ANNUAL REPORT

1985

RESOURCES SUPPORT SERVICES AGREEMENT
BETWEEN
U.S. AGENCY FOR INTERNATIONAL DEVELOPMENT
AND
U.S. DEPARTMENT OF AGRICULTURE
AGRICULTURAL RESEARCH SERVICE

RSSA : BST-4109-R-AG-3164

"WORLD RHIZOBIUM STUDY AND COLLECTION CENTER"

Nitrogen Fixation & Soybean Genetics Laboratory
Beltsville Agricultural Research Center
Beltsville, Maryland

ANNUAL PROGRESS REPORT
1985
AID-USDA RSSA
WORLD RHIZOBIUM STUDY
& COLLECTION CENTER

WORLD RHIZOBIUM STUDY & COLLECTION CENTER
ANNUAL REPORT
1985

INTRODUCTION

In 1976, the U.S. Agency for International Development (AID) contracted with the U.S. Department of Agriculture to establish the "World Rhizobium Study and Collection Center" within the Agricultural Research Service at Beltsville, Maryland. The purpose of the center is to provide a safe and perpetual depository and collection center for Rhizobium and Bradyrhizobium cultures, which are available to users worldwide, and to provide research and training opportunities in the area of legume-Rhizobium technology to scientists from developing countries. The overall goal of this project is to increase legume productivity through enhanced nitrogen fixation.

Since 1912 the Agricultural Research Service has maintained a collection of Rhizobium, and, despite limited funds, has continued to distribute this germplasm worldwide. The establishment of this project with supplemental funds from AID represents an investment on behalf of lesser developed countries. Benefits to them include increased collection, evaluation and distribution of rhizobial germplasm for legumes commonly grown in less developed countries, and opportunities for their scientists to work with the excellent rersearch staff in the Nitrogen Fixation and Soybean Genetics Laboratory. The combination of an existing Rhizobium collection (the USDA Rhizobium Culture Collection), experienced personnel and international connections makes the ARS, its facilities and programs, the best qualified instsitution for establishing and maintaining a truly international center for the collection and study of rhizobia. The center is housed within the Nitrogen Fixation & Soybean Genetics Laboratory, where a multi-disciplinary staff of research scientists working on nitrogen fixation is available for collaborative efforts.

The World Rhizobium Study and Collection Center is linked to numerous programs and institutions worldwide which promote utilization of symbiotic nitrogen fixation including NifTAL, INTSOY, the UNESCO/UNEP/ICRO MIRCENs, the International Research Centers sponsored by CGIAR, Limiting Factors in BNF (AID/CSRS).

SCOPE OF WORK

The specific objectives of the project are to:

1. Collect isolates of Rhizobium which fix nitrogen efficiently in association with legumes.
2. Collect other organisms which fix nitrogen when associated with other plants.
3. Characterize the performance of newly acquired microbial germplasm with reference to specificity of host, cultivar-Rhizobium interctions, nitrogen fixing efficiency and environmental variables.
4. Enumerate and cataloge germplasm accessions for distribution to researchers worldwide.
5. Serve as a depository of microbial germplasm.

6. Distribute upon request stock cultures for research and commercial inoculum production.

7. Train researchers and technicians from developing countries in techniques and subject matter for applied research and technology of Rhizobium.

8. Consult with and provide technical guidance to researchers in the area of symbiotic nitrogen fixation in developing countries.

9. Modify exisiting methods of preservation of rhizobia to determine the best inexpensive method which can be used in developing countries.

10. Study inoculation methods in relation to persistence and competition among strains of *Rhizobium*.

PROJECT PERSONNEL

Deane F. Weber, Ph.D.	Microbiologist, Principal Investigator
Harold H. Keyser, Ph.D.	Microbiologist, Curator
Richard F. Griffin, B.S.	Biological Laboratory Technician, Collection Manager
Ed Zebovitch	Biological Aid (half time)
Martin Reinhart	Biological Aid (half time)

* * * * * 1985 ACTIVITIES AND ACCOMPLISHMENTS * * * * *

GERMPLASM

In 1985 we distributed 852 cultures from 111 requests for germplasm of *Rhizobium*. Requests from foreign countries accounted for 41 % of the total. Cultures were sent to the following countries:

Ethiopia	Iraq
Tunisia	Canada
Italy	France
Senegal	India
Australia	Morocco
Turkey	Switzerland
United Kingdom	Egypt
West Germany	South Korea
Brazil	Mexico
Spain	Argentina
Romania	Japan

A summary of the 1985 requests by *Rhizobium* species follows:

Bradyrhizobium japonicum	371 cultures
Bradyrhizobium sp.	170 cultures
Rhizobium meliloti	31 cultures
Rhizobium fredii	139 cultures
Rhizobium leguminosarum	
bv *phaseoli*	36 cultures
bv *trifolii*	54 cultures
bv *viceae*	45 cultures
Rhizobium loti	6 cultures

Germplasm Deposited

In 1985 the Nitragin Company of Milwaukee, Wisconsin (a leading rhizobial inoculant manufacturer), deposited approximately 1,110 strains of rhizobia in our culture collection. The company decided not to continue maintenance of this large research collection that had been asembled by the former director of research, Dr. Joe Burton. This collection contained strains isolated from a wide range of legume hosts, and added 84 legume species to our list of hosts represented in our collection. This germplasm would be very difficult to replace, as it was accumulated from isolations made throughout the world, including from uncultivated legumes. These isolates were received on agar slants in cotton-plugged tubes. Several months were spent transferring the cultures to fresh slants, checking for contamination, and freezing cultures in glycerin at -80°C for permanent preservation. This will allow us to characterize these cultures at our leisure in the future. Also, staff members H. H. Keyser and R. F. Griffin visited the Nitragin Co. for 3 days in June, 1985, to copy strain records of those cultures deposited in the USDA *Rhizobium* Culture Collection.

I would like to add that Mr. Griffin has done an extraordinary job on the above strains under difficult conditions.

TRAINING

Miss Beatrice Anyango, from the University of Nairobi, Microbiological Resource Center, Nairobi, Kenya, served as a trainee in our group from April through August, 1985. A program tailored to her specific needs included techniques for *Rhizobium* strain identification (serological and antibiotic techniques), evaluation of symbiotic effectiveness of strains for *Phaseolus vulgaris*, *Albizzia* and *Acacia* species, and design of a program for evaluation of survival and competitiveness among *Rhizobium phaseoli* in Kenya. Also, she visited Kalo Inc. inoculant manufacturer in Columbus, Ohio, and the Boyce Thompson Institute, Ithaca, New York, as part of her training program. A brief report on her program is attached.

ASSISTANCE AND COLLABORATION

Assistance and collaboration was provided to other programs, both national and international, dealing with the use and promotion of biological nitrogen fixation:

Dr. Keyser was invited to participate in the First U.S.-Spain Program Development Workshop on Nitrogen Fixation, Granada, Spain, May 27-29. He presented the issue paper "Agronomic Implications of Competition Among Rhizobia", and met with researchers F. Temprano Vera and Rafael Orive of Sevilla about their inoculant production activities, and future collaboration with the group at Beltsville.

Dr. Weber and Dr. Keyser reviewed grant proposals for the Board on Science and Technology for International Development's program "Grants for Research on Biological Nitrogen Fixation".

Dr. Keyser served on the Soil Chemistry/Agronomy panel of AID's Program in Science and Technology Cooperation, reviewing grant proposals for the program.

Dr. Weber served on the Limiting Factors Panel of AID's Program in Science and Technology Cooperation, reviewing and prioritizing proposals for the program.

RESEARCH ACCOMPLISHMENTS

The Staff of the World *Rhizobium* Study and Collection Center conduct research with other scientists of the Nitrogen Fixation & Soybean Genetics Laboratory, using the collection as an exploitable base for research on important issues. Highlights of the research in 1985 follow:

Soybean rhizobia were tested for their ability to grow anaerobically in the presence of NO_3^- and for differences in final product formation from anaerobic NO_3^- metabolism. Anaerobic growth in the presence of NO_3^- was observed in 270 of 321 strains. None of the strains in serogroup 135 grew anaerobically in the presence of NO_3^-. Serogroups 31, 46, 76, and 94 Predominately liberated NO_2^- as the final product of denitrification, whereas the evolution of N_2 was prevelant in serogroups 110 and 122.

The relationship between ureide N and N_2 fixation was evaluated in greenhouse-grown soybean and lima bean, and in field studies with soybean. In the greenhouse, plant N accumulation from N_2 fixation in soybean and lima bean correlated with ureide N. Ureide N determinations in field studies with soybean correlated with N_2 fixation, aboveground N accumulation, nodule weight, and acetylene reduction. Ureide-N measurements may be useful to rank strains of *B. japonicum* for effectiveness of N_2 fixation.

Soybean genotypes have been identified which restrict nodulation by strain USDA 123, which is representative of indigenous bradyrhizobia in the midwest U.S. This restricted nodulation of the otherwise competitive strain USDA 123 permits an inoculant strain to form the majority of the nodules on these unique soybeans. The goal of this research is to breed this symbiotic characteristic into production cultivars, such as Williams.

A genotype of wild soybean, *Glycine soja*, was previously identified which is preferentially effective at nitrogen fixation with the fast -growing *Rhizobium fredii* as compared to the slow-growing *Bradyrhizobium japoncium*. In further tests, we have found that the *R. fredii* strain USDA 193 is also very competitive at forming nodules on this wild soybean when competing against USDA 123, while just the opposite outcome is found on the soybean cultivar Williams. Currently we are evaluating crosses of these two genotypes in an effort to develop a productive soybean that is effective and competitive with the new *R. fredii* strains.

ABSTRACTS AND PUBLICATIONS

Abstracts and publications by the center's staff in 1985 follow:

Keyser, H. H. and P. B. Cregan. 1985. Diversity of nodulation by serogroup 123 isolated in cultivated and exotic soybeans. Proceedings 10th N. Am. Rhizobium Conf. p. 41.

Keyser, H. H. and P. B. Cregan. 1985. Host influence on nodulation ability within Bradyrhizobium japonicum serogroup 123. Am. Soc. Agron., Abstr. p. 158.

Cregan, P. B. and H. H. Keyser. 1985. Host determined restricted nodulation of strain USDA 123. Proceedings 10th N. Am. Rhizobium Conf. p. 35.

Cregan, P. B. and H. H. Keyser. 1985. Use of the soybean host genotype to alter nodule occupancy by specific strains of rhizobia. Am. Soc. Agron. Abstr. p. 51.

van Berkum, P., and H.H. Keyser. 1985. Anaerobic growth and denitrification among different serogroups of soybean rhizobia. Appl. Environ. Microbiol. 49:772-777.

van Berkum, P., C. Sloger, D.F. Weber, P.B. Cregan and H.H. Keyser. 1985. Relationship between ureide N and N_2 fixation, aboveground N accumulation, acetylene reduction, and nodule mass in greenhouse and field studies with Glycine max L. (Merr). Plant Physiol. 77:53-58.

Keyser, H.H., M.J. Sadowsky and B.B. Bohlool. 1985. Fast-growing soybean rhizobia, pp.926-934. In R. Shibles (ed) World Soybean Research Conference III: Proceedings. Westview Press, Inc., Boulder, CO.

Keyser, H.H. 1985. Promising developments in rhizobia inoculant research, pp. 53-58. In Report of Fourteenth Soybean Seed Research Conference, American Seed Trade Association. ASTA, Washington, D.C.

Keyser, H.H. 1985. Agronomic implications of competition among rhizobia, pp. 33-34. In T. Ruiz-Argueso and D.A. Phillips (ed) Report on the First U.S.-Spain Program Development Workshop on Nitrogen Fixation. U.S.-Spain Joint Committee for Scientific and Technological Cooperation, Madrid, Spain.

van Berkum, P., H.H. Keyser and D.F. Weber. 1985. Examination of Hup expression by soybean bradyrhizobia belonging to different serogroup phenotypes. In H.J. Evans, P.J. Bottomley and W.E. Newton (eds) Proceedings of the 6th International Symposium on Nitrogen Fixation, Corvallis, OR. Martinus Nijhoff Publishers, Dordrecht.

Weber, D.F. 1985. Biological nitrogen fixation in agriculture. In R. Stevenson (ed) Proceedings of the Fifth International Culture Collection Congress, Bangkok, Thailand.

Weber, D.F. and H.H. Keyser. 1985. Rhizobium Microbiological Resource Centers. In R. Stevenson (ed) Proceedings of the Fifth International Culture Collection Congress, Bangkok, Thailand.

Kluson, R.A., W.J. Kenworthy and D.F. Weber. 1985. Soil temperature effects on competitiveness and growth of Rhizobium japonicum and on Rhizobium-induced chlorosis on soybeans. Plant and Soil. 95:201-207.

APPENDIX

Attached are reports, abstracts and publications from 1985.

UNIVERSITY OF NAIROBI

COLLEGE OF AGRICULTURE & VETERINARY SCIENCES
DEPARTMENT OF SOIL SCIENCE
(KABETE CAMPUS)

Telegrams: "VARSITY" Nairobi
Telephone: 592211

P.O. Box 30197
NAIROBI
KENYA

Ref:

9th September, 1985

Officer-In-Charge
USDA/OICD Training Programs
Auditors Building
Washington, D.C 20250
U S A

Dear Sir,

Please convey my sincere gratitude to USAID for sponsoring my short training course at the USDA - Nitrogen fixation and soy bean genetics laboratory in Beltsville and the Global Impacts of Applied Microbiology VII (GIAM VII) Conference in Helsinki, Finland. The report attached gives a brief summary of the achievements of the course. The course was well organized and it was possible to meet most of our objectives. I found most of the people from the USDA and OICD very helpful and nice to work with.

The GIAM VII was very interesting, it exposed me to new findings in the field of Biological Nitrogen Fixation in legumes and the role of Mycorrhizae in Nitrogen fixation in legumes and in non-legumes like Casuarina spp. It was also a place for meeting other scientists for exchange of ideas and opening new avenues for future collaboration. It is my sincere hope that I will continue to get support from USAID either directly or indirectly through other bodies like US National Academy of Sciences which is currently supporting a bean project in our laboratory.

Sincerely,

Anyango

Beatrice Anyango

BRIEF REPORT

ON

SHORT TRAINING COURSE

AT

THE SOYBEAN GENETICS AND NITROGEN FIXATION LABORATORY

UNITED STATES DEPARTMENT OF AGRICULTURE

AGRICULTURAL RESEARCH SERVICE

BELTSVILLE, MARYLAND

APRIL 11 - AUGUST 9, 1985

BY

BEATRICE ANYANGO

Introduction

The course was initiated by Professor S. O. Keya, University of Nairobi, MIRCEN project, Nairobi, Kenya and Dr. H. H. Keyser of Nitrogen Fixation and Soybean Genetics Laboratory, USDA, Beltsville, Maryland, USA. The course was a fulfillment of one of the aims of a US AID funded programme in the nitrogen fixation laboratory to have trainees and Scientists from developing countries visit the laboratory and acquire some techniques for the study of *Rhizobium* ecology.

The following techniques were acquired; agglutination, fluorescent antibody and antibiotic resistance markers. Enzyme linked immunosorbent assay (ELISA) and bacteriopha ge could not be covered due to shortage of time. Apart from the laboratory techniquesthere were two *Rhizobium* strain effectiveness experiments with common beans, Acacia spp and Albizia spp. A week's field trip was organized to enable visits to an inoculant production firm and a plant research institute.

Serological techniques

In order to study the persistence of Rhizobium in the soil in the presence of other microorganisms including rhizobia, the strains must be distinguishable. The availability of serological techniques for strain identification permits the success and persistance of inoculant strains to be determined with considerable precision provided that the appropriate controls are used.

Serology has been used extensively to identify different strains of *Rhizobium* and to measure the survival of inoculant strains, competition among

inoculant strains and the nature of the indigenous soil rhizobia. Several serological techniques have been developed, these are, agglutination, immunoflorescence. immunodiffusion and enzyme-linked immunosorbent assay (ELISA), all of which are based on the genetic uniqueness of microorganisms.

The immunofluorescence (IF) or fluorescent antibody (FA) techniques makes use of fluorescent markers which conjugate to antibody proteins and enable visualization of antibodies as they participate in antigen-antibody reactions. Agglutination is a simpler method which like the FA technique is based on the reaction of specific somatic antigens. The preparation of FA from antiserum and microscopic examination was covered. Crushed nodule suspension test with specific FAs was also done.

Antibiotic Resistance Markers

The use of serological techniques for strain differentation is reliable only if the strains do not cross react to an extent that it cannot be removed by heterologous adsorption. However, other methods of differentation can be used. Antibiotic resistance markers provide one possible alternative to serological markers. Many bacteria are intrinsically resistant to low levels of various antibiotics. The range and concentrations of antibiotics to which these bacteria are resistant, however, vary considerably even among strains. This unique pattern of intrinsic antibiotic resistance can be applied as a genetic fingerprint of an organism and used to recognize it.

Bacteria can also readily develop resistance to fairly high levels of antibiotics through sponteneous or induced mutation. If such mutations are stable then they can be used as markers for recognition and isolation of that strain from a mixed population. The methods involved in strain screening for antibiotic resistance were acquired. This was done on some 30 *Rhizobium* strains

from the Nairobi Rhizobium MIRCEN. The host plants for the strains were common beans, Soybeans, cow peas, pigeon pea, Lucerne, Desmodium, Leuceana leucocephala, Sesbania sesban, Albizia falcataria, Dalbergia melanoxylon and Acacia albida.

Strains of common beans and Acacia albida were subjected to a second marker. Some double marked strains were used for effectiveness tests with the respective hosts on the 26.7.85. The plants will be harvested for nodules after six weeks, this test is being carried out in a growth chamber.

Experiments

Apart from the laboratory techniques, there were two experiments carried out with beans and tree legumes. The bean experiment involved the screening of 23 Rhizobium phaseoli L.(6 Nairobi MIRCEN and 18 USDA strains) with Phaseolus vulgaris cv pinto. The plants have been harvested and the raw data indicate that four out of five strains from the Nairobi collection caused good nodulation but were poor at nitrogen fixation while the fifth strain showed good nodulation and intermediate nitrogen fixation. Three of the USDA strains were very effective in nitrogen fixation, but had few big nodules which were scattered along the root length but not necessarily at the crown region. Eight strains showed intermediate fixation rates, while four were very poor, at nitrogen fixation, and three of the strains hardly nodulated the plants at all.

The second experiment was carried out with tree legumes namely Acacia Albida and three species of Albizia. The response of Acacia Albida to inoculation with nine Rhizobium strains, four from Kenya and five from USDA, was tested. A similar test was carried out with Albizia julibrissin, A.falcataria and A.lebbek using five Rhizobium strains, two from Kenya and three from USDA. Due to the

germination failure in some of the seeds, only one seedling of Albizia lebbek and eighteen seedlings of A. falcataria were transplanted together with A. julibrissin in a "Rhizobium free soil" at Marlboro, 15 miles from Beltsville. The tree seedlings were transplanted after seven weeks and they will be harvested at the end of September for laboratory analysis.

There was one week's field trip organized for visits to a firm and a research institute like Kalo Inc., an inoculant production firm at Columbus, Ohio. Kalo Inc. is one of the three largest inoculant production firms in the United States. The processes involved in commercial inoculant production were explained and demonstrated where possible. Boyce Thomson Institute for plant research at Cornell University, Ithaca, New York, was the next and the last institute visited after Kalo Inc. I found the visits at both places quite interesting and educational.

On the whole the programme was well coordinated and I found the people very helpful and nice to work with.

I wish to thank Prof. S. O. Keya for initiating the training and Dr. H. H. Keyser for his cooperation in organizing the training programme and for devoting his precious time in training me. The financial support from US AID is gratefully acknowledged. Thanks to the Mycology Laboratory Chief, Dr. E. Rossman, Dr. McKnight and J. Plaskowitz for their cooperation in assisting me to examine Rhizobium melilotii in filter mud based legume inoculant from Kenya using scanning electron microscope. Special thanks to all staff members of the Nitrogen Fixation and Soybean Genetics Laboratory for their direct or indirect contribution in making my stay in the USA comfortable.

Agronomic Implications of Competition Among Rhizobia

Prepared for the Spain-U.S. Program
Development Workshop on Nitrogen Fixation

Harold H. Keyser
USDA Agricultural Research Service
Nitrogen Fixation & Soybean Genetics Lab.
Beltsville, Maryland USDA 20705

A major effort in the field of nitrogen fixation research has been the selection and application of the most effective strains of rhizobia. Recent efforts include the genetic development of strains for superior effectiveness and efficiency. However, the establishment of superior strains in a significant portion of the nodules formed by a field-grown legume has been, and remains, a critical problem. Faced with competition from indigenous rhizobia, even strains conditioning the most highly effective N_2-fixation are destined to form few nodules and therefore have a minimal effect upon N_2 fixation.

In the alfalfa-R. meliloti system, Weber and Leggett (1966) demonstrated that 'alfalfa sickness' in Washington and Idaho was related to the presence of completely ineffective, indigenous rhizobia. In this case the application of high numbers of recommended strains solved the problem. In California, a similar problem in the subclover-R. trifolii system was overcome only after the selection of effective strains for competitiveness and persistence (Jones, Burton and Vaughn, 1978). Such success in overcoming indigenous competiton is indeed difficult to find in the literature. This is certainly the case with soybeans in the U.S. (Ham, 1976; 1980).

At Beltsville, our research effort on competition in the soybean R.-japonicum system involves the collaboration of soil microbiology and soybean genetics. In 1966, Caldwell et al. defined the R_{j2} gene in the cultivar Hardee as a single, dominant gene which restricts nodulation by R. japonicum serogroups 6 and 122. Further, the gene r_{j1} is responsible for nonodulation in soybean, while genes R_{j3} and R_{j4} restrict only a single strain each. Given the precedent of such host controlled nodulation ability in soybean, we (P. B. Cregan and myself) undertook a program to determine if genes could be found which restrict nodulation by some of the major indigenous R. japonicum populations in the U.S.

In the major soybean production area of the midwestern U.S., R. japonicum serogroup 123 is the dominant competitor for nodule sites. Numerous studies have confirmed the tenacious competitive ability of serogroup 123 and the inability to displace this serogroup with an inoculant strain in an economically feasible manner. Using the serogroup's type strain, USDA 123, we screened over 1300 soybean plant introductions and identified 10 genotypes which do not nodulate, or nodulate very sparsely. These genotypes do nodulate and fix N_2 with other highly effective strains. Competition studies in soil show that these genotypes restrict occupancy by USDA 123 in favor of the superior inoculant strain. Studies with soils from the midwest U.S., containing a diverse serogroup 123 population, show that some isolates of this serogroup do nodulate these genotypes, and further screening is underway. These genotypes have been crossed with commercial soybean genotypes to determine the inheritance of this trait, and to develop near isogenic lines for subsequent field testing.

Competition among rhizobia can have many agronomic implications. In the previously mentioned cases of alfalfa and subclover, overcoming competition meant the difference between the success and failure of the crop. In the case of soybean, where the indigenous strains are rarely completely ineffective, overcoming competition could lead to enhanced N_2-fixation and yield through the displacement of a mediocre strain by a superior one. Certainly more research is needed in the area of competition, lest it remain a bottleneck in applying the research products of many Rhizobium workers and realizing the benefits from such.

References

Caldwell, B. E., K. Hinson, and H. W. Johnson. 1966. Crop Sic. 6:495-496.

Ham, G. E. 1976. p 144-150. In L. O. Hill (ed.) World Soybean Research. Interstate Printers and Publishers, Danville, Ill.

Ham, G. E. 1980. p 131-138. In W. E. Newton and W. H. Orme - Johnson (eds.). Nitrogen Fixation, Vol II. University Park Press, Baltimore.

Jones, M. B., J. C. Burton, and C. E. Vaughn. 1978. Agronomy Journal 70:1081-1085.

Weber, D. F., and G. E. Leggett. 1966. ARS 41-117, Agricultural Research Service, USDA.

Diversity of Nodulation by Serogroup 123 Isolates in Cultivated and Exotic Soybeans.

H. H. Keyser* and P. B. Cregan, USDA, ARS, Beltsville, Maryland.

Genotypes of exotic soybeans have been identified which restrict nodulation by Bradyrhizobium japonicum strain USDA 123. Tests were undertaken to examine the diversity among serogroup 123 isolates for restricted nodulation with these genotypes. In the first test, 20 isolates from the U.S. and China were compared for nodule mass production in soil on two USDA 123-restricting genotypes and on cv. 'Williams'. On the restricting genotypes, five of the isolates gave reduced nodulation (less than one-third of the mass produced by a mixture of effective strains) comparable to USDA 123, six isolates gave an intermediate response, and the ramainder produced normal nodulation. All 20 isolates produced normal nodulation with Williams. In a second study, six soils from five midwestern states were tested in the greenhouse for comparative nodule occupancy by indigenous 123 isolates on the two USDA 123-restricting genotypes and on cvs. 'Chippewa 64' and Williams. In five of six soils, nodule occupancy by serogroup 123 with the restricting genotypes was 50% or less than that found with Chippewa 64 and Williams. In a soil from Ohio, little difference in nodule occupancy was apparent between the USDA 123-restricting genotypes and the cultivars. These results demonstrate the diversity among the serogroup 123 population in ability to nodulate two USDA 123-restricting soybeans, and indicate the need to further screen exotic genotypes with 123 isolates to find additional gene(s) which can be combined to restrict most or all of the serogroup.

O

Rhizobitoxine Production and Soybean Nodulation by Colony-type Derivatives of Bradyrhizobium japonicum USDA 76.

J. S. LA FAVRE*, A. K. LA FAVRE and A. R. J. EAGLESHAM, Boyce Thompson Inst. at Cornell Univ.

Previous studies have suggested that the ability to nodulate "non-nodulating" (rj_1 rj_1) soybeans [Glycine max (L.) Merr.] is restricted to Bradyrhizobium japonicum strains able to synthesize the phytotoxin rhizobitoxine. In research reported here, colony-type derivatives differing in the ability to produce rhizobitoxine were isolated from B. japonicum USDA 76. Rhizobitoxine was detected in the culture supernatant of a small colony-type derivative (I4) but not in the supernatant of a large colony type (L4). Rhizobitoxine was not detected in cell extracts of L4 produced by sonication, autoclaving or French press treatments. Utilizing the Indian ink method with phase microscopy, cells from agar cultures of each derivative were examined for extracellular polysaccharide (EPS). Large amounts of EPS were found surrounding cells of L4 but none was observed around cells of I4. Several small and large colony-type derivatives were tested for nodulation ability on nodulating (Rj_1 Rj_1) Harosoy and Lee and rj_1 rj_1 Harosoy soybeans. Both sets of colony types were effective on Rj_1 Rj_1 soybeans and equally poorly infective (8-31 nodules plant^{-1} at 37 days) on rj_1 rj_1 soybeans. Nodule isolates typed true to the inoculum colony type. These results failed to support a role for rhizobitoxine in nodulation of non-nodulating soybeans by B. japonicum.

Host Determined Restricted Nodulation of Strain USDA 123.

P. B. Cregan* and H. H. Keyser, USDA, ARS, Beltsville, Maryland.

In the midwestern U.S., strains of serogroup USDA 123 often form 65% or more of the nodules on soybeans [Glycine max (L.) Merr.] in production fields. Strains of this serogroup are very competitive but relatively inefficient in the fixation of N_2 when compared to highly efficient soybean rhizobia already available or those forthcoming from genetic engineering. One approach to altering nodule occupancy in favor of a highly efficient introduced strain is the development of a host plant which restricts nodulation by strains of serogroup 123. We initiated an effort to isolate G. max genotypes which restricted nodulation by strain USDA 123 and identified 12 such accessions in the USDA germplasm collection. Further tests with two of these genotypes demonstrated their ability to effectively fix N_2 with Bradyrhizobium japonicum strains USDA 6, 110, and 122. These two genotypes and the cv. 'Williams' were grown in B. japonicum-free soil from Marlboro, MD in which USDA 123 was homogeneously mixed to a level of 10^6 cells/g soil. At planting seeds were inoculated with 10^9 cells of either USDA 110, 122, or 138. In the 123/110 treatment, USDA 123 occupied 79% of the nodules of Williams versus 7% of the nodules of the test genotypes. Similar frequencies were obtained in the 123/122 and 123/138 treatments. These results suggest that the host may provide an important vehicle by which to alter nodule occupancy despite the presence of highly competitive indigenous rhizobia.

O

Adsorption and Movement of Bradyrhizobium japonicum in Soils.

H. YANG and E. P. DUNIGAN*, Louisiana State Univ.

Adsorption and movement of Bradyrhizobium japonicum in soils was studied under laboratory conditions. B. japonicum moved vertically with the addition of water to columns of sand or silt loam soil. They were uniformly distributed through the 40 cm depth in a sand but movement decreased with depth in the silt loam. Movement decreased as clay content increased from 0 to 12%. Lateral movement also followed the water flow pattern with more cell movement in a sand than in a silt loam. Montmorillonitic clay adsorbed more cells than did kaolin. When the two clays were saturated with either Na^+, K^+, NH_4^+, Ca^{++}, Mg^{++}, Fe^{+++} or Al^{+++}, the order of increasing adsorption was monovalent cations < divalent < trivalent with the exception of Al^{+++}. Humic acid, corn starch, rice straw and calcium phytate exhibited low adsorptive capacities for the B. japonicum compared to the clays.

Nitrogen Fixation Reserach Progress. 1985. H.J. Evans, P.J. Bottomley and W.E. Newton (eds). Proceedings of the 6th international symposium on Nitrogen Fixation, Corvallis, OR. Martinus Nijhoff Publishers, Dordrecht.

EXAMINATION OF HUP EXPRESSION BY SOYBEAN BRADYRHIZOBIA BELONGING TO DIFFERENT SEROGROUP PHENOTYPES

Peter van Berkum, Harold H. Keyser, Deane F. Weber
USDA-ARS, BARC-W, Beltsville, MD 20705, U.S.A.

1. INTRODUCTION

Eisbrenner,Evans (1983) reported that 25% or less soybean bradyrhizobia oxidize H_2. Hup appears to be predominantly associated with strains of the 110 and 122 serogroups. Keyser et al. (1984) reported a low frequency of Hup^+ phenotypes among other serogroups. However, the Hup characterization used free-living cultures under conditions for expression of ex planta nitrogenase activity and tritium exchange. Emerich et al. (1980) reported no measurable isotope exchange with bradyrhizobial Hup, and Robson, Postgate (1980) suggested the presence of nitrogenase activity to cause an apparent exchange reaction. Therefore, we have used amperometry with bacteria and bacteroids to evaluate selected isolates for Hup.

2. PROCEDURE

The origin maintainance and purity checks of cultures, preparation of inoculum, plant culture, and C_2H_2 reduction were as described by van Berkum, Keyser (1985). H_2 was determined by gas chromatography and amperometry (Hanus et al. 1980). Chemoautotrophic growth was determined in liquid medium (Hanus et al. 1979). Cell suspensions in buffer of heterotrophically grown bacteria were subjected to Hup measurement according to Maier (1981).

3. RESULTS

Fifty two isolates of eight serogroups were selected according to their reported Hup phenotype. All were effective, evolving H2 except for the Hup+ 110 and 122 serogroups. Eleven strains (21%) were Hup^+ as bacteroids and bacteria, although 33 (63%) should have been Hup^+. Our analysis identified wild-type Hup^+ 6,31 and 123 serogroup phenotypes, and wild-type Hup^- 110 and 122 serogroup phenotypes. Chemoautotrophic growth was observed by the Hup^+ 6 and 31 isolates, but not by the Hup^+ 123 strains.

4. CONCLUSIONS

Our results show that most of the Hup^+ survey isolates did not oxidize H_2 as bacteroids or as cell suspensions in buffer. The disparity in the results between the two studies may indicate a lower incidence of strains in soils across the USA capable of forming Hup^+ symbioses.

5. REFERENCES

Eisbrenner G and Evans HJ (1983) Ann Rev Plant Physiol 34,105-136
Emerich DW et al. (1980) Plant Physiol 66,1061-1066
Hanus FJ et al. (1979) Proc Natl Acad Sci USDA 76,1788-1792
Hanus FJ et al. (1980. Methods Enzymol 69,731-739
Keyser HH et al. (1984) Appl Environ Microbiol 47,613-615
Maier RJ (1981) J Bacteriol 145,533-540
Robson RL and Postgate JR (1980) Ann Rev Microbiol 34,183-207
van Berkum P and Keyser HH (1985) Appl Environ Microbiol 49,772-777

6. ACKNOWLEDGEMENTS

We wish to express our gratitude to Dr R Hauck for the gift of an amperometric chamber, and to Dr R Maier and Mr F Moshiri for useful discussions concerning Hup measurement.

(Nicotiana tabacum L.) population (Co). One hundred random maternal dihaploid (MDH), S_1 and full-sib (FS) families were produced and evaluated for 9 parameters in one environment. The top 10% of the families for 2 parameters were identified and intercrossed to produce C_1 populations for each procedure and parameter. Based upon an additive genetic variance model, theoretical gain from MDH selection was expected to be 4 X and 2 X that of FS and S_1 selection, respectively. Genetic variance estimates for yield lead to predicted gains of 5.7% for FS, 8.1% for S_1 and 15.1% for MDH family selection. Predicted gains for increased total alkaloids were 6.5%, 12.7% and 21.1% for the same family selection procedures.

Testcross Selection Theory. N.M. Cowen, University of Missouri.
If a population initially in random mating equilibrium is inbred by self pollination until, the inbreeding coefficient, F=F', followed by crossing individuals to an inbred tester, then the genetic components of variance expressed among and within testcross families are linear functions of the additive genetic variance in the population, the covariance of additive effects and testcross dominance deviations, and the variance of testcross dominance deviations. The population response to testcross selection is also a function of these three variances or quadratic components. When a population is used as a tester the variance among testcross families is a linear function of the population additive genetic variance, the covariance of additive effects and mean testcross dominance deviations and the variance of the mean testcross domiance deviation. The variance within testcross families is a linear function of 6 variances or quadratic components. The population response to testcross selection with a population tester is a function of the 3 components expressed among testcross families. When the population under improvement is also used as the tester, the variances among and within testcross families reduce to the formulae for half-sib families.

Gamete and Half-gamete Evaluation in the Use of Exotic Germplasm. T. S. COX* and P. BRAMEL-COX, USDA-ARS Kansas State University.
A mating design for estimating the additive genetic variance within a population, and other genetic variances involving allelic differences and interactions between the population and an inbred line, is described. Individual plants from the population are crossed to the line and individual F_1 plants are backcrossed and selfed. Individual B_1F_1 plants are also selfed. Genetic variances and covariances involving B_0F_1 and B_1F_1 testcrosses, and F_2-derived lines among and within B_0F_1 and B_1F_1 families are computed. Because testcrosses, lines, and families are related through single gametes or half-gametes derived from the population, the variances and covariances can be formulated in terms of six genetic parameters, σ^2_a, σ_{ad}, σ^2_d, σ^2_A, σ_{AD}, and σ^2_D. The first three parameters occur because of allelic variation within loci in the population. The last three occur because of differences between the inbred line's allele and the mean of the population's alleles. The analysis is useful for detailed study of germplasm populations to be improved through selection or crossed to specific adapted lines.

An Evaluation of the HOPE Corn Breeding System. M. M. CRAMER* and L. W. KANNENBERG, Univ. of Guelph.
HOPE is a hierarchical, open-ended maize breeding system designed to increase genetic diversity in corn by incorporating a wide range of germplasm into breeding populations, and of manipulating this variability to generate useful inbreds for hybrid production. At the end of five seasons of HOPE, two cycles of modified reciprocal recurrent selection at the elite level and five cycles of both modified ear-to-row selection at the high and intermediate levels and mass selection at the low level were completed. During this period, 457 new components were incorporated into the system and improved material was introgressed from lower to higher levels in the hierarchy. A performance index of grain yield/percent grain moisture at harvest was the primary selection criterion. Results from a study to evaluate HOPE indicated significant reductions in percent moisture in seven of the eight populations and in the population cross Elite "A" X Elite "B". Significant increases in yield were observed in four of the populations but not in the population cross. This cross was equal in maturity and yielded 75% of the average of six adapted commercial hybrids used as checks. A study of selection experiments indicated no apparent relationship between genetic variability and cycle of selection.

Use of the Soybean Host Genotype to Alter Nodule Occupancy by Specific Strains of Rhizobium. P. B. CREGAN* and H. H. KEYSER, USDA-ARS, Beltsville, Maryland.
Strains of Bradyrhizobium japonicum serogroup USDA 123 occupy a very high proportion of the nodules of soybeans grown in the northern midwest. Strains of serogroup USDA 123 are reported to be highly competitive for nodule sites but relatively inefficient dinitrogen fixers. The objective of this work was to examine a previously identified Glycine soja genotype (PI 468397) to determine if it would preferentially nodulate with Rhizobium fredii (fast-growing soybean rhizobia) when an R. fredii strain and USDA 123 were both present in the rhizosphere. A sterilized sandy loam soil was pre-inoculated with USDA 123 to a level of 8.1×10^5 cells g^{-1} soil. Seeds of 'Williams' soybean, PI 468397, and four sublines of PI 468397 were planted in the USDA 123 infested soil and inoculated with R. fredii strain USDA 193. A total of 98% of the nodules of Williams soybean were occupied by USDA 123 whereas 86% of the nodules produced by one G. soja subline were occupied by the R. fredii strain. These results suggest that the G. soja subline may be used to exclude strains of serogroup USDA 123 in favor of more efficient strains of soybean rhizobia.

Grainfill of Opaque-2 Maize Strains Selected for Variable R-nj Expression. H. Z. CROSS* and H. DOSSO, North Dakota State Univ.
An experiment was designed to investigate changes in rate and duration of grainfilling associated with low and high R-nj aleurone color expression in an opaque-2 maize (Zea mays L.) synthetic. The xenia effects of one dose of normal pollen on these growth components also were studied. Results pooled over two years indicated that one cycle of selection for high R-nj expression had increased the grainfill rate in an opaque-2 synthetic. Opaque kernels had shorter effective filling periods than normal kernels. In general, the R-nj selection procedure produced changes in the opaque-2 population which were similar to those which were observed in a previous study with a normal dent synthetic.

Estimation of Genetic and Genetic x Environment Variances in Six Tropical Maize Populations. J. CROSSA and J. DEUTSCH*. CIMMYT.
The main objectives of this study are:(1) to summarize grain yield data for six of CIMMYT's tropical maize populations collected over the last 10 years for the various cycles of selection and (2) to obtain estimates of genetic and genetic x environment interaction variance components. Mean yield in the last cycle was higher than those in early ones for most of the populations. This suggests that the overall mean of the progenies has improved throughout the selection process. The F-test for families and families x environment interaction were significant for all populations. Low estimates of genetic variances were found for Antigua-Veracruz in cycle 3 (.09), Cogollero in cycle 4 (.08), Tuxpeño Caribe in cycle 3 and 4 (.08 and .09 respectively) and for Amarillo Dentado in cycles 3 and 6 (.07 and .09 respectively). Generally interaction components are high in early cycles and tend to slow down in later cycles. Antigua-Veracruz and Cogollero had large components in cycles 2, decreasing their magnitudes to .07 and .11 respectively for the last cycle. Tuxpeño Caribe had the highest interaction component in cycle 1 (1.74) and it decreased to .82 and .16 in cycles 2 and 5 respectively.

Reciprocal Differences for Several Quantitative Characters in Mungbeans.
T. B. CUPKA* and L. H. EDWARDS, Oklahoma State University
Reciprocals within a cross are often ignored by many breeding programs. Our objective was to determine if cytoplasmic effects were present in several mungbean crosses. Four crosses were made to the homozygous tester line M-1-77-OT-4. The lines crossed to M-1-77-OT-4 were MG-50-10A(Y), 52-22, 3-1, and 48-14. Significant reciprocal differences were found for days to flower, grain yield, plant weight, vegetative weight, and pods per plant in both individual crosses and the combined F_1 data. The F_1 progenies with M-1-77-OT-4 as the maternal parent consistently flowered later, produced lower grain yields, plant weights, vegetative weights, and fewer pods per plant, than did the F_1 progenies in which the other parental

BEST AVAILABLE COPY

Effect of Carbon Source on Immobilization and Chemical Distribution of Fertilizer N in Soil. K. R. KELLEY,* TVA, F. J. STEVENSON, Univ. of Illinois.
Glucose, a simple carbohydrate, catechol, a diphenol which readily undergoes polymerization reactions to produce humic-type compounds, and a phenolic glycoside, containing carbohydrate and phenolic constituents, were added to soil along with ^{15}N-labeled $(NH_4)_2SO_4$. The soils were sampled over a 120-day period, and the amounts of labeled and unlabeled N mineralized during incubation and contained in several inorganic and organic N fractions were determined. Net immobilization of the added N reached a maximum after 6, 10, and 20 days of incubation with glucose, phenolic glycoside, and catechol, respectively. In contrast to the catechol-amended soil, remineralization of the immobilized ^{15}N occurred more rapidly and to a greater extent in the glucose- and phenolic glycoside-amended soil. Net mineralization of ^{15}N was accompanied by a decrease in amino acid ^{15}N. After 120 days, higher proportions of the immobilized ^{15}N in the glucose- and phenolic glycoside-amended soil were determined as amino acids, NH_3-N, and acid-insoluble N than in the glucose-amended soil. Distinct differences were observed in the distribution of immobilized ^{15}N and native soil N.

Characterization of Antibiotic Resistant Isolates of Bradyrhizobium japonicum. A. C. KENNEDY* and A. G. WOLLUM II, North Carolina State Univ.
Double antibiotic resistant isolates of *Bradyrhizobium japonicum* strains 3I1b123, 3I1b24, NC1005, NC1028, NC1033, and BR587 were studied to determine similarities to the parental population with respect to their growth, temperature characteristics, saprophytic competence, competitiveness, and symbiotic capability. The antibiotics used were spectinomycin (Spe), streptomycin (Str), erythromycin (Ery), and kanamycin (Kan). No differences in survival of the isolates were seen when the isolates were added to a sandy loam soil and incubated at 28, 33, or 37°C. Isolates resistant to Kan were equal to or less competitive than the parent in forming nodules when placed in the rhizosphere with equal quantities of the parent . Isolates resistant to Ery tended to be more competitive than the parent. Shoot dry weight, and weight per nodule were generally lower in plants nodulated with Kan resistant isolates, while plants nodulated with isolates resistant to Spe, Str, or Ery combinations had shoot and nodule dry weight values equal to or greater than the parent.

Host Influence on Nodulation Ability Within Bradyrhizobium japonicum Serogroup 123. H.H. KEYSER* and P.B. CREGAN, USDA-ARS, Beltsville, Maryland.
Genotypes of soybean have been identified which restrict nodulation by *Bradyrhizobium japonicum* strain USDA 123. Strains belonging to serogroup 123 are dominant competitors for nodulation in the midwest soybean production area. Tests were performed in soil to evaluate the diversity among 20 serogroup 123 isolates for nodulation and competitive ability on two of the restrictive genotypes. Screening for nodule mass production revealed three classes; low, medium and high. Selected 123 isolates were mixed with the soil to about 10^5 cells g^{-1} prior to planting the two genotypes and cv. Williams which received USDA 110 peat inoculation. Typing of nodules showed that USDA 110 could out-compete 123 isolates from the low nodulation class (80% of the nodules formed by USDA 110), whereas USDA 110 was not competitive with isolates from the medium and high nodulation classes (less than 20% of the nodules formed by USDA 110). In contrast, all 123 isolates formed at least 94% of the nodules on cv. Williams. Serogroup 123 contains isolates with a diversity of nodulation and competitive abilities on specific soybean genotypes.

Effects of Organic Acids on Seed Germination and Seedling Growth in Soil. M. J. KROGMEIER* and J. M. BREMNER, Iowa State University.
It is commonly assumed that the adverse effect of plant residues on crop yields is largely, or partly, due to phytotoxic compounds leached from, or produced by decomposition of, these residues, and it has been suggested that the phytotoxic compounds responsible for reduced crop yields are organic acids such as acetic acid, butyric acid, and propionic acid. To test the validity of this hypothesis, we studied the effect of different amounts of acetic acid, butyric acid, and propionic acid on seed germination and seedling growth of corn (*Zea mays* L.), barley (*Hordeum vulgare* L.), and wheat (*Triticum aestivum* L.) in soil. The data obtained showed that, although the organic acids tested had an adverse effect on seed germination and seedling growth in soil when the amounts applied were substantially greater than the amounts detected in soil, they had no effect on seed germination or seedling growth when the amounts applied were similar to the amounts detected in soil.

Microbial Populations Involved in the Oxidation of Elemental Sulfur in Saskatchewan Agricultural Soils. J.R. LAWRENCE* and J.J. GERMIDA, Univ. of Saskatchewan.
Sulfur deficient soils in western Canada require fertilization, but the use elemental sulfur(S°) fertilizers is limited by the low rate of microbial oxidation to plant available SO_4. This study enumerated the populations of heterotrophic and autotrophic microorganisms which oxidized S° in agricultural soils and assessed the physical, chemical and biological factors affecting oxidation. A survey of 35 soils detected substantial populations of heterotrophic microorganisms which oxidized S°. These microorganisms produced either S_2O_3 or SO_4 as the predominant end product of S° oxidation. No autotrophic S° oxidizing thiobacilli were detected, however nine soils contained detectable populations of autotrophic S_2O_3 oxidizing microorganisms. The oxidation of S° in soil was significantly correlated with microbial biomass carbon ($r = 0.61$, $P < 0.01$) and respiratory activity ($r = 0.88$, $P < 0.01$), but not with any of the physical or chemical parameters measured.

Determination of an Average Structural Model of the Suwannee Standard Fulvic Acid by Convergent Independent Analyses. J. A. LEENHEER, U.S. Geological Survey, Denver, Colorado.
An attempt was made to perform organic structural analyses on the complex mixture of compounds that comprise the Suwannee standard fulvic acid of the International Humic Substances Society. If all independent data of various structural moieties converged, a hypothetical structural model could be written that would represent the average structures and properties of the fulvic-acid mixture. Data for the elemental composition, molecular weight and size, non-exchangeable proton distributions, and carbonyl-group content were reasonably consistent for independent analytical determinations. Data for exchangeable-proton, carboxyl, ester, phenolic-hydroxyl, and aliphatic-hydroxyl contents were variable. This variability was attributed to ester formation or hydrolysis during analyses. Determination of carbon distribution, by ^{13}C-nuclear-magnetic-resonance-spectroscopy, gave three markedly different distributions, depending on whether the sample was in the solid state, was in the salt form solvated by water, or was in the free-acid form solvated in organic solvents. An average structural model of the Suwannee standard fulvic acid is presented for the best convergence of the fixed and variable data.

Mineralization of Organic Nitrogen from Sludge Amended Field Soils. W. C. LINDEMANN* and J. GUITAR, New Mexico State University.
The objective of this study was to follow organic nitrogen mineralization in sludge amended soils. Gamma irradiated sewage sludge was applied to a southern New Mexico, clay loam soil at the rates of 33.6 Mg/ha and 67.3 Mg/ha in 1982 and 1983. Organic -N, NH_4^+-N, and NO_2^-+NO_3^--N levels were followed through the fall of 1984. After one cropping season, the 1982 sludge amended plots mineralized 43% and the 1983 sludge amended plots mineralized 41% of the organic -N applied in the sludge. After two cropping seasons, 74% and 67% of the organic -N was mineralized from the 1982 and 1983 amendments, respectively. After three cropping seasons, 82% of the organic -N was mineralized from the 1982 amendment. About 30% of the organic -N remained in sludge amended soils after two cropping seasons, but mineralization after the first two cropping seasons appears to be very slow.

Preliminary Estimates of Fungal Biomass and Nutrient Stores in the Litter and Soil of a Tropical Rainforest. D. J. LODGE, Center for Energy and Env. Res., San Juan, P.R.
Fungal biomass and nutrient stores have not previously been studied in tropical rainforests. These data are needed to estimate nutrient immobilization. Five 1131 cm^2 random samples of litter, and soil to 10 cm depth were taken every two weeks from a 1 ha plot at El Verde in Puerto Rico. The Jones-Mollison direct-count method was used to determine total fungal volume, but a micropipeter was used instead of a hemacytometer. A line-intercept technique was used to estimate hyphal lengths and diameter distributions. Data are from four dates in Feb. and Mar. of 1985. Dry fungal biomass was 1.6-4.3 g m^{-2} in litter, and 140.4 g m^{-2} in the

BEST AVAILABLE COPY

REPORT OF
FOURTEENTH SOYBEAN SEED RESEARCH CONFERENCE

Organized and Sponsored by the

Soybean Seed Division

of the
AMERICAN SEED TRADE ASSOCIATION

asta
first - the seed®

Held in Chicago, Illinois
December 4-5, 1984

Publication No. 14

Edited and Published by
Dolores Wilkinson

AMERICAN SEED TRADE ASSOCIATION
1030 15th Street, N.W.—Suite 964
Washington, D.C. 20005-1593

21

PROMISING DEVELOPMENTS IN RHIZOBIA INOCULANT RESEARCH

HAROLD H. KEYSER
USDA-Agricultural Research Service
Beltsville, MD 20705

Rhizobia are the soil bacteria which nodulate and fix nitrogen in symbiosis with legumes. In soybean, this process usually accounts for 40% or more of the plant's total nitrogen. Since rhizobia were discovered, almost 100 years ago, continual efforts have been made to improve upon the symbiotic system. In this paper, I will briefly summarize recent research efforts which are promising, including those that have been field tested as well as those that are still in the developmental phase.

Genetic Improvement of Rhizobium and Host

The last decade has seen steadily increasing efforts to improve rhizobia through genetic manipulation using the techniques of molecular biology. The enhanced functions sought include those of the nitrogenase enzyme complex, hydrogenase enzyme, fungicide resistance, host range, and competitiveness. With the soybean, attempts have been made to select for genotypes with increased N_2 fixation per se and to use host genotypes which restrict nodulation by certain rhizobia to influence competition for nodule occupancy.

The identification of mutants of *Rhizobium japonicum* with enhanced nitrogen fixing activity has been reported (1,2). More field tests with these strains will be required to demonstrate whether they are superior to the most effective natural strains in common use as inoculants.

The hydrogenase (Hup) enzyme in rhizobia enables the bacteria to recycle H_2 gas which is formed during N_2 fixation. The recycling benefits the symbiosis because it gains electrons and ATP in the process. In *R. japonicum* only 25% of field isolates have this enzyme (3). Since strains which possess the enzyme are considered to be more energy efficient, efforts are underway to find the gene(s) and transfer it to strains lacking the enzyme. This has been accomplished with pea rhizobia (4), and for soybean rhizobia a Hup gene bank has been developed (5). Field tests comparing Hup$^+$

BEST AVAILABLE COPY

and Hup$^-$ strains (Table 1) have shown that the Hup$^+$ strains increased the nitrogen percentage of soybean seed (6). In our USDA *Rhizobium* Collection, six of the seven most effective *R. japonicum* are Hup$^+$. While the extent of the benefit to the soybean from symbiotic association with a Hup$^+$ strain is yet to be shown consistently at the field level, selection and transfer of this characteristic to otherwise effective but Hup$^-$ strains is a worthwhile effort in improving inoculant strains.

Species of *Rhizobium*, and groups within species, normally have a well defined range of legume hosts with which they will nodulate and fix nitrogen. However, among some of the fast-growing rhizobia (for peas, clover, beans, and alfalfa) genetic manipulation has changed their host range. Transfer among rhizobia of plasmid genes containing the nitrogenase, host range, and hydrogenase functions has been accomplished predominately with the fast-growing rhizobia (4,7). Such advances have been sparse with the slow-growing soybean rhizobia, partly due to the difficulty of finding and isolating their plasmids. However, a recently discovered group of fast-growing soybean rhizobia from China possesses plasmids which carry some of the genes for nodulation and N_2 fixation (8,9). While this initial group of Chinese isolates was relatively ineffective with several North American soybean cultivars (10), another group has been screened which contains effective isolates (11). Genetic analysis and manipulation of these fast growers should accelerate research on genetic improvement of *R. japonicum*.

A major problem exists in the ability of introduced inoculant rhizobia to form a significant proportion of the nodules on soybeans in the U.S. The

Summary of effect of Hup+ and Hup- inoculants on N content of soybean seed

Experiment	N content of seeds Hup+	Hup-
	% of dry matter	
Corvallis, 1977	6.02**	5.69
Hermiston, 1979	6.38*	5.75
Corvallis, 1979A	7.04**	6.65
Corvallis, 1979B	6.59**	5.92

from Hanus et al, 1981

inoculant strains do not compete well with the resident strains and, therefore, a response to inoculation is difficult to achieve except on new land. Any improved strain selected for desirable symbiotic traits will face the same difficulty when it is eventually utilized in the field. With the exception of one report, *Rhizobium* geneticists have not attacked the problem of competition. Berwin et al. (12) found that the transfer of a plasmid between strains of *R. leguminosarum* can influence competitive ability, though the recepient's genotype is just as influential. Given the importance of the problem, surely more genetic studies on competition will be forthcoming.

The selection of mutants of *Rhizobium* which are resistant to common seed applied fungicides is being pursued in Dr. M. Alexander's lab at Cornell University (13). These strains are compatible with the seed applied fungicide. In addition, the fungicides decrease the population level of protozoa, which prey on rhizobia. This has been demonstrated with several rhizobia including *R. japonicum* though results from field trials have not been reported.

Efforts to improve the soybean host for symbiotic properties per se have been meager as compared with the efforts to improve *Rhizobium*. The emphasis of soybean breeders (and a well justified one) has been to select for high yield and associated characteristics such as pest and disease resistance. However, at the recent World Soybean Research Conference III, researchers from the University of Maryland (W. J. Kenworthy, M. C. Bolgiano, and J. J. Meisinger) and others at the University of Florida (A. R. Soffes, T. R. Sinclair, and K. Hinson) reported on efforts to select soybeans with high nitrogen fixation. These efforts are at least a beginning and should provide some answers as to the value and potential of this approach.

Inoculant Delivery Systems

The most common form of rhizobial inoculant for soybean is peat, to which is added a liquid containing the live bacteria. Powdered peat is applied directly to seed just prior to planting or granular peat can be applied to the soil along with seed. Research on an improved inoculant formulation has been reported by Kremer and Peterson (14). They have been testing the lyophilized cells of *Rhizobium* suspended in oil. Compared with peat base inoculant, the oil base formula provides more protection to the rhizobia against drought and high temperatures (Table 2) and has a longer shelf life. Field evaluation of this oil based formula on peanut, cowpea, and bean showed it compared favorably with peat inoculant. Further field studies with soybean are warranted, as the oil base inoculant could be a valuable alternative to peat under certain conditions.

BEST AVAILABLE COPY

Survival of peanut Rhizobium CAOO1 at 60° C

Days	Log MPN per g		
	Lyo	Oil	Peat
0	10.12	9.36	8.32
7	5.85	5.22	2.25
56	2.37	3.95	1.00

from Kremer and Peterson, 1983

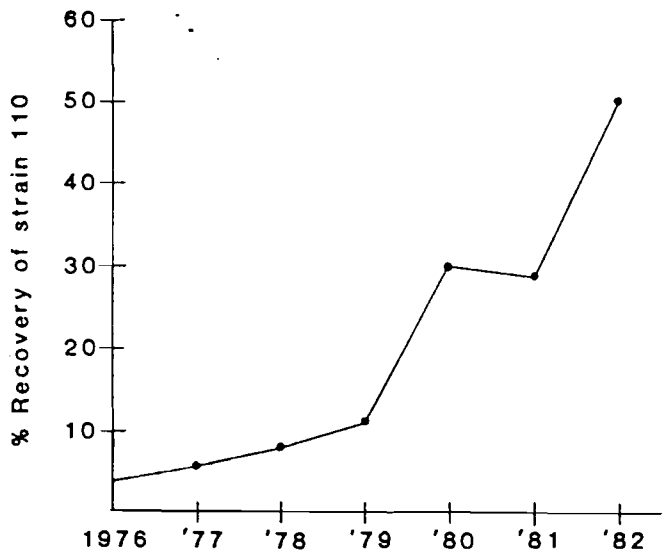
The results of a 7-year field trial by Dunigan et al. (15) relates to competition as affected by repeated inoculation using high rates of a liquid inoculum. Against an indigenous competing population of rhizobia they found that by the 7th year the inoculant strain had formed an average of 54% of the nodules on four soybean cultivars (Figure 1). This is quite an improvement over the 0–15% commonly found in 1-year inoculation studies, and this is indeed promising for the eventual delivery and establishment of improved rhizobia or other plant-beneficial microorganisms.

Competition among Rhizobia

As previously mentioned, competition of inoculant rhizobia with those in the soil represents the bottleneck in attaining the benefits from superior strains of rhizobia. This problem in U.S. soybean fields is well documented (16). Much research has focused on serogroup 123, a subgroup of *R. japonicum*, that is the major indigenous competitor in the Midwest states. It is indeed tenacious in its competitive ability against other strains (17,18). Kvien, Ham, and Lambert (19) attempted to use the plant genotype to influence this situation by screening soybeans for high nodulation affinity with an inoculant strain as compared to the indigenous 123 serogroup. Such types were identified, though one of them failed to show any enhanced affinity in another situation (17).

Another approach to competition using host control has been put forth by Devine (20). The soybean gene rj_1 prevents nodulation by all but a few strains of *R. japonicum*. Even these few can only sparsely nodulate rj_1 soybeans in the field. If these strains could be manipulated to nodulate well and to possess high N_2 fixing potential, then a unique soybean genotype-

Increase in nodule occupancy of 110 over seven years



from Dunigan et al, 1984

Rhizobium strain match would theoretically eliminate competition from indigenous strains. To date no success has been reported with this approach.

Using the diversity present in soybean germplasm to influence competition or other symbiotic functions remains intriguing especially since our developed cultivars represent a very narrow base of genotypes compared to what is available (21). Even unique features found in the wild soybean, *G. soja*, may be useful since it usually produces fertile hybrids in crosses with *G. max*. In our laboratory we have found *G. soja* genotypes which are very effective at N_2 fixation with the fast-growing rhizobia from China while they are ineffective with several typical slow growers (22). This same genotype is very ineffective with strain 123, and an effective fast grower inoculated in the presence of strain 123 produces a large nitrogen response in the plant. Such reactions may be useful in developing soybeans which express specific affinity for certain rhizobia, thereby, influencing competition for nod· ·ion.

In summary, many exciting, new developments in the genetics and genetic improvement of rhizobia have occurred in recent years. Efforts to improve the soybean for N_2 fixation per se and inoculant delivery systems are also receiving attention. Competition among rhizobia remains a complex problem which may thwart the successful use of improved rhizobia, host genotypes, or inoculation techniques. Therefore, more emphasis is needed on the soil and rhizosphere ecology of rhizobia and on the extent to which the soybean genotype can influence competition. In this last regard, coordinated research efforts by soil microbiologists and plant breeders need to be pursued.

BEST AVAILABLE COPY

Literature Cited

1. Williams, L. E., and D. A. Phillips. 1983. Increased soybean productivity with a *Rhizobium japonicum* mutant. Crop Sci. 23:246-250.
2. Maier, R. J., and W. J. Brill. 1978. Mutant strains of *Rhizobium japonicum* with increased ability to fix nitrogen for soybean. Science 201:448-450.
3. Uratsu, S. L., H. H. Keyser, D. F. Weber, and S. T. Lim. 1982. Hydrogen uptake (Hup) activity of *Rhizobium japonicum* from major U.S. soybean production areas. Crop Sci. 22:600-602.
4. DeJong, T. M., J. J. Berwin, A. W. B. Johnston, and D. A. Phillips. 1982. Improvement of symbiotic properties in *Rhizobium leguminosarum* by plasmid transfer. J. Gen Microbiol. 128:1829-1838.
5. Cantrell, M. A., R. A. Haugland, and H. J. Evans. 1983. Construction of a *Rhizobium japonicum* gene bank and use in isolation of a hydrogen uptake gene. Proc. Natl. Acad. Sci. USA 80:181-185.
6. Hanus, F. J., S. L. Albrecht, R. M. Zablotowicz, D. W. Emerich, S. A. Russell, and H. J. Evans. 1981. Yield and N content of soybean seed as influenced by *Rhizobium japonicum* inoculants possessing the hydrogenase characteristic. Agron. J. 73:368-372.
7. Berwin, N. J., J. E. Beringer, A. W. B. Johnston. 1980. Plasmid-mediated transfer of host-range specificity between two strains of *Rhizobium leguminosarum*. J. Gen. Micro. 120:413-420.
8. Masterson, R. V., P. R. Russell, and A. G. Atherly. 1982. Nitrogen fixation (nif) genes and large plasmids of *Rhizobium japonicum*. J. Bacteriol. 152(2):928-931.
9. Sadowsky, M. J., and B. B. Bohlool. 1983. Possible involvement of a megaplasmid in nodulation of soybeans by fast-growing rhizobia from China. Appl. and Env. Micro. 46:906-911.
10. Keyser, H. H., B. B. Bohlool, T. S. Hu, and D. F. Weber. 1982. Fast-growing rhizobia isolated from root nodules of soybean. Science 215:1631-1632.
11. Dowdle, S. F., and B. B. Bohlool. 1984. Predominance of indigenous fast-growing *Rhizobium japonicum* in a soybean field in the Peoples Republic of China. Agronomy Abstracts, p. 185.
12. Berwin, N. J., E. A. Wood, and J. P. W. Young. 1983. Contribution of symbiotic plasmid to the competitiveness of *Rhizobium leguminosarum*. J. Gen. Micro. 129:2973-2977.
13. Lennox, L. B., and M. Alexander. 1981. Fungicide enhancement of nitrogen fixation and colonization of *Phaseolus vulgaris* by *Rhizobium phaseoli*. Appl. Environ. Microbiol. 41:404-411.
14. Kremer, R. J., and H. L. Peterson. 1983. Field evaluation of selected *Rhizobium* in an improved legume inoculant. Agron. J. 75:139-143.
15. Dunigan, E. P., P. K. Bollich, R. L. Hutchinson, P. M. Hicks, F. Zounbrecher, S. G. Scott, and R. P. Mowers. 1984. Introduction and survival of an i. ulant strain of *Rhi-*

16. Ham, G. E. 1976. Competition among strains of rhizobia, p. 144-150. *In* L. D. Hill (ed.). World Soybean Research. Interstate Printers and Publishers, Danville, Ill.
17. Moawad, H. A., W. R. Ellis, and E. L. Schmidt. 1984: Rhizosphere response as a factor in competition among three serogroups of indigenous *Rhizobium japonicum* for nodulation of field grown soybeans. Appl. Environ. Microbiol. 47:607-612.
18. Ellis, W. R., G. E. Ham, and E. L. Schmidt. 1984. Persistence and recovery of *Rhizobium japonicum* inoculum in a field soil. Agron. J. 76:573-576.
19. Kvien, C. S., G. E. Ham, and J. W. Lambert. 1981. Recovery of introduced *Rhizobium japonicum* by soybean genotypes. Agron J. 73:900-905.
20. Devine, T. E. 1977. Genetic control of symbiotic nitrogen fixation in soybeans. p. 417-418. *In* A. Hollaender (ed.). Genetic Engineering for Nitrogen Fixation. Plenum Press, New York, N.Y.
21. Committee on Genetic Vulnerability of Major Crops. 1972. Genetic vulnerability of major crops. National Academy of Sciences, Washington, D.C.
22. Keyser, H. H., and P. B. Cregan. 1984. Interactions of selected *Glycine soja* Sieb. & Zucc. genotypes with fast- and slow-growing soybean rhizobia. Crop Sci. 24:1059-1062.

25

Plant and Soil 95, 201–207 (1986). Ms. 6162
© 1986 *Martinus Nijhoff Publishers, Dordrecht. Printed in the Netherlands.*

Soil temperature effects on competitiveness and growth of *Rhizobium japonicum* and on Rhizobium-induced chlorosis of soybeans*

R. A. KLUSON,
Department of Agronomy and Soils, University of Puerto Rico, Mayaquez, Puerto Rico

W. J. KENWORTHY
Department of Agronomy, University of Maryland, College Park, MD 20742, USA

and D. F. WEBER
Nitrogen Fixation and Soybean Genetics Laboratory, USDA, Beltsville, MD 20705, USA

Received 7 November 1984. Revised January 1986

Key words Chlorosis Competition Enumeration *Glycine max* Growth *Rhizobium japonicum* Serology Temperature

Summary The effects of temperature on growth in broth and soil and on competition for nodule formation between *Rhizobium japonicum* serotypes USDA 76 and 94 compared to 6 and 110 were studied. Increasing root temperatures of Lee soybean from 20 to 35°C increased the competitiveness of 76 and 94 relative to 6 and 110 for all inoculum ratios such that at 30 and 35°C symptoms of *Rhizobium*-induced chlorosis appeared. Tolerance to elevated temperatures was exhibited by 76 and 110, but not 94 and 6 in broth and soil which suggested that increased competitiveness of 76 and 94 at high soil temperatures was not dependent upon growth at elevated temperatures. Nodulation and vegetative growth of Lee soybeans were at a minimum at 20°C and optimum at 30°C. Differences in competitiveness of 6 to previous studies indicated the need to standardize temperatures of assays. Differences in growth responses of 76 and 94 to temperature from a previous study suggested a confounding effect on different carbon sources in growth media.

Introduction

Soil temperature is one component of the environment affecting the successful nodulation of a legume by compatible rhizobia[6] and can alter strain competition for nodulation on cultivars of soybean, *Glycine max* L. (Merr.), and white clover, *Trifolium repens*[3,7]. The mode of action of the effect of soil temperature on serotype competition is unknown, however as rhizobia differ in their survival and growth responses to elevated temperature[1,4] it can be hypothesized that such differences may explain the changed competitiveness between strains as soil temperatures are varied.

Erdman *et al.*[5] described *Rhizobium*-induced chlorosis (RIC) of

* Scientific Article No. A-3721 Contribution No. 6697 of the Maryland Agric Exp Sta, Dept of Agronomy, College Park, MD 20742, and the USDA, ARS, Beltsville, MD 20705. Part of a thesis submitted by the senior author in partial fulfillment of the requirements for the M.S. Degree.

26

soybeans nodulated by USDA 76 and 94, and Weber and Miller[16] correlated its occurrence at elevated root temperatures with increased competitiveness of 94. The objectives of this research were to investigate the effects of soil temperature on competitiveness and growth of serotypes 76 and 94.

Materials and methods

Effects of temperature on competition of serotypes 76 and 94 in nonsterile soil

Bulked samples were obtained from random cores of the surface plough layer (0–20 cm) of an Adelphia sandy loam (Mesic Aquic Hapludult) with no previous history of soybeans and negligible level of indigenous *R. japonicum* indicated by the most probable number method of enumeration. Lime amendments of 0.9 g Ca $(CO)_3$/kg dry soil increased the pH from 4.8 to 6.0. No additional fertilizers were used because a soil test indicated adequate fertility.

A mixture of soil and autoclaved perlite (3:1 ratio by volume) was placed in 20-cm diamter pots which were then partially submerged in thermostatically controlled water baths. The temperature of the soil in the pots was monitored daily from thermocouples at depths of 2.5, 6.5 and 13 cm.

The pots of soil were incubated in the water baths for one week before seeding to permit equilibration to the desired soil temperatures of 20, 25, 30 and 35°C. In order to minimize any extra heating of the soil surface by solar radiation, the water baths were covered with plastic shading material until after seedling emergence. Air temperature in the greenhouse was maintained between 24 to 32°C, and natural photoperiod was extended by incandescent lights to maintain vegetative growth. Seeds of the cultivar 'Lee', which had been identified as highly susceptible to RIC[5], were surface sterilized with washings of ethanol and $Hg(Cl_2)$[15]. Eight seeds were planted per pot and thinned to final stands of four plants per pot at emergence. At planting, the seeds were inoculated with 10 ml of inoculum of one of four different 1:1 serotype combinations: USDA76/USDA6, USDA76/USDA110, USDA94/USDA6, and USDA94/USDA110.

The experimental design was a split-plot with soil temperature as the main plot and inoculum as the subplot, with three replications. Observations and recordings of RIC foliar symtoms were scored on a daily basis[5] and considered the intensity of the foliar symptoms and their duration. Plant tops were harvested at 60 days after planting, dried at 60°C for 48 hours and measured for dry weight/plant. Nodules were collected from the roots of plants, washed clean of soil, nodule fresh weight/plant determined, and stored frozen for serotyping. Using antisera against the inoculum strains, the tube agglutination method was used to serotype twenty-five nodules randomly selected from the total nodule harvest per pot[12,15]. The antisera had been produced by injecting rabbits with prepared suspensions of serotypes 6, 76, 94 and 110[14]. In the statistical analysis the percentage of nodulation was adjusted with the arcsine transformation of the square root of the value for considerations of homogeneity of variances. Nodulation by 76 or 94 was noted as either due to single or double infection. Nodulation by indigenous rhizobia was negligible.

Growth study in broth and soil of serotypes 76 and 94 at different temperatures

For the broth experiment, screw-capped test tubes were placed inside temperature-controlled incubators calibrated at either 20, 25, 30 or 35°C. Inoculum treatments of serotypes 76, 94, 6 and 110 consisted of pure stock cultures diluted to a concentration of 2.5×10^5 cells/ml, and 1 ml of diluted culture was transferred to individual test tubes of 9 ml of sterile YEM broth which had been equilibrated to the desired temperature. The shaker of each incubator was set at 100 rpm in order to uniformly aerate and stir the inoculated broth.

For the soil experiment, snap-capped test tubes were placed inside temperature-controlled incubators calibrated at 20 or 35°C. Inoculum treatments consisted of pure stock cultures which were washed clean of YEM broth by centrifugation with normal saline and adjusted to a

BEST AVAILABLE COPY

27

Table 1. The orthogonal comparisons for soil temperature (T) effects and the F test for inoculum (I) and T × I interaction effects for nodule fresh weight, vegetative dry weight and nodulation of frequencies of serotypes USDA 76 and 94 and of double infections on Lee soybeans grown at four temperatures

Component	Nodule fresh weight	Vegetative dry weight	Nodulation frequency	
			Serotypes 76 and 94	Double infections
T_L	°°	°°	°	ns
T_Q	ns	°°	ns	ns
T_C	ns	°	ns	ns
I	ns	ns	°	°°
T × I	ns	ns	ns	ns

°, °°Significance at the 0.05 and 0.01 levels, respectively T_L, T_Q, T_C Linear, quadratic & cubic components of temperature, respectively I
Temperature X inoculum interaction (F test respectively).

concentration of 1.0×10^8 cells/ml. At incoulation 1 ml was added to 10 g of autoclaved Adelphia soil which had been moistened to 75% water holding capacity (WHC). The test tubes were placed in a plastic bag in order to control dessication and the moisture content of the soil was maintained at 60 to 75% WHC with the addition of sterile, distilled water whenever required.

Population levels of the serotypes were measured with a destructive sampling method at 2, 4, 10 and 14 days (broth experiment), and 3, 10, 20, 30 and 40 days (soil experiment) after inoculation. Enumeration was done with the Miles-Misra micropipet drop technique[15] using a 20 ul Eppendorf Pipet 4700. Serial dilutions were performed with sterile water amended with Tween 80 at a rate of 4 drops per liter. The plate counts were grown on YEM agar at 25°C, and selected for a range of 20 to 30 colonies per count. Uninoculated controls were included for each harvest date to insure against contamination. With the soil experiment, the enumerations were expressed on a per gram of dry soil weight basis. In the statistical analysis the recorded enumerations of rhizobial populations were all calculated with a $\log^{10}$ transformation for considerations of homegeneity of variances.

Both experiments were conducted in a completely randomized design. The main treatments in this study were inoculum, harvest time (four and five for the broth and soil experiments, respectively), and replication (five and four for the broth and soil experiments, respectively). Separate experiments were done for each specified temperature. Comparisons across temperature were done with calculated 95% confidence limits of population at the final harvest time.

Results and discussion

Temperature and inoculum effects on vegetative dry weight and nodule fresh weight for the cultivar Lee are shown in Table 1. Soil temperature demonstrated a significant positive trend which fit a cubic equation on vegetative growth and a significant linear trend on nodule fresh weight. Figure 1 shows that the minimum and maximum values for both variables were produced at 20 and 30°C, respectively. Weber and Miller[16] have also reported that the minimum and optimum temperatures were 20 and 30°C, respectively, for both nodule and vegetative weight of soybean cultivar Lee.

-28

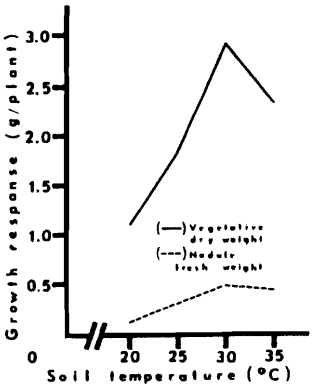


Fig. 1. Soil temperature effects on nodule fresh weight/plant and vegetative dry weight/plant of Lee soybeans grown in soil and inoculated with 1:1 mixtures of *R. japonicum* serotypes USDA 76 or 94 with 6 or 110.

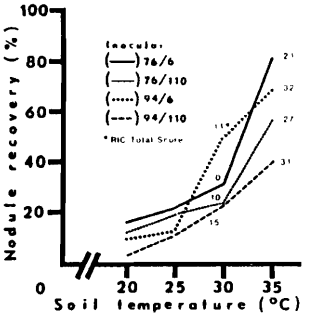


Fig. 2. Soil temperature effects on the competitiveness for nodulation of *R. japonicum* serotypes USDA 76 and 94 in a 1:1 mixture inocula with serotypes USDA 6 or 110 and on the RIC total scores for foliar symptoms observed on Lee soybeans grown in rhizobia-free Adelphia soil.

The mean frequencies of nodulation of serotypes 76 and 94 for each 1:1 inoculum mixture at different temperatures are shown in Figure 2. A significant positive linear increase in these frequencies was observed with maximum values produced at 35°C. The increased presence of serotypes 76 and 94 in the nodules was directly related to the occurrence of foliar symptoms of RIC. The total scores for RIC foliar symptoms shown in Figure 2 represent the means of three repetitions. Note that foliar symptoms of RIC only appeared at soil temperatures of 30 and 35°C. The inoculum combination of 76/6 did not produce chlorosis at 30°C even though approximately one third of the nodules contained 76. Chlorosis scores increased for all inocular at 35°C.

Elevated soil temperatures, therefore, increased the competitiveness of serotypes 76 and 94 in competition with 6 and 110. Serotype 76 was more competitive than 94 when each was in competition with 6 and

BEST AVAILABLE COPY

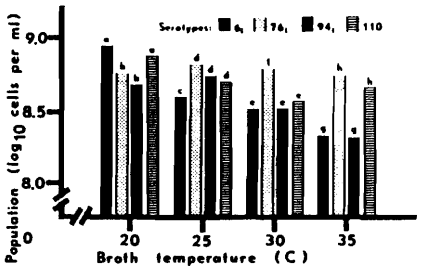


Fig. 3. Temperature effects on growth of *R. japonicum* serotypes USDA 76 and 94 compared to 6 or 110 in YEM broth shake culture. Population levels represent 14 days of incubation in separate experiments at each temperature. Values within temperature with same letter do not differ significantly $P = 0.05$).

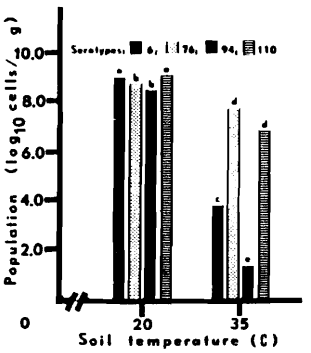


Fig. 4. Soil temperature effects on growth in *R. japonicum* serotypes USDA 76 and 94 compared to 6 and 110 in sterile Adelphia soil. Population levels represent 40 days of incubation in separate experiments at each temperature. Values within temperatures with same letter do not differ significantly ($P = 0.05$).

110 at all soil temperatures except 30°C, and this agrees with Means *et al.*[11] The dominance of 110 over 76 at 20, 25 and 30°C agreed with the results at unspecified temperatures reported by Caldwell[2] and Johnson and Means[8]. However, the dominance of 6 over 76 at the same temperatures was not in agreement with Johnson and Means[8] who reported 6 to be a very poor competitor in greenhouse and field studies. This suggested that competition studies should be carried out under strictly controlled temperature conditions.

The occurrences of double infections were observed at low frequencies and were unaffected by temperature. Mean values were averaged over temperatures ranged from 0 to 5%. There was an effect of inocula as the 5% double infections of inoculum 76/6 was significantly greater than the 1 and 0% frequencies of inocula 94/6 and 94/110, respectively, but was not different than the 2% frequency of inoculum 76/110.

Growth of all strains in broth and soil was affected by increasing

temperatures. At each of the temperatures for both the broth and soil experiments, the individual analyses of variance indicated significant population differences due to serotype, date of enumeration, and serotype X date interaction (data not shown). All strains exhibited either growth or death responses, and the final enumerations are illustrated because they are representative of the temperature effects on each serotype. In the broth experiment (Figure 3), the population of serotype 76 was relatively constant at all temperatures in contrast to 94 and exceeded 94 at the highest temperatures. In the soil experiment, similar growth responses to the broth cultures occurred with serotypes 76 and 94 for both 20 and 35°C (Fig. 4). At the high soil temperature, the population level of serotype 76 was again significantly greater than 94.

Munevar[13] reported slightly different growth response for serotypes 76 and 94 using optical density readings of cultures in HEPES-MES-Gluconante (HMG) broth medium. It was reported that serotype 94 had the greater maximum permissive temperature for growth of 35.2°C compared to 33.7°C for serotype 76. This discrepancy may be due to the different carbon sources of the broth growth medium as previously shown by Kuykendall and Elkan[10] who observed that isolates of the same *R. japonicum* stock serotype can exhibit differing carbohydrate utilization for growth. Therefore, growth responses in broth to temperature could be confounded with the effects of different carbon sources. It is noteworthy that in this study the temperature effects on all serotypes were consistent in broth and soil.

The differences in survival of 76 compared to 94 at a soil temperature of 35°C did not support the hypothesis that the effect of the temperature on competition for nodule formation was entirely due to survival or growth at elevated temperatures. In addition, increased dominance of serotypes of 76 and 94 were not necessarily shown to mean a greater population compared to the competitor serotype (*i.e.* 6 or 110). Jones and Morley[9] reported a correlation between growth and competitiveness for two serotypes of *R. trifolii* at different pH levels that resulted in greater growth for the more competitive serotype and, therefore, significantly linked the two responses. In this research, however, the two responses of growth and competitiveness were not correlated, and it was concluded that a more complex mode of action is involved in the soil temperature effects on changing competitiveness of *Rhizobium* serotypes.

Acknowledgements The principal author thanks the entire team of researchers at the USDA Nitrogen Fixation and Soybean Genetics Laboratory, including Drs. Sloger, Keyser and van Berkum, for their counsel and guidance. The assistance and friendship of technicians Jeff, Paul and Rich are also gratefully acknowledged.

References

1 Boonkerd N and Weaver R W 1982 Survival of cowpea rhizobia in soil as affected by soil temperature and moisture. Appl. Environ. Microbiol. 43, 585–589.

2 Caldwell B E 1969 Initial competition of root-nodule bacteria on soybeans in a field environment. Agron. J. 61, 813–815.

3 Caldwell B E and Weber D F 1970 Distribution of *Rhizobium japonicum* serotypes in soybean nodules as affected by planting dates. Agron. J. 62, 12–14.

4 Danso S K A and Alexander M 1974 Survival of two strains of *Rhizobium* in soil. Soil Sci. Soc. Am. Proc. 38, 86–89.

5 Erdman L W, Johnson H W and Clark F 1957 Varietal responses of soybeans to a bacterial-induced chlorosis. Agron. J. 49, 267–271.

6 Gibson A H 1977 The influence of the environment and managerial practices on the legume-rhizobium symbiosis. *In* A Treatise on Dinitrogen Fixation. Section IV Agronomy and ecology. Eds R W F Hardy and A H Gibson. Wiley-Interscience, New York pp 393–450.

7 Hardarson G and Jones D G 1979 Effect of temperature on competition amongst strains of *Rhizobium trifolii* for nodulation of two white clover varieties. Annals of Appl. Biol. 92, 229–236.

8 Johnson H W and Means U M 1964 Selection of competitive strains of soybean nodulating bacteria. Agron. J. 56, 60–62

9 Jones D G and Morley S J 1981 The effect of pH on host plant preference for strains of *Rhizobium trifolii* using fluorescent ELISA for strain identification. Annals of Appl. Biol. 97, 183–190.

10 Kuykendall L D and Elkan G H 1976 *Rhizobium japonicum* derivatives differing in nitrogen fixing efficiency and carbohydrate utilization. Appl. Environ. Microbiol. 32, 511–519.

11 Means U M, Johnson H W and Erdman L W 1961 Competition between bacterial strains effecting nodulation in soybeans. Soil Sci. Soc. Am. Proc. 25, 105–108.

12 Means U M, Johnson H W and Date R A 1964 Quick serological method of classifying strains of *Rhizobium japonicum* in nodules. J Bacteriol. 87, 547–553.

13 Munevar F 1981 Studies of the Soybean-Rhizobium Association as Affected by High Root Temperature. Ph.D. thesis, North Carolina State University.

14 Roberson R S 1978 Laboratory Exercises in Immunology. Department of Microbiol. Publication, University of Maryland.

15 Vincent J M 1970 A Manual for the Practical Study of the Root Nodule Bacteria. IBP Handbook 15. Blackwell Publishers, Oxford.

16 Weber D F and Miller V L 1972 Effect of soil temperature on *Rhizobium japonicum* serogroup distribution in soybean nodules. Agron. J. 64, 796–798.

32

REPRINTED FROM:

World Soybean Research Conference III:

Proceedings

edited by

Richard Shibles

Copyright 1985 by Westview Press, Inc.

Westview Press / Boulder and London

33

FAST-GROWING SOYBEAN RHIZOBIA

H. H. Keyser, M. J. Sadowsky, and B. B. Bohlool

Rhizobium are the bacteria capable of forming a nitrogen-fixing symbiosis with leguminous plants. Traditionally, their classification has been based on specific host infectivity with the recognition of two broad groups of fast- and slow-growing strains (Jordan and Allen, 1974). In addition to growth rate, the two groups are distinguished by several important differences, which has led to the proposed establishment of a separate genus for slow growers (*Bradyrhizobium* gen. nov.), while retaining fast growers in the genus *Rhizobium* (Jordan, 1982).

The soybean-nodulating bacteria, *R. japonicum*, have been classified as slow growers. Recently, however, Keyser et al. (1982) reported the existence of fast-growing isolates obtained from soil and nodules collected in China. Examination of the 11 fast-growing isolates for microbiological, genetic, and symbiotic properties has shown them to be distinct from the slow growers. They represent a unique and valuable addition of *Rhizobium* germplasm. In this article we present an overview of microbiological and symbiotic properties of the fast-growing soybean rhizobia. Results from genetic studies are presented elsewhere in this volume by Atherly et al. Several investigators are currently examining these fast growers for various attributes, and knowledge of them should increase rapidly.

ISOLATION

The 11 fast-growing strains were obtained from three separate collection expeditions in China (Table 1). Strain USDA 191 was isolated by J. C. Burton (Nitragin Co., Milwaukee) and the others by T. S. Hu and S. N. May in the USDA Nitrogen Fixation and Soybean Genetics Laboratory at Beltsville, Md. The isolates were authenticated by three cycles of plant passage through *G. max* cv. Peking and reisolation of single colonies. Careful examination showed no slow-growing colonies that could have been masked by the fast-growing type. Subsequent use of fluorescent antibodies confirmed their purity.

CULTURAL CHARACTERISTICS

The fast growers have generation (doubling) times of 2.9 to 4.8 h at 28 C in yeast extract-mannitol-salts (YEM) broth with a concomitant acidic reaction (Keyser et al., 1982). This is in contrast to slow-growing *R. japonicum*, which have generation times of 6 or more hours and a neutral to alkaline reaction in YEM media (Vincent, 1974). On YEM agar plates the fast growers gave 1 to 5 mm diameter colonies after 6 to 7

-34-

Table 1. Strain history of fast-growing soybean rhizobia from China.

USDA accession no. and synonyms	Geographic origin of isolate	Source material from which isolation was made
191 (=440)	Shanghai, Jiangsu Province	Soil: isolated from nodule of trap host *G. max* cv. Jupiter[a]
192 (=0B2=PRC 192)	Tsinan, Shantung Province	Desiccated root system of *G. soja*: isolated from nodule of trap host *G. max* cv. Peking[b]
193 (=0B3=PRC 193)	Taiyuanchen, Shansi Province	Desiccated root system of *G. soja* PI 468397: isolated from nodule of trap host *G. max* cv. Peking[b]
194 (=PRC 194)	Chengchou, Honan Province	Desiccated nodule of *G. max*: direct isolation[c]
201 (= PRC 201)	Same as 194	Same as 194
205 (=PRC 205)	Same as 194	Same as 194
206 (=PRC 206)	Sinsiang, Honan Province	Same as 194
208 (=PRC 208)	Same as 206	Same as 194
214 (=PRC 214)	Same as 206	Same as 194
217 (=PRC 217)	Same as 206	Same as 194
257 (=PRC 257)	Wukung, Shensi Province	Same as 194

[a]Collected by J. Tanner, Univ. of Guelph, 1979.
[b]Collected by T. S. Hu, Chinese Academy of Agric. Sciences, Peking, 1980.
[c]Collected by W. Fehr, Iowa State Univ., and K. Hinson, Univ. of Florida, 1979.

days, whereas colonies of slow growers are only 0.5 to 1 mm. The fast growers produce typical circular, convex, white colonies on YEM agar, with little production of extracellular polysaccharide (Sadowsky et al., 1983). In liquid media, cells from late-log-phase to stationary-phase became enlarged and exhibited marked pleomorphism, while slow growers remained as typical rods. The growth rate difference between the two types of soybean rhizobia is maintained in sterile soil (Sadowsky, 1983). This information indicates that the cultural characteristics of the fast-growing soybean rhizobia are similar to other fast-growing *Rhizobium* species (e.g., *R. leguminosarum* and *R. trifolii*).

GROWTH RESPONSES

The fast-growing soybean rhizobia do not require vitamins (biotin, thiamine, pantothenate) for growth in a defined medium (Stowers and Eaglesham, 1984), a characteristic shared by slow-growing rhizobia. However, unlike slow growers they are tolerant of pH 9.5 and sensitive to pH 4.5 (Sadowsky et al., 1983). As with other fast growers, they are more sensitive to antibiotics than are the slow growers (Sadowsky, 1983; Stowers and Eaglesham, 1984). Also, in contrast to slow-growing *R. japonicum*, the fast growers are tolerant of high levels of NaCl (Sadowsky et al., 1983; Yelton et al., 1983; Stowers and Eaglesham, 1984). Likewise, such tolerance is also found

35

among the fast-growing *R. meliloti* (Graham and Parker, 1964). Yelton et al. (1983) demonstrated that tolerance of 0.4 M NaCl is accompanied by increased levels of K^+ and glutamate in USDA 191. Stowers and Eaglesham (1984) found the fast growers, with one exception, to be tolerant of 0.17 M NaCl, but completely sensitive to 0.34 M NaCl. Sadowsky et al. (1983) found that all 11 of the fast growers made growth at 0.34 M NaCl, although some strains had growth limited to a few tolerant colonies after 14 days. None of the slow-growing *R. japonicum* tested by Sadowsky et al. (1983) produced any colonies at 0.34 M NaCl.

CARBOHYDRATE UTILIZATION

Fast-growing species of *Rhizobium* can use many sugars and organic acids for growth, whereas the slow growers are more specialized and prefer pentoses (Fred et al., 1932). Studies of the carbohydrate utilization by the fast-growing soybean rhizobia have shown they match the pattern of other fast-growing rhizobia (Sadowsky et al., 1983; Yelton et al., 1983; Stowers and Eaglesham, 1984). Unlike the slow-growing *R. japonicum* the fast growers utilized cellobiose, inositol, lactose, maltose, raffinose, glucitol, sucrose, and lactose. The ability to use lactose for growth requires sufficient activity of β-Galactosidase. Sadowsky et al. (1983) demonstrated large differences between the fast- and slow-growing soybean rhizobia in levels of β-Galactosidase activity (Table 2).

Consistent with the division of the rhizobia into fast- and slow-growing groups based on carbohydrate utilization is the division of these organisms based on the

Table 2. Enzyme activities of fast- and slow-growing soybean rhizobia (Sadowsky et al., 1983).

Strain	Enzyme activities	
	6-PGD[a]	β-Galactosidase[b]
Slow-growing rhizobia		
USDA 31	<0.5	7.1
USDA 110	<0.5	3.8
USDA 123	<0.5	3.0
USDA 136	<0.5	7.6
USDA 138	<0.5	3.4
PRC 005	<0.5	4.2
PRC 113-2	<0.5	3.3
PRC 121-6	<0.5	4.8
PRC B15	<0.5	4.8
Fast-growing rhizobia		
USDA 191	97	–
USDA 192	181	459
USDA 193	140	183
USDA 194	126	101
USDA 201	50	345
USDA 205	159	97
USDA 206	59	261
USDA 208	147	383
USDA 214	160	245

[a]6-phosphogluconate dehydrogenase, expressed as nanomoles of reduced NADP produced per min per mg of protein.

[b]Expressed as micromoles of o-nitrophenol produced per min per mg of protein.

-36

presence and absence of enzymes of the pentose phosphate pathway. Only fast-growing rhizobia have NADP-linked 6-phosphogluconate dehydrogenase (6-PGD) activity, a key enzyme of that pathway (Keele et al., 1969; Martinez-de Drets and Arias, 1972). Table 2 shows the clear differences between the two types of soybean rhizobia in 6-PGD activity, again linking these fast growers to other fast-growing rhizobia. Studies by Yelton et al. (1983) and Stowers and Eaglesham (1984) also showed the presence of 6-PGD activity in these fast-growers.

Of the 30 strains examined from 6 species of *Rhizobium*, only the fast-growing soybean rhizobia were capable of substantial growth in ethanol (Sadowsky, 1983). While the fast growers grew to final cell densities of 10^8-10^9 cells/mL on 0.2% ethanol, the slow-growing *R. japonicum* never reached densities greater than 10^7 cells/mL on any concentration of ethanol. No activity of NAD- or NADP-linked alcohol dehydrogenase was found.

BIOCHEMICAL ATTRIBUTES

In comparing the 11 fast-growing soybean rhizobia with 7 isolates of slow-growing *R. japonicum*, Sadowsky et al. (1983) found both groups to be positive for catalase, oxidase, urease, penicillinase, and nitrate reductase. Neither group produced H_2S or 3-Ketolactose. Bernaerts and DeLey (1963) found that production of 3-Ketolactose from lactose is limited to species of *Agrobacterium*, a genus closely related to the fast-growing *Rhizobium*. Graham and Parker (1964) found that production of penicillinase was more common in slow-growing rhizobia, though some isolates of the fast-growing *R. leguminosarum* possessed this attribute.

Gelatinase activity clearly differentiated the two groups. The slow growers were negative while the fast growers and an isolate from *Leucaena* were positive (Sadowsky et al., 1983).

In a test of litmus milk reactions, 20 of 21 slow growing *R. japonicum* produced an alkaline pH change with no peptonization (Sadowsky et al., 1983). By comparison, 10 of 11 fast growers produced peptonization of the medium, though pH changes were variable. This response of the fast-growing soybean isolates is typical of other fast-growing *Rhizobium* species (Graham and Parker, 1964).

The fast growers apparently lack hydrogenase activity, as determined in culture and in bacteroid preparations. All seven of the fast growers tested for hydrogenase in free-living culture were negative (S. Uratsu, pers. commun.). Yelton et al. (1983) found USDA 191 to lack hydrogenase activity in culture as well as in symbiosis with *G. max* cv. Clark and cowpea (*Vigna unguiculata* [L.] Walp.) cv. Calif. Blackeye 5. We have found all 11 of the fast growers also to be negative in symbiosis with *G. max* cv. Peking and *G. soja* PI 468398.

SEROLOGICAL RELATIONSHIPS

The fast-growing soybean rhizobia are serologically related (Sadowsky, 1983). Tests with whole-cell antisera indicated that the fast growers shared at least one heat-labile antigen in common. Examination with the more specific somatic antigens showed at least three serological groups among the fast growers; USDA 192 (3 strains), USDA 194 (2 strains), and USDA 205 (6 strains).

In agglutination tests with somatic antisera from 14 non-cross-reacting strains of slow-growing *R. japonicum*, the only cross reaction we found with the fast growers occurred between the USDA 194 group and the USDA 122 group (representing the

37

slow growers USDA 122, USDA 136, CB 1809, and others). Sadowsky (1983) found the same degree of difference between the fast and slow growers. He also found that while USDA 136 cross-reacted with USDA 194 in immunofluorescence reactions, this was not detected in immunodiffusion reactions using somatic antisera.

Immunodiffusion tests with species of *Rhizobium* other than *R. japonicum* with antisera from three of the fast growers showed some very interesting relationships. Fast-growing isolates of *R. meliloti*, an isolate from *Sesbania*, and an isolate from *Lablab purpureus* (L.) Sweet had whole-cell and somatic antigens in common with the fast-growing soybean rhizobia (Sadowsky, 1983). Slow growers from *R. lupini* and the 'cowpea' groups did not cross react. The fast-growing soybean rhizobia appear to be fairly distinct among *Rhizobium* in their serological relatedness to both slow growers (*R. japonicum*) and fast growers (*R. meliloti* and 'cowpea' types), though Trinick (1980) reported the same for isolates of *Leucaena*.

SYMBIOSIS WITH LEGUMES

The fast-growing soybean rhizobia nodulate and fix N_2, to varying degrees of effectiveness, with *G. max* and *G. soja* genotypes and with some legumes of the cowpea cross inoculation group. Similar host range is found with the slow-growing *R. japonicum* (Leonard, 1923; Van Rensburg et al., 1976). The fast growers do not nodulate hosts of other fast-growing species (*R. meliloti; R. trifolii; R. phaseoli; R. leguminosarum*) (Keyser et al., 1982; unpublished data). Many adapted genotypes of North American soybeans form ineffective symbioses with the fast growers (Keyser et al., 1982). Table 3 shows the response of four cultivars. In most cases the fast-growers

Table 3. Top dry weight of soybean cultivars inoculated with fast- and slow-growing soybean rhizobia.[a]

Strain	Cultivars of *G. max*			
	Wilson-6	Bedford	Clark	Hardee
		– g/jar[b] –		
Slow growers				
USDA 110		4.8 r		6.1 r
USDA 122	6.4 r		4.6 r	
Fast growers				
USDA 191		1.9 t		2.4 s
USDA 192	1.9 u		2.7 s	2.4 s
USDA 193	1.5 uv		2.2 s	
USDA 194	1.7 uv	3.0 s	2.9 s	
USDA 201			2.8 s	2.5 s
USDA 205	2.6 t	1.9 t	2.8 s	2.2 s
USDA 206		2.0 t		
USDA 208	3.3 s		3.1 s	2.4 s
USDA 217	1.8 uv		2.3 s	2.6 s
Uninoculated	1.3 v	1.8 t	2.6 s	1.9 s

[a]Tests conducted in vermiculite-Leonard jar assemblies, grown for 5-6 weeks in a growth chamber.

[b]Each value is a mean of 3 replicates, 3 plants per replicate. Values followed by the same letter within a column are not significantly different ($P < .05$) according to Duncan's New Multiple Range Test.

-38-

were not significantly better than an uninoculated control, as well as being significantly inferior to the reference slow grower. Nodule production on these cultivars ranges from normal (with cultivars Bedford and Hardee) to sparse and atypical (with cultivars Clark, Kent, and Lee). Similar ineffective responses by North American soybeans have been reported by Van Rensburg et al. (1983) and Stowers and Eaglesham (1984). This general ineffective response is not without exception as Yelton et al. (1983) reported USDA 191 to be effective with Clark, and Dowdle (pers. commun.) found three of the fast growers to be effective with other cultivars, based on acetylene-reduction.

Peking forms an effective symbiosis with the fast growers (Table 4). Peking is an unimproved selection of a line from China. Also, Stowers and Eaglesham (1984) found the fast growers to be very effective with three "Asian-type" soybeans. Van Rensburg et al. (1983) found an effective symbiosis between the fast growers and two South African soybeans (Table 5). Cultivar Geduld is a selection from a cross of Dixie and a line from China, while Usutu was selected from crosses in which Geduld was involved. Perhaps, the low frequence of effectiveness with the U.S. cultivars is atypical of soybean germplasm in general and simply results from the narrow germplasm base of U.S. cultivars, which may be built upon accessions with a very low frequency of effectiveness with the fast growers.

Cowpea forms an effective symbiosis with the fast growers (Table 4). This has been confirmed by Stowers and Eaglesham (1984) and Yelton et al. (1983). While they are

Table 4. Shoot nitrogen content in three legumes inoculated with fast- and slow-growing soybean rhizobia.[a]

Strain	Host legume		
	G. max cv. Peking	*G. soja* PI 468398	*Vigna unguniculata* cv. Calif. Blackeye-5
		– mg N/jar[b] –	
Fast growers			
USDA 191	65.3 r	6.9 rs	97.6 rstu
USDA 192	37.7 u	8.5 r	88.8 rstuv
USDA 193		6.8 rs	103.0 rst
USDA 194	46.3 stu	7.1 rs	63.5 vw
USDA 201	63.8 r	7.0 rs	78.3 tuv
USDA 205	50.4 s	6.0 rs	93.6 rstu
USDA 206	38.7 tu	5.8 s	49.5 w
USDA 208	45.9 stu	6.9 rs	78.8 tuv
USDA 214	45.3 stu	7.6 rs	73.2 uvw
USDA 217	49.1 st	7.0 rs	82.9 stuv
USDA 257		7.0 rs	104.9 rs
Slow growers			
USDA 122	52.6 s	6.2 rs	
176A22[c]			116.1 r
Uninoculated	6.9 v	1.1 t	8.7 x

[a]See footnote a, Table 3.

[b]See footnote b, Table 3.

[c]A slow-growing reference strain of *R. sp.* 'cowpea miscellany'.

39

Table 5. Dry mass and nodulation of cultivars of *G. max* inoculated with slow- and fast-growing strains of *R. japonicum* (from Van Rensburg et al., 1983).

Strain	Cultivar of American origin	Cultivars of South African origin		
	Forrest	SSS3	Geduld	Usutu
		– g/plant[a] –		
Slow grower				
WB1	2.72	2.66	2.11	1.87
Fast growers				
USDA 193	0.56[b]	0.58	2.14	1.46
USDA 194	0.64[b]	0.52	1.59	1.33
USDA 201	0.67[b]	0.49	2.07	1.79
USDA 205	0.67[b]	0.80	2.29	1.38
USDA 206	0.71[b]	0.52	1.57	1.47
Uninoculated	0.82[b]	0.68[b]	0.56[b]	0.54[b]

[a]Average of 3 replicates LSD ($P < 0.01$) = 0.52; c.v. = 28.4%.
[b]No nodules formed.

also effective with pigeon pea (*Cajanus cajan* [L.] Huth) the fast growers are ineffective with mung bean (*Vigna radiata* [L.]) and species of *Sesbania* and *Macroptilium* (Keyser et al., 1982; Stowers and Eaglesham, 1984).

The most interesting comparison of host preference between the fast- and slow-growing soybean rhizobia is found in their reaction with different genotypes of wild soybean (*G. soja*). Keyser et al. (1982) reported the fast growers to be effective at N_2-fixation with a line from China (Table 4). Subsequent tests with seven other *G. soja* genotypes demonstrated significant effectiveness interactions with both rhizobia groups (Keyser and Cregan, 1984a). The response of two genotypes with opposite group preferences is shown in Figure 1. PI 468397 exhibits a general specificity for effectiveness with the fast growers as opposed to slow growers. This very unusual reaction has not yet been found in *G. max*, and indicates there is a large range of symbiotic adaptations in *G. soja* germplasm.

CONCLUSIONS

The fast-growing soybean rhizobia from China are distinct from the slow-growing *R. japonicum*. Several distinguishing bacteriological properties used to separate the slow-growing *Bradyrhizobium* from the fast-growing *Rhizobium* place these new isolates in the *Rhizobium* genus. However, the similar host range with the slow growers points out the difficulty of including both the bacteriological and the host related properties in the classification system of root nodule bacteria.

China is considered to be the gene center of the *G. max* and *G. soja*, and apparently it is also the gene center of two types of soybean rhizobia. The occurrence of the fast growers in China is not rare, as they were found in five provinces. In the collection made by Fehr and Hinson, 8 of 69 nodules contained fast-growing isolates. In contrast, the occurrence of the fast growers in U.S. soils is apparently very low at best, though most workers would probably discard such isolates as suspected contaminants.

Genetic studies of the fast growers have also shown them to be distinct from the

40

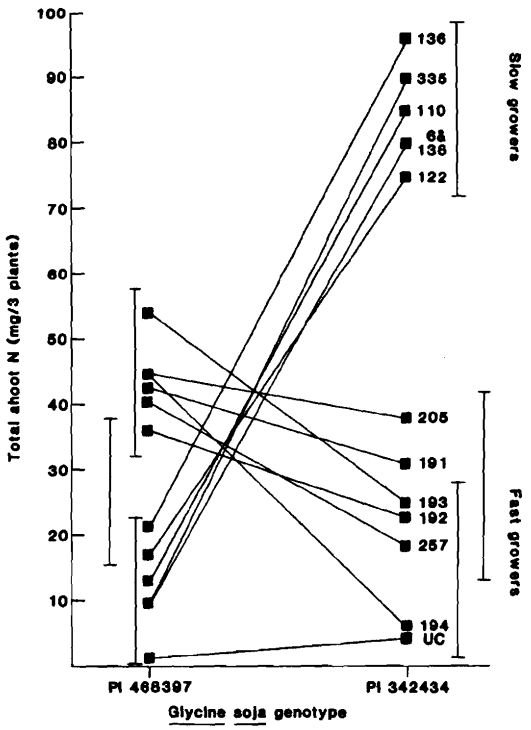


Figure 1. Total shoot N in two *G. soja* genotypes in symbiosis with fast- and slow-growing strains of rhizobia. Strains are listed by USDA number, UC = uninoculated control. Values within a *G. soja* genotype encompassed by the vertical bar are not significantly different ($P \leq 0.05$) as tested by Duncan's New Multiple Range Test from Keyser and Cregan, 1984b).

slow growers (see the chapter by Atherly et al., this volume). Their potential for genetic manipulation, combined with their differential symbiotic reactions with *G. max* and *G. soja* genotypes, provides researchers with valuable organisms for use in increasing our understanding of the soybean-rhizobia symbiosis.

NOTES

H. H. Keyser, USDA, ARS, Nitrogen Fixation and Soybean Genetics Laboratory, Beltsville, Maryland 20705; M. J. Sadowsky, Department of Biology, Genetic Manipulation Research Group, McGill University, Montreal, Canada H3A 1B1; B. B. Bohlool, Department of Microbiology, University of Hawaii, Honolulu 96822.

REFERENCES

Bernaerts, M. J. and J. DeLey. 1963. A biochemical test for crown gall bacteria. Nature (London) 197:406-407.

Fred, E. B., I. L. Baldwin and E. McCoy. 1932. Root nodule bacteria and leguminous plants. University of Wisconsin Studies in Science. No. 5, University of Wisconsin, Madison.

Graham, P. H. and C. A. Parker. 1964. Diagnostic features in the characterization of the root-nodule bacteria of legumes. Plant Soil 20:383-396.

41

Jordan, C. D. 1982. Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing root nodule bacteria from leguminous plants. Int. J. Syst. Bacteriol. 32:136-139.

Jordan, D. C. and O. N. Allen. 1974. Genus II. *Rhizobium*, p. 262-264. *In* R. E. Buchanan and N. E. Gibbons (eds.) Bergey's manual of determinative bacteriology. 8th ed. The Williams & Wilkins Co., Baltimore.

Keele, B. B., P. B. Hamilton and G. H. Elkan. 1969. Glucose catabolism in *Rhizobium japonicum*. J. Bacteriol. 97:1184-1191.

Keyser, H. H., B. B. Bohlool, T. S. Hu and D. F. Weber. 1982. Fast-growing rhizobia isolated from root nodules of soybean. Science 215:1631-1632.

Keyser, H. H. and P. B. Cregan. 1984a. Interaction of *Glycine soja* genotypes with fast and slow growing soybean rhizobia. p. 598. *In* C. Veeger and W. E. Newton (eds.) Adv. in nitrogen fixation research. Martinus Nijhoff, The Hague, The Netherlands.

Keyser, H. H. and P. B. Cregan. 1984b. Interaction of selected *Glycine soja* Sieb & Zucc. genotypes with fast- and slow-growing soybean rhizobia. Crop Sci. 24:1059-1062.

Leonard, L. T. 1923. Nodule-production kinship between the soybean and the cowpea. Soil Sci. 15:277-283.

Martinez-de Drets, G. and A. Arias. 1972. Enzymatic basis for the differentiation of *Rhizobium* into fast- and slow-growing groups. J. of Bacteriol. 109:467-470.

Sadowsky, M. J. 1983. Physiological, serological, and plasmid characterization of fast-growing rhizobia that nodulate soybeans. Ph.D. Dissertation, University of Hawaii, Honolulu.

Sadowsky, M. J., H. H. Keyser and B. B. Bohlool. 1983. Biochemical characterization of fast- and slow-growing rhizobia that nodulate soybeans. Int. J. Syst. Bacteriol. 33:716-722.

Stowers, M. D. and A. R. J. Eaglesham. 1984. Physiological and symbiotic characteristics of fast-growing *Rhizobium japonicum*. Plant Soil 77:3-14.

Trinick, M. J. 1980. Relationships amongst the fast-growing rhizobia of *Lablab purpureus, Leucaena leucocephala, Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinity with other rhizobial groups. J. Appl. Bacteriol. 49:39-53.

Van Rensburg, H. J., B. W. Strijdom and M. M. Kriel. 1976. Necessity for seed inoculation of soybeans in South Africa. Phytophylactica 8:91-95.

Van Rensburg, H. J., B. W. Strijdom and C. J. Otto. 1983. Effective nodulation of soybeans by fast-growing strains of *Rhizobium japonicum*. South African J. Sci. 79:251-252.

Vincent, J. M. 1974. Root-nodule symbiosis with *Rhizobium*. p. 265-347. *In* A. Quispel (ed.) Biology of nitrogen fixation. North-Holland Publishing Co., Amsterdam.

Yelton, M. M., S. S. Yang, S. A. Edie and S. T. Lim. 1983. Characterization of an effective salt-tolerant, fast-growing strain of *Rhizobium japonicum*. J. Gen. Microbiol. 129:1537-1547.

42

Plant Physiol. (1985) 77, 53–58
0032-0889/85/77/0053/06/$01.00/0

Relationship between Ureide N and N_2 Fixation, Aboveground N Accumulation, Acetylene Reduction, and Nodule Mass in Greenhouse and Field Studies with *Glycine max* L. (Merr)[1]

Received for publication May 30, 1984 and in revised form August 28, 1984

PETER VAN BERKUM*[2], CHARLES SLOGER, DEANE F. WEBER, PERRY B. CREGAN, AND HAROLD H. KEYSER[3]
Nitrogen Fixation and Soybean Genetics Laboratory, United States Department of Agriculture, Beltsville Agricultural Research Center, Building 011, HH-19, Beltsville, Maryland 20705

ABSTRACT

The relationship between ureide N and N_2 fixation was evaluated in greenhouse-grown soybean (*Glycine max* L. Merr.) and lima bean (*Phaseolus lunatus* L.) and in field studies with soybean. In the greenhouse, plant N accumulation from N_2 fixation in soybean and lima bean correlated with ureide N. In soybean, N_2 fixation, ureide N, acetylene reduction, and nodule mass were correlated when N_2 fixation was inhibited by applying KNO_3 solutions to the plants. The ureide-N concentrations of different plant tissues and of total plant ureide N varied according to the effectiveness of the strain of *Bradyrhizobium japonicum* used to inoculate plants. The ureide-N concentrations in the different plant tissues correlated with N_2 fixation. Ureide N determinations in field studies with soybean correlated with N_2 fixation, aboveground N accumulation, nodule weight, and acetylene reduction. N_2 fixation was estimated by ^{15}N isotope dilution with nine and ten soybean genotypes in 1979 and 1980, respectively, at the V9, R2, and R5 growth stages. In 1981, we investigated the relationship between ureide N, aboveground N accumulation, acetylene reduction, and nodule mass using four soybean genotypes harvested at the V4, V6, R2, R4, R5, and R6 growth stages. Ureide N concentrations of young stem tissues or plants or aboveground ureide N content of the four soybean genotypes varied throughout growth correlating with acetylene reduction, nodule mass, and aboveground N accumulation. The ureide-N concentrations of young stem tissues or plants or aboveground ureide-N content in three soybean genotypes varied across inoculation treatments of 14 and 13 strains of *Bradyrhizobium japonicum* in 1981 and 1982, respectively, and correlated with nodule mass and acetylene reduction. In the greenhouse, results correlating nodule mass with N_2 fixation and ureide N across strains were variable. Acetylene reduction in soybean across host-strain combinations did not correlate with N_2 fixation and ureide N. N_2 fixation, ureide N, acetylene reduction, and nodule mass correlated across inoculation treatments with strains of *Bradyrhizobium* spp. varying in effectiveness on lima beans. Our data indicate that ureide-N determinations may be used as an additional method to acetylene reduction in studies of the physiology of N_2 fixation in soybean. Ureide-N measurements also may be useful to rank strains of *B. japonicum* for effectiveness of N_2 fixation.

Soybeans and lima beans are able to derive N from N_2 by the active participation of *Bradyrhizobium* spp. (8) in the plant root nodules. Various methods are used to estimate the magnitude of N_2 fixation, which principally depends upon the effectiveness of the host-strain combination and/or the quantity of combined N available to the plants (15). Acetylene reduction has been extensively used in the study of physiology of N_2 fixation in soybeans. Host and strain symbiotic performance has usually been evaluated by acetylene reduction in combination with determinations for nodule mass, plant dry matter, and total N. Estimates of N_2 fixation based on methods with ^{15}N have also been made, but the expense incurred and the complex experimental designs have prohibited widespread use of this technique.

In several legumes, allantoin and allantoic acid, originating predominantly from N_2 fixation (4), are the principal forms of N transported (8). The relative ureide-N concentration in greenhouse-grown soybeans has been shown to decline with the addition of combined N to the plants, which was reported to indicate a quantitative relationship between ureide-N concentration and N_2 fixation (6, 11–13). However, ureide-N concentrations relative to measurements for N_2 fixation made by comparing the total N of N_2 fixing and control plants in greenhouse studies have not been reported. Patterson and LaRue (17) reported a quantitative relationship between ureide-N concentration and acetylene reduction in studies with field-grown soybeans. However, ureide-N concentration relative to measurements for ^{15}N isotope dilution, aboveground N accumulation, and nodule mass have not been reported. Our objectives were to evaluate ureide N measurements in greenhouse- and field-grown plants as estimates of N_2 fixation based on total N accumulation, ^{15}N isotope dilution, acetylene reduction, and nodule mass determinations.

MATERIALS AND METHODS

Plant Material. Seed of *Glycine max* cv 'Clark' nodulating and nonnodulating (Clark rj_1rj_1) isolines, and *Phaseolus lunatus* cv 'Jackson Wonder' were surface sterilized with acidified 0.1% (w/v) $HgCl_2$ (22) for 3 min and washed five times with sterile distilled H_2O. Surface sterile seeds were sown in sterile vermiculite moistened with 1.0 L nutrient solution (14), covered with a 2-cm layer

[1] Supported partly by United States Department of Agriculture Grant 82-CRCR-1-1039.

[2] Supported by United States Department of Agriculture, Nitrogen Fixation and Soybean Genetics Laboratory, Beltsville, MD 20705, and the Agronomy Department, University of Maryland, College Park, MD 20742, under Cooperative Agreement 58-32UA-3-370. Scientific Article No. A-3860, Contribution No. 6840, of the Maryland Agricultural Experiment Station, Department of Agronomy, College Park, MD 20742.

[3] Supported by United States Agency for International Development-United States Department of Agriculture RSSA 4-76.

43

Table I. *Correlation (r) between Measurements for Ureides, Acetylene Reduction, Nodule Mass, and Nitrogen Fixation Determined with G. max cv 'Clark' × USDA122 Grown with Eight Different Rates of KNO_3 Addition*

All correlation coefficients are significant at P = 0.01. Determinations were made with plants 47 d after sowing.

	Plant Ureide-N Concn.	Plant Top Ureide-N Concn.	Nodule Mass	Acetylene Reduction
	mg N/g dry wt		*mg/plant*	*μmol/plant·h*
N from N_2 fixation[a]	0.88	0.89	0.84	0.91
Plant ureide-N concn.[b]		1.00	0.94	0.97
Plant top ureide-N concn.[b]			0.95	0.98
Nodule mass				0.93
Acetylene reduction				

[a] N_2 fixation was determined from the difference in total N between the nodulating and nonnodulating isolines.

[b] Ureide-N concentrations were corrected by subtracting the values obtained with the nonnodulating isoline.

of sterile perlite, in 23-cm-diameter pots rinsed in 1% Clorox[4] solution followed by three rinses of water. The nutrient solution was N free except for a combined N experiment in which KNO_3 and KCl were added to final concentrations of 0, 0.5, 1.0, 1.5, 3.0, 4.5, 6.0, and 12.0 mM NO_3^- and 12.0 mM K^+. Each pot was inoculated with 10 ml of a culture of approximately 10^9 cells/ml of *Bradyrhizobium*, which were grown for 7 d in yeast-mannitol salts broth (21). The strains of *Bradyrhizobium* were obtained from the USDA Rhizobium Collection at Beltsville (9). The pots were placed in saucers and watered from below. In the combined N experiment, the plants were watered with distilled H_2O to which KNO_3 and KCl had been added in the same proportions as the initial additions in nutrient solution. Four plants were grown per pot in a greenhouse without supplemental lighting and each of the treatments was tested in triplicate. The pots of the NO_3^- experiment contained two nodulating and two nonnodulating isolines of Clark rj_1 rj_1 inoculated with USDA122 at each level of NO_3^- addition.

Nitrogen fixation of soybean grown in ^{15}N-enriched field plots was determined during the 1979 and 1980 seasons using the ^{15}N dilution technique (19). The soil organic matter was enriched with ^{15}N in 1977 (19). The experimental design was randomized complete block with a split plot arrangement of the treatments. Whole plots consisted of harvest dates corresponding to the V9, R2, and R5 growth stages (3). Subplots were the *G. max* genotypes, 'Ware', 'Bonus', 'Emerald', 'Kent', L74-914, A 75-302017, 'Wabash', D66-5566, 'Delmar', and 'Clark'. In 1979, the plots consisted of single 0.6 m rows spaced 0.7 m apart. Each plot was bordered by a 0.6 m row of the nonnodulating genotype 'Clark rj_1rj_1'. In 1980, plots consisted of a single 0.3 m row with 0.7 m between rows. A 0.3 m plot of Clark rj_1rj_1 was planted within the row adjacent to each nodulating genotype. At each harvest date, aboveground plant tissues from the plots were harvested for determinations of N_2 fixation and ureide N.

Soybean genotypes Bonus, Emerald, Clark, and Delmar were grown in a low N sandy loam soil at the Beltsville Agricultural Research Center in 1981 to examine the relationships among aboveground N accumulation, ureide-N, acetylene reduction, and nodule mass. The plants were grown in six replicate 15 m long rows spaced 0.6 m apart. Inoculum was not applied to the seed at sowing because the soil contained *B. japonicum*. At specific growth stages (V4, V6, R4, R5, and R6), aboveground N, ureide N, acetylene reduction, and nodule mass were determined using five replicate plants from each row.

[4] Mention of a trademark, proprietory product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Table II. *Correlation (r) between Measurements for Ureides, Per Cent N, and N_2 Fixation with Greenhouse-Grown G. max cv 'Clark' Inoculated with 18 Strains of B. japonicum*

All correlation coefficients are significant at P = 0.01. Determinations were made 55 d after sowing. The strains of *B. japonicum* used were: USDA6, USDA24, USDA31, USDA33, USDA46, USDA61, USDA71a, USDA76, USDA110, USDA117, USDA122, USDA123, USDA136, USDA138, USDA140, USDA205, USDA243, USDA309.

Ureide-N	Plant N Concn.	Nitrogen Fixation[a]
	% N	*mg N/plant*
Concn. in young stem tissue (mg/g dry wt)	0.63	0.73
Concn. in plant tops (mg/g dry wt)	0.85	0.93
Concn. in roots and nodules (mg/g dry wt)	0.78	0.83
Concn. in whole plants (mg/g dry wt)	0.84	0.85
Total (mg/plant)	0.81	ND[b]
Concn. in the xylem sap (μg/ml)	0.72	0.71
Flow rates in xylem (μg/h)	0.75	0.83

[a] N_2 fixation was determined from the difference in total N between the nodulating and nonnondulating isolines.

[b] ND = not determined.

Soybean genotypes Williams, Kent, and Essex were grown on assayed *B. japonicum* free-soil at the University of Maryland Tobacco farm station at Upper Marlboro. Plants were grown in a randomized complete block design, each plot consisting of three 6 m rows spaced 0.6 m apart. The seed was coated with 45% neutralized gum arabic and peat inoculum was applied at a rate of 0.2 g/200 seeds before sowing. The peat inoculum contained approximately 10^9 cells of *B. japonicum*/g dry weight. Three plants were taken from three replicate plots at R4 in 1981 and R3 in 1982 to determine ureide N, acetylene reduction, and nodule mass. Several of the strains used in this study (J507, PRC3, PRC43, PRC127, PRC143, PRC205, PRC160, SIa, YIa, WB19, WB69, R54a, 315, 29W, 587, PRCB-15, THA-1, and PRC113-2) have recently been assigned USDA accession numbers (USDA145, USDSA146, USDA159, USDA184, USDA189, USDA205, USDA221, USDA243, USDA244, USDA270, USDA273, USDA277, USDA285, USDA309, USDA310, USDA322, USDA325, and USDA335, respectively).

Measurements. Ureide-N concentration was determined with an autoanalytical procedure (20) with xylem sap collected ac-

44

Table III. *Correlation (r) between Measurements for Ureides and Acetylene Reduction, Nodule Mass, Per Cent N, and N_2 Fixation Determined with Greenhouse-Grown G. max cv 'Clark' across 15 Strains of B. japonicum*

Determinations were made 55 d after sowing. The strains of *B. japonicum* used were: USDA122, USDA145, USDA146, USDA184, USDA189, USDA221, USDA270, USDA273, USDA277, USDA285, USDA309, USDA310, USDA322, USDA325, USDA335.

	Young Stem Ureide-N Concn.	Plant Ureide-N Concn.	Total Plant Ureide-N
	mg N/g dry wt		*mgN/ plant*
% N	0.75**	0.71**	0.80**
Nitrogen fixation[a] (mg N/plant)	0.79**	0.82**	0.85**
Acetylene reduction (μmol/plant·h)	0.22 NS	0.11 NS	0.23 NS
Nodule mass (mg/plant)	0.17 NS	0.13 NS	0.24 NS

[a] N_2 fixation was determined from the difference in total N between the nodulating and nonnodulating isolines.
[b] **, Significant at P = 0.01.

Table IV. *Correlation (r) between Measurements for Ureides and Acetylene Reduction, Nodule Mass, Per Cent N, and N_2 Fixation Determined with Greenhouse-Grown G. max cv 'Clark' across 18 Strains of B. Japonicum*

Determinations were made 77 days after sowing. The strains of *B. japonicum* used were: USDA6, USDA24, USDA31, USDA33, USDA46, USDA61, USDA716, USDA7b, USDA110, USDA117, USDA122, USDA123, USDA136, USDA138, USDA140, USDA205, USDA243, USDA309.

	Young Stem Ureide-N Concn.	Plant Ureide-N Concn.	Total Plant Ureide-N
	mg N/g dry wt		*mg N/ plant*
% N	0.79**	0.96**	0.87**
Nitrogen fixation[a] (mg N/plant)	0.60**	0.90**	0.78**
Acetylene reduction (μmol/plant·h)	0.24 NS	0.39 NS	0.42 NS
Nodule mass (mg/plant)	0.29 NS	0.52*	0.66**

[a] N_2 fixation was determined from the difference in total N between the nodulating and nonnodulating isolines.
[b] **, Significant at P = 0.01; *, significant at P = 0.05.

cording to the method described by McClure *et al.* (13) or with plant material dried at 60°C for 2 d and milled with a Udy Cyclone Sample Mill (Udy Corporation, Boulder, CO). Young stems were obtained by cutting the main stem above the node of the second uppermost, fully expanded trifoliate and removing the leaves. Extracts of the ground plant material used for ureide determination were prepared according to the method described by van Berkum and Sloger (20). Total N of the milled dry plant tissues were determined by Kjeldahl digestion (1) followed by measurement of NH_4^+ with an autoanalytical procedure (2). N_2 fixation in the greenhouse experiments was derived from the difference in total N between nodulated plants and the nonnodulated controls. N_2 fixation was determined by ^{15}N dilution in soybean tissue using the nonnodulating isoline of Clark rj_1rj_1 as control and with the samples prepared and analyzed for ^{15}N according to the methods described by Talbott *et al.* (19). An autoanalytical procedure was used to determine NO_3^- concentration (7) of dried-milled soybean tissue extracted in distilled H_2O at 80°C for 30 min. Acetylene reduction was determined with the roots from detopped plants according to the method

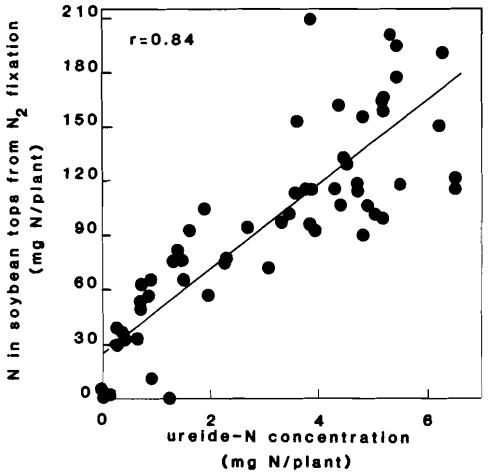


FIG. 1. Relationship between plant top ureide-N concentration and N in the plant top originating from N_2 fixation ($y = 22.0x + 28.4$). The data were obtained by combining the results from all the soybean experiments.

described by Keyser *et al.* (10). The nodules were removed from the roots after acetylene reduction, dried at 60°C for 2 d, weighed, and in greenhouse studies returned to the dried root tissues for milling.

RESULTS

The addition of NO_3^- to greenhouse-grown Clark soybean inoculated with USDA122 significantly decreased N_2 fixation determined from the difference in total N between the nodulating and nonnodulating isolines at each rate of application. Total plant N originating from N_2 fixation ranged from 124 to 0 mg N/plant for the control and 12 mM NO_3^- treatments, respectively. The total N of the nodulating soybean plants among NO_3^- treatments were similar and of the nonnodulating controls increased significantly with higher rates of NO_3^- application. The NO_3^- treatment decreased nodule development, the rate of nitrogenase activity, and the concentration of ureide N of the nodulating plants. The ureide-N concentrations of the nonnodulating plants without NO_3^- and with 12 mM NO_3^- addition were similar. The variation in ureide-N concentration of the nodulating plants across the NO_3^- additions correlated with nitrogen fixation, nodulation, and nitrogenase activity (Table I). Nitrogen fixation, ureide-N concentration, nodulation, and nitrogenase activity were negatively correlated with the NO_3^- concentrations of the nodulating plants across the NO_3^- treatments ($r = -0.99, -0.89, -0.85$, and -0.93, respectively; P = 0.01).

N_2 fixation, determined 55 d after sowing from the difference in total N between nodulated and nonnodulated Clark soybean inoculated with 18 strains of *B. japonicum* ranging from 0 to 138 mg N/plant correlated with ureide concentrations in young stem tissues, plant tops, roots and nodules, xylem sap, and whole plants, and with total plant ureide contents and ureide-N flow rates in the xylem (Table II). The per cent N of the plants ranged from 0.8 to 2.5 across treatments and also correlated with ureide N in the different plant parts. Ureide N in the young stem tissues correlated with ureide-N concentrations in the plant tops, whole plants, xylem sap and with total plant ureide N and with ureide N flow rates in the xylem ($r = 0.97, 0.96, 0.72, 0.95$, and 0.63, respectively; P = 0.01).

Ureide-N concentration in the young stems and plant tops, and total plant ureide-N contents correlated with N_2 fixation and per cent N in a second examination with 55-d-old Clark soybean inoculated with different strains of *B. japonicum* (Table III). Total plant N accumulation originating from N_2 fixation ranged

45'

Table V. *Correlation (r) between Measurements for Ureides, N_2 Fixation, Acetylene Reduction, Nodule Mass, and Per Cent N Determined with Greenhouse-Grown Phaseolus lunatus cv 'Jackson Wonder' across 19 Strains of Bradyrhizobium sp.*

All correlation coefficients are significant at P = 0.01. Determinations were made 33 d after sowing. The strains of *Bradyrhizobium* used were: USDA3250, USDA3251, USDA3252, USDA3252, USDA3254, USDA3255, USDA3256, USDA3257, USDA3258, USDA3259, USDA3260, USDA3261, USDA3313, E12, E16, E17, A4, A5, TAL22.

	N	Nodule Mass	Acetylene Reduction	Plant Ureide-N Concn.
	%	*mg/plant*	*μmol/plant·h*	*mg N/ g dry wt*
Nitrogen fixation[a]	0.96	0.80	0.60	0.91
% N		0.86	0.64	0.96
Nodule mass			0.76	0.80
Acetylene reduction				0.56
Ureide-N concentration				

[a] N_2 fixation was determined from the differences in total N between the inoculated plants and the uninoculated control.

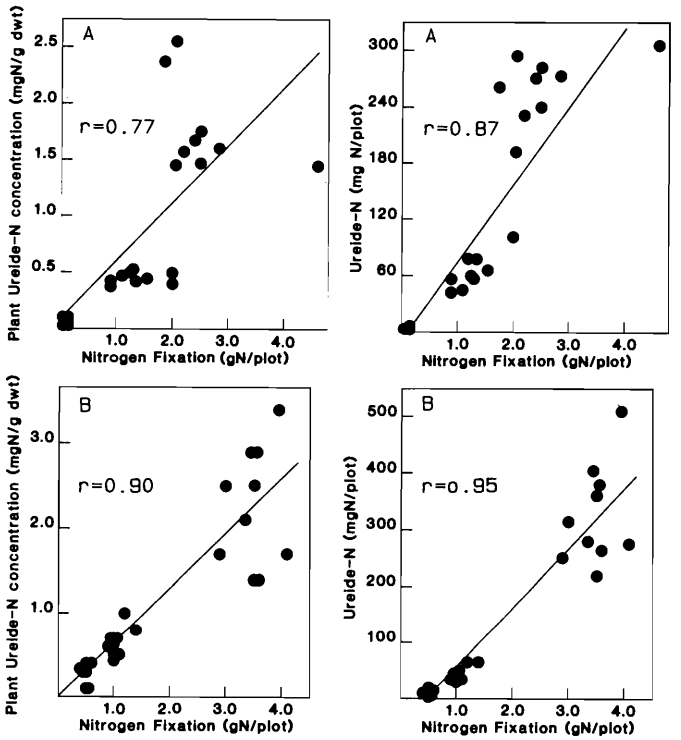


FIG. 2. Comparison of nitrogen fixation and ureide N determined with nine or ten field-grown soybean genotypes at V9, R2, and R5 growth stages during the 1979 and 1980 seasons (A and B, respectively). The determinations were made with total aboveground plant tissues.

from 4 to 132 mg N/plant from the poorest to most effective host × strain combinations, respectively. Acetylene reduction did not correlate with the three ureide-N determinations nor with N_2 fixation and per cent N (r = 0.33 and 0.39, respectively). Similarly, nodule mass did not correlate with the three ureide-N measurements (Table III), nor with N_2 fixation and per cent N (r = 0.29 and 0.42, respectively). However, acetylene reduction correlated with nodule mass (r = 0.79; P = 0.01).

Ureide-N concentration in the young stems and plant tops and total plant ureide-N contents correlated with N_2 fixation and per cent N in a third examination with 77-d-old soybean plants (Table IV). Total plant N accumulation originating from N_2 fixation ranged from 5 to 210 mg N/plant from the poorest to most effective host × strain combinations, respectively. However, rates of acetylene reduction did not correlate with the three ureide-N determinations. Acetylene reduction, also did not correlate with N_2 fixation and per cent N (r = 0.37 and 0.42, respectively). Nodule mass correlated with plant ureide-N concentration and total plant ureide-N content, but not with young stem ureide-N concentration (Table IV). Nodule mass also correlated with N_2 fixation (r = 0.67; P = 0.01), and acetylene reduction (r = 0.51; P = 0.05), but not with % N (r = 0.37).

The results obtained from the investigations with soybean were combined to test whether the ureide-N concentration determined under variable conditions correlated with the N in the plant tops originating from N_2 fixation. Ureide-N concentration and N_2 fixation correlated (r = 0.84; P = 0.01) and a regression line was used to describe the relationship (Fig. 1).

Correlations between measurements for N_2 fixation, per cent N, plant ureide-N concentrations, acetylene reduction, and nodule mass were observed in *P. lunatus* cv Jackson Wonder inoculated with 19 strains of *Bradyrhizobium* sp. (Table V). Total plant N accumulation originating from N_2 fixation ranged from 0 to 34 mg N/plant from the poorest to most effective host × strain combinations, respectively.

The relationship between ureide-N and N_2 fixation determined by ^{15}N dilution was examined with aboveground soybean tissue using nine or ten genotypes and the nonnodulating (rj_1rj_1) isoline of Clark as control during the seasons of 1979 and 1980. Correlations between N_2 fixation and ureide N were derived across three harvest dates during late vegetative and reproductive growth stages (Fig. 2). The measurements for N_2 fixation among nine or ten genotypes at each harvest date were not significantly different.

The relationship between ureide-N content of soybean and acetylene reduction, nodule mass, or total aboveground N accumulation was investigated using four genotypes of soybean. The ureide-N concentrations in the young stem tissues and plants and aboveground ureide-N content during plant development correlated with the rate of acetylene reduction, nodule mass, and aboveground N accumulation (Table VI). The plant ureide-N concentration, aboveground N accumulation, acetylene reduction, and nodule mass varied across the growing season of the four soybean genotypes with 1.11 mg ureide N/g dry weight, 35 mg/plant, 9.6 μmol C_2H_4/plant·h, and 69 mg/plant at the first harvest date ranging to 2.65 mg ureide N/g dry weight, 1396 mg/plant, 47.6 μmol C_2H_4/plant·h, and 900 mg/plant for the highest recorded levels, respectively. Aboveground N accumulation across the six dates correlated with acetylene reduction and nodule mass (r = 0.77 and 0.91, respectively, P = 0.01), and the

46

Table VI. *Correlation (r) between Measurements for Ureides and Acetylene Reduction, Nodule Dry Weight, and Aboveground N Accumulation Determined Six Times across the Growing Season of Four Genotypes of G. max*

Growth stages of aboveground N accumulation determinations were V4, V6, R2, R4, R5, R6.

	Aboveground Ureide-N Content	Plant Ureide-N Concn.	Young Stem Ureide-N Concn.
	mg N/plant	*mg N/g dry wt*	
Above ground N (g N/plant)	0.92[a]**	0.61**	0.67**
Acetylene reduction (μmol/plant·h)	0.71**	0.62**	0.61**
Nodule mass (mg dry wt/plant)	0.81**	0.66**	0.69**

[a] **, Significant at P = 0.01.

Table VII. *Correlation (r) of Ureide Analysis with Acetylene Reduction and Nodule Dry Weight Determinations with Field-Grown Glycine max cv 'Williams' and 'Kent' at R4 in the 1981 Season and 'Kent' and 'Essex' at R3 in the 1982 Season Across Inoculation Treatments of B. japonicum*

In 1981, the treatments were: USDA110, USDA145, USDA146, USDA159, USDA241, USDA243, USDA270, USDA277, USDA285, USDA309, USDA310, USDA322, USDA325, USDA335, and uninoculated control. In 1982, the treatments were: USDA31, USDA110, USDA122, USDA145, USDA184, USDA189, USDA221, USDA243, USDA273, USDA277, USDA309, USDA310, USDA335, and uninoculated control.

		Young Stem Ureide-N Concn.		Plant Ureide-N Concn.		Aboveground Ureide N	
	1981	Wiliams	Kent	Williams	Kent	Williams	Kent
	1982	Kent	Essex	Kent	Essex	Kent	Essex
		mg/g dry wt				*mg/plant*	
Rate of acetylene reduction	1981	0.52**	0.75**	0.70**	0.64**	0.67**	0.77**
(μmol/plant·h)	1982	0.54*	0.73**	0.61**	0.75**	0.81**	0.77**
	1981	0.61**	0.65**	0.87**	0.66**	0.68**	0.87**
Nodule mass (g dry wt/plant)	1982	0.70**	0.69**	0.73**	0.58*	0.68**	0.60*

*, Significant at P = 0.05; **, significant at P = 0.01.

rate of acetylene reduction and nodule mass across the six dates also correlated ($r = 0.85$; P = 0.01).

The relationship between the variation of ureide N and acetylene reduction or nodule mass at one sampling date was investigated two successive years using three genotypes of soybean across inoculation treatments of *B. japonicum* varying in effectiveness for N_2 fixation. The concentration of ureide N in the young stem and plant tissues and aboveground ureide-N content of Williams and Kent in the 1981 season correlated with acetylene reduction and nodule mass (Table VII). Correlations were also observed for each genotype between acetylene reduction and nodulation across inoculation treatments ($r = 0.68$ and 0.91, respectively; P = 0.01). The analyses of ureide N of Kent and Essex in the 1982 season also correlated with acetylene reduction and nodulation (Table VII). Correlations were also observed for each genotype between acetylene reduction and nodulation across inoculation treatments ($r = 0.85$ and 0.81, respectively; P = 0.01).

DISCUSSION

We report that in a greenhouse study with soybean and lima bean, plant N accumulation from N_2 fixation correlated with ureide N, that ureide N in field studies with soybean genotypes correlated with N_2 fixation estimated by ^{15}N isotope dilution, and that across inoculation treatments of *B. japonicum* ureide N correlated with nodule weight and acetylene reduction. N_2 fixation was modulated in our study by using different strains of *Bradyrhizobium* varying in their effectiveness for N_2 fixation, by sampling across growth season, or by inhibiting N_2 fixation with solutions of KNO_3.

The relative ureide content of xylem sap (12, 13) or plant tissue (6, 11) has been suggested to indicate N_2 fixation in greenhouse-grown soybean plants supplied with solutions of combined N. However, in these studies N_2 fixation was estimated from the enrichment of xylem sap with ^{15}N from $K^{15}NO_3$ (13), nodule mass determinations (11, 12), or measurements of acetylene reduction (6). Our approach was to measure N_2 fixation across the NO_3^- treatments by determining the difference in total N between nodulated and nonnodulated plants grown in the same pots. N_2 fixation, nodulation, acetylene reduction, and the ureide-N concentrations decreased proportionately as the NO_3^- concentrations supplied to the plants were increased. Therefore, the data from our study support the suggestions of a quantitative relationship between ureide N and N_2 fixation in greenhouse-grown soybeans supplied with nutrient solutions containing different concentrations of combined N.

McClure *et al.* (13) proposed widespread applicability for the use of relative ureide content of xylem sap as indicators of N_2 fixation in greenhouse-grown soybean. The ureide-N analyses of soybean xylem sap and different plant tissues in our study correlated with each other as well as with measurements for N_2 fixation among inoculation treatments with 18 strains of *B. japonicum*. Therefore, either the measurement of ureide-N in the tissues or xylem sap may be used to indicate N_2 fixation in greenhouse studies with soybean. Plant ureide-N concentration correlated with the total N in the plant tops originating from N_2 fixation when we combined the greenhouse data of the experiments with soybeans. Therefore, the ureide-N concentration can probably be used to estimate N_2 fixation in greenhouse-grown Clark soybean. Similar regression lines could possibly be used with other soybean genotypes to estimate N_2 fixation.

In field studies with soybean, we determined that ureide N and acetylene reduction were correlated similar to the observations reported by Patterson and LaRue (17). We also have shown that the ureide-N concentration in field-grown soybean increased

47

as N accumulated from N_2 fixation when estimated by ^{15}N dilution or aboveground N accumulation. Patterson and LaRue (17) suggested that the ureide-N concentration of plants ranked genotypes of soybean for nitrogenase activity. We were unable to determine whether ureide-N concentration of plants ranked genotypes of soybean for N_2 fixation because estimates at each date of sampling were similar. However, our data indicate that the ureide-N concentration of field-grown soybeans may rank strains of *B. japonicum* for nitrogenase activity and nodulation.

Acetylene reduction and nodule mass measurements did not always correspond with N_2 fixation by soybeans in greenhouse studies indicating these parameters by themselves to be unsuitable for evaluation of strain effectiveness of *B. japonicum*. The spectrum of effectiveness among strains used in the field was narrower than in the greenhouse, probably causing the disparity between the two results. Ureide N and acetylene reduction in greenhouse-grown lima beans correlated indicating that the symbiotic characteristics among strains may influence the relationship between different methods to evaluate N_2 fixation. Nevertheless, tissue ureide N may be more appropriate than nodule mass or acetylene reduction to evaluate strain effectiveness in the greenhouse, because ureide N correlated with N_2 fixation.

Our data indicate that ureide-N determinations may be used as an additional method to acetylene reduction in studies of the physiology of N_2 fixation of soybean. Ureide-N measurements also may be useful to rank strains of *B. japonicum* for effectiveness of N_2 fixation. Patterson and LaRue (17) have previously suggested that ureide-N concentration also ranks soybean genotypes for N_2-fixing ability. The potential advantages of the ureide analysis in N_2 fixation research are that many determinations can be made inexpensively and plants need not be destroyed if a portion of the young stem is sampled. Furthermore, the methodology used to determine plant tissue ureide N (20) is convenient because the simple analyses are made rapidly with samples which can be stored without significantly affecting the results (6, 16).

Acknowledgments—We wish to thank Dr. John Meisinger for the ^{15}N determinations. The technical assistance of Michael B. McMahon, Deborah J. Thibeau, Jeffrey N. Powers, and Richard F. Griffin is gratefully appreciated.

LITERATURE CITED

1. Eastin EF 1978 Total nitrogen determination of plant material containing nitrate. Anal Biochem 85: 591–594
2. Eastin EF 1978 Use of an autoanalyzer for total nitrogen determination in plants. Commun Soil Sci Plant Anal 9: 107–113
3. Fehr WR, CE Caviness, DT Burmood, JS Pennington 1971 Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. Crop Sci 11: 929–931
4. Fujihara S, M Yamaguchi 1978 Probable site of allantoin formation in nodulating soybean plants. Phytochemistry 17: 1239–1243
5. Harper JE, RH Hageman 1972 Canopy and seasonal profiles of nitrate reductase in soybeans (*Glycine max* L. Merr.) Plant Physiol 49: 146–154
6. Herridge DF 1982 Relative abundance of ureides and nitrate in plant tissues of soybean as a quantitative assay of nitrogen fixation. Plant Physiol 70: 1–6
7. Jackson WA, CE Frost, DM Hildreth 1975 Versatile multirange analytical manifold for automated analysis of nitrate-nitrogen. Soil Sci Am Proc 39: 592–593
8. Jordan DE 1982 Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants Int J Syst Bacteriol 32: 136–139
9. Keyser HH, DF Weber 1979 USDA-Beltsville Rhizobium Culture Collection Catalogue. Plant Physiology Institute Report 16. United States Department of Agriculture, Agricultural Research Service, Beltsville, MD
10. Keyser HH, P van Berkum, DF Weber 1982 A comparative study of the physiology of symbioses formed by *Rhizobium japonicum* with *Glycine max*, *Vigna unguiculata*, and *Macroptilium atropurpureum*. Plant Physiol 70: 1626–1630
11. Matsumoto T, M Yatazawa, Y Yamamoto 1977 Effects of exogenous nitrogen compounds on the concentrations of allantoin and various constituents in several organs of soybean plants. Plant Cell Physiol 18: 613–624
12. McClure PR, DW Israel 1979 Transport of nitrogen in the xylem of soybean plants. Plant Physiol 64: 411–416
13. McClure PR, DW Israel, RJ Volk 1980 Evaluation of the relative ureide content of xylem sap as an indicator of N_2 fixation in soybeans. Plant Physiol 66: 720–725
14. Norris DO 1964 Techniques used in work with *Rhizobium*. Commonw Bur Pastures Field Crops Hurley Berkshire Bull 47: 186–198
15. Pate JS 1977 Functional biology of dinitrogen fixation by legumes. *In* RWF Hardy, WS Silver, eds, A Treatise on Dinitrogen Fixation, Section III, Biology. John Wiley & Sons, New York, pp 473–518
16. Patterson TG, TA LaRue 1983 N_2 fixation (C_2H_2) and ureide content of soybeans: environmental effects and source-sink manipulations. Crop Sci 23: 819–824
17. Patterson TG, TA LaRue 1983 N_2 fixation (C_2H_2) and ureide content of soybeans: ureides as an index of fixation. Crop Sci 23: 825–831
18. Reinbothe H, K Mothes 1962 Urea, ureides, and guanidines in plants. Annu Rev Plant Physiol 13: 129–150
19. Talbott HJ, WJ Kenworthy, JO Legg 1982 Field comparison of the nitrogen-15 and difference methods of measuring nitrogen fixation. Agron J 74: 799–804
20. van Berkum P, C Sloger 1983 Autoanalytical procedure for the determination of allantoin and allantoic acid in soybean tissue. Plant Physiol 73: 511–513
21. Vincent JM 1970 A Manual for the Practical Study of Root Nodule Bacteria. IBP Handbook No. 15. Blackwell Scientific Publications, Oxford
22. Wright WH 1925 The nodule bacteria of soybeans. I. Bacteriology of strains. Soil Sci 20: 95–129

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1985, p. 772–777
0099-2240/85/040772-06$02.00/0
Copyright © 1985, American Society for Microbiology
Vol. 49, No. 4

Anaerobic Growth and Denitrification among Different Serogroups of Soybean Rhizobia†

PETER VAN BERKUM* AND HAROLD H. KEYSER

Nitrogen Fixation and Soybean Genetics Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

Received 7 September 1984/Accepted 11 January 1985

We screened soybean rhizobia originating from three germplasm collections for the ability to grow anaerobically in the presence of NO_3^- and for differences in final product formation from anaerobic NO_3^- metabolism. Denitrification abilities of selected strains as free-living bacteria and as bacteroids were compared. Anaerobic growth in the presence of NO_3^- was observed in 270 of 321 strains of soybean rhizobia. All strains belonging to the 135 serogroup did not grow anaerobically in the presence of NO_3^-. An investigation with several strains indicated that bacteria not growing anaerobically in the presence of NO_3^- also did not utilize NO_3^- as the sole N source aerobically. An exception was strain USDA 33, which grew on NO_3^- but failed to denitrify. Dissimilation of NO_3^- by the free-living cultures proceeded without the significant release of intermediate products. Nitrous oxide reductase was inhibited by C_2H_2, but preceding steps of denitrification were not affected. Final products of denitrification were NO_2^-, N_2O, or N_2; serogroups 31, 46, 76, and 94 predominantly liberated NO_2^-, whereas evolution of N_2 was prevalent in serogroups 110 and 122, and all three were formed as final products by strains belonging to serogroups 6 and 123. Anaerobic metabolism of NO_3^- by bacteroid preparations of *Bradyrhizobium japonicum* proceeded without delay and was evident by NO_2^- accumulation irrespective of which final product was formed by the strain as free-living bacteria. Anaerobic C_2H_2 reduction in the presence of NO_3^- was observed in bacteroid preparations capable of NO_3^- respiration but was absent in bacteria that were determined to be deficient in dissimilatory nitrate reductase.

Anaerobic metabolism of nitrate (NO_3^-) by free-living cells and bacteroid preparations of *Rhizobium* sp. and *Bradyrhizobium* sp. has been reported (2–4, 7, 14, 18, 20, 25, 26). Most bradyrhizobia grow anaerobically in the presence of NO_3^-; growth is mediated by the ability of the organism to denitrify (2, 3, 14). However, denitrification among rhizobia is rare (3, 25), and few strains have been reported to grow anaerobically in the presence of NO_3^- (3, 7). The dissimilation of NO_3^- to gaseous products (N_2O or N_2) by *Bradyrhizobium japonicum* has been suggested as a characteristic of uptake hydrogenase-positive (Hup^+) phenotypes, whereas uptake hydrogenase-negative (Hup^-) cultures produced nitrite (NO_2^-) from NO_3^- (14). However, inferences of the relationship between growth or Hup character and denitrification in *B. japonicum* have been made from the analysis of only 20 different strains.

Several studies have indicated the absence of nitrite reductase in bacteroids of *B. japonicum* (2, 20), although most of the strains tested anaerobically as free-living cells produce gaseous products from NO_3^- (3, 25). A methodical examination of *B. japonicum* relating the characteristics of denitrification by free-living cells with those of denitrification by bacteroid preparations of the same strains has not been reported.

Isolations made from root nodules and soil samples of the People's Republic of China have yielded soybean-nodulating rhizobia which are physiologically and biochemically different from *B. japonicum* (9, 10, 19). Furthermore, a survey of *B. japonicum* distribution in 12 states of the United States (12) has furnished strains belonging to nine different serogroups charactertized for Hup phenotype. Our objectives were (i) to characterize anaerobic growth in the presence of NO_3^- and determine products of denitrification in strains of soybean rhizobia and bradyrhizobia from the People's Republic of China, the U.S. survey, and the U.S. Department of Agriculture collection; (ii) to relate the product of anaerobic NO_3^- metabolism with the serogroup of each organism; (iii) to determine the relationship between denitrification and anaerobic growth by the soybean rhizobia from China; (iv) to investigate Hup phenotype and product of anaerobic NO_3^- metabolism in the U.S. survey germplasm; and (v) to compare denitrification abilities in free-living and bacteroid preparations of selected characterized strains of bradyrhizobia.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of soybean rhizobia and bradyrhizobia used in this study originated from three germplasm collections; the U.S. Department of Agriculture (USDA) culture collection (11), isolates from the People's Republic of China (9, 10), and a survey of serogroup identity and hydrogenase phenotype distribution in the United States (12). Of the U.S. survey isolates used in this study, hydrogenase-positive and -negative (Hup^+ and Hup^-) representatives for each serogroup, except for serogroup 135, were chosen to test for a relationship between hydrogenase and denitrification. Stock cultures of each strain were maintained at 4°C on yeast-salts-mannitol agar (22). Yeast-salts-mannitol broth (YMB) was used to prepare inocula for experiments. In the case of strains belonging to serogroup 135, the YMB was modified by replacing the 10 g of mannitol per liter with 2.5 g each of mannitol, arabinose, and sodium glutamate per liter. Anaerobic growth in the

* Corresponding author.
† Scientific article no. A-3908, contribution no. 6889, of the Maryland Agricultural Experiment Station.

49

presence of 6 mM KNO_3 was determined at 30°C in 3 ml of YMB or modified YMB under 1 ml of sterile mineral oil in 10-ml stoppered serum vials inoculated with approximately 10^7 cells as described by Daniel et al. (3). Products formed by anaerobic metabolism of NO_3^- were determined with 19-ml turbid broth cultures (approximately 5×10^8 cells per ml) in 36-ml serum vials 3 days after the addition of 20 μmol of sterile KNO_3 per ml and 3 ml of sterile mineral oil. The formation of N_2 from NO_3^- was detected by the disappearance of NO_3^- and by the formation of N_2O in the presence of 1% (vol/vol) C_2H_2 (24). Aerobic growth with KNO_3 as the sole N source in a defined growth medium (22) was determined at 30°C with strains failing to grow anaerobically in the presence of NO_3^-. Strain USDA 33 was tested for growth in the presence of $KClO_3$ by the method of Stephens and Neyra (20) to select for dissimilatory nitrate reductase-negative mutants of *B. japonicum*.

Serogroup determination. Isolates were grown as described to a density of approximately 10^9 cells per ml, and the heat-labile flagellar antigens were destroyed by heating the cultures to 100°C for 30 min. Rabbit antisera prepared against serotype strains USDA 6, USDA 31, USDA 46, USDA 76, USDA 94, USDA 110, USDA 122, USDA 123, and USDA 135 were used in agglutination tests at a 1:100 final concentration. Each isolate was tested against all antisera with the short agglutination procedure (22).

Plant culture and bacterial preparation. Seeds of soybean (*Glycine max* L. Merr.) cv. Williams were surface sterilized with acidified 0.1% (wt/vol) $HgCl_2$ (22) for 3 min and washed five times with sterile distilled water. Surface sterile seed was sown in vermiculite moistened with 100 ml of an N-free nutrient solution (17) in modified Leonard jars (13) sterilized with steam for 4 h. Each jar was inoculated with 4 ml of approximately 10^9 *B. japonicum* cells per ml, grown as described above for 7 days. The strains used were USDA 6, USDA 24, USDA 33, USDA 61, USDA 110, USDA 135, USDA 147, USDA 154, USDA 176, USDA 221, USDA 227, LA3 2a, and NC4 5c, selected on the basis of their characteristic of anaerobic NO_3^- utilization as determined in vegetative cells. Bacteroids were prepared at 4°C under Ar with a Sorvall homogenizer with approximately 2 g (fresh weight) of nodules in 50 ml of phosphate-ascorbate buffer (pH 7.4) containing 4 g of acid-washed polyvinylpolypyrrolidone and 30 mg of sodium dithionite (5). The homogenates were passed through four layers of cheesecloth and centrifuged at $5,000 \times g$ for 10 min. The bacteroids were washed once with and resuspended in 50 ml of Tris buffer (pH 8.0) under Ar. Nitrate respiration and NO_3^--supported anaerobic C_2H_2 reduction were determined at 25°C with 36-ml serum vials containing 2 ml of bacteroid preparation, 2 ml of 250 mM sodium succinate–50 mM KNO_3, and 12 ml of 50 mM Tris buffer (pH 8.0) under Ar with or without 12% (vol/vol) C_2H_2.

Measurements. In all experiments, samples of liquid (0.1 ml) and gas (0.2 ml) were taken for analyses. The qualitative analysis for NO_2^- was as described by Neyra and van Berkum (16). Quantitative analyses for NO_3^- and NO_2^- were accomplished by using an automated procedure (8). Denitrification of NO_3^- to N_2O by cultures with or without the addition of 1% (vol/vol) C_2H_2 was determined by gas chromatography (26). The analyses of C_2H_2 and C_2H_4 were as described by van Berkum and Sloger (21). Bacteroid preparations were lysed by boiling for 5 min in 0.1 M NaOH (20), and protein concentrations were determined as described by Bradford (1) with bovine serum albumin as standard.

RESULTS

B. japonicum USDA 31, USDA 122, USDA 138, USDA 147, USDA 154, and USDA 221 grown in YMB without NO_3^- supplement were tested for denitrification with or without C_2H_2 addition by time course analyses (Fig. 1). Acetylene was used to inhibit nitrous oxide reductase to identify denitrification of NO_3^- to N_2 as the final product in preparations without C_2H_2 (24). The results with USDA 138, USDA 122, and USDA 31 were similar to those with USDA 147, USDA 154, and USDA 221, respectively. The metabolism of NO_3^- after a delay of approximately 4 h was evident in all of the strains tested by the disappearance of NO_3^- from the culture solution. Accumulation of NO_2^- was observed with USDA 31 and USDA 221, whereas significant concentrations of NO_2^- were not detectable in the culture solutions of the other four strains. USDA 138 and USDA 147 formed N_2O from NO_3^-, whereas USDA 122 and USDA 154 accumulated N_2O only in the presence of C_2H_2. The kinetics of NO_3^- disappearance from culture solution in all the strains and final product formation in USDA 31, USDA 138, USDA 147, and USDA 221 were not affected by C_2H_2. Agglutination tests indicated that USDA 31, USDA 122, USDA 138, and USDA 147 belong to serogroups 31, 122, 6, and 6, respectively. USDA 154 and USDA 221 did not agglutinate with any of the nine antisera used.

Anaerobic growth in the presence of KNO_3 was observed with 26 of the 28 strains of *B. japonicum* tested belonging to the USDA culture collection (Table 1). Anaerobic growth in the presence of KNO_3 by USDA 33 was poor, and no growth was detected with USDA 135. Aerobic growth was observed with USDA 33 but not with USDA 135 when KNO_3 was supplied as the sole N source. USDA 33 also grew at reduced pO_2 on yeast-salts-mannitol agar supplemented with 10 mM $KClO_3$. Among the 26 strains able to grow anaerobically in the presence of KNO_3, 42% produced NO_2^-, 23% evolved N_2O, and 35% liberated N_2 as final products of NO_3^- metabolism. With the exception of USDA 145, USDA 273, USDA 277, and USDA 325, the 28 strains were observed to have antigens in common with the nine serogroups tested. Strains in serogroups 31, 46, 76, and 94 predominantly produced NO_2^- from KNO_3, whereas those in serogroups 6, 110, 122, and 123 evolved N_2O or N_2, except for USDA 3, which produced NO_2^-.

Anaerobic growth in the presence of KNO_3 was observed with 150 of the 195 isolates of *B. japonicum* which originated from a survey study in the United States (Table 2). All isolates belonging to serogroup 135, two in serogroup 110, and one each in serogroups 31 and 6 did not grow anaerobically in the presence of KNO_3. Five of these strains were tested aerobically with KNO_3 as the sole N source and did not grow. Isolates in serogroups 31, 46, 76, and 94 predominantly produced NO_2^- from KNO_3. Two isolates each in serogroups 31, 76, and 94 evolved N_2, and one isolate in serogroup 94 liberated N_2O during denitrification. Isolates in serogroups 110 and 122 predominantly evolved N_2 during denitrification except for two serogroup 122 isolates which produced NO_2^-. All three products of NO_3^- respiration were detected with different isolates belonging to serogroups 6 and 123. Isolates belonging to serogroup 135 did not respire NO_3^- anaerobically, since the NO_3^- concentrations in the culture solutions at the end of the experiment were unchanged from the initial addition. We studied 70 Hup^+ survey isolates, of which 40, 11, and 46% produced NO_2^-, N_2O, and N_2, respectively, and 3% liberated no product. The survey also included 125 Hup^- isolates, of which 46, 9,

50

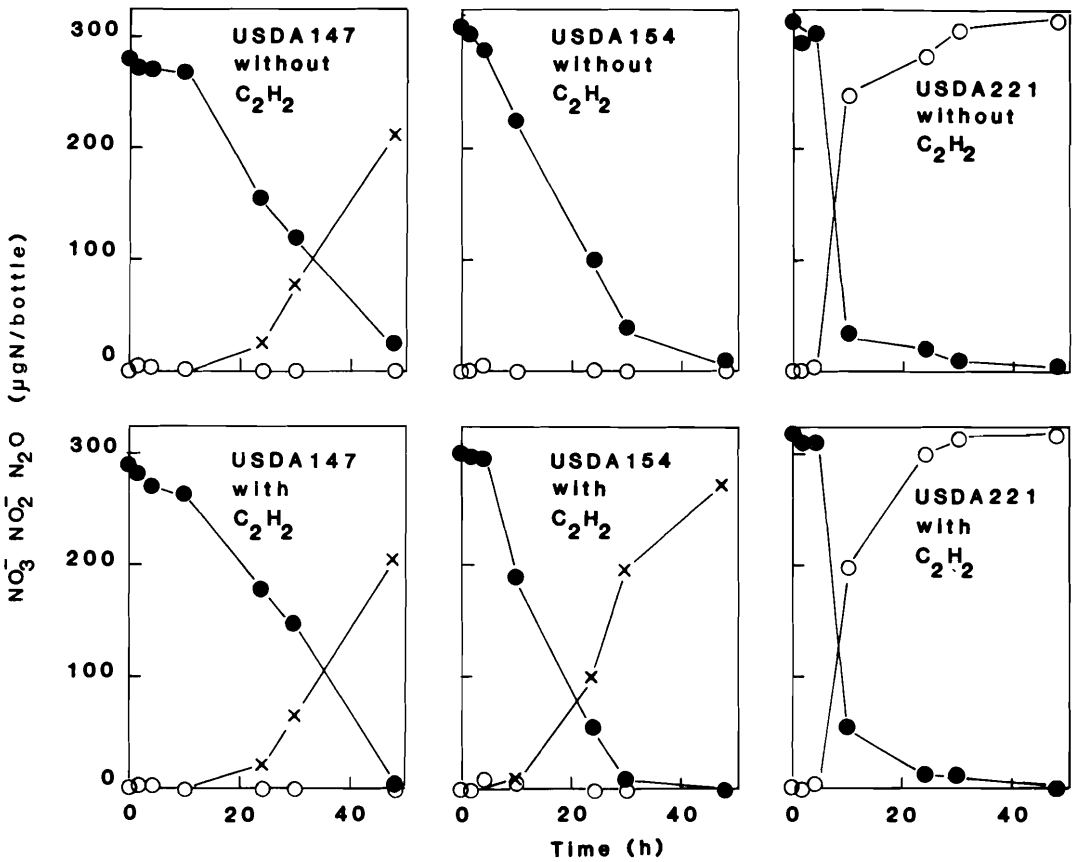


FIG. 1. Time course analyses of NO_3^- N (●), NO_2^- N (○), and N_2O N (×) concentrations during denitrification by free-living *B. japonicum* USDA 147, USDA 154, and USDA 221 incubated with or without 1% (vol/vol) C_2H_2. Cultures (20 ml; approximately 5×10^8 cells per ml) were incubated in 36-ml stoppered serum bottles under 3 ml of mineral oil at 25°C. Subsamples (0.1 ml for liquid and 0.2 ml for gas) were removed periodically for the determination of NO_3^-, NO_2^-, and N_2O. Values are the means of four determinations.

and 10% produced NO_2^-, N_2O, and N_2, respectively; no product was formed by 34%.

Ninety-eight isolates of soybean rhizobia and bradyrhizobia from the People's Republic of China were tested for anaerobic growth in the presence of KNO_3. Eleven of the isolates belonging to this germplasm have been reported to be more closely related to the rhizobia than to bradyrhizobia, based on growth rate in culture (9) and biochemical characterization (19). Agglutination tests of the bradyrhizobia indicated that the majority have antigens in common with serogroups 6, 76, and 123. Only three and one of the strains reacted with antisera prepared for serogroups 31 and 110, respectively. Eleven of the strains were determined to have antigens in common with two serogroups, whereas 34 isolates did not react with the nine antisera used (Table 3). Anaerobic growth in the presence of KNO_3 was observed with all but four of the strains, which were also unable to grow aerobically with KNO_3 as sole N source. Final products of denitrification by the bradyrhizobia were observed to be predominantly N_2O or N_2, except for the three isolates in serogroup 31 and six isolates of undetermined serogroups, which produced NO_2^- from KNO_3. All 11 soybean rhizobia grew anaerobically in the presence of KNO_3 with N_2O as the final product, except for USDA 257, which liberated N_2.

Selected strains of *B. japonicum* were used to inoculate soybean to compare characteristics of denitrification between bacteroidal and free-living states. Time course analyses for NO_3^- respiration and anaerobic NO_3^--supported C_2H_2 reduction were made with bacteroids of USDA 147, USDA 154, and USDA 221. The results obtained with the three strains were similar. The concentration of NO_3^- declined immediately upon its addition to the bacteroid preparations, and NO_2^- accumulated proportionately as the NO_3^- was used (Fig. 2). The concentration of accumulated NO_2^- remained constant for more than 24 h after the NO_3^- had been completely used by the bacteroids (Fig. 2, inset). Anaerobic NO_3^- respiration by USDA 147, USDA 154, and USDA 221 supported C_2H_2 reduction. The rate of C_2H_2 reduction declined when the bacteroids had converted most of the NO_3^- to NO_2^-.

Fixed-time assays were used to investigate the relationship between the characteristics of denitrification by USDA 6, USDA 24, USDA 33, USDA 61, USDA 110, USDA 135, USDA 176, USDA 227, LA3 2a, and NC4 5c in the free-living state and those of anaerobic NO_3^- respiration-supported C_2H_2 reduction in the bacteroids (Table 4). With the exception of USDA 24, bacteroids from the strains capable of NO_3^- respiration under free-living conditions reduced C_2H_2 anaerobically in the presence of NO_3^- and produced NO_2^-. Respiration of NO_3^- and C_2H_2 reduction did not occur with bacteroid preparations of USDA 24 or the strains not capable of growing aerobically or anaerobically with KNO_3. Anaerobic growth in the presence of NO_3^- and products of denitrification by free-living USDA 33 were not detected, although anaerobic bacteroid preparations respired NO_3^- to NO_2^-, supporting C_2H_2 reduction.

51

TABLE 1. Anaerobic growth on nitrate and products of nitrate reduction by USDA culture collection strains of *B. japonicum*

Strain	Serogroup	Anaerobic growth on NO_3[a]	Production of the following by NO_3^- reduction:		
			NO_2^-	N_2O	N_2
USDA 3	6	+	+	−	−
USDA 6	6	+	−	+	−
USDA 24	6	+	−	+	−
USDA 31	31	+	+	−	−
USDA 33	31	+/−	−	−	−
USDA 44	6	+	−	−	+
USDA 46	46	+	+	−	−
USDA 61	31	+	+	−	−
USDA 71a	46	+	+	−	−
USDA 76	76	+	+	−	−
USDA 94	94	+	+	−	−
USDA 110	110	+	−	−	+
USDA 117	76	+	+	−	−
USDA 122	122	+	−	−	+
USDA 123	123	+	−	−	+
USDA 135	135	−	−	−	−
USDA 136	122	+	−	−	+
USDA 138	6	+	−	+	−
USDA 140	62	+	−	−	+
USDA 143	122	+	−	−	+
USDA 145	—[b]	+	−		+
USDA 270	31	+	+	−	−
USDA 273	—	+	−	+	−
USDA 277	—	+	−	+	−
USDA 285	6	+	−	+	−
USDA 309	76	+	+	−	−
USDA 310	31	+	+	−	−
USDA 325	—	+	−	−	+

[a] Growth was determined by scoring turbidity after 21 days of incubation at 30°C. +, Positive result; −, negative result; +/−, trace of growth.
[b] No agglutination with the nine antisera used.

DISCUSSION

We studied 321 strains of soybean rhizobia and bradyrhizobia originating from three germplasm collections for the ability to grow anaerobically in the presence of NO_3^- and for differences in product formation from anaerobic NO_3^- metabolism. The ability to grow anaerobically in the presence of NO_3^- varied among strains and serogroups. In general, free-living cultures of *B. japonicum* capable of anaerobic growth in the presence of NO_3^- also metabolized NO_3^- anaerobically as bacteroids, but differences were observed in final product formation.

Most bradyrhizobia are capable of anaerobic growth in the presence of NO_3^-, whereas denitrification is rare among the rhizobia (3, 7, 25). In our study, 270 strains were capable of anaerobic growth in the presence of NO_3^-, including the 11 soybean rhizobia from China. The soybean rhizobia have been shown to express nitrate reductase (19), which probably operates under anaerobic conditions in the presence of NO_3^- to promote growth. Among the other rhizobia, only *R. meliloti* has been shown to denitrify (3), and *R. trifolii* TA1 was reported to respire NO_3^- (7). The 51 strains in our study that were not capable of anaerobic growth in the presence of NO_3^- are bradyrhizobia and include all of serogroup 135.

TABLE 2. Anaerobic growth on nitrate and products of nitrate reduction by U.S. survey isolates of *B. japonicum*

Serogroup	No. of isolates tested	Anaerobic growth on NO_3^- (% positive)[a]	% Production of the following by NO_3^- reduction:		
			NO_2^-	N_2O	N_2
6	24	96	30	52	13
31	28	96	93	0	7
46	12	100	100	0	0
76	24	100	92	0	8
94	18	100	83	6	11
110	13	85	0	0	100
122	14	100	14	0	86
123	21	100	14	29	57
135	41	0	0	0	0

[a] Growth was determined by scoring turbidity after 21 days of incubation at 30°C. All isolates grew aerobically in the same media.

TABLE 3. Anaerobic growth on nitrate and products of nitrate reduction by *B. japonicum* isolates obtained from the People's Republic of China

Serogroup	No. of isolates tested	Anaerobic growth on NO_3^- (% positive)[a]	% Production of the following by NO_3^- reduction:		
			NO_2^-	N_2O	N_2
6	26	100	0	85	15
31	3	100	100	0	0
76	1	0	0	0	0
110	1	100	0	0	100
123	11	100	0	55	45
76/123	11	82	0	78	22
Undetermined[b]	34	97	18	32	50

[a] Growth was determined by scoring turbidity after 21 days of incubation at 30°C.
[b] No agglutination was observed with the nine antisera used.

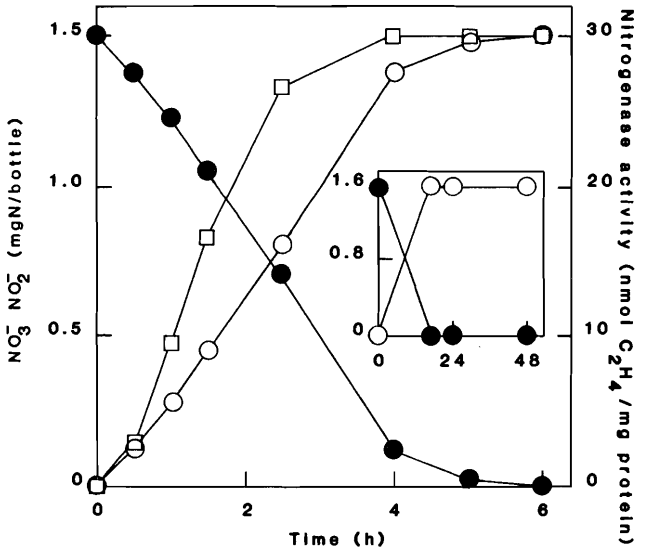


FIG. 2. Time course analysis of NO_3^- N (●), NO_2^- N (○), and C_2H_4 (□) concentrations during denitrification by anaerobic bacteroid preparations of *B. japonicum* USDA 154 incubated with 12% (vol/vol) C_2H_2. The inset shows a time course analysis of NO_3^- N (●) and NO_2^- N (○) in anaerobic bacteroid preparations of USDA 147. Stoppered serum vials (36 ml) contained 2 ml each of the bacteroid preparations plus 50 mM KNO_3 in 250 mM sodium succinate–50 mM Tris buffer (12 ml; pH 8.0) under Ar atmospheres with or without 12% (vol/vol) C_2H_2. The USDA 147 and USDA 154 bacteroid preparations contained 460 and 450 μg of protein per ml, respectively. Values are the means of three determinations.

52

Failure to grow anaerobically in the presence of NO_3^-, together with the inability to aerobically use NO_3^- as the sole N source, indicates the lack of nitrate reductase(s) or gene products essential for NO_3^- metabolism. Although USDA 33 grew aerobically with NO_3^- as the sole N source, little anaerobic growth in the presence of NO_3^- was detected. Similarly, Zablotowicz et al. (25) reported USDA 33 to be negative for denitrification and NO_3^- respiration. Although USDA 33 is dissimilatory nitrate reductase deficient, our results indicate that this strain does express assimilatory nitrate reductase.

The dissimilation of NO_3^- by *B. japonicum* proceeded without the significant release of intermediate products. Denitrification was evident by the disappearance of substrate and the accumulation of product. We observed C_2H_2 to inhibit nitrous oxide reductase in soybean rhizobia and bradyrhizobia, as has been reported in *Pseudomonas denitrificans*, *Pseudomonas aeruginosa*, *Micrococcus denitrificans*, and *Azospirillum* spp. (16, 24), and was useful for characterizing strains producing N_2 anaerobically from NO_3^-.

We observed distinct differences among serogroups 110, 135, and 31, 46, 76, and 94 combined for product of denitrification, a characteristic which indicates physiological similarities for NO_3^- metabolism within isolates of specific serogroups. Several of the isolates were different, with respect to denitrification, from the majority of the strains within each serogroup tested, similar to the reported variation in characteristics among the members within the 123 serogroup (6). Vincent (23) cautions that relationships between serogroup affinity and other properties may be coincidental, and we wish to emphasize that this may be the case also with our data.

The anaerobic metabolism of NO_3^- by bacteroid preparations of *B. japonicum* proceeded without delay upon the addition of substrate, although plants had been grown without supplemental KNO_3. The presence of nitrate reductase in nodule homogenates of soybean grown without a source of combined N has been established by others (2, 4, 7), indicating that bacteroids in soybean nodules express nitrate reductase constitutively. The anaerobic metabolism of NO_3^- by soybean bacteroids was evident by the disappearance of substrate and the accumulation of NO_2^- irrespective of which final product was formed by the strain when tested as a free-living culture. Nitrite accumulated as the final product of anaerobic NO_3^- metabolism in soybean bacteroid preparations because the absence of nitrite reductase activity (2, 20) prevents further metabolism of this intermediate.

We have observed that anaerobic C_2H_2 reduction is supported by the reduction of NO_3^- in soybean bacteroid preparations similar to that described by Rigaud et al. (18). Our data show that anaerobic C_2H_2 reduction is dependent upon NO_3^- respiration because strains characterized to be deficient in dissimilatory nitrate reductase under free-living conditions did not respire NO_3^- and reduce C_2H_2 anaerobically as bacteroids. Similarly, mutant strains selected for nitrate reductase deficiency under free-living conditions reportedly did not accumulate NO_2^- from NO_3^- as bacteroids (7), but effects on anaerobic C_2H_2 reduction were not communicated.

The results obtained with strains USDA 24 and USDA 33 have further implications for the relationship between the symbiosis and nitrogen fixation or NO_3^- respiration by soybean bacteroids. USDA 24 formed ineffective nitrogen-fixing symbioses with soybean and denitrified under free-living conditions. The bacteroid preparations of USDA 24 did not metabolize NO_3^- anaerobically, indicating that an effective symbiosis may be a prerequisite for the expression of constitutive nitrate reductase by the bacteroids. Bacteroids of USDA 33 produced NO_2^- anaerobically from NO_3^-, although under free-living conditions this strain only expresses assimilatory nitrate reductase. Our observations with USDA 33 may indicate that dissimilatory nitrate reductase is expressed by USDA 33 in symbiosis with soybean or that assimilatory nitrate reductase operates producing NO_2^- from NO_3^- because nitrite reductase is reportedly absent (2, 20).

TABLE 4. Dissimilation of NO_3^- by bacteroid preparations from nodules formed by strains of *Bradyrhizobium japonicum* with *Glycine max* cv. Williams

Strain[a]	Serogroup	Final product of anaerobic NO_3^- reduction by free-living cells	NO_3^- N produced[b]	NO_2^- N produced[b]	Acetylene reduction[c]
USDA 6	6	N_2O	0	1.5	8.1
USDA 24	6	N_2O	1.5	0	0
USDA 33	31	—[d]	0	1.5	22.5
USDA 61	31	NO_2	0	1.5	32.8
USDA 110	110	N_2	0.6	0.9	11.8
USDA 135	135	—	1.5	0	0
USDA 176	76/123	—	1.5	0	0
USDA 227	76	—	1.5	0	0
LA3 2a	135	—	1.5	0	0
NC4 5c	6	N_2O	0	1.5	27.7

[a] Bacteroid preparations contained 190, 38, 158, 368, 290, 250, 338, 220, and 263 μg of protein/ml, respectively. Data are the means of three determinations.

[b] Expressed as milligrams of N per bottle.

[c] Expressed as nanomoles of C_2H_4 per milligram of protein per milligram of NO_2^- formed.

[d] —, No product was detected with free-living cells cultured anaerobically with 6 mM KNO_3.

ACKNOWLEDGMENTS

We thank Michael B. McMahon for technical assistance.

This study was supported in part by the U.S. Department of Agriculture, the University of Maryland under cooperative agreement 58-32U4-3-370, and by the U.S. Agency for International Development RSSA 4-76.

LITERATURE CITED

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
2. **Daniel, R. M., and C. A. Appleby.** 1972. Anaerobic-nitrate, symbiotic and aerobic growth of *Rhizobium japonicum*: effects on cytochrome P450, other haemproteins, nitrate and nitrite reductases. Biochim. Biophys. Acta **275**:344–354.
3. **Daniel, R. M., A. W. Limmer, K. W. Steele, and I. M. Smith.** 1982. Anaerobic growth, nitrate reduction and denitrification in 46 *Rhizobium* strains. J. Gen. Microbiol. **128**:1811–1815.
4. **Evans, H. J.** 1954. Diphosphopyridine nucleotide-nitrate reductase from soybean nodules. Plant Physiol. **29**:298–301.
5. **Evans, H. J., B. Koch, and R. Klucas.** 1972. Preparation of nitrogenase from nodules and separation into components. Methods Enzymol. **24**:470–476.
6. **Gibson, A. H., W. F. Dudman, R. W. Weaver, J. C. Horton, and I. C. Anderson.** 1971. Variation within serogroup of 123 of *Rhizobium japonicum*. Plant Soil Special Volume, p. 33–37.
7. **Gibson, A. H., and J. D. Pagan.** 1977. Nitrate effects on the nodulation of legumes inoculated with nitrate-reductase-defi-

cient mutants of *Rhizobium*. Planta **134**:17–22.

8. **Jackson, W. A., C. E. Frost, D. M. Hildreth.** 1975. Versatile multirange analytical manifold for automated analysis of nitrate-nitrogen. Soil Sci. Am. Proc. **39**:592–593.
9. **Keyser, H. H., B. B. Bohlool, T. S. Hu, and D. F. Weber.** 1982. Fast-growing rhizobia isolated from root-nodules of soybean. Science **215**:1631–1632.
10. **Keyser, H. H., P. van Berkum, and D. F. Weber.** 1981. Nitrogenase activity and hydrogen evolution by *Rhizobium japonicum* in symbiosis with siratro and cowpea, p. 374. *In* A. H. Gibson and W. E. Newton (ed.), Current perspectives in nitrogen fixation. Australian Academy of Science, Canberra.
11. **Keyser, H. H., and D. F. Weber.** 1979. USDA Beltsville *Rhizobium* Culture Collection Catalogue. Plant Physiology Institute Report 16. Agriculture Research Service, U.S. Department of Agriculture, Beltsville, Md.
12. **Keyser, H. H., D. F. Weber, and S. L. Uratsu.** 1984. *Rhizobium japonicum* serogroup and hydrogenase phenotype distribution in 12 states. Appl. Environ. Microbiol. **47**:613–615.
13. **Leonard, L. T.** 1923. Nodule production kinship between the soybean and the cowpea. Soil Sci. **15**:277–283.
14. **Neal, J. L., G. C. Allen, R. D. Morse, and D. D. Wolf.** 1983. Anaerobic nitrate-dependent chemolithotrophic growth by *Rhizobium japonicum*. Can. J. Microbiol. **29**:316–320.
15. **Neyra, C. A., J. Dobereiner, R. Lalande, and R. Knowles.** 1977. Denitrification by N_2-fixing *Spirillum lipoferum*. Can. J. Microbiol. **23**:300–305.
16. **Neyra, C. A., and P. van Berkum.** 1977. Nitrate reduction and nitrogenase activity in *Spirillum lipoferum*. Can. J. Microbiol. **23**:306–310.
17. **Norris, D. O.** 1964. Techniques used in work with *Rhizobium*. Commw. Bur. Pastures Field Crops Hurley Berkshire Bull. **47**:186–198.
18. **Rigaud, J., F. J. Bergersen, G. L. Turner, and R. M. Daniel.** 1973. Nitrate dependent anaerobic acetylene-reduction and nitrogen fixation by soybean bacteroids. J. Gen. Microbiol. **77**:137–144.
19. **Sadowsky, M. T., H. H. Keyser, and B. B. Bohlool.** 1983. Biochemical characterization of fast- and slow-growing rhizobia that nodulate soybeans. Int. J. Syst. Bacteriol. **33**:716–722.
20. **Stephens, B. D., and C. A. Neyra.** 1983. Nitrate and nitrite reduction in relation to nitrogenase activity in soybean nodules and *Rhizobium japonicum* bacteroids. Plant Physiol. **71**:731–735.
21. **van Berkum, P., and C. Sloger.** 1979. Immediate acetylene reduction by excised grass roots not previously preincubated at low oxygen tensions. Plant Physiol. **64**:739–743.
22. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. IBP Handbook no. 15. Blackwell Scientific Publications, Oxford.
23. **Vincent, J. M.** 1982. Serology, p. 235–273. *In* W. J. Broughton (ed.), Nitrogen fixation, vol. 2. Rhizobium. Oxford University Press, New York.
24. **Yoshinari, T., and R. Knowles.** 1976. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Commun. **69**:705–710.
25. **Zablotowicz, R. M., D. L. Eskew, and D. D. Focht.** 1978. Denitrification in *Rhizobium*. Can. J. Microbiol. **24**:757–760.
26. **Zablotowicz, R. M., and D. D. Focht.** 1979. Denitrification and anaerobic, nitrate-dependent acetylene reduction in cowpea *Rhizobium*. J. Gen. Microbiol. **111**:445–448.

54