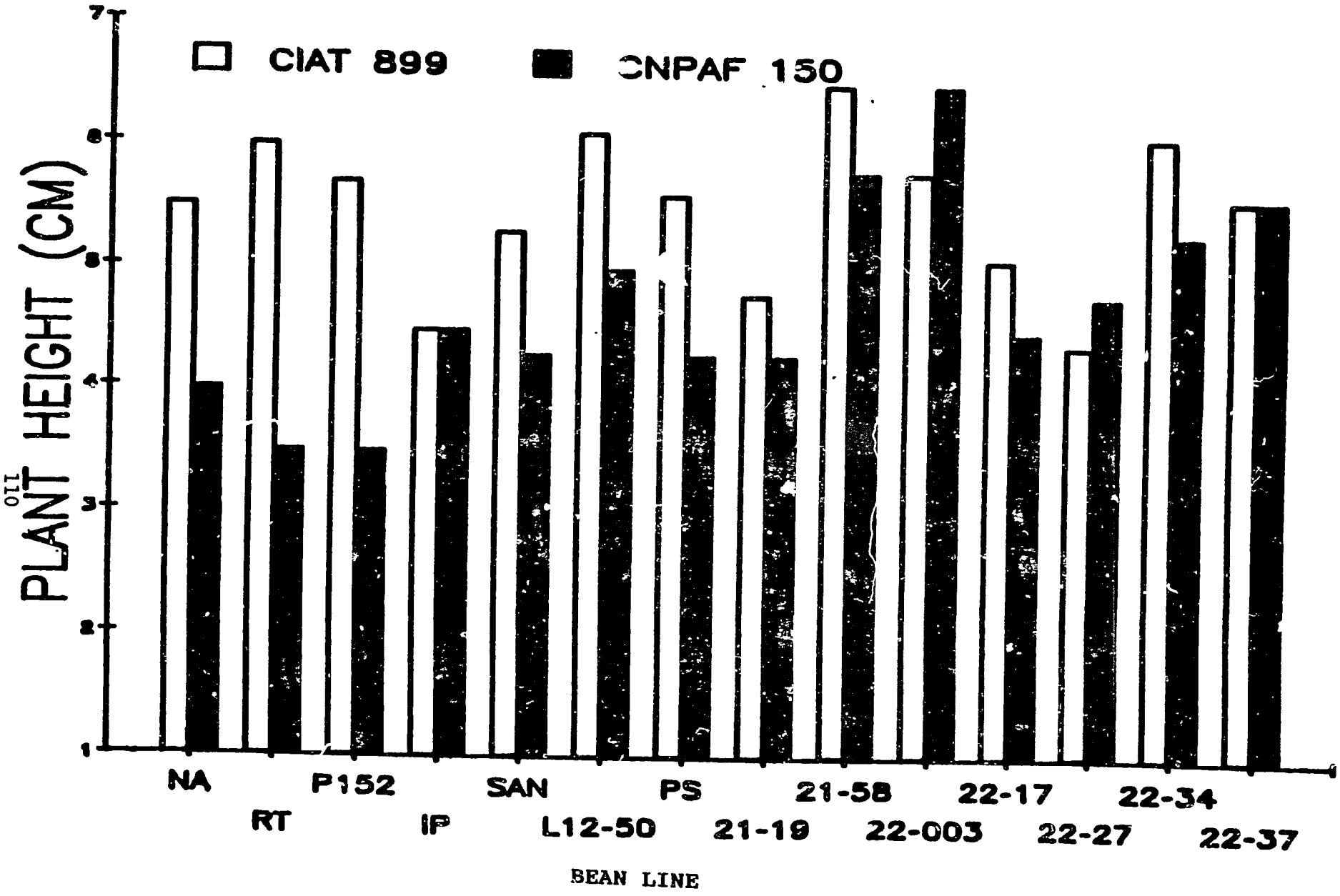


Figure 6-5. Bean top height after ten days growth in sand culture with R. phaseoli.

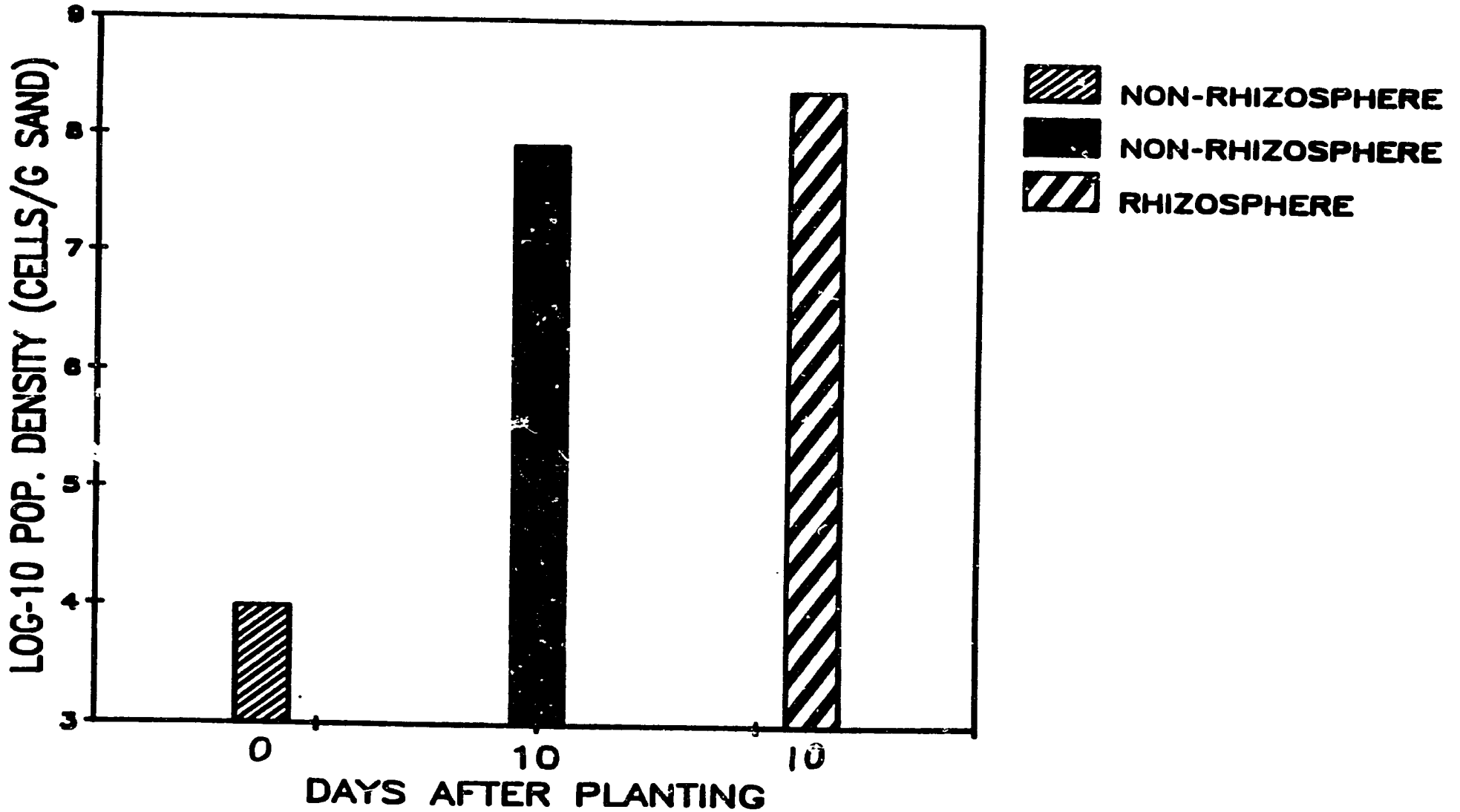


Figure 6-6. Development of R. phaseoli CIAT 899 in the rhizosphere and non-rhizosphere of beans (Composite of 14 lines).

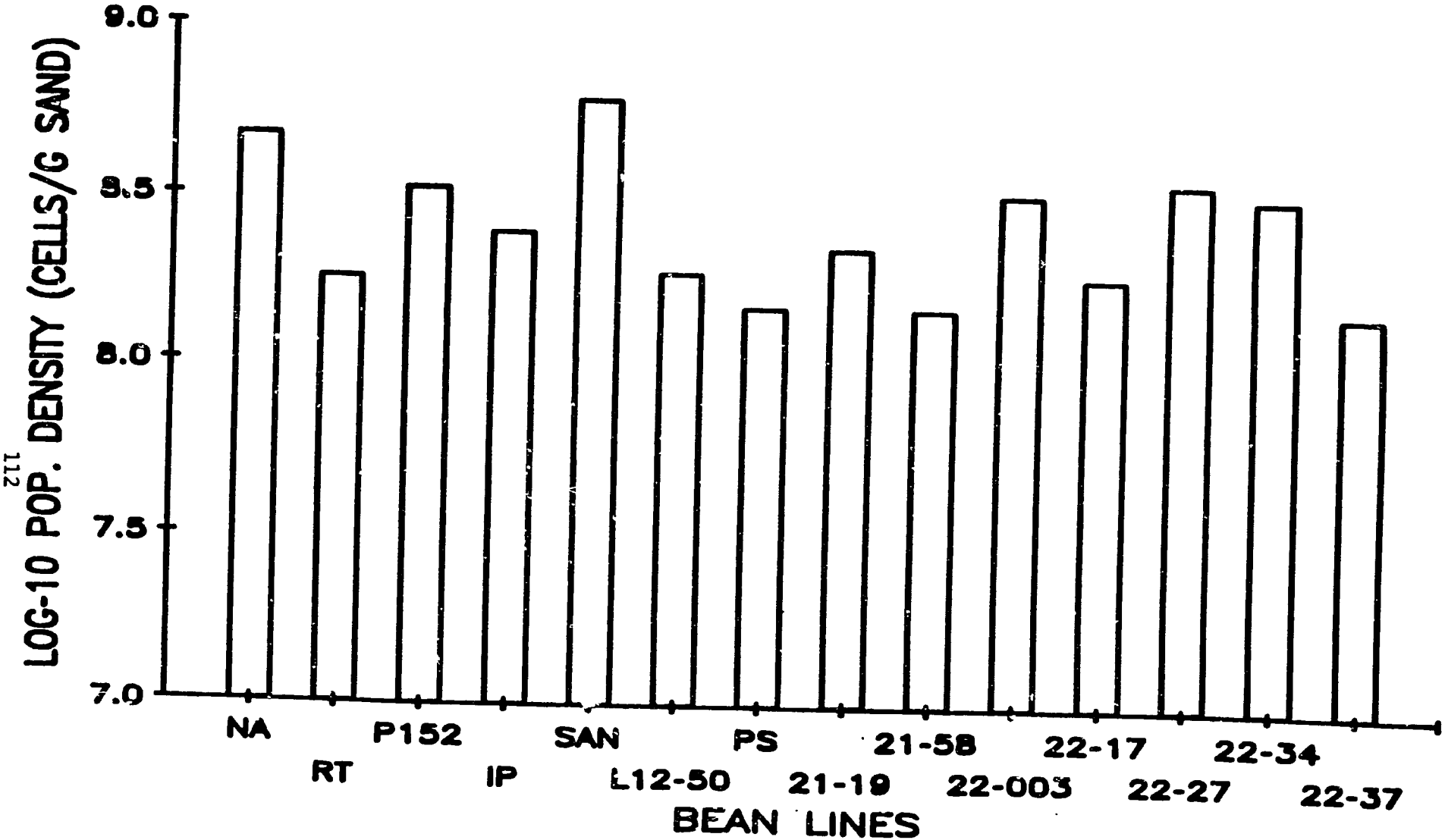


Figure 6-7. Density of R. phaseoli CIAT 899 in the rhizosphere of different bean lines ten days after planting.

indicate the root imprint, and incubated so that bacterial growth would indicate the extent of root colonization (Fig. 6-8). Within the limits of resolution of the method, the results suggested that CIAT 899 had colonized the entire root system of each bean line, as there were no obvious gaps on the place imprint which failed to develop bacterial growth carrying the spectinomycin resistance and pearl-white mucoid markers of this bacterial strain.

After imprinting on the sterile agar surface, the cleaned roots were photographed, and enlarged prints were analyzed for total root length and extent of branching by digitizing morphometry using the R & M Biometrics Bioquant System (Dazzo and Peterson, 1989). Root lengths were significantly longer for several of the bean lines inoculated with strain CNPAF 150 (Fig. 6-9). For example, there was greater development of roots for the bean cv. ICA Pijao when grown with CNPAF 150 vs. CIAT 899 (Fig. 6-10). Development of branched lateral roots varied among bean lines and was influenced by the inoculant strain, with more branched roots developing in association with CNPAF 150 (Fig. 6-11). Thus, the bacterial symbiont exerts a major impact on expression of different growth characteristics among the various bean lines, and these differences in plant growth could feasibly affect the evaluation of bean lines in breeding programs. They also may contribute to the basis for different bean yields among the various bean lines under field conditions.

Interestingly, we have obtained field results in Mexico which suggest that CNPAF 150 can benefit production of certain bean

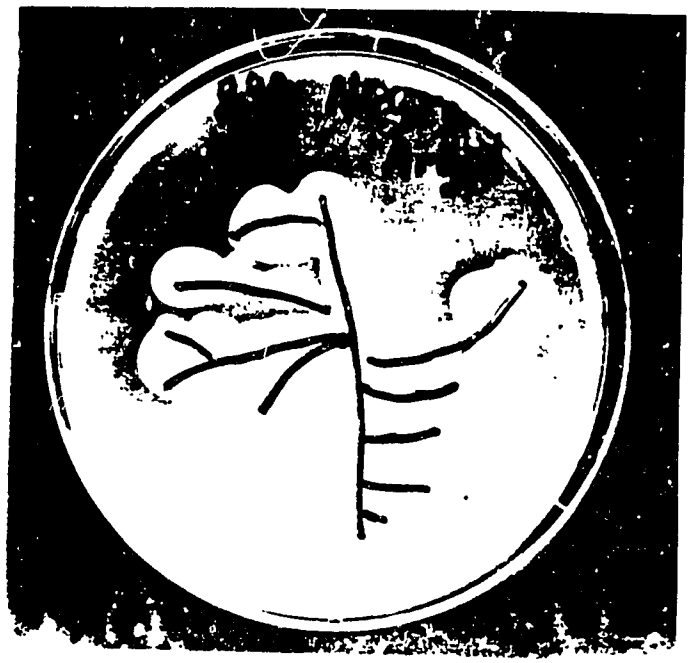


Figure 6-8. B III plate imprint of bean root to show extent of colonization by strain CIAT 899.

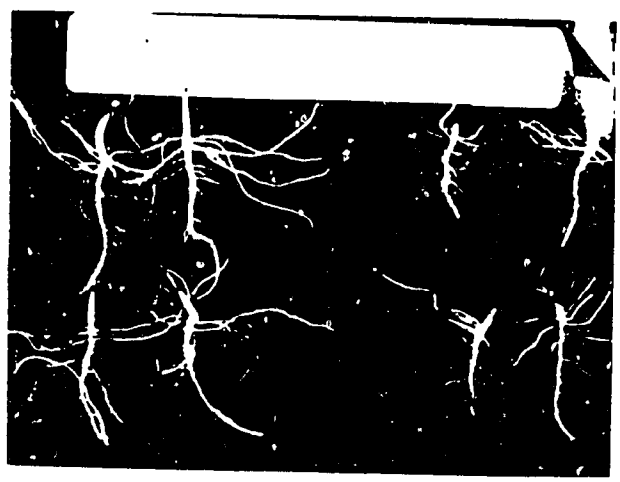


Figure 6-10. Root development of cv. ICA Pijao inoculated with either CIAT 899 or CNPAF 150.

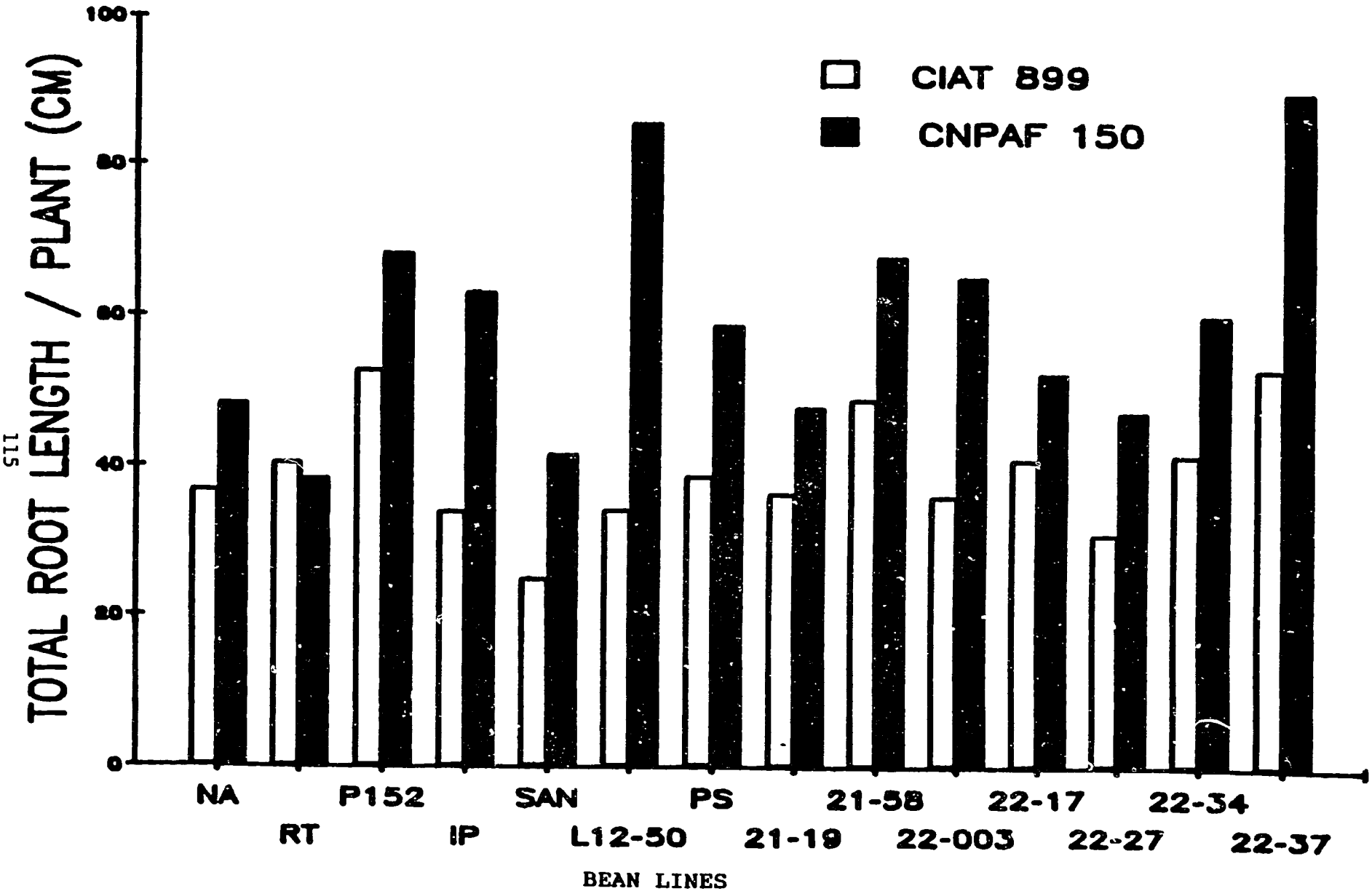


Figure 6-9. Bean root length after ten days growth in sand culture with R. phaseoli.

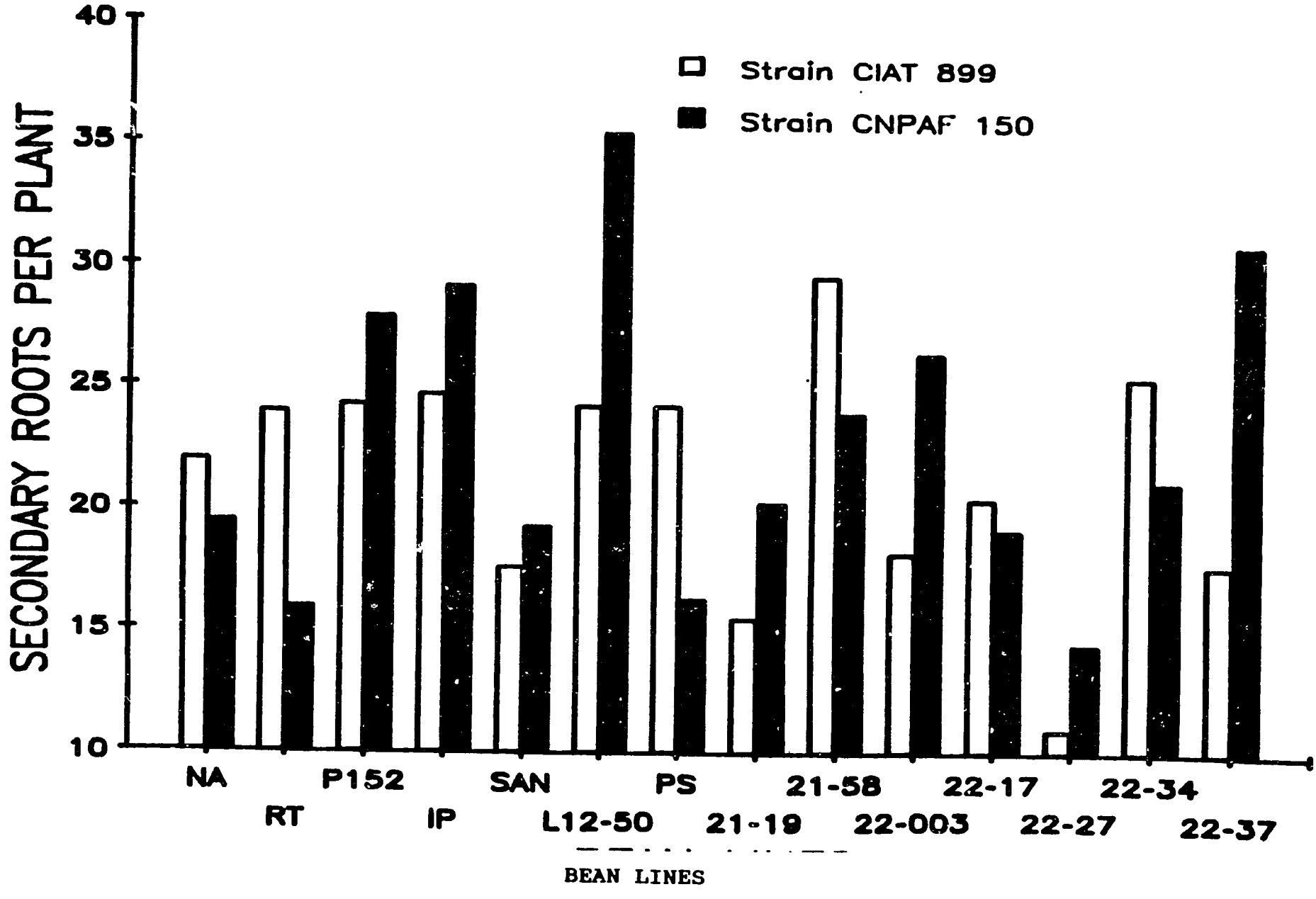


Figure 6-11. Root branching in beans grown ten days in sand culture with R. phaseoli.

varieties even when their production is not enhanced by adding N fertilizer (Maya-Flores, Valdes, and Dazzo, unpublished results). These results suggest that Rhizobium is a plant growth-promoting rhizobacterium which can benefit legume production in more ways than biological nitrogen fixation alone.

Finally, the extent of root nodulation by the different bean lines was compared. Roots were cleared under vacuum in 24% Chlorox, strained for 2 min. with 0.01% methylene blue, rinsed in water, and examined by light microscopy. This method visualized nodules at many different stages of development, including early cortical cell divisions and primordial nodules which have not emerged from the root. As a result, these nodule counts are far more accurate than what would be obtained by counting externally visible nodules only. As expected, wide differences in nodule development occurred with the various bean lines, and this was very dependent upon the inoculant strain (Fig. 6-12). Line WBR 22-34 stands out as the superior host for early root nodulation and this finding helps explain why this line performs well under low nitrogen field conditions. It would be interesting to compare these data with the field evaluations of root nodulation for the same bean lines. It must be emphasized that these data only reflect early nodulation (10 days), and not the potential for nodulation throughout the life cycle of the plant. The greater nodulation response of lines 21-19 and 22-03 with strain CIAT 899 is also an interesting finding which warrants further investigation.

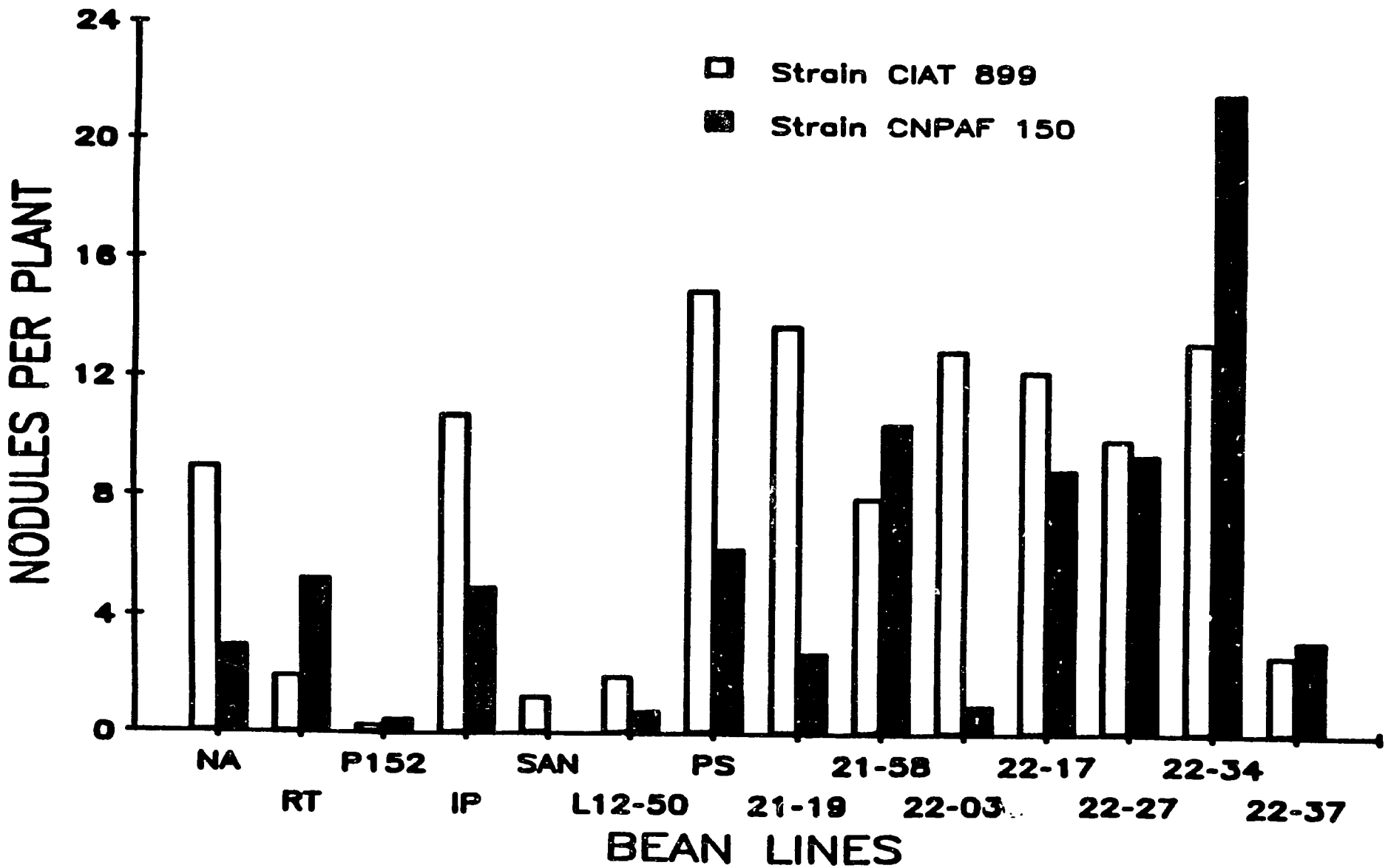


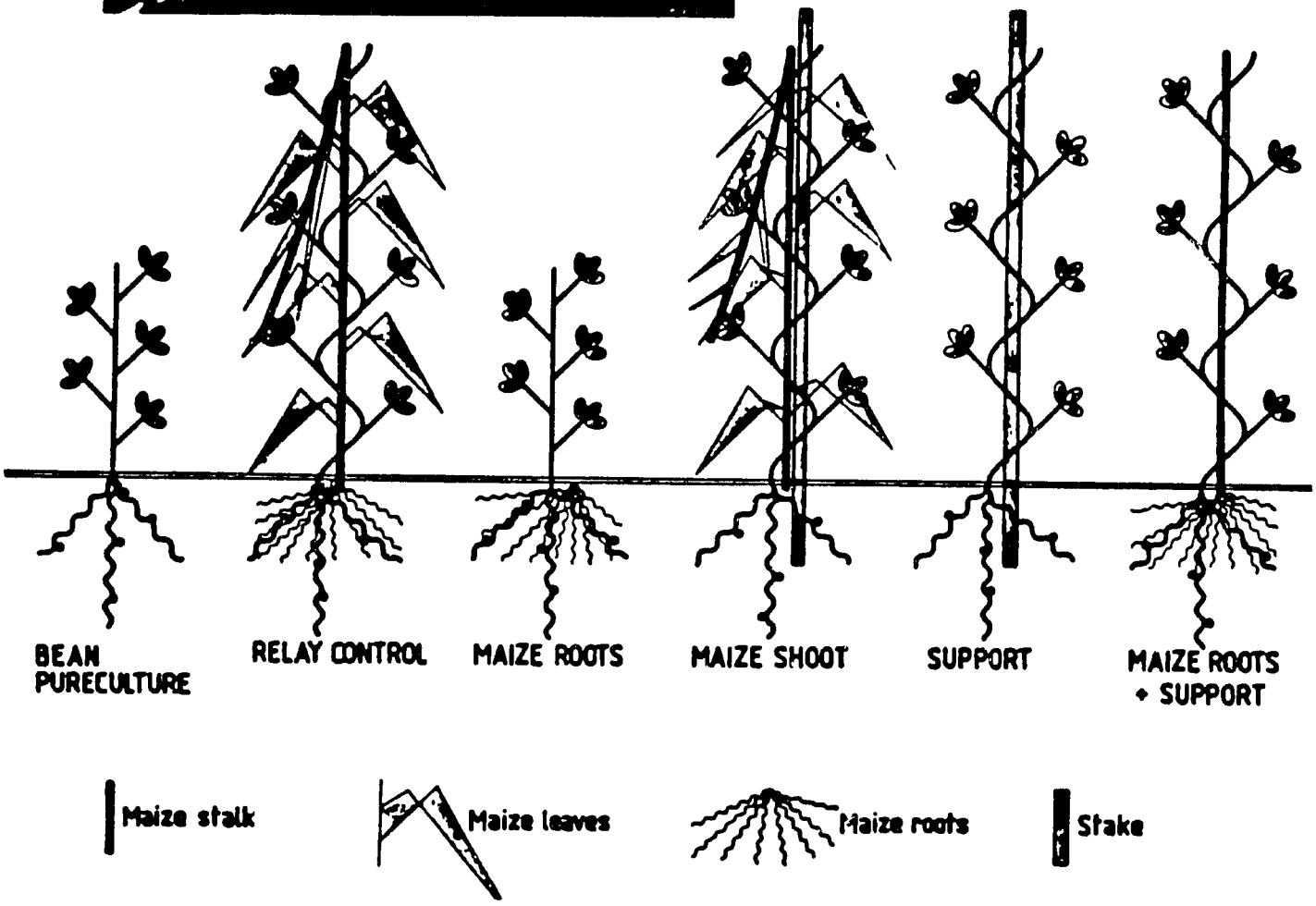
Figure 6-12. Nodule induction in beans grown ten days in sand culture with R. phaseoli.

Maize/Bean Relay Cropping

Relay Cropping Effects on Nitrogen Fixation. Approximately 69% of the Brazilian bean crop is produced in multiple cropping systems (Fundacao IBGE, 1980), most often relay cropped or intercropped with maize (*Zea mays* L.) (Vieira, 1985). In the relay system, a pure stand of maize is planted during the rainy season. At maturity, beans are planted between the maize rows, maize plants are doubled-over below the ear, and the beans climb up the maize plants (Fig. 6-13a). This system is characteristic of small farms, where price, availability, and level of technology often limit fertilizer usage (Pinchinat et al., 1976), and insufficient nitrogen (N) often limits yields (e.g. Duque et al., 1985).

Under such conditions, increasing N_2 fixation by the bean-rhizobia symbiosis provides an attractive alternative in supplying additional N for crop growth requirements. However, N_2 fixation in the relay system has been given little research attention considering the importance of this system in Latin America and the potential benefits to be gained. Early experiments by the CNPAF Microbiology Project showed differences in nodulation, nodule activity, and yield between beans grown as a sole crop (pureculture) and grown in the maize/bean relay system (Pereira et al., 1984). In 1981, grain yield was significantly higher in pureculture than in relay in all treatments, but the duration of nodule mass was significantly prolonged in the relay system. In 1983, nodule dry weight was again maintained longer into podfilling in

Figure 6-1[illegible]. Maize and bean plant combinations providing comparisons with and without shade, support, and maize roots; left, corn-bean intercropping in the field at CNPAF, below, diagram showing different combinations of culture, support and roots.



relay than in pureculture, and grain production was also significantly higher in relay.

In these experiments, the relative yield ranking of the two cropping systems varied, but in both years a significantly higher nodule mass was observed in the relay system during podfilling, suggesting that some factor(s) in this system is conducive to prolonged nodulation and, presumably, N_2 fixation.

The microclimate of a bean plant grown in the relay system is quite different from that of one grown in pureculture. In relay, shading by the maize plant alters incident light striking the bean plant as well as soil temperature and moisture content. This may result in a more favorable or more stable microclimate for nodulation. Perhaps the presence of a support results in a better bean leaf distribution and light interception, or reduced pest incidence. Decomposition products from the maize roots may stimulate soil rhizobial activity or may raise the C:N ratio in the soil, in either case possibly increasing N_2 fixation.

A 2-year experiment was conducted at CNPAF in 1984 and 1985 to test the effects of shading by the maize plant, support for the bean plant, and the presence of maize roots in the soil on parameters related to N_2 fixation. A split-plot format was adopted with six main plot factors designed to separate factors being studied. After cropping the entire experimental area to maize during the rainy season, the following main plot treatments were installed: bean pureculture, relay control, maize roots (no shoot), stake + maize shoot (no roots), stake, and maize roots +

stalk without leaves (Fig. 6-13b). The subplot factor was bean line: Rio Tibagi and Carioca, commonly grown Brazilian cultivars, and Negro Argel and WBR 22-34, lines selected for increased N_2 fixation under this project. Large plots were used to reduce border effects from the adjacent cropping system.

Bean seeds were inoculated at planting with a mixture of four R. phaseoli strains (CNPAF, 150, CNPAF 170, CNPAF 180, and CNPAF 189) selected at CNPAF. Samples were taken at 21 DAE (vegetative), 39 DAE (full bloom), and 53 DAE (mid-podfill), to measure shoot, root, and nodule dry matter, shoot and root N content, and acetylene reduction activity (ARA). Grain and pod yields and seed test weight were measured at maturity. In addition to plant samples, data were collected from main plots on incident light (top of bean canopy at 11, 25, and 48 DAE), soil moisture content (0-15 cm at 28 and 39 DAE), and soil temperature (18 cm at 36, 39, and 48 DAE). Disease ratings and plant height were noted at the full bloom stage. The experiment was conducted in 1985 as in the first year, but with data on incident light, soil moisture, and soil temperature taken at 10 and 17 DAE.

The data were analyzed by combining main plot treatments and testing single degree of freedom comparisons to obtain the effects of ± shade, ± support, and ± maize roots on the parameters measured. Comparisons among bean lines were made by Tukey's HSD.

In 1984, nodule dry matter and grain yield were the only parameters which showed a consistent, significantly different response to shade, support, or maize roots. Across genotypes,

shading decreased nodule dry matter, while support increased nodulation at each sampling date (Fig. 6-14). The presence of maize roots resulted in slightly higher nodule dry matter on each date, and prolonged nodule duration during podfill.

The data were re-analyzed within each bean line to determine genotypic variability in the observed response. Although the magnitude varied among genotypes, nodule dry matter was consistently reduced with shading and increased with support (Fig. 6-15). The genotypic response to maize roots, however, was more variable. Carioca showed reduced nodulation with maize roots, while Negro Argel showed a small and variable response. Nodulation of Rio Tibagi and WBR 22-34 was consistently increased in the presence of maize roots, especially at the full bloom and podfill samples. Unlike nodulation, grain yield across lines was increased by shading and decreased by maize roots (Fig. 6-16). Like nodulation, support increased grain yield.

Grain yield response to shade, support, and maize roots was similar among lines but the magnitude of the response varied (Fig. 6-14). Carioca and Negro Argel produced a significantly higher yield with shade, while that of Negro Argel and WBR 22-34 was significantly higher with support. A general yield decrease was observed with maize roots, but was only significant for Negro Argel.

Conditions which favored increased or decreased nodulation usually had the opposite effect on grain yield, supporting the theory of nodules and seeds as competing sinks. However, genotypic

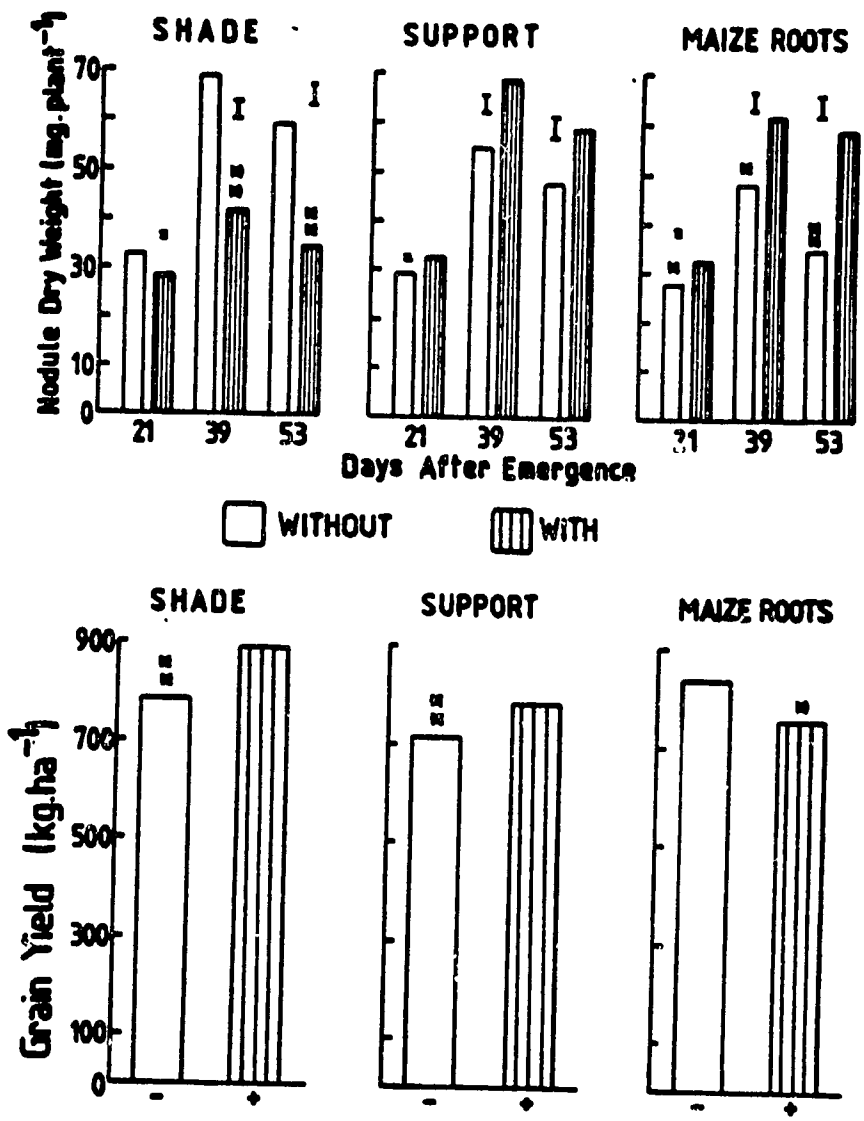


Figure 6-14. Nodulation and grain yield of bean lines with and without shade, support and maize roots in the first year experiment at CNPAF, 1984.

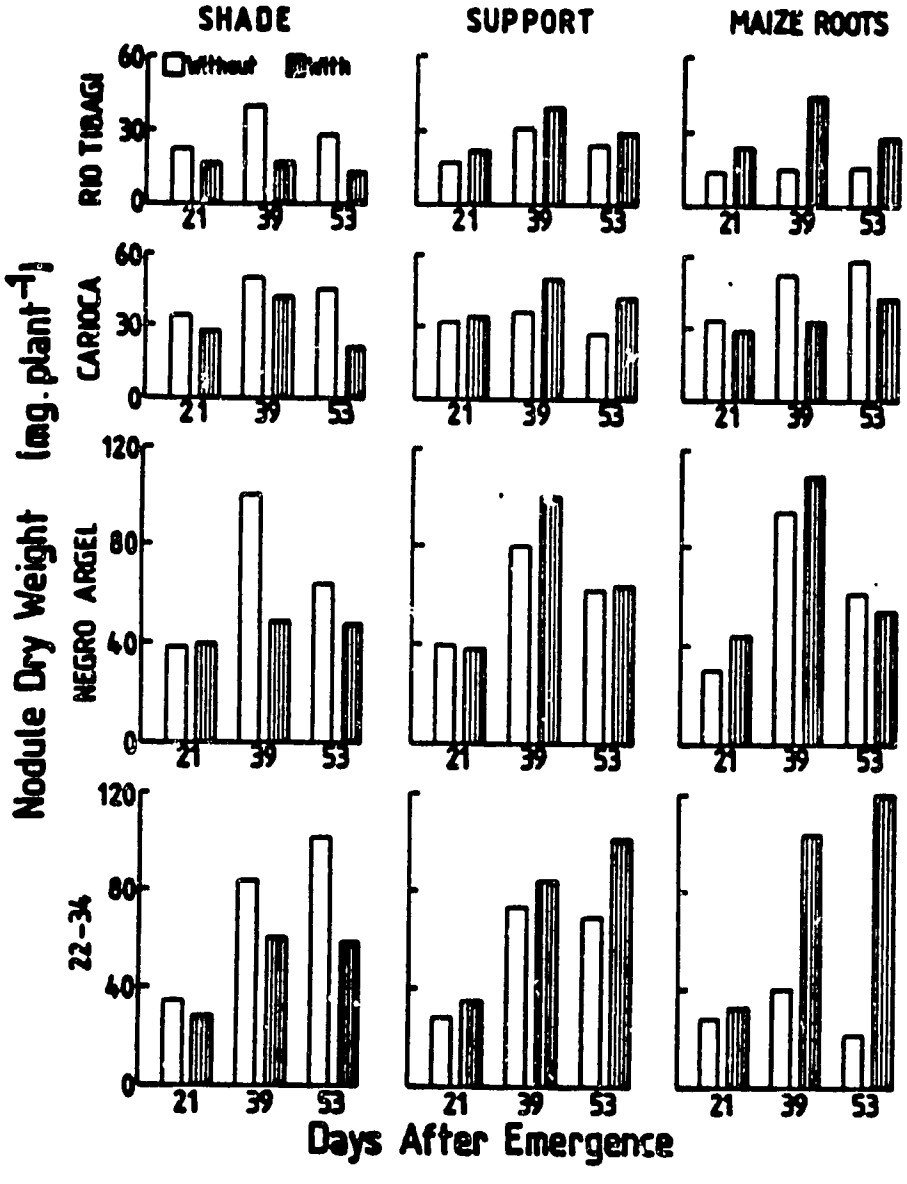


Figure 6-15. Nodule dry weight of bean lines with and without shade, support and maize roots in the first year experiment at CNPAF, 1984.

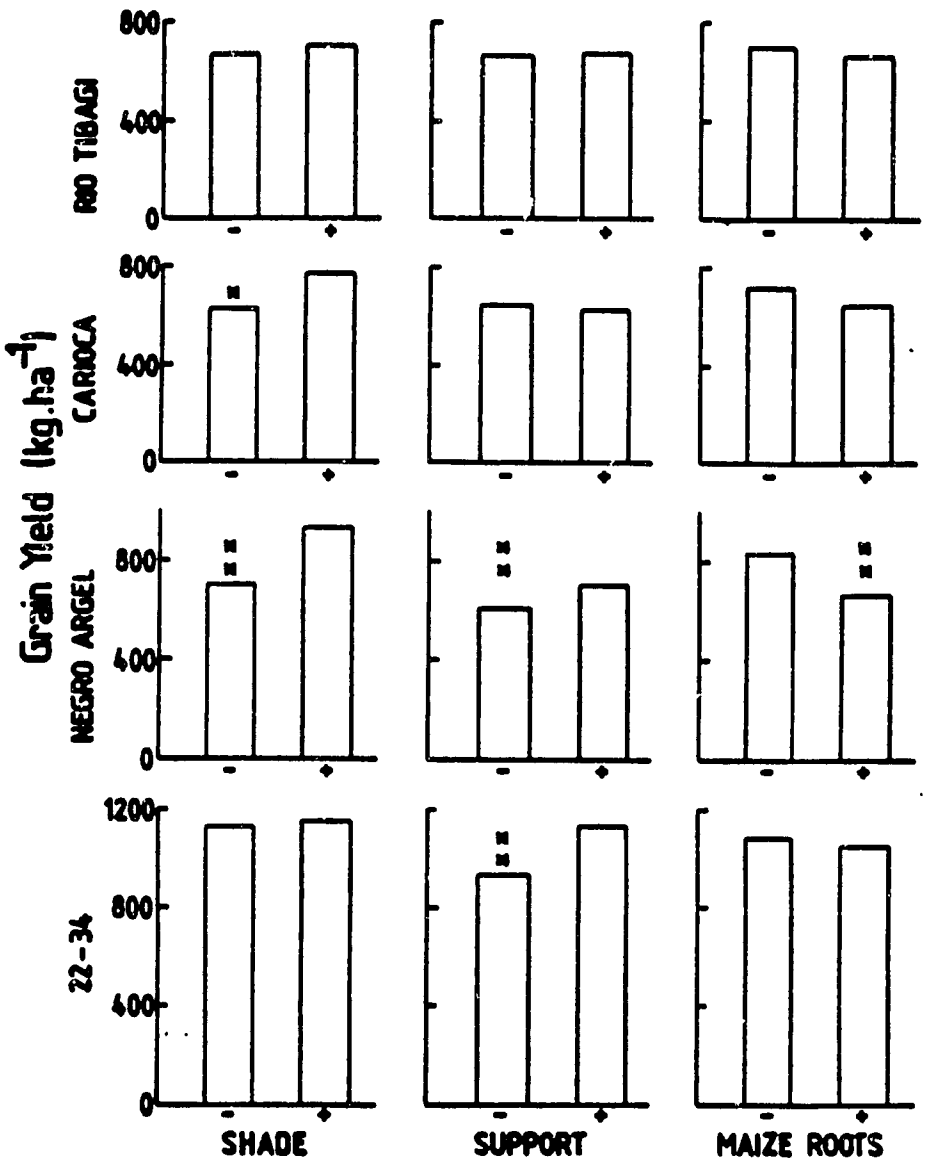


Figure 6-16. Grain yield of bean lines with and without shade, support and maize roots in the first year experiment at CNPAF, 1984.

variability existed in the responses (e.g. WBR 22-34, where maize roots increased nodulation greatly, but had almost no effect on yield). The benefit of maize roots to nodulation seems to be greatest during the reproductive period of legumes. The responses to shade and support were more uniform across bean lines.

A marked treatment reduction in incident light at the top of the bean canopy was observed in relay cropping. Soil temperature at 18 cm showed the same pattern, but with smaller differences. At shallower depths, the differences were probably much more pronounced. Soil moisture from 0-15 cm showed the relay control to retain moisture better than the other treatments. The 28 DAE moisture sample was collected 8 days after the previous irrigation (more than 5 days at CNPAF is considered stressful). In 1985, nodule dry matter was reduced by shading, by support and by maize roots, but no significant differences were observed in the single-degree-of-freedom comparisons of nodulation and yield (Fig. 6-17). However, the majority of nodules senesced before flowering, and results in 1985 do not reflect the normal duration of nodulation during the bean growth cycle. Some genotype variability existed, but the effects were quite uniform.

Two facts are notable in trying to explain differences in results between results the two years. Enhanced nodulation observed in the relay system in previous experiments may be a real effect, but unfavorable environmental conditions, such as cool temperatures, may override this effect in some years. Second, for undetermined reasons, most nodules senesced before full bloom in

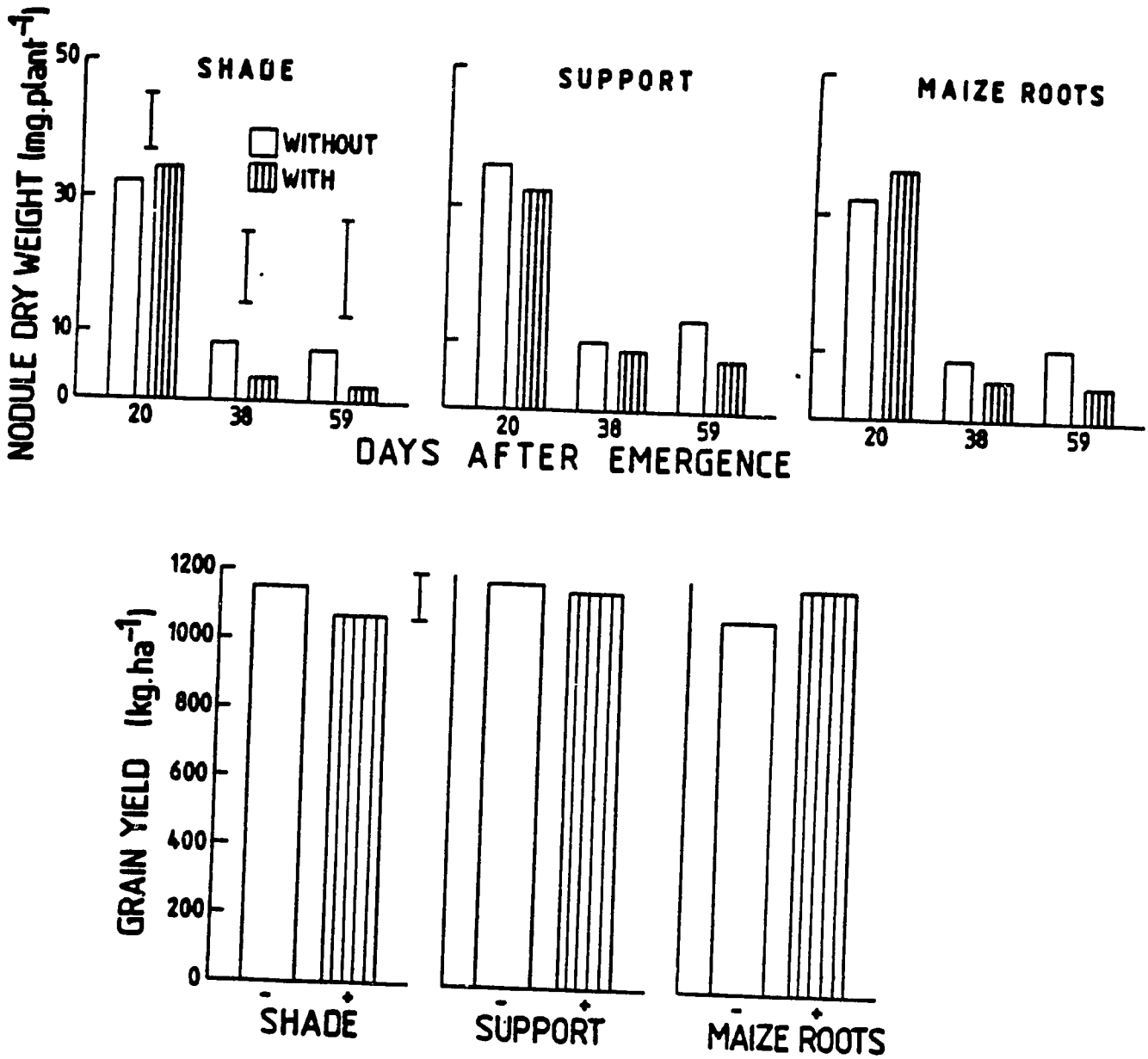


Figure 6-17. Nodulation and grain yield of bean lines, with and without shade, support and maize roots in the second year experiment at CNPAF, 1985.

1985 (Fig. 6-17). Thus, 38 and 59 DAE observations are not based on normally nodulated root systems.

Studies at MSU on Relay Cropping. These studies took into account physical, chemical, and biological changes in the bean growing environment -- above and below ground -- when beans are grown in relay instead of in pure culture. The following are results of studies performed at MSU.

Physical and chemical analyses were performed on soil samples taken at bean pure culture and adjacent corn relay growing sites at CNPAF. The soil was classified as an oxisol red clay loam (35% sand, 18% silt, 47% clay). X-ray diffraction patterns on the isolated clay fraction showed that the major clay was kaolinite; other clays included gibbsite and a trace of genthite.

Other chemical analyses of soil samples from the CNPAF bean growing area are presented in Table 6-36. Perhaps the most important finding of these analyses was that the mature corn rhizosphere at the time of planting beans in relay can have higher levels of several plant nutrients than the neighboring nonrhizosphere soil prepared for beans in pureculture. The analyses rule out the possibility that beans nodulate better in the corn rhizosphere because this environment has lower levels of nitrate.

The difference in pH between these two representative soils could contribute to better nodulation in relay cropping. It is well known that there are essential acid-sensitive steps of early legume root infection by *Rhizobium*. The higher pH (6.2) of the maize rhizosphere as compared to the nonrhizosphere soil (5.6)

Table 6-36. Chemical Analysis of Soil Samples from fields at CNPAF, Goiania, Brazil, March 1985.

Soil Analysis	Nonrhizosphere at bean growing site (average ± std. dev. of 4 composite samples)	Mature corn rizosphere
pH	5.6 + 0.0	6.2 ± 0.2
N (%)	0.13 ± 0.0	0.16 ± 0.0
P (lb/acre, top 9")	42 ± 8	71 ± 46
K (")	177 ± 28	609 ± 427
Ca (")	1000 ± 85	1768 ± 69
Mg (")	168 ± 6	263 ± 43
Zn (ppm)	4 ± 1	12 ± 8
Mn (ppm)	8 ± 1	16 ± 2
Cu (ppm)	7 ± 1	9 ± 2
Fe (ppm)	7 ± 1	5 ± 1
NO_3^- (ppm)	4 ± 1	4 ± 1
Organic matter (%)	2.60	2.90
Soluble salts (mmhos)	0.1 ± 0.01	0.15 ± 0.06
% of total exchangeable bases:		
K	6.6 ± 0.9	11.9 ± 7.2
Ca	72.9 ± 1.7	70.6 ± 7.8
Mg	20.5 ± 1.1	17.4 ± 1.6

would be less inhibitory to root infection and nodulation of beans. This pH is crucial biologically in the 4-6 range.

Fixation of CO_2 leading to production of energy-yielding photosynthate is very important for nodule formation and symbiotic nitrogen fixation, and enrichment of atmospheric CO_2 significantly stimulates nodulation and nitrogen fixation in legumes. Therefore, atmospheric CO_2 and rates of CO_2 evolution from the soil surface were measured from field samples obtained at CNPAF, Goiania, Brazil (Fig. 6-18), and analyzed by infrared spectrophotometry at MSU. The results (Table 6-37) are suggestive of a CO_2 enrichment in the maize relay cropping system. Probably the greater organic matter input into the soil and the wind-breaking effect of the dense maize stand contribute to improved bean nodulation and nitrogen fixation under maize relay cropping.

Soils at CNPAF are low in phosphorus, and lines selected for nitrogen fixation respond to P fertilization while gaining N through the *Rhizobium phaseoli*/bean symbiosis (Pereira and Bliss, 1987). In soils limited in both P and N, legumes develop dual symbioses consisting of root nodules containing rhizobia and other areas of the root developing vesicular-arbuscular mycorrhizae (VAM) containing endomycorrhizal fungi which act synergistically in mutual associations. Both beans and maize can form VAM under field conditions with endomycorrhizal fungi including numerous species of *Glomus* and other genera. Thus, one of our hypotheses to explain enhanced root nodulation of beans grown in relay cropping next to maize is that the maize root may enrich the rhizosphere with VAM

Table 6-37. Concentration of Carbon Dioxide in field samples at CNPAF, Goiania, Brazil, March 1985.

Description of Sample	Carbon dioxide, ppm
Calibration of analyzer with 356 ppm standard	357 ± 7
Ambient, CNPAF circular driveway pavement (AM)	653 ± 68
Atmosphere at soil level, 1 month bean monoculture (AM)	648 ± 43
Atmosphere at soil level, matured corn monoculture (AM)	739 ± 84
Atmosphere at 4 ft height, 1 month bean monoculture (AM)	562 ± 22
Atmosphere at 4 ft height, matured corn monoculture (AM)	730 ± 237
Atmosphere at 4 ft height, 1 month bean monoculture (PM)	591 ± 60
Atmosphere at 4 ft height, matured corn monoculture (PM)	677 ± 71
CO_2 trapped from soil, 3 hr, 1 month bean monoculture (PM)	1,252 ± 85
CO_2 trapped from soil, 3 hr, matured corn monoculture (PM)	2,044 ± 173

Gas sampled into vacutainers, sealed with parafin, and analyzed by infrared spectrophotometry (Dr. Alvin Smucker, MSU Dept. Crop & Soil Sci.)

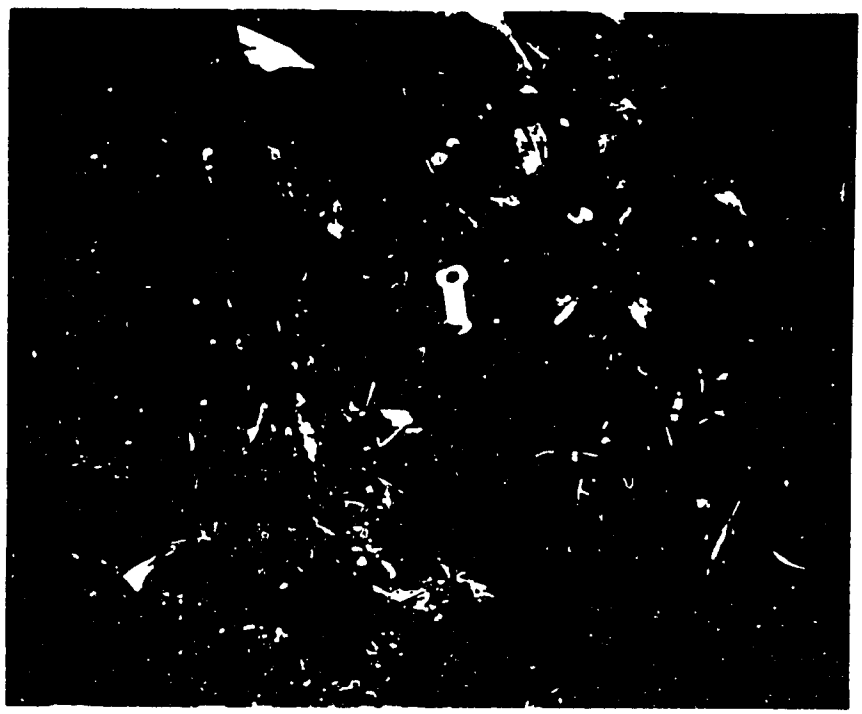


Figure 6-18. Apparatus for trapping and sampling CO_2 from soil in bean-maize intercropping.

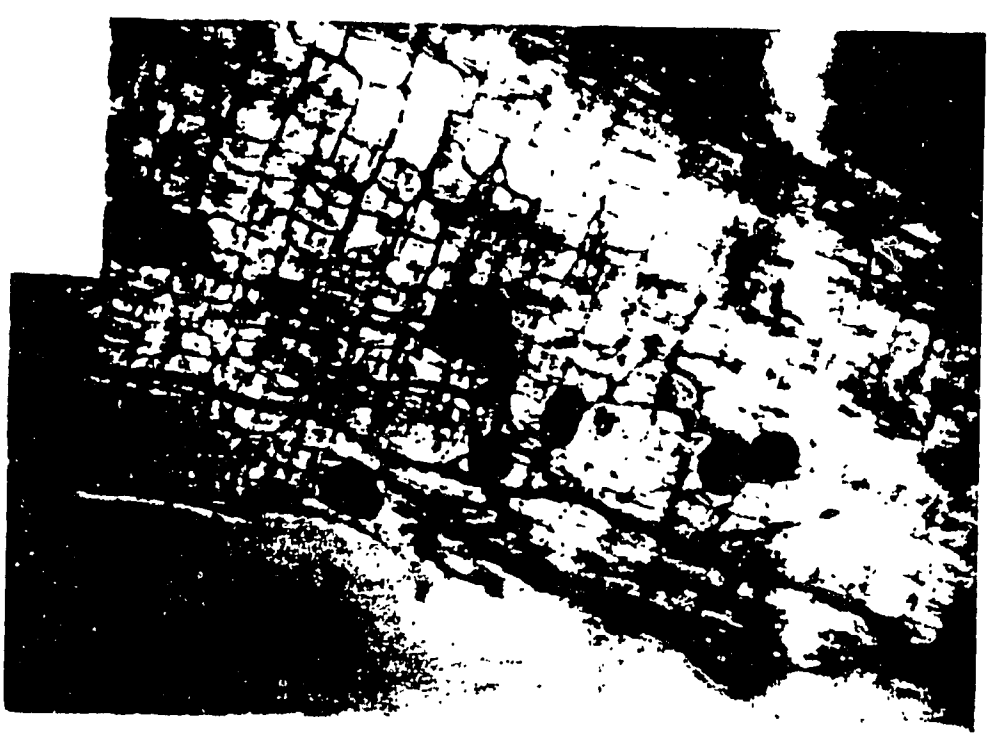


Figure 6-19. Bean root segment infected with vesicular-arbuscular endomycorrhizal fungi and stained with Basic Fuchsin.

fungi which can cross-infect bean roots. To test this hypothesis, we first decided to examine bean roots (Black Turtle Soup, Rio Tibagi, WBR 22-34) grown in the fields of CNPAF for the presence of VAM. We evaluated the three following methods to clear the roots and three different stains for the fungi.

Method 1: Bean roots were gently washed to remove coarse soil, immersed in 10% KOH, heated at 85°C for 1 h, washed in water to remove the KOH, acidified for 1 h in 0.1 M HCl solution, and stained overnight. The roots were then destained over 48 h (2 changes) in fresh lactophenol without stain, mounted in lactophenol on microscope slides and observed by light microscopy.

Method 2: Same as above except autoclaved with 10% KOH for 5 min at 121°C and then autoclaved for 5 min with the stain.

Method 3: Washed roots were heated in 10% KOH at 90°C for 2 h, washed in fresh 10% KOH, immersed in an alkaline solution of H_2O_2 for 1 hr, washed in water to remove the excess H_2O_2 and acidified with 0.1 M HCl solution. Acidified roots were stained at 90°C, washed in fresh lactophenol, mounted and examined. The stains used were 0.1% acid fuchsin, trypan blue, and aniline blue dissolved in lactophenol.

All methods gave positive results for VAM on all bean root samples. The best result was obtained by clearing the roots using Method 1 and staining with trypan blue. This result provided the best clearing and contrast of staining, although basic fuchsin also worked well (Fig. 6-19). We conclude that beans grown at CNPAF are infected with VAM.

Bean roots grown for 1, 2, 3, 4 and 6 weeks in pureculture and in relay at CNPAF were sampled, cleared, stained, and mailed to MSU. The degree of endomycorrhizal infection was assessed using two different methods of scoring. It was clear that bean roots in both cropping systems developed endomycorrhizae, however, the extent of VAM infection was approximately the same in relay and in pureculture (Table 6-38).

Soil samples were also analyzed for endomycorrhizal spore counts. Spores were isolated by selective filtering and flotation in sucrose during centrifugation. The results (spores/5 g of soil, four replicate soil samples per site, eight composite rhizospheres per replicate soil sample) were: 53.5 for non-rhizosphere soil at bean growing sites; 77.2 for mature maize rhizosphere at relay cropping site; 31.0 for rhizosphere on uninoculated pureculture bean line WBR 22-34; 27.0 for rhizosphere of uninoculated bean varieties Rio Tibagi, Manteigo, and Venezuela 350 PS grown one month next to maize. Thus, all soils had endomycorrhizal fungal spores which decreased in density when planted with beans. Presumably this reduction in dormant spore counts results from their germination into vegetative hyphae in the bean rhizosphere. The spore count in the mature maize rhizosphere was slightly higher than the nonrhizosphere and at least twice as high as in the 1-month old bean rhizosphere. Thus, bean roots would encounter more VAM spore propagules when grown in the mature maize rhizosphere as in relay cropping than in non-rhizosphere soil used for purecultures. A comparison which should have been done was to measure the

Table 6-38. Endomycorrhizal infection of bean roots grown at CNPAF, Goiania, Brazil, March 1985.

Mycorrhizal infection index after growth for:	Cropping System	
	Bean monoculture	Beans/corn relay
1 week	1.16 ± 0.40	1.27 ± 0.49
2 weeks	1.38 ± 0.49	1.35 ± 0.58
3 weeks	1.07 ± 0.05	1.11 ± 0.52
4 weeks	1.18 ± 0.45	1.24 ± 0.18
6 weeks	1.53 ± 0.67	1.31 ± 0.56

population of total VAM propagules capable of infecting bean roots in these different environments.

We were interested in comparing the mineral content of shoots of beans grown in maize relay vs. pureculture, but were only able to obtain samples in maize relay and at one plant age (one month). Four bean lines were examined; two (WBR 22-34 and Mantiegao) nodulate well next to maize, whereas two others (Rio Tibagi and Venezuela 350 PS) do not Table 6-39). Two conclusions could be made. The aluminum content was above the critical high level for Rio Tibagi and WBR 22-34, and may be adversely affecting the plants. The N content was below the critical low level for all four lines and was probably a limiting factor.

Two experiments were done to assess how diffusible compounds from maize residues influence bean growth and nodulation by R. phaseoli. This information would contribute to a better understanding of how chemicals associated with relay cropping with mature maize plants can influence bean growth and development of the root-nodule symbiosis.

Root and leaf samples of maize (Great Lakes 5922) were collected from brown, mature plants at the MSU Campus Farms. The lateral roots were cut into 1" segments. The brown leaves were dried at room temperature and then ground. Ground leaves (200 g) and chopped roots (50 g) were shaken separately overnight in 1 L deionized water. The aqueous extracts were filtered through cheese cloth, clarified by centrifugation, lyophilized, and stored in sealed bottles at 4°C. The water-insoluble plant material was

Table 6-39. Shoot mineral content of bean varieties grown 1 month in corn relay at CNPAF, Goiania, Brazil, March 1985.

Nutrient	Critical low level (estimation only)[a]	Bean cultivars/lines			
		22-34	Rio Tibagi	Venezuela 350 PS	Manteigao
N (%)	3	1.64	1.92	2.33	2.13
P (%)	0.2	0.30	0.33	0.37	0.30
K (%)	2	3.55	3.68	3.88	2.98
Mg (%)	0.2	0.34	0.37	0.36	0.25
Ca (%)	1.44	1.59	1.71	1.09	0.86
B (ppm)	20	23	24	23	15
Zn (ppm)	15	46	54	56	37
Fe (ppm)	not known	430	485	340	320
Mn (ppm)	30	51	55	56	43
Cu (ppm)	15	12	17	10	9
Al (ppm)[b]	260	1,330	1,285	625	660

[a] Personal communication, Dr. Al Smucker, MSU. These estimates may vary with varieties.

[b] Critical high level for Al is 688 ppm.

dried, re-extracted with a 1:1 mixture of chloroform:methanol, centrifuged, flash evaporated to dryness, and stored in sealed bottles at 4°C.

In the first experiment, we examined the effect of maize leaf and root extracts on bean root growth in enclosed sand/flask cultures. Acid-washed quartz sand was rinsed with deionized water and dried in an oven. Flasks were filled with 300 g of sand mixed with 20 mg of dried maize extract and inoculated with 2.7×10^6 cells of R. phaseoli strain Viking 1. Two sterile seedlings of bean line WBR 22-3 were planted per flask, enclosed with foam plug closures, and covered with aluminum foil. This bacterial/plant combination showed good growth in relay crops at CNPAF in 1986 (R. Araujo and R. Henson, personal communication). Cultures were grown in the growth chamber, irrigated with Smucker's medium as necessary, and the tops allowed to protrude out of the flask with the foam closure in place. Root biomass was measured after 30 days incubation. The results indicated that extracts from maize residues enhanced bean root growth, especially the water-soluble fraction from mature maize roots (Fig. 6-20).

The next experiment was designed to examine the effect of aqueous extracts of maize roots and leaves on bean growth and nodulation by R. phaseoli in open sand culture to more closely approximate field conditions. Plastic containers were filled with dry sand mixed with the lyophilized maize extract at 1 mg/15 g sand, planted with two seeds of bean line 21-58 which had been surface-sterilized and inoculated with R. phaseoli strain Viking

CORN EXTRACT TREATMENT 10 MG / PLANT	BEAN ROOT DRY WT (MG / PLANT)
NONE	221
LEAVES, ORGANIC	386
LEAVES, AQUEOUS	354
ROOTS, ORGANIC	414
ROOTS, AQUEOUS	492

EACH FLASK CONTAINED 2 PLANTS, 300 G SAND, AND 20 MG CORN EXTRACT

Figure 6-20. Corn extract improves bean root growth. Flask-sand cultures of the bean line WBR22-3 were inoculated with R. phaseoli Viking 1 grown 30 days in the growth chamber.

1. The peat-based inoculum dose was 4.5 x 10^4 cells/seed. The containers were irrigated with 100 ml of Smucker's medium supplemented where indicated with 70 ppm KNO_3. Each treatment was replicated with four containers (eight plants/treatment). Plants were grown in a growth chamber for five weeks, photographed, harvested, and their tops and roots compared (Fig. 6-21 and 6-22). The maize root extract increased bean root growth. The ability of R. phaseoli inoculation to enhance bean top growth was annulled by both maize leaf and root extracts. The ability of R. phaseoli inoculation to enhance bean root growth is increased by maize leaf and root extracts. In summary, these results confirm the first experiment and show that water-soluble chemicals from maize residues have an important impact on the growth of beans, especially their roots in symbiosis with Rhizobium. We propose, therefore, that growth-promoting substances from maize residues may contribute to the benefit of relay cropping on bean root growth and symbiosis with R. phaseoli.

Line/Strain Combinations in Monoculture and Relay with Maize. Due to the possibility of bean genotype x rhizobium strain interactions, the best symbiotic combination may not always involve the same host with the same bacteria. By 1986, a set of elite black lines had been bred and selected for N_2 fixation. Twelve inoculum strains selected at MSU had also been individually field-tested with Rio Tibagi and with WBR 22-34 at CNPAF. A 2-year experiment was begun to test six elite black bean sister lines (WBR 22-3, WBR 22-8, WBR 22-34, WBR 22-50, WBR 22-52, and WBR 22-55) in

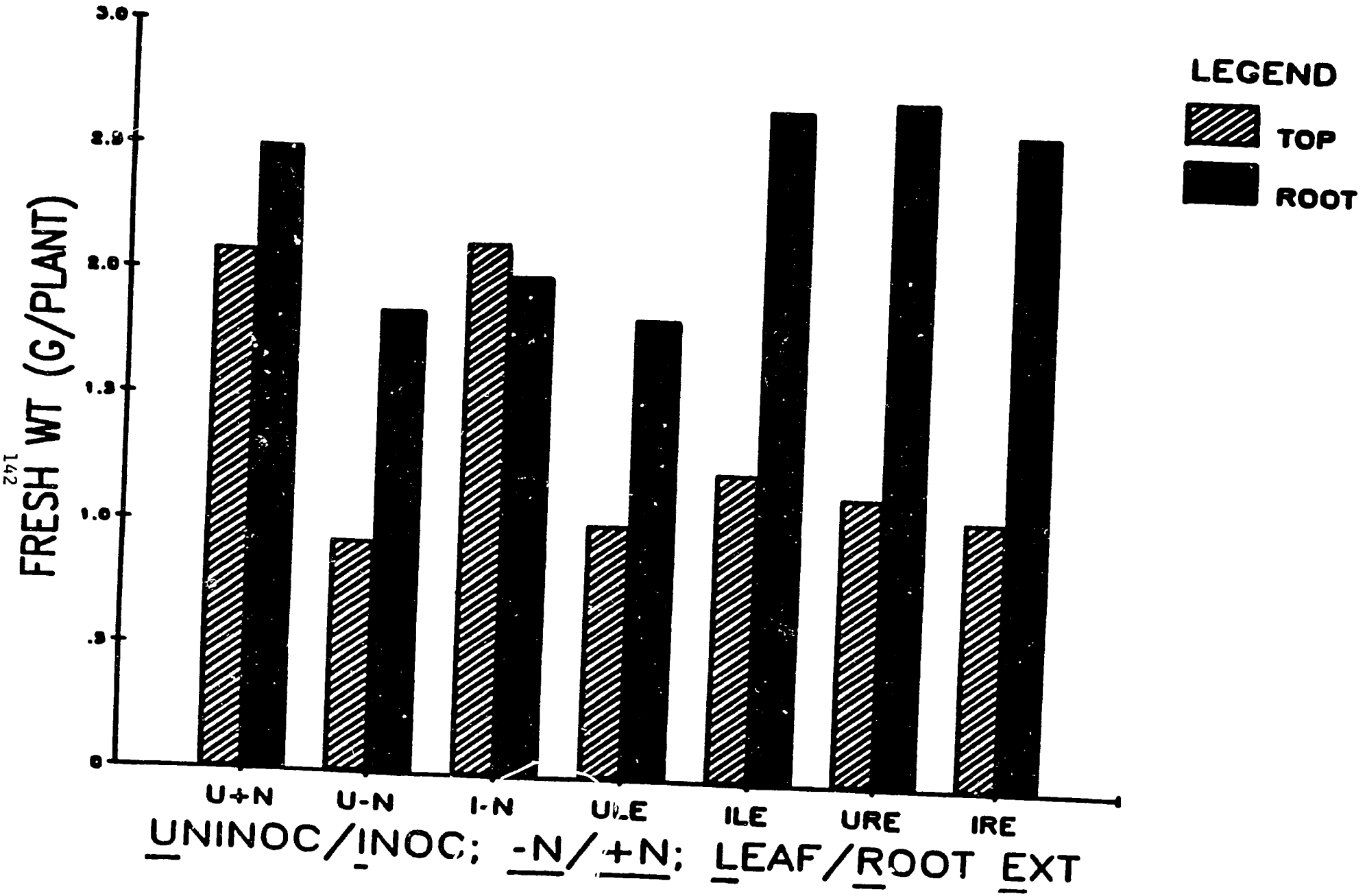


Figure 6-21. Effect of corn extract on growth of beans in symbiosis with Rhizobium.

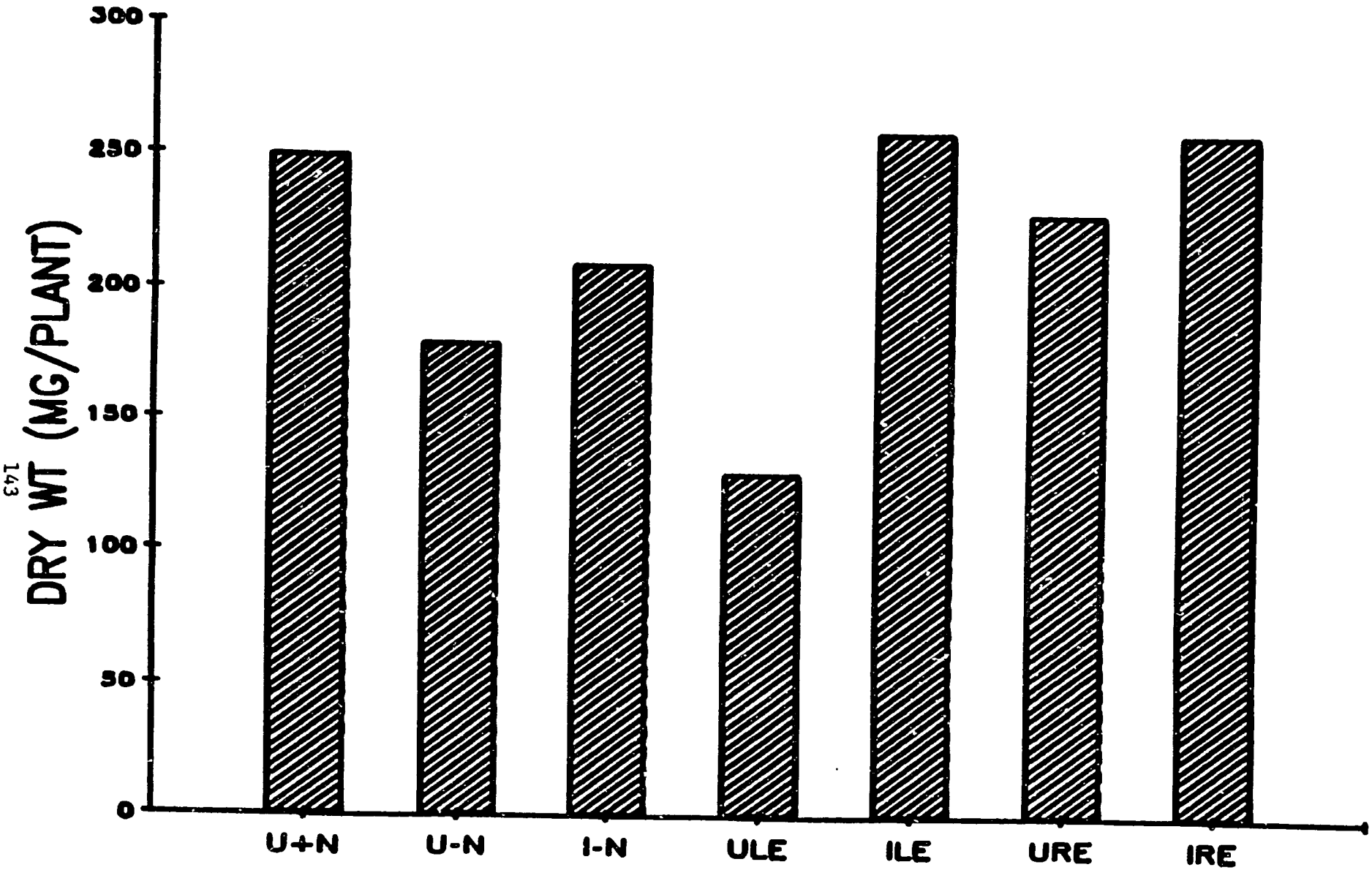


Figure 6-22. Corn extract affects growth of bean roots in symbiosis with R. phaseoli. Same abbreviations used as in Fig. 6-21.

combination with four selected rhizobial strains (CIAT 899, CNPAF 150, Cias, and Viking 1. Since previous results suggested a more favorable environment for N_2 fixation in the relay cropping system, these combinations were compared in pureculture and in relay. A four-replicate, split-plot layout was used with cropping system as the mainplot factor. The subplot factor was the combination of one line inoculated with one strain. Due to the size of the experiment, an incomplete block design was used. Thus, each strain was individually inoculated on three lines and each line was seed-inoculated with two strains. Each line was also planted with N and with neither N fertilizer nor inoculum. Peat-based inocula were prepared at MSU.

The entire experimental area was cropped to maize during the rainy season. When mature, maize shoots and roots were removed from the pureculture main plots and beans were planted. During growth, weeds were controlled by hoeing and hoes were washed with alcohol between inoculum treatments to reduce cross-contamination of plots. Shoot, root, and nodule dry matter samples were taken at R1 and at R7. Shoots were analyzed for total N concentration and N content was calculated. Pod and seed yields and seed test weight were measured at maturity.

At approximately weekly intervals, incident light and soil moisture were measured in all main plots and soil temperature was measured in three replicates of each main plot.

This experiment was repeated in 1987. Due to MSU enthusiasm over strain ITC 53, it was substituted for Cias. In the second

year, measurements of incident light and soil temperature and moisture were not taken.

Results of the two-year experiment showed higher accumulation of dry matter and N in pureculture than in relay and with N fertilizer than without (data not shown). No significant responses to inoculation were seen when compared to the uninoculated controls.

In 1985, soil moisture, soil temperature, and light penetration data collected from 7 DAE to 63 DAE (R7) are presented in Table 6-40. The data were taken at random in relation to irrigation applications, so the mean increase of 1.5% H_2O in the relay system probably signifies an important difference in periods when rainfall is lacking. Beginning at 36 DAE (R1), when the cropping system effect was more pronounced even though the canopy had closed, samples were taken under considerably drier conditions (see Pureculture values throughout the season). Early in the season, soil temperature at 15 cm was reduced slightly in the relay system, but the differences were small.

Light penetration measurements were made between 9 and 10 am (midpoint between sunrise and midday). On most days the sky was clear, but values were also recorded on hazy and partially overcast days. Light penetration was reduced in both the more shaded (dark) and less shaded (light) rows in relay. The effect was more pronounced early in the season and decreased as the plants grew taller.

Table 6-40. Comparison between microclimatological parameters in bean pureculture (P) and in maize/bean relay cropping (R). Soil moisture and temperature values are means of 4 and 3 replicates, respectively, measured at the midpoint between maize rows. Light penetration values are means of 4 replicates measured directly above bean rows.

	Soil Moisture[1] (%)				Soil Temperature[2] (°C)				Light penetration[3] (μEinsteins.m^{-2}.s^{-1})											
									Dark Row				Light Row				$\bar{x}$			
DAE	P	R	% Δ	$\bar{x}$	P	R	% Δ	$\bar{x}$	P	R	% Δ	$\bar{x}$	P	R	% Δ	$\bar{x}$	P	R	% Δ	$\bar{x}$
7	27.0	27.4	1.5	27.2	22.9	22.5	-1.7	22.7	4162	1762	-58	2962	4178	3045	-27	3611	4170	2404	-42	3287
15	25.8	27.8	7.8	26.8	22.2	21.3	-4.1	21.8	3810	2190	-43	3000	3870	3128	-19	3499	3840	2659	-31	3250
20	26.8	27.4	2.2	27.1	22.1	21.9	-0.9	22.0	628	358	-43	492	610	520	-15	565	619	439	-29	529
28	30.8	31.2	1.3	31.0	22.3	22.0	-1.3	22.2	2182	1538	-30	1860	2392	2100	-12	2246	2287	1819	-20	2053
36	23.8	26.0	9.2	24.9	22.0	22.0	0	22.0	4050	324[illegible]	-20	364[illegible]	4005	3968	- 1	3986	4028	3608	-10	3818
42	23.0	25.4	10.4	24.2	21.5	21.4	-0.5	21.4	3870	3172	-18	3521	3735	3862	3	3799	3802	3517	- 7	3660
49	23.3	25.4	9.0	24.4	20.1	20.2	0.5	20.2	- [4]	-	-	-	-	-	-	-	-	-	-	-
56	23.8	24.8	4.2	24.3	18.1	18.0	0.6	18.0	3645	2738	-25	3191	3645	3368	- 8	3506	3645	3053	-16	3349
63	23.4	25.9	10.7	24.6	18.3	18.2	0.5	18.2	3338	2610	-22	2974	3248	3285	1	3266	3293	2948	-10	3120
$\bar{x}$	25.3	26.8	6.3	26.1	21.1	20.8	-0.8	20.9	3211	2202	-32	2706	3210	2910	-10	3060	3210	2556	-21	2883

[1] 0-15 cm depth. [2] 15 cm depth. [3] Top of bean canopy. [4] Light meter didn't function.

Maize Cultivar Effects in Relay Cropping. Several experiments comparing beans grown in pureculture and in relay with maize showed that the relay environment increased the duration of nodulation in the podfilling phase of growth. In 1984, this effect was shown to be largely due to the presence of decomposing maize roots in the soil and dependent upon bean line. Project line WBR 22-34 showed the best nodulation response and also outperformed traditional cultivars. Rio Tabagi and Carioca, and another selection for N_2 fixation, Negro Argel, in other growth and yield parameters.

However, instances of almost total nodule sloughage have been observed. In both cropping systems in 1984 (Table 6-17) and in pureculture in 1983 (Pereira et al., 1984), nodules have senesced before the R2 stage. The influence of year and cropping system suggests a weather or microclimatic effect, but data from the CNPAF weather station and the use of irrigation to supplement rainfall do not indicate the stress which caused nodule sloughage. In 1984, when the nodules in the relay cropping experiment did not slough off, and in 1985, when they did, different maize cultivars were used in association with the beans. Since the presence of decomposing roots of some maize varieties has been shown to increase the duration of nodulation, perhaps other varieties stimulate nodule senescence. A field experiment was installed at CNPAF to test this hypothesis in a split-splitplot layout with four replicates. The mainplot factor was cropping system: pureculture or relay. Subplots were maize cultivars (AG 162, AG 301, BR 301, Cargill 111-S, or Agroman), which were planted at the onset of the

rainy season. At maturity, maize shoots and roots were removed from the pureculture mainplots and maize cultivar subplots were split into sub-subplots of bean lines Rio Tibagi and WBR 22-34. Bean samples were collected at the R3 and R7 growth stages for measurement of shoot, root, and nodule dry matter content and determination of shoot N concentration and content. At maturity, pod and seed yields and seed test weight and moisture content were measured.

Bean pureculture was superior to the relay system in shoot and root dry matter production (data not shown), shoot N accumulation (Table 6-41), and grain and pod yields (Table 6-42). Line WBR 22-34 was superior to Rio Tibagi. Across cropping systems and bean lines, none of the parameters measured were significantly affected by maize cultivar. However, nodule dry matter data at the R3 stage (Table 6-43) suggests improved nodulation by both bean lines when associated with AG 162 than with the other maize cultivars tested. It is not known if the generally poor nodulation at R3 was a result of nodule senescence prior to sampling or failure of the plants to produce nodules, but improved nodulation with AG 162 was not translated into higher N accumulation (Table 6-41) or grain yield (Table 6-42). Additional work is merited on AG 162 to further test for stimulatory effects on bean nodulation and nodule duration.

Nitrogen Fertilizer Application to Beans

Timing of N Fertilizer Application. Under certain growing conditions, some bean lines yield as well with inoculum as with N

Table 6-41. Shoot N content (mg plant $^{-1}$) in bean pureculture (P) and relay cropped (R) after various maize cultivars, CNPAF, April-July 1987. Values are means of 4 replicates.

Bean Genotype	Cropping System	Maize Cultivar: AG 162	AG 301	BR 301	Cargill 111-S	Agroman	$\bar{x}$
		R3					
Rio Tibagi	P	178	140	176	163	169	165
	R	128	103	108	98	112	110
	$\bar{x}$	153	122	142	131	140	138
22-34	P	160	186	172	163	159	168
	R	130	137	150	110	96	125
	$\bar{x}$	145	162	161	137	128	146
$\bar{x}$	P	169	163	174	163	164	167***
	R	129	120	129	104	104	117***
	$\bar{x}$	149 a	142 a	152 a	134 a	134 a	142
		R7					
Rio Tibagi	P	205	211	210	254	201	216
	R	134	136	141	149	136	139
	$\bar{x}$	169	173	176	208	168	178***
22-34	P	280	284	223	229	224	248
	R	203	163	224	182	202	195
	$\bar{x}$	242	223	224	206	213	221***
$\bar{x}$	P	242	247	217	241	212	232**
	R	168	150	182	168	169	168**
	$\bar{x}$	205 a	199 a	200 a	207 a	191 a	200

** and *** denote significant differences ($P<0.01$ and $P<0.001$, respectively) between overall genotype and cropping system means.

Table 6-42. Pod and grain yield in bean pureculture (P) and relay cropped (R) after various maze cultivars, CNPAF, April-July 1987. Values are means of 4 replicates.

Bean Genotype	Cropping System	Maize Cultivar: AG 162	AG 301	BR 301	Cargill 111-S	Agroman	$\bar{x}$
		Pods ($n\ m^{-2}$)					
Rio Tibagi	P	204	170	213	211	198	199
	R	117	137	153	104	137	129
	$\bar{x}$	161	154	183	157	167	164
22-34	P	140	149	156	161	143	150
	R	114	114	128	124	117	119
	$\bar{x}$	127	132	142	142	130	135
$\bar{x}$	P	172	160	185	186	170	174**
	R	115	126	141	114	127	124**
	$\bar{x}$	144[1]	143	163	150	148	149
		Grain (kg ha^{-1})					
Rio Tibagi	P	960	798	957	949	861	905
	R	588	653	690	438	637	601
	$\bar{x}$	774	726	823	693	749	753
22-34	P	1400	1276	1392	1511	1230	1344
	R	1202	1069	1265	1201	1050	1157
	$\bar{x}$	1256	1172	1329	1356	1140	1250
$\bar{x}$	P	1135	1036	1174	1230	1046	1124**
	R	895	861	977	819	844	879**
	$\bar{x}$	1015[1]	949	1076	1024	945	1002

[1] Overall maize cultivar means do not differ significantly by the Waller-Duncan K-ratio t-test.

** denotes significant difference ($P<0.01$) between overall cropping system means.

Table 6-43. Nodule dry weight (mg plant^{-1}) in bean pureculture (P) and relay cropped (r) after various maize cultivars, CNPAF, April-July 1987. Values are means of 4 replicates.

Bean Genotype	Cropping System	Maize Cultivar: AG 162	AG 301	BR 301	Cargill 111-S	Agroman	$\bar{x}$
		R3					
Rio Tibagi	P	16	5	2	9	2	7
	R	26	2	1	1	4	7
	$\bar{x}$	21	4	2	5	3	7
22-34	P	38	15	9	6	16	17
	R	6	3	16	4	7	7
	$\bar{x}$	22	9	12	5	11	12
$\bar{x}$	P	27	10	5	8	9	12[1]
	R	16	2	8	2	5	7
	$\bar{x}$	21[1]	6	7	5	7	9
		R7					
Rio Tibagi	P	0	0	0	0	0	0
	R	0	0	0	0	0	0
	$\bar{x}$	0	0	0	0	0	0
22-34	P	2	0	1	1	1	0
	R	0	0	1	1	1	1
	$\bar{x}$	1	0	1	1	1	1
$\bar{x}$	P	1	0	0	0	1	0[1]
	R	0	0	0	1	0	0
	$\bar{x}$	0[1]	0	0	0	0	0

[1] Overall maize cultivar and cropping system means do not differ significantly ($P<0.05$) by the Waller-Duncan K-ratio t-test and by F-test, respectively.

fertilizer, but in general, seed yield with N fertilizer is superior to that when no N is added. In 1984, research was begun to determine the stage(s) of plant development when N fertilizer application produces the greatest yield response. The objectives were to identify which growth stage(s) should be emphasized when selecting for N_2 fixation, to study possible crop management systems using N_2 fixation and N fertilizer in combination to maximize yield, and to determine genotypic effects on the above factors.

Experiments were installed at CNPAF in the seed multiplication plots of Rio Tibagi, Carioca, and WBR 22-34, with five replicates. All plots were seed-inoculated at planting with a mixture of 4 strains of R. phaseoli selected at CNPAF. In 1984, treatments included an application of 50 kg N ha^{-1} either at the V3-V4 vegetative stage, at flowering, or during podfill, and a control to which no N fertilizer was added. In 1985 and 1987, this experiment was repeated with a 60 kg ha^{-1} application of N fertilizer and two additional treatments: N at planting and a split application of 30 kg N ha^{-1} at planting + 30 kg N side-dressed at the V3-V4 growth stage. Due to space limitations, the split application treatment was not applied to Carioca in 1985.

Grain yield, pod yield and seed test weight were measured at maturity in all experiments. Samples were taken also to study the effect of applying N fertilizer to a functioning, N_2 fixing system. At flowering time, shoot, root, and nodule dry matter, shoot:root dry matter ratio, and shoot N concentration and content were

determined for control plots and plots which had received a vegetative N application. At 0, 5, and 11 (1984) and at 0, 5, 11 and 20 (1985 and 1987) days after the application of N at flowering, the same measurements were taken on the control and on the N application at flowering treatments.

Data were analyzed statistically using MSTAT. Due to unequal treatment combinations in all three years, maturity data were grouped to maximize the number of years, N application treatments, or bean lines in each of the three analyses. Although mid-season measurements comparing control plots to those which received N fertilizer were complete factorials, three samples were taken in 1984 and four in 1985 and 1987. Thus, separate analyses were performed for 3-sample, 3-year and for 4-sample, 2-year time courses.

Analyses of the maturity data from the four N application treatments included in all three experiments showed that grain yield was significantly higher when N was applied at the vegetative stage than for any other treatment (Table 6-44). The application of N at flowering also produced significantly higher yields than the control. Pod yield was also highest with the vegetative N application, while seed test weight increased with all N applications, although they did not differ significantly among themselves.

Results of the two experiments which also included N fertilization at planting (Table 6-45) were similar to those above: yield from the vegetative N application was significantly higher than all other treatments and all N application times except that

Table 6-44. Maturity parameters in the Time of N Application experiments, CNPAF, 1984, 1985 and 1987. Values are means of 5 replicates in each year.

Genotype	N Application Time				
	None	Vegetative	Flowering	Podfill	$\bar{x}$
	Grain Yield (kg ha^{-1})				
Rio Tibagi	1359	1729	1594	1540	1555
Carioca	1705	2005	1897	1669	1819
WBR 22-34	1494	1963	1726	1631	1703
$\bar{x}$	1519 C[1]	1899 A	1739 B	1613 BC	1693
	Pod Yield (no m^{-2})				
Rio Tibagi	245	279	274	262	265
Carioca	242	260	254	230	246
WBR 22-34	186	224	193	194	199
$\bar{x}$	224 B	254 A	240 AB	228 B	237
	50-seed Field Test Wt. (g)				
Rio Tibagi	7.86	8.22	8.41	8.31	8.20
Carioca	11.22	11.44	11.53	11.28	11.37
WBR 22-34	10.01	10.32	10.37	10.42	10.28
$\bar{x}$	9.70 B	10.00 A	10.10 A	10.00 A	9.95

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-45. Maturity parameters in the Time of N Application experiments, CNPAF, 1985 and 1987. Values are means of 5 replicates in each year.

Genotype	N Application Time					
	None	Planting	Vegetative	Flowering	Podfill	$\bar{x}$
	Grain Yield (kg ha^{-1})					
Rio Tibagi	1469	1729	1863	1671	1617	1670
Carioca	1701	1825	2024	1851	1635	1807
WBR 22-34	1535	1781	2005	1845	1662	1766
$\bar{x}$	1568 C[1]	1778 B	1964 A	1789 B	1638 BC	1748
	Pod Yield (no.m^{-2})					
Rio Tibagi	230	256	247	229	220	237
Carioca	205	215	220	205	176	204
WBR 22-34	154	181	188	162	148	167
$\bar{x}$	196 BC	217 A	218 A	199 B	182 C	202
	50-seed Field Test Wt. (g)					
Rio Tibagi	7.67	7.75	8.09	8.16	8.02	7.94
Carioca	10.75	10.40	11.03	11.20	10.77	10.83
WBR 22-34	9.83	9.43	10.38	10.29	10.13	10.01
$\bar{x}$	9.41 BC	9.19 C	9.83 A	9.88 A	9.64 AB	9.59

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

during podfill produced significantly higher yields than the control. Fertilizing with N at planting or during the vegetative phase gave a significantly higher pod yield than the other treatments, while N fertilizer applied during the vegetative phase or later increased seed test weight. Data analyses from the two years and two lines which also included the split N application treatment showed that the split application resulted in the highest pod set, but grain yield and seed test weight were intermediate (Table 6-46). Across lines at flowering, plots which had received a vegetative N application contained a significantly higher shoot dry matter and N content and shoot:root dry matter ratio (Table 6-47). Root dry matter and shoot N concentration were not affected significantly, but nodule dry matter was reduced.

Based on the time course study of plant dry matter accumulation in 1985 and 1987, there were no significant effects across lines on shoot (Table 6-48), root (Table 6-49), and shoot:root ratio (Table 6-50) within 20 days after applying 60 kg N ha^{-1} at flowering. At five days after adding N, formation of nodule mass (Table 6-51) was slightly lower in the plots receiving N fertilizer than on the control plants. After this sample, N fertilizer apparently caused more rapid nodule senescence than in the control plots, with a significant difference between N treatments being observed at 20 days after N addition. Within lines, Rio Tibagi showed the best late-season nodulation without N, while nodulation of WBR 22-34 was least affected by N fertilizer.

Table 6-46. Maturity parameters in the Time of N Application experiments, CNPAF, 1985 and 1987. Values are means of 5 replicates in each year.

Genotype	N Application Time						
	None	Planting	Vegetative	Flowering	Podfill	Split	$\bar{x}$
	Grain Yield (kg ha^{-1})						
Rio Tibagi	1469	1729	1863	1671	1617	1547	1649
WBR 22-34	1535	1781	2005	1845	1662	1847	1779
$\bar{x}$	1502 B[1]	1755 AB	1934 A	1758 AB	1640 B	1697 AB	1714
	Pod Yield (no.m^{-2})						
Rio Tibagi	230	256	247	229	220	259	240
WBR 22-34	154	181	188	162	148	183	169
$\bar{x}$	192 C	219 AB	218 AB	196 BC	184 C	221 A	205
	50-seed Field Test Wt. (g)						
Rio Tibagi	7.67	7.75	8.09	8.16	8.02	7.75	7.91
WBR 22-34	9.83	9.43	10.38	10.29	10.13	9.88	9.99
$\bar{x}$	8.75 BC	8.59 C	9.24 A	9.22 A	9.08 AB	8.82 BC	8.95

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-47. Dry matter and N parameters at flowering of control plots (-N) and plots fertilized with N 22 days previously (+N), CNPAF, 1984, 1985 and 1987. Values are means of 5 replicates in each year and are on a per plant basis.

Genotype	Treat-ment	Dry Matter				N	
		Shoot	Root	Shoot :Root	Nodule	Concen-tration	Mass
		----- g -----			- mg -	-- % --	-mg-
Rio Tibagi	-N	4.02	0.79	5.00	44	3.35	133
	+N	4.80	0.82	5.73	33	3.50	165
	$\bar{x}$	4.41	0.81	5.37	39	3.43	149
Carioca	-N	3.84	0.60	6.42	79	3.47	133
	+N	4.27	0.58	7.08	32	3.68	152
	$\bar{x}$	4.06	0.59	6.75	55	3.57	143
WBR 22-34	-N	4.21	0.82	5.13	78	3.41	143
	+N	4.98	0.95	5.10	53	3.38	165
	$\bar{x}$	4.59	0.88	5.12	65	3.40	154
$\bar{x}$	-N	4.02*	0.74	5.52*	67***	3.41	136*
	+N	4.68*	0.78	5.97*	39***	3.52	161*
	$\bar{x}$	4.35	0.76	5.74	53	3.47	149

* and ** denotes significant differences between N treatments at $P<0.05$ and $P<0.001$, respectively.

Table 6-48. Shoot dry matter content in control plots (-N) and those fertilized with N at flowering (+N), CNPAF, 1985 and 1987. Values are means of 5 replicates in each year.

Genotype	Treat-ment	Days After N			
		0	5	11	20
		g plant $^{-1}$			
Rio Tibagi	-N	4.65	7.02	10.05	13.17
	+N	--	6.52	9.43	13.25
	$\bar{x}$	--	6.77	9.74	13.21
Carioca	-N	4.33	7.34	9.98	14.12
	+N	--	6.42	9.62	13.38
	$\bar{x}$	--	6.88	9.80	13.75
WBR 22-34	-N	4.26	5.31	8.42	12.20
	+N	--	5.94	8.45	12.46
	$\bar{x}$	--	5.62	8.44	12.33
$\bar{x}$	-N	4.41	6.55	9.48	13.17
	+N	--	6.29	9.17	13.03
	$\bar{x}$	--	6.42	9.33	13.10

Table 6-49. Root dry matter content in control plots (-N) and those fertilized with N at flowering (+N), CNPAF, 1985 and 1987. Values are means of 5 replicates in each year.

Genotype	Treat-ment	Days After N			
		0	5	11	20
		g plant^{-1}			
Rio Tibagi	-N	0.87	1.14	1.35	1.35
	+N	--	1.08	1.22	1.24
	$\bar{x}$	--	1.11	1.29	1.30
Carioca	-N	0.70	0.80	0.83	0.71
	+N	--	0.73	0.81	0.74
	$\bar{x}$	--	0.77	0.82	0.73
WBR 22-34	-N	0.80	0.96	1.04	1.02
	+N	--	1.04	1.08	1.04
	$\bar{x}$	--	1.00	1.06	1.03
$\bar{x}$	-N	0.84	0.97	1.07	1.03
	+N	--	0.95	1.04	1.01
	$\bar{x}$	--	0.96	1.05	1.02

Table 6-50. Shoot:root dry matter ratio in control plots (-N) and those fertilized with N at flowering (+N), CNPAF, 1985 and 1987. Values are means of 5 replicates in each year.

Genotype	Treat-ment	Days After N			
		0	5	11	20
Rio Tibagi	-N	5.38	6.16	7.53	9.77
	+N	--	6.04	7.76	10.54
	$\bar{x}$	--	6.10	7.65	10.16
Carioca	-N	6.12	9.17	11.96	18.93
	+N	--	9.00	12.08	17.88
	$\bar{x}$	--	9.08	12.02	18.41
WBR 22-34	-N	4.99	5.62	8.19	11.89
	+N	--	5.68	7.73	11.56
	$\bar{x}$	--	5.65	7.96	11.73
$\bar{x}$	-N	5.50	6.99	9.23	13.53
	+N	--	6.90	9.19	13.33
	$\bar{x}$	--	6.95	9.21	13.43

Table 6-51. Nodule dry matter content in control plots (-N) and those fertilized with N at flowering (+N), CNPAF, 1985 and 1987. Values are means of 5 replicates in each year.

Genotype	Treat-ment	Days After N			
		0	5	11	20
		mg plant^{-1}			
Rio Tibagi	-N	49	73	85	110
	+N	-	72	66	37
	$\bar{x}$	-	73	76	74
Carioca	-N	101	112	85	67
	+N	-	86	61	32
	$\bar{x}$	-	99	73	49
WBR 22-34	-N	81	133	113	96
	+N	-	117	64	73
	$\bar{x}$	-	125	88	85
$\bar{x}$	-N	77	106	94	91*
	+N	-	92	64	47*
	$\bar{x}$	-	99	79	69

* denotes significant difference ($P<0.05$) between N treatment means.

Within the 20-day period sampled, shoot N concentration of the N fertilized plants increased significantly over that of plants in the control plots (Table 6-52). Although shoot N content values also increased with N fertilizer over the same period, differences between N treatments were not significant (Table 6-53). Analysis of the data from all three years at 0, 5 and 11 days after N addition yielded the same statistical results as for two years, with the exception that N fertilizer at flowering significantly increased shoot N concentration by 11 days after the N application (data not shown).

Two of the principal components of bean grain yield are the number of pods produced and seed test weight. In this study, yield was increased by addition of N fertilizer (Tables 6-44 thru 6-46). Fertilization with N at planting or during the vegetative stage increased significantly the number of pods produced. Applying N during the vegetative stage, at flowering, or, in some years, midway during podfilling significantly increased seed test weight. Thus, the vegetative stage (in this study V3-V4, approximately four weeks after emergence for winter plantings in Central Brazil), appears to be the most efficient time to apply N because of increases in both pod-set and seed test weight.

In this study an application of N fertilizer during the vegetative phase had a pronounced negative effect on nodulation and, presumably on N_2 fixation at flowering (Table 6-51), while N fertilization at flowering was much less deleterious to nodulation and seasonal N_2 fixation. Additional research is needed to compare

Table 6-52. Shoot N concentration in control plots (-N) and those fertilized with N at flowering (+N), CNPAF, 1985 and 1987. Values are means of 5 replicates in each year.

Genotype	Treat-ment	Days After N			
		0	5	11	20
		%			
Rio Tibagi	-N	3.23	2.80	2.84	2.07
	+N	--	2.90	2.61	2.29
	$\bar{x}$	--	2.85	2.73	2.18
Carioca	-N	3.41	2.98	2.69	2.30
	+N	--	3.22	2.87	2.52
	$\bar{x}$	--	3.10	2.78	2.41
WBR 22-34	-N	3.42	2.96	2.50	2.18
	+N	--	2.92	2.97	2.36
	$\bar{x}$	--	2.94	2.74	2.27
$\bar{x}$	-N	3.35	2.91	2.68	2.18**
	+N	--	3.01	2.82	2.39**
	$\bar{x}$	--	2.96	2.75	2.29

** denotes significant difference ($P<0.05$) between N treatment means.

Table 6-53. Shoot N content in control plots (-N) and those fertilized with N at flowering (+N), CNPAF, 1985 and 1987. Values are means of 5 replicates in each year.

Genotype	Treat-ment	Days After N			
		0	5	11	20
		mg plant^{-1}			
Rio Tibagi	-N	151	196	283	271
	+N	-	189	247	303
	$\bar{x}$	-	193	265	287
Carioca	-N	148	221	268	311
	+N	-	209	274	331
	$\bar{x}$	-	215	271	321
WBR 22-34	-N	144	156	207	260
	+N	-	172	244	280
	$\bar{x}$	-	164	225	270
$\bar{x}$	-N	148	191	253	280
	+N	-	190	255	304
	$\bar{x}$	-	190	254	292

the results from these winter seasons to planting in February-March, when nodule duration into the podfilling phase is greatly reduced. Studies are also needed to determine the optimum amount of N fertilizer to maximize biological and economic yields as well as pinpointing more precisely the optimum time during the vegetative phase when N fertilizer should be applied. The latter studies should emphasize stage of plant development, rather than days after emergence.

Nitrogen Level at Planting. Most Brazilian research on the effects of N fertilizer on bean nodulation and N_2 fixation has been done using relatively large doses of N at planting (30-100 kg N ha^{-1}), while farmers who use N generally apply from 0-20 kg ha^{-1} at planting. Data are lacking on the effects of realistic doses of N, those used in production systems, on nodulation and N_2 fixation.

To begin collecting such data, parts of the 1986 and 1987 seed multiplication areas were divided into the following treatments: 0, 5, 10 and 20 kg N ha^{-1} at planting for lines CNF 178, 21-58, 22-27, 22-14, and 22-52. Due to a smaller area planted, lines 22-14 and 22-52 received only the 0 and 10 kg N ha^{-1} levels in 1986. All seeds were inoculated with the standard CNPAF inoculum mixture at planting, and no side-dress N was applied.

Samples were taken at V2-3, R1, and R7 for shoot, root, and nodule dry matter and shoot N content. At maturity, pod, seed, and stover yield and seed test weight were measured. Statistical analyses across years were performed combining the five lines and

two N level treatments and also the three lines and four N treatments.

Across three lines all levels of N increased shoot dry weight over the control in the vegetative phase, at flowering, and at R7, but differences in the last sampling were no longer significant (Table 6-54). Across all five lines, 10 kg N ha^{-1} at planting produced a significantly higher shoot dry weight at R7 than the control (Table 6-55). Root dry matter showed a similar tendency except at R7, when a significant difference was observed across three lines between the control and 20 by N ha^{-1} at planting (Table 6-56) and across five lines the difference between 1 and 10 kg N was not significant (Table 6-57). Nodule dry matter in the early vegetative stage was significantly reduced by applying 10 or more kg N ha^{-1} at planting, but across lines no significant differences due to N level were observed later in the season (Tables 6-58 and 6-59). Grain yield and seed test weight across lines were not significantly increased by applying up to 20 kg N ha^{-1} at planting, while pod yield was significantly increased with added N (Table 6-60 and 6-61).

Results of this experiment over two years show no statistically significant yield advantage in applying up to 20 kg N ha^{-1} at planting. Benefits from applying N fertilizer are apparently offset by a reduction in N_2 fixation during vegetative growth (Tables 6-58 and 6-59). Although results may vary with year and bean genotype, the year x N level interaction was not significant

Table 6-54. Shoot dry matter content (g plant^{-1}) of 3 bean lines with 4 levels of N at planting, CNPAF, 1986 and 1987. Values are means of 3 replicates each year.

Line	Kg N Ha^{-1} at Planting				
	0	5	10	20	$\bar{x}$
	V2-V3 (19 DAE)				
CNF 178	0.51	0.66	0.76	0.78	0.67 A[1]
WBR 21-58	0.60	0.78	0.83	0.84	0.76 A
WBR 22-27	0.57	0.87	0.85	0.75	0.76 A
$\bar{x}$	0.56 b[1]	0.77 a	0.81 a	0.79 a	0.73
	R1				
CNF 178	3.02	3.64	3.68	4.00	3.59 B
WBR 21-58	3.34	3.85	4.83	5.27	4.32 A
WBR 22-27	3.14	4.24	4.52	5.58	4.37 A
$\bar{x}$	3.17 c	3.91 b	4.34 ab	4.95 a	4.09
	R7				
CNF 178	12.98	12.42	12.60	15.00	13.25 A
WBR 21-58	9.74	11.94	11.99	12.49	11.54 B
WBR 22-27	11.51	14.54	13.39	12.28	12.93 AB
$\bar{x}$	11.41 a	12.97 a	12.66 a	13.26 a	12.57

[1] Means within a column followed by the same capital letter and within a row followed by the same small letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-55. Shoot dry matter content (g plant^{-1}) of 5 bean lines at 2 levels of N, CNPAF, 1986 and 1987. Values are means of 3 replicates in each year.

Line	Kg N Ha^{-1} at Planting		
	0	10	$\bar{x}$
	V2-V3 (19 DAE)		
CNF 178	0.51	0.76	0.63 A[1]
WBR 21-58	0.60	0.83	0.72 A
WBR 22-27	0.57	0.85	0.71 A
WBR 22-14	0.61	0.81	0.71 A
WBR 22-52	0.54	0.79	0.67 A
$\bar{x}$	0.57***	0.81***	0.69
	R1		
CNF 178	3.02	3.68	3.35 C
WBR 21-58	3.34	4.83	4.08 AB
WBR 22-27	3.14	4.52	3.83 BC
WBR 22-14	4.04	5.25	4.65 A
WBR 22-52	3.08	3.59	3.33 C
$\bar{x}$	3.32***	4.37***	3.85
	R7		
CNF 178	12.98	12.60	12.79 AB
WBR 21-58	9.74	11.99	10.86 B
WBR 22-27	11.51	13.39	12.45 AB
WBR 22-14	13.67	15.46	14.56 A
WBR 22-52	12.38	11.95	12.17 B
$\bar{x}$	12.06*	13.08*	12.57

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

* and *** denote significant differences between N treatment means at $P<0.05$ and $P<0.001$, respectively.

Table 6-56. Root dry matter content (g plant^{-1}) of 3 bean lines with 4 levels of N at planting, CNPAF, 1986 and 1987. Values are means of 3 replicates each year.

Line	Kg N Ha^{-1} at Planting				
	0	5	10	20	$\bar{x}$
	V2-V3 (19 DAE)				
CNF 178	0.16	0.22	0.22	0.27	0.22 AB[1]
WBR 21-58	0.15	0.20	0.18	0.22	0.19 B
WBR 22-27	0.19	0.26	0.28	0.24	0.24 A
$\bar{x}$	0.17 b[1]	0.23 a	0.23 a	0.24 a	0.22
	R1				
CNF 178	0.51	0.68	0.63	0.76	0.65 B
WBR 21-58	0.47	0.58	0.70	0.75	0.62 B
WBR 22-57	0.62	0.88	0.94	1.08	0.86 A
$\bar{x}$	0.53 c	0.71 b	0.76 ab	0.86 a	0.72
	R7				
CNF 178	0.90	0.88	0.90	1.08	0.94 B
WBR 21-58	0.70	0.90	0.89	0.98	0.87 B
WBR 22-27	1.01	1.26	1.14	1.20	1.15 A
$\bar{x}$	0.87 b	1.01 ab	0.98 ab	1.08 a	0.99

[1] Means within a column followed by the same capital letter and within a row followed by the same small letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-57. Root dry matter content (g plant^{-1}) of 5 bean lines at 2 levels of N, CNPAF, 1986 and 1987. Values are means of 3 replicates in each year.

Line	Kg N Ha^{-1} at Planting		
	0	10	$\bar{x}$
	V2-V3 (19 DAE)		
CNF 178	0.16	0.22	0.19 BC[1]
WBR 21-58	0.15	0.18	0.17 C
WBR 22-27	0.19	0.28	0.23 A
WBR 22-14	0.16	0.23	0.20 ABC
WBR 22-52	0.16	0.25	0.21 AB
$\bar{x}$	0.17***	0.23***	0.20
	R1		
CNF 178	0.51	0.63	0.57 B
WBR 21-58	0.47	0.70	0.58 B
WBR 22-27	0.62	0.94	0.78 A
WBR 22-14	0.65	0.89	0.77 A
WBR 22-52	0.52	0.72	0.62 B
$\bar{x}$	0.55***	0.78***	0.66
	R7		
CNF 178	0.90	0.90	0.90 BC
WBR 21-58	0.70	0.89	0.80 C
WBR 22-27	1.01	1.14	1.07 AB
WBR 22-14	1.13	1.18	1.16 A
WBR 22-52	1.11	1.06	1.09 A
$\bar{x}$	0.97	1.04	1.00

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

*** denotes a significant difference between N treatment means at $P<0.001$.

Table 6-58. Nodule dry matter content (mg plant^{-1}) of 3 bean lines with 4 levels of N at planting, CNPAF, 1986 and 1987. Values are means of 3 replicates each year.

Line	Kg N Ha^{-1} at Planting				
	0	5	10	20	$\bar{x}$
	V2-V3 (19 DAE)				
CNF 178	12	12	7	6	9 A[1]
WBR 21-58	13	10	6	4	8 A
WBR 22-27	11	9	8	4	8 A
$\bar{x}$	12 a[1]	10 ab	7 bc	5 c	9
	R1				
CNF 178	56	77	68	70	68 A
WBR 21-58	54	65	49	43	55 A
WBR 22-27	47	64	63	46	55 A
$\bar{x}$	56 a	69 a	60 a	53 a	59
	R7				
CNF 178	55	34	58	44	48 AB
WBR 21-58	49	67	26	77	55 A
WBR 22-27	27	28	29	16	25 B
$\bar{x}$	44 a	43 a	38 a	45 a	43

[1] Means within a column followed by the same capital letter and within a row followed by the same small letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-59. Nodule dry matter content (mg plant^{-1}) of 5 bean lines at 2 levels of N, CNPAF, 1986 and 1987. Values are means of 3 replicates in each year.

Line	Kg N Ha^{-1} at Planting		
	0	10	$\bar{x}$
	V2-V3 (19 DAE)		
CNF 178	12	7	9 B[1]
WBR 21-58	13	6	10 B
WBR 22-27	11	8	10 B
WBR 22-14	11	6	8 B
WBR 22-52	19	11	15 A
$\bar{x}$	13***	8***	10
	R1		
CNF 178	56	68	62 AB
WBR 21-58	64	49	56 AB
WBR 22-27	47	63	55 AB
WBR 22-14	80	84	82 A
WBR 22-52	56	52	54 B
$\bar{x}$	60	63	62
	R7		
CNF 178	55	58	57 B
WBR 21-58	49	26	38 B
WBR 22-27	27	29	28 B
WBR 22-14	145	93	119 A
WBR 22-52	50	45	48 B
$\bar{x}$	65	51	58

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

*** denotes a significant difference between N treatment means at $P<0.001$.

Table 6-60. Maturity parameters of 3 bean lines with 4 levels of N at planting, CNPAF, 1986 and 1987. Values are means of 3 replicates each year.

Line	Kg N Ha^{-1} at Planting				
	0	5	10	20	$\bar{x}$
	Grain Yield (kg ha^{-1})				
CNF 178	1569	1524	1557	1613	1566 A[1]
WBR 21-58	1368	1370	1385	1444	1392 B
WBR 22-27	1282	1308	1315	1297	1300 C
$\bar{x}$	1406 a[1]	1400 a	1419 a	1451 a	1419
	Pod Yield (no m^{-2})				
CNF 178	181	199	187	203	192 A
WBR 21-58	135	141	145	143	141 C
WBR 22-57	150	166	162	165	161 B
$\bar{x}$	155 b	169 a	165 ab	170 a	165
	50-seed Test Weight (g)				
CNF 178	9.57	9.50	9.25	9.64	9.49 AB
WBR 21-58	9.61	9.51	9.85	9.52	9.62 A
WBR 22-27	9.43	9.42	9.55	9.14	9.38 B
$\bar{x}$	9.54 a	9.47 a	9.55 a	9.43 a	9.50

[1] Means within a column followed by the same capital letter and within a row followed by the same small letter do not differ significantly ($P<0.05$) by Tukey's HSD.

Table 6-61. Maturity parameters of 5 bean lines at 2 levels of N, CNPAF, 1986 and 1987. Values are means of 3 replicates in each year.

Line	Kg N Ha^{-1} at Planting		
	0	10	$\bar{x}$
	Grain Yield (kg ha^{-1})		
CNF 178	1569	1557	1563 A[1]
WBR 21-58	1368	1385	1377 BC
WBR 22-27	1282	1315	1298 CD
WBR 22-14	1465	1499	1482 AB
WBR 22-52	1132	1198	1165 D
$\bar{x}$	1363	1391	1377
	Pod Yield (no m^{-2})		
CNF 178	181	187	184 A
WBR 21-58	135	145	140 C
WBR 22-27	150	162	156 B
WBR 22-14	152	157	154 B
WBR 22-52	132	141	136 C
$\bar{x}$	150**	158**	154
	50-seed Test Weight (g)		
CNF 178	9.57	9.25	9.41 B
WBR 21-58	9.61	9.85	9.73 AB
WBR 22-27	9.43	9.55	9.49 B
WBR 22-14	9.97	9.99	9.98 A
WBR 22-52	9.48	9.14	9.31 B
$\bar{x}$	9.61	9.56	9.58

[1] Means followed by the same letter do not differ significantly ($P<0.05$) by Tukey's HSD.

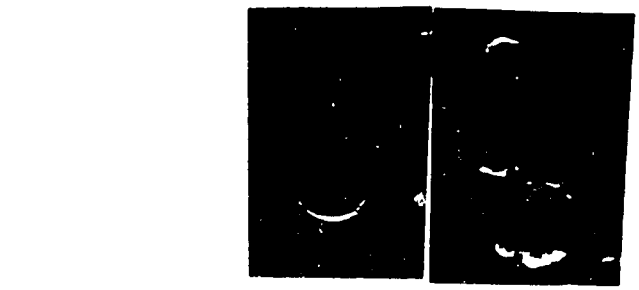
** denotes a significant difference between N treatment means at $P<0.01$.

for grain yield and no significant line x N level interactions were observed for any of the parameters measured.

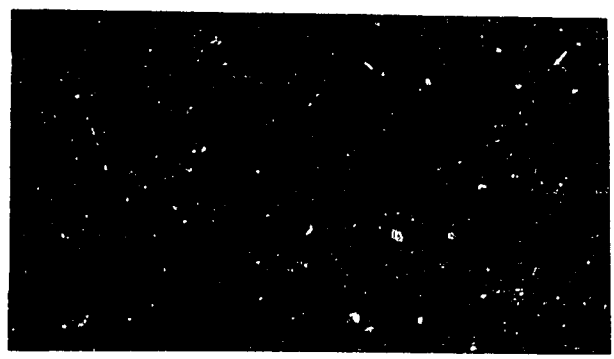
Nodule Senescence

Premature nodule senescence is a major constraint to nitrogen fixation in the R. phaseoli-P. vulgaris symbiosis. We have studied this phenomenon at MSU using selected strains of bean rhizobia and bean line 21-58. In our general evaluation of nitrogen fixation potential by R. phaseoli isolates, we found that bean line 21-58 inoculated with R. phaseoli UMR 1024 induces a high proportion of nodules which prematurely senesce. This occurs despite the fact that 21-58 is a superior bean line suited for enhanced nitrogen fixation and was grown under non-stressful environmental conditions in the growth chamber. This is a clear indication that the bacterial symbiont plays a major, contributing role in determining whether nodules will prematurely senesce. Thus, we used this combination of symbionts as a model system to study premature nodule senescence under microbiologically controlled conditions.

Qualitative observations of flask cultures indicated that nodules emerged from inoculated plants within two weeks. During the third week, the nodules were red and fixed nitrogen as measured by reduction of acetylene and $^{15}N_2$. After three weeks, some of the nodules displayed the first visible signs of senescence, which is a greening of the nodule beginning in the center and later spreading outward (Fig. 6-23a). By five weeks, some of the leaves had turned yellow with brown edges toward their tips (Fig. 6-23b).



(A)



(B)

(C)

Figure 6-23. Nodules (A) and leaves (B) of bean plants (C) of line UW21-58 undergoing premature nodule senescence with R. phaseoli UMR 1024.

Some nodules on the same root system remained red throughout the vegetative growth period of the plant. Because this sequence begins in the center of the nodule, it is not possible to score for the ratio of senescing/active (red) nodules without dissection. Other "effective" bean rhizobia (e.g., CIAT 899 and KIM-5) also induced some nodules which eventually turn green (5-6 weeks). However, in general, a larger proportion of the nodules formed by these effective strains remain red longer and fewer green nodules eventually develop. Thus, the process of normal bean nodule senescence can be accelerated by some bacterial symbionts, resulting in a premature cessation of the active phase of nitrogen fixation.

Microbiological studies of the nodule occupants re-isolated after induction of premature senescence have ruled out the possibility that UMR 1024 was a mixed culture containing one strain which produces red nodules only and another strain producing green nodules only. The same results were also obtained with the effective strain KIM-5. Regardless of whether the inoculum was prepared from isolates of red or green nodules, the re-isolates of either nodule occupant produced both red and green nodules on new plants.

Histological, cytological, and ultrastructural studies were performed on red and green nodules induced by UMR 1024 and CIAT 899. Light microscopy of 1 um cross-sections of nodules indicated that the senescing green region stains less intensely with toluidine blue and contains fewer infected plant cells which are

highly vacuolate (Fig. 6-24a and b) in contrast to the red nodules (Fig. 6-24c and d). Similar results distinguishing green vs. red nodules were induced by both bacterial strains.

Transmission electron microscopy of red nodules induced by UMR 1024 (Fig. 6-25a and b) contained many amyloplasts in uninfected cells and many cytoplasmic organelles such as endoplasmic reticulum, Golgi bodies, and well-formed peribacteroid membranes in infected cells. Often the uninfected cells had a single, large central vacuole and cytoplasm which was more prominent towards the periphery, containing many mitochondria and plastids, as reported for effective nodules by others. Green nodules induced by UMR 1024, on the other hand, displayed a typical picture of nodule senescence, including displaced and empty plant cytoplasm, extensive lysis, and membrane fragmentation (Fig. 6-25c and d). The bacteria were frequently plasmolyzed with cytoplasm shrunken from their outer membranes, tended to have less polybetahydroxybutyrate (PBHB) granules, and were surrounded by an incomplete peribacteroid membrane or devoid of this organelle altogether. Uninfected cells contained many smaller vacuoles and generally lacked starch granules.

The ultrastructure of nodules induced by CIAT 899 displayed similar differences between red and green nodules (Fig. 6-26a-d). The major differences in appearance between these two bacterial strains within infected cells were that UMR 1024 accumulated more PBHB granules than did CIAT 899, and unlike UMR 1024, CIAT 899 bacteroids were embedded in a distinct fibrous network of electron-

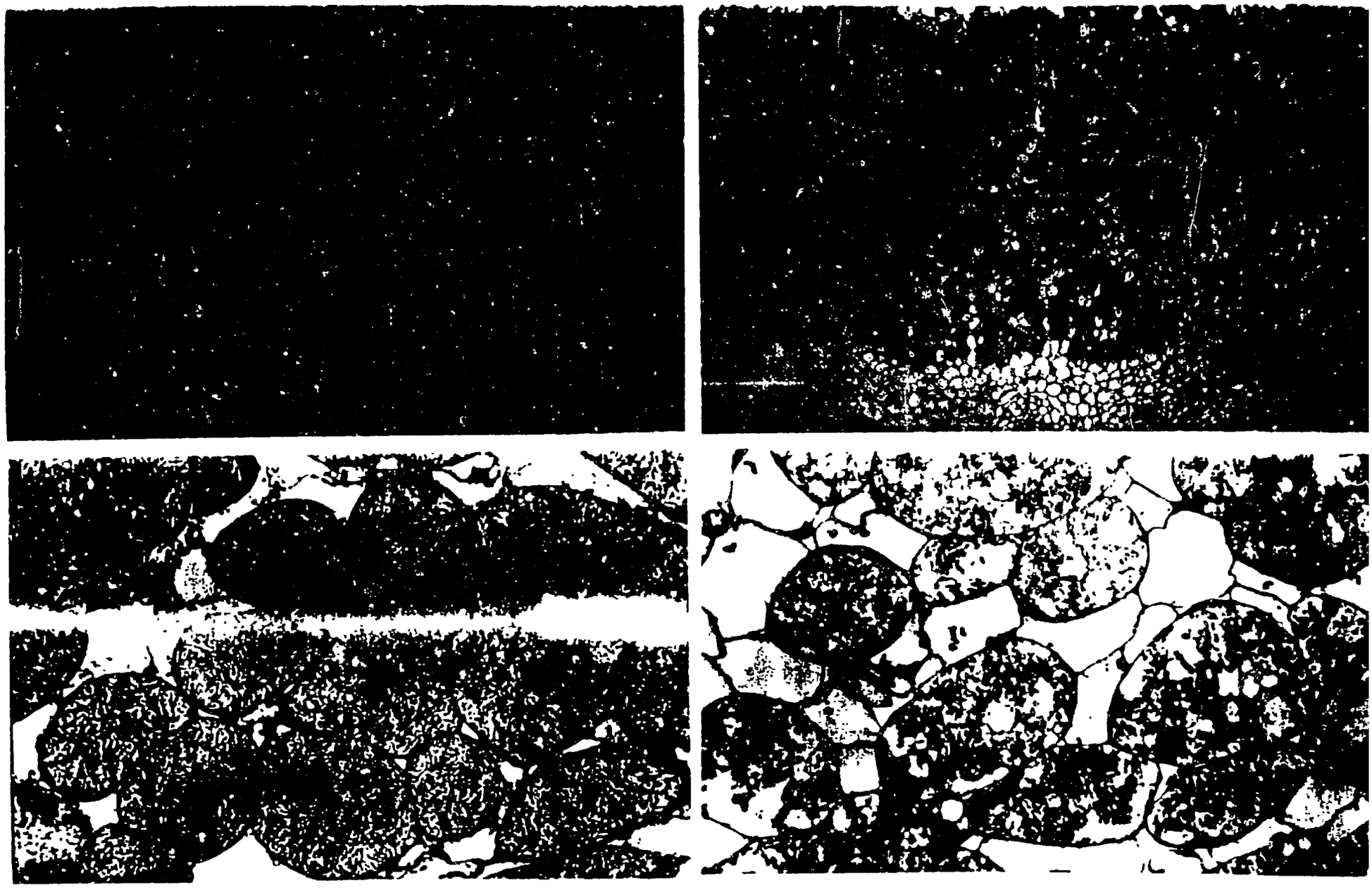


Figure 6-24. Histological stained sections of bean nodules showing; prematurally-senescing nodule (A and B), effective nodule (C and D).

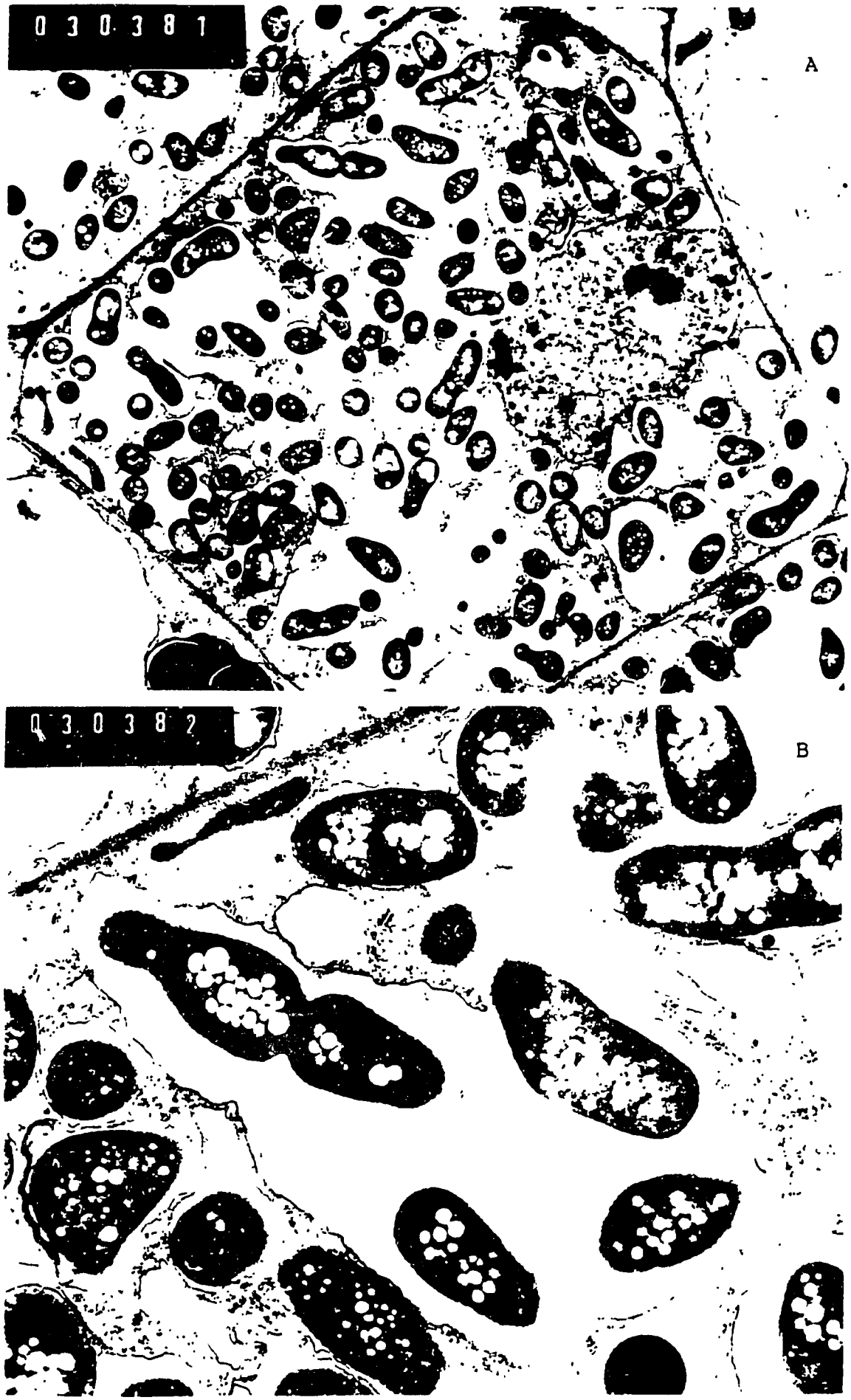


Figure 6-25. Transmission electron micrograph of the ultrastructure within root nodules infected with R. phaseoli UMR 1024; A and B are red nodules, C and D are green senescing nodules.

Figure 6-25. (Continued).

Figure 6-26. Transmission electron micrographs of bean nodules infected with R. phaseoli CIAT 899; A and B are red nodules, C and D are green nodules.

Figure 6-26. (Continued).

dense material within peribacteroid vesicles of red nodules. This fibrous material surrounding CIAT 899 cells appears reminiscent of extracellular polysaccharide.

One mm thick, longitudinal sections of red and green nodules induced by CIAT 899 were examined by scanning electron microscopy (Fig. 6-27a-d) and analyzed for elemental composition by energy-dispersive X-ray microanalysis using a 1.08 mm^2 window. The density of the red vs. green nodules were different, the red being more dense and compact in the center. No enlarged Fe, Co, or Cu peaks were found, suggesting that the green coloration is not due to an accumulation of these elements. Since only four nodules could be examined and the results varied somewhat between replicate samples, the data are considered suggestive but not conclusive. With this limitation understood, it appeared that the green nodules may have accumulated more Ca (and possibly K), but not more S and P than red nodules (Table 6-62).

The ATP content and acidity of freshly removed, 4-week old whole root nodules of bean line 21-58 induced by UMR 1024 were examined to get an indication of their overall metabolic activity (Ching et al., 1978). *In vivo* ^{31}P-NMR spectroscopy indicated discrete peaks of Pi (inorganic phosphate), (nucleotide monophosphates), (nucleotide diphosphates), and (nucleotide triphosphates) within red nodules whereas only inorganic phosphate produced a sufficiently strong signal with green nodules (Fig. 6-28). The shift to a higher ppm of the major peak corresponding to Pi for the red nodules (Fig. 6-28) indicated that they have higher internal

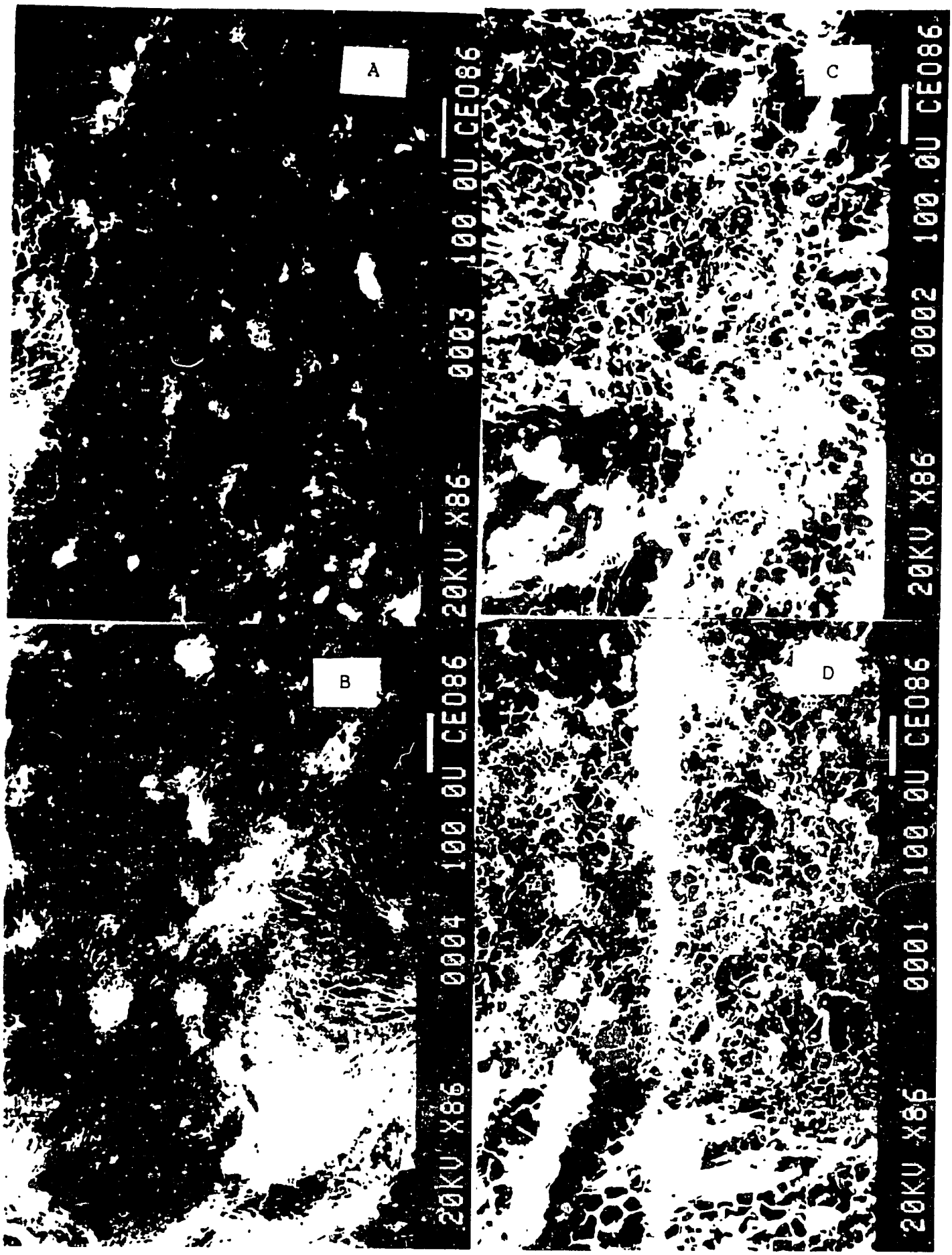


Figure 6-27. Scanning electron micrographs of 1 mm thick sections of bean root nodules infected with CIAT 899; A and B are red nodules, C and D are green nodules.

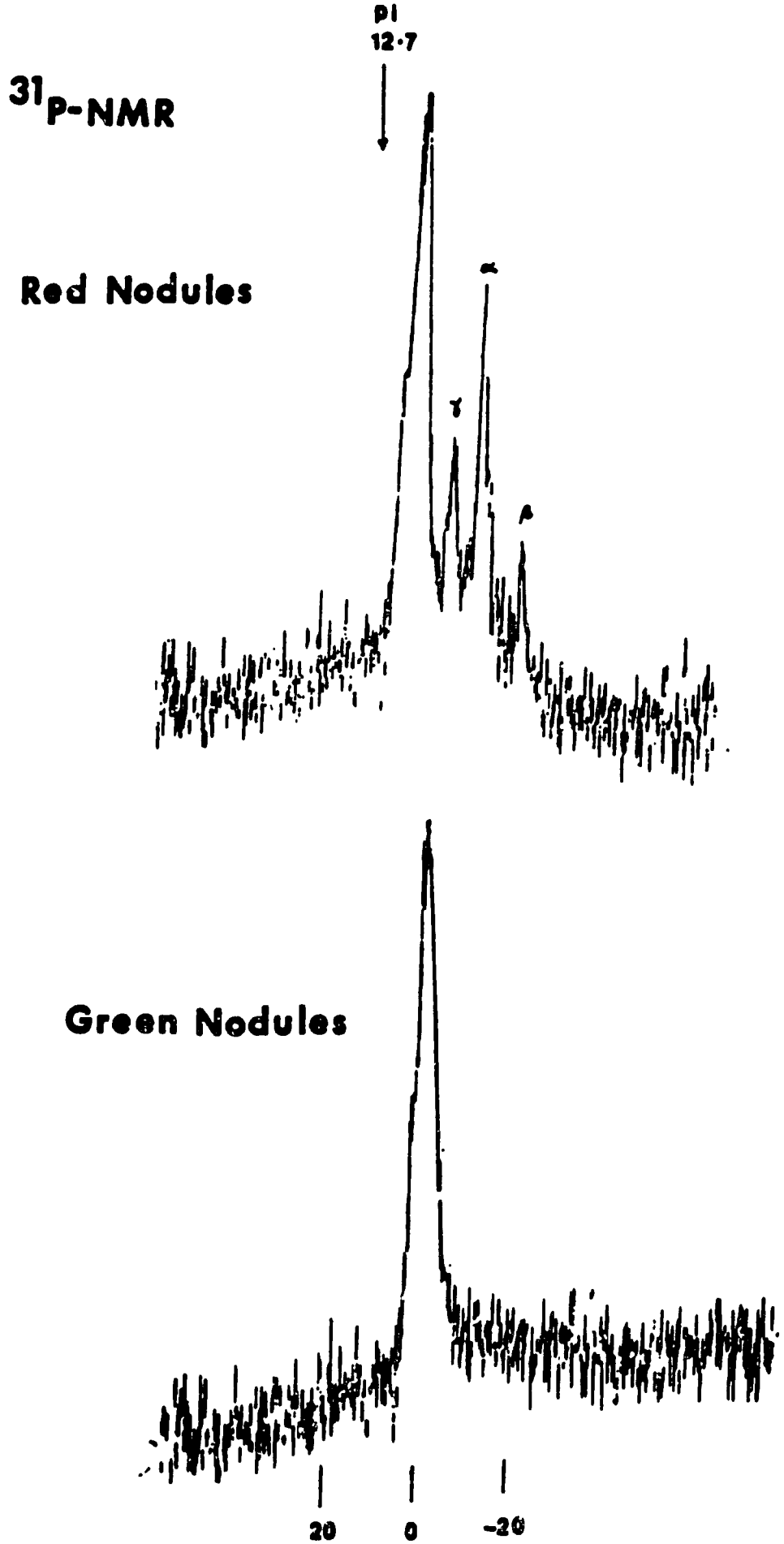


Figure 6-28. ^{31}P-NMR spectroscopy of red (active and green (senescing) bean nodules infected with R. phaseoli UMR 1024.

acidity. This result was confirmed by in vivo ^{1}H-NMR measurement of fresh, whole root nodules, which showed distinct lobes indicating resolvable domains or compartments of different acidities within the nodules (Fig. 6-29). The major lobe at the higher 8.0 ppm corresponds to a more acidic domain at an approximate pH 5 within the red nodule. This acidic domain is not detected in the green senescing nodules. We speculate that this latter result indicates a greater pool of organic acids (e.g., TCA intermediates) which serve as carbon and energy sources for respiring bacteroids during nitrogen fixation. The luciferase bioluminescence assay showed that levels of ATP were higher in extracts of red than of green nodules (Fig. 6-30). For this latter study, several methods to extract ATP from root nodules were compared, and 60 mM phosphate buffer was found to be superior to alternatives (trichloric acid, perchloric acid, boiling water, boiling ethanol). This is because the phosphate buffer inhibits endogenous alkaline phosphatases (which hydrolyze ATP) through end product inhibition and the pH 7.4 is optimum for the luciferase-mediated bioluminescence reaction. These results suggest that the red nodules are more active in ATP-generating metabolism than are green nodules, which should have a major impact on biological nitrogen fixing activity.

Biological nitrogen fixation in the Rhizobium-legume root nodule symbiosis is intimately dependent on allocation of photosynthate from leaves for energy to reduce dinitrogen and fixed carbon to assimilate the ammonia product. Since our NMR studies indicated more acidic domains in the red nodules, we wondered whether

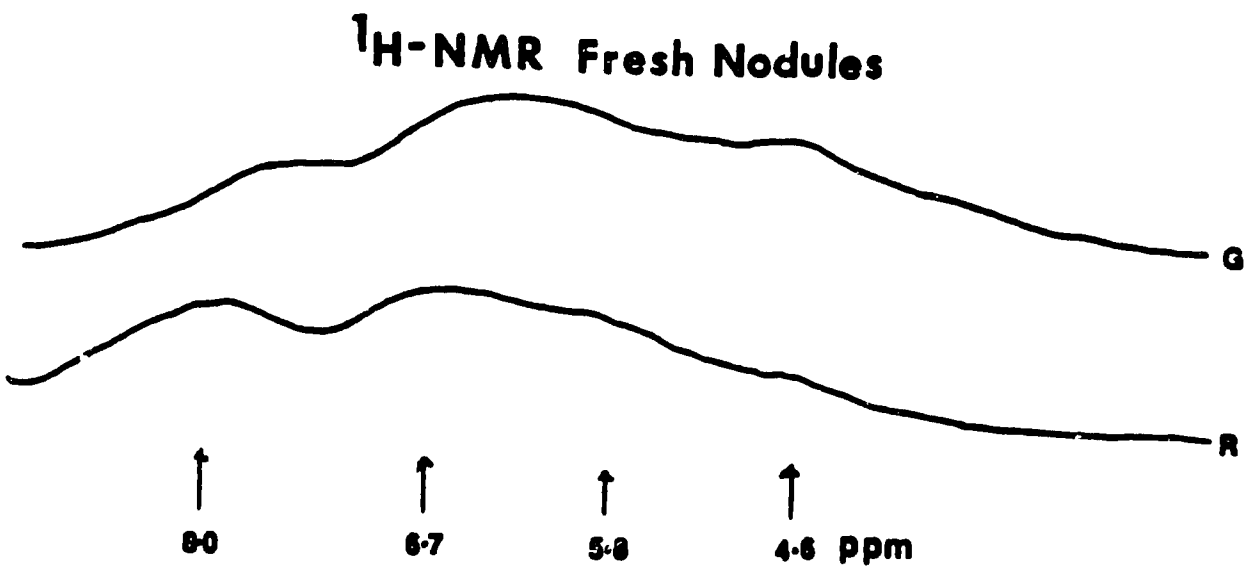


Figure 6-29. ^{1}H-NMR of 4-week old bean nodules infected with R. phaseoli UMR 1024. G= green nodules, R= red nodules.

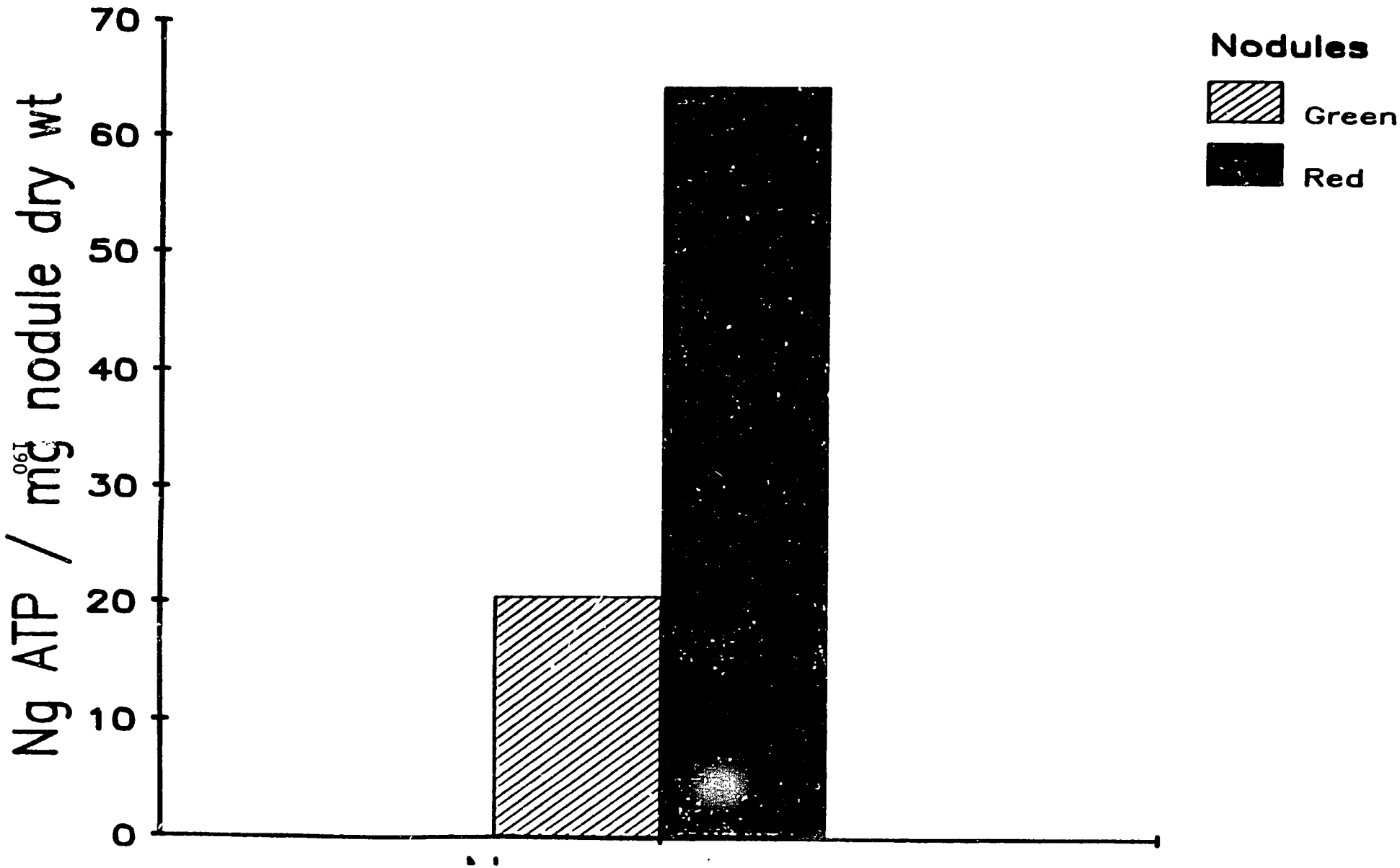


Figure 6-30. ATP content in 4-week old red and green nodules of the bean line UW21-58 inoculated with R. phaseoli UMR 1024.

deprivation of photosynthate to root nodules was a feature of senescing nodules, perhaps even being a cause. Therefore, we designed experiments to test the hypothesis that premature nodule senescence (as recognized by green nodules) is initiated by blockage of photosynthate transport to root nodules. This hypothesis was tested in a double labelling experiment utilizing $^{14}CO_2$ and $^{15}N_2$.

Seeds of bean line 21-58 were surface sterilized, germinated, and grown in Erlenmeyer flask culture containing Smucker's nitrogen-free agar medium. Flasks were equipped with a sidearm port which permitted introduction and removal of gas samples and a foam plug at the top. Each flask received two seedlings, each inoculated with a 0.5 ml suspension of the bacteria (10^8 cells). The flask cultures were incubated in the growth chamber and irrigated on days 7 and 14 with sterile water, day 21 with 1:4 strength Smucker mineral nutrient medium, days 28 and 35 with sterile water. This schedule was designed to avoid water and mineral nutrient limitation. When of sufficient length, the stem and attached leaves were removed from the flask and the foam plug replaced to exclude microbial contamination from the root system.

A flask culture assembly containing a significant nodule population that was green and thought to be senescent (5-6 wk) is shown in Fig. 6-31. At this time, the neck of the flask was sealed around the plant stem to isolate the root/nodule atmosphere. The gas within the flask was replaced with an $^{15}N_2/O_2$ mixture (80:20, 60 atom % ^{15}N). The plants were placed in a sealed chamber (30 x 20

Figure 6-31. Conical side arm flask culture of beans nodulated with R. phaseoli UMR 1024 and used for $^{14}C/^{15}N$ labelling experiments.

x 20) and illuminated at a PAR of 560 uEm/m/sec. The atmosphere within the chamber was purged of $^{12}CO_2$ and this was replaced with $^{14}CO_2$ (6 mBq/mg C). The concentration of $^{14}CO_2$ was maintained at 350 ppm by controlled addition for the labelling pulse of 1 hr. After 1 hr of labelling, the $^{14}CO_2$ and $^{15}N_2$ mixtures were replaced with normal air. Plants were harvested 0, 1, 2, and 3 hr after the beginning of the chase period. All nodules greater than 1 mm diameter were removed from the roots, cut in half, and grouped according to internal color (red, green, brown). The plants and nodules were dried and weighed prior to ^{15}N and ^{14}C analyses. Individual nodules and subsamples of plant material were analyzed for ^{15}N and ^{14}C using a CN analyzer in line with a continuous flow isotope ratio mass spectrometer fitted with an automatic CO_2 collector (Harris and Paul, 1989).

The atom % ^{15}N in green and red nodules, in leaves of the plants incubated with $^{15}N_2$ and in control leaves incubated without label is shown in Fig. 6-32. The control value is typical and representative of the natural abundance of ^{15}N and thus this background control was not replicated. The results indicate that both red and green nodules were fixing nitrogen and that some of the fixed nitrogen is transported from the nodulated root system to the leaves of these bean plants. The data are averages from several samples (indicated above bars). It should be noted that most green nodules showed negligible increases in atom % ^{15}N, but some accumulated higher levels (1.9691; 1.1250; 1.0166) than the average for the red nodules.

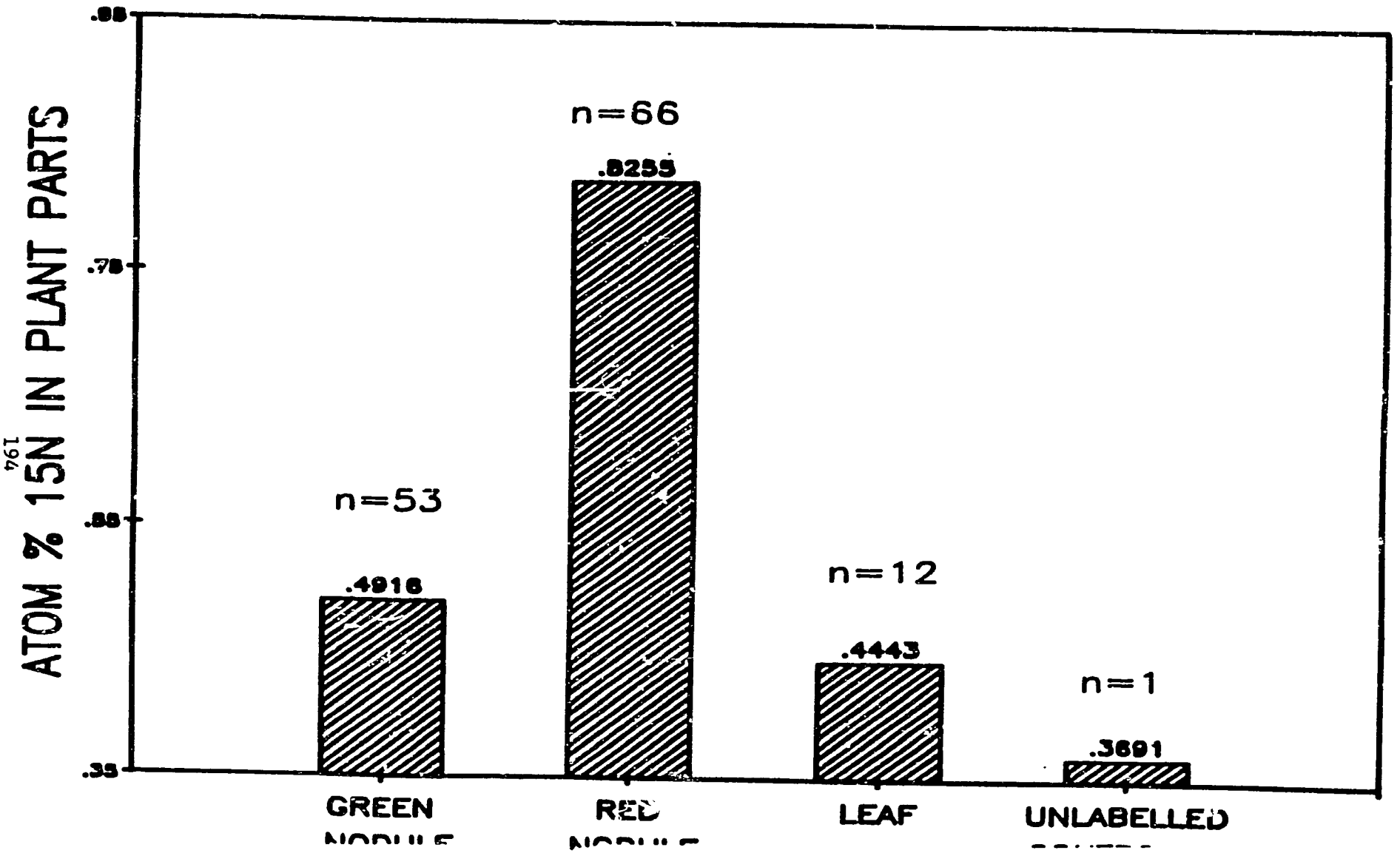


Figure 6-32. Distribution of nitrogen fixed in the bean line UW21-58 nodulated with R. phaseoli UMR 1024.

The results of a pulse-chase experiment designed to measure allocation of photosynthate to red and green nodules, respectively, are shown in Figs. 6-33 and 6-34. The mean specific activity of ^{14}C in red nodules increased with the length of the chase period (Fig. 6-33). By three hours, the specific activity was about 20% of that in the leaves, indicating that a significant quantity of photosynthate was allocated to the active red nodules. Allocation of photosynthate to green nodules fell into two categories (Fig. 6-34): some had very low ^{14}C label, whereas several others had a ^{14}C content equal to or greater than the average for red nodules on the same plant.

This experiment refutes the hypothesis that blockage of photosynthate allocation to bean nodules triggers senescence since it is clear that some green nodules accumulated ^{14}C and ^{15}N.

In normally active red nodules, the C and N pools turn over rapidly and fixed N is continually exported. This is why the ^{15}N content of these nodules never became very high. A disruption of the fixed N export system has been shown to be associated with nodule senescence (Atkins et al., 1984) and could explain the accumulation of ^{15}N found in some green nodules which were still active in the nitrogen fixation. These data suggest that the formation of green pigment and loss of the N export system occur before complete loss of nitrogenase activity in bean nodules undergoing premature senescence.

We attempted to isolate and identify the green pigment(s) which accumulate in the senescing nodules. Since these pigments

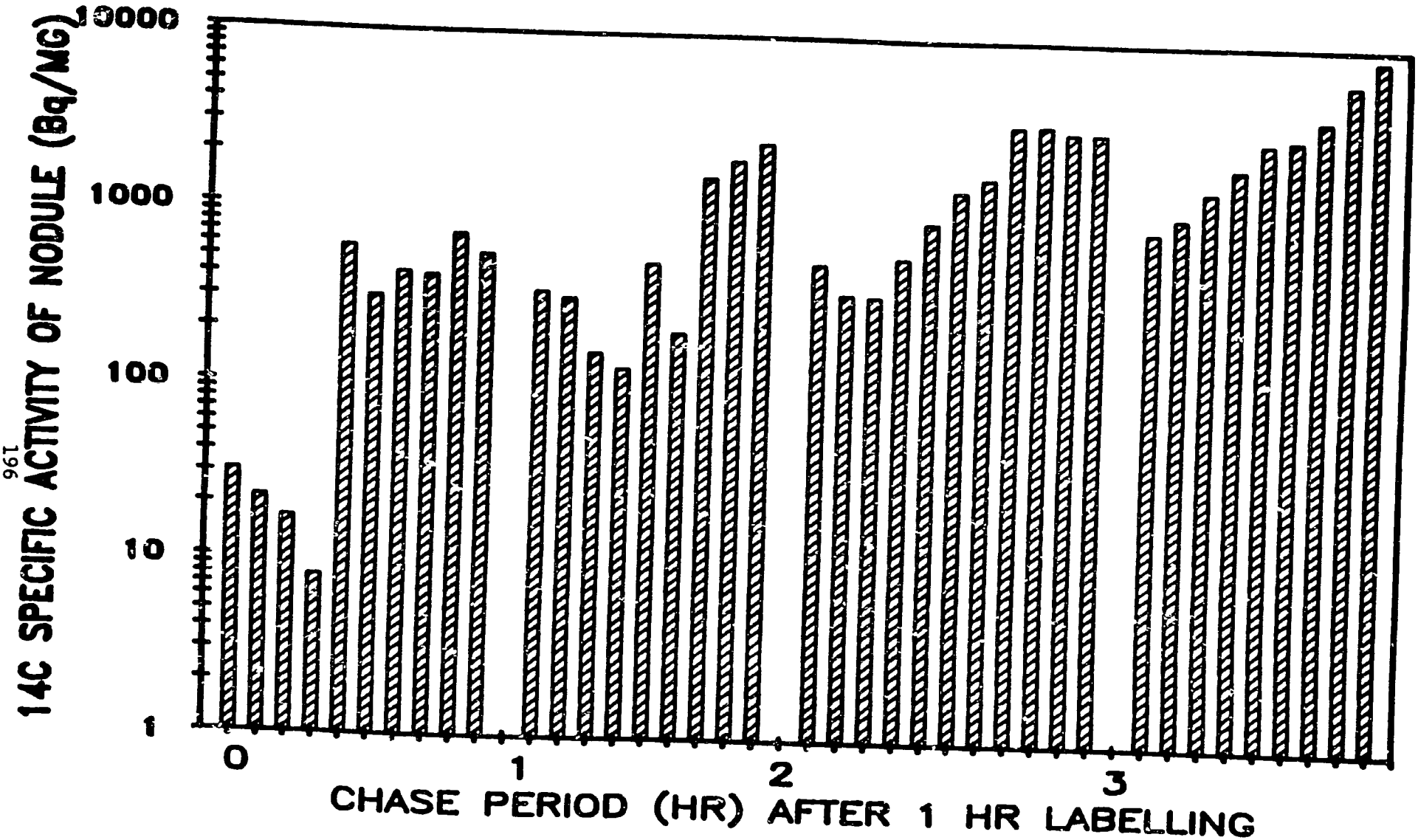


Figure 6-33. ^{14}C-labelled photosynthate allocation to red nodules of the bean line UW2i-58 inoculated with R. phaseoli UMR 1024.

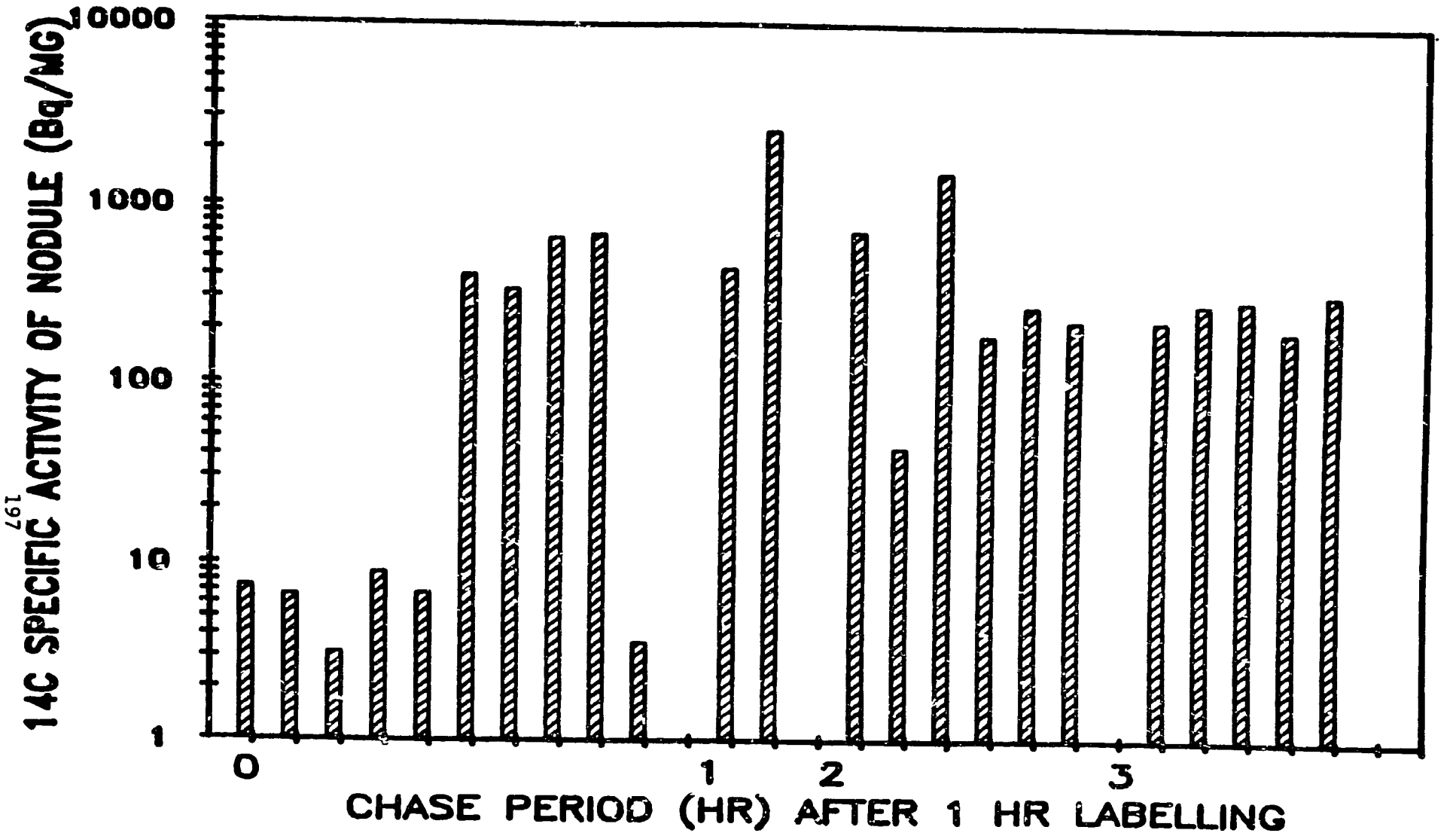


Figure 6-34. ^{14}C-labelled photosynthate allocation to senescent green nodules of the bean line UW21-58 inoculated with R. phaseoli UMR 1024.

are characteristic of the premature nodule senescence syndrome, knowledge of their structure would help to direct research at understanding the physiological basis and solution of the problem. We began by considering the suggestion of W. Rudiger (Univ. Munich, West Germany) based on TLC analysis that the green pigment is biliverdin, the first decomposition product of heme (from leghemoglobin) which arises from its oxidation via heme oxygenase. We optimized the extraction and methyl derivatization of biliverdin standard added to bean root tissue. The best extraction and derivatization of the biliverdin dimethyl ester (BVDM) was accomplished with MeOH:HCl (85:15). Lyophilization to remove water from the mascerated root tissue before adding the methanolic HCl also improved methyl esterification and extraction.

Having developed a reliable method to extract and methylate biliverdin from green nodules, we began to analyze green nodules from bean line 21-58 grown for five weeks with R. phaseoli UMR 1024. Methylated nodule extracts were green and shown by TLC to contain at least three green pigments having different mobilities in two different solvent systems. Additionally, these green pigments had different fluorescence properties under UV light: the major one fluoresced bluish white and the two minor pigments fluoresced bright red. We then developed a solvent system for TLC that would produce an Rf for BVDM of 0.5. Using that solvent system, we fractionated the concentrated mixture of extracted green pigments by absorption chromatography through a silica column and collected green fractions having bluish-white and red fluorescence.

These isolated green pigments ran as single spots on TLC using two different solvent systems. The BVDM standard had the same Rf as the major green pigment BUT did not fluoresce in the visible region. Also unlike BVDM, the major green nodule pigment produced a molecular ion of 736 amu when analyzed by fast-atom-bombardment mass spectrometry. UV-visible absorption spectra of the three green nodule pigments indicated absorption maxima characteristic of tetrapyrrole rings in the red region. Spectrofluorimetry showed unequivocally that the three green pigments are different, since they had different wavelength excitation maxima and produced different emission spectra (Fig. 6-35). Solubility in phosphate buffer argues against the nodule green pigments as being chlorophyll. We have also checked senescing green nodules of field-grown soybean, and TLC studies showed the same diversity of pigments in these nodules. Although we were unable to complete these studies, we can nevertheless conclude that the green coloration of senescing root nodules is due to a mixture of greenish and bluish-green tetrapyrrole pigments, and not biliverdin. A major complication of this work was the instability of the isolated pigments, which can rapidly undergo photooxidation. Thus, purification steps had to be conducted anaerobically with the lab lights off. It would be necessary to identify the pigments to link their production to decomposition of leghemoglobin.

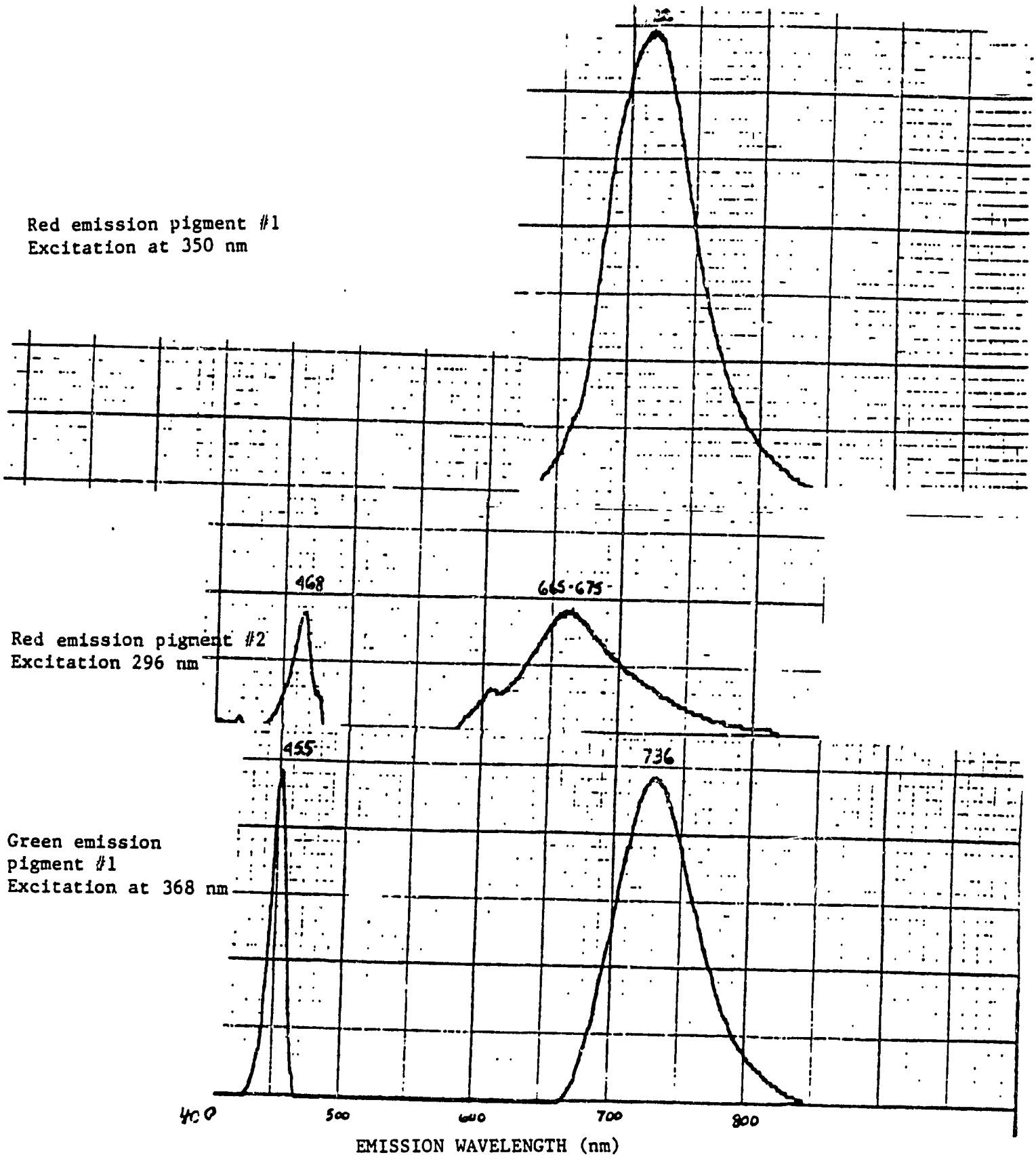


Figure 6-35. Fluorescence emission spectra of the three green pigments from root nodule extracts of P. vulgaris line UW21-58 infected with R. phaseoli UMR 1024. Extracts partially purified by TLC. Excitation and emission wavelengths are indicated.

7.0 PROJECT ADMINISTRATION

The lead U.S. institution for this project was the University of Wisconsin-Madison. During the first 5-year period, sub-contracts with CNPAF/EMBRAPA and Michigan State University were in effect. The sub-sub-contract back to Michigan State University caused considerable problems. Reports of expenditures from the M.S.U. Accounting Office usually were sent late to U.W-Madison, causing difficulties in reporting and budgeting for subsequent years. During the 3-year extension period, the work on rhizobia at M.S.U. was considered to be an intergral part of the budget and the project, but the money to Dr. Dazzo was sent directly to him from the Bean/Cowpea Management Office rather than through a subcontract of the U.W.-Madison. This latter arrangement eliminated the accounting problems of the first 5-year period.

During the entire project, a subcontract to CNPAF/EMBRAPA was in effect. Contrary to most contracts to Host Countries in which funds were sent in advance, funds for the project were first spent by the H.C., who was then reimbursed. The reason for this was the continuing high rate of inflation. Had the usual approach been used, the value of the money sent to Brazil would have been considerably less. This approach was not without some disadvantages, primary of which was that sometimes their were budget restrictions that resulted from lack of available funds to continue the work until reimbursement. Overall this procedure worked very well. Detailed accounts of expenditures were sent to the U.W.-Madison Research Administration on a monthly basis,

without fail. The administrators at CNPAF/EMBRAPA are to be commended on their precision and promptness. The only difficulties that occurred were those attributable to the accounting offices at the U.W.-Madison.

During the early years of this program, the budgeting process including carry-over funds, was very difficult for the P.I. and accounting personnel to understand. During the 3-year extension period, it seemed to become more like standard approaches to budgeting and accounting.

The timing of extensions by USAID seemed to have no rhyme or reason. Even though the projects were on an October through September basis, funding extensions were on a different time-frame, making budget control very difficult.

The review process sometimes seemed to be continuous. During one 6-month period there were at least three review teams at CNPAF/EMBRAPA. During the last 3-year period of the project, the reviews were more organized and reasonable.

In general, administration of the project at the U.W.-Madison went quite well. Ms. Barb Keenan in Research Administration worked with me very closely and was patient and understanding.

Counstraints Encountered

Methodology. The lack of good quality seeds of some lines in our evaluations sometimes resulted in a diminished useable plot are and consequent omission of sampling, possibly distorted comparisons, and diminished return on our time, material inputs, and energy for conducting field research. Also, some requests were

received for from 1/2 to 5 kg of seed of specific lines to test without N fertilizer. When inadequate seed was available, opportunities were missed. This problem was reduced by producing our own seed stocks at CNFAP and storing them in the CNPAF cold storage area.

Due to breakage and problems in making new distillation units, Kjeldahl total N analyses were often delayed, resulting in selection of entries for some experiments without N data. The arrival of the Grain Quality Analyzer for automated N analyses could eliminate this constraint.

Brazilian bean line selection programs not studying N_2 fixation uniformly fertilize trials with N. Our lines have done well because they yield well with or without N, but materials selected for N_2 fixation do not show their true potential unless they can be evaluated under N-stress conditions. More trials for evaluation without fertilizer N are needed at the regional and state levels to identify and study the best lines for N_2 fixation in the different Brazilian environments and farming systems.

Personnel. The Brazilian assistants hired on CRSP funds did not receive all the benefits of people contracted by EMBRAPA. Also they often got paid several days late, which sometimes resulted in two days-off to pay bills and penalties for overdue time payments. We were not allowed to pay them more in compensation than EMBRAPA employees.

More serious than these disadvantages was the continuing uncertainty of whether or not the CRSP project would be refunded each year and the question of whether the project would end in

September 1988 or May 1989. This lack of a permanent position resulted in some workers quitting to take a less desirable, but more secure, position in another field. Such departures not only meant a loss of the time spent training those people, but also research program interruptions until replacements were found and trained. Since we were not allowed to pay more than EMBRAPA, we could not motivate or reward CRSP-supported employees with additional salary increases.

Financial. Inadequate money at EMBRAPA and the general lack of a smoothly-functioning economy in Brazil at times resulted in severe shortges of field laborers, functioning machinery, and transportation at CNPAF.

Bureaucracy. The delay in releasing budget funds at the beginning of the 3-year extension resulted in a delay in ^{15}N sample analyses. For a period, USAID approval of international travel was not received until the last minute, sometimes 1 or 2 days before departure, which made travel preparations uncertain and unnecessarily stressful.

The signed contract between EMBRAPA and the International Atomic Energy Agency (IAEA) concerning our participation in the Agency's program to improve N_2 fixation in beans in Latin America was due in Vienna in January 1988. A proposal for renewal of that contract was sent to EMBRAPA/Brasilia in March 1988. As of July, 1988 it had not been signed and submitted to IAEA. Since participation in the IAEA program furnished additional research money under no obligation, it is unfortunate that contractual details have not been completed in a more timely fashion.

At CNPAF, EMBRAPA vehicles circulate to transport personnel and equipment to and from experimental field areas. Since considerable time was sometimes lost waiting for transportation, CRSP funds to buy a project vehicle were requested. Although the yearly budget had been approved, additional USAID approval was needed to purchase a vehicle. After many months, authorization was received, but by that time EMBRAPA had placed a freeze on buying all vehicles, even with reimbursement. When the freeze was lifted, the current budget year had expired and reapproval by USAID was required. This time authorization took even longer and came only after EMBRAPA had issued a policy of not buying any large equipment items for reimbursement. After 3 years of having funds for a vehicle in the approved CRSP budget and not being able to buy one, the idea was discarded as a waste of time. The Bureaucrats finally won! Unfortunately, the only feasible way of expanding research into the area of on-farm trials involved having a project vehicle.

8.0 RECOMMENDATIONS

Research

It is important that this project continue to be as fully integrated as possible into the ongoing program at CNPAF at the termination of the formal support of the Bean/Cowpea CRSP. We believe that the concept of breeding for increased N_2 fixation has been institutionalized into CNPAF, and that with continuing support from EMBRAPA, substantial improvement in beans for ability to fix atmospheric nitrogen will be realized. Continuing support should be in the form of:

1) A staff scientist trained in agronomy/physiology to work with the plant breeder and rhizobiologist as a research team at CNPAF focused on improving N_2 fixation in beans for Brazil.
2) The technical support staff previously supported by Bean/Cowpea funds should be added to the permanent staff of CNPAF to support the work on N_2 fixation in beans.
3) Staff support is needed for the further development of packages of technology to farmers that will allow the potential of biological N_2 fixation in beans to be fully utilized in appropriate production systems for small farms able to utilize limited purchased inputs.

Delivery of Technology

Most of the research information components needed to improve N_2 fixation in beans in Brazil have been identified. The variability among lines for fixation ability is evident, the

methods of breeding for improved fixation have been developed, efficient rhizobial strains are available, and timing of inoculant addition and supplemental N fertilization have been determined. Now it is essential that these components be assembled in a package for delivery to the farmers' fields.

We recommend that CNPAF seek additional support for a program to deliver this technology. This program should emphasize the application rather than the need to gather more data. The ongoing program at CNPAF and State research groups should back-up the delivery program. This program should be very practical and emphasize on-farm testing. Evaluation trials of new technology should be planned to allow comparisons in farmers' fields and on experiment stations, and the use of levels of N fertilizer application that are unrealistic for the low-input farmer should be avoided.

One possible source of funding for a new delivery program might be the UNDP of FAO of the United Nations.

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