Cereal Nitrogen Fixation

International Crops Research Institute for the Semi-Arid Tropics

The International Crops Research Institute for the Semi-Arid Tropics is a nonprofit scientific educational institute receiving support from donors through the Consultative Group on International Agricultural Research. Donors to ICRISAT include governments and agencies of Australia, Belgium, Canada, Federal Republic of Germany, Finland, France, India, Italy, Japan, Netherlands, Nigeria, Norway, People's Republic of China, Sweden, Switzerland, United Kingdom, United States of America, and the following international and private organizations: Arab Bank for Economic Development in Africa, Asian Development Bank, International Development Research Centre, International Fertilizer Development Center, International Fund for Agricultural Development, The European Economic Community, The Ford Foundation, The Leverhulme Trust, The Opec Fund for International Development, The Population Council, The Rockefeller Foundation, The World Bank, and the United Nations Development Programme. Information and conclusions in this publication do not necessarily reflect the position of the aforementioned governments, agencies, and international and private organizations.

Cover: *Mucigel-secreting brace roots of sorghum grown at ICRISAT Center during the rainy season. Inset shows an electron micrograph of a nitrogen-fixing bacterium* Azospirillum brasilense *(x 19 800).*

Cereal Nitrogen Fixation

Proceedings of the Working Group Meeting held at ICRISAT Center, India, 9-12 October 1984

International Crops Research Institute for the Semi-Arid Tropics
Patancheru, Andhra Pradesh 502 324, India

1986

Correct citation: ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502324, India: ICRISAT.

Workshop Coordinator and Scientific Editor

S. P. Wani

The opinions in this publication are those of the authors and not necessarily those of ICRISAT. The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of ICRISAT concerning the legal status of any country, territory, city, or area, or of its authorities, or concerning the delimitation of its frontiers or boundaries. Where trade names are used this does not constitute endorsement of or discrimination against any product by the Institute.

ISBN 92-9066-111-9

Contents

Foreword

Among cereals, sorghum and millets together provide the highest calorie intake in African diets and rank third in Asia. These crops are grown largely on rainfed, marginal soils by resource-poor farmers who cannot bear the cost of nitrogenous fertilizers that can boost their yields.

Although an inexhaustible store of nitrogen exists in the atmosphere, most plants are not able to use it directly. Legumes have been known to make use of this nitrogen through association of nitrogen-fixing bacteria, which form nodules in the roots of these plants and have a symbiotic relationship with them. In recent years, research has indicated that certain bacteria living near the roots of sorghum and millet can also fix the atmospheric nitrogen nonsymbiotically and partially meet the nitrogen requirement of these crops.

Consequently, the complex process of nitrogen fixation in sorghum and millet has attracted the attention of many scientists in India and abroad. The studies are still in their preliminary stages and the methodology is still being perfected. With a view to share experiences, discuss methods and techniques, and arrive at an agreed basis for assessing results, a working group meeting was convened at ICRISAT Center during 9-12 October 1984. A total of 30 scientists took part in the deliberations, 19 from Indian institutions and 11 from ICRISAT.

Meetings such as this play an important role, developing interpersonal and professional relationships among the scientists involved, which can lead to more meaningful research and development strategies. I am pleased that ICRISAT hosted this meeting on cereal nitrogen fixation, and I believe these proceedings will be a valuable source of information and inspiration to all concerned.

L. D. Swindale
Director General

Welcome Address

C. R. Jackson

Ladies and Gentlemen:

We are pleased to have you here today. I want to welcome you on behalf of ICRISAT, its management, and our staff to this important working group meeting on cereal nitrogen fixation.

Most of my concern as Director of International Cooperation in ICRISAT is aimed at places outside India, particularly in Africa. So this meeting is of particular importance to me, because I believe it helps aid the development of technology that will mean a great deal to India, to Africa, and to all countries where the cultivation of crops is in some part marginal and of a subsistence nature.

As you all know, nitrogen is one of the most important factors limiting cereal production in the world and biological nitrogen fixation needs to be fully exploited. We need to understand it as well as possible, but not to the extent that we understand everything before we go to the field in attempting to use this phenomenon.

There are many parts of the world that are in difficulty from lagging food supplies, growing populations, etc. Here I want to emphasize the problem of Africa, particularly West Africa, in attempting to feed itself. The Sahelian region, and the region a bit further South from the Sahel are in such great distress that we must contemplate the movement of people on a continental scale to places where conditions are better for growing food.

I am going to make an appeal to you to give your very best thoughts to this particular subject of nitrogen fixation in cereal crops. We must make early progress in this subject. It is not just an academic subject. I want to transmit to you the urgency of the problem of developing this technology, so that it can be applied on a broad scale and with dependability in India, in Africa, and in other parts of the world. You must give your best thought to it and see that it works in the field. You must expend every effort to see that something useful for humankind comes out of your research and out of the deliberations of conferences like this.

We cannot continue to rely solely on plant breeding to give us bigger and better plants that will grow on less and less water. There are other factors in the equation. Soil fertility is one of them and a very important one. We certainly need to put to use every increment of knowledge that we have, such as the practical use of N_2-fixing microorganisms, to give us an additional increment of growth in these very harsh environments where people are trying desperately to survive.

With those few words of encouragement, and with the noting that your program is going to cover some very important topics and perhaps reveal things never before revealed to humankind, I wish you all luck in this conference. Welcome. Give us your best efforts.

Inaugural Address

J. S. Kanwar

It gives me immense pleasure to extend my warm welcome to the participants of this meeting, who are actively engaged in research on N_2 fixation by cereals, specifically sorghum and millets. I feel this meeting is timely and important. Sorghum and millet together provide food to 700 million people of the semi-arid tropics. They are grown on 70 million ha, mostly under rainfed conditions, often without nitrogenous fertilizers, and by resource-poor farmers who cannot afford monetary inputs. Nearly 95% of millet and 60% of sorghum grain is produced under environments where stress from lack of moisture, nutrient stress, and other physical and biotic stresses seriously constrain yields.

Nitrogen is a key element in increased crop production, and any technique that will enhance nitrogen nutrition of the crop will go a long way in increasing crop yields. The Green Revolution in wheat and rice is nothing but the conversion of chemical energy supplied through fertilizers into biological products where high-yielding varieties act as vehicles of this change. The greater the capacity of the variety to respond to heavier doses of nitrogen, greater is the yield obtained. Thus, it is no exaggeration that the problem of food deficit and malnutrition in the world is really the problem of nitrogen starvation of the cereal crops.

Despite the fact that an inexhaustible store of N_2 exists in the atmosphere, most plants are not able to use it directly. Industrial plants are converting a small fraction of available N_2 into nitrogenous fertilizers, but it is recognized that despite man's ingenuity and resources, the world is not able to meet more than a fraction of the nitrogen requirement of the crops. Thus we have to look to other sources, of which biological nitrogen fixation (BNF) is an important one. The dependance of fertilizer nitrogen production on fossil energy resources and the prospects of diminished availability of this costly input for fertilizer in the years to come have added an urgency to the study of BNF.

We recognize that crops like sorghum and millets are very poor competitors for the available fertilizers, and more so under dry-farming conditions, because of the greater risks involved and lower payoff. Thus, for more than 60 million ha of sorghum and millet grown in the SAT, any breakthrough in BNF could bring in a revolution in production.

The finding by Dobereiner of nonsymbiotic N_2 fixation by associated bacteria in cereals, stimulated research in this subject all over the world. With the establishment of ICRISAT, a special emphasis was laid on N_2-fixation studies in sorghum and millets. Considerable progress has been made in our understanding of this process, but there are numerous aspects that need thorough investigation before we can take this technology to farmers' fields. The standardization of techniques to measure N_2 fixation is the first problem needing attention. It is recognized that unless reliable, repeatable, and comparable results are obtained by different workers, no valid conclusions can be drawn.

After 10 years of painstaking research, ICRISAT microbiologists are now reasonably confident that the methodologies they have developed can give reproducible results for other scientists as well. Whether it is a measurement of nitrogenase activity with acetylene reduction method on seedlings grown in test tubes or pots, or ^{15}N dilution techniques, similar results have been claimed. However, the question still remains whether the results of the controlled environments correlate well with the field performance of different genotypes in different environments.

Dr Wani will give you the details of our BNF work. I will just make a mention of the broad objectives:

- to standardize research techniques.
- to study relative efficiency of N_2 fixation by different genotypes of sorghum and millets.
- to study environmental factors affecting N_2 fixation.
- to increase N_2 fixation by genetic manipulation of the plant, by agronomic manipulation of the crop, or by inoculating with effective bacterial cultures.

In short, we are interested in incorporating the desirable traits of N_2 fixation in high-yielding varieties and developing techniques and practices for enhancing yields of sorghum and millets in farmers' fields. Our studies are not restricted to N, as we are also studying differences in activities of mycorrhizae associated with different genotypes for release of phosphates and its interactions with N_2 fixation.

An External Program Review panel that evaluated ICRISAT's work earlier this year, while appreciating our work to date on BNF, observed that it is innovative but speculative and needs to be intensified to test and select the most effective plant-microorganism associations. We realize that to accomplish this task, we need the support of many competent microbiologists working under different environments, using the same methodology.

I consider this meeting very timely as you can share your experiences with ICRISAT scientists and develop a common approach for further studies on N_2 fixation by sorghum and millets. We are now entering a second phase in the study of N_2 fixation by cereals. Dr Wani and his colleagues have screened some germplasm lines and rated them for their N_2-fixing efficiency. However, there is a large germplasm resource that still needs to be screened. Secondly, we are interested in transferring these useful traits to agronomically superior lines for achieving the yield advantage. There are some indications that this can be done, but we would like to have some critical studies made cooperatively so as to be sure about the findings. This Working Group Meeting should help us achieve the following objectives :

- to share knowledge and experience of nonsymbiotic N_2 fixation in sorghum and millet;
- to accept standardized techniques for making measurements of N_2 fixation;
- to develop a coordinated approach for conducting research for transferring desirable traits to agronomically superior genotypes; and
- to study the effect of environments on nonsymbiotic N_2 fixation in cereals.

For achieving these objectives, it is essential that this meeting identifies teams of scientists working in different institutes to take up coordinated research, who agree to

share experiences and materials. These should be interdisciplinary teams consisting of geneticists, microbiologists, and agronomists/soil scientists. I am convinced that the high hopes that have arisen—about improving the nitrogen nutrition of sorghum and millet and enhancing their production—can be realized only through interdisciplinary teamwork.

I understand that all of you assembled here today are interested in such teamwork, but while studying N_2 fixation you should not forget the interaction of the environment and the effect of other nutrients involved in this process. The effect of vesicular arbuscular mycorrhiza in stimulating N_2 fixation through enhanced availability of phosphates has been observed. Whether this synergism can be enhanced through management of environments or genetic engineering is a question that needs investigation. There is a lot of basic work that needs to be done. I find in this group some scientists who are involved in such basic studies.

Quite often the experiments are conducted under controlled environments without any moisture constraints. Whether the same results are possible under conditions of drought stress and high temperature needs investigation. The effect of physical soil environments and initial soil-fertility level on N_2 fixation also needs to be studied. It is my impression that there are more unknown factors than the known ones influencing nonsymbiotic N_2 fixation by crops like sorghum and millets and we need to investigate further.

Judging from the program that my colleagues have drawn up for this meeting, I get the feeling that you would be looking at all these problems. I realize that many of you are interested in N_2 fixation by cereals other than sorghum and millets. There are some papers relating to rice. Since the environments in which rice is grown are very different from those of sorghum and millets, the transferability of the information from rice to sorghum and millets and vice versa needs investigation. It would be desirable for you to extend your discussions to N_2 fixation by sorghum and millets under conditions of drought stress as well. I understand that the best estimates show that about 20 kg N ha^{-1} could be added through bacterial association with sorghum and millets. Even an increase of 20 kg N ha^{-1} in all the sorghum- and millet-producing areas can mean a tremendous increase in the production of these cereals. We will be doing a great service to humanity if we can demonstrate this effect on farmers' fields, under the environments where these crops are grown.

It will be a real achievement if we succeed in transferring this trait to agronomically superior genotypes. I need hardly emphasize that Africa, more than India, is badly in need of this technology and we are keenly interested in the outcome of your researches.

I wish you all success in your deliberations. I have great pleasure in inaugurating this Working Group Meeting.

Cereal Nitrogen Fixation: Problems and Potentialities

S. P. Wani[1]

Summary

Estimates of nonsymbiotic and associative nitrogen fixation indicate that nitrogen fixation occurs at magnitudes that may be of agronomic significance.

Long-term N balances for crop production, although difficult to measure, are necessary for estimating the amounts of N_2 fixed. Techniques using ^{15}N directly to measure nitrogen fixation and problems involved in employing these techniques are discussed. Acetylene-reduction assays (ARA) are very sensitive but there are limitations to their use in quantification of nitrogen fixation as well as infield studies.

Current understanding about the source of energy for cereal nitrogen fixation and the effect of light, temperature, soil moisture, plant genotype, plant age, and combined nitrogen on nitrogen fixation is illustrated with examples. Possibilities of improving the ability of cereals to support nitrogen fixation through plant breeding are discussed.

Types of bacteria involved and methods used to isolate, count, and test their nitrogenase activity influence the results of such studies. Problems associated with selecting bacteria for field studies, their performance, and mode of benefiting crops from inoculations are discussed. Future areas of work are highlighted.

Introduction

Nitrogen is the most limiting nutrient in food production. The biological nitrogen cycle (Fig. 1) is responsible for a turnover of 10^8-10^9 t N a^{-1} on earth in which biologically fixed N_2 is one of the inputs. Nonsymbiotic and associative N_2 fixation is considered to occur at magnitudes that may be of agronomic significance (Dobereiner 1978, Knowles 1976, Moore 1966, Dart and Wani 1982, Wanietal. 1984). The apparent potential for biological nitrogen fixation (BNF) associated with cereals exceeds its present utilization, but knowledge in this field is not enough to exploit these associations fully. There appear to be many ways of increasing the contribution from cereal nitrogen fixation. The aim of this review is to evaluate the problems and potentialities of cereal nitrogen fixation and to indicate the areas needing further investigation.

Microbiology of the Association

Since Winogradsky (1893) established that *Clostridium pasteurianum* could fix atmospheric N_2 and Beijerinck (1901) described the first *Azotobacter,* the list of nitrogen-fixing bacteria has gone on increasing (Balandreau 1983). Many different genera and strains of N_2-fixing bacteria can be isolated from the soil and the roots. The difficulty in studying the ecology of N_2-fixing bacteria is in devising selective media and new isolation procedures to count the populations of particular organisms. Each labora-

1. Microbiologist, Pearl Millet Improvement Program, ICRISAT, Patancheru, A.P. 502 324, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

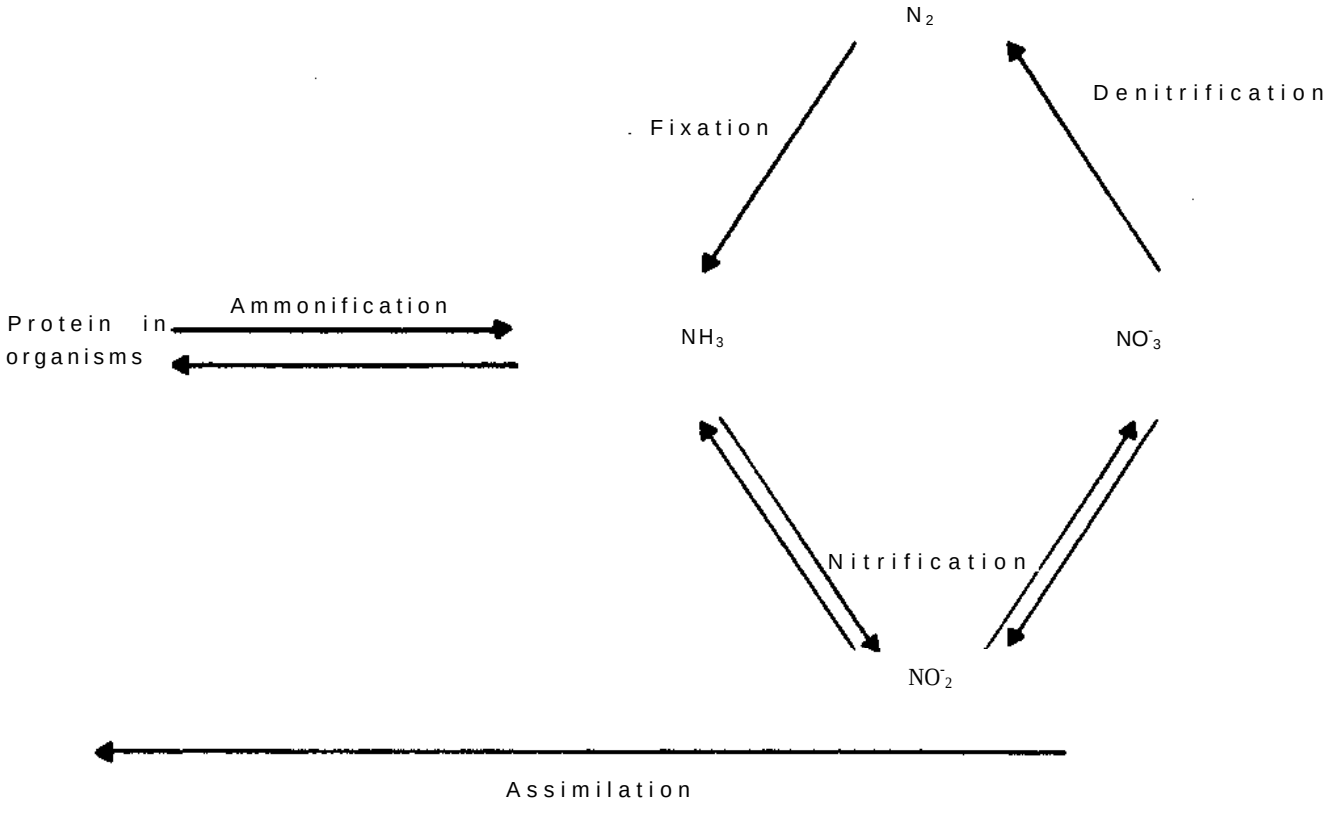


Figure 1. The nitrogen cycle.

tory uses a particular set of techniques for growing, isolating, and counting N_2-fixing bacteria. Consequently, each laboratory has a tendency to consider that its own bacterium has a dominant role in N_2 fixation.

Isolation and Enumeration of N_2-fixing Bacteria

To overcome the problem of selective carbon source in the medium as far as rhizosphere bacteria are concerned, use of carbon sources similar to those present in the rhizosphere, i.e, root exudates, would be better. Use ofthe 'spermosphere model' is promising for counting and isolating N_2-fixing bacteria (Thomas-Bauzon et al. 1982).

Another difficulty in comparing the results of various groups is the way of expressing the number of bacteria. These are generally expressed per g of rhizospheric soil. However, there is no clear definition of rhizospheric soil. Expressing the results per unit mass of root does not solve the problem either, considering the different types of roots found on the same plant, sampling method, age of the plant at sampling, distance of roots from the crown, and difficulty in recovering all the roots from the soil. However, expressing the number of bacteria per unit mass of above-ground plant parts produced may give a better understanding and uniformity in expression.

Testing for N_2-fixing Efficiency

Sometimes reports of new organisms capable of fixing atmospheric nitrogen are later proved to be untrue because the culture under test may not be pure and even slight contamination by a N_2-fixing organism could be sufficient to indicate fixation. Growth on a N-free medium is not a sufficient criterion for nitrogenase activity (Hill and Postgate 1969) as some of the N-scavenging bacteria can grow on traces of N present in the medium and some N_2-fixing bacteria cannot grow on a medium completely free of combined N (Watanabe and Barraquio 1979). The $^{15}N_2$ incorporation, even though a definitive test to measure N_2 fixation is too expensive and generally C_2H_2 reduction as an indirect assay technique is used. This technique poses problems due to the short

exposure period to C_2H_2 reduction and also due to oxidation of C_2H_4 by some bacteria (Knowles 1981). Many N_2-fixing bacteria express nitrogenase activity only when they are in sufficient numbers (Hauke-Pacewiczowa et al. 1970, Brouzes et al. 1971) and in enumeration experiments with high dilutions this can take as long as 3 weeks (Villemin et al. 1974). The established nitrogenase activity can be suppressed by carbon or oxygen limitation so that the overall period for expression of activity is very short. This difficulty can be overcome by waiting long enough for each tube containing N_2-fixing bacteria to develop a sufficiently large population and then adding fresh medium, and incubating under C_2H_2 (Villemin et al. 1974). Another possibility is to incubate the replicate tubes under 1% C_2H_2 as soon as they are inoculated (Balandreau 1983).

Identification of Bacteria

A detailed identification of bacteria isolated is generally overlooked by soil microbiologists. As indicated by Balandreau (1983) "many soil microbiologists are not very keen on taxonomy". Sometimes organisms are identified up to the generic level based on common tests, e.g., organisms forming a pellicle in malate semisolid medium are called *Azospirillum,* when there are other N_2-fixing bacteria such as some members of enterobacteriaceae and species of *Pseudomonas* capable of forming pellicle in semisolid malate medium. Either way, workers should not overlook detailed taxonomy and should avoid naming unconfirmed cultures (Balandreau 1983).

Occurrence of N_2-fixing Bacteria

Large populations of heterotrophic bacteria capable of growing on N-free media exist in soils of the semi-arid tropics (Wani, in press). In general, multiplication of such bacteria as well as selective proliferation of particular types occurs in the rhizosphere. Nitrogen-fixing bacteria have been observed to adhere very closely to the roots and considerable numbers were obtained from root pieces surface-sterilized in 1% chloramine T for 1 h (Dart and Wani 1982). Whether the association between bacteria and plant roots is external or the bacteria invade the root tissues is not clearly known. Umali-Garcia et al. (1980) found that adsorption of three strains of *A. brasilense* to millet root hairs was better than the adsorption of *Rhizobium trifolii* and *Pseudomonas fluorescens.* Electron micrographs of *P. maximum* and millet roots and optical micrographs of tetrazolium-reducing bacteria in the roots of maize, wheat, and sorghum suggest that infection of the cortex and stale of these roots by *Azospirillum* is at the point of emergence of lateral roots (Patriquin and Dobereiner 1978; Umali-Garcia et al 1978, 1980; Magalhase et al. 1979). Bacteria have been observed in torn or disrupted root cells (Schank et al. 1983) and also observed intercellularly but not within living root cells (Umali-Garcia et al. 1980).

Estimates of Nonsymbiotic and Associative N_2 Fixation

Some of the most-convincing evidence that nonsymbiotic nitrogen fixation may be important under field conditions has come from nitrogen-balance studies. The long-term N-balance studies at Rothamsted, England estimated nonsymbiotic nitrogen fixation up to 18-20 kg N ha^{-1} a^{-1} in plots continuously cropped to wheat since 1943 and receiving no nitrogen fertilizer, and more than 39 kg N ha^{-1} in plots left to develop natural vegetation (Jenkinson 1977, Witty et al. 1977) (Table 1).

Nitrogen-balance studies are also available in the tropics. In the old long-term, permanent-manurial experiment at Coimbatore, India, there was a net gain of N in both control (no fertilizer) plots and plots with N- and P- fertilizer application (Krishnamoorthy and Ravikumar 1973). Several pot experiments with sorghum, pearl millet, finger millet

Table 1. Some estimates of nitrogen fixed in association with cereals and grasses based on N balance.

Crop	Nitrogen fixed (kg N ha^{-1} a^{-1})	Reference
Maize	11.2	Smith et al. 1954
Wheat	18-23	Dart and Day 1975
Rice	30-60	Firth et al. 1973
		Koyama and App 1979
		Walcott et al 1977
Legume-free grass sod	34	Smith et al. 1954
Grasses	45	White et al. 1945
Noncultivated (legume-free)	49	Dart and Day 1975
Rye grass	63	Parker 1957
Finger millet	112-148	Moore 1963

(*Eleucine coracana*), and Napier bajra 21 (*Pennisetum purpureum x P. americanum*) have shown substantial positive balances for N (Dart and Wani 1982; Wani, in press; Upadhyaya et al. 1986). A positive N balance over a 4-month period for the soil plant system in pot experiments with finger millet was found, which extrapolated to a gain of 112-148 kg N ha^{-1} (Moore 1963). Similarly, positive N balance for flooded soils in pots planted to rice have been reported (App et al. 1980). The substantial contribution of BNF to the N economy of the rice crop is well documented. *Azolla-Anabaena* association/blue-green algae, and photosynthetic bacteria account for substantial contributions to total N input for the rice crop (Venkataraman 1975, Watanabe 1981, Singh 1981). At the International Rice Research Institute (IRRI), 23 rice crops were grown over 11 years without addition of N with no apparent decline in soil N fertility. About 45-60 kg N ha^{-1} $crop^{-1}$ were removed through grain and straw (Watanabe and Lee 1977). Using $^{15}N_2$ incorporation by rhizospheric soil, it has been demonstrated that rhizospheric soil fixed four-fold higher nitrogen than the nonrhizospheric soil. The $^{15}N_2$ incorporation in the rhizosphere also varied significantly depending on the variety (Charyulu et al. 1981).

Generally, low rates of dinitrogen fixation (<6 kg N ha^{-1} $season^{-1}$) have been reported for grasses, in temperate climates (Table 2) (Nelson et al. 1976, Tjepkema and Burris 1976, Pedersen et al. 1978). Higher fixation rates of up to 33 kg N ha^{-1} in 100 days for *Cynodon dactylon* (L.) pers. were reported from Texas (Weaver et al. 1980). From the Oregon wetlands *Juncus ballicus* plants were reported to fix up to 0.8 kg N ha^{-1} d^{-1} (Tjepkema and Evans 1976).

In the tropics, high rates of dinitrogen fixation associated with grasses have been reported. For example, 90 kg N ha^{-1} a^{-1} with *Paspalum notatum* Flugge (Dobereiner et al. 1972), 2 kg N ha^{-1} d^{-1} with *Zea mays* L. (von Bulow and Dobereiner 1975), 3-63 kg N ha^{-1} $season^{-1}$ with flooded rice (Yoshida and Ancajas 1973), and 70 kg N ha^{-1} a^{-1} also with rice (Balandreau et al. 1976). The highest rates (2 kg N ha^{-1} d^{-1}) of dinitrogen fixation in the tropics were obtained by using preincubated excised-root assays (von Bulow and Dobereiner 1975, Dobereiner 1978). The shortfalls in this assay method are discussed under ARA methods.

There are few reports (Table 3) indicating incorporation of $^{15}N_2$ into cereal plants that provide an unequivocal proof of biological nitrogen fixation (Ruschel et al. 1975, De-Polli et al. 1977, Ito et al. 1980, Giller et al. 1984). There is a need to collect more data in the tropics to estimate the nitrogen fixation with different cereals. This can be achieved by conducting long term N-balance trials in the field and also by $^{15}N_2$ incorporation studies.

Methodology for Measurement of N_2 Fixation

Techniques used for measurement of nitrogen fixation associated with field- and pot-grown plants can be broadly classified as direct and indirect.

Direct Techniques

Total N by Kjeldahl analysis. Total-N measure-

Table 2. Some estimates of nitrogen fixed, based on acetylene-reduction activity (ARA).

Plant	Period	kg N ha^{-1}	Reference
Grasses in California	Season	<6	Steyn and Delwiche 1970
Oklahoma Oregon			Kapustka and Rice 1978
Wisconsin and New Zealand			Line and Loutit 1973
Wheat and sorghum in Nebraska			Nelson et al. 1976
			Tjepkema and Burris 1976
			Pedersen et al. 1978
Sporobolis heterolepsis	Year	9	Tjepkema and Burris 1976
Cynodon dactylon	100 Days	33	Weaver et al. 1980
Juncus balticus	Day	0.8	Tjepkema and Evans 1976
Paspalum notatum	Season	90	Dobereiner et al. 1972
Zea mays	Day	2	von Bulow and Dobereiner 1975
Oryza sativa	Season	3.6	Yoshida and Ancajas 1973
	Year	70	Balandreau et al. 1976
Pasture soils	Year	32	Koch and Oya 1974

Table 3. Incorporation of $^{15}N_2$ by nonlegumes.

Crop	Incubation time	% Ndfa			N fixed (µg plant^{-1})			Reference
		Shoot	Root	Soil	Shoot	Root	Soil	
D.decumbens	78 h	0.01	0.12	·	1	7	-	De-Polli et al. 1977
P. notatum	30 h	0.001	0.02	-	0	1	-	De-Polli et al. 1977
S. officinarum	30 h	4.66	4.14	-	160	52	-	Ruschel et al. 1975
	24 h	0.001	0.03	-	0	3	-	Ruschel et al. 1978
	72 h	0.15	0.27	-	124	46	-	Ruschel et al. 1981
0. sativa	7 d	3.26	1.43	-	961	1005	-	Ito et al. 1980
	7 d	0.08	0.37	0.07	65	44	510	Yoshida and Yoneyama 1980
	3 d	0.003	0.35	0.01	6	95	16	Eskew et al. 1981
S. vulgare	3 d	0.406	0.242	-	33	13	-	Giller et al. 1984
P. americanum	3 d	0.05	0.089	-	1.4	1.1	-	
	Grown further							
	5 d				2.0	1.5	6.01	

ments with the Kjeldahl method with small subsampies of a particular system enable N-accretion to be determined with ease. However, as the Kjeldahl analysis does not distinguish N fraction within the total, it is essential to construct an N-balance sheet for estimating N input from N_2 fixation. Under field conditions such experiments are difficult to conduct, as they need to run for more than one season and require a rigorous sampling of the soil if they are to reliably measure soil-N changes of 20-50 kg N ha^{-1} a^{-1} (Vallis 1973). Also, estimation of N_2 loss by denitrification is difficult under field conditions, although it is believed to be small under normal field situations with low doses of N fertilizer applications (Greenland 1962). The lysimeter, although a disturbed system, enables measurements of N-accretion and loss with more precision. However, it is difficult to regulate the water content of lysimeters, because the soil within the lysimeter is detached from the water table.

The use of N isotopes. The use of isotope ^{13}N is restricted because of its short half-life of 11 min. The stable isotope ^{15}N is preferred for measuring nitrogen fixation. It has been used with sugarcane, tropical grasses, and rice, using chambers to enclose both the plants and growth media (Ruschel et al 1975, De-Polli et al. 1977, Ito et al. 1980). Major difficulties with such experiments are the enclosure of plants and changes in environmental conditions with the necessary long-term incubations requiring complex control equipment. Incubation chambers have also been evacuated to remove the air before introduction of gas mixture (De-Polli et al. 1977; Ruschel et al. 1975, 1981). This could lead to disturbance of rhizospheric integrity and unrepresentative nitrogen uptake by the plant. These problems have been overcome recently with a simple, inexpensive apparatus developed at ICRISAT Center for exposing the plants to $^{15}N_2$ (Giller et al. 1984). This method can be used to establish the ratio of C_2H_2 reduction to N_2 fixation but is not relevant to field experiments. It is obviously difficult to extrapolate amounts of nitrogen fixed from $^{15}N_2$ incorporation studies over a short period to amounts fixed on a per plant or per hectare basis.

The above limitation can be overcome using the ^{15}N isotope-dilution technique. Using this technique lines of sorghum and millet grown in pots containing vermiculite were screened for their potential to fix N_2 (Giller et al., 1986). In such experiments, extra care has to be taken to prevent the systems from getting contaminated with ^{14}N from other sources, such as water or the growth medium.

An alternative method to measure N_2 fixation would be to determine differences in natural abundance of ^{15}N arising from mass discrimination effects resulting from N_2 fixation, NH_4 assimilation, and ^{15}N transport. But as the $\delta^{15}N$ has been reported to vary considerably with soil depth (Karamanos and Rennie 1980), its use to determine N_2 fixation may be limited.

Indirect Techniques

Acetylene-reduction assays (ARA). Nitrogenase reduces numerous chemical analoges of nitrogen, small molecules containing a triple bond (Table 4). All biological dinitrogen-fixing systems tested to

Table 4. Some substrates reduced by nitrogenase.

Substrate		Products	
Dinitrogen	N=N	NH_3	Ammonia
Acetylene	HC=CH	$H_2C=CH_2$	Ethylene
Hydrogen cyanide	H-C=N	CH_4+NH_3	Methane, ammonia
Methyl isocyanide	CH_3-N=C	$CH_3NH_2+CH_4$	Methylamine, methane[1]
Hydrogen azide	H-N-N=N	NH_3+N_2	Ammonia, nitrogen
Nitrous oxide	N=N-O	N_2+H_20	Nitrogen, water
Hydrogen ion	H_30+	H_2+H_20	Hydrogen, water

1. Ethylene, ethane, and propylene formed as minor products.
All reductions require ATP. For more detailed discussion and references, see Postgate (1972).

date have also reduced acetylene to ethylene. The use of flame ionisation detector gas chromatography to measure the ethylene produced was first proposed by Hardy and Knight (1967). The ARA is a simple but indirect method to test nitrogenase activity (Bergersen 1970). The ARA is about 10^3 times more sensitive than ^{15}N techniques and 10^6 times more sensitive than the Kjeldahl method. Ethylene can be separated completely from C_2H_2, CH_4, and all other gases and C_2H_2 and C_2H_4 are easily and rapidly detected using gas chromatography. As the ARA does not measure transfer of fixed N_2 from the diazotroph to the associated crop plant, it can only identify whether or not nitrogenase activity is present in a particular system. Experiments with ^{15}N are still necessary to demonstrate that agricultural crops derive significant benefit from N_2 fixation. Being an indirect assay, the major difficulty with the ARA is in quantifying the amounts of N_2 fixed over time. The ratio of C_2H_2:N_2 reducing activity cannot be assumed with much accuracy without actual tests and is seldom experimentally determined (Knowles 1981). Theoretically, three moles of C_2H_2 are reduced per mole of N_2 reduced, however, C_2H_2: N_2 ratios varying from 1.5 to 6.9 for different systems have been reported (Bergersen 1970, Knowles 1981). The solubilities of C_2H_2 and N_2 in water are different which makes it difficult to interpret the C_2H_2 data. Problems with the ARA can also be encountered when low N_2-fixation rates are measured in soils. Ethylene produced by anaerobic bacteria can overestimate N_2 fixation, and bacterial oxidation of ethylene can reduce estimates of fixation (de Bont 1976, Harvey and Unscott 1978, Nohrstedt 1975, Witty 1979). The problem of endogenous production of ethylene interfering in ARA could be overcome using $^{14}C_2H_2$ (Witty 1979) or the endogenous production of ethylene can be measured by suppressing nitrogenase activity using CO, which stops nitrogenase functioning without damaging the plants (Nohrstedt 1983).

The excised root assay involves preincubation for 8-18 h under reduced oxygen tension before exposure to C_2H_2 (Dobereiner and Day 1975, Neyra and Dobereiner 1977). During preincubation of roots, considerable fermentation and proliferation of bacteria takes place resulting in overestimation of nitrogenase activity (Okon et al. 1977, van Berkum and Bohlool 1980, Barber et al. 1976). However, immediate reduction of acetylene by excised roots from several grasses (van Berkum and Sloger 1979) and sorghum and millets (Dart and Wani 1982) has been reported. The difficulties in complete recovery of the plant roots under field conditions complicates the interpretation and comparison of data collected by different groups.

In-situ assays with intact plants are cumbersome and the measurements are difficult to interpret (Balandreau and Dommergues 1973, Lee et al. 1977, Tjepkema and van Berkum 1977). Soil-root cores removed from the field at harvest have been used for measuring nitrogenase activity of both grasses and grain crops (Day et al. 1975b; van Berkum and Day 1980; Wani et al. 1983; Wani, in press). The initial lag period varies from 1 to 30 h with soil cores, depending on the time required for diffusion of C_2H_2 through different soil types (van Berkum and Day 1980, Wani et al. 1984). However, large plant-to-plant variability has been reported using this technique (Dart and Wani 1982, Wani et al. 1983, Upadhyaya 1984) and it is not clear whether this reflects the natural variation. However, such variability has been reported with *in-situ* assays (Balandreau 1979) as well as intact plant assays in the greenhouse (Wani et al. 1984). The factors responsible have been studied and as a result improvements

in the soil-core assay technique have been made (Wani et al. 1983).

With the improved soil-core (planted core) assay, which involves growing the plant in cores in the field from 20 days after sowing (DAS) till assayed, significantly higher activity has been recorded than for the plants grown and sampled in the normal (disturbed core) way (Wani et al. 1983). In some cases results of soil-core assays are extrapolated to hectare basis on the basis of core area (Nelson et al. 1976, Weaver et al. 1980) or plant population (Pedersen et al. 1978). However, such estimates can be correct only when factors like seasonal and diurnal variations in the activity, soil moisture, soil temperature, and fertility status of the soil are taken into account. If it is essential to extrapolate the soil-core assay results to hectare basis, then several cores at each assay time should be taken and the plants should be assayed at regular intervals throughout the growth period. The activity at different crop-growth stages can be plotted, and by considering the period under each activity point, necessary corrections for diurnal variation and C_2H_2:N_2 reduction ratio can be made.

An intact-plant assay for pot-grown plants overcomes the problems faced with soil-core assays, e.g., destruction of the plants, mechanical disturbance, tedious and time-consuming operations, etc. (Wani et al. 1984). Using this technique, genotypes can be screened for their potential to stimulate rhizospheric nitrogenase activity and/or various environmental and biological factors affecting the activity can be studied. Similarly, for tube-grown seedlings, intact-plant assays have been used for screening lines of crops or bacterial strains in association with the plants for their nitrogenase activity (Wani, in press). These intact-plant assays being nondestructive are promising for screening plants with high activity. Selected plants can then be used in breeding programs.

Current Understanding about Factors Affecting Nonsymbiotic and Associative N_2 Fixation

Energy Source

The basic unsolved problem concerning associative nitrogen fixation is the supply of an adequate energy source. The types and numbers of microorganisms present in the rhizosphere are largely determined by energy sources available through root exudates and plant debris (Rovira 1965). Root exudates play an important role for rhizospheric microflora of young seedlings. As roots age and die, cell debris becomes the dominant energy source. Plant-root exudation is affected by plant species, cultivar, plant age, light, temperature, plant nutrition, soil moisture, microorganisms, and root damage (Rovira 1965, 1969). These are generally the same factors that affect associative nitrogen fixation (Dobereiner and Day 1975; Balandreau et al. 1978; Wani et al. 1983, 1984). The total loss of carbon from roots is much greater when compared to the organic carbon exuded (Rovira 1969, Martin 1977, Barber and Martin 1976). Using growth and nitrogenase activity of azospirilla as criteria, qualitative differences in the root exudates of sorghum (ICRISAT 1983) and millet genotypes (Rao and Venkateswarlu 1986) have been shown.

Photosynthesis

Several-fold higher nitrogenase activity has been recorded with intact sorghum plants, as compared to those whose tops were removed prior to assay (Wani et al. 1984). However, it is not clear whether this effect is directly related to photosynthate supply.

Diurnal variations in nitrogenase activity associated with grasses, sorghum, millet, finger millet, *Panicum maximum,* and *Lolium perenne* have been reported (Dobereiner and Day 1975; Balandreau 1975; Wani et al. 1983; Wani, in press; Upadhyayaet al. 1986). However, these studies do not show any clear relationship between photosynthesis and root-associated N_2 fixation because fluctuations in soil temperature coincide with the cycle of nitrogenase activity during the day-night cycle (Dart and Wani 1982, van Berkum and Bohlool 1980). Further experiments conducted at ICRISAT Center with controlled soil temperature did not show diurnal variation in nitrogenase activity of intact sorghum and millet plants (Wani, unpublished results). Significant changes in the rate of root-associated C_2H_2 reduction within 15 min of transferring plants from sunlight into the dark or vice versa have been reported (Van Berkum and Sloger 1981), whichsuggests a strong link between nitrogenase activity and light incidence in rice. However, the nitrogenase activity of the above-reported crops may be indirectly related to photosynthesis, as is evident from studies of Dobereiner et al. (1973) who found that even though no diurnal cycle of nitrogenase was observed in *P. notatum,* prolonged incubations of plants in the dark reduced the activity.

Seasonal Variation

Seasonal variations in the nitrogenase activity of forage grasses, corn, sorghum, millet, *Setaria italica*, and *Eleucine coracana* have been reported using excised-root or soil-core assays (Dobereiner and Day 1975, von Bulow and Dobereiner 1975, Balandreau 1975, Kapulnik et al. 1981, Wani et al. 1983, Upadhyaya et al. 1986). With corn maximum activities were recorded at the 75% silking stage (von Bulow and Dobereiner 1975), and with sorghum and millet at the late flowering/early grain-filling stage. The activity was related to ontogenetic development of the plant (Wani et al. 1983). While studying seasonal profiles of nitrogenase activity, fertilizer nitrogen should be taken into consideration as it has been shown that higher rates of N application inhibit the nitrogenase activity associated with cereals.

Temperature

A linear response to temperature has been observed from 10°C to 35°C for C_2H_2 reduction activity of *Clostridium pasteurianum* in culture and for *Azotobacter* cell-free nitrogenase (Hardy et al. 1968). Increased C_2H_2 reduction over time by grass cores was attributed to warming of the soil (Nelson et al. 1976). With intact millet and sorghum plants grown in pots, significantly higher C_2H_2 reduction activities were recorded at 34° and 40°C than with the plants incubated at 29°C (Wani et al. 1984).

Soil Moisture

The rate of nitrogenase activity by the soil cores was positively correlated with soil moisture and the rate of acetylene reduction increased exponentially with linear increases in soil moisture (Day et al. 1975a). Similar correlations have been reported in soil cores of grasslands (Vlassak et al. 1973) and sorghum and millet (Wani et al. 1983), as also in pot-grown sorghum and millet plants (Wani et al. 1984). It is difficult to pinpoint how soil moisture affects nitrogenase activity as many plant processes that may influence this activity are also affected by soil-moisture levels. Day et al. (1975a) hypothesized that as the level of anaerobiosis in soil crumbs and the rhizosphere increases with higher soil moisture, the pO_2 affects nitrogenase activity.

Combined Nitrogen and Phosphorus

The presence of combined N affects the enzyme nitrogenase. Manipulating the times and methods of N application and selection of the proper form of fertilizer N, like slow-release formulations, may help to harness maximum nitrogen fixation associated with these crops, without reducing yields.

Phosphorus fertilizer application is required for optimum growth and nitrogen-fixing activity by azolla and blue-green algae (De and Mandal 1956, Watanabe et al. 1977). It is necessary to study the effect of levels and forms of P and K and other elements on nitrogenase activity of cereals.

Plant Breeding

The major thrust in N_2 fixation has been microbiological in orientation. Even though the role of plant genotype in N_2 fixation has been recognized, there is a paucity of information on the nature of the genetic involvement of the host. The use of nondestructive intact-plant assays for measuring C_2H_2 reduction activity in the greenhouse coupled with ^{15}N isotope dilution technique and further testing for yield potential under low-fertility field conditions seems a prospective proposal for such studies.

Before breeding methods can produce cereal lines with increased potential for nitrogen fixation, it is essential to understand the associations governing N_2 fixation traits in particular crops. There are several reports indicating differences amongst genotypes of sorghum, millet, minor millets, rice and forage grasses, and wheat, (Bouton et al. 1979; Dobereiner 1966, 1970,1977; von Bulow and Dobereiner 1975; Pederson et al. 1978; Watanabe et al. 1979; Charyulu et al. 1981; Dart and Wani 1982; Upadhyaya et al. 1986; Wani, in press). At ICRISAT Center, 18 out of 248 millet lines tested showed significantly high nitrogenase activity (>460 nmol C_2H_4 15 cm diam core^{-1} h^{-1}) and two lines, Gam 73 and J 1407, were consistently active over several seasons. Similarly, 15 out of 334 lines of field-grown sorghum were consistently active in three or more seasons though not on each assay occasion. This may have been due to unfavorable soil moisture or other conditions during the season.

In the Ex-Bornu population of millet, large plant-to-plant variability for stimulating nitrogenase activity ranging from 0 to 1900 nmol C_2H_4 plant^{-1} h^{-1} has been observed using intact pot assay technique.

Work on stabilizing the character of high and low nitrogenase activity in this population is under way to study the inheritance of this trait (ICRISAT 1983). There is an urgent need to pursue breeding research for producing lines with increased potential for nitrogen fixation.

Crop Responses to Inoculation

There are several reports about field- and pot-grown cereals inoculated with N_2-fixing bacteria and these have been reviewed (Boddey and Dobereiner 1982). Many reports show statistically significant increases in cereal yields or otherwise and also negative responses. The mechanism by which the cereals inoculated with nitrogen-fixing bacteria derive the benefit is not clearly understood. However, knowledge has accumulated to indicate the possible mechanisms involved. It has been shown that N_2-fixing azospirilla and *Azotobacter* benefit the inoculated plants through biological nitrogen fixation (Cohen et al. 1980, Dart and Wani 1982, Hegazi et al. 1983, Nur et al. 1980, Okon 1982) and also by enhancing root-hair formation and therefore, increased root uptake capacity caused by the secretion of growth hormones (Tien et al. 1979, Vlassak and Reynders 1981). The extent to which each of the various processes contributes to yield increase of inoculated cereal plants remains to be assessed.

Areas of Future Research

These associative N_2-fixing systems need to be understood in detail, in order to fully harness their potential benefits. Work in several areas needs to be continued with vigor so as to put the systems to work and improve them further. More precise estimates of the quantity of nitrogen fixed are essential by conducting experiments using careful nitrogen-balance studies and ^{15}N -based techniques.

Methodologies for studying associative nitrogen fixation with improved methods of measuring nitrogenase activity (acetylene reduction), e.g., planted-core assays for field-grown plants, intact-plant assays for tube-grown seedlings and potted greenhouse plants, etc., and greater use of ^{15}N-based techniques have been developed. However, this area needs more attention as it is important from the point of view of better screening and selection methods.

There is a need to look at the bacterial systems involved in associative symbiosis systems. By manipulating the culture media and cultural conditions like O_2 concentration, pH, temperature, and concentrations of carbon and other nutrient sources, it will be possible to identify many unknown organisms involved in these associations. In particular, we need to give thought to the role of nonnitrogen fixers present in the rhizosphere, as reports have shown synergism amongst nitrogen fixers and nonnitrogen fixers. These organisms may play an important role in manipulating oxygen concentration in the rhizosphere, pH changes and moreover, may provide energy substances by metabolizing the compounds which N_2-fixing bacteria cannot use directly.

More critical methods of isolation and identification are required, as it is apparent that there are still little-known or new forms of rhizosphere bacteria. Greater support for taxonomic studies is essential. The role of bacteria in associative symbiotic systems needs to be better understood at the basic level so as to seek information on (1) location of the bacteria on roots, (2) source of energy for N_2 fixation, (3) role of plant affinity beyond that of carbohydrate supplier, (4) ecological factors governing such associations, (5) types of bacteria involved, and (6) criteria to be used for selecting bacterial strains for field-inoculation studies.

Although several reports have indicated significant positive responses to crop inoculations in fields, the data are highly variable. The reasons for the failure to obtain positive responses in some cases must be studied. Increased yields of field-grown crops inoculated with N_2-fixing bacteria and the possibility of interaction between host cultivars and bacterial strains indicate the need to select the most-suitable combinations of host cultivars and bacterial strains. There is little agreement on several points. For example: (1) Is there a host specificity for bacteria? (2) Which bacteria should be used as inoculum and should it be a single species or a mixture of bacteria? (3) Which method of inoculation should be used? (4) What should be the nature of a suitable carrier? (5) What criteria should be used for checking the quality of the inoculants produced?

Information is also essential to put the system to work. More emphasis needs to be given to studies pertaining to establishment and survival of the added inoculum in the rhizosphere and also the factors that might affect the performance of the added inoculum. What is the exact role of inoculated bacteria in increasing crop yields? Are these solely due to N_2-fixation or hormonal effects or because of protection against plant pathogens?

Knowledge about the agronomic practices that could help increase N_2 fixation under normal situations, as well as with inoculation, will go a long way in improving N_2 fixation. The information on the role of organic amendments, synergistic levels of combined N, appropriate form and method of application of combined N, effect of other macro- and microelements on N_2 fixation, and interaction with other rhizospheric microorganisms like mycorrhiza, will help to derive maximum possible benefits from associative N_2 fixation.

Based on the available literature, it seems possible to improve plant genotypes in a practical way for increased associative N_2 fixation by following routine plant breeding methods. Not many efforts have been directed in this line, probably because of lack of routine assay methods to measure N_2 fixation, which can be used for selection, and also lack of information on the inheritance of this particular trait in host plant. More information is required on basic aspects, such as mechanism of inheritance of the N_2 fixation trait in host plants, criteria to be used for selecting lines with high N_2 fixation potential, and breeding methods to be adopted. Through concerted efforts in this direction, it will be possible to select or breed host lines with increased associative N_2-fixing ability.

References

App, A., Bouldin, *D.R.*, Dart, P.J., and Watanabe, I. 1980. Constraints to biological nitrogen fixation in soils of *the* tropics. Pages 319-337 *in* Priorities for alleviating soil-related constraints to food production in the tropics. Los Banos, Laguna, Philippines: International Rice Research Institute.

Balandreau, J. 1975. Mesure de l'activite nitrogenasique des microorganismes fixateurs libres d'azote de la rhizosphere de quelques graminees. (In Fr. Summary in En.) Revue d'Ecologie et de Biologie du Sol 12:273-290.

Balandreau, J. 1979. Evaluation and factors affecting rhizospheric nitrogen fixation. Pages 166-189 *in* Recent advances in biological nitrogen fixation (Subba Rao, N.S., ed.). New Delhi, India: Oxford and IBH Publishing Co.

Balandreau, J. 1983. Microbiology of the association. Canadian Journal of Microbiology 29:851-859.

Balandreau, J., and Dommergues, Y. 1973. Assaying nitrogenase (C_2H_2) activity in the field. Pages 247-254 *in* Modern methods in the study of microbial ecology (Roswall, T, ed.). Bulletins from the Ecological Research Committee no. 17. Stockholm, Sweden: Swedish Natural Science Research Council.

Balandreau, J., Ducerf, P., Hamad-Fares, I., Weinhard, P., Rinaudo, G., Millier, C., and Dommergues, Y. 1978. Limiting factors in grass nitrogen fixation. Pages 275-302 *in* Limitations and potentials for biological nitrogen fixation in the tropics (Dobereiner, J., Burris, R.H., Hollaender, A., France, A.A., Neyra, C.A., and Scott, D.B., eds.). New York, USA: Plenum Press.

Balandreau, J., Rinaudo, G., Oumarov, M.M., and Dommergues, Y. 1976. Asymbiotic N_2 fixation in paddy soils. Pages 611-628 *in* Proceedings of the First International Symposium on Nitrogen Fixation, 3-7 Jun 1974, Pullman, Washington, USA (Newton, W.E., and Nyman, *C.J.*, eds.). Vol.2. Pullman, Washington, USA: Washington State University Press.

Barber, L.E., and Martin, J.K. 1976. The release of organic substances by cereal roots into soil. New Phytology 76:69-80.

Barber, L.E., Tjepkema, J.D., Pussell, S.A., and Evans, H.J. 1976. Acetylene reduction (nitrogen fixation) associated with corn inoculated with *Spirillum.* Applied and Environmental Microbiology 32:108-113,

Beijerinck, M.W. 1901. Ueber Oligonitrophile Mikroben. (In De.) Zentralblatt fuer Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene,II 7:561-582.

Bergersen, F.J. 1970. The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. Australian Journal of Biological Sciences 23:1015-1025.

Boddey, R.M., and Dobereiner, J. 1982. Association of *Azospirillum* and other diazotrophs with tropical Graminae. Pages 28-47 *in* Non-symbiotic nitrogen fixation and organic matter in the tropics. Symposia papers 1. Transactions of the 12th International Congress of Soil Science, 8-16 Feb 1982, New Delhi, India. New Delhi, India: Indian Agricultural Research Institute.

Bouton, J.H., Smith, R.L., Schank, S.C., Burton, G.W., Tyler, M.E., Littell, R.C., Gallaner, R.N., and Quesenberry, K.H. 1979. Response of pearl millet inbreds and hybrids to inoculation with *Azospirillum brasilense.* Crop Science 19:12-16.

Brouzes, R., Mayfield, C.L., and Knowles, R. 1971. Effect of oxygen partial pressure on nitrogen fixation and acetylene reduction in a sandy loam soil amended with glucose. Plant and Soil, Special volume, pp. 481-494.

Charyulu, P.B.B.N., Nayak, D.N., and Rao, V.R. 1981. $^{15}N_2$ incorporation by rhizosphere soil: influence of rice variety, organic matter and combined nitrogen. Plant and Soil 59:399-405.

Cohen, E., Okon, Y., Kigel, J., Nur, I., and Henis, Y. 1980. Increase in dry weight and total nitrogen content in *Zea mays* and *Setaria italica* associated with nitrogen fixing *Azospirillum* spp. Plant Physiology 66:746-749.

Dart, P.J., and Day, J.M. 1975. Non-symbiotic nitrogen fixation in soils. Pages 225-252 *in* Soil microbiology: a critical review (Walker, N., ed.). London, UK: Butterworths.

Dart, P.J., and Wani, S.P. 1982. Non-symbiotic nitrogen fixation and soil fertility. Pages 3-27 *in* Non-symbiotic nitrogen fixation and organic matter in the tropics. Symposia paper I, Transactions of the 12th International Congress of Soil Science, 8-16 Feb 1982, New Delhi, India. New Delhi, India: Indian Agricultural Research Institute.

Day, J.M., Harris, D., Dart, P.J., and van Berkum, P. 1975a. The Broadbalk experiment. An investigation of nitrogen gains from non-symbiotic fixation. Pages 71-84 *in* Nitrogen fixation by free-living micro-organisms (Stewart, W.D.P., ed.). Cambridge, UK: Cambridge University Press.

Day, J.M., Neves, M.C.P., and Dobereiner, J. 1975b. Nitrogenase activity on the roots of tropical forage grasses. Soil Biology and Biochemistry 7:107-112.

De, P.K., and Mandal, K.A. 1956. Fixation of nitrogen by algae in rice soils. Soil Science 81:453-458.

de Bont, J.A.M. 1976. Bacterial degradation of ethylene and the acetylene reduction test. Canadian Journal of Microbiology 22:1060-1062.

De-Polli, H., Matsui, E., Dobereiner, J., and Salati, E. 1977. Confirmation of nitrogen fixation in two tropical grasses by $^{15}N_2$ incorporation. Soil Biology and Biochemistry 9:119-123.

Dobereiner, J. 1966. *Azotobacter paspali* n. sp. uma bacteria flxadora de nitrogenio na rhizosphera de *paspalum.* (In Pt.) Pesquisa Agropecuaria Brasileira 1:357-365.

Dobereiner, J. 1970. Further research on *Azotobacter paspali* and its variety specific occurrence in the rhizosphere of *Paspalum notatum,* Flugge. Zentralblatt fuer Bakteriologie, Parasitenkunde, Infektionskrankheiten and Hygiene, II 124:224-230.

Dobereiner, J. 1977. Plant genotype effects on nitrogen fixation in grasses. Pages 325-334 *in* Genetic diversity in plants (Muhammed, A., Aksel, R., and von Borstel, R.C, eds.). New York, USA: Plenum Press.

Dobereiner, J. 1978. Nitrogen fixation in grass-bacteria associations in the tropics. Pages 51-68 *in* Isotopes in biological dinitrogen fixation: Proceedings of an Advisory Group Meeting organized by the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, 21-25 Nov 1977, Vienna, Austria. Vienna, Austria: International Atomic Energy Agency.

Dobereiner, J., and Day, J.M. 1975. Nitrogen fixation in the rhizosphere of tropical grasses. Pages 39-56 *in* Nitrogen fixation by free-living micro-organisms (Stewart, W.D.P., ed.). Cambridge, UK: Cambridge University Press.

Dobereiner, J., Day, J.M., and Dart, P.J. 1972. Nitrogenase activity and oxygen sensitivity of the *Paspalum notatum-Azotobacter paspali* association. Journal of General Microbiology 71:103-116.

Dobereiner, J., Day, J.M., and Dart, P.J. 1973. Rhizosphere associations between grasses and nitrogen-fixing bacteria: effect of 0_2 on nitrogenase activity in the rhizosphere of *Paspalum notatum.* Soil Biology and Biochemistry 5:157-159.

Eskew, D.L, Eaglesham, A.R.J., and App, A.A. 1981. Heterotrophic $^{15}N_2$ fixation and distribution of newly fixed nitrogen in a rice-flooded soil system. Plant Physiology 68:48-52.

Firth, P., Thitipoca, H., Suthipradit,S., Wetselaar, R.,and Beech, *D.F.* 1973. Nitrogen balance studies in the Central Plain of Thailand. Soil Biology and Biochemistry 5:41-46.

Giller, K.E., Day, J.M., Dart, P.J., and Wani, S.P. 1984. A method for measuring the transfer of fixed nitrogen from free-living bacteria to higher plants using $^{15}N_2$. Journal of Microbiological Methods 2(6):307-316.

Giller, K.E., Wani, S.P., and Day, J.M. 1986. Use of isotope dilution to measure nitrogen fixation associated with the roots of sorghum and millet genotypes. Plant and Soil 90:255-263.

Greenland, D.J. 1962. Denitrification in some tropical soils. Journal of Agricultural Science (UK) 58:227-233.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K., and Burns, R.C. 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiology 43:1185-1207.

Hardy, R.W.F., and Knight, E.J. 1967. ATP-dependent reduction of azide and HCN by N_2-fixing enzymes of *Azotobacter vinelandii* and *Clostridium pasteurianum.* Biochemica et Biophysica Acta 139:69-90.

Harvey, R.G., and Linscott, J.J. 1978. Ethylene production in soil containing quackgrass rhizomes and other plant materials. Soil Science Society of America Journal 42:721-724.

Hauke-Pacewiczowa, T., Balandreau, J., and Dommergues, Y. 1970. Fixation microbienne de l'azote dans un sol salin tunisien. (In Fr. Summary in En.) Soil Biology and Biochemistry 2:47-53.

Hegazi, N.A., Monib, M., Amer, H.,and Shokr, E. 1983. Response of maize plants to inoculation with azospirilla and (or) straw amendment in Egypt. Canadian Journal of Microbiology 29:888-894.

Hill, S., and Postgate, J.R. 1969. Failure of putative nitrogen-fixing bacteria to fix nitrogen. Journal of General Microbiology 58:277-283.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), 1983. Annual report 1982. Patancheru, A.P. 502 324, India; ICRISAT.

Ito, O., Cabrera, D., and Watanabe, I. 1980. Fixation of dinitrogen-I5 associated with rice plants. Applied and Environmental Microbiology 39:554-558.

Jenkinson, D.S. 1977. The nitrogen economy of the Broadbalk experiments. I. Nitrogen balance in the experiments. Report for 1976, Rothamsted Experimental Station 2:103-109.

Kapulnik, Y., Sang, S., Nur, I., Okon, Y., Kigel, J., and Henis, Y. 1981. Yield increases in summer cereal crops of Israel in field inoculated with *Azospirillum,* Experimental Agriculture 17:171-178.

Kapustka, L.A., and Rice, E.L. 1978. Symbiotic and asymbiotic N_2-fixation in a tall grass prairie. Soil Biology and Biochemistry 10:553-554.

Karamanos, R.E., and Rennie, D.A. 1980. Changes in natural 15N abundance associated with pedogenic processes in soil. II. Changes on different slope positions. Canadian Journal of Soil Science 60:365-372.

Knowles, R. 1976. Factors affecting dinitrogen fixation by bacteria in natural and agricultural systems. Pages 539-555 *in* Proceedings of the First International Symposium on Nitrogen Fixation, 3-7 Jun 1974, Pullman, Washington, USA (Newton, W.E., and Nyman, C.J., eds.). Vol. 2. Pullman, Washington, USA: Washington State University Press.

Knowles, R. 1981. The measurement of nitrogen fixation. Pages 327-333 *in* Current perspectives in nitrogen fixation: proceedings of the Fourth International Symposium on Nitrogen Fixation, 1-5 Dec 1980, Canberra, Australia (Gibson, A.K., and Newton, W.E., eds.). Canberra, Australia: Australian Academy of Science.

Koch, B.L., and Oya, J. 1974. Non-symbiotic nitrogen fixation in some Hawaii pasture soils. Soil Biology and Biochemistry 6:363-367.

Koyama, T., and App, A. 1979. Nitrogen balance in flooded rice soils. Pages 95-104 *in* Nitrogen and rice. Los Banos, Laguna, Philippines: International Rice Research Institute.

Krishnamoorthy, K.K., and Ravikumar, T.V. 1973. Permanent manurial experiments conducted at Coimbatore. Coimbatore, Tamil Nadu, India: Tamil Nadu Agricultural University.

Lee, K.K., Alimagno, B., and Yoshida, T. 1977. Field technique using the acetylene reduction method to assay nitrogenase activity and its association with the rice rhizosphere. Plant and Soil 47:519-526.

Line, M.A., and Loutit, M.W. 1973. Studies on non-symbiotic nitrogen fixation in New Zealand fussock-grass land soils. New Zealand Journal of Agricultural Research 16:87-94.

Magalhase, F.M.M., Patriquin, D., and Dobereiner, J. 1979. Infection of field grown maize with *Azospirillum* spp. Revista Brasileira de Biologia 39:587-596.

Martin, J.K. 1977. Factors influencing the loss of organic carbon from wheat roots. Soil Biology and Biochemistry 9:1-7.

Moore, A.W. 1963. Nitrogen fixation in latosolic soil under grass. Plant and Soil 19:127-138.

Moore, A.W. 1966. Non-symbiotic nitrogen fixation in soil and soil-plant systems. Soils and Fertilizers 29:113-128.

Nelson, A.D., Barber, L.E., Tjepkema, J., Russell, S.A., Powelson, R., Evans, H.J., and Seidler, R.J. 1976. Nitrogen fixation associated with grasses in Oregon. Canadian Journal of Microbiology 22:523-530.

Neyra, C.A., and Dobereiner, J. 1977. Nitrogen fixation in grasses. Advances in Agronomy 29:1-38.

Nohrstedt, H.O. 1975. Decomposition of ethylene in soils and its relevance in the measurement of nitrogenase activity with the acetylene reduction method. Grundforbattring 27:171-178.

Nohrstedt, H.O. 1983. Natural formation of ethylene in forest soils and methods to correct results given by the acetylene-reduction assay. Soil Biology and Biochemistry 15(3):281-286.

Nur, I., Okon, Y., and Henis, Y. 1980. An increase in nitrogen content of *Setaria italica* and *Zea mays* inoculated with *Azospirillum.* Canadian Journal of Microbiology 26:482-485.

Okon, Y. 1982. *Azospirillum:* physiological properties, mode of association with roots and its application for the benefit of cereal and forage grass crops. Israel Journal of Botany 31:214-220.

Okon, Y., Albrecht, S.L., and Burris, R.H. 1977. Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Applied and Environmental Microbiology 33:85-88.

Parker, C.A. 1957. Non-symbiotic nitrogen-fixing bacteria in soil. III. Total nitrogen changes in a field soil. Journal of Soil Science 8:48-59.

Patriquin, D.G., and Dobereiner, J. 1978. Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. Canadian Journal of Microbiology 24:734-742.

Pedersen, W.L., Chakrabarty, K., Klucas, R.V.,and Vandiver, A.K. 1978. Nitrogen fixation (acetylene reduction) associated with roots of winter wheat and sorghum in Nebraska. Applied and Environmental Microbiology 35:129-135.

Postgate, J.R. 1972. The acetylene reduction test for nitrogen fixation. Pages 343-356 *in* Methods in microbiology (Norris, J.R., and Ribbons, D.W., eds.). Vol. 6B. London, UK: Academic Press.

Rao, A.V., and Venkateswarlu, B. 1986. Studies on the interactions between *Azospirillum* and Pearl millet. Pages 37-42 *in* Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics.

Rovira, A.D. 1965. Plant root exudates and their influence upon soil microorganisms. Pages 170-186 *in* Ecology of soil-borne plant pathogens: prelude to biological control (Barker, K.F., and Snyder, W.C., eds.). Berkley, California, USA: University of California Press.

Rovira, A.D. 1969. Plant root exudates. Botanical Review 35:35-57.

Ruschel, A.P., Henis, Y., and Salati, E. 1975. Nitrogen-15 tracing of N_2-Fixation with soil-grown sugarcane seedlings. Soil Biology and Biochemistry 7:181-182.

Ruschel, A.P., Matsui E., Salati, E., and Vose, P.B. 1981. Potential N_2-fixation by sugar cane (*Saccharum* sp.) in solution culture. II. Effect of inoculation and dinitrogen fixation as directly measured by $^{15}N_2$. Pages 127-132 *in* Associative N_2-fixation: proceedings of the International Workshop on Associative N_2-Fixation, 2-6 Jul 1979, Piracicaba, Brazil (Vose, P.B., and Ruschel, A.P., eds.). Vol.2. Boca Raton, Florida, USA: CRC Press.

Ruschel, A.P., Victoria, R.L., Salati, E., and Henis, Y. 1978. Nitrogen fixation in sugarcane (*Saccharum officinarum* L.). Pages 297-303 *in* Environmental role of nitrogen-fixing blue-green algae and asymbiotic bacteria (Granhall, U., ed.). Ecological Bulletins no.26. Stockholm, Sweden: Swedish Natural Science Research Council.

Schank, S.C., Smith, R.L., and Littell, R.C. 1983. Establishment of associative N_2-fixing systems. Proceedings, Soil and Crop Science Society of Florida 42:113-117.

Singh, P.K. 1981. Use of *Azolla* and blue-green algae in rice cultivation in India. Pages 183-196 in Associative N_2-fixation: proceedings of the International Workshop on Associative N_2-Fixation, 2-6 Jul 1979, Piracicaba, Brazil (Vose P.B., and Ruschel, A.P., eds.). Vol.2. Boca Raton, Florida, USA: CRC Press.

Smith, R.M., Thompson, D.O., Collier, J. W., and Hervey, RJ. 1954. Soil organic matter, crop yields, and land use in the Texas Blackland. Soil Science 77:377-388.

Steyn, P.L., and Delwiche, C.C. 1970. Nitrogen fixation by non-symbiotic micro-organisms in some southern California soils. Environmental Science and Technology 4:1122-1128.

Thomas-Bauzon, D., Weinhard, P., Villecourt, P., and Balandreau, J. 1982. The spermosphere model. I. Its use in growing, counting and isolating N_2-fixing bacteria from the rhizosphere of rice. Canadian Journal of Microbiology 28:922-928.

Tien, T.M., Gaskins, M.H., and Hubbell, D.H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Applied and Environmental Microbiology 37:1016-1024.

Tjepkema, J.D., and Burris, R.H. 1976. Nitrogenase activity associated with some Wisconsin prairie grasses. Plant and Soil 45:81-94.

Tjepkema, J.D., and Evans, HJ. 1976. Nitrogen fixation associated with *Juncus balticus* and other plants of Oregan wetlands. Soil Biology and Biochemistry 8:505-509.

Tjepkema, J.D., and van Berkum, P. 1977. Acetylene reduction by soil cores of maize and sorghum in Brazil. Applied and Environmental Microbiology 33:626-639.

Umali-Garcia, M., Hubbell, D.H., and Gaskins, M.H. 1978. Process of infection of *Panicum maximum* by *Spirillum lipoferum.* Pages 373-379 *in* Environmental role of nitrogen-fixing blue-green algae and asymbiotic bacteria (Granhall, U., ed.). Ecologica Bulletins no.26. Stockholm, Sweden: Swedish Natural Science Research Council.

Umali-Garcia, M., Hubbell, D.H., Gaskins, M.H., and Dazzo, F.B. 1980. Association of *Azospirillum* with grass roots. Applied and Environmental Microbiology 39:219-226.

Upadhyaya, M.N. 1984. Root associated nitrogen fixation in finger millet (*Eleusine coracana* Gaertn.). M.Sc. thesis, University of Agricultural Sciences, Bangalore, Karnataka, India.

Upadhyaya, M.N., Hegde, S.V., Rai, P.V., and Wani, S.P. 1986. Root associated nitrogen fixation in finger millet. Pages 93-101 *in* Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics.

Vallis, I. 1973. Sampling for soil nitrogen changes in large areas of grazed pastures. Communications in Soil Science and Plant Analysis 4:163-170.

van Berkum, P., and Bohlool, B.B. 1980. Evaluation of nitrogen fixation by bacteria in association with roots of tropical grasses. Microbiological Reviews 44(3):491-517.

van Berkum, P., and Day, J.M. 1980. Nitrogenase activity associated with soil cores of grasses in Brazil. Soil Biology and Biochemistry 12:137-140.

van Berkum, P., and Sloger, C. 1979. Immediate acetylene reduction by excised grass roots not previously preincubated at low oxygen tensions. Plant Physiology 64:739-745.

van Berkum, P., and Sloger, C. 1981. Physiology of root-associated nitrogenase activity in *Oryza saliva* L. Page 490 *in* Current perspectives in nitrogen fixation: proceedings of the Fourth International Symposium on Nitrogen Fixation, 1-5 Dec 1980, Canberra, Australia (Gibson, A.H., and Newton, W.E., eds.). Canberra, Australia: Australian Academy of Science.

Venkataraman, G.S. 1975. The role of blue-green algae in tropical rice cultivation. Pages 207-218 *in* Nitrogen fixation by free-living micro-organisms (Stewart, W.D.P., ed.). Cambridge, UK: Cambridge University Press.

Villemin, G., Balandreau, J., and Dommergues, Y. 1974. Utilisation du test de reduction de l'acetylene pour la numeration des bacteries libres fixatrices d'azote. (In Fr.) Annales de Microbiologic 24:87-94.

Vlassak, K., and Reynders, L. 1981. *Azospirillum* rhizocoenoses in agricultural practice. Page 494 *in* Current perspectives in nitrogen fixation: proceedings of the International Symposium on Nitrogen Fixation, 1-5 Dec 1980, Canberra, Australia (Gibson A.H., and Newton, W.E., eds.). Canberra, Australia: Australian Academy of Science.

Vlassak, K., Paul, E.A., and Harris, R.E. 1973. Assessment of biological nitrogen fixation in grassland and associated sites. Plant and Soil 38:637-649.

von Bulow, J.F.W., and Dobereiner, J. 1975. Potential for nitrogen fixation in maize genotypes in Brazil. Proceedings of the National Academy of Sciences of the United States of America 72:2329-2393.

Walcott, J J., Chanviroj, M., Chinchest, A., Choticheny, P., Ferraris, R., and Norman, B.W. 1977. Long term productivity of intensive rice cropping systems on the central plain of Thailand. Experimental Agriculture 13:305-315.

Wani, S.P. (In press.) Nitrogen fixation potentialities of sorghum and millet. *In* New trends in biological nitrogen fixation (Subba Rao, N.S., ed.). New Delhi, India: Oxford and IBH Publishing Co.

Wani, S.P., Dart, P.J., and Upadhyaya, M.N. 1983. Factors affecting nitrogenase activity (C_2H_2 reduction) associated with sorghum and millet estimated using the soil core assay. Canadian Journal of Microbiology 29:1063-1069.

Wani, S.P., Upadhyaya, M.N., and Dart, P.J, 1984. An intact plant assay for estimating nitrogenase activity (C_2H_2 reduction) of sorghum and millet plants grown in pots. Plant and Soil 82:15-29.

Watanabe, I. 1981. Biological nitrogen fixation associated with wetland rice. Pages 313-316 *in* Current perspectives in nitrogen fixation: proceedings of the Fourth International Symposium on Nitrogen Fixation, 1-5 Dec 1980, Canberra, Australia (Gibson, A.H., and Newton, W.E., eds.). Canberra, Australia: Australian Academy of Science.

Watanabe, L, and Barraquio, W.L. 1979. Low levels of fixed nitrogen required for isolation of free-living N_2-fixing microorganisms from rice roots. Nature 277:565-566.

Watanabe, I., and Lee, K.K. 1977. Non-symbiotic nitrogen fixation in rice and rice fields. Pages 289-305 *in* Biological nitrogen fixation in farming systems of the tropics (Ayanaba, A., and Dart, P.J., eds.). New York, USA: John Wiley.

Watanabe, L, Barraquio, W.L., De Guzman, M.R., and Cabrera, D.A. 1979. Nitrogen-fixing (acetylene reduction) activity and population of aerobic heterotrophic nitrogen-fixing bacteria associated with wetland rice. Applied and Environmental Microbiology 37:813-819.

Watanabe, L, Espinas, C.R., Berja, N.S., and Alimagno, B.V. 1977. Utilization of the *Azolla-Anabaena* complex as a nitrogen fertilizer for rice. Research Paper series 11-15. Los Banos, Leguna, Philippines: International Rice Research Institute.

Weaver, R.W., Wright, S.F., Varanka, M.W., Smith, U.E., and Holt, E.C. 1980. Dinitrogen fixation (C_2H_2) reduction by established forage grasses. Texas Agronomy Journal 72:965-968.

White, J.W., Holben, F.J., and Richer, A.C. 1945. Maintenance level of nitrogen and organic matter in grassland and cultivated soils over periods of 54 and 72 years. Journal of the American Society of Agronomy 37:21-31.

Winogradsky, S. 1893. Sur l'assimilation de l'azote gazeux de l'atmosphere par les microbes. (In Fr.) Compte Rendu Hebdomadaire des Seances de l'Academie des Sciences 116:1385-1388.

Witty, J.F. 1979. Acetylene reduction assay can overestimate nitrogen-fixation in soil. Soil Biology and Biochemistry 11:209-210.

Witty, J.F., Day, J.M., and Dart, P.J. 1977. The nitrogen economy of the Broadbalk experiments. II. Biological nitrogen fixation. Report for 1976, Rothamsted Experimental Station 2:111-118.

Yoshida, T., and Ancajas, R.R. 1973. Nitrogen-fixing activity in upland and flooded rice fields. Proceedings, Soil Science Society of America 37:42-46.

Yoshida, T., and Yoneyama, T. 1980. Atmospheric dinitrogen fixation in the flooded rice rhizosphere as determined by the 15-N isotope technique. Soil Science and Plant Nutrition 26:551-559.

Discussion

H.L.S. Tandon:
Why do you tag the inoculation with the availability of a seed drill, which introduces another cost factor? Farmers not having a drill may still be interested in the proven inoculum.

S.P.Wani:
The seed drill is not a must, but if it is already in use we are suggesting the use of the slurry inoculator. Otherwise hand application of slurry is perfectly all right.

H.L.S.Tandon:
When do you expect the BNF technology to enter on-farm research programs, before it is available for the SAT farmers?

S.P.Wani:
Inoculation technology should be at the on-farm test stage in 3-4 years.

P.Tauro:
When light has no effect, why should decapitation have an effect on ARA?

S.P.Wani:
As I mentioned in the presentation, the mechanism of reduced activity soon after decapitation is not understood yet. Because of injury to the plant, the roots may be secreting some compounds that might be detrimental to the bacteria, resulting in reduced activity.

G.S. Jadhav:
As stated in the presentation, the moisture and N in the soil should be kept constant. However, they are dynamic with time. How can the response of different strains in the field be compared, when both moisture and N are changing within the season as well as between seasons?

S.P.Wani:
I referred to constant moisture and N only for the experiments involving germplasm screening or seasonal measurement of nitrogenase activity, and not for the inoculation experiments. It will be impossible to obtain uniform moisture and N during the season in the field. This is suggested for controlled greenhouse experiments alone.

G.S.Jadhav:
Has the water-suspension method of inoculation application been compared with seed inoculation, soil application, and FYM-mixed furrow application?

S.P.Wani:
No.

S.V.Hegde:
Did you examine the roots/rhizosphere of inoculated and noninoculated pearl millet regarding the establishment of inoculated *Azospirillum* or the counts of the bacterium, to prove that the beneficial effects are due to inoculated *Azospirillum?*

S.P. Wani:
No, it is not possible to study the establishment of the inoculated strains in the field unless we have specific marker strains as inoculants. Neither MPN nor ordinary plating can give the desired results. At present we are standardizing the ELISA technique, and if we can use this technique successfully we intend to study the establishment and survival of the inoculated strain in the field.

B.K. Konde:
It is reported by Egyptian scientists that liquid inoculation is superior to seed inoculation in groundnuts where the seeds are treated with fungicides. In order to avoid the direct contact of fungicides with rhizobia, liquid inoculation proved to be superior, but methods of azospirilla inoculation have not been tried and evaluated. They, therefore, need to be studied.

S.P.Wani:
I agree with your views that work on inoculation methods of azospirilla needs to be done. About slurry inoculation at ICRISAT Center, Dr Nambiar has observed that for groundnuts slurry inoculation gives better results than seed coating, and this is mainly because the groundnut seed cotyledons get separated because of wetting, resulting in reduced germination. In addition, fungicides can be used at sowing along with rhizobial culture application.

Cereal Nitrogen Fixation Research under the BNF Coordinated Project of the ICAR

N.S. Subba Rao[1]

Summary

The paper highlights the results of field experiments carried out under the Biological Nitrogen Fixation (BNF) project of the Indian Council of Agricultural Research (ICAR) on inoculation effects of sorghum and millets by Azospirillum. *The review also encompasses the initial results obtained on the isolation and establishment of* Azospirillum *in roots, and it briefly indicates the lacunae existing in our knowledge and the future possibilities of research under the project.*

Introduction

The BNF coordinated project has the following mandate.

1. Intensification of research on biological nitrogen fixation in a coordinated manner.
2. Survey and ecology of N_2-fixing microorganisms, evaluation of benefits of inoculation and interaction with host germplasm, pesticides, and inorganic fertilizers.
3. Genetic studies to enlarge host spectrum by mutation, conjugation, transduction, and transformation.
4. Somatic hybridization, tissue-culture techniques, and fusion of protoplasts using host plants and microbial cultures.
5. Physiological studies to transfer available knowledge at the fundamental level on nitrogen-fixing and related enzymes to the applied side.

The project has 11 centers located all over India (Fig. 1).

The present report summarizes the fieldwork done on cereal nitrogen fixation under the project, with particular reference to sorghum and millets.

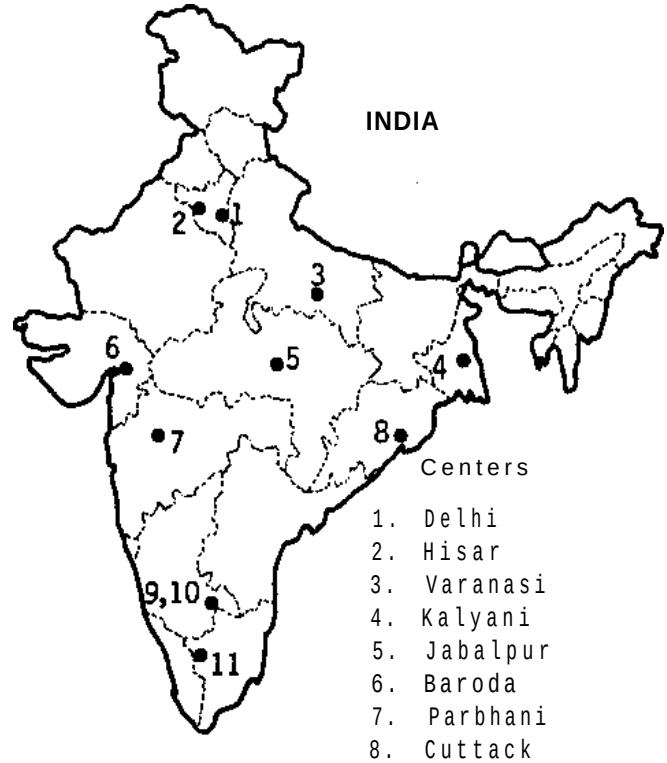


Figure 1. Locations of the centers of ICAR's coordinated project on biological nitrogen fixation.

1. Project Director, Agro-Energy Center, Indian Agricultural Research Institute, New Delhi 110 012, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

Ecology of Diazotrophic Bacteria

Several centers under the BNF project initiated work on the isolation and characterization of nitrogen-fixing bacteria from rice, wheat, barley, sorghum, and millets. Greater attention was paid to the isolation and characterization of *Azospirillum* into *A. brasilense* and *A. lipoferum.* Nitrogenase activities of intact cultivated plants and weeds associated with them were measured. *Azospirillum* isolates were purified and acetylene-reduction assay (ARA) measurements in pure cultures were made. The amount of nitrogen fixed per gram of carbon source was also quantified by the Kjeldahl method.

In root colonization studies with known strains of *Azospirillum* in association with specially grown plants, the bacteria were found in the root hairs and were found to traverse upwards in the stem, as observed under a light microscope (Lakshmi et al. 1977). In wheat, *Azospirillum* could be isolated from surface-sterilized roots and stem segments (Kavimandan et al. 1978). Transverse sections of stem showed the presence of bacteria prominently in protoxylem and often in metaxylem vessels (Lakshmi et al. 1977). In rice seedlings grown under aseptic nitrogen-free conditions, *Azospirillum* inoculation increased root biomass (Dewan and Subba Rao 1979).

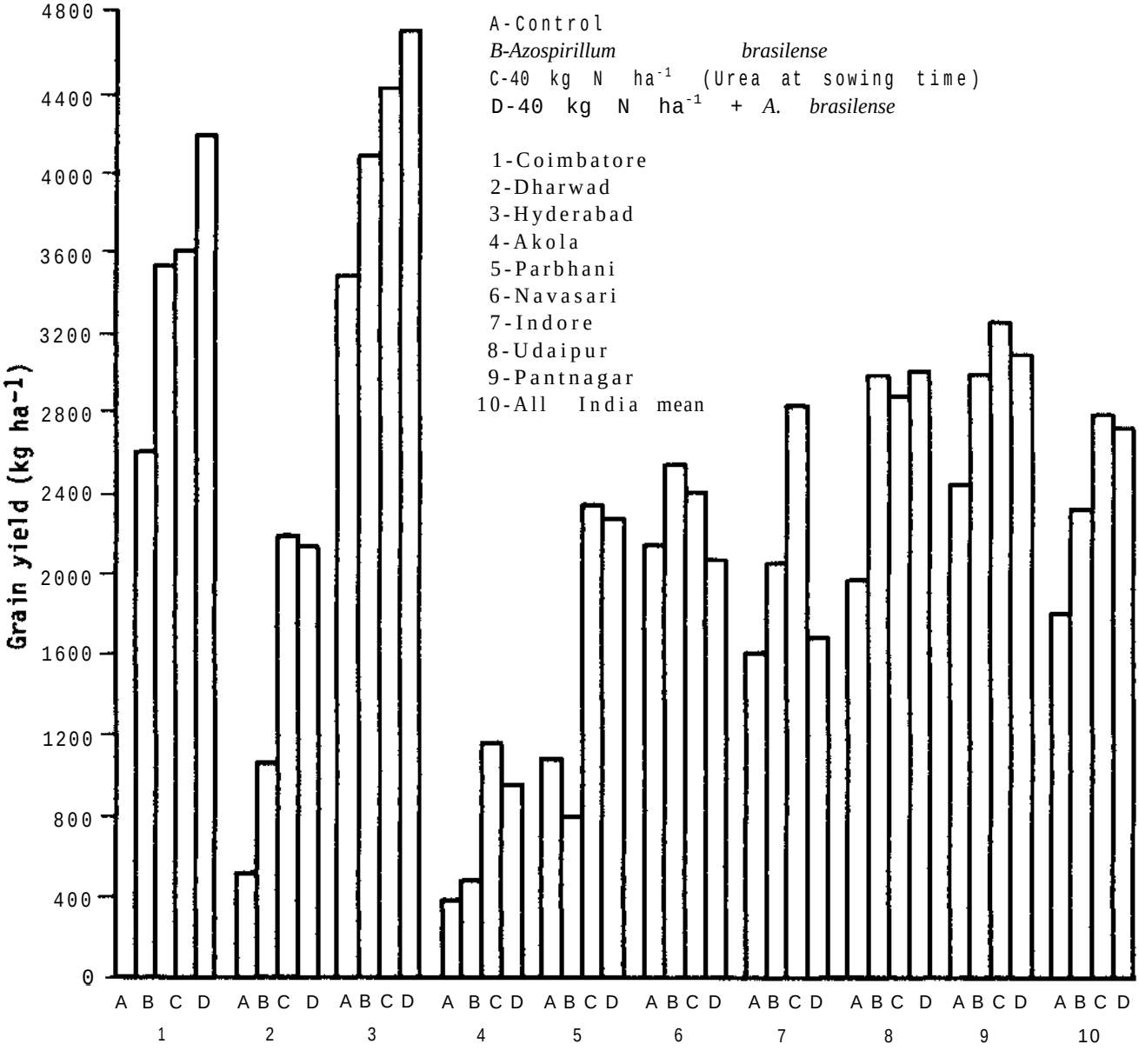


Figure 2. Response of sorghum to inoculation with *A. brasilense* at different locations (average of 4 years' field trials), 1979-82.

Response of Crops to *Azospirillum* Inoculation

Having isolated several strains of *Azospirillum* (Lakshmikumari et al. 1976), systematic trials in the pot-culture house were carried out to determine the efficiency of different strains in increasing crop yields (Subba Rao et al. 1979), particularly of sorghum, pearl millet, finger millet (*Eleucine coracana* Gaertn.), and barley. Efficient strains of *A. brasilense* capable of enhancing yields of these crops were later tested under field conditions under the All India Coordinated Crop Improvement Projects of the ICAR for barley, sorghum, and millets. The results obtained so far have been summarized in Figure 2 for sorghum, Figure 3 for pearl millet, and Figure 4 for finger millet.

For most crops, the yield response to inoculation varied between locations. In pearl millet also, the yields were variable with location but inoculation proved to be beneficial even at a basal dose of 40 kg N ha^{-1} except at Jodhpur, where fertilizer N did not provide additional benefits in conjunction with bacterial inoculation. In sorghum also, the yields varied with locations but simple *Azospirillum* inoculations increased yields in six locations with or without addition of 40 kg N ha^{-1}. At four locations (Akola, Parbhani, Navsari, and Indore), addition of 40 kg N ha^{-1} decreased the benefits due to inoculation. Attempts are under way to understand the factors responsible for the negative response to inoculation

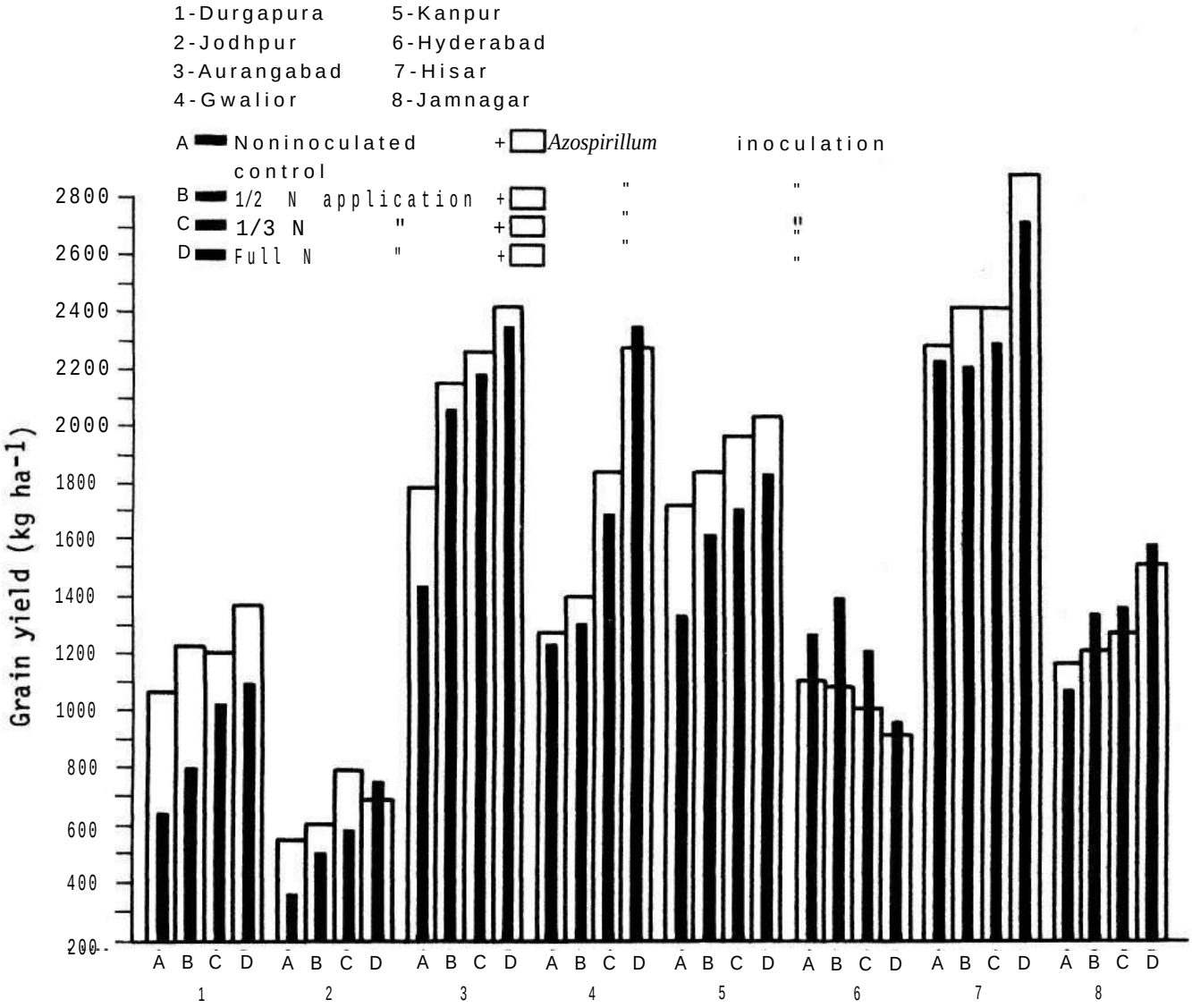


Figure 3. Response of pearl millet to inoculation with *A. brasilense* at different locations (average of 3 years' field trials), 1979-81.

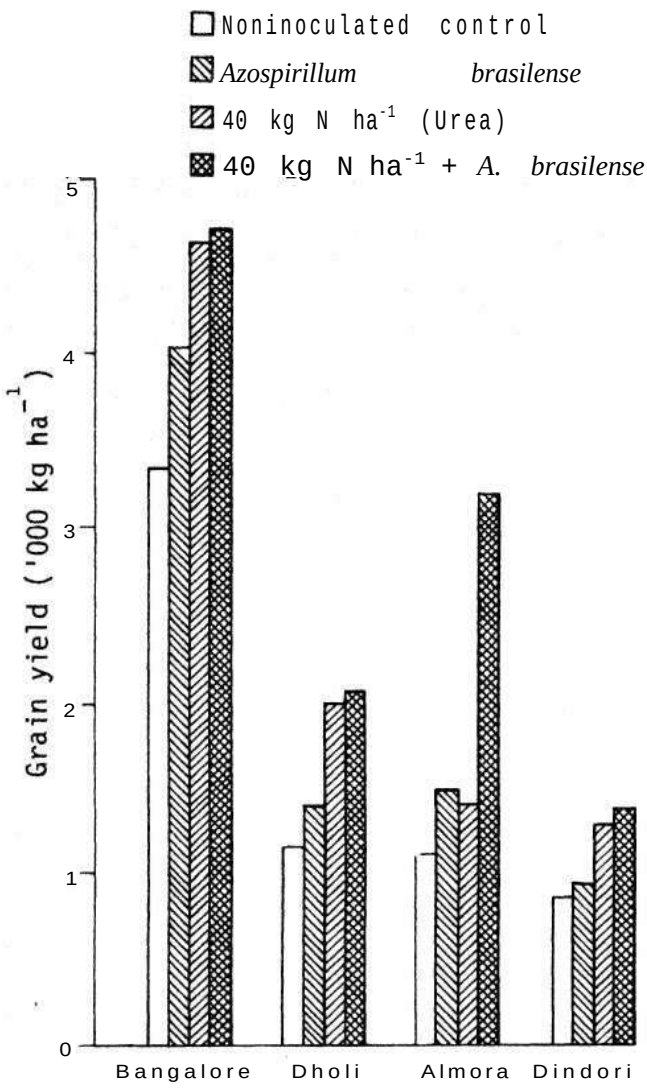


Figure 4. Response of finger millet to inoculation with *A. brasilense.*

in the presence of fertilizer N at certain locations.

Experiments under field conditions by cooperators at Coimbatore and Hisar centers of the project have also shown that *Azospirillum* inoculations increased pearl millet yield.

Effect of *Azotobacter* and *AzospiriUum* on Root and Shoot Measurements

It is now recognized that nonsymbiotic nitrogen-fixing bacteria produce growth-promoting substances (Tien et al. 1979) in addition to fixing atmospheric nitrogen. One question has often been posed: will seed inoculation help in increasing the root biomass under field conditions? This question was answered by conducting a field experiment at the Parbhani center with sorghum and pearl millet during the 1983 rainy season. A summary of the data of the 1982/83 experiments is presented in Tables 1 and 2. The following conclusions were drawn.

Table 1. Root mass, shoot mass, root:shoot ratio, root volume, and yield of field-grown rainy-season sorghum *(Sorghum bicolor)*, as influenced by seed inoculation with *Azotobacter* and *Azospirillum* at different stages of crop growth in a field experiment, Parbhani, rainy season 1983.[1]

Parameter/ Days of sampling	Non-inoculated control	*Azoto-bacter*	*Azospi-rillum*	CD at 5%
Root mass (g plant^{-1})				
20	3.40	4.34[2]	6.41[2]	0.89
35	12.20	I6.41[2]	18.53[2]	2.03
50	16.05	20.77[2]	21.77[2]	2.70
65	17.82	20.47	22.86[2]	3.77
80	15.20	15.90	15.26	NS[3]
Shoot mass (g plant^{-1})				
20	32.66	37.60	50.93[2]	7.64
35	111.80	139.33[2]	156.26[2]	15.84
50	165.13	184.86[2]	I90.49[2]	12.76
65	167.66	187.73	203.86[2]	23.01
80	156.46	167.86[2]	182.40[2]	10.09
Root:shoot ratio				
20	9.60	8.66	7.94	
35	9.16	8.49	8.43	
50	10.28	8.90	8.75	
65	9.40	9.16	8.91	
80	10.29	10.55	11.95	
Root volume (mL plant^{-1})				
20	2.79	3.40	4.76[2]	0.89
35	9.05	12.21[2]	13.68[2]	1.62
50	13.85	17.09[2]	17.76[2]	2.20
65	14.50	16.36	19.26[2]	2.11
80	13.18	14.06	13.93	NS
Grain yield (kg ha^{-1})	3237	3632	3817	NS
Fodder yield (kg ha^{-1})	4581	5950[2]	6515[2]	802

1. Mean of five plants, each from three replicated plots.
2. Significant increase over control.
3. NS = Not significant.

Table 2. Shoot mass, root biomass, and yield of field-grown pearl millet (*Pennisetum americanum*), as influenced by seed inoculation with *Azotobacter* and *Azospirillum* at different stages of crop growth in a field experiment, Parbhani, rainy season 1983.[1]

Parameter/ Days of sampling	Non-inoculated control	*Azotobacter*	*Azospirillum*	CD at 5%
Shoot mass (g plant^{-1})				
20	22.27	31.65[2]	32.382	6.62
35	72.46	94.41[2]	88.00[2]	9.01
50	89.38	103.432	97.78	10.48
65	67.58	86.83	78.98	NS[3]
80	59.66	79.752	67.662	9.44
Root mass (g plant^{-1})				
20	2.13	3.162	3.31[2]	0.61
35	5.56	7.902	6.662	0.83
50	6.51	8.802	8.15	1.79
65	5.08	8.532	7.832	0.97
80	4.60	8.01[2]	6.18[2]	0.92
Root:shoot ratio				
20	10.45	10.01	9.78	
35	13.00	11.82	13.21	
50	13.72	11.75	11.99	
65	13.30	10.77	10.08	
80	12.96	9.95	10.94	
Root volume (mL plant^{-1})				
20	1.96	2.612	2.732	0.58
35	4.38	6.582	6.032	0.83
50	5.08	7.082	6.352	1.56
65	5.38	8.382	7.062	0.83
80	5.23	9.752	6.802	1.03
Grain yield (kg ha^{-1})	1036	1469	1175	NS
Fodder yield (kg ha^{-1})	4497	74722	50262	732

1. Mean of five plants, each from three replicated plots.
2. Significant increase over noninoculated control.
3. NS = Not significant.

Sorghum

1. Seed inoculation with *Azotobacter* or *Azospirillum* significantly increases shoot mass and root biomass at different stages of crop growth. However, *Azospirillum* had a more pronounced effect on root biomass and shoot dry mass than *Azotobacter.*
2. The grain yield increase due to seed inoculation over the noninoculated control was 12.2% with *Azotobacter* and 17.9% with *Azospirillum,* but the results were not significant. However, both the organisms individually brought about a significant increase in fodder yield over the noninoculated control.

Pearl Millet

1. Seed inoculation with either *Azotobacter* or *Azospirillum* increased shoot mass and root biomass significantly over the noninoculated control at all stages of crop growth. In pearl millet the response with *Azotobacter* was better than with *Azospirillum* inoculation.
2. The grain yield increase due to seed inoculation over the noninoculated control was 41.8 with *Azotobacter,* and 13.4% with *Azospirillum* inoculation, but the results were not significant.
3. Seed inoculation with *Azotobacter* or *Azospirillum* significantly increased fodder yield over the noninoculated control.

These results indicate that bacterial inoculation results in increased root biomass with the possible consequent improved uptake of nutrients from the soil. Therefore, the observed enhanced yields due to inoculation may be attributable to factors such as growth-promoting substances elaborated by the bacteria, in addition to their ability to fix elemental nitrogen.

The ability of *A. brasilense* to improve root biomass has also been shown in pot experiments with finger millet, kodo millet (*Paspalum scrobiculatum*), and Italian millet (*Setaria italica*).

Response of Pearl Millet to Inoculation with VAM Fungi and *Azospirillum brasilense*

In these pot experiments the vesicular arbuscular mycorrhiza (VAM) fungi used for soil inoculation were *Acaulospora* sp, *Gigaspora margarita,* and *Glomus fasciculatum,* with *A. brasilense* combinations, over the noninoculated control. The dry-

matter content of shoots, root biomass, and P-uptake of the crop were studied (Table 3).

Table 3. Response of pearl millet to inoculation with *A. brasilense* and VAM fungi in potted plants[1], IARI, New Delhi.

Treatment	Shoot mass (g plant^{-1})	Root mass (g plant^{-1})	Total P content (%)	P uptake (mg plant^{-1})
Noninoculated control (No VAM, no *A. brasilense)*	13.3	0.16	0.10	13.7
A. brasilense	14.4	0.35[2]	0.142	20.1
Acaulospora sp	16.4	0.28[2]	0.142	21.1
G. margarita	16.4	0.25	0.142	21.2
G. fasciculatum	16.8	0.28	0.11	20.2
Acaulospora sp + *A. brasilense*	17.4	0.38[2]	0.09	16.6
G. margarita + *A. brasilense*	19.8[2]	0.362	0,12	23.2
G. fasciculatum + *A. brasilense*	20.62	0.382	0.13	25.92
L.S.D. (P<0.05)	NS[3]	0.14	0.012	NS
1. *Azospirillum*	NS	0.14	0.012	NS
2. VAM fungi	NS	NS	0.012	NS
3. VAM x *Azospirillum*	3.12	0.10	NS	5.2

1. Average of six replications.
2. Significant increase over corresponding control.
3. NS = Not significant.

Effect of Dual Inoculation with *A.brasilense* and VAM Fungi on Sorghum Growth and Yield

In a field experiment at Parbhani, it was observed that the mean root and shoot mass of sorghum plants increased significantly due to *Azospirillum* and VAM inoculation. The results also showed that grain and fodder yield increased significantly due to combined inoculation (Table 4).

Table 4. Influence of *A. brasilense* and VAM (*Glomus fasciculatum*) inoculation on root and shoot mass, root volume, and yield of sorghum CSH 8R in a field experiment.

Treatments	Root mass[1] (g plant^{-1})	Shoot mass[1] (g plant^{-1})	Root volume (mL plant^{-1})	Grain yield (kg ha^{-1})	Fodder yield (kg ha^{-1})
Noninoculated control	4.1	21.5	12.6	1990	4280
A. brasilense	4.8[2]	23.22	13.9	2150	4610
VAM (G. *fasciculatum)*	4.3	22.32	13.1	2100	4480
A. brasilense + *(G. fasciculatum)*	6.8[2]	28.12	15.4	2660[2]	56802
CD (P<0.05)	0.5	0.9	NS[3]	390	630

1. Sampled at 80 days of plant growth.
2. Significant increase over noninoculated control.
3. NS = Not significant.

Future Program

The areas of research that need attention with regard to BNF aspects of sorghum and millets are: (1) The mode of entry of the associated bacteria into plant roots has to be clearly understood. (2) The procedures for enumeration of bacteria in the rhizosphere, on the root surface, and inside the roots have to be standardized. (3) The best method of seed inoculation has to be standardized. (4) The relative benefits by growth factors and N_2 fixation in plant growth have to be quantitatively assessed. (5) What are the growth factors elaborated by the bacteria in the root system and in the rhizosphere, both qualitatively and quantitatively? (6) Are host-cultivar responses to inoculants repeatable at different locations and, likewise, can the intrinsic ability of cultivars to show high ARA values in intact-plant systems be reproduced under different conditions? Are these characteristics heritable? And, if so, can they be transferred genetically to other cultivars?

In developing countries such as India, where sorghum and millets are grown under low-input conditions, even marginal increases in crop yields to the

equivalents obtainable by the addition of 20-30 kg N ha^{-1}, are indeed worthwhile. Hence, intensive future efforts on BNF research aspects of sorghum and millets, at both the fundamental and applied levels, would be rewarding. The fundamental work in future requires emphasis on the genetics and molecular biology of diazotrophic bacteria associated with plant roots, on experiments designed to integrate engineered nitrogen-fixing bacteria with plant protoplasts and tissue cultures, so as to impart higher nitrogen-fixation potential to sorghum and millet cultivars.

Acknowledgements

The author wishes to thank workers at different centers for their cooperation and, more particularly, those involved in field work at the Parbhani center—Drs R.S.Raut, G.B.Rudraksha, K.V. B.R. Tilak, and C.S.Singh, and the project coordinators of the sorghum and millet programs of the ICAR.

References

Dewan, G.I., and Subba Rao, N.S. 1979. Seed inoculation with *Azospirillum brasilense* and *Azotobacter chroococcum* and the root biomass of rice (*Oryza sativa* L.). Plant and Soil 53:295-302.

Kavimandan, S.K., Subba Rao, N.S., and Mohrir, A.V. 1978. Isolation of *Spirillum lipoferum* from the stems of wheat and nitrogen fixation in enrichment cultures. Current Science 47:96-98.

Lakshmi, V., Rao, A.S., Vijayalakshmi, K., Lakshmikumari, M., Tilak, K.Y.B.R., and Subba Rao, N.S. 1977. Establishment and survival of *Spirillum lipoferum.* Proceedings of the Indian Academy of Sciences, Section B 86:397-405.

Lakshmikumari, M., Kavimandan, S.K., and Subba Rao, N.S. 1976. Occurrence of nitrogen fixing *Spirillum* in roots of rice, sorghum, maize and other plants. Indian Journal of Experimental Biology 14:638-639.

Subba Rao, N.S., Tilak, K.V.B.R., Singh, C.S., and Lakshmikumari, M. 1979. Response of a few economic species of graminaceous plants to inoculation with *Azospirillum brasilense.* Current Science 48:133-134.

Tien, T.M., Gaskins, M.H., and Hubbell, D.H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Applied and Environmental Microbiology 37:1016-1024.

Discussion

O.P. Rupela:
You report that at Parbhani, increase in root mass was noticed due to inoculation consistently for 3 years. Was it a pot or field trial? If it was a field trial, how was the root mass recovered and measured?

N.S. Subba Rao:
Yes, it was a field trial. The root system was carefully dug out and washed, and lateral roots were recovered and measured by displacement of water in a measuring cylinder for volume, and later dry mass data were obtained.

G.S. Jadhav:
To realize the agronomic significance of N_2 fixation by nonsymbiotic bacteria it is very essential to:

1. Report all supplementary information, such as initial soil fertility, rainfall pattern, irrigation, soil type, and variety or cultivar used.
2. In determining responses we should analyze yield data both in terms of statistical significance and economic benefits, in terms of N economy and yield gain.
3. Adaptive trials on farmers' fields should be conducted to take N_2-fixation technology to farmers quickly, it being low cost and economically and ecologically profitable.

On the basis of present knowledge of inoculation methods *of Azospirillum,* which is the best and most economical method of inoculation in pearl millet?

N.S. Subba Rao:
Seed inoculation as practiced for legume inoculation with rhizobia.

J.V.D.K. Kumar Rao:
You have reported significant responses to *Azospirillum* inoculation at six out of nine centers under the All India BNF project. What is the method of inoculation used in these trials? What is the form of inoculant and the nature of the carrier? What is the viable-cell count in the inoculant and on the seed after inoculation?

N.S. Subba Rao:
Seed inoculation was done from a finely powdered farmyard manure and soil inoculant in the ratio of 1:1, by making an aqueous slurry and also using carboxy methyl cellulose as an adhesive (same as

rhizobial seed inoculation). We did not make viable-cell counts on the seed, but we monitored inoculant survival in this carrier. It had 10^9 cells even after 9 months of storage under room conditions.

S.V. Hegde:
You mentioned nitrogenase activity of some cucurbits in tissue culture in the absence of any N_2-fixing microorganisms.

1. How was the cucurbit plant made free of microorganisms?
2. What was the level of activity in the axenic culture?

N.S. Subba Rao:
The calli of cucurbits exhibited high nitrogenase activity without any bacteria in the cells. The possibility of *nif* gene incorporation into the chromozome of the plant cell has been postulated by Prof. Sen and his students at Kalyani University, West Bengal.

P. Tauro:
Regarding the Kalyani experiment, what was the level of acetylene reduction in tissue culture?

N.S. Subba Rao:
Endogenous ethylene production is ruled out.

S.P. Wani:
Is there any coordinated trial on inoculation response?

N.S. Subba Rao:
There are no coordinated trials. But we are conducting experiments at Parbhani and Coimbatore. We have no facilities for large-scale testing.

P.A. Shinde:
During the VII Five Year Plan, positions of Microbiologists should be proposed in Millet and Sorghum projects of the ICAR.

Associative Biological Nitrogen Fixation Research at Haryana Agricultural University

B.S. Kundu, K.R. Dadarwal, and P. Tauro[1]

Summary

Several different types of bacteria with the ability to reduce acetylene have been found to be associated with sorghum, pearl millet, and wheat grown at Hisar. The quantity of nitrogen fixed, as determined by their acetylene-reduction activity (ARA), and the conditions for optimal A RA vary widely with the isolates. Among the several hundred isolates tested, one sorghum isolate, 12S, has been found to have high ARA and has been identified as a strain of Azospirillum brasilense. *It also possesses a strong hydrogenase, reduces* $C0_2$, *and carries out a nitrate-dependent nitrogen fixation. Its ARA is insensitive to the levels of inorganic nitrogen generally used in the cultivation of the above crops.*

Introduction

Our interest in nitrogen fixation at Haryana Agricultural University (HAU) began in the late 1970s when we isolated a strain of *Pseudomonas azotogensis* from the roots of pearl millet that could reduce nitrogen (Prabha et al. 1980). Since then this work has been strengthened as part of an ICAR coordinated research project on Biological Nitrogen Fixation (BNF) in the Department of Microbiology. Our major interests are centered around the isolation and quantification of nitrogen fixation by the various bacteria from the roots of sorghum, pearl millet, and wheat, three major crops grown in or around Haryana.

Nitrogen Fixation in Pearl Millet and Wheat

We carried out some initial studies to determine the time of day when maximum nitrogen fixation occurs and also the stage of crop growth during which fixation is maximum. In all studies reported herein, nitrogen fixation was determined by measuring the acetylene-reduction activity (ARA).

In pearl millet, the *in situ* nitrogenase activity was estimated at 50 days after sowing (flowering stage) by the core assay method. For this, soil cores of 15 cm diameter and 22 cm depth were removed along with the plant and transferred to 5 L airtight plastic buckets with the provision for injecting and sampling acetylene. The cores were then incubated in 10% acetylene atmosphere for 18 h at 30-32°C, after which gas samples were withdrawn and analyzed for the extent of acetylene reduction. In wheat, similar soil cores around the plants were taken and the adhering soil was removed by gentle shaking. The top was cut, and the roots were transferred to 500 mL assay jars and assayed as before for ARA.

In experiments to determine the diurnal variation in nitrogen fixation, we found that more than 25 µ moles of acetylene was reduced per plant soil core in 24 h when the plants were assayed around noon, and fixation was less either before or after. It was found that N_2 fixation was maximum during the

1. Associate Professor, National Fellow, and Professor of Eminence, Department of Microbiology, College of Basic Sciences and Humanities, Haryana Agricultural University, Hisar, Haryana 125 004, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502324, India: ICRISAT.

preflowering stage and less thereafter. These parameters were found to be similar in wheat. It was also found that the extent of ARA varied significantly with the wheat variety; the high-yielding varieties were generally (but not always) found to display a higher level of ARA.

Determination of Most Probable Number (MPN) of Nitrogen-fixing Bacteria Associated with Pearl Millet and Wheat

For the estimation of MPN, 10 varieties of wheat and 3 varieties of pearl millet were selected. Plant samples were collected from the nonfertilized area of the university farm during the preflowering stage and MPN estimated both from the root washings and from the root macerates. Details of the procedures followed are available from the authors. The sterilized Dobereiner's semisolid medium was used (Dobereiner et al. 1976) and the MPN was calculated from the reference table of Harrigan and McCance (1956), The roots were washed gently for removing the adhering soil, cut into 2-3 cm long pieces, and 5-g root pieces were transferred to 45 mL sterile saline and shaken at room temperature (28-30°C) for lh. The washed root pieces were then macerated in sterilized mortar and pestle and the volume was made up to 50 mL. Ten-fold serial dilutions from root washing and root macerate were prepared and used for MPN using Dobereiner's semisolid medium (Dobereiner et al. 1976). Five replicated tubes from each dilution were incubated for 48 h at 30°C and then 10% of the gas phase was replaced with acetylene.

In wheat, the MPN in root washings varied from 1.7 x 10^6 g^{-1} of fresh roots to 13 x 10^6 g^{-1}, while in the root macerates, the number varied between 0.6-5.5 x 10^6 g^{-1} of fresh root weight. The high-yielding cultivars WH 147, WH 157, HD 2009, and P 1030 had a higher number of nitrogen-fixing bacteria associated with their roots. In contrast to this, in pearl millet (a rainy-season crop), the number of bacteria found in the root washings were less than in the root macerates suggesting a possible relationship between the number of bacteria in the root zone and the environmental temperature during the growth period of pearl millet. It is likely that because of the high temperature, a greater number of bacteria are found inside the roots than outside. In wheat it is the reverse, since the growth period corresponds to a time when the temperatures are moderate. In both crops, however, there is a relationship between the number of bacteria found, the extent of nitrogen fixation, and the yield potential of the variety. These results allowed us to conclude that a large number of bacteria associate with the roots of cereals grown in this area.

From the tubes that showed ARA from the above experiment, isolations were made on N-free agar medium, and 216 isolates from pearl millet were selected based on colony morphology, out of which 183 were selected for further study. Similarly, from wheat and sorghum, a total of 146 cultures were isolated for further study. The isolates included both Gram-positive and Gram-negative bacteria but surprisingly, except for one or two cultures, azospirilla were conspicuously absent. These cultures were found to vary widely in their ARA, and the media conditions required for their optimal ARA expression also varied (B.S.Kundu, unpublished data). Table 1 shows the ARA of some of the bacterial isolates from the roots of the above three crops. Isolate 12S from sorghum showed the highest ARA under the assay conditions; but the value was much less than the values reported for other bacteria isolated from the roots of cereals. To check the potential of this organism with the other cultures, standard cultures were obtained from other laboratories and cultured similarly along with the two best cultures from each of three crops. It was found that the level of ARA was highest when cultured in semisolid medium and on the 3rd or 4th day of incubation (Table 2). Among all the cultures tested, isolate 12S had the highest activity and was considered to be

Table 1. The ARA (nmoles C_2H_4 $tube^{-1}$ d^{-1}) of some isolates from sorghum, pearl millet, and wheat.

Pearl millet		Wheat		Sorghum	
Isolate	ARA	Isolate	ARA	Isolate	ARA
PM 1B	31	W 1	12	S2	192
2B	84	2	168	3	ND[1]
3B	906	3	12	4	480
4B	996	4	24	5	384
5B	756	5	19	6	384
6B	276	6	29	7	612
7B	252	7	17	8	552
8B	ND	8	190	9	156
9B	4	9	20	10	300
10B	ND	10	12	11	720
MB	4	11	14	12	2160

1. ND = not detected.

Table 2. ARA (nmoles C_2H_4 $tube^{-1}$ d^{-1}) level of various nitrogen-fixing bacteria.[1]

Bacteria	ARA, days after inoculation					
	2	3	4	5	6	7
S. itersonii	125	140	140	135		95
A. brasilense, strain SP 7	1373	2420	2005	1595	861	602
A. brasilense, strain 12S	1889	2595	2715	2990	1610	1365
Isolate 8S from sorghum	9	30	72	82	72	24
Isolate 4B from pearl millet	6	11	14	6	3	2
Isolate 22B from pearl millet	-	3	6	5	2	
Isolate 2W from wheat	8	12	74	58	42	4
Isolate 8W from wheat	50	141	161	168	-	68

1. *Azotobacter* and Rhizobia showed no ARA in this medium.

a superior culture. It has been subsequently tested for its ARA, compared with *A. brasilense* SP7, and found superior under all conditions.

It has been found that ARA expression of this organism is highest when it is cultured in a semisolid medium. Further, for maximal expression the media should contain malate as the carbon source and either vitamins or yeast extract. Optimum pH should be 6-7 and the optimum temperature 30°±2°C under semisolid conditions. Highest ARA is detected within 4-5 days.

To estimate the nitrogen-fixing potential of isolate 12S, we have tried to grow it in liquid medium for 24 h and then transfer the cells to semisolid medium. By this it was possible to use a larger cell mass and induce ARA earlier. Under these conditions, it was possible to record higher ARA values, for both SP7 and 12S strains. We believe that this strain has capacities higher than 103 µm of ARA per OD, which is considerably higher than any other culture tested so far.

Physiological Studies with Isolate 12S

Isolate 12S was subjected to various tests, and it has been found similar to *A. brasilense* except in its antibiotic resistance pattern. While strain SP7 is resistant to streptomycin, strain 12S is resistant to chloramphenicol.

Table 3. Growth rates *of A. brasilense* isolates 12S and SP7.

Treatment	O.D. 600 nm after 6 days at 30°C	
	12S	SP7
Control (-Malate)	0.02	0.09
+Malate	0.04	0.27
+Malate +Urea	0.05	0.38
+Malate +KNO_3	0.09	0.45
+Malate +NH_4C1	0.09	0.48

Malate concentration: 5 gm L^{-1}; N source: 5 µm.

Unlike SP7, the isolate 12S displays poor growth under all cultural conditions (Table 3). Until recently, we had no medium in which this could grow uniformly, but now we have developed one containing galactose, in which this organism grows uniformly as well as possesses ARA activity (B.S.Kundu, unpublished data). This strain, like other strains of azospirilla, also possesses a strong hydrogen-oxidizing system that appears to be associated with nitrogen fixation, since ARA activity is enhanced by 31% in the presence of 10% hydrogen (Table 4). This culture has also been found to show autotrophic growth and the ability to reduce carbon dioxide. The ability to fix atmospheric nitrogen, oxidize hydrogen, as well as reduce carbon dioxide, makes this organism an ideal choice for future studies in associative symbiosis. The only problem is its extremely slow rate of growth.

Associative symbionts perhaps have a role in soils with low nitrogen levels or under subsistence agriculture. Although NO_3-dependent N_2 reduction is known in this organism, the nitrogen metabolism of these organisms under conditions of low soil N levels becomes important. We have examined its ability to utilize different inorganic forms of nitrogen and its relation to nitrogen fixation. When cultured in

Table 4. Enhancement of ARA in strain 12S and SP7 of *A. brasilense* by hydrogen.

Strain	ARA[1]		H_2 ase[2]	% increase in ARA
	C_2H_2	$C_2H_2+H_2$		
12S	4220	5570	34	31
SP7	2564	2830	15	10

1. ARA: nm C_2H_4 $tube^{-1}$ d^{-1}
2. H_2ase: µm H_2 consumed d^{-1}.

media containing 300 ppm nitrate-N, this organism carries out denitrification. However, when cultured under lower levels of nitrate N (60 ppm), it not only utilizes nitrate but also fixes nitrogen. As compared to this, ARA is not detected at concentrations of ammonium-N as low as 24 ppm. In view of the fact that soils rarely contain such levels of either nitrate or ammonical nitrogen, we can safely conclude that under low-nitrogen regimes, nitrogen-fixing ability of this organism is not affected.

Pot-house and Field Studies

Since isolate 12S was found to be very similar to SP7 but had higher ARA, it was decided to test whether it could associate with the roots of some of the crops and fix nitrogen. In pot-house experiments with two varieties of pearl millet, a significant response was seen both in root and shoot weight to the seed application of these bacteria (Table 5). These observations led us to test the usefulness of this bacterium under field conditions.

Our studies have not shown conclusively whether these bacteria excrete any growth-promoting substances. Our estimates of malate in the roots of sorghum and pearl millet suggest a greater accumulation of malate during later stages of growth when nitrogen fixation is minimum.

Future Research Priorities

Future studies will include evaluation of strain 12S under field conditions in sorghum, pearl millet, and wheat. At present we find that its survival following seed application is poor, and we have had difficulties in establishing its association with any part of the roots. We also have plans to continue our biochemical studies with this organism and to understand this organism genetically, with a view to explore its usefulness in the cultivation of cereals.

Acknowledgements

This research was financed by the Indian Council of Agricultural Research (ICAR), New Delhi, and the authors gratefully acknowledge the financial assistance.

References

Dobreiner, J., Marrel I.E., and Nery, M. 1976. Ecological distribution of *Spirillum lipoferum* Beijerinck. Canadian Journal of Microbiology 22:1464-1476.

Table 5. Effect of seed inoculation in pearl millet cultivars.

Parameter	Treatment: Cultivars	Control	Strain 12S	Strain 4B	Strain 4B+22B	CD (P<0.05)
N_2-fixers x 10^4 g^{-1}	HS 1	8	27	129	43	-
(Rhizosphere)	HS 4	54	246	129	183	
N_2-fixers x 10^4 g^{-1}	HS 1	2	16	154	12	-
(Rhizosplane)	HS 4	134	134	162	222	-
Root ARA	HS 1	ND	6.0	1.2	1.8	-
(C_2H_4 g^{-1} root mass)	HS 4	8.2	24.1	28.6	15.9	-
Dry matter	HS 1(R)[1]	203	305	353	348	80.7
(mg/3 plants)	HS 1(S)[2]	580	726	883	773	130
	HS 4(R)	435	643	570	630	72
	HS 4(S)	683	900	883	953	240

12S: *A. brasilense,*
4B and 22B: Bacterial isolates from pearl millet.
1. R = Root weight.
2. S = Shoot weight.

Harrigan, W.F., and McCance, M.E. 1956. Probability tables for the estimation of bacterial numbers by the dilution tube technique. *In* Laboratory methods in microbiology. London, UK: Academic Press.

Prabha, S., Nair, S.K., Dadarwal, K.R., and Tauro, P. 1980. *Pseudomonas azotogensis:* an associate symbiont of *Pennisetum americanum.* Plant and Soil 49:657-659.

Discussion

C.P. Ghonsikar:
Which carbohydrate compounds were identified besides malate at a time of high ARA activity?

P. Tauro:
We estimated malate enzymatically, and we were interested only in malate since this carbon source has been found to support optimum N_2 fixation by *Azospirillum.*

N.S. Subba Rao:
Other Kreb's cycle intermediates also need to be studied.

P. Tauro:
Other carbon sources will be tested. But malate can be easily assayed enzymatically.

N.N. Prasad:
When malate is less in root extract, it increases with age while nitrogenase activity decreases with age. What may be the possible source of carbon for *Azospirillum* to fix atmospheric nitrogen? In rice crop, without C4 pathway also, *Azospirillum* is effective as reported by TNAU. So, I feel there is a need to ascertain the nature of carbon compounds used by *Azospirillum* to fix atmospheric nitrogen.

P. Tauro:
We didn't study these aspects.

O.P. Rupela:
In your presentation you mentioned that there was a log 2 decrease in population of inoculant strain, in pots, within a week. Could you please give us some figures? How did you estimate these populations?

P. Tauro
There was a decrease of log 2 (from 10^6 to 10^4) during the 1st week, which further decreased. At the end of 2 weeks, the survival was only about 10^2. The bacteria introduced had a chloramphenicol marker.

Studies on the Interactions Between *Azospirillum* and Pearl Millet

A.V. Rao and B. Venkateswarlu[1]

Summary

Biologically active compounds—such as sugars, amino acids, and organic acids—are present in seed and root exudates of pearl millet. The growth and nitrogenase activity of A. brasilense, *stimulated by seed and root exudates, was generally related to the quantity of organic compounds present in the exudates. Inoculation significantly enhanced the exudation of various compounds by the roots of axenically grown pearl millet plants, and the effect was more pronounced in the older plants. A marked and consistent increase in cell permeability of roots was observed upon inoculation/ impregnation with* A. brasilense. *Axenically grown pearl millet plants, upon inoculation, exhibited nitrogenase activity that varied with cultivars. The activity was related to the amounts of carbon compounds. However, the carbon compounds present in the root exudates were found inadequate for the optimum growth of the organism in the root zone, as there was a marked increase in the population and nitrogenase activity upon addition of sucrose. Maximum increase in* Azospirillum *population was observed in the rhizoplane following inoculation in a sandy loam soil.*

Introduction

Pearl millet, an important crop of the semi-arid tropics (SAT), has been reported to derive agronomically significant amounts of nitrogen from the *Azospirillum* association (Smith et al. 1976). However, results of the inoculation experiments have not always been consistent (Barber et al. 1979, Nur et al. 1980, Vlassak and Reynders 1981, Venkateswarlu and Rao 1983). The causes for the plant-growth response following inoculation are not clearly known. There is also a lack of information on the physiological interactions between the host plant and the microsymbiont. In the present study, an attempt has been made to investigate the interactions between the seed and root exudates of pearl millet with *Azospirillum* and its implications in the formation of the associative symbiosis.

Materials and Methods

The bacterial strain used in these experiments was isolated from the roots of pearl millet and identified as *Azospirillum brasilense,* following the method used by Tarrand et al. (1978). The inoculum used in these studies is a pure-cell suspension of 4-day-old *A. brasilense* (OD=0.4 grown in nutrient broth) suspended in phosphate buffer.

Seed exudates were collected by soaking surface-sterilized seeds in distilled water for 24 h and thoroughly rinsed with distilled water. The exudates were sterilized by filtration and stored at 4°C. For collection of root exudates, 12-day-old plants, grown axenically in test tubes (25 x 200 mm) containing acid-washed quartz sand and watered with Hoagland's nutrient solution containing 50 ppm ammonium nitrate, were used. The exudates were

1. Microbiologists, Central Arid Zone Research Institute, Jodhpur, Rajasthan 342003, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986, Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

collected by rinsing the roots and the associated sand in distilled water, pooled, purified, and concentrated to represent 1 mL for every 10 seedlings.

The effect of inoculation with *A. brasilense* on the pattern of root exudation was studied on 3-day-old plants by adding 1 mL pure-cell suspension to each tube. The control plants received the same amount of autoclaved cells. Root exudates were periodically collected and analyzed for organic carbon by Walkley and Black rapid titration method, total nitrogen by micro-Kjeldahl method, reducing sugars by Nelson's arseno-molybdate method, and amino nitrogen by the ninhydrin method.

To study the effect of seed and root exudates on the growth and nitrogenase activity of *A. brasilense*, the exudates were incorporated in the culture media in different concentrations. For seed exudates, Dobereiner's semisolid malate medium (Dobereiner and Day 1976) with 0.08% agar was used for growth, and with 0.175% agar for nitrogenase activity. The tubes after inoculation were incubated at 30°C for 72 h, and the growth was measured as OD at 520 nm. For nitrogenase activity 2 days after incubation, the cotton plugs were replaced by Subaseals and 10% of the air was replaced with C_2H_2. Ethylene produced 24 h after incubation was estimated by an AIMIL-Nucon gas chromatograph, employing a Poropak-T column (2 m * 0.003 m) with N_2 as carrier gas. For root exudates, a malate liquid medium, containing only 50% of the malic acid recommended but supplemented with 50 ppm ammonium sulfate, was used, while for nitrogenase activity a semisolid malate medium containing 50% of the recommended level of malic acid was utilized.

To study the permeability of pearl millet roots, one set of tubes was inoculated with 1 mL cell suspension of *A. brasilense* on the 3rd day, while the control received the same amount of autoclaved cells. At 15 days after sowing (DAS), samples of fresh roots were wrapped in a cheese cloth and kept in distilled water. The contents were shaken for 5 min and the conductivity of the bathing solution was measured at 1 h intervals. Further the root material from control plants was impregnated with pure cell suspension of *A. brasilense* under vacuum, and the permeability was tested as described above.

To measure nitrogenase activity of pearl millet plants, 1 mL of inoculum and 4 mL of 1% sucrose in nutrient solution were added to one set of 3-day-old seedlings in a sterilized sand and vermiculite growth medium, while the second set received the inoculum and 4 mL of nutrient solution. Nitrogenase activity (C_2H_2 reduction) of 21-day-old plants was measured after incubation for 24 h with 10% C_2H_2. Appropriate controls were maintained to get absolute activity.

To estimate *Azospirillum* populations, the 3-day-old plants were inoculated with 1 mL of cell suspension (10^8 cells mL^{-1}). The populations of *Azospirillum* in both the rhizosphere and nonrhizosphere (tubes containing the growth medium but without plants) were determined at 3-day intervals by dilution plate technique, using a malate-agar medium supplemented with 500 ppm ammonium sulfate. To assess the establishment of this bacteria following inoculation, the numbers of *Azospirillum* in different root zones, i.e., rhizosphere, rhizoplane, washed and crushed roots, and surface-sterilized and crushed roots were estimated by the MPN method from 30-day-old plants growing in a sandy loam soil.

Results

Pearl millet seed exudates contained variable amounts of sugars and amino acids, and the differences among cultivars were not significant (Table 1). Reducing sugars were much higher than amino acids in all the cultivars.

Seed exudates stimulated the growth of *A. brasilense* (Table 2). The increase in growth was not directly proportional to the concentration of the exudates. The growth stimulation appeared to be due to the C and N compounds present in the exudates. The nitrogenase activity also increased with the addition of exudates upto 9% (Table 2). There was a profuse growth in the semisolid medium, pos-

Table 1. Biochemical composition of seed exudates of three pearl millet cultivars.

Compound (mg/2.5 mL of seed exudates)	Pearl millet cultivars		
	BJ 104	M 78	V.No. 2
Organic carbon	3.10 ± 0.85[1]	3.60 ± 1.12	3.50 ± 1.09
Reducing sugars	0.62 ± 0.028	0.66 ± 0.024	0.52 ± 0.037
Amino nitrogen	0.32 ± 0.018	0.26 ± 0.017	0.31 ± 0.021
Total nitrogen	0.53 ± 0.031	0.47 ± 0.024	0.54 ± 0.027

1. Mean ± Standard deviation.

Table 2. Effect of seed exudates from pearl millet cultivars on growth and nitrogenase activity of *A. brasilense*.

Parameter	Concentration of seed exudates (%)	Control	Pearl millet cultivar			Mean
			BJ 104	M 78	V No.2	
Growth (OD)	3	0.22	0.28	0.28	0.33	0.296
	6	-	0.29	0.35	0.37	0.337
	9	-	0.30	0.35	0.41	0.353
	12	-	0.31	0.36	0.41	0.360
	Mean	-	0.295	0.355	0.380	0.349
Nitrogenase activity ($nmol\ C_2H_4\ tube^{-1}\ h^{-1}$)	3	76	139	142	140	140.4
	6	-	160	186	198	181.3
	9	-	14!	212	170	174.3
	12	-	110	245	168	174.5
	Mean	-	137.5	196.0	169.0	167.6
			Growth		N_2 ase	
CD (*P*< 0.05)	Cultivar		0.016		12.0	
	Concentration		0.018		13.8	
	Interaction		NS[1]		23.9	
	Control vs Rest		0.023		17.6	

1. NS = Not significant.

sibly due to the presence of considerable nitrogenous compounds in the exudates. Similar effects were observed on the growth of *A. brasilense* with individual amino acids (Rao and Venkateswarlu 1982). At concentrations exceeding 9%, the activity was reduced but growth was unaffected, indicating the repression of the activity by the amino nitrogen. This is evident from the fact that the activity was not repressed even at higher concentrations with cultivar M 78, whose exudates contained relatively less of total and amino nitrogen.

The data indicated that pearl millet seed exudates have a favorable effect on the growth of *A. brasilense* and might help in the multiplication of the inoculated bacteria on the seed. Hence, soaking seeds before inoculation, as recommended for rhizobial inoculation, may not be necessary for pearl millet.

The data on exudation of various organic compounds by pearl millet roots indicated that marked quantitative differences exist among cultivars in the exudation pattern (Fig. 1). The exudates of cultivar BJ 104 contained the highest amounts of organic carbon and reducing sugars, followed by cultivar M B H 110. Qualitative analysis of the exudates by thin-layer chromatography showed the presence of amino acids like glutamic acid, tryptophane, cysteine, and asparagine, and sugars like glucose, fruc-

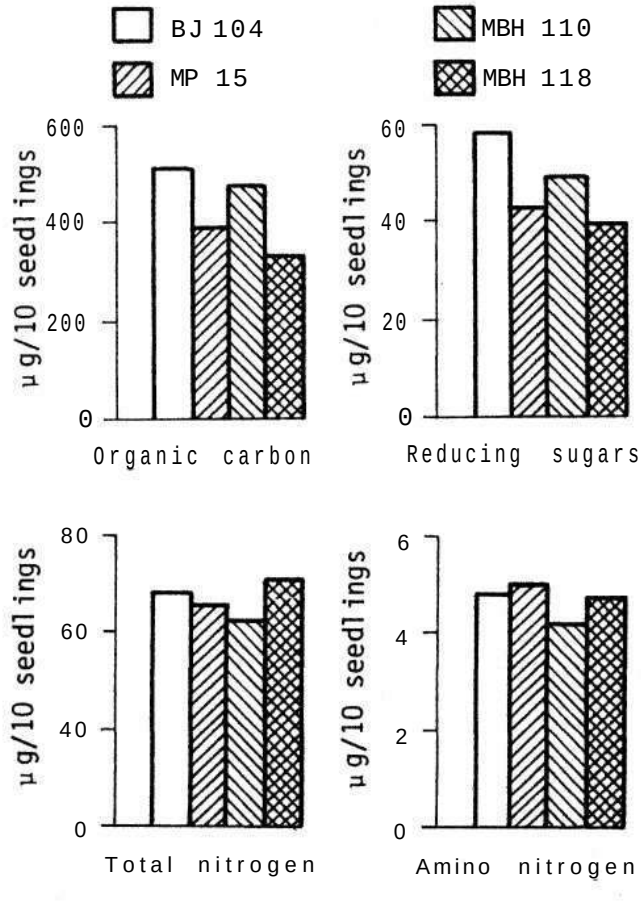


Figure 1. Chemical composition of root exudates from four pearl millet varieties.

tose, and sucrose. Organic acids like citrate, malate, and succinate were also detected, but in very small quantities.

The growth and nitrogenase activity of *A. brasilense* were stimulated by the incorporation of root exudates in a medium containing a suboptimal level of carbon source. The growth enhancement varied with the variety and was generally related to the amount of carbon compounds present in the exudates (Table 3).

The exudation of organic compounds by the roots of axenically grown pearl millet was greatly increased in the presence of *A. brasilense* (Table 4). Inoculated plants exuded markedly higher amounts of organic carbon, reducing sugars, and total and amino nitrogen. Increased root exudation in the presence of N_2-fixing bacteria has also been found in sorghum (Lee and Gaskins 1982) and in wheat (Beck and Gilmour 1983). Changes in the permeability and alteration in root metabolism might be some of the reasons for the enhanced root exudation upon inoculation (Rovira 1965), The enhancement in the root exudation of various compounds in the present study was essentially due to the increased permeability of the root cells, which increased by 8.5% over the control, while with the direct impregnation with pure cells of *A. brasilense,* the increase was about 40%, indicating some physiological interaction between roots and the bacterium at the cellular level and/or injury to the roots caused due to vacuum impregnation.

The population of *Azospirillum* increased markedly and remained much higher in the rhizosphere as compared to the nonrhizosphere, indicating that the organic compounds in the root exudates aided the multiplication of *Azospirillum.* The nitrogenase activity of pearl millet cultivars was generally related to the organic carbon and reducing sugars released in the root exudates. However, these results are from two different sets of experiments. The close relationship observed between the organic carbon in the exudates and the growth *of Azospirillum* in vitro and nitrogenase activity of the intact plants emphasized the role of root exudates in the genetic variation in nitrogenase activity. The carbon compounds present in the exudates were, however, found to be inadequate for optimum activity as there was a spurt in the activity in all the 4 cultivars when an extraneous carbon source (sucrose) was supplied in the root zone (Table 5). Similar results have been reported with wheat (Lethbridge and Davidson 1983).

Studies under field conditions in a sandy loam soil have also indicated that *Azospirillum* establishes in the roots of pearl millet upon inoculation, and maximum MPN counts were found in the rhizosphere

Table 4. Changes in root exudation pattern in pearl millet, as influenced by inoculation with *A. brasilense.*

Compound (µg/10 seedlings)	Age of the plant (DAS)				
	Treatment	8	12	15	Mean	SD
Organic carbon	Control	412	466	492	456.7	36.64
	Inoculated	481	548	590	539.7	49.40
Total nitrogen[1]	Control	59	67	75	67.0	9.91
	Inoculated	68	79	88	78.3	9.99
Reducing sugars[1]	Control	41	52	66	53	12.07
	Inoculated	58	56	75	63	10.08
Amino nitrogen[1]	Control	3.9	4.8	5.6	4.77	0.97
	Inoculated	4.6	5.9	6.8	5.77	1.12

1. Significant at 5% level using Kruskal Wallis test.

Table 3. Growth of *A. brasilense* as influenced by root exudates of pearl millet cultivars.

Concentration of root exudates (%)	Growth (OD) with cultivar			
	BJ 104	MP 15	MBH 110	MBH 118
0 (Control)	0.202	-	-	-
5	0.218	0.209	0.215	0.210
10	0.236	0.221	0.231	0.225
15	0.259	0.235	0.250	0.242
20	0.281	0.262	0.273	0.269
SE±	0.033			

Table 5. Nitrogenase activity of pearl millet cultivars, as influenced by sucrose.

Cultivar	N_2ase activity (nmoles C_2H_4 h^{-1} $tube^{-1}$)	
	Without sucrose	With sucrose
BJ 104	21	49
MP 15	13	33
MBH 110	14	45
MBH 118	13	46

Table 6. Localization of *Azospirillum* in different root zones of pearl millet, as influenced by inoculation.

Root zone	MPN of *Azospirillum* (x 10^4) g^{-1} dry soil	Plant-'	g^{-1} dry root
Rhizosphere			
Control	3.46 ±0.47[1]	5.50 ± 0.93	42.20 ± 4.57
Inoculated	9.17 ± 1.41	11.00 ± 2.10	72.60 ± 8.78
Rhizoplane			
Control	-	0.39 ± 0.07	3.03 ± 0.58
Inoculated	-	4.25 ± 0.96	28.30 ± 3.89
Washed and crushed roots			
Control	-	1.02 ± 0.13	7.90 ± 1.03
Inoculated	-	6.00 ± 1.13	40.10 ± 5.79
Surface-sterilized crushed roots			
Control	-	0.33 ± 0.05	2.46 ± 0.39
Inoculated	-	0.45 ± 0.09	3.01 ± 0.47

1. ± standard deviation.

(Table 6). The population was much higher in washed roots than in surface-sterilized roots, indicating a greater concentration of the organisms on the root surface. Similar observations were recorded in maize (Okon et al. 1977). Inoculation significantly increased the numbers in all the root zones, but the highest response was noted in the rhizoplane. There was a nine-fold increase in the population in the rhizoplane on unit root dry weight basis. This again indicates that the population buildup largely takes place on the root surface.

From these results, it may be concluded that the root exudates of pearl millet play an important role in the establishment *of A. brasilense* in the root zone, resulting in the successful formation of the associative symbiosis.

References

Barber, L.E., Russel, S.A.,and Evans, H.J. 1979. Inoculation of millet with *Azospirillum.* Plant and Soil 52:49-57.

Beck, S.M., and Gilmour, C M , 1983. Role of wheat root exudates in associative nitrogen fixation. Soil Biology and Biochemistry 15:33-38.

Dobereiner, J., and Day, J.M. 1976. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. Pages 518-538 *in* Proceedings of First International Symposium on Nitrogen Fixation, 3-7 Jun 1974, Pullman, Washington, USA (Newton, W.E., and Nyman, C.J., eds.). Vol.2. Pullman, Washington, USA: Washington State University Press.

Lee, K.J., and Gaskins, M.H. 1982. Increased root exudation of ^{14}C compounds by sorghum seedlings inoculated with nitrogen-fixing bacteria. Plant and Soil 69:391-399.

Lethbridge, G., and Davidson, M.S. 1983. Root associated nitrogen fixing bacteria and their role in the nitrogen nutrition of wheat estimated by ^{15}N-isotope dilution. Soil Biology and Biochemistry 15:365-374.

Nur, I., Okon, Y., and Henis, Y. 1980. Comparative studies of nitrogen fixing bacteria associated with grasses in Israel with *Azospirillum brasilense.* Canadian Journal of Microbiology 26:714-718.

Okon, Y., Albrecht, S.L., and Burris, R.H. 1977. Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Applied and Environmental Microbiology 33:85-88.

Rao, A.V., and Venkateswarlu, B. 1982. Nitrogen fixation by *Azospirillum* isolated from tropical grasses native to Indian desert. Indian Journal of Experimental Biology 20:316-318.

Rovira, A.D. 1965. Plant root exudates and their influence upon soil microorganisms. Pages 170-186 *in* Ecology of soil borne plant pathogens: prelude to biological control (Baker, K.F., and Snyder, W.C.,eds.). Berkley, California, USA: University of California Press.

Smith, R.L., Bouton, J.H., Schank, S.C., Quesenberry, K.H., Tyler, M.E., Milam, J.R., Gaskins, M.H., and Littell, R.C. 1976. Nitrogen fixation in grasses inoculated with *Spirillum lipoferum.* Science 193:1003-1005.

Tarrand, J.J., Krieg, N.R., and Dobereiner, J. 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Canadian Journal of Microbiology 24:967-980.

Venkateswarlu, B., and Rao, A.V. 1983. Response of pearl millet to inoculation with different strains *of Azospirillum brasilense.* Plant and Soil 74:379-386.

Vlassak, K., and Reynders, L. 1981. Agronomic aspects of biological dinitrogen fixation by *Azospirillum* spp. in temperate region. Pages 93-100 *in* Associative N_2-fixation: proceedings of the International Workshop on Associative N_2-fixation, 2-6 Jul 1979, Piracicaba, Brazil (Vose, P.B., and Ruschel, A.P., eds.). Vol. 1. Boca Raton, Florida, USA: CRC Press.

Discussion

N.S. Subba Rao:
What will be the story in root exudates of any other plant?

A.V.Rao:
We have not studied the root exudates of other plants. However, there are differences in exudation among plants because of the variation in photosynthetic pathways among these plants.

P.V. Rai:
While the concentration of seed exudate is linear to *Azospirillum* population increase, why does it inhibit the nitrogenase activity at 6% concentration of exudate?

A.V.Rao:
Probably the nitrogen content of seed exudate at proportions higher than 6% is inhibitory to nitrogenase activity. Because of the N concentration, the population of *Azospirillum* is more but the nitrogenase is inhibited.

P.V.Rai:
What is the difference between seed exudate and root exudate?

A.V.Rao:
Washed seeds were soaked in water and the chemicals that leaked from seeds were called seed exudates. Root exudates were collected after germination.

P.V. Rai:
When the *Azospirillum* population is so well maintained by the root exudate, why does it enter into the root?

A.V.Rao:
It is the character of *Azospirillum* to enter and colonize the endo-rhizosphere (Histosphere). Unlike *Azotobacter*, it is an associative symbiotic bacterium. This organism is observed under the epidermal layers and within the cortex.

B.S.Kundu:
N_2-fixation efficiency is not correlated to dry weight but growth-promoting substance. So we should look for organisms secreting more of growth-promoting substance, and not for N_2 fixation.

A.V.Rao:
In case of *Azospirillum*, growth regulators in addition to N_2 fixation are responsible for enhanced dry matter and grain yield. Even in legumes, there is no correlation between the nitrogenase activity at any stage and the yield increase upon inoculation.

B.K. Konde:
At what stage of plant growth do you estimate *Azospirillum* population to determine its localization?

A.V.Rao:
At 30 days of plant growth.

Response of Pearl Millet to Inoculation with *Azospirillum brasilense* at Varying Levels of Nitrogen

G.S. Jadhav, A.N. Giri, and N.B. Pawar[1]

Summary

Investigations carried out during 1979 to 1983 on black soils at Aurangabad and mixed red and black soils at Vaijapur revealed that Azospirillum inoculation of seed alone increased the yield of pearl millet by 14-20% over the control. A synergistic effect of Azospirillum inoculation with nitrogen on increased yield was also observed, though it was not significant. On both soil types, the highest yield increase was due to A. brasilense inoculation of seed at sowing + 40 kg N ha^{-1} applied in two splits. The effect of rainfall on response is discussed. The future strategy of research for realizing the agronomic significance of Azospirillum inoculation in pearl millet-based cropping systems is presented.

Introduction

In experiments conducted under the All India Co-ordinated Millets Improvement Project (AICMIP) with *Azospirillum brasilense,* the yields of pearl millet increased significantly due to inoculation at Durgapura, were nonsignificant at Hisar, and did not increase at Kanpur and Jamnagar (AICMIP 1982). Thus, the results of field experiments have been extremely variable. The information on pearl millet response to *Azospirillum* inoculation is meager, especially with and without N application in Maharashtra state. In this paper, research on pearl millet response to inoculation with *A. brasilense* conducted at the AICMIP Center, Aurangabad, and at Vaijapur in Maharashtra state is presented.

Materials and Methods

Field experiments were laid out during 1979-81 on black soils at Aurangabad and during 1982-83 on mixed red and black soils at Vaijapur, to study the effect of *A. brasilense* and fertilizer nitrogen on grain yield of pearl millet hybrid BJ 104 and its economics.

The initial soil-fertility status of experimental soils is given in Table 1.

All the experiments reported here were conducted during the rainy season. Eight treatments (Table 2) were replicated four times in a randomized-block

Table 1. Initial fertility status of experimental soils in Aurangabad and Vaijapur, Maharashtra.

	Aurangabad			Vaijapur
Parameter	1979	1980	1981	1983
Organic carbon (%)	0.18	0.38	0.49	0.27
pH	8.2	8.31	8.3	8.2
Available phosphorus (kg ha^{-1})	11.6	14.4	32.4	35.0
Available potassium (kg ha^{-1})	448	448	347	350

1. Agronomist, Seed Production Specialist, and Plant Pathologist, Bajra Research Station, Marathwada Agricultural University, Paithan Road, Aurangabad, Maharashtra, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

Table 2. Effect *of Azospirillum brasilense* and nitrogen on grain yield (kg ha^{-1}) of pearl millet at Aurangabad and Vaijapur, Maharashtra.

	Aurangabad					Vaijapur			
Treatments	1979	1980	1981	Pooled average	Increase over control (%)	1982	1983	Pooled average	Increase over control (%)
1. Control[1]	1431	1861	1834	1702	—	726	630	678	—
2. *A. brasilense*	1789	2128	1882	1933	14	904	732	818	20.0
3. 13.3 kg N ha^{-1}	2053	1899	1998	1983	17	867	940	903	33.0
4. 13.3 kg N ha^{-1} + *A. brasilense*	2151	2083	1825	2020	19	859	897	878	30.0
5. 20 kg N ha-'	2173	2081	2254	2084	22	770	980	875	30.0
6. 20 kg N ha^{-1} + *A., brasilense*	2464	2185	2102	2184	28	844	1147	996	47.0
7. 40 kg N ha-'	2339	2250	2254	2284	34	807	1170	955	41.0
8. 40 kg N ha-' + *A., brasilense*	2421	2513	2085	2340	38	659	1250	955	41.0
SE	±77	±193	±163	±166		±33	±88		
CV (%)	6.4	15.8	16.30	—	—	8.2	18.3		

1. Noninoculated and no nitrogen.

design. The crop was sown at a 45 cm x 10 cm spacing. Nitrogen was applied as urea in two equal splits. Half the dose of N as per the treatment and 30 kg P_2O_5 ha^{-1} was applied at sowing. The remaining half N was applied at tillering. Pearl millet seeds were inoculated with carrier-based inoculant of *A. brasilense* prior to sowing (as per the procedure of Subba Rao 1981). Cultural operations were carried out as and when required.

Results

Aurangabad

In 1979, inoculation with *Azospirillum* without N application enhanced grain yield significantly over the noninoculated control with no nitrogen. The application of 40 kg N ha^{-1} + *Azospirillum* produced the highest grain yield, which was on par with 40 kg N ha^{-1} and 20 kg N ha^{-1} + *Azospirillum* inoculation. Inoculation with *A. brasilense* produced significantly higher grain yield over the respective noninoculated controls only in combination with 20 kg N ha^{-1}.

With few exceptions, similar but nonsignificant increases in yield were observed due to various treatments during 1980 and 1981 (Table 2). Pooled data analysis for three years revealed that the *Azospirillum* inoculation alone increased the grain yield by 14% over the control. The interaction of *Azospirillum* with nitrogen was not statistically significant.

Seasonal rainfall at Aurangabad during the three years ranged between 605 and 690 mm. However, the response to *Azospirillum* inoculation along with N application was significant only during 1979. This may be due to the low organic carbon and general low fertility of the field selected in 1979 (Table 1), as *A. brasilense* is active in low-fertility soil (Dobereiner and Day 1976).

Vaijapur

The treatment differences were significant during both the years (Table 2). In 1982 *Azospirillum* inoculation of seed at the time of sowing recorded the highest grain yield, but the results were rather inconsistent.

During 1983 the grain yields due to 20 and 40 kg N ha^{-1} with *Azospirillum,* as well as 40 kg N ha^{-1} without *Azospirillum,* were comparable and significantly higher than all other treatments. Though there is inconsistency in the results, data for two years indicated that *Azospirillum* inoculation of seed at the time of sowing produced 20% increase in grain yield over the control; the response to *Azospirillum* inoculation with the application of 20 and 40 kg N ha^{-1} was positive. The percent yield increase due to *Azospirillum* inoculation with or without N

application was more marked in the low-fertility, mixed red and black soil at Vaijapur than on the relatively fertile black soil at Aurangabad.

Economics of *Azospirillum* Inoculation

Data from Aurangabad on *Azospirillum* inoculation reveal that, under prevailing prices of different inputs, application of 40 kg N ha^{-1} in two equal splits + *Azospirillum* inoculation of seed is economical; the highest gross returns per ha (Rs 957) and net profit (Rs 752) were obtained from this treatment.

Thus inoculation with *A. brasilense* significantly increased pearl millet yield in some years but not in others. With few exceptions, the synergistic effect of *Azospirillum* and nitrogen was observed at 20 and 40 kg N ha^{-1}. The initial soil-fertility status, rainfall, soil moisture, and possibly the nitrogen-transformation processes in the soil are responsible for varying degrees of responses observed in this study. The increases in crop yields when inoculated with *Azospirillum* species and further increases when this is supplemented with 20 and 40 kg N ha^{-1} finds support from the studies by Smith et al. (1978), Subba Rao (1981), and Dart and Wani (1982). The synergistic effect of *Azospirillum* and fertilizer N may be attributed to additional N_2 fixation and possible production of plant harmones by *A. brasilense* at small doses of nitrogen application (Tien et al. 1979).

Problems and Future Research Needs

Most of the farmers in the SAT practice different forms of intercropping with pearl millet. Pigeonpea and groundnut are established intercrops with pearl millet. The possibility of using *Azospirillum* and *Rhizobium* inoculants, along with a basal starter dose of fertilizer nitrogen, needs to be studied in millet-legume intercropping systems. The main agronomic problem with the use of *Azospirillum* or any other inoculant is the competition it will face from physical, chemical, and biological factors in field situations. Thus, there is a need to conduct a large number of farm trials to study the effects of environmental factors on the efficiency of nitrogen fixation by *Azospirillum*, so that a viable agrotechnology for better manipulation of these novel plant-bacteria associations for increased yields can be developed.

Acknowledgement

The authors wish to thank ICAR, New Delhi, for financial support and the Head, Division of Microbiology, IARI, New Delhi, for supplying *Azospirillum* culture. They also acknowledge the technical assistance received from S.D.Dhawandkar, B.G.Hiwale, and D.T.Garad.

References

AICMIP (All India Coordinated Millets Improvement Project). 1982. Progress report of the All India Coordinated Millets Improvement Project (ICAR). Pune, Maharashtra, India: College of Agriculture.

Dart, P J., and Wani, S.P. 1982. Non-symbiotic nitrogen fixation and soil fertility. Pages 3-27 *in* Non-symbiotic nitrogen fixation and organic matter in the tropics. Symposia paper 1. Transactions of the 12th International Congress of Soil Science, 8-16 Feb 1982, New Delhi, India. New Delhi, India: Indian Agricultural Research Institute,

Dobereiner, J., and Day, J.M. 1976. Associative symbioses in tropical grasses: characterisation of microorganisms and dinitrogen-fixing sites. Pages 518-538 *in* Proceedings of the First International Symposium on Nitrogen Fixation, 3-7 Jun 1974, Pullman, Washington, USA (Newton, W.E., and Nyamn, C.J., eds.). Vol. 2. Pullman, Washington, USA: Washington State University Press.

Smith, R.L., Schank, S.C., Bouton, J.H., and Quesenberry, K.H. 1978. Yield increases of tropical grasses after inoculation with *Spirillum lipoferum.* Pages 380-385 *in* Environmental role of nitrogen-fixing blue-green algae and asymbiotic bacteria (Granhall, U., ed.). Ecological Bulletins no. 26. Stockholm, Sweden: Swedish Natural Research Council.

Subba Rao, N.S. 1981. Response of crops to *Azospirillum* inoculation in India. Pages 137-144 *in* Associative N_2-fixation: proceedings of the International Workshop on Associative N_2-fixation, 2-6 Jul 1979, Piracicaba, Brazil (Vose, P.B., and Ruschel, A.P., eds.). Vol. 1. Boca Raton, Florida, USA: CRC Press.

Tien, T.M., Gaskins, M.H., and Hubbel, D.H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Applied and Environmental Microbiology 37:1016-1024.

Rhizosphere Ecology and Nitrogen Fixation of *Azospirillum* in Pearl Millet

D. Purushothaman and K. Govindarajan[1]

Summary

This paper describes the qualitative and quantitative occurrence of nitrogen-fixing bacteria, particularly Azospirillum, *in the root environments of pearl millet. In the rhizosphere, organisms like* Azotobacter, Beijerinckia, ard Derxia *were present in low density; however, the population of* Azospirillum *was considerably high in the rhizoplane and rhizosphere.* Azospirillum *colonized to the extent of 1-8% of the total heterotrophic bacteria. An analysis of 400 isolates of* Azospirillum *revealed that both* A. lipoferum *(79%) and* A. brasilense *(21%) were present in the root environments.*

The nitrogen-fixing ability of these isolates ranged from 3 to 24.8 mg N g^{-1} *malic acid, which corresponded to 50-450 n moles of ARA. The data on the population dynamics of* Azospirillum *in the roots of pearl millet revealed that certain drug-resistant mutants were more competitive than the native strain of* Azospirillum *in colonizing the rhizoplane and rhizosphere. In general, the density of* Azospirillum *was maximum at the tillering and flowering stages of the crop. The root exudates of pearl millet contained fairly high concentrations of soluble sugars and amino nitrogen.*

Introduction

Divergent views have been expressed on the use of *Azospirillum* as an inoculant in agriculture (Brown 1982). However, it is generally believed that *Azospirillum,* living in rhizocoenotic association with several graminaceous crops, has a considerable potential for improving yields in extensive low-cost farming systems in the tropics. In this paper, the rhizosphere ecology of *Azospirillum* spp and the nitrogen-fixing potential of its isolates are described with relevance to pearl millet.

Materials and Methods

The drug-resistant mutants, str^r and CAM^r were developed in our laboratory by exposure to the chemical mutagen, ethyl methane sulfonate (EMS). They were resistant to 500 µg ml^{-1} of streptomycin and chloramphenicol. The nonnitrogen-fixing mutant, S3, was developed from the parent culture, CD (ATCC: 29729, a pink isolate) at the Microbiology and Cell Science Laboratory, University of Florida, USA.

A large number of *Azospirillum* cultures were isolated from the root tissues of pearl millet grown at the Millet Breeding Station of the TNAU following the enrichment technique detailed by Dobereiner and Day (1976).

Young and fresh root bits, surface-sterilized with 80% ethanol and washed with sterile distilled water, were plunged into malate semisolid medium (Baldani and Dobereiner 1980) and supplemented with 50 mg L^{-1} of yeast extract (NFb). A loopful of the characteristic subsurface pellicle was streaked on

1. Associate Professor and Assistant Professor, Center of Advanced Studies in Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu 641 003, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

NFb agar plates. The single colonies were purified further by streaking on potato agar (BMS) and the typical pink, wrinkled colonies were examined microscopically and transferred to trypticase soy-agar slants. The species of *Azospirillum* isolates were identified as per Tarrand et al. (1978).

Enumeration of the total heterotrophic bacteria in the root environments of pearl millet was done following the technique of Pramer and Schmidt (1966). Enumeration of *Azotobacter* was done by using Waksman medium no.77, of *Beijerinckia* by using Jensen's medium, and of *Derxia* sp by using Compelo and Dobereiner's medium. The total diazotrophs were enumerated by using the medium of Watanabe and Barraquio (1979). The most probable number (MPN) technique was used for determining the populations of *Azospirillum* (Hegazi et al. 1979).

Earthen pots (30 cm dia) containing red soil:compost 1:1 (v/v) at 14 kg pot^{-1} were used for pot culture studies (organic carbon 0.580%, total nitrogen 1.12%, pH 7.0 and E.C. 0.46 m mhos cm^{-1}). The soil received a basal dose of 20 kg ha^{-1} of N, 30 kg ha^{-1} of P_2O_5, and 30 kg ha^{-1} of K_2O.

The seeds of the pearl millet cultivar, X3, were surface sterilized with 0.1% sodium hypochlorite and treated with the peat-based inoculum of *Azospirillum* (containing ca. 10×10^8 cells g^{-1}), using jaggery solution as an adhesive. Air-dried seeds were sown in the pots. Each pot also received a soil inoculum of 2 g of *Azospirillum* at the time of sowing. Standard agronomic and cultivation practices were followed. At desired intervals, the plants were uprooted and rhizosphere and rhizoplane samples were collected (Pramer and Schmidt 1966).

The drug-resistant mutants used in the study were enumerated on Okon's medium containing 500 µg mL^{-1} of the respective antibiotic (Okon et al. 1977). As S3 was a naturally pink pigmented mutant, it was easy to count them on trypticase soy agar medium.

For the estimation of N_2 fixation, the isolates were grown in the NFb semisolid medium (supplemented with 100 mg of glutamic acid L^{-1}) for 7 days at 30°C. Total nitrogen was determined by the micro-Kjeldahl method (Humphries 1956).

Acetylene reduction activity (ARA) of 3-day-old cultures, grown in 30 ml of N free-semisolid malate medium, was estimated by incubating the cultures under 1% acetylene for 6 h. In the case of the root-associated ARA, freshly collected roots free from soil particles were taken in the flasks and the assay conducted similarly (Purushothaman et al. 1979).

Root exudates were collected by the method described by Dazzo and Hrabak(1981). After 7, 14, and 21 days of seed germination, the solution was aseptically collected, clarified by centrifugation, and the volume was suitably reduced. Total sugars and amino nitrogen in the root exudate were determined following the methods of Nelson (1944) and Moore and Stein (1948).

Results

A number of organisms like *Azotobacter, Beijerinckia, Derxia,* and *Azospirillum* were observed in the rhizosphere of pearl millet. Excepting *Azospirillum,* the populations of *Azotobacter, Beijerinckia,* and *Derxia* were very poor in the rhizosphere and in the rhizoplane (Table 1). *Azospirillum* was found to occupy the roots in the rhizosphere and the rhizoplane to the extent of 8% (Table 2). The rhizoplane had higher colonization by *Azospirillum* than the rhizosphere. Perhaps the abundant mucigel on the root surface is helpful in the attachment of microorganisms on to the rhizoplane. One of the reasons for the intense microbial activity in the root zone is the exudation of energy-rich and growth-promoting organic compounds (Newman 1978, Brown 1982). It has been estimated that 12-18% of the photosynthetically fixed carbon is released as organic compounds in the root exudation (Beck and Gilmour 1983). In our study, the amount of total soluble sugars in the root exudates ranged from 0.6 to 2.25% and the amino nitrogen varied from 0.21 to 0.43%. It is evident from the study that the rhizosphere and rhizoplane are potential sites for the colonization of *Azospirillum.* The populations of diazotrophs in general and *Azospirillum* in particular, were high at the tillering and flowering stages of the crop.

The species distribution of *Azospirillum* in the

Table 1. Distribution of nitrogen-fixing organisms in pearl millet at flowering.

Organisms	No.of bacteria g^{-1} dry wt.	
	Rhizosphere (soil)	Rhizoplane (roots)
Azotobacter sp	7.27×10^3	2.8×10^3
Azospirillum sp	6.30×10^5	$12.8 \times 10.^5$
Beijerinckia sp	14.2×10^3	26.4×10^3
Derxia sp	2.6×10^2	1.3×10^2
Others (unidentified)	15.25×10^5	6.8×10^5
Total heterotrophs	28.6×10^7	42.0×10^6

Table 2. Percentage distribution of *Azospirillum* and other diazotrophs to total bacteria in the roots of pearl millet.

Root environment	Growth phase of crop				Mean
	Seedling	Tillering	Flowering	Harvest	
Rhizosphere					
Azospirillum	1.6	2.39	6.14	0.69	2.70
Other diazotrophs	22.7	39.8	24.9	24.6	28.0
Rhizoplane					
Azospirillum	0.8	4.2	8.62	0.22	3.46
Other diazotrophs	3.8	12.6	18.12	10.8	11.33

roots of pearl millet revealed that *A. lipoferum* constituted 79% and *A. brasilense* 21%. Both nir+ and nir strains were present and the nir+ isolates predominated (Fig.1).

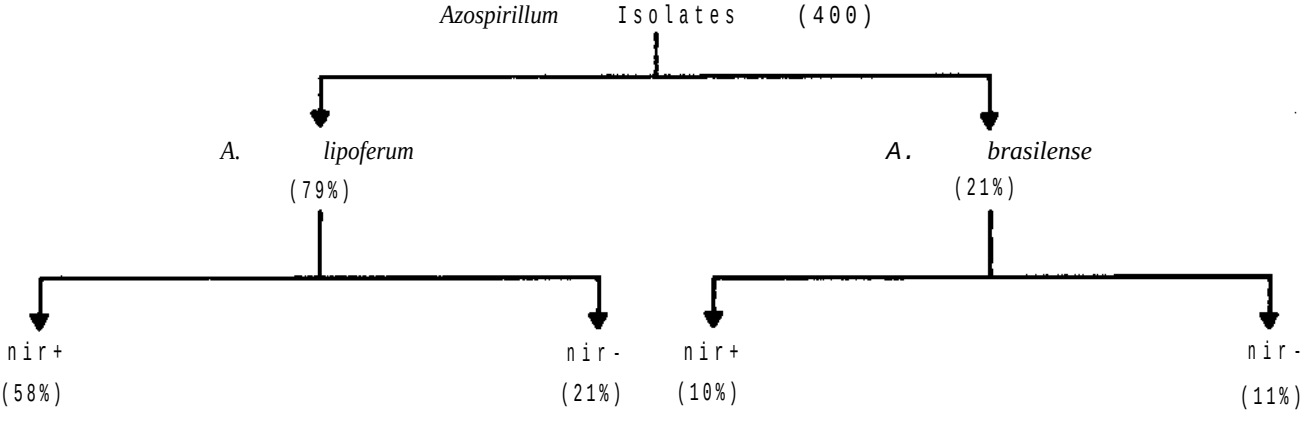
About 80% of the isolates had very poor N_2-fixing capacity (3-10 mg N g^{-1} malic acid)); while for 0.8% isolates N_2 fixed was in the range of 21-25 mg N g^{-1} of malic acid (Table 3). The ARA, which also varied considerably from 50 to 450 nmoles C_2H_4 $isolate^{-1}$ h^{-1}, was low in 79% of the isolates and high in only 9% isolates.

The drug-resistant mutants in general exhibited a much higher colonizing ability both in the rhizosphere and in the rhizoplane (Table 4). The native population was poor in competitive colonization in the rhizosphere; however, on the rhizoplane their number was fairly high. It is of interest to observe that the nonnitrogen-fixing mutant, S3, also possessed a much better colonizing ability than the native isolates. The root-associated ARA of pearl millet inoculated with CAM^r is also found to be significantly higher (Table 5).

Recently, increased colonization of *Rhizobium phaseoli* in the rhizosphere of bean has been achieved by the use of fungicide-resistant strains and

Table 3. Nitrogen fixation and ARA by 400 isolates of *Azospirillum* spp from the roots of pearl millet.

Group	N_2 fixation range (mg N g^{-1} malic acid)	Distribution (%)	ARA (n mol C_2H_4 $tube^{-1}$ h^{-1})	Distributrion <%)
1	1-5	56.8	10-50	46.5
II	6-10	21.6	51-100	33.0
III	11-15	12.5	101-200	12.5
IV	16-20	8.3	201-400	6.8
V	21-25	0.8	401-500	2.2



nir + : Converts NO_3 to NO_2 and beyond NO_2

nir - : Converts NO_3 to NO_2 and not beyond

Figure 1. Distribution of *Azospirillum* spp in the rhizosphere of pearl millet.

also by applying fungicides to the soil (Mendez-Castro and Alexander 1983).

The striking increase in the root-associated ARA of pearl millet, inoculated with the mutant strains, is no doubt due to the increased colonization of the root environment with efficient nitrogen-fixing strains. This opens up newer hopes for exploiting the potentials of *Azospirillum* in nitrogen fixation. Though we could not trace the nutrient release in the root exudates over a long period, the data collected over 21 days clearly illustrate the availability of an abundant energy source in the vicinity of roots. Recent studies on the surface colonization of root by microorganisms have indicated that only 4-10% of the root area is colonized by microorganisms (Russel 1977).

Table 4. Percent colonization of rhizosphere and rhizoplane of pearl millet by drug-resistant mutants and native *Azospirillum*.

Locus and stage of crop	Native isolate	Drug-resistant mutants			Mean
		Str^r	CAM^r	N_2ase-S3	
Rhizosphere					
seedling	0.20	14.24	13.26	3.7	7.85
tillering	3.60	14.92	29.09	5.5	13.27
flowering	3.60	14.92	27.12	43.1	22.18
harvest	0.31	12.19	14.30	25.0	12.95
Mean	1.92	14.06	20.94	19.32	
Rhizoplane					
seedling	2.7	24.2	3.7	4.3	17.05
tillering	14.8	61.4	58.9	20.5	19.85
flowering	17.8	44.7	40.2	10.0	23.72
harvest	6.9	25.0	24.6	3.7	15.05
Mean	10.55	28.82	31.85	9.62	

Table 5. The ARA of pearl millet root as influenced by inoculation with different *Azospirillum* strains.

Strain	ARA (n moles C_2H_4 g^{-1} root h^{-1})
CAM^r	168.76[1]
Str^r	185.80[1]
S3	22.60
Noninocuiated control	23.35

1. Significantly different from the control ($P<0.05$).

References

Baldani, V.L., and Dobereiner, J. 1980. Host plant specificity in the infection of cereals with *Azospirillum* spp. Soil Biology and Biochemistry 12:433-439.

Beck, S.M., and Gilmour, C.M. 1983. Role of wheat root exudates in associative nitrogen fixation. Soil Biology and Biochemistry 15:33-38.

Brown, M.E. 1982. Nitrogen fixation by free-living bacteria associated with plants: fact or fiction? Pages 25-41 *in* Bacteria and plants (Rhodes-Robert, M.E., and Skinner, F.A., eds.). London, UK: Academic Press.

Dazzo, F.B., and Hrabak, E.M. 1981. Presence of Trifolin-A, a *Rhizobium* binding lectin in clover root exudate. Journal of Supermolecular Structure and Cellular Biochemistry 16:133-138.

Dobereiner, J., and Day, J.M. 1976. Associative symbioses in tropical grasses: characterisation of microorganisms and dinitrogen-fixing sites. Pages 518-538 *in* Proceedings of the First International Symposium on Nitrogen Fixation, 3-7 Jun 1974, Pullman, Washington, USA (Newton, W.E., and Nyman, C.J., eds.). Vol.2. Pullman, Washington, USA: Washington State University Press.

Hegazi, N.A., Amer, H., and Monib, M. 1979. Enumeration of N_2-fixing spirilla. Soil Biology and Biochemistry 11:437-438.

Humphries, E.C. 1956. Mineral components and ash analysis. Pages 468-502 *in* Modern methods of plant analysis (Paech, K., and Tracey, M.V., eds.). Vol. 1. Berlin, Federal Republic of Germany: Springer Verlag.

Mendez-Castro, F.A., and Alexander, M. 1983. Method for establishing a bacterial inoculum on corn root. Applied and Environmental Microbiology 45:248-254.

Moore, S., and Stein, W.H. 1948. A photometric adaptation of ninhydrin method for use in the chromatography of amino acids. Journal of Biological Chemistry 176:367-388.

Nelson, N. 1944. A photometric adaptation of Somogyi method for the determination of glucose. Journal of Biological Chemistry 153:375-380.

Newman, E.I. 1978. Root microorganisms, their significance in the ecosystem. Biological Reviews 53:521-554.

Okon, Y., Houchins, J.P., Albrecht, S.A., and Burris, R.H. 1977. Growth of *Spirillum lipoferum* at constant partial pressures of oxygen, and the properties of its nitrogenase in cell-free extracts. Journal of General Microbiology 98:87-93.

Pramer, D., and Schmidt, EX. 1966. Experimental soil microbiology. Minneapolis, Minnesota, USA: Burgess Publishing Co.

Purushothaman, D., Gunasekaran, S., and Oblisami, G. 1979. Nitrogen fixation by *Azospirillum* in some tropical plants. Proceedings of the Indian National Science Academy, Part B 46:713-717.

Russell, R.S. 1977. Plant root systems: their function and interaction with the soil. London, UK: McGraw Hill. 298 pp.

Tarrand, J.J., Krieg, N.R., and Dobereiner, J. 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Canadian Journal of Microbiology 24:967-980.

Watanabe, I., and Barraquio, W.L. 1979. Low levels of fixed nitrogen required for isolation of free-living N_2-fixing microorganisms from rice roots. Nature 277: 565-566.

Response of Sorghum and Pearl Millet Genotypes to *Azospirillum* and *Azotobacter* Inoculations

C.P. Ghonsikar, R.S. Raut, and G.B. Rudraksha[1]

Summary

Sorghum and pearl millet genotypes were tested in afield trial for their response to Azospirillum *and* Azotobacter *inoculations.* Azospirillum *inoculation increased grain yield of sorghum by 18% over the control as compared to 12% yield increase with* Azotobactcr. *Fodder yields in sorghum and pearl millet reflected a significant influence of* Azotobacter *and* Azospirillum *inoculations. Sorghum hybrid CSH 9 and pearl millet genotypes MP 21 and Bangaon showed relatively better response to* Azotobacter *and* Azospirillum *inoculation than other genotypes. The results imply that nitrogen-fixing organisms like* Azotobacter *and* Azospirillum *showed an ability to enhance biomass production in sorghum and pearl millet. However, it is necessary to locate specificity of interaction between an isolate and host-plant species.*

Introduction

Nitrogen supply to sorghum and pearl millet is generally a limiting factor, particularly when they are grown on low-fertility soils, and the exploitation of rhizospheric nitrogen fixation may prove beneficial for crop improvement. Field experiments have provided limited information and there is a great variability in the responses to inoculations. Inoculation resulted in increased yields, although such responses were variable depending on the N status of the soil and variety of the crop planted. This paper reports the effect of *Azotobacter* and *Azospirillum* inoculation on growth (root and shoot mass) and yield of sorghum and pearl millet under field conditions.

Materials and Methods

Sorghum cultivars CSH 1, CSH 5, CSH 6, CSH 9, and SPV 297 were sown on a typic Vertisol field with pH 8.2, O.C. 0.62%, C.E.C. 42 meq 100 g^{-1} soil and high moisture-retention capacity. The seeds were inoculated either with *Azotobacter chroococcum* or *Azospirillum brasilense,* and noninoculated plants served as a check treatment. The crop received 40 kg N ha^{-1} and 60 kg P_2O_5 ha^{-1} at the time of sowing. In another field trial, pearl millet cultivars Bangaon, BJ 104, BK 560, and MP 21 were planted with similar treatments. The crop received 25 kg N ha^{-1} and 50 kg P_2O_5 ha^{-1}. These two trials were conducted during the rainy season (Jun-Oct) in 6.0 m x 4.5 m plots organized in a factorial RBD with three replicates. Root and shoot masses were measured periodically to assess biomass production. Root volume was determined by displacement of water volume in a measuring cylinder. Finally, grain and fodder yields were recorded at harvest.

Results

Sorghum

Inoculation of sorghum plants with *Azospirillum* increased root mass by 29%, shoot mass by 30%, and root volume by 39% over the respective noninoculated controls. The *Azotobacter* inoculation in-

1. Associate Dean, PG School, and Microbiologists, Department of Microbiology, Marathwada Agricultural University, Parbhani, Maharashtra 431 402, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502324, India: ICRISAT.

Table 1. Effect of inoculation *with Azotobacter* and *Azospiriiium* on the growth of sorghum during the rainy season.

Treatment	Days after sowing 20	35	50	65	80
	Mean root mass (g plant^{-1})				
No inoculation	3.4	12.2	16.0	17.8	15.2
Azotobacter chroccoccum	4.3	16.4	20.7	20.5	15.9
Azospirillum brasilense	6.4	18.5	21.7	22.9	15.3
CD (P<0.05)	0.89	2.0	2.7	3.7	NS[1]
	Meanshootmass(gplant^{-1})				
No inoculation	32.6	111.8	165.1	167.6	156.0
Azotobacter chroccoccum	37.6	139.3	184.8	187.3	167.8
Azospirillum brasilense	50.9	156.2	190.5	203.8	182.5
CD (P<0.05)	7.6	15.8	12.7	23.0	10.1

1, NS = Not significant.

Table 2. Effect of inoculation with *Azotobacter* and *Azospirillum* on the growth of pearl millet, Parbhani, rainy season.

Treatment	Days after sowing 20	35	50	65	80
	Mean root mass (g plant^{-1})				
No inoculation	2.1	5.5	6.5	5.1	4.6
Azotobacter chroccoccum	3.2	7.9	8.8	8.5	8.01
Azospirillum hrasilense	3.3	6.6	8.1	7.8	6.18
CD (P<0.05)	0.6	0.83	1.72	0.99	0.91
	Mean shoot mass (g plant^{-1})				
No inoculation	22.3	72.7	89.4	67.6	59.6
Azotobacter chroccoccum	31.6	94.4	103.4	86.8	79.7
Azospirillwn brasilense	32.4	88.0	97.8	78.9	67.6
CD (P<0.05)	8.6	9.0	10.4	NS[1]	9.4

1. NS = Not significant.

creased the root mass by 18%, shoot mass by 15%, and root volume by 14% over the control (Table 1). The observed enhancement in root and shoot mass of sorghum, particularly in early growth stages as a result of *Azotobacter* and *Azospirillum* inoculation, may be ascribed to growth-promoting substances (Lakshmikumari et al. 1976, Shende et al. 1977). The 12.2% increase in grain yield of sorghum by *Azotobacter* and 18.0% increase with *Azospirillum* inoculation was, however, statistically nonsignificant. The mean increases in fodder yield of sorghum genotypes due to *Azotobacter* (32%) and *Azospirillum* (43%) inoculations were significantly greater than noninoculated sorghum plots. Apparently *Azotobacter* and *Azospirillum* inoculations influence biomass production greatly, but it is not significantly reflected in the grain yield.

Pearl Millet

Both the bacteria stimulated root growth significantly and the effect of *Azotobacter* on root mass was more than that of *Azospirillum* (Table 2). The inoculated plants also reflected higher root volume than noninoculated plants. The inoculated plants produced nearly 14% higher shoot mass than noninoculated checks. The benefits from such microorganisms may be viewed as effects of plant growth substances (Mishustin and Shilinikova 1969), in addition to their known nitrogen-fixing ability. *Azotobacter* inoculation in general produced 0.23 t ha^{-1} additional grain over its noninoculated check, while *Azospirillum* gave 0.14 t ha^{-1} additional grain. This advantage however, was not statistically significant. *Azotobacter* inoculation increased fodder yield of pearl millet significantly and its effect was better than that of *Azospirillum.*

This report has shown positive effects of *Azotobacter* and *Azospirillum* inoculations to sorghum and pearl millet. It may be necessary to identify specificity between an isolate and host-plant species to realize the potential of rhizospheric nitrogen fixers for increasing crop yields.

References

Lakshmikumari, M., Kavimandan, S.K., and Subba Rao, N.S. 1976. Occurrence of nitrogen fixing *Spirillum* in roots of rice, sorghum, maize and other plants. Indian Journal of Experimental Biology 14:638-639.

Mishustin, E.N., and Shilnikova, V.K. 1969. The biological fixation of atmospheric nitrogen by free-living bacteria. Pages 82-109 *in* Soil biology: reviews of research. Paris, France: UNESCO.

Shende, S.T., Apte, R.G., and Singh, T. 1977. Influence of *Azotobacter* on germination of rice and cotton seeds. Current Science 46:675.

Research on Cereal Nitrogen Fixation at ICRISAT

S.P. Wani[1]

Summary

The acetylene-reduction assay for nitrogenase activity has shown that the roots of sorghum and pearl millet stimulate N_2 fixation, A soil-core assay used for measuring nitrogenase activity of field-grown plants showed large plant-to-plant variability. An improved core assay (planted core assay) developed at ICRISAT Center showed higher activity than that recorded by regular core assay. In greenhouse assay methods plants with shoots sustained higher activity than the ones whose tops were cut before the assay, Nitrogenase activity varied throughout the day, as well as over the season. Activity was maximum at the late flowering or early grain-filling stage and it was related to the ontogenetic development of the plant. Activity was favored in moist, warm (ca 35° C) soil and decreased with high levels of fertilizer N.

Genotypic variation in sorghum and pearl millet lines for stimulating rhizospheric nitrogenase activity was observed. Out of 284 pearl millet lines tested, 18 lines stimulated high nitrogenase activity (>460 nmol C_2H_4 h^{-1} 15 cm diam $core^{-1}$) in the rhizosphere. Two lines, Gam 73 and J 1407, were consistently active over several seasons. Similarly, 28 of 334 sorghum lines tested had high nitrogenase activity (>460 nmol C_2H_4 h^{-1} 15 cm diam $core^{-1}$).

At ICRISAT Center, pot-culture experiments with sorghum and pearl millet grown in a low-fertility Alfisol, or in unsterilized washed vermiculite, showed substantial positive balances for N. Long-term N balance studies in the field with sorghum and pearl millet cultivars are continuing.

Using $^{l5}N_2$ it has been shown that 20-days-old sorghum seedlings fix N_2 in the rhizosphere, and part of it is taken up by the plant within 3 days after exposure. The ^{15}N isotope dilution technique has been evaluated for studying genotypic variation in sorghum and pearl millet cultivars for N_2 fixation.

Many different kinds of bacteria closely associated with the roots of sorghum and pearl millet plants show nitrogenase activity. Responses to inoculation in terms of increased dry-matter production and N uptake have been observed in pot experiments with sorghum and pearl millet grown in Alfisols and vermiculite. In five out of nine field trials with pearl millet cultivars, inoculations with N_2-fixing bacteria significantly increased the grain and plant dry-matter yields in all the cultivars.

Introduction

The semi-arid tropics (SAT) produce 60% of the world's sorghum, and 95% of the world's millets, from a total cropped area of about 70 million ha. Both cereals respond to inputs of N, yet almost all the production is without the use of fertilizer. The soil fertility with respect to N depends on the rate of biological turnover of nitrogen and the amounts of nitrogen added to soil through fertilizer, manure,

1. Microbiologist, Pearl Millet Improvement Program, ICRISAT, Patancheru, A.P. 502 324, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation, Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502324, India: ICRISAT.

and biological nitrogen fixation.

The overall objectives of the ICRISAT research program that concentrates on biological N_2 fixation associated with sorghum and millet are

1. To quantify the amounts of N_2-fixation and to gauge its importance in relation to other N inputs that sustain cereal crop production in the SAT.
2. To seek ways of enhancing this activity by genetic, agronomic, or microbiological methods.

Development of Acetylene Reduction Assay (ARA) for Measuring Nitrogenase Activity

Central to our research is the development of suitable assay techniques to measure nitrogenase activity.

A soil-core assay method has been standardized for measuring acetylene-reduction activity (ARA) associated with field-grown plants. Soil cores containing plant roots are taken in metal cylinders (15 cm diameter, 22 cm length) with as little soil disturbance as possible, and incubated in sealed 6 L plastic vessels under an atmosphere of ca 15% acetylene in air (Fig. 1). Gas samples are taken after 1 and 6 h and later analyzed for ethylene production by gas chromatography. The activity of such cores is usually linear with little or no lag period prior to the onset of nitrogenase activity. With this assay technique, we encountered large plant-to-plant variability in nitrogenase activity. We have studied the factors responsible for such plant-to-plant variability and modified our techniques accordingly.

Mechanical disturbance. The cores sampled and transported with precautions had three-fold greater activity than the cores sampled and transported normally.

Time lag between severing the plant tops and injecting C_2H_2. The highest activity was recorded when the time lag was least, i.e., at 0.5 h. An increase in the time taken to inject the C_2H_2 significantly reduced the activity recorded, with a significant negative correlation ($r = -0.421$, $P<0.01$).

Temperature. Most activity was recorded when the soil cores were incubated at 35°C (Wani et al. 1983).

Core size. The activity varied from field to field and the cores taken over the crown of the plant contained between 13 and 50% of the total activity of the plant depending on its age (Rao and Dart 1981).

Time of sampling. This significantly affected nitrogenase activity of field-grown pearl millet hybrid NHB 3. Nitrogenase activity increased from 0915 to 1815 h during the photoperiod and then declined during the night until 0715 h the next morning. The activity changed little between 0915 and 1500 h. However, significantly higher nitrogenase activity was recorded for the plants sampled between 1715 and 2115 h than at 1115 h.

Soil moisture. The effect of soil moisture on nitrogenase activity was studied in two ways: (1) by using the line-source irrigation system and (2) by adding water to cores just before assay. Both methods gave significant correlations between soil moisture and nitrogenase activity.

Obviously, it is necessary to control as many of the above variables as possible if an assay of field-grown plants is to work.

The activity recorded with the improved soil-core assay technique was significantly higher than that recorded with the regular soil-core assay technique. Nitrogenase activity of field-grown pearl millet hybrid NHB 3 estimated by both the assay methods indicated that activities of most of the plants estimated by the regular core assay ranged from 0 to 20 nmol C_2H_4 plant^{-1} h^{-1} compared with 100-250 nmol C_2H_4 plant^{-1} h^{-1}, for the improved planted-core assay. Mean activity recorded for planted cores was 167 nmol C_2H_4 plant^{-1} h^{-1}, significantly higher than the regular core activity of 18 nmol C_2H_4 plant^{-1} h^{-1}. As plants aged, activity of the regular cores declined more than that of planted cores (Wani et al. 1983). Similar results were obtained with sorghum lines as shown in Table 1 (ICRISAT 1983).

Because soil-core assays are time consuming and somewhat variable, we have developed alternative assay systems — one where seedlings are grown in test tubes, and another where plants are grown in pots.

The test tube culture technique has been developed mainly to test the effect of host genotype and bacterial culture on nitrogenase activity. Plants are grown in 25 mm ^{x} 200 mm tubes, with a small tube attached to the side near the bottom. The tubes are filled with 20-25 mL of growth medium, soil, sand, sand:FYM, vermiculite, or nitrogen-free agar, covered and painted to keep light from the root medium. Plants are grown inside the tube, which is plugged with cotton wool until the assay, then with a

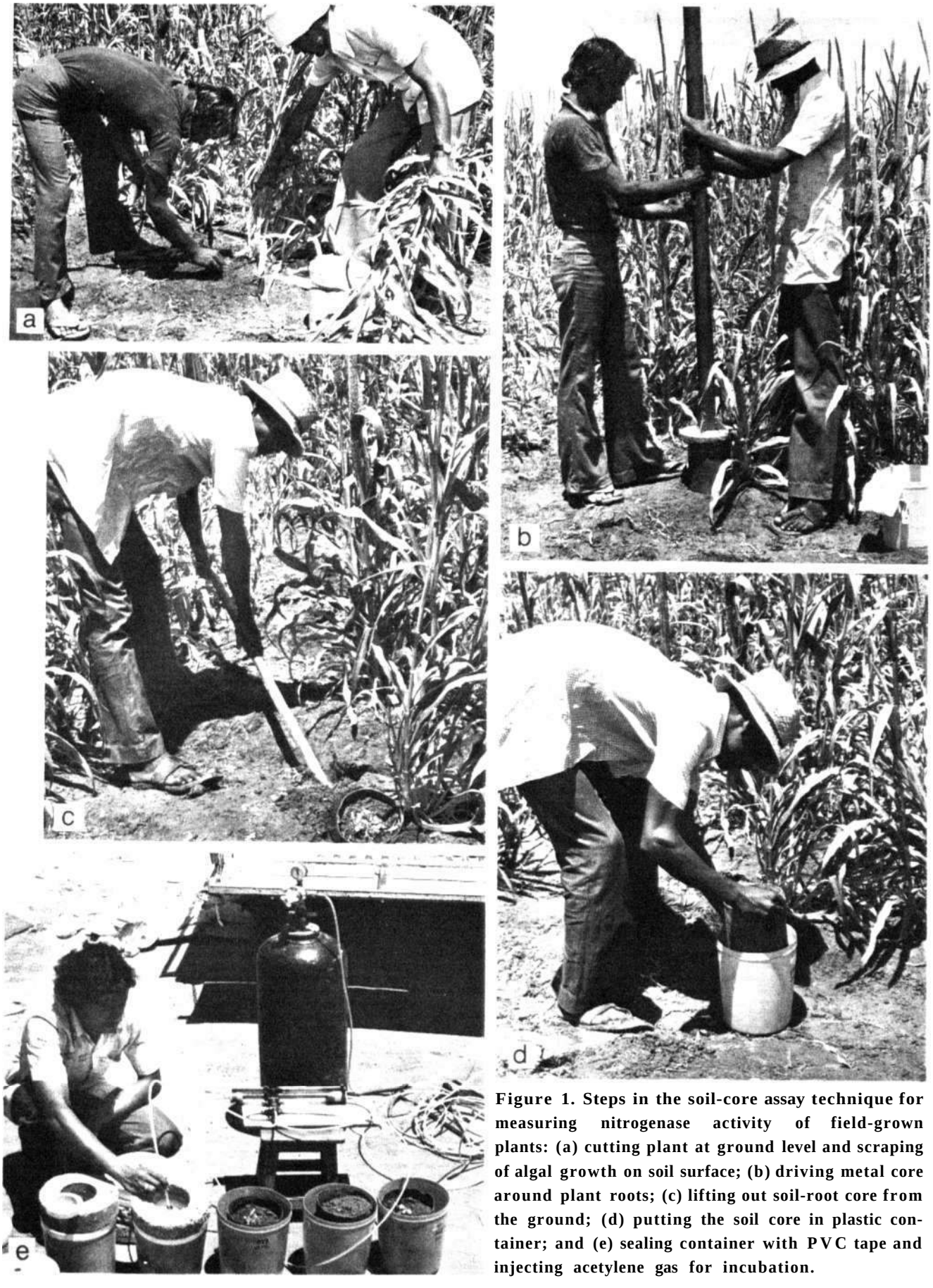


Figure 1. Steps in the soil-core assay technique for measuring nitrogenase activity of field-grown plants: (a) cutting plant at ground level and scraping of algal growth on soil surface; (b) driving metal core around plant roots; (c) lifting out soil-root core from the ground; (d) putting the soil core in plastic container; and (e) sealing container with PVC tape and injecting acetylene gas for incubation.

Table 1. Nitrogenase activity (nmoles C_2H_4 plant^{-1} h^{-1}) of sorghum cultivars as estimated by regular-core and planted-core assay methods at ICRISAT Center.[1]

Cultivar	Regular-core assay	Planted-core assay
IS 1057	24	2101
IS 2207	41	253
IS 9180	33	295
IS 2638	33	316
IS 2391	38	682
IS 3951	37	448
IS 3949	30	267
CSV 5	61	335
Soil	20	119
Mean	35 b	535 c

1. Average of 4 replicated cores. Log transformation (of nmoles C_2H_4+1) used to analyze data. Figures appended with letters vary significantly (P<0.05) from each other.

rubber Suba seal; the side tube is also closed with a Suba seal. Acetylene gas, equivalent to 15% of the free volume in the tube, is injected through the bottom Suba seal.

Eleven lines of pearl millet were sown and one control tube without seed inoculated with a rhizospheric extract of field-grown millet to provide a mixed inoculum. Differences between cultivars in stimulating nitrogenase activity were apparent by 20 days after sowing (DAS) and rankings were similar in different growth media. The nitrogenase activity associated with pearl millet seedlings grown in vermiculite test tubes varies with the culture of organisms used. At 14 DAS a maximum activity of 21 nmoles C_2H_4 plant^{-1} d^{-1} was obtained with a culture of *Derxia* sp. The variability among plants in this system was much less than for field-grown plants. It may be possible to screen pearl millet lines more reliably for differences in their ability to stimulate nitrogenase activity in such a system.

Similarly, the activity of sorghum CSH 6 seedlings inoculated with *Azospirillum lipoferum. A, brasilense, Azotobacter chroococcum,* and *Derxia* spp at 8 DAS was examined. The highest activity of 364 nmol C_2H_4 plant^{-1} d^{-1} was obtained with plants inoculated with *Azotobacter chroococcum.* The activity in the noninoculated control plants was 1.2 nmol C_2H_4 plant^{-1} d^{-1} (ICRISAT 1980).

The above technique was further improved by allowing the shoots to grow outside the test tube. At assay time, Suba seal and silicone rubber were used to seal the gas phase around the root medium, with the shoot remaining free in the air. This method again permitted differences to be detected between pearl millet lines by 20 DAS. Likewise, maximum field activity of nine pearl millet lines recorded with regular soil-core assays over three to six seasons and the activity recorded with seedlings grown in tubes filled with sand:farmyard manure (97:3 w/w) and vermiculite were positively correlated (r=0.65, r=0.67, P<0.05) (ICRISAT 1984).

We have also developed a nitrogenase assay method for intact plants in pots, where only the root system in the pot is exposed to acetylene. Quicker and better diffusion of C_2H_2 and several times greater nitrogenase activity was observed when acetylene was injected at the bottom of the pot than at the top. Intact-plant assays with sorghum and millet showed linear rates of C_2H_4 production up to 16 h with a small lag in the beginning (Fig. 2). The time-course assays indicated the feasibility of measuring C_2H_2 reduction by intact sorghum and millet plants grown in pots. After 16 h C_2H_4 production increased slightly, suggesting that the incubation period for estimating ARA by intact-plant assays should be less than 16 h. In our assays we use 6-h incubation for estimating ARA. The initial lag period for C_2H_4 production was possibly because of the time

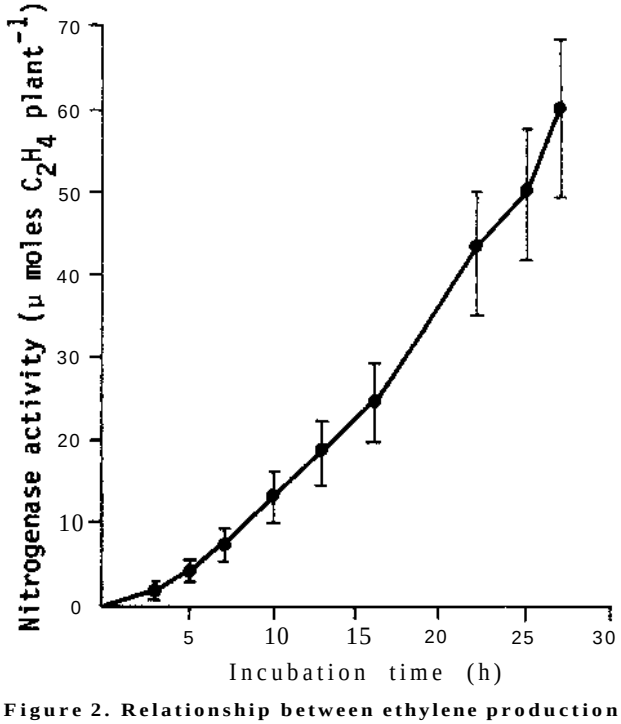


Figure 2. Relationship between ethylene production and incubation time (h) during the assay of intact sorghum plants. Bars represent ±SE.

required for C_2H_2 diffusion throughout the root medium in the pot.

Higher ARA was associated with the intact plants than with decapitated plants. Mean nitrogenase activity of 49-day-old plants across the 15 sorghum cultivars with intact shoots was 625 nmoles C_2H_4 $plant^{-1}$ h^{-1} significantly higher ($P < 0.05$) than the activity with decapitated plants (247 nmoles C_2H_4 $plant^{-1}$ h^{-1}). There was no significant interaction between the assay methods and the sorghum cultivars. There was a 26-times reduction in the activity of these cultivars due to decapitation of their shoots with 76-day-old plants as compared with 2.5 times reduction with 49-day-old plants. This higher activity might be due to a continuous supply of photosynthate to the roots from the intact shoot, or the physiological processes of the roots might have been disturbed because of decapitation. Further studies will be required (Wani et al. 1984).

With plants grown in sand, higher nitrogenase activity was observed with increasing FYM concentrations. The increased activity may be due to better plant growth, with increased FYM concentrations providing more root-surface area and more root exudates (derived from an increased root mass) for colonization by N_2-fixing bacteria. Further experimentation is required to understand the role of FYM in stimulating nitrogenase activity associated with plants. Increased activity was recorded with the temperature increasing from 27°C to 33°C. Soil moisture had a large effect on nitrogenase activity of sorghum and millet in the intact-plant assay.

The intact-plant assay was tested for estimating nitrogenase activity associated with 15 sorghum cultivars, and we could differentiate cultivars with high and low associated activity. The variability between plants grown and assayed in pots was much less than between field-grown plants (Wani et al. 1984). This suggested the promise of using this technique for screening sorghum and millet cultivars for their potential to fix atmospheric nitrogen biologically, after studying the relationship between intact-plant assay and field assay methods. It permits a study of the activity at different growth stages of the plant, collection of selfed seed from the plants, and crossing between plants that could be used for producing test hybrids. Maximum expressions of the activity could be obtained by growing plants in 3% FYM mixed with sand or Alfisol soil, by watering the plants to 60-70% water-holding capacity (WHC), by maintaining the temperature of the plant growth medium at around 33° C, and by not decapitating the shoot.

Host Genetic Differences in Promoting N_2-fixation

Different genotypes of both sorghum and pearl millet show differences in stimulating nitrogenase activity. At ICRISAT Center, 135 out of the 284 millet lines tested stimulated nitrogenase activity that was more than twice that of soil without plant roots. Eighteen lines stimulated high nitrogenase activity (>460 nmol C_2H_4 15 cm diam $core^{-1}$ h^{-1}). Two lines, Gam 73 and J 1407, were consistently active over several seasons (ICRISAT 1978, 1980, 1981; Dart and Wani 1982). Large plant-to-plant variability in nitrogenase activity was observed in the Ex-Bornu population, ranging from 0 to 1900 nmol C_2H_4 $plant^{-1}$ h^{-1}. Work on stabilizing the character of high and low nitrogenase activity in this population is under way to study the inheritance of this trait (ICRISAT 1984).

In sorghum, 28 out of 334 field-grown lines tested had high nitrogenase activity (>460 nmol C_2H_4 15 cm diam $core^{-1}$ h^{-1}) associated with their roots. The active lines came from India (12 out of 104 tested), West Africa (6 of 36), East and Central Africa (5 of 63), South Africa (6 of 29), USA (2 of 39), Thailand (1 of 2), and Japan (1 of 3). However, 167 lines stimulated nitrogenase activity of at least 100 nmol C_2H_4 $core^{-1}$ h^{-1}, which was more than twice the mean activity of soil cores without plant roots (range 0-40 nmol C_2H_4 $core^{-1}$ h^{-1}). Fifteen lines have been consistently active in three or more seasons, though they are not consistently active on each assay occasion during the season (ICRISAT 1978, Dart and Wani 1982).

Several lines of minor millets including *Eleusine coracana, Panicum* sp, *P. miliaceurn,* and *Setaria italica* have been screened for nitrogenase activity. The proportion of active lines in minor millets was more than that with pearl millet lines (ICRISAT 1979, 1980). Several tropical grasses belonging to the genera *Brachiaria, Cenchrus, Chloris, Cymbopogon, Dicanthium, Euchlaena, Panicum, Pennisetum, Setaria,* and *Sorghum* have been grown without addition of any nitrogen fertilizer over 7 years in an observation plot. Thirty-four out of 48 entries were very active in stimulating N_2-fixation, as observed from the data obtained from soil-core assays with these grasses. Some of the entries, e.g., *Pennisetum purpureum* and a cross between *P. purpureum* and *P. americanum* (Napier bajra), had remarkably high nitrogenase activity, reaching as much as 300 g N ha^{-1} d^{-1} (ICRISAT 1978).

Measurement of N_2-fixation

Use of ^{15}N techniques

$^{15}N_2$ Incorporation. In the $^{15}N_2$ incorporation technique a plant enclosed in a container is exposed to $^{15}N_2$, and after incubation, plant tissues are examined for above-normal concentrations of the heavy isotope. Sorghum CSH 5 seedlings grown in tubes in sand:FYM (97:3 w/w) mixture using an intact-tube assay system were exposed to $^{15}N_2$, by exchanging the gas in the root medium in the test tubes by water displacement (ICRISAT 1983). The oxygen content of the root zone was monitored and maintained at 20%. After exposing 20-day-old seedlings to labelled $^{15}N_2$ in this fashion for 3 days, ^{15}N was detected in the growth medium (0.005 ^{15}N atom % excess). Seven days after the labelled gas was removed, the ^{15}N atom % excess in the plants had increased considerably with 0.029 ^{15}N atom % excess in the roots and 0.019 ^{15}N atom % excess in shoots (ICRISAT 1983). Variation in ^{15}N due to differences in both natural abundance and analytical errors can account for at the most ± 0.010 atom % ^{15}N excess (Bergersen 1970).

An apparatus has been developed to introduce gas mixture into the root-growth chamber by purging with CO_2 followed by absorption of the CO_2 with soda lime (Giller et al. 1984) as suggested by Witty and Day (1978), thus avoiding flooding the root zone with water to transfer the gas used in the previous experiment. Using this device 24-day-old sorghum seedlings were exposed to $^{15}N_2$. The results clearly demonstrated that (1) detectable amounts of $^{15}N_2$ were fixed in the rhizosphere of sorghum seedlings and that (2) fixed ^{15}N was incorporated into the plant roots and shoots within 3 days of initial exposure to the gas (Table 2). After a further week of growth the incorporation of ^{15}N in the roots and in the shoots had almost doubled. The actual amount of $^{15}N_2$ fixed during the exposure period and the amount which was incorporated into the plant roots and shoots can be estimated by converting the results into µg N fixed, using the following formula:

$$\text{N fixed } (\mu g) = \text{Total plant N } (\mu g) \times \frac{^{15}\text{N atom \% excess in plant}}{^{15}\text{N atom \% excess in gas phase}}$$

^{15}N dilution. The isotope dilution principle has been used in pot experiments for differentiating lines of sorghum and millet for their potential to fix atmospheric nitrogen. Six lines each of sorghum and millet were grown in pots in a washed vermiculite:sand mixture (1:1 w/w) and watered with 10 ppm N solution with 10 ^{15}N atom % excess daily as required. The percentage of N_2 fixed by an individual line was calculated by comparing it to the line showing the highest ^{15}N atom % content as control (ICRISAT 1984, Giller et al. 1986). Sorghum line IS 801 derived 27%, and millet line D 180 17%, of total plant N from BNF, in comparison with low nitrogenase stimulating sorghum line IS 3003 and pearl millet ICH 107. However, in these experiments considerable dilution of added ^{15}N was observed in all the lines in comparison with the ^{15}N content of the solution added, suggesting that a source of ^{14}N other than fixation may be present in the system (seed or vermiculite). It was noted that ^{14}N released from vermiculite diluted

Table 2. ^{15}N incorporated into 21-day-old sorghum CSH 5 plants after exposure of root systems to $^{15}N_2$[1] for 72 h.

Plant age at harvest (days)	Dry mass (mg plant^{-1})	Total N (mg plant^{-1})	Atom% ^{15}N excess	Fixed N[2] incorporated (µg plant^{-1})
24				
Shoot	264 ± 7.2[3]	7.0 ± 0.28	0.056 ± 0.006	10 ± 1.2
Root	246 ± 17.4	4.2 ± 0.26	0.056 ± 0.003	6 ±0.5
Total	510 ± 19.1	11.2 ± 0.33		16 ± 1.5
33				
Shoot	400 ± 13.4	5.9 ± 0.54	0.102 ± 0.022	16 ±4.7
Root	673 ± 56.5	5.8 ± 0.52	0.073 ± 0.016	11 ±3.2
Total	1073 ± 62.7	11.7 ± 0.96		27 ± 7.9

1. Mean enrichment 40.3 atom % ^{15}N excess.
2. Calculated as total N x atom % ^{15}N excess in the plant divided by atom % ^{15}N excess in gas.
3. Values are means of five and four replicates for the 24- and 33-day harvests, respectively, ± S.E.

^{15}N added. Therefore, the comparison was made to the line with the highest ^{15}N content. In such experiments extra care has to be taken to prevent the systems getting contaminated with ^{14}N from other sources such as water or the growth medium. An alternative approach is to use uniformly ^{15}N labelled soil in pots for growing lines to screen for their potential to fix atmospheric nitrogen (ICRISAT 1983).

Nitrogen-balance Studies

Another aspect of our work deals with measurement of N_2 fixation associated with sorghum, millet, and related species, and transferred to the crop. We are approaching this by experiments using ^{15}N enrichment, by nitrogen-balance experiments in pot culture, and by the much more difficult, long-term field experiments.

Pot Experiments

We have concentrated so far on establishing nitrogen balances associated with sorghum, millet, and Napier bajra (*Pennisetum purpureum x P. americanum*) grown in pot culture in vermiculite media with and without added N fertilizer, and in soil low in nitrogen. These experiments have established that there is a small but measurable N_2 fixation by bacteria associated with the roots of these plants, which is taken up by the plant during the growing season, and some of which also remains in the rooting medium.

In one experiment sorghum was grown to maturity without fertilizer N in washed vermiculite. The plants were ratooned twice and grain harvests taken, with total dry-matter production of 195 g pot^{-1} of 10 plants originally sown. In another experiment with sorghum grown in vermiculite for 49 days, the N balance showed a considerable increase in N in planted but not in unplanted pots (Dart and Wani 1982, ICRISAT 1980). The positive N balance across all inoculation treatments was 269 mg pot^{-1} of five plants when no fertilizer N was applied, and 124 mg pot^{-1} when 53 mg N pot^{-1} was applied. A substantial proportion of this N was gained by the root medium (33% of the total N balance for zero-N treatment), although this may result from fine roots missed from the root sample.

In another pot experiment with sorghum CSH 5 grown in an unsterilized Alfisol with three different levels of added nitrogen, a considerable balance for N due to inoculation over the control was observed (Table 3). A maximum mean balance across the nitrogen levels for N over unplanted treatment of 331 mg pot^{-1} was observed in case of inoculation with *A. lipoferum* (ICM 1001). Addition of 20 kg N ha^{-1} equivalent resulted in higher balance for N across the inoculation treatments compared to no N addition and addition of 40 kg N ha^{-1} equivalent (ICRISAT 1984).

Pearl millet grown in vermiculite in pots also attained a positive N balance of 109 mg pot^{-1} containing five plants without added N fertilizer. There was a positive N balance of 96 mg por^{-1} with nitrogen added equivalent to 20 kg N ha^{-1} (Dart and Wani 1982). Similarly, in a pot trial with millet BJ 104 grown in an unsterilized Alfisol there was a significantly higher positive nitrogen balance (over unplanted treatments) due to inoculation with N_2-fixing bacteria over the noninoculated control. A

Table 3. Nitrogen balance with sorghum CSH 5 inoculated with nitrogen-fixing bacteria.[1]

Culture	Grain mass (g pot^{1})	Total dry matter (g pot^{-1})	Nitrogen in total dry matter (mg pot^{-1})	Net nitrogen balance (mg pot^{-1})
Azospirillum lipoferum	13.9	79.9	307	331
Azotobacter chroococcum	12.1	75.4	259	226
Napier bajra root extract (NBRE)	14.9	79.9	270	150
Control (noninoculated)	11.6	66.9	223	113
SE±	0.64	2.52	8.7	56.5
c v %	16.8	11.6	11.8	95

1. Plants were grown in the greenhouse in pots containing nonsterilized Alfisol. Along with inoculation with bacteria, three nitrogen levels (0, 20, and 40 kg N ha^{-1}) were used and each treatment was replicated four times.

positive nitrogen balance of 322 mg pot^{-1} over an unplanted control was recorded in a treatment inoculated with *Azospirillum lipoferum* across the nitrogen levels. A maximum N balance of 240 mg pot^{-1} was observed across the inoculation treatments that received 20 kg N ha^{-1} equivalent. Addition of 40 kg N ha^{-1} reduced the balance significantly (ICRISAT 1984). These findings indicated that addition of nitrogen at lower rates enhanced N_2 fixation that resulted in higher positive balance, and higher rates of N application inhibited N_2 fixation.

Cuttings of the Napier bajra hybrid NB 21 were grown in vermiculite and an Alfisol. The cuttings grew without added N fertilizer to about 150 cm in 72 days before being ratooned. At final harvest at 194 days, the extra N accumulated in the soil amounted to 216 mg N pot^{-1} for a single plant without added N with total positive balance of 539 mg N, and 368 mg N $plant^{-1}$ with 20 kg ha^{-1} added N with the total positive balance of 657 mg N. For the vermiculite rooting medium, N accumulation with zero N treatment was 167 mg N in the medium and 361 mg N positive overall balance (Dart and Wani 1982).

Field Experiments

A long-term field experiment was started in 1978 in collaboration with the Soil Chemistry and Fertility subprogram to measure the N balance in sorghum production in an Alfisol under rainfed conditions. The same eight cultivars with either high nitrogenase activity or high N uptake under low fertility, are grown each year on the same plots. Fertilizer N is added at the rate of 0, 20, and 40 kg ha^{-1}. Mean initial N content of the top 0-15 cm of the soil without fertilizer N was 0.040% N, 0.056% in the 15-30 cm zone, and 0.053% at 30-90 cm. All aboveground plant material is removed at harvest. There were significant differences between the cultivars in grain yield and N uptake from the second season onwards. During the 6th year of this experiment (rainy season 1983), cultivars also varied significantly across the N levels in total dry-matter production. During the 7th year of the experiment (rainy season 1984), a uniform crop of pearl millet was grown. The total dry-matter yield of pearl millet on plots where cultivars CSH 5 and IS 2333 were grown previously was at par across the nitrogen levels.

The cumulative nitrogen uptake through aboveground plant parts from 1978 to 1983 (except 1981) indicated that highest nitrogen uptake amongst the sorghum cultivars across the applied nitrogen levels was in the case of CSH 5 (230 kg ha^{-1}) and the lowest was in the case of IS 2333 (180 kg ha^{-1}) (ICRISAT 1985). These results suggest that sorghum cultivars do vary for their N_2-fixing ability. However, we will have a clearer picture of this when soil-N changes over the first years of the experiment are measured. Samples are currently being processed. Leaching losses and inputs of N through rainfall are likely to be small.

In another long-term N-balance trial at ICRISAT Center, several tropical grasses are grown for 7 years without adding any fertilizer N. The crop receives 40 kg P_2O_5 ha^{-1} a^{-1} and is irrigated during the dry season. The maximum dry-matter production has been obtained with the Napier bajra hybrid NB 21 where an equivalent of 370 t ha^{-1} dry matter, containing an equivalent of 2752 kg N have been harvested in 7 years (Table 4). A preliminary soil sampling up to a depth of 90 cm after 5.5 years showed no difference in N content of the soil from the plots where NB 21 was grown and where the low dry-matter-producing entry was grown. These results suggest that entries like NB 21 are deriving some of their requirement of N through BNF in addition to the soil-N pool. Thorough soil sampling in these plots will allow computation of the exact amounts of N fixed by these entries over the years.

Contribution to N Balance by Blue-green Algae

Crusts of blue-green algae develop on the soil surface of many cropped fields during the rainy season and after irrigation (Dart and Wani 1982, ICRISAT 1983). The predominant heterocystous algae, seen forming mats on fields at ICRISAT Center, were two *Anabaena* species and *Nostocmuscorum.* Other *Nostoc* species as well as algae belonging to the genera *Calothrix, Aphanothece, Microsystis, Lyngbya,* and *Oscillatoria* were also observed (ICRISAT 1983). On Alfisols, the growth and N_2-fixing activity of those algae under sorghum and millet was generally low, but under tropical grasses such as *Pennisetum purpureum,* the mats may be very active, depending on the wetness of the soil surface, and the extent of the plant canopy. Activity decreases rapidly as the soil surface dries out, virtually ceasing 3 days after wetting of the soil surface if the radiation levels are high. Nitrogenase activity of these algal

Table 4. Dry-matter production and nitrogen uptake by forage grass spp in long-term nitrogen-balance trial.[1]

Grass	Age (years)	Dry matter cumulative (t ha^{-1})	Production per day (kg ha^{-1})	Nitrogen cumulative (kg ha^{-1})	Uptake per day (kg ha^{-1})
Pennisetum americanum x P. purpureum NB 21	6.94	370.7	146.3	2752	1.09
P. purpureum JVM-2	6.94	211.0	83.3	1760	0.69
P. squamulatum	6.94	165.5	65.3	1189	0.47
Cenchrus ciliaris	6.94	164.3	64.9	1235	0.49
Panicum maximum	6.00	152.0	69.0	1080	0.49
Setaria anceps	6.94	119.0	47.0	836	0.33
Pennisetum mezinium	6.94	98.9	39.0	617	0.24
Chloris gayana	6.31	96.3	41.8	706.6	0.31
Panicum antidotale	6.5	43.0	18.1	318	0.13
Pennisetum rupellii	6.5	41.7	17.6	312	0.13

1. Estimated from 6 m^2 net harvest area, harvested 19 times, crop is irrigated during the dry season and 40 kg P_2O_5 ha^{-1} is added every year.

mats when extrapolated to a surface-area basis ranged from 24 to 119 mg N fixed m^{-2} d^{-1}, compared with only 0.5 to 1.6 mg N m^{-2} d^{-1} for surface soil without visible algal growth. The algal mat covered up to 29% of the soil surface (Dart and Wani 1982), at which level this extrapolates to an upper estimate of fixation of 28 kg N ha^{-1} a^{-1}, assuming 80 days at the above level of activity for an irrigated perennial grass crop.

Factors Affecting Associative N_2 Fixation

Nitrogen fixation associated with sorghum and millet is affected by plant genotype, root exudates, seasonal and diurnal variation, soil type, soil moisture, temperature, levels of combined nitrogen, and organic carbon in the soil. Some of these factors have been discussed under various assay techniques and only those not discussed earlier are described below.

Root Exudates

Qualitative differences in the soluble exudates of seedlings of different sorghum genotypes were demonstrated by variation in growth and nitrogenase activity of a given bacterial culture in semisolid synthetic media containing root exudates as the sole carbon source. Only the azospirilla cultures grew well; the other organisms tested grew poorly, with little nitrogenase activity. No correlation was observed between the amount of exudate and root and/or shoot growth of seedlings grown in axenic liquid culture, and the ranking of the cultivars inoculated with *A. lipoferum* for nitrogenase activity did not correlate with the amounts of soluble exudate measured. Similarly, this ranking also differed from that for growth and activity of *A. lipoferum* in synthetic media (ICRISAT 1984).

Combined Nitrogen

The presence of combined N affects the enzyme nitrogenase. With sorghum plants grown in tubes filled with washed sand, nitrogenase activity was drastically reduced when the plants were fed with above 15 ppm N in solution (ICRISAT 1984). Nitrogenase activity of sorghum plants in the field was stimulated due to addition of 20 kg N ha^{-1} over the no-N addition treatment, but 40 kg N ha^{-1} reduced the activity. With millet BJ 104 plants grown in pots filled with Alfisol, mean nitrogenase activity throughout the growth period was significantly higher when the plants were watered with 10 and 25 ppm N in solution daily, than with the plants which received no nitrogen and also at 100 ppm N solution daily. Application of a basal dose of 20, 40, and 80 kg N ha^{-1} equivalent did not affect the activity significantly.

Nitrogen-fixing Bacteria

The marked differences in nitrogenase activity of sorghum and millet between fields may be partly due

to differences in their microbial populations, suggesting that it may be possible to obtain responses to inoculation with bacteria. We compared different methods of estimating bacterial populations and isolating N_2-fixing bacteria from root or soil samples. The N-free media that contained sucrose and malate as the carbon source were used to compare three methods of isolating and quantifying the bacteria. Counts of presumptive nitrogen fixers (4.5×10^8 in sucrose and 2.5×10^8 in malate media g^{-1} of root) were higher under the dilution plate count method than counts estimated by most probable number (MPN) method in semisolid media (5×10^5 in sucrose and 5×10^4 g^{-1} root in malate medium), and higher than axenic plant tubes (5×10^5 g^{-1} root). Recovery of nitrogenase-positive bacterial isolates was 43% higher with sucrose and 30% higher with malate by the MPN method. Recovery of nitrogenase-positive bacterial isolates from MPN with axenic plants was 37% using malate medium (Table 5; ICRISAT 1984).

In the plant enrichment culture technique for isolating N_2 fixers, sterile seedlings of sorghum or millet are grown on a carbon and N-free medium in test tubes, and inoculated with a dilution series of the soil or culture under test. Most probable number estimates of N_2-fixing bacteria are made from the ARA of these tubes, and from direct plating of the highest positive dilutions. A second or third selection from this population can be made by again going through the process of dilution and reinoculation of sterile seedlings. With this technique, with the increasing enrichment during each generation, the number of colony types decreased and the proportion of nitrogenase-positive bacteria increased.

Large populations of bacteria capable of growing in air on N-free media exist in soil. Use of the acetylene-reduction assay indicated nitrogenase activity for about 60% of these presumptive N_2 fixers. The number of colony types and the population sizes depended on the selection of media and varied with the carbon source. About four times as many bacteria grew on a sucrose-based medium than on a malate-based medium. Adding a small amount of yeast extract (100 mg L^{-1}) doubled their number on both the media. The number in the top 40 cm of soil was about 10 times greater than that in the 40-to-60 cm zone. There were over a million Enterobacteriaceae g^{-1} soil, several species of which are known to fix nitrogen anaerobically.

Stimulation of presumptive, aerobic N_2 fixers occurs in the rhizosphere of several plants. The number of easily recognizable types of bacteria from the root surface that grew on N-free media was 10 times greater than the population growing in the soil away from the roots. A selection for particular types of bacteria also occurred in the root zone, resulting in less than half the types found in the bulk soil. Some bacteria were found very closely bound to the root and perhaps even in the root tissues. After shaking the root with glass beads to remove surface-attached bacteria and then thoroughly sterilizing the root surface with 1% chloromine T for 1 h, we recovered more than 400 000 bacteria g^{-1} of fresh root from the root macerate (Dart and Wani 1982).

A survey of 200 sites in the traditional pearl millet-growing areas in northwestern India indicated that total population of organisms capable of growing on a N-free sucrose medium supplemented with 50 mg L^{-1} yeast extract (YE), ranged from 10^7 to 10^8 g^{-1} soil (Dart and Wani 1982). Nitrogenase activity was detected in 42% of the 3760 isolates made from the highest-dilution plates. Every soil contained organisms which produced pellicles and reduced C_2H_2 on a malate medium, with a MPN of N_2 fixers varying from 10^3 to 10^5 g^{-1} soil. Some of these soils did not contain *Azospirillum*, and Enterobacteriaceae and *Pseudomonas* were commonly isolated from the malate enrichment cultures. The isolates from the sucrose-based medium could be classified into at

Table 5. Estimation and isolation of nitrogen-fixing bacteria from root samples by different methods.[1]

Method	Total counts	Number of isolates	
		Total	Active
Dilution and plating			
Sucrose medium	4.5×10^8	10	0
Malate medium	2.5×10^8	10	0
MPN in semisolid medium			
Sucrose	5×10^5	7	3
Malate	5×10^4	10	3
MPN with plants	5×10^5		
Isolations on			
Sucrose medium		4	0
		5	0
Malate medium		8	3
		6	1

1. Roots of active Ex-Bornu plants were macerated and serial dilutions were used for plating or inoculating semisolid media in bottles containing 6 mL medium or Ex-Bornu plants grown on Fahraeu's medium. The plants were grown in 6 mL Fahraeu's medium in 25 x 200 mm plant tubes.

least seven different genera of N_2-fixing bacteria, including types which are still to be identified. *Enterobacter cloacae* was the most-common isolate. Some *Pseudomonas* types were also nitrogenase positive. There were at least 10^6 actimonycete-like organisms g^{-1} soil in every sample and of the 229 isolates, 70 had nitrogenase activity on sucrose + YE medium but on subsequent purification they lost the activity. The isolates obtained from this study could be classified on colony morphology into at least 22 different groups. However, they represent only the most-numerous organisms able to grow on two media (Dart and Wani 1982, ICRISAT 1982).

Some of these cultures lose activity during purification and subculturing. In laboratory studies a synergistic effect on nitrogenase activity was found when two low-fixing pure cultures were mixed together. Cultures of *Erwinia herbicola* and *Enterobacter cloacae* each grew on N-free media, but were inactive when assayed for C_2H_2 reduction activity. When both cultures were grown together, a high activity was measured by C_2H_2 reduction (101 nmoles C_2H_4 bijou bottle^{-1} h^{-1}) (ICRISAT 1982). Similarly, *Azotobacter chroococcum* in pure culture alone had nitrogenase activity of 50 nmoles C_2H_4 bijou^{-1} h^{-1}, but when it was grown with *Erwinia herbicola* the activity increased to 121 nmoles C_2H_4 bijou^{-1} h^{-1} (ICRISAT 1982).

Responses to Inoculation

In pot-culture studies, the response of sorghum and millet to inoculation with N_2-fixing bacteria varied with the growth medium and amount of N fertilizer added. The responses of sorghum grown in pots in nonsterile Alfisol, to inoculation with *Azospirillum lipoferum* and NBRE, a mixed culture, were a grain yield increase of 22% and total dry matter increase of 29%. The yield increase occurred even when the equivalent of 40 kg N ha^{-1} was added (ICRISAT 1982). Nitrogen-balance studies indicated a positive N balance over the control due to inoculations (Table 3). In a field trial on an Alfisol with the three sorghum hybrids CSH 1, CSH 5, and CSH 9 inoculated with liquid peat culture suspension of N_2-fixing bacteria, we observed increased dry-matter production ($P < 0.1$) of all three hybrids inoculated with *A. lipoferum* (ICM 1001) and a NBRE culture (ICRISAT 1983).

Another field trial with three sorghum cultivars CSH 5, CSH 9, and SPV 351 and 10 inoculation treatments was conducted during the 1984 rainy season. Inoculation of sorghum cultivars with nitrogen-fixing bacteria resulted in 2-10% increase in grain yields across the cultivars over the noninoculated control. However the increases were not statistically significant. A similar trend was observed for total dry-matter production (ICRISAT 1985).

During 1982-1984, nine field trials were conducted at ICRISAT Center and different locations in India to study the response of millet cultivars to inoculation with nitrogen-fixing bacteria. In all the trials inoculation increased the grain and total dry-matter yields of millet. However significant yield increases due to inoculation were observed in five trials (Table 6). The summary table indicates that up to 30% increase in mean yield over the control was observed in the test cultivars due to inoculation with N_2-fixing bacteria.

In all the experiments, plant dry-matter yields also followed trends similar to that of grain yield. Inoculations increased plant N uptake in all the experiments except where *A. brasilense* (SP 7) was used. In a few cases grain N content was increased due to inoculations. In all the trials, trends for host cultivar and bacterial strain interactions for grain and plant dry-matter yields were observed; however, significant interaction for grain and plant dry-matter yields was observed in one trial each only.

Acknowledgements

Dr P.J. Dart initiated the Cereal Microbiology program at ICRISAT and Drs R.V. Subba Rao, D.B. Godse, and J.A. Kipe-Nolt initiated some of the studies discussed in this paper. The results for N balances from experiments are part of a collaboration with Drs K.L. Saharawat, T.J. Rego, and F.R Bidinger. Excellent technical assistance was received from S. Chandrapalaiah, U.K. Avalakki, M.N. Upadhyaya, and K. Prakash Hebbar.

References

Bergersen, F.J. 1970. The quantitative relationship between nitrogen fixation and the acetylene-reduction assay. Australian Journal of Biological Sciences 23:1015-1025.

Dart, P.J., and Wani, S.P. 1982. Non-symbiotic nitrogen fixation and soil fertility. Pages 3-27 *in* Non-symbiotic nitrogen fixation and organic matter in the tropics. Sympo-

Table 6. A summary of pearl millet inoculation experiments conducted at different locations during 1982-84.

Location and season	Soil type	Cultivars	Inoculation treatment	Percentage increase[1]	Remarks
ICRISAT Center Summer 1982	Alfisol	2. IP 2787 ICMS 7819	4. *A.lipoferum* (1) *A.brasilense* (SP 7) NBRE	6.05 -6.5	For plant dry matter significant interaction (P=0.05) between cultivar and culture was noted.
ICRISAT Center Rainy 1982	Alfisol	3. IP 2787 ICMS 7703 WC C 75	6. *A.lipoferum* (1) *A.lipoferum* (ICM 1001) NBRE *A.chroococcum* (ICM 2001) *A.brasilense* (SP 7)	14.5 21.0[2] 6.0 14.0 -11.8	For grain Nb content, significant interaction between cultivars and cultures was observed.
ICRISAT Center Summer 1983	Alfisol	3. BJ 104 MBH 110 MEBH 23/81	5. *A.lipoferum* (1) *A.lipoferum* (ICM 1001) *A.chroococcum* (ICM 2001) NBRE	5.0 6.0 6.0 12.4	
ICRISAT Center Rainy 1983	Alfisol	3. BJ 104 MBH 110 MEBH 23/81	5. *A.lipoferum* (1) *AMpoferum* (ICM 1001) *A.chroococcum* (ICM 2001) NBRE	- 5.5 -	
ICRISAT Center Rainy 1984	Alfisol	4. BJ 104 BK 560 WC C 75 ICMS 7703	7. *A.lipoferum* (ICM 1001) *A.brasilense* (1) *A.brasilense* (2) Abl + Ab2 *A.chroococcum* (ICM 2001) NBRE	10.7[2] 0.7 7.6 4.8 11.6[2]	Significant interaction for grain yield between cultivars and inoculants was noted.
Vaijapur[3] Rainy 1984	Mixed Red and black	4. BJ 104 BK 560 WC C 75 ICMS 7703	5. *A.lipoferum* (ICM 1001) *A.brasilense* (2) *A.chroococcum* (ICM 2001) NBRE	25.6[2] 6.1 6.1 17.4	
Bhavanisagar Rainy 1984	Alfisol	4. BJ 104 BK 560 WC C 75 ICMS 7703	5. *A.lipoferum* (ICM 1001) *A.brasilense* (SL 33) *A.chroococcum* (ICM 2001) NBRE	18.7[2] 16.5[2] 6,1 .	
Parbhani[4] Rainy 1984	Vertisol	4. BJ 104 MP 21 WC C 75 ICMS 7703	7. *A.lipoferum* (ICM 1001) *A.brasilense* (1) *A.brasilense* (2) Ab1 + Ab2 *A.chroococcum* NBRE	24.5[2] 19.3[2] 29.5[2] 10.5 30.6[2] 27.8[2]	
Rahuri[5] Rainy 1984	Vertisol	4. BK 560 RHR 1 WC C 75 ICMS 7703	5. *A.lipoferum* (ICM 1001) *A.brasilense* (SL 33) *A.chroococcum* (ICM 2001) NBRE	9.4 7.1 13.9 6.7	

1. Increase in mean grain yield across the cultivars.
2. Significantly (P = 0.05) higher over the control.
3. In cooperation with G.S. Jadhav, Marathwada Agricultural University, Parbhani.
4. In cooperation with N.S. Subba Rao and K.V.B.R. Tilak, IARI, New Delhi.
5. In cooperation with S.D. Ugale, Mahatma Phule Agricultural University, Rahuri.

sia papers 1. Transactions of the 12th International Congress of Soil Science, 8-16 Feb 1982, New Delhi, India. New Delhi, India: Indian Agricultural Research Institute.

Giller, K.E., Day, J.M., Dart, P.J., and Wani, S.P. 1984. A method for measuring the transfer of fixed nitrogen from free-living bacteria to higher plants using $^{15}N_2$. Journal of Microbiological Methods 2(6):307-316.

Giller, K.E., Wani, S.P., and Day, J.M. 1986. Use of isotope dilution to measure nitrogen fixation associated with the roots of sorghum and millet genotypes. Plant and Soil 90:255-263.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1978. Annual report 1977-78. Patancheru, A.P. 502 324, India: ICRISAT.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1979. Annual report 1978. Patancheru, A.P. 502 324, India: ICRISAT.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1980. Annual report 1979. Patancheru, A.P. 502324, India: ICRISAT.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1981. Annual report 1980. Patancheru, A.P. 502 324, India: ICRISAT.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1982. Annual report 1981. Patancheru, A.P. 502 324, India: ICRISAT.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1983. Annual report 1982. Patancheru, A.P. 502324, India: ICRISAT.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1984. Annual report 1983. Patancheru, A.P. 502324, India: ICRISAT.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1985. Annual report 1984. Patancheru, A.P. 502 324, India: ICRISAT.

Rao, R.V.S., and Dart, P.J. 1981. Nitrogen fixation associated with sorghum and millet. Pages 169-177 *in* Associative N_2-fixation: proceedings of the International Workshop on Associative N_2-fixation, 2-6 July 1979, Piracicaba, Brazil (Vose, P.B., and Ruschel, A.P., eds.). Vol. 1. Boca Raton, Florida, USA: CRC Press.

Wani, S.P. (In press.) Nitrogen fixation potentialities of sorghum and millet. *In* New trends in biological nitrogen fixation (Subba Rao, N.S., ed.). New Delhi, India: Oxford and 1BH Publishing Co.

Wani, S.P., Dart, P.J., and Upadhyaya, M.N. 1983. Factors affecting nitrogenase activity (C_2H_2 reduction) associated with sorghum and millet estimated using the soil core assay. Canadian Journal of Microbiology 29:1063-1069.

Want, S.P., Upadhyaya, M.N., and Dart, P.J. 1984. An intact plant assay for estimating nitrogenase activity (C_2H_2 reduction) of sorghum and millet plants grown in pots. Plant and Soil 82:15-29.

Witty, J.F., and Day, J.M. 1978. Use of $^{15}N_2$ in evaluating asymbiotic N_2 fixation. Pages 135-150 *in* Isotopes in biological dinitrogen fixation: proceedings of an Advisory Group Meeting organized by the Joint FAO/IAEA Division of Atomic Energy in Food and Agriculture, 21-25 Nov 1977, Vienna, Austria. Vienna, Austria: International Atomic Energy Agency.

Discussion

P.Tauro:
Considering that millets are grown with minimum agronomic practices, what inoculation technique would you recommend?

S.P.Wani:
Seed inoculation is the best, but if we find that slurry inoculation is superior we will have to opt for it. At present we are comparing various methods of inoculation.

P.Tauro:
Is anything known about the N-scavenging ability of different grasses?

S.P.Wani:
No. But with thorough soil sampling we should be in a position to find out the status of N source in the soil.

P.Tauro:
Could N recovery up to 2000 kg/5 yr be more due to scavenging than due to BNF?

S.P.Wani:
No. As the initial soil sampling and sampling after 5 years have not shown N differences to this extent, we can infer that soil N was not the source. Removal of 2000 kg N from the soil will be easily detected by Kjeldahl analysis.

P.Tauro:
Have you noticed any negative response to inoculation?

S.P.Wani:
Yes, in the case of inoculation with *Azospirillum brasilense.*

B.K. Konde:
What added advantages did you observe from slurry inoculum over seed inoculation?

S.P.Wani:
By using slurry inoculation (peat culture suspended in water), more bacterial inoculum can be added in the field as compared to seed inoculation. Another advantage is that in drought-prone areas, this additional moisture helps in the establishment of the plants in the early stage. Such an effect has been observed at Vaijapur where there were no rains at all after sowing, and on the entire station only the inoculation trial where liquid suspension was applied has come up well, whereas all other trials sown in the normal way resulted in patchy plant establishment.

B.K.Konde:
When do you inoculate the plants?

S.P.Wani:
Generally at the time of sowing if the trial is hand sown, otherwise soon after emergence, i.e., 5-6 days after sowing, if the trial is tractor sown.

G. Oblisami:
What is the cost of liquid inoculation of *Azospiril lum* to sorghum when compared to seed inoculation?

S.P.Wani:
We have not worked out the exact costs of slurry inoculation, but if sowing is done by a bullock-drawn implement, the cost will be about Rs. 30-40. A simple, inexpensive device has been developed by our Farm Power and Equipment scientists for this purpose in groundnut, and it can be used for other crops also.

O.P. Rupela:
In continuation of Dr. Tauro's questions, was the negative effect statistically significant?

S.P.Wani:
The negative effects with *A. brasilense* (SP 7) inoculation were consistent in two trials where 4 cultivars were tried, and in one trial the reduction was statistically significant.

G.S. Murthy:
When there is no increase in the grain yield but there is an increase in total biomass yield, perhaps other nutrients such as phosphorus and zinc are becoming limiting factors.

S.P.Wani:
I don't think so, as generally basal dressings with P_2O_5 are done in all the trials.

Joseph Thomas:
I am impressed by the painstaking experiments with improved field techniques. The quantity of nitrogen recovered by Napier grass works out to 465 kg ha^{-1}. Do you have any ^{15}N data to substantiate this?

S.P.Wani:
No.

S.V. Hegde:
Did you have an unplanted soil system for N-balance study? What was the N gain due to planting per se?

S.P.Wani:
Yes, we do maintain an unplanted control in all N-balance trials. The N gain due to planting alone was 113 mg N pot^{-1} sown with sorghum, and 27 mg N pot^{-1} sown with millet. These gains are across the 3 N levels used.

S.V.Hegde:
Is it appropriate scientifically to use Napier bajra root extract (NBRE), which is a mixture of several microorganisms and which could also change every time the culture is obtained from bajra root? Instead can we not isolate the organism or organisms responsible in pure culture and use them?

S.P.Wani:
I agree with you that NBRE is a complex culture and there will be problems in producing and maintaining the quality of such a complex inoculum. I don't think NBRE can be recommended for general use. We started using NBRE as a test mixture and as it gave positive responses we continued with it. It cannot be used unless known bacteria from NBRE mixed together perform equally well. We are studying the composition of NBRE in terms of microbial components and there are at least 14-15 different bacteria involved which should be equally good in performance. This will take more time and I am not sure whether in the near future it will be possible or not. Coming to the question of maintaining the same composition, we always use the starting material for preparing NBRE from our long-term N-balance trial and till now we have observed positive responses with NBRE prepared at different times.

Yield and Nitrogen Gains of Sorghum as Influenced by *Azospirillum brasilense*

N.S. Subba Rao, K.V.B.R. Tilak, C.S. Singh, and P.V. Nair[1]

Summary

Initial pot experiments on seed inoculation of sorghum (Sorghum bicolor) *with* Azospirilium brasilense *demonstrated increased dry-matter production, with the cultivar CSH 5 responding better than CSH 6. In field experiments conducted at nine centers in India for four successive years, the mean increase in grain yield due to inoculation over the noninoculated control (no nitrogen) was equivalent to that obtainable by the application of 15-20 kg N* ha^{-1}. *This trend was also noticed in treatments with increasing levels of N application.*

In afield experiment, inoculation of sorghum resulted in increased N uptake, which was more pronounced at low levels of applied nitrogen. In general, the increased N uptake by plants due to seed inoculation with A. brasilense *varied from 14.6 to 21.7 kg N* ha^{-1}.

Growth and P uptake of sorghum on a nonsterile, phosphorus-deficient soil were improved by soil inoculation with the vesicular-arbuscular mycorrhizal (VAM) fungi (Acaulospora, Giga-spora margarita, *and* Glomus fasciculatum), *in combination with seed inoculation with* Azospi-rilium brasilense. *The responses were significant with* A. brasilense + G. margarita *and A.* brasilense + G. fasciculatum.

Introduction

The present study was conducted to (1) evaluate the response of sorghum to *Azospirilium brasilense* inoculation, alone and in combination with graded levels of fertilizer nitrogen in pots and in field conditions and (2) to find out the combined effects of seed inoculation with *A. brasilense* and soil inoculation with VAM fungi on the growth and P uptake of sorghum.

Materials and Methods

Azospirilium was isolated from the roots of 18 cultivated sorghum varieties grown in breeders' plots by following the methods described by von Bulow and Dobereiner (1975) and Subba Rao et al. (1979a). The strains were identified as *A. brasilense* according to Tarrand et al. (1978). The nitrogen-fixing ability of different strains was estimated by the micro-Kjeldahl method (Jackson 1967).

Seeds of sorghum hybrids CSH 5 and CSH 6 were inoculated with a carrier-based (soil + FYM 1:1) culture of *A. brasilense* containing efficient strains, following the method outlined by Subba Rao et al (1979a,b). Noninoculated seeds were coated with sterilized (heat-killed) carrier-based culture.

Two pot culture experiments were conducted using 12 kg nonsterilized alluvial soil of pH 7.3.

In the first experiment, an interaction of two sorghum cultivars (CSH 5 and CSH 6) with three strains of *A. brasilense* (S_3 S_{12}, and S_{18}) singly and in combination was studied. A basal dose of 20 kg N

1. Project Director, Agro-Energy Center, Senior Microbiologist, Scientist, and PhD Student, Division of Microbiology, Indian Agricultural Research Institute, New Delhi 110 012, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

ha^{-1} was applied to all the treatments. Phosphorus (P_2O_5) and potassium (K_2O) was applied at the rate of 40 kg ha^{-1}. In the second experiment, the effect of inoculation on CSH 6 was studied with five levels of fertilizer N (0, 20, 40, 60, and 80 kg ha^{-1}) applied at seeding. In the third experiment, the combined effects of seed inoculation with *A. brasilense* and soil inoculation with different V A M fungi (*Acaulospora* sp, *Gigaspora margarita,* and *Glomus fasciculatum*) were studied on sorghum cultivar CSH 5. Nitrogen was applied at the rate of 20 kg ha^{-1}, and potash (K_2O) at 60 kg ha^{-1}, at the time of seeding. In all pot experiments, dry matter produced by 75-day-old plants (preflowering stage) was determined. In all experiments, N was applied as urea, P_2O_5 as single superphosphate (SSP), and K_2O as potassium chloride.

Field experiments were conducted at different locations in India on a simple randomized-block design. The effect of seed inoculation of sorghum cultivar CSH 5 with a carrier-based (soil: FYM-1:1) culture of *A. brasilense* was tested at all locations. The inoculation effects were studied in the presence and absence of graded levels of N at the time of sowing. Phosphorus (P_2O_5) and potassium (K_2O) were applied as single superphosphate and potassium chloride at the rate of 40 kg ha^{-1} each at the time of sowing. Grain and straw yields at the time of crop maturity (120 days after sowing) were determined. At the Delhi center also, a field experiment was laid out on a sandy-loam soil of pH 7.3. The plot size was 10 m^2 (4x2.5m) and the treatments were replicated four times.

Enumeration of the *Azospirillum* was done 20 days after sowing in the rhizosphere-soil samples and roots separately, following the most-probable number (MPN) method as per the tables reproduced by Alexander (1965).

The nitrogen content in root, shoot, and grain samples was determined by the modified Kjeldahl method (Jackson 1967) and expressed as a percentage on oven-dry basis.

Results

A total of 18 strains (S_1-S_{18}) were isolated from the roots of different sorghum cultivars using semisolid malate medium. The efficiency of N_2 fixation among the isolates ranged from 8 to 17 mg N g^{-1} calcium malate. Maximum N_2 fixation rate of 16.8 mg N g^{-1} calcium malate was estimated in three cultures S_3, S_{12}, and S_{18} grown for 72 h.

The maximum mean yield increase was recorded by the culture S_{12}, closely followed by S_3+S_{12}, even though there was no significant difference between these two treatments. Similarly, no significant difference could be noted among the treatments S_3, S_{18}, S_3+S_{18}, S_{12}+S_{18}, and S_3+S_{12}+S_{18}, indicating that there was no added effect on the growth of sorghum plants by using a mixed inoculum containing more than one isolate *of Azospirillum.* The response of cultivar CSH 5 to *Azospirillum* inoculation was better than that of CSH 6. This type of genotypic differences have already been reported with lines of maize (von Bulow and Dobereiner 1975) and *Digitaria decumbens* and *Panicum maximum* (Smith et al. 1978).

The response of sorghum to N was linear up to 80 kg N ha^{-1} under no-inoculation treatments while with inoculation, yield increases were noticed only up to the 60 kg N ha^{-1} level (Table 1). No differences could be noticed between the inoculated and non-inoculated treatments under the highest level of N (80 kg ha^{-1}), suggesting the possibility of an interference with functioning of nitrogenase in the presence of combined N (Day et al. 1975, Weier 1980). The data in Table 1 indicate that the increased dry-matter yield obtained by the application of 20 kg N ha^{-1} could also be achieved by inexpensive inoculation.

Inoculations with azospirilla brought about an 8-fold increase in its rhizospheric population (Table 2). Similarly plant roots of inoculated plants contain

Table 1. Dry matter (g pot^{-1}) of sorghum as affected by inoculation with *Azospirillum* strains in combination with fertilizer nitrogen.[1]

	Nitrogen (kg ha^{-1})					
Treatment	0	20	40	60	80	Mean
Without inoculation	135	147	158	166	173	156
With inoculation	146	152	172	178	170	164[2]
Mean levels of nitrogen	140.5	149.5	165.0	172.0	171.5	
CD (P<0.05)		12.60				5.68
CV(%)		7.3				

1. Average of four replication.
2. Significantly (P <0.05) different from corresponding noninoculated controls.

Table 2. Numbers of *Azospirillum* associated with the rhizosphere and roots of sorghum as affected by inoculation with *Azospirillum* strain S_{12}.

Treatment	MPN g^{-1} soil
Rhizosphere	
N_0 1_0[1]	2.8×10^3
N_0 1_1[2]	2.2×10^4
Root	
N_0 1_0 (nonsterilized)	2.1×10^4
N_0 1_0 (sterilized)	1.8×10^2
N_0 1_1 (nonsterilized)	3.5×10^5
N_0 1_1 (sterilized)	3.5×10^2

1. N_0 1_0: Noninoculated control without fertilizer nitrogen.
2. N_0 1_1: Without fertilizer N but inoculated with *Azospirillum.*

17-fold higher bacterial population than the roots of noninoculated plants. Sterilization of the roots with chloramine-T for 30 min sharply decreased the number of *Azospirilla,* indicating that many of the bacteria were associated with the root tissue. That this decrease was of the order of a 1000-fold in treated plants as against a 100-fold in nontreated ones showed that the roots of inoculated plants harbored a substantial number of bacterial cells on the outer root tissue.

The evidence from this study indicated that *Azospirillum* may be favored in the rhizoplane of an inoculated sorghum crop. It is admitted that the criteria for identifying the organism was not perfect in the absence of acetylene-reduction assay (ARA) or fluorescence antibody (FA) techniques. But the overall analysis of data points to the possibility of increasing the populations of nitrogen-fixing *Azospirillum* in the rhizosphere as well as in the roots of a sorghum crop by population of selected strains.

In multilocational trials under different agroclimatic conditions of India, the increase in yield due to inoculation was significant at 4 out of 9 centers during the years 1979-1982 (Table 3). The mean increase in grain yield due to seed inoculation over the control, averaged over all trials over 4 years, amounted to 18.7%, almost equivalent to the application of 15-20 kg N ha^{-1} as urea. Inoculation in the presence of 40 kg N ha^{-1} significantly increased grain yield over the corresponding control at two out of nine centers (Coimbatore and Pantnagar).

In a field trial at Delhi, 60 kg N ha^{-1} + inoculation was found superior to all other treatments (Table 4). Inoculation could not bring significant benefits if no N was applied. Stover yield in sorghum obtained with 20 kg N ha^{-1} application could also be achieved by *Azospirillum* inoculation. It was also observed that inoculation along with graded levels of N resulted in 7.6-13.5% increase in stover yields. The effect was more significant (15.6%) in combination with 20 kg N ha^{-1}.

Several workers reported N gains by free-living

Table 3. Grain yield (kg ha^{-1}) of sorghum CSH 5 as affected by inoculation with *Azospirillum brasilense and* fertilizer N at nine locations in India.

Location	Noninoculated control	*Azospirillum brasilense*	40 kg N ha^{-1}	40 kg N ha^{-1} + *Azospirillum brasilense*	CD (P<0.05)
Udaipur	2640	3190[1]	4480	4580	3250
Coimbatore	3190[1]	3410[1]	4170	4780[1]	475
Dharwad	1650	2070[1]	3020	3160	405
Hyderabad	3410	4010	4340	4390	875
Akola	690	770	2070	1990	250
Parbhani	880	1150	2510	2180	285
Navsari	1670	1830	1960	1860	350
Indore	1470	1660	2450	2180	450
Pantnagar	1950	2400	2430	2810	375
Mean	1920	2280	3050	3110	-
CV(%)			9.5		

1. Significant increase over corresponding noninoculated control.

Table 4. Grain and stover yields (kg ha^{-1}) of sorghum, as affected by inoculation *with Azospirillum* in combination with fertilizer nitrogen, IARI, New Delhi.

Parameter	Treatment	Nitrogen (kg ha^{-1}) 0	20	40	60	Mean
Grain yield	Without inoculation	3260	3920	4460	5119	4190
	With inoculation	3400	4790[1]	5380[1]	5820[1]	4850[1]
	Mean	3330	4360	4920	5469	
	CD (P<0.05)		325			1.62
	CV (%)		14.5			
Stover yield	Without inoculation	7120	9110	10240	11460	9480
	With inoculation	7990	10530[1]	11 370[t]	12340	10550[1]
	Mean	7550	9810	10810	11890	
	CD (P<0.05)		961		481	
	CV (%)		11.5			

1. Significantly different from corresponding noninoculated treatments.

bacteria ranging from 34-165 kg N ha^{-1} a^{-1} (Baltensperger et al. 1978). The basic questions with regard to the contribution of *Azospirillum* to the increased N status of inoculated plants still remain to be satisfactorily answered. The data do indicate that N uptake was more in inoculated sorghum than in the corresponding noninoculated ones, and this effect was more pronounced in combination with 20 kg N ha^{-1} (Table 5).

A maximum gain of 21.7 kg N ha^{-1} by the plant was obtained by inoculation with 20 kg N ha^{-1} over the corresponding noninoculated treatment. Inoculation also resulted in a gain of about 15 kg N ha^{-1} both under the the zero N and 60 kg N levels. Though the above figures represent only approximate values, these rates of N_2 fixation could very well be correlated with the N savings noted from increased grain yields due to inoculation.

Soil inoculation with mycorrhizal fungi increased the dry matter of roots, shoots, phosphorus concentration in plants, and phosphorus uptake by sorghum. Seed inoculation with *A. brasilense* in conjunction with *Gigaspora margarita* or *Glomus fasciculatum* produced significantly more dry-

Table 5. Total nitrogen uptake in sorghum under different combinations of *Azospirillum brasilense* and fertilizer nitrogen, IARI, New Delhi.

Treatment kg N ha^{-1}	*A. brasilense*	Nitrogen uptake (kg ha^{-1}) Root	Stover	Grain	Total	Increase in N uptake by inoculation (kg ha^{-1})
0	N[1]	3.0	31.9	41.6	76.5	-
0	I[2]	3.7	40.2	47.2	91.1	14.6
20	N	3.8	48.5	53.3	105.6	-
20	I	4.1	57.5	65.7	127.3	21.7
40	N	4.0	55.9	61.8	121.7	-
40	I	3.8	63.1	73.8	141.7	20.0
60	N	4.5	69.00	73.7	147.1	-
60	I	5.2	70.75	86.3	162.3	15.2

1, N = Noninoculated.

2. I = Inoculated.

Table 6. Response of sorghum CSH 5 to inoculation with *Azospirillum brasilense* and VAM fungi.

Treatment	Shoot mass (g plant^{-1})	Root mass (g plant^{-1})[1]	Total phosphorus content (%)	Phosphorus uptake (mg plant^{-1})
Noninoculated control (no VAM and no *A. brasilense)*	14.5	0.18	0.10	14.5
A. brasilense	18.5	0.32[2]	0.12	21.9
Acaulospora sp	17.2	0.22	0.15	25.8
Gigaspora margarita	17.5	0.25	0.15[2]	25.5
Glomus fasciculatum	17.8	0.22	0.16*	26.7
Acaulospora + *A. brasilense*	17.8	0.25	0.15[2]	26.5
G. margarita + *A. brasilense*	20.5[2]	0.34[2]	0.15[2]	28.5[2]
G. fasciculatum + *A. brasilense*	20.8[2]	0.37[2]	0.17[2]	32.5[2]
		CD (P<0.05)		
Azospirilium brasilense	NS[3]	0.12	NS	NS
VAM fungi	NS	NS	0.04	NS
A.brasilense x VAM fungi interaction	2.12	0.11	0.03	5.3

1. Average of six replicated pots; each pot had four plants.
2. Significant increase over corresponding control.
3. NS = Not significant.

matter content of roots, shoots, P content of shoots, and P uptake of sorghum than the nonmycorrhizal treatments (Table 6).

Azospirillum inoculation has been shown to increase the root biomass (Dewan and Subba Rao 1979) and therefore, the significant increase in dry-matter production and root biomass by a combination of seed inoculation with *A. brasilense* and soil inoculation with *Gigaspora margarita* and/or *Glomus fasciculatum* could be attributed to an increased P transport by the mycorrhizal fungus aided by an increased root biomass (Table 6).

References

Alexander, M. 1965. Most-probable-number method for microbial populations. Pages 1467-1472 *in* Methods of soil analysis, (Black, C.A., Evans, D.D., White, J.L., Ensminger, L.E., and Clark, F.E., eds.). [In Pt.] Madison, Wisconsin, USA: American Society of Agronomy.

Baltensperger, A.A., Schank, S.C., Smith, R.L., Littell, R.C, Bouton, J.H., and Dudeck, A.E. 1978. Effect of inoculation with *Azospirillum* and *Azotobacter* on turf-type bermuda genotypes. Crop Science 18:1043-1045.

Day, J.M., Harris, D., Dart, PJ., and van Berkum, P. 1975. The Broadbalk experiment. An investigation of nitrogen gains from non-symbiotic fixation. Pages 71-84 *in* Nitrogen fixation by free-living micro-organisms (Stewart, W.D.P.,ed.). Cambridge, UK: Cambridge University Press.

Dewan, G.I., and Subba Rao, N.S. 1979. Seed inoculation with *Azospirillum brasilense* and *Azotobacter chroococcum* and the root biomass of rice *(Oryza sativa* L.). Plant and Soil 53:295-302.

Jackson, M X. 1967. Soil chemical analysis. New Delhi, India: Prentice Hall.

Smith, R.L., Schank, S.C., Bouton, J.H., and Quesenberry, K.H. 1978. Yield increases of tropical grasses after inoculation with *Spirillum lipoferum.* Pages 380-385 *in* Environmental role of nitrogen-fixing blue-green algae and asymbiotic bacteria (Granhall, U., ed.). Ecological Bulletins no. 26. Stockholm, Sweden: Swedish Natural Science Research Council.

Subba Rao, N.S., Tilak, K.V.B.R., Lakshmikumari, M., and Singh, C.S. 1979a. *Azospirillum* a new bacterial fertilizer. Indian Farming 30:3-5.

Subba Rao, N.S, Tilak, K.V.B.R., Singh, C.S.,and Lakshmikumari, M. 1979b. Response of a few economic species of graminaceous plants to inoculation with *Azospirillum brasilense.* Current Science 48:133-134.

Tarrand, J.J., Krieg, N.R., and Dobereiner, J. 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Canadian Journal of Microbiology 24:967-980.

von Bulow, J.F.W., and Dobereiner, J. 1975. Potential for nitrogen fixation in maize genotypes in Brazil. Proceedings of the National Academy of Sciences of the United States of America 72:2389-2393.

Weier, K.L. 1980. Nitrogen fixation associated with grasses. Tropical Grasslands 14:194-201.

Discussion

G. Oblisami:
What is the load of VAM-fungal inoculant to the soil inoculation for sorghum?

K.V.B.R. Tilak:
The inoculum carried 100-125 chlamydospores per 50 g of inoculum.

Joseph Thomas:
The data of Dr Tilak showing consistent increase in root mass on *Azospirillum* inoculation are significant. This is similar to a mycorrhyzal effect leading to enhanced uptake of nutrients. That this is really so can be demonstrated by appropriate work at centers like IARI and ICRISAT.

K.V.B.R. Tilak:
VAM is known to increase other nutrients like Zn, Fe, Cu, etc., apart from Phosphorus. So it needs to be examined.

S.P. Wani:
In your data you refer to N balance, which is not correct. It is increased N uptake and not the N gain or N balance.

K.V.B.R. Tilak:
Yes, this may be considered as N uptake.

P.V. Rai:
What was the inoculation method followed in the dual inoculum of *Azospirillum brasilense* and VAM?

K.V.B.R. Tilak:
Seed inoculation of *A. brasilense* and VAM was mixed with soil prior to sowing.

B.K. Konde:
Did you inoculate *Azospirillum* and VAM at the same time?

K.V.B.R. Tilak:
Yes, at the time of sowing.

H.L.S. Tandon:
Why is there so much emphasis on studying N balance or N gain by total N analysis of the soil when the N changes are extremely small as compared to total N present in soil, and it would be very difficult to separate real gains from analytical errors or variability?

K.V.B.R. Tilak:
We have not given much emphasis on soil N. But soil N content is to be taken into consideration while calculating the benefits of total N status due to inoculation with the N_2 fixer.

Azotobacter Inoculation: Nitrogen Economy and Response of Sorghum CSH 1

S.T. Shende[1], G.B. Rudraksha[2], Rajani Apte[1], and R.S. Raut[2]

Summary

Field trials conducted during the 1979, 1980, and 1981 rainy seasons with sorghum CSH 1 revealed that inoculation with Azotobacter chroococcum *(M_4), increased grain and fodder yields substantially and consistently. The inoculation effect was more at lower N-fertilizer levels and decreased gradually with increased doses of N-fertilizer. But at all levels of nitrogen application, e.g., 30, 60, and 90 kg N ha^{-1}, inoculation with* Azotobacter *showed a complementary effect and appeared to reveal a saving of 30 kg N ha^{-1}.*

Introduction

Inconsistent results with *Azotobacter chroococcum,* particularly in agronomical experiments, have been attributed to the low population of the organism both in soil and crop rhizosphere (Mishustin and Shilnikova 1969, Brown 1974). However, there arc reports that inoculation with free-living nitrogen-fixing bacteria enhanced crop yields, including that of cereals (Meshram and Shende 1982, Shende and Apte 1982, Subba Rao et al. 1982, Wani et al. 1985).

This paper reports the results of field trials conducted with sorghum under agroclimatic conditions for three consecutive rainy seasons at Parbhani in Maharashtra.

Materials and Methods

Field trials were conducted at Parbhani during the 1979, 1980, and 1981 rainy seasons. The experiments were laid out in randomized-block designs including four levels of nitrogen, i.e., 0, 30, 60, and 90 kg N ha^{-1}. In all these experiments, sorghum hybrid variety CSH 1 was used. A uniform basal dose of 40 kg P_2O_5 and 40 kg K_2O ha^{-1} was applied. Nitrogen was given as urea in two split applications.

The strain of *Azotobacter chroococcum* (M_4) used was isolated from the rhizosphere of maize plant. This strain is chromogenic and was found to fix 1.76 mg N g^{-1} sucrose and synthesize indole acetic acid (IAA) and GLS compounds.

Results

During 1979, the fodder yield of 8830 kg ha^{-1}, obtained by a combined treatment of 60 kg N ha^{-1} and inoculation with *Azotobacter,* was significantly higher than the 8020 kg ha^{-1} obtained with application of 60 kg N ha^{-1} alone, and was at par with the fodder yield obtained with the application of 90 kg N ha^{-1} (Table 1).

Inoculation with *Azotobacter* alone increased grain yield from 3180 to 3680 kg ha^{-1} (15.7%), which was statistically at par with the 3890 kg ha^{-1} obtained with the application of 30 kg N ha^{-1}. Thus, there is an indication of nitrogen saving up to 30 kg N ha^{-1}. At higher levels of applied N, the yield increases due to *Azotobacter* were not statistically significant. The combined treatment appeared to be advantageous even at the 90 kg N ha^{-1} application.

A similar trend was seen during 1980. Application of *Azotobacter* significantly raised the grain yield

1. Senior Microbiologist and Scientist, Division of Microbiology, Indian Agricultural Research Institute, New Delhi 110 012, India,
2. Microbiologists, Department of Microbiology, Marathwada Agricultural University, Parbhani, Maharashtra 431 402, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

Table 1. Effect of *Azotobacter* (M_4) inoculation and levels of nitrogen on grain and fodder yields of sorghum CSH 1, Parbhani, rainy seasons 1979-81.

Treatment	Grain yield (kg ha^{-1})	Fodder yield (kg ha^{-1})	Increase in grain yield (%)
	Rainy season 1979		
Control	3180	5980	-
Azotobacter	3680	6540	15.7
30 kg N. ha^{-1}	3890	6970	-
30 kg N ha^{-1} + *Azotobacter*	4220	7600	8.6
60 kg N ha^{-1}	4100	8020	-
60 kg N ha^{-1} + *Azotobacter*	4280	8830	4.53
90 kg N ha^{-1}	4670	8760	-
90 kg N ha^{-1} + *Azotobacter*	4960	9070	6.4
SE	±137	±201	
	Rainy season 1980		
Control	1960	5510	-
Azotobacter	2620	7200	33.6
30 kg N ha^{-1}	2830	8400	-
30 kg N ha^{-1} + *Azotobacter*	3170	9080	12.1
60 kg N ha^{-1}	3510	9600	-
60 kg N ha^{-1} + *Azotobacter*	3620	10460	3.0
90 kg N ha^{-1}	4170	10710	-
90 kg N ha^{-1} + *Azotobacter*	4320	12680	3.6
SE	±170	±406	
	Rainy season 1981		
Control	1660	4890	-
Azotobacter	2050	5740	23.2
30 kg N ha^{-1}	1980	5740	-
30 kg N ha^{-1} + *Azotobacter*	2930	7370	47.7
60 kg N ha^{-1}	2100	7370	-
60 kg N ha^{-1} + *Azotobacter*	3390	8660	61.3
90 kg N ha^{-1}	3460	8490	-
90 kg N ha^{-1} + *Azotobacter*	3810	9600	3.5
SE	±150	±400	

from 1960 to 2620 kg ha^{-1} (33.6% increase), when fertilizer N was not added. In combinations with N, *Azotobacter* increased the yield but differences were again not significant. But it could be seen very clearly that 30 kg N ha^{-1} could be saved at each level of nitrogen application if seed inoculation with *Azotobacter* was practiced.

During 1981, grain as well as fodder yields increased markedly with *Azotobacter* inoculation. Fodder yield increased with inoculation at all levels of nitrogen application, but increases were high with 30-60 kg N ha^{-1}. Grain yield increased significantly with inoculation, giving 390 kg ha^{-1} or 23.2% more than the control. Inoculation alone was comparable to application of 30 or 60 kg N ha^{-1}. As in the previous 2 years, a combined treatment of inoculation together with application of 30 and 60 N kg ha^{-1} was the best treatment both for fodder and grain yields.

Thus, the combined application of *Azotobacter* with 60 kg N ha^{-1} in the form of urea was the best treatment, to harness the effect of *Azotobacter* in order to increase grain and fodder yields and at the same time to save about 30 kg ha^{-1} of fertilizer N.

Acknowledgements

The authors wish to thank Dr N.S. Subba Rao, Head, Division of Microbiology, IARI, and Marathwada Agricultural University for providing the facilities for these experiments.

References

Brown, M.E. 1974. Seed bacterization. Annual Review of Phytopathology 12:181-197.

Meshram, S.U., and Shende, S.T. 1982. Response of maize to *Azotobacter chroococcum.* Plant and Soil 69:265-273.

Mishustin, E.N., and Shilnikova, V.K. 1969. The biological fixation of atmospheric nitrogen by free-living bacteria. Pages 82-109 *in* Soil biology: reviews of research. Paris, France: UNESCO.

Shende, S.T., and Apte, R.G. 1982. *Azotobacter* inoculation—a remunerative input for agricultural crops. Pages 532-543 *in* Proceedings of the National Symposium on Biological Nitrogen Fixation, 25-27 Feb 1982, New Delhi, India. Bombay, India: Bhabha Atomic Research Centre.

Subba Rao, N.S., Tilak, K.V.B.R., Singh, C.S., and Gautam, R.C. 1982. Crop yield and nitrogen content of pearl millet *(Pennisetum americanum)* in response to *Azopirillium brasilense.* Pages 507-516 *in* Proceedings of the National Symposium on Biological Nitrogen Fixation, 25-27 Feb 1982, New Delhi, India. Bombay, India: Bhabha Atomic Research Centre.

Wani, S.P., Chandrapalaiah, S., and Dart, P.J. 1985. Response of pearl millet cultivars to inoculation with nitrogen-fixing bacteria. Experimental Agriculture 21:175-182.

Response of Sorghum Cultivars to Inoculation with *Azospirillum*

D. Purushothaman and G. Oblisami[1]

Summary

This study presents evidence of wide variations among sorghum genotypes in supporting nitrogen fixation. Quantitatively and qualitatively, the isolates of Azospirillum *differ in their interaction with cultivars. Based on the root-associated acetylene-reduction activity (ARA), 19 sorghum cultivars were classified into high ARA and low ARA genotypes. Inoculation with an efficient strain (CSH 24) of* Azospirillum *enhanced the ARA of only certain cultivars. Some cultivars when inoculated exhibited negative response in ARA. In root-bit preincubation test also, the cultivars CSH 5 and Co.24 recorded high ARA while K.tall and USH 1 showed negligible increase. In afield study,* Azospirillum *inoculation significantly increased the grain yield (13.1%) of cultivar Co. 24.*

Introduction

Of the many cereals grown in India, sorghum has been consistently found to respond to *Azospirillum* inoculation (A1CRPBNF 1980). The response has been much greater at low levels of nitrogen than at higher levels. Perhaps one of the most significant and encouraging findings in relation to associative N_2 fixation is that there appear to be varietal differences in responses to inoculation. In this paper, the responses of different genotypes of sorghum *[Sorghum bicolor* L. (Moench)] to inoculation with *Azospirillum* are presented.

Materials and Methods

The isolates of *Azospirillum* were cultured following the root-tissue enrichment technique. The isolates were purified by streaking over potato-malic acid medium. The enumeration of *Azospirillum* was done following the most-probable number (MPN) technique using N-free malic acid medium (Hegazi et al. 1979).

Mud pots of 36 cm diameter were filled with 5 kg pot^{-1} redloam soil:sand:farmyard manure (FYM) (5:5: lv/v) mixture (pH 7.2; total nitrogen 0.085%, organic carbon 0.48%). Before raising the crop, 20 kg N ha^{-1} as urea, 40 kg P_20_5 ha^{-1} as superphosphate, and 40 kg K_20 ha^{-1} as potassium chloride were applied.

Soil samples from the rhizosphere and rhizoplane were collected following standard procedures (Pramer and Schmidt 1966, Lynch 1982).

Azospirillum isolates grown in 30 mL malic acid semisolid medium for 72 h in special 100 mL restricted-neck Erlenmeyer flasks were assayed for ARA by incubating under 1% acetylene for 6 h at 28°C

In the case of root-associated ARA, freshly collected root samples were placed in 100 mL Erlen-

1. Associate Professor and Head, Center of Advanced Studies in Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu 641 003, India.

ICR1SAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

meyer flask (roots to occupy 20-25% of the volume) and similarly incubated under 1% acetylene for 6 h (Purushothaman et al. 1979).

The promising isolate CSH 24 was grown under static conditions in malic acid broth medium, supplemented with 100 mg glutamic acid L^{-1}. After 5 days of growth the broth was mixed with sterilized peat soil neutralized with calcium carbonate. The peat-based inoculum contained 12×10^8 cells g^{-1} on dry mass basis. Surface-sterilized seeds of the sorghum genotypes were treated with the inoculum, using jaggery solution as the sticker. Each seed after treatment carried 32×10^6 cells.

The field experiment was conducted at the Millet Breeding Station, Tamil Nadu Agricultural University (TNAU), Coimbatore, on a sandy loam soil with a pH of 6.9, 0.026% total nitrogen, and 0.64% organic carbon. The plot size of 6 m x 5 m was replicated thrice in a randomized-block design. Ten packets (2.5 kg) of the inoculum ha^{-1} were used for the field study. At the time of thinning the crop (15-20 days after sowing), the biofertilizer was side-dressed. The crop received two life-saving irrigations.

Results

Ten out of 19 sorghum genotypes differed significantly in total *Azospirillum* counts in the rhizosphere at the flowering stage. Cultivars Co.24 and Co. 19 registered a higher count of *Azospirillum* in the rhizoplane (Table 1). These differences may arise from the qualitative and quantitative changes in the root exudates upon which the energy-demanding diazotrophs are dependant (Hubbell 1977). The mean ARA of isolates from genotypes ranged from 16 to 360 nmoles of C_2H_4 h^{-1}, showing over 20-fold variation. In general, isolates from CSH 5, Co. 19, Co. 24, USH 1, and Co. 18 recorded substantially higher ARA than the others (Table 2). Though the isolates were maintained on N-rich medium for several generations, the variation in ARA could only be traceable to the host genotypes. Although *in vitro* determinations of ARA do not reflect the field performance of these isolates, these data suggest the variation among the isolates. The genotypes differed significantly in respect of the root-associated ARA determined under noninoculated conditions at the flowering stage (Table 3). Genotypes that supported high ARA were Co. 18, Co. 24, Co. 20, CSH 5, Co. 19, and USH 1. The reason for low ARA observed with some varieties might be the presence of inefficient strains. It should be pointed out that cultivars Co.24, CSH 5, Co.20, Co. 18, and Co. 19 possessed high ARA and also a reasonably large number of Azospirilla.

All the 10 cultivars tested responded to *Azospirillum* inoculation. However, inoculation did not increase the ARA significantly, except in genotypes Co.24, CSH 5, Co. 18, and Co.20. Cultivars Co.21, CSH 4, and USH 1 registered negative responses in ARA with inoculation (Table 4). In general, preincubation of roots with energy source for 6 h greatly increased ARA. The maximum ARA was observed with Co.24 and CSH 5 while K.tall and USH 1 had low ARA. Without preincubation the cultivars recorded poor ARA (Table 5). As the tissues were not surface-sterilized but were uniformly washed to remove the adhering soil particles, *Azospirillum*

Table 1. Most-probable number (MPN) of *Azospirillum* sp in the roots of some sorghum cultivars.

Cultivar	Rhizosphere (x 10^4)g^{-1}	Rhizoplane (x 10^4) g^{-1}
Co.19	22.6	42.6
Co.20	56.2	7.2
Co.24	84.6	24.6
CSH 4	4.8	15.8
CD(P<0.05)	11.84	6.16

Table 2. The *ARA of Azospirillum* isolates from different sorghum cultivars.

Cultivar	No.of isolates tested	Mean ARA (nmole C_2H_4 h^{-1} $flask^{-1}$)
CSH 5	10	360 ± 26
Co.19	20	312 ± 18
Co.24	25	280 ± 21
USH 1	15	220 ± 26
Co.18	16	211 ± 23
Co.20	12	190 ± 16
Co.21	15	105 ± 12
CSH 6	10	99 ± 12
Co.22	15	87 ± 16
CSV 3	20	65 ± 11
CSV 4	15	65 ± 18
CSH 4	10	18 ± 4
K.tall	10	16 ± 8

found entrapped in the mucigel would not have been lost; also, those dwelling within the cortical cells would respond to the energy-source supply and the cell number would have considerably increased. It could be argued that the cultivars Co.24 and CSH 5 permitted a higher rate of multiplication of the diazotroph during the incubation period than the other genotypes. Alternatively, as indicated earlier, the presence of more efficient strains with Co.24 and CSH 5 would have reflected in the increased ARA. Though preincubation leads to overestimation of nitrogen fixation, it does give us an idea of the behavior of varieties.

In a field experiment, the grain yield increased significantly due to *Azospirillum* inoculation. On an average, the increase in grain yield was 13.1%. Grain yield with 20 or 30 kg N ha^{-1} + *Azospirillum* inoculation was 2.4 t ha^{-1}, which was equivalent to that obtained with 50 kg N ha^{-1} without inoculation (Table 6).

In conclusion, it may be said that sorghum genotypes differ markedly in harboring diazotrophs both in quantity and in quality. In a combination of potential genotypes with appropriate, efficient strains of *Azospirillum*, one can definitely get good responses.

Table 3. Root-associated nitrogenase activity of sorghum cultivars at the flowering stage.

Cultivar	Nitrogenase activity (nmole C_2H_4 g^{-1} dry root h^{-1})
Co. 18	327
Co. 24	318
Co. 20	268
CSH 5	241
Co. 19	216
USH 1	212
Co. 21	120
CSH 6	112
USV 3	105
CSV 3	92
Co. 22	68
296	68
K.tall	48
CSH 4	42
USV 1	38
CSV 5	36
CSV 4	35
269	34
AS 3280	30
CD *(P<0.05)*	12.72

Table 4. The ARA of sorghum cultivars as influenced by *Azospirillum* inoculation.

Cultivar	Root associated ARA (nmole C_2H_4 g^{-1} root h^{-1})		
	Noninoculated	Inoculated	Change (%)
Co. 18	217	268	+ 23.7
Co. 19	126	176	+ 39.0
Co. 20	168	186	+ 10.7
Co. 21	146	123	- 5.8
Co. 22	62	84	+ 34.8
Co. 24	308	426	+ 38.0
K.tall	65	68	+ 4.9
CSH 4	44	38	- 13.6
CSH 5	198	268	+ 35.2
USH 1	142	112	- 21.1

Table 5. Effect of preincubation of washed sorghum root bits with malic acid on ARA.

Cultivar	ARA (nmole C_2H_4 g^{-1} dry root h^{-1})	
	Preincubation	No preincubation
Co.24	288 ± 19	143 ± 8
CSH 5	320 ± 16	76 ± 7
K tall	60 ± 8	43 ± 12
USH 1	75 ± 10	68 ± 8

1. ± Standard deviation of the mean.

Table 6. Grain yield (t ha^{1}) of sorghum Co. 24 as influenced by *Azospirillum* inoculation and fertilizer N.

Nitrogen applied kg ha^{-1}	Grain yield(t ha^{-1})			
	Non-inoculated	Inoculated	Mean	% increase
0	1.7	1.9	1.8	+ 12.7
10	2.0	2.1	2.04	+ 7.0
20	2.0	2.3	2.15	+ 13.7
30	2.3	2.5	2.44	+ 11.5
40	2.4	2.7	2.55	+ 15.2
50	2.4	2.8	2.6	+ 16.3
Mean	2.1	2.4	-	+ 13.1

References

AICRPBNF (All India Coordinated Research Project on Biological Nitrogen Fixation). 1980. Project co-ordinators' report. New Delhi, India: Indian Council of Agricultural Research.

Hegazi, N.A., Amer, H., and Monib, M. 1979. Enumeration of N_2-fixing spirilla. Soil Biology and Biochemistry 11:437-438.

Hubbell, D.H. 1977. Plant root and biological nitrogen fixation. Proceedings, Soil and Crop Science Society of Florida 36:37-40.

Lynch, J.M. 1982. Interactions between bacteria and plants in the root environment. Pages 1-23 *in* Bacteria and plants (Rhodes-Robert, M.E., and Skinner, F.A., eds.). London, UK: Academic Press.

Pramer, D, and Schmidt, E.L. 1966. Experimental soil microbiology. Minneapolis, Minnesota, USA: Burgess Publishing Co.

Purushothaman, D., Gunasekaran, S. and Oblisami, G. 1979. Nitrogen fixation by *Azospirillum* in some tropical plants. Proceedings of the Indian National Science Academy, Part B 46:713-717.

Nitrogen Transformations by *A. brasilense* Strain 12S from Sorghum Roots

Bela Shukla and B.S. Kundu[1]

Summary

One of the isolates of A. brasilense (12S) from sorghum roots showed enhanced nitrogenase activity and cell growth with NO^-_3 (<60 µg NO^-_3 mL^{-1}) and NO^-_2 (<30 µg NO^-_2 mL^{-1}) under microaerophilic conditions. Both denitrification and ammonification were observed simultaneously in this organism. At low levels of ammonium (<20 µg NH^+_4 mL^{-1}) and urea (60 µg mL^{-1}), growth and nitrogenase activity were improved. These compounds were also transformed to gaseous forms of nitrogen.

Introduction

Azospirillum spp are capable of several nitrogen transformations: (1) nitrate can be utilized by assimilatory nitrate and nitrite reductases; (2) dissimilatory nitrate reduction is carried out under anaerobic conditions, with NO^-_3 as an electron acceptor, and is reduced to nitrite, nitrous oxide, nitrogen, or ammonium; and (3) nitrate-dependent nitrogen fixation occurs under severe oxygen limitations. In the light of these observations, the present investigation was undertaken to study the nitrogen transformations by *A. brasilense* (12S), effect of nitrogen sources on cell growth, total nitrogen under microaerophilic conditions, and levels which are critical for expression of nitrogenase activity.

Materials and Methods

The sorghum root isolate *A. brasilense* (12S) was a local isolate maintained by regular transfers on modified (0.2 g yeast extract replaced vitamins) malate medium agar slants (Dobereiner and Day 1976). The culture was multiplied in broth medium incubated at 30° ± 2°C and 0.1 mL culture having 10^8 cells mL^{-1} was used as inoculum for the studies.

The nitrogen transformations studies were conducted using variable concentrations of NO^-_3, NO^-_2, NH^+_4, and NH^+_2 (urea) in malate semisolid medium. The tubes containing 5 mL medium were inoculated with 0.1 mL inoculum and incubated at 30 ° ± 2°C. The presence of NO^-_3, NO^-_2, NH^-_4 urea, N_2O, total nitrogen, and cell protein were determined at 0, 3, and 6 days after incubation.

For the estimation of various forms of nitrogen, the culture medium after ARA and N_2O assay was centrifuged at 10 000 rpm, and the supernatent was used for various estimations. Nitrate concentrations were measured by using salicylic acid (Cataldo et al. 1975) and NO_2 by developing color with sulfanilic acid and a-N-naphthyl ethylene diaminehydrochloride (Nicholas and Nason 1957). The ammonium estimations were made by Nesselerization (Jackson 1967) and urea by the procedure of Douglas and Bremner (1970). Total nitrogen was determined by the conventional micro-Kjeldhal method (Jackson 1967). Total cell protein was measured by further

1. MSc Student and Associate Professor, Department of Microbiology, College of Basic Sciences and Humanities, Haryana Agricultural University, Hisar, Haryana 125 004, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

digesting the cell mass in IN NaOH and later determining the amount of solubilized protein by the procedure of Lowry et al. (1951).

The nitrogenase activity was measured by reduction of acetylene to ethylene using a dual-column Nucon 5500 gas chromatograph. The tubes were incubated under 10% C_2H_2 for 24 h and the samples were analyzed for C_2H_4 produced.

Nitrous oxide was analyzed from the tubes that were subjected to ARA assay using gas TCD chromatograph (fitted with porapak N column), using H_2 as carrier gas (30 mL min^{-1}), at a oven temperature of 100° ± 5°C. Nitrous oxide for reference was prepared by reacting zinc with dilute HNO_3 (1:1) in an airtight container. The N_2O peaks were detected by comparing with reported results and retention time was recorded.

Results

A. brasilense isolate 12S showed higher ARA than *A. brasilense* (Sp7a) and differed physiologically from it. The added nitrate nitrogen was denitrified to nitrite at all levels (60-1200 µg NO_3 mL^{-1}) and further reduction was noticed only at lower levels on longer incubation (Table 1). Similar observations have been reported earlier using azospirilla strains (Scott et al. 1979, Neyra and Van Berkum 1977, Nelson and Knowles 1978). Nitrogenase activity was slightly increased by nitrate additions (<60 µg NO_3 mL^{-1}), The inhibition of nitrogenase at higher levels of NO_3 maybe due to NO_2 accumulation that subsequently blocks NO_3-dependent ARA. A slight gain in total cell protein was noticed only at lower levels of NO_3 (<60 µg mL^{-1}, Table 2). Nitrate did not increase the total cell protein appreciably, which indicates the nonutilization of this compound under microaerophilic conditions for cell growth, but is used as an electron acceptor. Total nitrogen showed a marginal gain at low levels and a small loss at higher levels. A small increase in cell mass and total nitrogen at the end of the experiment maybe due to nitrogen fixation. At higher concentrations of nitrate, the loss in total nitrogen was due to further degradation of NO_3 to N_2O and N_2.

It was found that NO_2 was reduced to N_2O at 60 µg NO_2 mL^{-1} and ARA was improved, but at higher levels the nitrogenase was repressed. At lower levels, slight increase in total cell protein was also noticed. The increase in cell mass was less after 3 days than at the earlier stage. Total nitrogen showed a marginal gain up to 6 days of incubation only at low levels; at higher concentrations a slight loss was observed.

Table 1. Effect of nitrate, nitrite, ammonium, and urea on nitrogenase activity (n moles C_2H_4 d^{-1} mL^{-1}).

	Levels	Days of incubation	
	(µg mL^{-1})	3	6
Nitrate	0	698	282
(NOT)	60	710	380
	300	ND[1]	ND
	600	ND	ND
	1200	ND	ND
Nitrite	0	792	334
(NOT)	30	870	351
	60	910	380
	90	ND	ND
	120	ND	ND
	150	ND	ND
Ammonium	0	647	430
(NH^+_4)	10	699	499
	20	676	460
	40	ND	395
	100	ND	ND
Amide	0	726	423
(NH^+_2)	30	759	480
	60	752	468
	120	ND	256
	300	ND	ND

1. ND = Not detected.

Precise quantitative determinations of N_2O could not be made because of nonavailability of a standard nitrous oxide. Ammonium was also found at the end of the experiment, suggesting conversion of NO^-_3 to NH^+_4. In view of the fact that nitogen fixation does not occur under these conditions, the presence of ammonium is most likely due to ammonification. Similar observations have also been reported by Scott et al. (1979). Nitrogen-fixing bacteria with the ability to assimilate NO^-_3 will not use this compound for nitrogen fixation. However under oxygen levels too low to allow respiration, nitrate-dependent acetylene reduction can occur. In the experiments where nitrite was used as a substrate, similar results were found and nitrite was used as electron acceptor. It therefore appears that both nitrate and nitrite respiration in this organism are coupled to ARA.

The added ammonical nitrogen was utilized by *A. brasilense* 12S rapidly, and on the 3rd day of incubation no residual ammonium was found in the

Table 2. Effect of nitrate, nitrite, ammonium, and amide-N on cell growth, protein, and total nitrogen.

	Levels	Total cell protein (µg mL^{-1})			Total nitrogen (µg mL^{-1})		
	(µg mL^{-1})	Days			Days		
		0	3	6	0	3	6
Nitrate	0	100	282	325	36	46	53
(NO^-_3)	60	100	310	340	50	60	69
	300	98	108	113	96	93	91
	600	98	115	120	170	160	154
	1200	110	100	120	312	312	296
Nitrite	0	100	318	355	32	46	55
(NO^-_2)	30	120	357	423	39	55	62
	60	110	350	370	48	60	65
	90	110	116	120	57	55	50
	120	130	147	147	66	59	54
	150	100	104	95	75	66	54
Ammonium	0	100	286	352	36	48	53
(NH^+_4)	10	90	378	396	48	66	68
	20	90	437	468	67	76	80
	40	100	450	494	74	81	83
	100	110	512	520	111	115	110
Amide	0	100	270	310	42	44	50
(NH^+_2)	30	110	407	470	58	68	78
	60	90	475	535	73	77	89
	120	90	568	600	103	104	98
	300	100	619	660	193	191	182

medium at lower concentrations. An appreciable increase in ARA was noticed upto 20 µg NH^+_4 mL^{-1} over the control. This may be because of more cell mass available for acetylene reduction. The ammonium added at higher levels showed a loss in total nitrogen, indicating oxidation of ammonium to gaseous nitrogen which was confirmed by gas chromatography. At the 40 µg NH^+_4 mL^{-1} level, nitrogenase was depressed on the 6th day and no activity was detected on the 3rd day. Marked increase in total cell protein was noticed at all levels of ammonium compared to the control. However, the effect was less at higher levels. A small gain in total nitrogen was found at less than 20 µg NHJ mL^{-1} with a loss at 100 µg NH^+_4 mL^{-1}.

Urea was converted to ammonium and no residual urea was detected on the 6th day even at a concentration of 300 µg mL^{-1}. The presence of active urease was noticed as the added urea was converted into ammonium even on the 3rd day of incubation. Ammonium formed further showed transformations similar to those reported above. ARA was enhanced over the control with added urea upto 60 µg on the 3rd day, and was detected at 120 µg on the 6th day. At all concentrations, an appreciable increase in cell protein was noticed. The total nitrogen content increased marginally at low levels, but at higher concentrations a small loss was found.

All the nitrogenous compounds tested showed an increase in ARA at lower levels, which is an additional benefit of using this organism in the presence of added nitrogen. The inhibitory levels are above the recommended field doses, therefore, a loss in added nitrogen will not occur. Rather, an increase in populations will help in more nitrogen fixation. Use of *A. hrasilense* 12S can therefore enrich nitrogen content by dinitrogen fixation, even when the levels of combined nitrogen are low and the oxygen level is optimal in the soil. Alternatively, this may contribute to losses of nitrogen by denitrification when the concentration of combined nitrogen is high and oxygen is limiting.

References

Cataldo, D.A., Haroon, ML, Schrader, L.W., and Youngs, V.L. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Communications in Soil Science and Plant Analysis 6:71-80.

Dobereiner, J., and Day, J.M. 1976. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. Pages 518-538 *in* Proceedings of the First International Symposium on Nitrogen Fixation, 3-7 Jun 1974, Pullman, Washington, USA (Newton, W.E., and Nyman, C.J., eds.). Vol.2. Pullman, Washington, USA: Washington State University Press.

Douglas, L.A., and Bremner, J.M. 1970. Extraction and colorimetric determination of urea in soils. Proceedings, Soil Science Society of America 34:859-862.

Jackson, M.L. 1967. Soil chemical analysis. New Delhi, India: Prentice Hall.

Lowry, P.H., Rosenbrough, N.J., Farr, A.L., and Randall, RJ. 1951. Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193:265-275.

Nelson, L.M., and Knowles, R. 1978. Effect of oxygen and nitrate on nitrogen fixation and denitrification by *Azospirillum brasilense* grown in continuous culture. Canadian Journal of Microbiology 24:1395-1403.

Neyra, C.A., and van Berkum, P. 1977. Nitrate reduction and nitrogenase activity in *Spirillum lipoferum.* Canadian Journal of Microbiology 23:306-310.

Nicholas, D.J.D., and Nason, A. 1957. Determination of nitrate and nitrite. Methods in Enzymology 3:981-984.

Scott, D.B., Scott, C.A., and Dobereiner, J. 1979. Nitrogenase activity and nitrate respiration in *Azospirillum* spp. Archives of Microbiology 121:141-145.

Effect of *Azotobacter chroococcum* and *Azospirillum brasilense* Inoculations and Nitrogen on Yields of Sorghum, Maize, Pearl Millet, and Wheat

B.K. Konde and P.A. Shinde[1]

Summary

Field experiments were conducted during the 1979-82 rainy and postrainy seasons on Vertisols. In general inoculation with Azotobacter chroococcum or Azospirillum brasilense, along with intermediate levels of fertilizer N, significantly increased grain and stover yields of sorghum, maize, pearl millet, and wheat over the fully fertilized noninoculated treatments. Either culture, along with one-third or two-third level of recommended N, generally resulted in increased crop yields, thus saving a third of recommended fertilizer N. Inoculation with either of the cultures was significantly superior to the noninoculated control. In some cases, A. brasilense was superior to A. chroococcum, and in others both cultures were at par with each other.

Introduction

Several workers have reported that field inoculation with *Azospirillum* under diverse soil and environmental conditions resulted in 10-81% increase in cereal crop yields. Yields obtained with inoculation, along with intermediate levels of fertilizer N, were reported to be higher than in fully fertilized noninoculated plots (Desale 1980, Kapulnik et al. 1982, Subba Rao et al. 1982, Patil 1983, Desale and Konde 1984, Zambre et al. 1984). These results suggest that it is possible to save a part of valuable fertilizer N by *Azospirillum* inoculation.

The present paper reports the results of field experiments conducted at Mahatma Phule Agricultural University (MPAU), Rahuri, India, on the effects of *Azotobacter chroococcum* and *Azospirillum brasilense* inoculation with graded levels of fertilizer N on grain and stover yields of sorghum, maize, pearl millet, and wheat.

Materials and Methods

The carrier-based inoculant of *Azospirillum brasilense* (Ab) was obtained from the Department of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi. The lignite-based inoculants of *Azospirillum brasilense* (AMP 5) isolated from the rhizosphere of pearl millet and *A. brasilense* (C2) (10^9 cells g^{-1}) isolated from the roots of *Cynodon dactylon* were prepared in this department. The carrier-based inoculant of *Azotobacter chroococcum* (10^8 cells g^{-1}) was obtained from the Agricultural Bacteriologist, College of Agriculture, Pune, Maharashtra, India.

The seeds were treated with the inoculants of respective organisms at the rate of 250 g (10 kg seed)$^{-1}$ as per the method described by Subba Rao (1981).

The experiments were conducted on Vertisols (pH 7.0-7.5, total N 0.03-0.05%, organic C 0.60%) during

1. Associate Professor and Head, Department of Agricultural Microbiology and Plant Pathology, Mahatma Phule Agricultural University, Rahuri, Maharashtra 413 722, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center. India. Patancheru, A.P. 502 324, India: ICRISAT.

the 1979-82 rainy and postrainy seasons, using factorial randomized-block designs (RBD) with three to four replications. The plot size varied from 9.2 m^2 to 20.26 m^2, depending upon the crop. For sorghum and maize, 62 kg ha^{-1} each of P_2O_5 and K_2O were applied at sowing to all plots. The nitrogen levels 0, 33, 66, and 100 kg N ha^{-1} were applied in two split doses, i.e., two-third at sowing and the remaining one-third one month after sowing.

In the pearl millet experiment, a basal dose of 30 kg P_2O_5 ha^{-1} was applied, while the nitrogen at 20, 40, 60, 80, and 100% of recommended dose (i.e., 12, 24, 36, 48, and 60 kg N ha^{-1}) was applied in two equal doses, one at sowing and the other one month later.

In the wheat experiment, P_2O_5 (60 kg ha^{-1}) and K_2O (50 kg ha^{-1}) were applied at sowing, while N was applied in two split doses, i.e., half at sowing and the rest 25 days after sowing (DAS).

In all cases, N was applied as urea, P_2O_5 as single superphosphate, and K_2O as muriate of potash. All crops received a light irrigation soon after sowing. Weeding, interculturing, and plant-protection operations were carried out as and when required. The stover and grain yields were recorded from each plot at harvest.

Results

Sorghum

Increases in grain yields along with bacterization obtained at the 66 and 100 kg N ha^{-1} levels were not statistically significant. Maximum percentage increase in grain yield was observed at the 33 kg N ha^{-1} level along with bacterization than at higher N levels (Table 1). This showed that higher level of N fertilizer application was detrimental to nitrogen-fixing bacteria. However, higher grain yield was obtained at 66 kg N ha^{-1} along with bacterization than with 100 kg N ha^{-1} alone. This indicated that 66 kg N ha^{-1} could be compatible with either cultures, and there appeared to be a possibility of saving 33 kg N ha^{-1}. Across the nitrogen levels, inoculations with both the cultures significantly increased grain yield over the control and no significant difference could

Table 1. Grain and stover yields of sorghum as influenced by inoculation with nitrogen-fixing bacteria and fertilizer N.

Culture	N level (kg ha^{-1}) 0	33	66	100	Mean
	Grain yield (t ha^{-1})				
Control	4.00	4.19	4.67	5.20	4.52
A chroococcum (Ac)	5.04	5.65	5.94	6.01	5.66
	(26.00)[1]	(34.84)	(27.19)	(15.58)	(25.22)
A. brasilense (Ab)	5.14	5.76	6.09	6.17	5.79
	(28.50)	(37.47)	(30.41)	(18.65)	(28.09)
CD			NS[2]		0.52
Mean	4.72	5.20	5.57	5.80	
CD (P<0.05)			0.52		
	Stover yield (t ha^{-1})				
Control	5.50	6.00	8.00	8.01	6.88
A. chroococcum (AC)	6.67	8.59	8.81	9.02	8.27
	(21.12)	(43.16)	(10.12)	(12.60)	(20.20)
A. brasilense (Ac)	7.51	9.76	11.01	12.01	10.07
	(36.54)	(62.66)	(36.62)	(49.93)	(46.36)
CD (P<0.05)			1.43		0.72
Mean	6.56	8.12	9.27	9.68	
CD (P<0.05)			0.72		

1. % increase over control.
2. NS = Not significant.

be noticed between the two bacteria. The results for the interaction between cultures and N levels were also not statistically significant. In general, trends similar to grain yields were also observed in the case of stover yields. The *Azospirillum* inoculation was found to be significantly superior to *Azotobacter*, since it produced two tonnes more stover yield than *Azotobacter*. The results for interaction between cultures and N levels were significant.

Maize

A significant increase in grain yield could be obtained with 66 and 100 kg N ha^{-1} along with inoculation, but not with 33 kg N ha^{-1} application along with inoculation (Table 2). Differences between 66 kg and 100 kg N along with bacterization were not significant. This showed that both the cultures were compatible at the 66 kg N level and indicated the possibility of saving 33 kg N ha^{-1}. *Azotobacter* and *Azospirillum* produced 49% and 55% higher grain yields over the noninoculated control, and no significant difference could be noticed between them. The results of interaction for cultures and N levels were statistically significant. The results for stover yield also showed a similar trend to those for grain yields.

Pearl Millet

Bacterization under intermediate levels (36, 48, and 60 kg ha^{-1}) of N fertilizer application significantly increased grain yield (Table 3). Highest percentage increase in grain yield was obtained at the 48 kg N level, along with bacterization, with either of the *Azospirillum* strains. The yields obtained at 48 kg N along with bacterization were higher than with 60 kg N ha^{-1} alone. Thus both strains had better compatibility with the 48 kg N level and showed the possibility of saving 12 kg N ha^{-1}. Differences between the two strains as also for the interaction between the strains and N levels were statistically insignificant. The results for stover yields showed a similar trend to those obtained for grain yields.

Table 2. Grain and stover yields of maize as influenced by inoculation with nitrogen-fixing bacteria and fertilizer N.

	N level (kg ha^{-1})				
Culture	0	33	66	100	Mean
	Grain yield (t ha^{-1})				
Control	3.43	3.95	3.98	4.15	3.88
A. chroococcum (Ac)	3.60	5.60	6.98	7.07	5.81
	(4.95)[1]	(41.86)	(75.38)	(70.36)	(49.74)
A. brasilense (Ab)	3.90	5.81	7.20	7.27	6.40
	(13.70)	(47.18)	(80.90)	(75.18)	(55.67)
CD (P<0.05)			2.65		1.33
Mean	3.64	5.12	6.05	6.16	
CD (P<0.05)			1.33		
	Stover yields (t ha^{-1})				
Control	3.90	5.12	6.08	7.22	5.58
A. chroococcum (Ac)	4.71	6.22	7.47	7.94	6.58
	(5.36)	(21.48)	(22.86)	(9.97)	(17.92)
A. hrasilense (Ab)	4.80	6.53	7.94	8.55	6.95
	(7.38)	(27.53)	(30.60)	(18.42)	(24.55)
CD (P<0.05)			1.04		0.52
Mean	4.47	5.%	7.16	7.90	
CD (P<0.05)			NS[2]		

1. % increase over control.

2. NS = Not significant.

Table 3. Grain and stover yields of pearl millet as influenced by inoculation with nitrogen-fixing bacteria and fertilizer N.

Culture	N level (kg ha^{-1}) 12	24	36	48	60	Mean
	Grain yield (t ha^{-1})					
Control	1.70	1.82	2.19	2.30	2.48	2.10
A. brasilense (AMP 5)	1.80	1.94	2.32	2.65	2.60	2.26
	(5.88)[1]	(6.59)	(5.94)	(15.22)	(4.84)	(7.62)
A. brasilense (Ab)	1.78	1.92	2.30	2.72	2.63	2.27
	(4.77)	(5.49)	(5.02)	(18.26)	(6.05)	(8.09)
CD (P<0.05)				NS[2]		0.16
Mean	1.76	1.89	2.27	2.56	2.57	-
CD(P<0.05)				0.26		
	Stover yield (t ha^{-1})					
Control	2.53	2.55	2.60	3.07	3.22	2.79
A. brasilense (AMP 5)	2.60	2.64	2.73	3.36	3.42	2.95
	(2.77)	(3.53)	(5.00)	(9.44)	(6.21)	(5.74)
A. brasilense (Ab)	2.59	2.63	2.75	3.50	3.50	2.99
	(2.37)	(3.14)	(5.76)	(14.01)	(6.69)	(7.17)
CD				NS		NS
Mean	2.57	2.61	2.69	3.31	3.38	
CD (P<0.05)				0.16		

1. % increase over control.
2. NS = Not significant.

Wheat

The *Azospirillum* inoculation recorded a mean grain yield of 3.04 t ha^{-1} over different levels of N fertilizer application, followed by *Azotobacter,* 2.90 t ha^{-1}, as compared to 2.50 t ha^{-1} in the noninoculated control (Table 4). The interaction effects were found to be statistically significant. The interaction effect of the 120 kg N ha^{-1}, level with *Azospirillum* (4.19 t ha^{-1}) and *Azotobacter* (4.01 t ha^{-1}) was at par and superior to all other remaining treatment combinations. The highest grain yield was obtained at 120 kg N ha^{-1}, but

Table 4. Grain yield of wheat as influenced by inoculation with nitrogen-fixing bacteria and fertilizer N.

Culture	N level (kg ha-') 12	24	36	48	60	Mean
	Grain yield (t ha^{-1})					
Control	1.58	2.07	2.53	2.91	3.41	2.54
A. chroococcum (Ac)	1.66	2.40	2.97	3.47	4.01	2.90
	(5.06)'	(15.94)	(17.39)	(19.24)	(17.59)	(16.00)
A. brasilense (Ab)	1.76	2.49	3.12	3.66	4.19	3.04
	(11.39)	(20.28)	(23.32)	(25.77)	(22.87)	(21.60)
CD (P<0.05)				0.18		0.093
Mean	1.67	2.32	2.87	3.35	3.87	
CD (P<0.05)				0.093		

1. % increase over control.

highest increase over noninoculated control due to *Azotobacter* was 19.24% and due to *Azospirillum* 25.77%, in combination with 90 kg N ha^{-1}. This is obvious, since increase in fertilizer doses at a certain level decreases a crop's response. Inoculation with *Azotobacter* and *Azospirillum* over N levels produced 16 and 21% higher grain yields on an average over the noninoculated control.

Conclusions

The results indicate that about one-third of the recommended level of fertilizer N could be saved if seed bacterization with either cultures is done before sowing. This is of particular significance to those farmers who are not in a position to invest in fertilizers. In addition to the known benefits from associative nitrogen fixation (Wani 1986), the increase in crop yields have also been attributed to the production of growth-promoting substances by either of these organisms (Tien et al. 1979, Vlassak and Reynders 1981) and also due to the positive effects of *Azospirillum* root development and root uptake of minerals and water (Kapulnik et al. 1982). In the present investigation *Azospirillum brasilense* was found to be either superior to *A. chroococcum* or at par with it. The interaction effects on grain yields between N fertilizer levels and seed bacterization with either cultures were observed to be insignificant in sorghum and pearl millet, but significant in maize and wheat.

Priorities for Future Research

Based on results of the present experiments the following lines of work are suggested:

1. Response of crop genotypes to *Azospirillum* should be tested under field conditions. This information would be useful for breeding genotypes having enhanced nitrogen-fixing ability.
2. Isolation and screening of diazotrophic azospirilla strains having high nitrogenase activity from a wide range of hosts including cereals, vegetables, grasses, sugarcane, potato, ginger, turmeric, etc., should be undertaken.
3. Compatibility of *Azospirillum* with seed-dressing fungicides, insecticides, and fertilizer nitrogen should be studied.

Acknowledgements

The authors express their sincere thanks to Dr N.S. Subba Rao, Head, Division of Microbiology, IARI, New Delhi, and Dr P.L. Patil, Agricultural Bacteriologist, College of Agriculture, Pune, for supply of *Azospirillum* and *Azotobacter* inoculants. They also express their thanks to Ms M.A. Zambre, Mr P.H. Patil, and late Mr A.G. Desale, postgraduate students on whose research this paper has been based, for their help in the present investigation.

References

Desale, A.G. 1980. Effect of seed bacterization with *Azotobacter* and *Azospirillum* cultures under various levels of nitrogen on growth, and yield of sorghum and maize. M.Sc. thesis, Mahatma Phule Agricultural University, Rahuri, Maharashtra, India.

Desale, A.G., and Konde, B.K. 1984. Response of sorghum to seed bacterization with nitrogen levels. Journal of Maharashtra Agricultural Universities 9:169-170.

Kapulnik, Y., Sarig, S., Nur, I., Okon, Y., and Henis, Y. 1982. The effect of *Azospirillum* inoculation on growth and yield of corn. Israel Journal of Botany 31:247-255.

Patil, P.H. 1983. Response of some pearl millet genotypes to *Azospirillum* inoculation and effect of inoculation under graded levels of nitrogen on growth and yield of pearl millet cv. BJ 104. M.Sc. thesis, Mahatma Phule Agricultural University, Rahuri, Maharashtra, India.

Subba Rao, N.S. 1981. Response of crops to *Azospirillum* inoculation in India. Pages 137-144 *in* Associative N_2-fixation: proceedings of the International Workshop on Associative N_2-fixation, 2-6 Jul 1979, Piracicaba, Brazil (Vose, P.B., and Ruschel, A.P., eds.). Vol.1. Boca Raton, Florida, USA: CRC Press.

Subba Rao, N.S., Tilak, K.V.B.R., Singh, C.S., and Gautam, R.C. 1982. Crop yield and nitrogen content of pearl millet (*Pennisetum americanum*) in response to *Azospirillum brasilense.* Pages 507-516 *in* Proceedings of the National Symposium on Biological Nitrogen Fixation, 25-27 Feb 1982, New Delhi, India. Bombay, India: Bhabha Atomic Research Centre.

Tien, T.M., Gaskins, M.H., and Hubbell, D.H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet *(Pennisetum americanum* L.). Applied and Environmental Microbiology 37:1016-1024.

Vlassak, K., and Reynders, L. 1981. *Azospirillum* rhizocoenoses in agricultural practice. Page 494 *in* Current perspectives in nitrogen fixation: proceedings of the International Symposium on Nitrogen Fixation, 1-5 Dec 1980, Canberra, Australia (Gibson, A.H., and Newton, W.E., eds.). Canberra, Australia: Australian Academy of Science.

Wani, S.P. 1986. Cereal nitrogen fixation : problems and potentialities. Pages 7-21 *in* Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: International Crops Research Institute for the Semi-Arid Tropics.

Zambre, M.A., Konde, B.K., and Sonar, K.R. 1984. Effect of *Azotobacter chroococcum* and *Azospirillum brasilense* inoculation under graded levels of nitrogen on growth and yield of wheat. Plant and Soil 79:61-67.

Discussion

G.S.Murty:
At what level of N-application was the saving of one-third to two-third of the recommended nitrogen dose observed, 20 kg ha^{-1}, 40 kg ha^{-1}, or more? The recommended dose for sorghum and maize is 100 kg N ha^{-1}.

B.K. Konde:
Saving of one-third N (33 kg N ha^{-1}) has been observed in sorghum and maize and a saving of 20% N (12 kg N ha^{-1}) in pearl millet and one-third N (30 kg N ha^{-1}) in wheat.

P.V. Rai:
We need to look for the quality of protein in the grains from the inoculated treatment, although it always gives higher protein.

B.K. Konde:
This may be included in our research projects. Yes, we have also already estimated the protein content of grains in all crops, but the data presented are confined to fodder and grain yields and N content in plant tissues, owing to limitation of time.

G.S. Jadhav:
Small plot size may be the reason for very high yield.

B.K. Konde:
The net plot size ranged from 9 to 20 m^2.

N.S. Subba Rao:
At the higher level of fertilizer nitrogen application, the amount of fertilizer N left behind unutilized is not known. Simple arithmetic calculation of savings is convenient to present, but deductions for residual nitrogen may have to be done.

B.K. Konde:
Well, we have not estimated the quantum of fertilizer nitrogen left behind; however, this suggestion will be taken into consideration while planning further experiments.

S.P. Wani:
Higher N percentage in plant tissues and increased biomass will result in very high N uptake. The data need to be rechecked for unusually high values.

B.K. Konde:
Yes, 1 agree with your comments but can't deny the facts that we observed. However, in future experiments, we shall take due care to ensure the proper N estimation of plant tissues.

V.R. Rao:
In your studies on sorghum, the percentage N in plant tissues due to inoculation increased from 0.3% in the control to 0.8%. Could you throw some light on this?

B.K. Konde:
This increase is attributed to biologically fixed N, besides the fertilizer nitrogen supplied through the soil in the form of urea. These data, as suggested by other members, will be checked.

S.T. Shende:
Why was the inoculation effect at par with high nitrogen application? Was it due to low efficiency of N application during that season?

B.K. Konde:
It is attributed to the reason that higher level of N fertilizer application might have depressed the nitrogenase activity of the organisms, although increased levels of N fertilizer alone also increased yields and their parameters.

G. Oblisami:

In your data on inoculation of maize with *A. chroococcum* and *Azospirillum brasilense,* the zero level of fertilizer nitrogen gave higher grain yields than 100% fertilizer nitrogen alone. How do you explain this phenomenon?

B.K. Konde:

I think the reasons might be (1) high organic matter (organic C 0.6%) content of the soil, (2) high rates of fertilizer nitrogen depressing nitrogenase activity, and (3) more growth-promoting substances being produced in the absence of higher levels of fertilizer N.

Root-Associated Nitrogen Fixation in Finger Millet

M.N. Upadhyaya[1], S.V. Hegde[2], P.V. Rai[2], and S.P. Wani[3]

Summary

This paper presents the results of investigations carried out on some aspects of root-associated nitrogen fixation in finger millet (Eleucine coracana Gaertn). Soil-root nitrogenase assay of 53 finger millet cultivars belonging to four maturity groups indicated (1) low levels of nitrogenase activity, (2) high plant-to-plant variability, (3) high variability between cultivars, (4) higher activity in hybrids over pure lines, (5) higher activity in medium-maturity group over early- or late-maturity groups, and (6) no apparent relationship between nitrogenase activity and dry-matter production. Nitrogenase activity of finger millet soil-root system was further affected by plant age, soil moisture, and diurnal changes. All cultivars had highest diazotroph counts during the reproductive stage in root-adhering soil, root wash, and unsterilized root macerate. The root-associated nitrogen fixers were species of Pseudomonas and Enterobacter. Inoculation of finger millet cultivar Indaf 7, with NBRE and SRI diazotroph cultures, increased above-ground yields and N uptake over the control in the greenhouse, but not in the field. Nitrogen-balance study with inoculation and three levels of applied nitrogen showed a net gain equivalent to 63-9.4 kg N ha^{-1} from inoculation.

Introduction

Finger millet *(Eleusine coracana* Gaertn) is an important food crop in the semi-arid tropics (SAT), particularly in India and Eastern Africa. The crop ranks fourth in area (5 million ha) and production (4.5 million t) among millets in the world. India accounts for 45% of the world production.

Contributions of nonsymbiotic nitrogen fixation associated with finger millet and other millets, though felt to be substantial, have been wide ranging (20-148 kg N ha^{-1}) due to different methodologies used by different workers (Moore 1963, Jenkinson 1977, Dart and Wani 1982). Studies were conducted at the University of Agricultural Sciences, Bangalore, on (1) root-associated nitrogenase activity of 53 finger millet cultivars, (2) occurrence of diazotrophs, (3) inoculation response, (4) factors affecting nitrogen fixation, and (5) nitrogen balance.

Screening Germplasm

Fifty-three cultivars representing both pure-line selections and hybrids in four maturity groups were screened for their ability to stimulate nitrogenase activity (C_2H_2 reduction) in the rhizosphere at the flowering and postflowering growth stages (Table 1). Unplanted soil and finger millet cultivars Indaf 5 were included as checks in each group. The plants were grown in polythene bags filled with 3.3 kg soil. The soil surface in each bag was covered with a 5-cm thick gravel, and the bags were kept immersed in a sand bed to avoid algal growth at the soil surface and

1. PhD Student, Australian National University, Canberra, Australia.
2. Senior Microbiologist, and Professor and Head, Department of Agricultural Microbiology, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra (GKVK), Bangalore, Karnataka 560 065, India.
3. Microbiologist, Pearl Millet improvement Program, ICRISAT, Patancheru, A.P. 502 324, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

at the interface of soil and bag. A basal dose of 50 kg each of N, P_2O_5, and K_2O ha^{-1} equivalent was applied through ammonium sulfate, superphosphate, and potassium chloride respectively. Plants were watered as required with tap water and one day before assay the moisture content of the bags was adjusted to 70-80% water-holding capacity (WHC). For nitrogenase assay, the method followed by Wani et al. (1983) was used.

The general level of nitrogenase activity associated with 53 finger millet cultivars was low, and the group means ranged from 2 to 267 nmoles C_2H_4 h^{-1} bag^{-1} or 1 to 59 nmoles h^{-1} g^{-1} root mass (Table 1). Ranking of cultivars based on root activity differed when expressed as g^{-1} root than on bag^{-1} basis. The mean nitrogenase activity of 0.2-13 nmoles h^{-1} bag^{-1} in the unplanted soil suggested stimulation of nitrogenase activity by the plant. Increased nitrogenase activity in Assay II over Assay I in maturity groups I and II indicated higher activity during post-flowering growth stages. The maximum nitrogenase activity of individual plants in this study was 488 nmoles C_2H_4 h^{-1} bag^{-1}. This is much lower than the activity of 1064 nmoles C_2H_4 h^{-1} $core^{-1}$ recorded for finger millet cultivar PR 202 at ICRISAT Center (ICRISAT 1978) and 374 nmoles C_2H_4 h^{-1} g^{-1} dry mass by Dommergues et al. (1973).

Within a cultivar, plant-to-plant variation in nitrogenase activity from 10 to 488 nmoles C_2H_4 h^{-1} bag^{-1} in cultivar TNAU 169 and 5-359 nmoles C_2H_4 h^{-1} bag^{-1} in cultivar Indaf 5 was observed. Such variations have also been recorded in sorghum and pearl millet (Wani et al. 1983).

Of the 53 cultivars tested, the activity was more

Table 1. Nitrogenase activity (nmoles C_2H_4 h^{-1})[1] of soil-root system of finger millet cultivars during two assays.[1]

Maturity group (Days to 50% flowering)	Cultivar	Assay 1			Assay II		
			Range/Cultivar[2]			Range/Cultivar[2]	
		DAS[3]	bag^{-1}	g^{-1} root	DAS	bag^{-1}	g^{-1} root
I (56-62)	VL 114,VL 115,VL 116 VL 118,VL 110, PES 83-2,PES 400 RAU 3,RAU 7,RAU 5,RAU 2, REC 45-1, REC 13-1	55	12-70 REC 13-1 REC 45-1 (38)	2-30 REC 13-1 VL 115	62	10-138 REC 45-1 PES 83-2 (68)	4-46 REC 45-1 PES 83-2
II (63-70)	VL 117,Indaf 9,HR 374, PR 177,PR 1103, PR 1091, PES 176, TNAU 169,TNAU 167, TNAU 193, TNAU 256, TNAU 160, TNAU 294	70	2-30 TNAU 160 TNAU 169 (13)	1-11 TNAU 160 TNAU 169	92	56-267 VL 117 TNAU 169 (131)	8-59 PR 1103 PR 1091
HI (71-78)	HR 919,HR 154,HR 2823 ROH 2, HR 1541, Purna,PR 230, PR 202, B 7-7-43,PES 110, JNR 981-1 JNR 852,JNR 1008		ND[4]	ND	94	21-166 PR 230 B 7-7-43 (85)	3-24 PR 230 B 7-7-43
IV (79-89)	Indaf 1, Indaf 3, VR 550, Indaf 7,Indaf 8, Indaf 10, Indaf 11,VR 530 KM 13, IE 1012, U 6, U 10 (51) A 104		ND	ND	99	19-12 Indaf 8 IE 1012	4-32 Indaf 8 Indaf 10

1. Mean of 4 replications. Indaf 5 and implanted soil were used as checks. Nitrogenase activity of unplanted control ranged from 0.2 to 13 nm C_2H_4 bag^{-1}.
2. Mean nitrogenase activity.
3. PAS = Days after sowing.
4. ND = Not determined.

than 300 nmole C_2H_4 h^{-1} bag^{-1} in 12 cultivars, between 200 to 300 in 8 cultivars, between 100 to 200 nmol in 20 cultivars, and less than 100 in 13 cultivars. Such variability for nitrogenase activity amongst finger millet cultivars has also been observed earlier (ICRISAT 1978). The top five cultivars, based on maximum activity, were TNAU 169, TNAU 256, Purna, IE 1012, and Indaf 9, in that order. Least activity was found in RAU 7. Generally, hybrid selections showed higher nitrogenase activity than pure-line selections (Table 2).

The medium-maturity cultivars (groups II and III) had higher nitrogenase activity than either early- (group I) or late-maturity cultivars (group IV). Mishra et al. (1980) had also found that medium-maturity cultivars were more productive than early or late types, based on their screening of 400 finger millet cultivars.

Seasonal Variation in Diazotroph Counts and Nitrogenase Activity

Plants of cultivar Indaf 5 were similarly grown in polythene bags, except that 2.75 kg soil was used in this experiment. The plants were inoculated with 50 mL rhizosphere-soil extract. This extract was prepared by mixing 1 kg rhizospheric soil collected from the field with 4 L N-free sucrose medium, incubated for 42 h at room temperature, filtered through a cheese cloth, and finally made up to 10 L using water. An unplanted treatment was also included. Six randomly selected plants were assayed for nitrogenase at 22, 32, 44, 59, 68, 81, 105, and 129 days after sowing (DAS), following the method mentioned earlier.

Nitrogenase activity increased from 22 DAS (9 nmol C_2H_4 h^{-1} bag^{-1}) to peak at 68 DAS (106 nmol C_2H_4 h^{-1} bag^{-1}) and then decreased to 36 nmoles C_2H_4 h^{-1} bag^{-1} at 105 DAS (Fig. 1).

For estimating the counts of diazotrophs, two randomly selected bags each, i.e., planted and unplanted, were selected each time after assay. Increase in nitrogenase activity of the soil-root system closely followed increase of diazotroph counts upto 68 days, particularly the diazotroph count of nonsterilized and sterilized root samples. Unlike the nitrogenase activity that decreased 68 DAS, the diazotroph count remained largely unchanged. Assay at 68 DAS gave both maximum nitrogenase activity (129 nmol C_2H_4 h^{-1} bag^{-1}) as well as maximum diazotroph count of sterilized (4×10^9 g^{-1} root) and nonsterilized (2×10^{11} g^{-1} root) macerate.

In studies with cultivar Indaf 5, the diazotroph counts were in general higher during the reproductive stage than during the vegetative stage. Root-adhering soil gave the maximum diazotroph count (8.5×10^9) g^{-1} dry root, followed by nonsterilized root macerate (3.4×10^9), and root wash (3.1×10^9). Surface-sterilization of root reduced the count from 3.4×10^9 to 1.6×10^8 (g root mass)$^{-1}$ for 5 min sterilization, and 1.3×10^6 (g root mass)$^{-1}$ for 60 min sterilization. The number of colony types of diazotrophs did not increase from the vegetative to repro-

Table 2. Screening of pure-line and hybrid selections of different origin for the root-associated nitrogenase activity.

Origin	Pedigree	No. of cultivars tested	No. of cultivars having nitrogenase activity (nmol C_2H_4 $plant^{-1}$ h^{-1}) >300	200-300	100-200	<100	Most active cultivar	Highest nitrogenase activity (nmol C_2H_4 $plant^{-1}$ h^{-1})
Karnataka	Hybrid	15	5	4	2	4	Indaf 9	361
Tamil Nadu	Hybrid	6	3	0	3	0	TNAU 169	488
Andhra Pradesh	Pure line	7	0	1	3	3	PR 1091	250
Madhya Pradesh	Pure line	5	1	1	2	1	JNR 852	354
Uttar Pradesh	Pure line	11	1	2	6	2	PES 83-2	324
Bihar	Pure line	4	0	0	2	2	RAU 5	183
Orissa	Pure line	2	1	0	1		B 7-7-43	314
Exotic collection	Pure line	3	1	0	1	1	IE 1012	403
Total		53	12 (23)*	8 (15)	20 (37)	13 (25)		

1. Figures in parentheses are percentages.

ductive growth stage. A maximum of 15 colony types were observed in nonrhizospheric soil, and surface-sterilized root macerate showed 5 to 6 colony types. A total of 40 isolates that grew consistently on N-free medium were finally obtained in pure cultures.

When the 40 diazotroph isolates from cultivar Indaf 5 were screened for ARA, all but 3 isolates

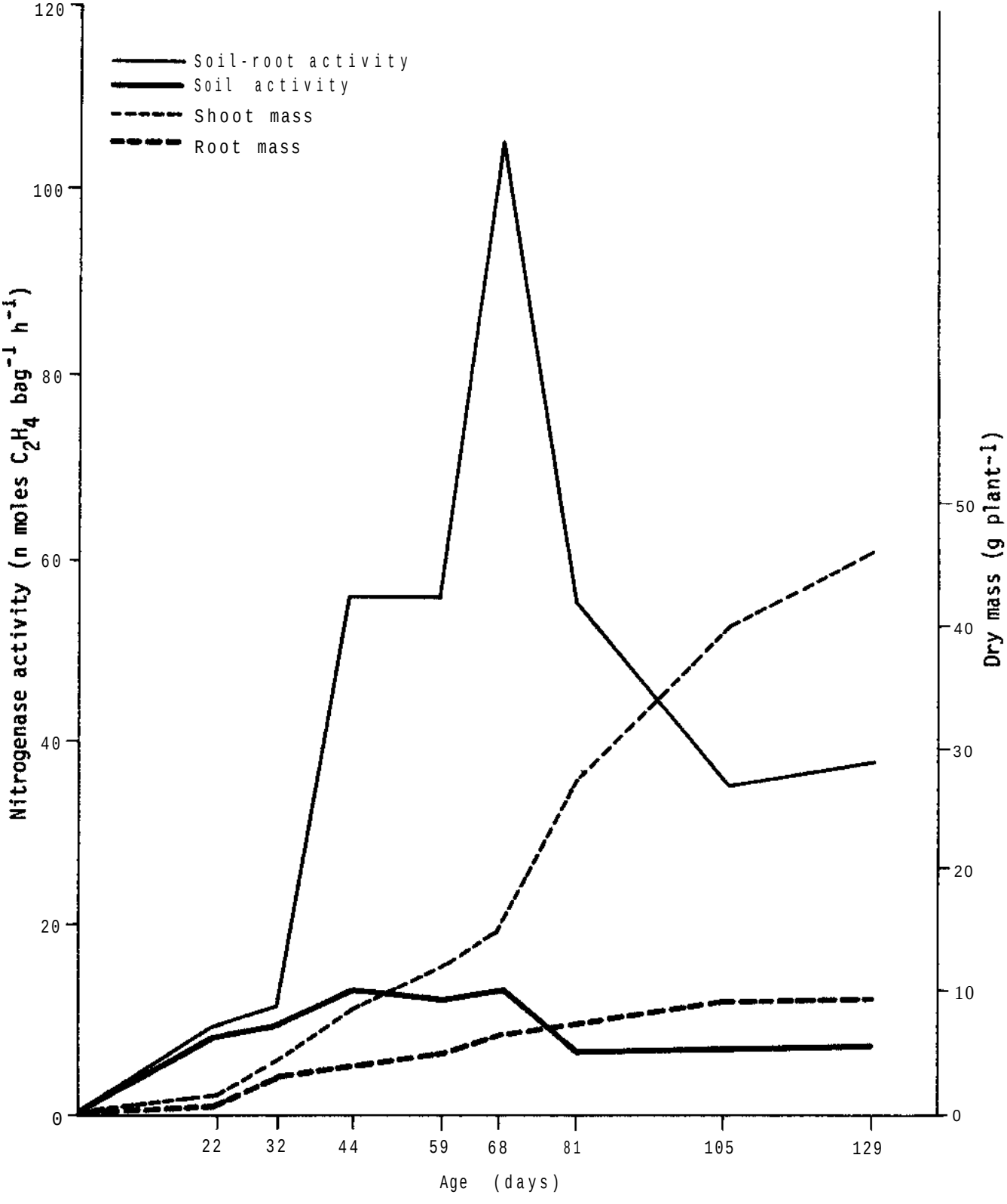


Figure 1. Seasonal pattern of nitrogenase activity and shoot and root mass of finger millet cultivar Indaf 5.

produced less than 2 nmoles C_2H_4 h^{-1} $tube^{-1}$. Of these three isolates, No. 38 and 45 were identified as *Enterobacter cloacae* and Isolate No. 23 was *Azospirillum-like.* The rest of the isolates were species of *Pseudomonas* or *Enterobacter cloacae.* Isolates from finger millet were compared with three reference cultures, i.e., NBRE (napier bajra root extract), *A. lipoferum* (4ABL), and *Azospirillum lipoferum* (ICM 1001) for *in-vitro* activity. The cultures NBRE and 4ABL showed four-fold more activity than the isolates from finger millet. However, E. *cloacae* showed higher activity than *A. lipoferum.* Highest activity (115 nmoles C_2H_4 h^{-1} $tube^{-1}$) was recorded in the NBRE culture.

Effect of Soil Moisture

Effect of moisture on nitrogenase activity of finger millet Indaf 5 was studied by manipulating the moisture regime in the soil and by using an intact-plant assay method (Wani et al. 1984). The plants were grown in soil in plastic bags, and a basal dose of 25 kg N and 50 kg each of P_2O_5 and K_2O ha^{-1} through urea, superphosphate, and potassium chloride was applied. Till the moisture-level treatments were imposed, all the plants were watered uniformly. Nitrogenase activity of the soil-root system as affected by six moisture levels (20% WHC to 90% WHC) increased with increasing soil moisture. There was no significant change in nitrogenase activity between moisture levels varying from 19% to 47% WHC. Linear regression of average nitrogenase activity against soil-moisture content indicated a significant correlation (r=0.788).

Diurnal Variation

Ten plants of cultivar Indaf 5 were grown in 6-L plastic pots containing 6 kg alluvial soil. A basal dose of 25 kg N and 50 kg each of P_2O_5 and K_2O was applied. The conditions for growing plants were similar to those described by Wani et al. (1984). Nitrogenase activity was estimated using intact-plant assay technique (Wani et al. 1984). Acetylene gas was injected at 1200 and gas samples were collected at 2 h intervals up to 2400. Soil temperature in the pot at each assay time was recorded. The diurnal pattern of nitrogenase activity is shown in Figure 2. Maximum activity of 104 nmol C_2H_4 h^{-1} $plant^{-1}$ was recorded between 10 am and 12 noon.

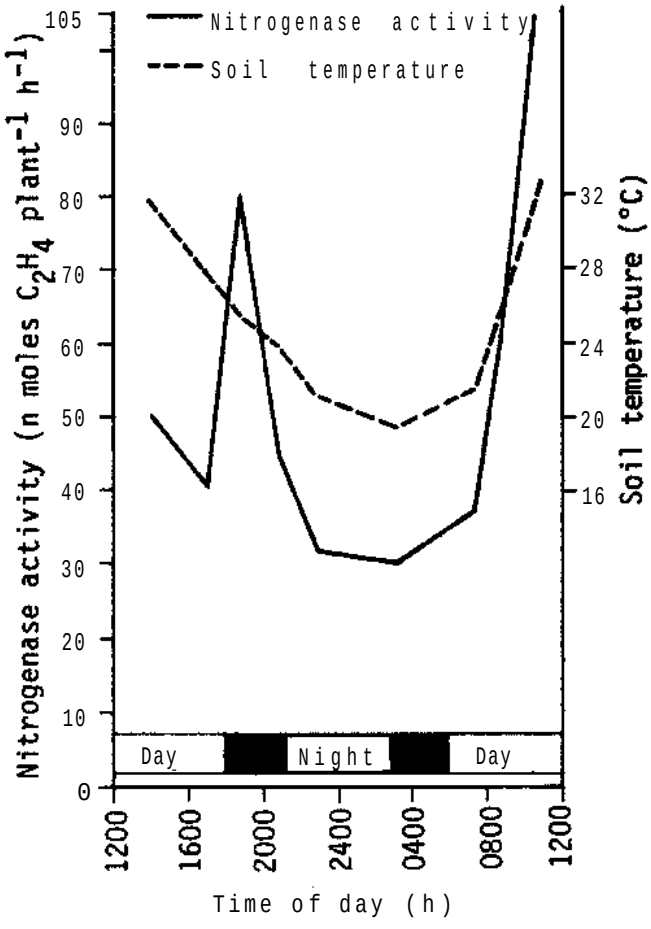


Figure 2. Diurnal pattern of nitrogenase activity of an intact-plant system of finger millet cultivar Indaf 5.

Response to Inoculation with Nitrogen-Fixing Bacteria

Response of finger millet cultivar Indaf 7 to seed inoculation with diazotroph cultures (obtained from ICRISAT Center) was studied at 25 and 50 kg ha^{-1} levels of applied nitrogen, under greenhouse and field situations. In the greenhouse, plants were raised in 25 cm x 15 cm iron cores filled with 3/75 kg alluvial soil. The basal dose consisted of a part of the N along with 50 kg each of P_2O_5 and K_2O. The remaining N was applied as a top dressing. In each case, a single plant was grown. The seeds were soaked for 18 h in broth culture of bacteria as per the treatment and dried. At sowing, each core was inoculated with 8 mL broth culture. The noninoculated broth served as the control. Surface soil in the cores was covered with gravel and the moisture content in the cores was maintained at around 70% WHC.

The grain and straw yields increased significantly

over the control due to inoculation with NBRE (37%) and SRI (35%) (Fig. 3). Inoculation with *A. lipoferum* (4 ABL) increased leaf number but not plant height over the control. Nitrogen alone or interaction of N x inoculation had no significant effect on yield parameters. Nitrogen content of grain increased 50% from inoculation with NBRE and 47% with SRI; total uptake of nitrogen by the plant increased 72% with NBRE and 66% with SRI. Inoculation had no effect on nitrogen content of straw. Interaction of N x inoculation (NBRE) increased N content of grain by 1%, while N alone had no effect.

In the field experiment, all the cultures used in the pot trial except the SRI isolate were used. The experiment was conducted following randomized-block design and 2 m x 2 m plots for each treatment, replicated four times. The N, P, and K treatments were as in the case of the pot experiment. Seeds were soaked in peat-inoculant suspension for 18 h, air dried and sown in rows 22.5 cm apart. Each plot was inoculated at sowing with peat-inoculant suspension (1 g peat inoculant suspended in 500 mL water) uniformly. Inoculation of finger millet with all the cultures of N_2-fixing bacteria increased grain or straw yields by 8-10% over the control plants, but the increase was not significant (Fig. 4). Application of N also gave nonsignificant yield increases over the control plants. Highest above-ground yield (5552 kg ha^{-1}) was obtained with *A. Lipoferum* (ICM 1001) inoculation + 50 kg N ha^{-1}, followed by NBRE culture + 25 kg N ha^{-1} (5168 kg ha^{-1}). Nitrogen content of finger millet straw increased by 0.3% due to inoculation with NBRE and N uptake increased by 12 kg ha^{-1} (Fig. 5). Application of 50 kg N ha^{-1} increased grain nitrogen uptake by 25 kg ha^{-1}, but had no effect on straw nitrogen content.

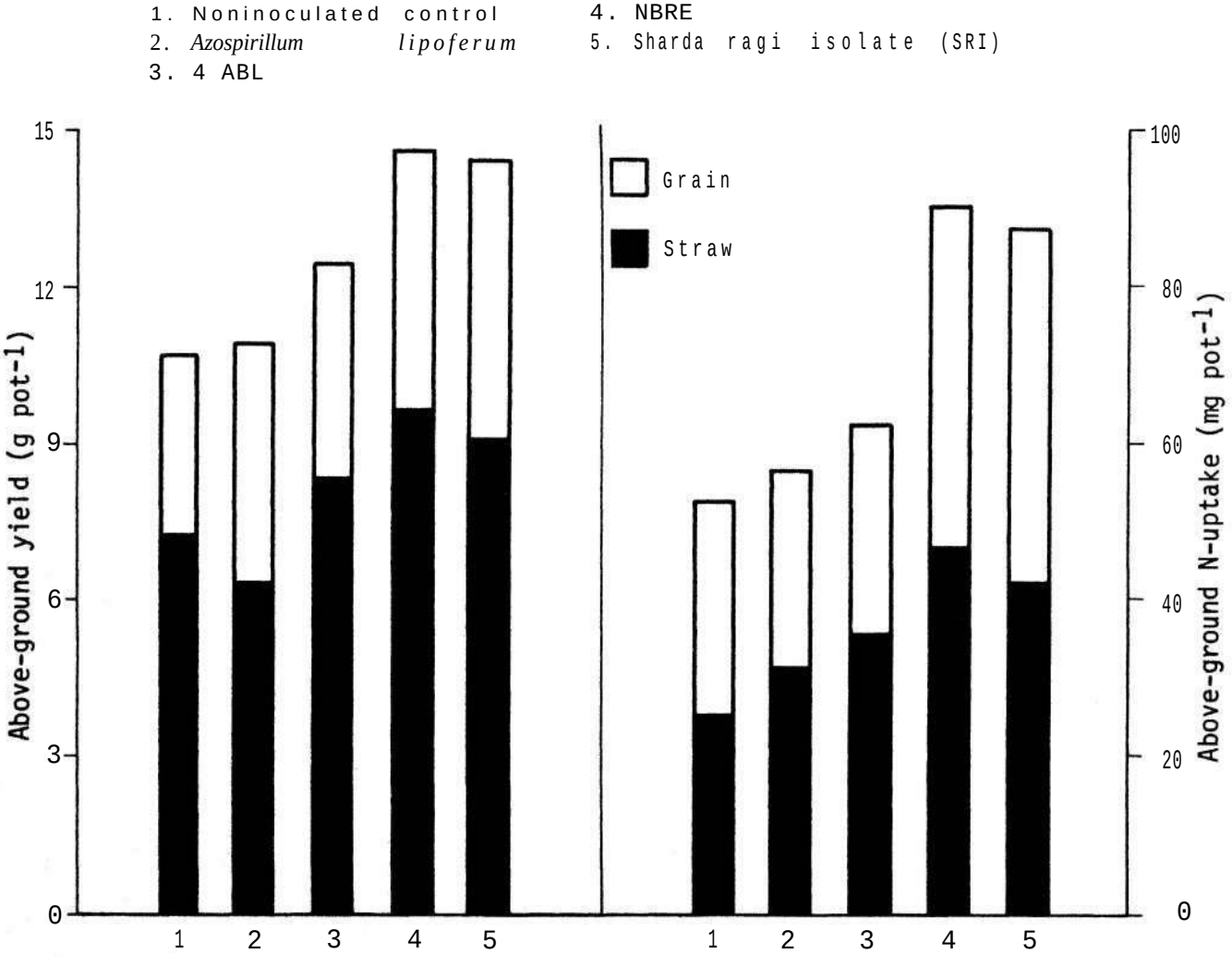


Figure 3. Response of finger millet cultivar Indaf 7 to inoculation with diazotrophs in a pot trial.

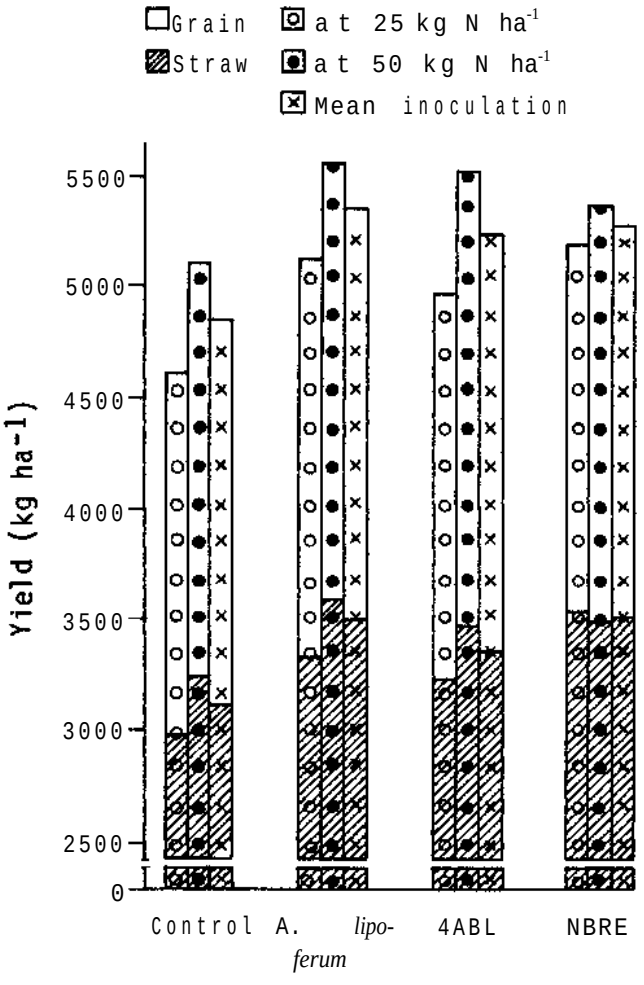


Figure 4. Response of field-grown finger millet cultivar Indaf 7 with nitrogen levels to inoculation with diazotrophs.

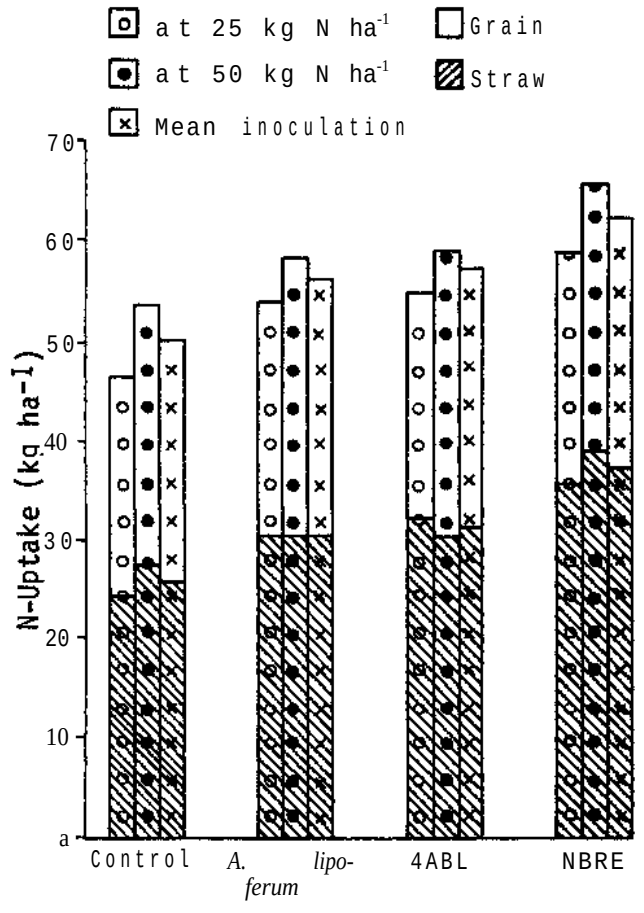


Figure 5. Nitrogen uptake by field-grown finger millet cultivar Indaf 7 as influenced by nitrogen levels and inoculations with diazotrophs.

Nitrogen Balance

Nitrogen balances (postharvest N-presowing N in soil) due to the growth of finger millet Indaf 5 as affected by planting, inoculation, and three levels of nitrogen (0, 25, and 50 kg ha^{-1}) were studied in pot culture in the greenhouse (Fig. 6). Maximum positive nitrogen balance (200 mg por^{-1}) was found in inoculated finger millet at the 0 level applied-N, which also showed the least loss of soil nitrogen (5 mg pot^{-1}). Maximum loss of soil nitrogen was found in the unplanted pot with 50 kg ha^{-1} applied N. Final N-balances in inoculated and planted systems were 200, 168, and 198 mg pot^{-1}. In the unplanted system, there were negative balances of 11, 58, and 93 mg pot^{-1} respectively at the 0, 25, and 50 kg applied-N levels. Even though inoculated pots showed a higher balance (188 mg por^{1}) than the noninoculated pot (161 mg pot^{-1}), the difference was not significant. Planting Indaf 5 resulted in an N gain of 175 mg pot^{--1}, whereas in the unplanted system there was a loss of 49 mg N pot^{-1}. The system gains of N in pots due to inoculation extrapolated to a net gain of 6.3, 7.6, and 9.4 kg N ha^{-1}, respectively, at the 0, 25, and 50 kg ha^{-1} applied-N levels.

References

Dart, P.J., and Wani, S.P. 1982. Non-symbiotic nitrogen fixation and soil fertility. Pages 3-27 *in* Non-symbiotic nitrogen fixation and organic matter in the tropics. Symposia papers 1. Transactions of the 12th International Congress of Soil Science, 8-16 Feb 1982, New Delhi, India. New Delhi, India: Indian Agricultural Research Institute.

Day, J.M., Neves, M.C.P., and Dobereiner, J. 1975. Nitrogenase activity on the roots of tropical forage grasses. Soil Biology and Biochemistry 7:107-112.

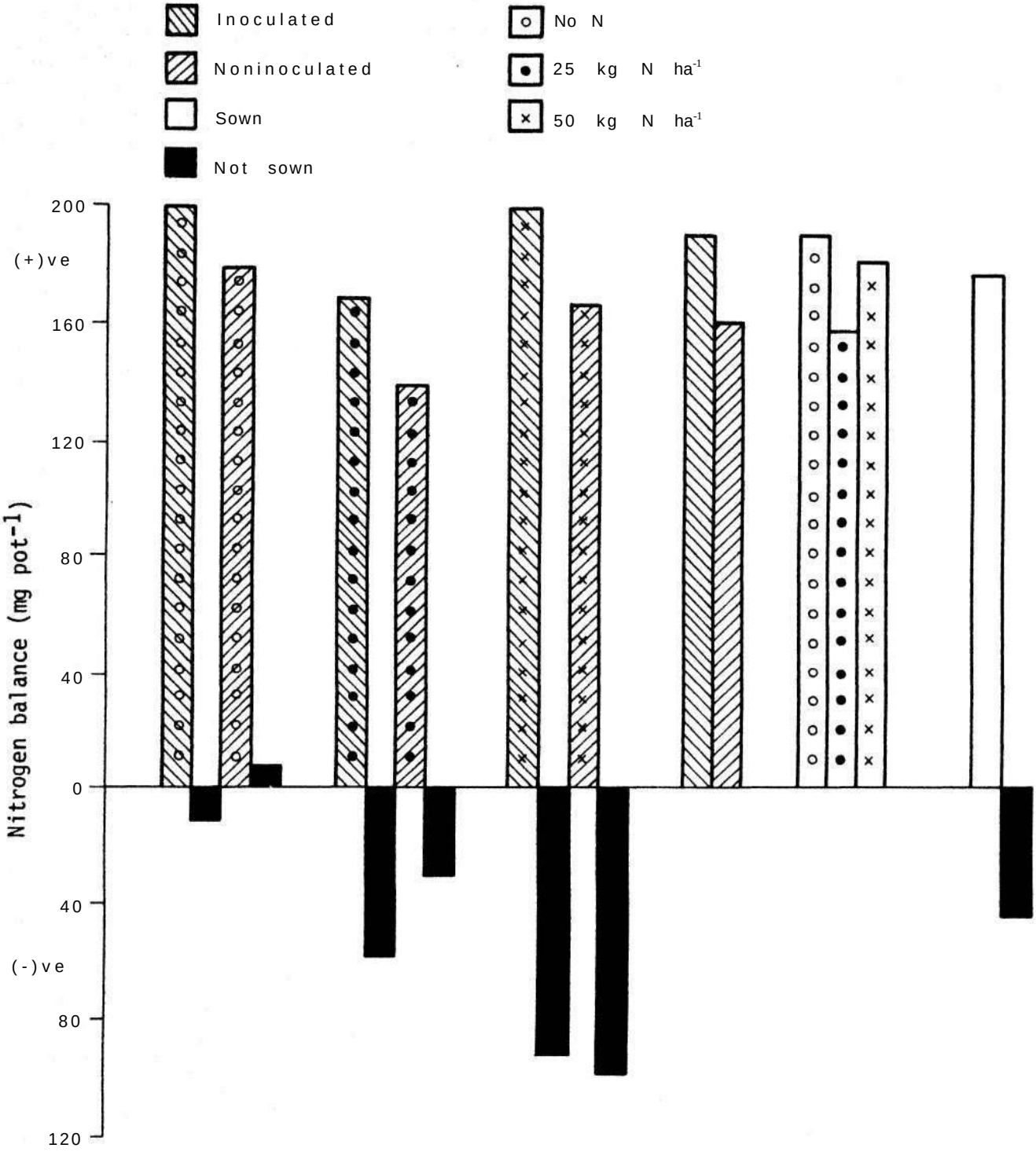


Figure 6. Nitrogen balance in pot-grown finger millet cultivar Indaf S as affected by nitrogen and inoculation (95-day-old plants).

Dommergues, Y., Balandreau, J., Rinaudo, G., and Weinhard, P. 1973. Non-symbiotic nitrogen fixation in the rhizosphere of rice, maize and different tropical grasses. Soil Biology and Biochemistry 5:83-89.

Hardy, R.W.F., Burns, R.C., and Holsten, R.D. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biology and Biochemistry 5:47-81.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1978. Annual report 1977-78. Patancheru, A.P 502 324, India: ICRISAT.

Jenkinson, D.S. 1977. The nitrogen economy of Broadbalk experiments. I. Nitrogen balance in the experiments. Report for 1976, Rothamsted Experimental Station 2:103-109.

Mishra, H.P., Patnaik, M.C., and Nayak, B.K. 1980. Variation in quantitative characters and their association with qualitative traits in finger millet. Indian Journal of Agricultural Sciences 50:298-301.

Moore, A.W. 1963. Nitrogen fixation in latosolic soil under grass. Plant and Soil 19:127-138.

Rennie, R.J. 1981. A single medium for the isolation of acetylene reducing (dinitrogen fixing) bacteria from soils. Canadian Journal of Microbiology 27:8-14.

Wani, S.P., Dart, P.J., and Upadhyaya, M.N. 1983. Factors affecting nitrogenase activity (C_2H_2 reduction) associated with sorghum and millet estimated using the soil core assay. Canadian Journal of Microbiology 29:1063-1069.

Wani, S.P., Upadhyaya, M.N., and Dart, P.J. 1984. An intact plant assay for estimating nitrogenase activity (C_2H_2 reduction) of sorghum and millet plants grown in pots. Plant and Soil 82:15-29.

Heterotrophic Nitrogen Fixation as Influenced by Fertilizers in Rice-Soil Systems

V. Rajaramamohan Rao, J.L.N. Rao, and T.K. Adhya[1]

Summary

The rice rhizosphere-soil nitrogenase is affected by several environmental and cultural factors. We investigated the effects of mineral fertilizers on soil nitrogenase under laboratory and field conditions, employing gas chromatographic acetylene-reduction assay. Studies indicate that the level form, and method of fertilizer nitrogen application influenced the nitrogenase associated with the rhizosphere. The application of urea briquettes significantly enhanced nitrogenase activity, the extent of stimulation varying with the method of application and the growth phase of the rice plant. Among the phosphate sources tested, superphosphate exerted stimulatory influence on nitrogenase, while rock phosphate was less effective. Based on this and earlier research from this laboratory, there is an urgent need to evaluate the overall contribution of N_2 fixation to the nitrogen economy under the impact of improved rice technology.

Introduction

Fertilizer is one of the key inputs in crop production, but the information on its influence on nitrogen fixation in the rice rhizosphere soil is rather limited. It has been well established that nitrogenase is depressed by fertilizer nitrogen. However, under field conditions, the effects of nitrogen fertilizer either singly or in combination with other nutrients could be complicated owing to plant absorption and other field factors. Information on the effect of phosphorus application on soil nitrogenase in lowland-rice soils is also not available. We have examined the potential nitrogen-fixing activity in rhizosphere soil as influenced by different sources of fertilizer nitrogen, phosphorus, and certain management practices under field and laboratory conditions.

Materials and Methods

A field experiment was conducted to evaluate the influence of forms and methods of application of urea nitrogen on the nitrogenase activity of the rhizospheric soil. Nitrogen was applied as urea prills and urea briquettes at a rate of 40 kg N ha^{-1}, in addition to 20 kg ha^{-1} each of P_2O_5 and K_2O, to all treatments. Urea briquettes were applied behind the plow or to shallow water between the rows a month after sowing. The treatments with prilled urea included broadcast and incorporation, urea applied behind the plow and between seed rows. Water was allowed to accumulate in the field a fortnight after germination, and the level reached about 50 cm by 60 days after sowing (DAS). The water level varied between 40 cm and 50 cm up to 120 DAS and gradually declined thereafter.

In all treatments, rhizospheric soil (2 g fresh wt) was collected periodically from three plants from each plot and transferred to 125 x 16 mm B-D Vacutainer (New Jersey) tubes for acetylene (C_2H_2) reduction analysis. The incubation and analysis were conducted as per the procedures described by Nayak et al. (1980), Mahapatra and Rao (1981), and Rao et al. (1983). High-purity nitrogen served as a carrier gas at a flow rate of 30 mL min^{-1}.

The effect of different levels and sources of phos-

1. Microbiologist, UGC Teacher Fellow, and Microbiologist, Central Rice Research Institute, Cuttack, Orissa 753 006, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986, Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502324, India: ICRISAT.

phorus on soil nitrogenase activity was evaluated in a laboratory incubation study. Alluvial and P-deficient alkaline soils were air dried, screened through <2 mm sieve, and 5 g placed in B-D vacutainer tubes. One set of soils was flooded (1.5 cm standing water) and the other was kept at 50% water-holding capacity (WHC). All treatments were replicated three times. Phosphorus was applied as K_2HPO_4 to provide 0, 10, 20, 40, 80, and 100 ppm P levels. In another experiment rock phosphate, dicalcium phosphate, and superphosphate were applied to yield final concentrations of 0, 20, 40, and 60 ppm P. Nitrogenase activity was determined at different intervals. The remaining available P was estimated as Olsen's P.

The populations of different groups of nitrogen-fixing microorganisms in the soils under different treatments were counted by conventional serial-dilution techniques on N-free media. *Azospirillum* sp was counted following the method suggested by Okon et al. (1977), and anaerobic nitrogen fixers and *Azotobacter* by the procedure of Rao et al (1973). Results presented are the means of five replicates for *Azospirillum* and anaerobic N_2 fixers, and three replicates for *Azotobacter.*

Results

Nitrogenase was, in general, stimulated by the application of 40 kg N ha^{-1}, particularly during the initial 54 DAS (Table 1). Nitrogenase activity was significantly ($P<0.05$) reduced when the urea briquettes were applied to standing shallow water, whereas it was high when the briquettes were applied either behind the plow or between the rows. Perhaps the increased N concentration in the rhizosphere solution could have reduced the nitrogen availability, resulting in a reduction in activity when the urea was applied to standing shallow water. In fact, application of urea briquettes to the root zone prevents nitrogen loss and results in the slower release of nitrogen. Thus, the slower supply of low levels of nitrogen might have favored nitrogenase activity. Added-N is known to stimulate soil nitrogenase (Knowles and Denike 1974, Trolledenier 1977, Charyulu et al. 1981).

Urea briquettes resulted in a significant ($P<0.05$) increase in grain yield, compared with the control, in all methods of application. The nitrogenase was, however, affected by the method of N application. Thus, no apparent correlation existed between the nitrogenase and the crop yield under these situations.

Table 1. Nitrogenase activity (n mol C_2H_4 g^{-1} soil d^{-1}) in the rhizosphere as influenced by form and method of nitrogen application.

Treatment (40 kg N ha^{-1})	Days after sowing					
	30	47	54	76	88	102
Without N fertilizer	580	420	390	250	150	210
Urea prills						
Applied between *seed* rows	640	510	450	320	230	170
Broadcast and incorporated	760	450	430	310	480	170
Applied behind the plough	740	430	400	140	150	190
Urea briquettes						
Applied in shallow water	570	130	200	180	170	190
Applied behind the plough	620	620	540	360	310	370
SE	±38	±25	±33	±27	±27	±20

Our results indicate that phosphorus addition stimulated nitrogenase activity in the two soils (Table 2), and the stimulation was more pronounced under nonflooded conditions. The stimulation by phosphorus was more apparent in the alluvial soil. Thus the effect of phosphorus on nitrogenase depended on the water regime and soil type. These differences could be attributed to the alterations in the available P under two water regimes in the soils. In fact, the available P disappeared faster in the P-deficient soil than in alluvial soil.

Addition of superphosphate and dicalcium phosphate stimulated nitrogenase activity almost throughout incubation, while the rock phosphate had an innocuous effect (Table 3). Superphosphate was superior over other P-sources in enhancing soil nitrogenase and crop yields on this alluvial soil.

Addition of phosphorus in alluvial soil had little effect on the populations of *Azospirillum*, anaerobic N_2-fixers, and *Azotobacter* (Table 4). In contrast, in a P-deficient soil, the addition of phosphate stimulated the population of *Azospirillum* and *Azotobacter.* The stimulation was more apparent at the 10 ppm P level. The P-deficient soil, however, had higher numbers of *Azotobacter* than the alluvial soil. Changes in the microbial populations and levels

of available P might be responsible for changes in nitrogenase activity in the soils.

Our results suggest that rhizosphere soil nitrogenase is influenced by the mode of application of fertilizer nitrogen. The level and source of phosphorus applied to rice soils under nonflooded and submerged conditions also exhibited profound influence on nitrogenase activity and the population of nitrogen fixers. This study opens up a further need to manipulate fertilizer-management practices to exploit the benefit of biological nitrogen fixation for rice under these situations.

Table 2. Influence of phosphate on soil nitrogenase activity (n mol C_2H_4 g^{-1} soil d^{-1}) in two soils under flooded and nonflooded conditions.

P-level	Days of incubation							
	11		16		25		30	
ppm	F[1]	NF[2]	F	NF	F	NF	F	NF
	Alluvial soil							
0	30	80	30	100	40	80		ND[3]
10	40	100	50	90	40	90		
20	70	590	40	200	50	70		
40	80	310	40	160	40	30		
80	90	290	50	170	80	20		
100	70	430	70	60	130	20		
LSD (*P*<0.05)	18	97	10	34	10	11		
(*P*<0.01)	25	136	15	48	15	15		
	P-deficient alkaline soil							
0	80	120	100	40	60	60	70	30
10	80	490	100	250	120	120	90	130
20	80	130	140	70	110	100	120	90
40	150	150	120	140	100	70	100	50
80	250	170	150	180	90	110	70	50
100	170	200	200	60	140	10	100	20
LSD (P<0.05)	48	105	30	37	33	21	17	24
(P<0.01)	67	147	43	52	46	30	24	34

1. F = Flooded.
2. NF = Nonflooded.
3. ND = Not determined.

Acknowledgement

The authors wish to thank Dr H.K. Pande, Director, CRRI, for providing the facilities to conduct this research.

Table 3. Effect of P-sources on soil nitrogenase (nmol C_2H_4 g^{-1} soil d^{-1}) in a submerged alluvial soil.

P-source	P-level ppm	Days of incubation					
		8	14	19	22	25	29
Control	0	40	150	90	40	40	30
Rock phosphate	20	20	150	110	50	50	40
	40	80	90	40	30	10	30
	60	70	130	60	50	30	30
Dicalcium phosphate	20	100	157	130	50	50	40
	40	90	160	130	40	40	30
	60	100	160	150	80	50	50
Super phosphate	20	100	110	150	50	40	40
	40	120	180	210	50	40	30
	60	150	160	140	40	40	40
LSD(P<0.05)		34	31	29	21	19	14
(P<0.01)		47	42	40	29	NS[1]	19

1. NS = Not significant.

Table 4. Population of N_2-fixing microorganisms as influenced by the phosphate level in flooded soils (12 days incubation).

Soil	P-level (ppm)	Nitrogen-fixing population g^{-1} dry soil					
		Azospirillum (x 10^6)		Anaerobic N fixers ($x10^5$)		*Azotobacter* ($x10^4$)	
		F[1]	NF[2]	F	NF	F	NF
Alluvial	0	16	0.14	2.4	1.4	5	7.5
	10	2.8	0.60	2.8	1.7	2	1.8
	80	16	0.12	2.8	2.4	3	6.5
P-deficient alkaline	0	0.5	0.45	1.4	0.24	47	9
	10	13.0	13.5	0.17	0.24	122	21
	80	2.8	0.18	1.7	0.17	98	14

1. F = Flooded.
2. NF = Nonflooded.

References

Charyulu, P.B.B.N., Nayak, D.N., and Rao, V.R. 1981. Nitrogen incorporation by rhizosphere soil: influence of rice variety, organic matter and combined nitrogen. Plant and Soil 59:399-405.

Knowles, R., and Denike, D. 1974. Effects of ammonium nitrite and nitrate-nitrogen on anaerobic nitrogenase activity in soil. Soil Biology and Biochemistry 6:353-358.

Mahapatra, R.N., and Rao, V.R. 1981. Influence of hexachlorocyclohexane on the nitrogenase activity of rice rhizosphere soil. Plant and Soil 59:473-477.

Nayak, D.N., Pasalu I.C., and Rao, V.R. 1980. Influence of natural and synthetic pesticides on nitrogen fixation (C_2H_2 reduction) in the rice rhizosphere. Current Science 49:118-119.

Okon, Y., Albrecht, S.L., and Bums, R.H. 1977. Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Applied and Environmental Microbiology 33:85-88.

Rao, J.L.N., Pasalu, I.C., and Rao, V.R. 1983. Nitrogen fixation (C_2H_2 reduction) in the rice rhizosphere soil as influenced by pesticides and methods of their application. Journal of Agricultural Science (UK) 100:637-642.

Rao, V.R., Kalininskaya, T.A., and Miller, Y.M. 1973. The activity of non-symbiotic nitrogen fixation in soils of rice fields studied with ^{15}N. Mikrobiologiya 42:729-734.

Trolledenier, G. 1977. Influence of some environmental factors on nitrogen fixation in the rhizosphere of rice. Plant and Soil 47:203-217.

Discussion

N.S. Subba Rao:
How do you estimate Rhizosphere in the rice plant in wet land?

V.R.Rao:
The rhizosphere collection and sampling from wetland soils often pose several problems. We consider the soil fraction attached to the rice roots as the rhizosphere.

G.S.Murthy:
Did you study the individual effects of N and P on nitrogenase activity, or their combined effect?

V.R.Rao:
We have information on the independent and combined effects of N and P on rice rhizosphere N_2 fixation. The deleterious effect of higher levels of N on nitrogenase is alleviated by the application of higher levels of P to the system.

D. Purushothaman:
Could you tell us the response of rice genotypes to inoculation with *Azospirillum'!*

V.R.Rao:
We have limited information on the genotype response to *Azospirillum* inoculation with regard to yield.

U.S. Kundu:
What is the percentage of azospirilla in the rice rhizosphere?

V.R.Rao:
Azospirillum is one of the N_2 fixers associated with rice. Convincing quantitative data on the percentage of azospirilla in rice rhizosphere are not available.

C.P. Ghonsikar:
What is the reliability of ARA methodology in wetland conditions of rice cultures? Any difficulties in assay techniques? If there are, what special techniques are adopted?

V.R.Rao:
ARA method in wetland conditions could at best be qualitative and is often employed for screening and evaluating the N_2-fixing potential under such conditions. There are several difficulties associated with this assay system like gas diffusion, initial lag, solubility, etc. This technique still needs perfection and improvements are in progress.

P. Tauro:
What is the number of azospirilla in the rice soil?

V.R.Rao:
10^6 g^{-1} soil.

Effect of Certain Organic Amendments and Potassium on the Bacterization of Rice with *Azotobacter chroococcum*

N.N. Prasad[1]

Summary

The effect of Sesbania, glyricidia and sunhemp (green manures), and paddy straw on the bacterization of Oryza sativa L. with Azotobacter chroococcum was studied. Among the treatments, Sesbania + Azotobacter gave a 31. 7% increase in grain yield and Glyricidia + Azotobacter gave 31.1% increase over the nonamended control. The results also showed the beneficial effects of neem cake application in combination with Azotobacter inoculation on rice yield. Neem cake application markedly augmented the Azotobacter population in the rhizosphere. Potassium application with Azotobacter indicated an increase in grain and straw yields of rice with an increase in K levels. The Azotobacter population in the rhizosphere of rice increased with an increase in K levels.

Introduction

Nitrogen fixation is an energy-consuming process and the availability of carbohydrate in soil assumes much significance for the fixation of atmospheric nitrogen. Under field conditions, application of organic amendments, therefore, becomes essential for the exploitation of the nitrogen-fixing potential of *Azotobacter*. Some of the data obtained on the bacterization of rice (*Oryza sativa* L.) with *A. chroococcum* are summarized below.

Results

In studies on the effect of green manures and paddy straw on the bacterization of rice with *Azotobacter* at Annamalai University, Sesbania + *Azotobacter* gave the maximum increase in grain yield (31.7%) over the control (Table 1), while glyricidia + *Azotobacter* gave a 31.1 % increase and sunhemp + *Azotobacter* gave a 23.1% increase in grain yields (Prakash and Prasad 1980).

Organic amendments along with *Azotobacter* have also increased the straw yield and 1000 grain mass. Interestingly, the addition of organic amendment combinations with *Azotobacter* inoculation have markedly augmented shoot nitrogen content, when compared to either *Azotobacter* inoculation or organic amendments alone (Prakash 1977).

Work done at Annamalai University revealed that with *Azotobacter* inoculation the optimum level of fertilizer N could be reduced from 120 to 90 kg ha^{-1} for the rice crop (Prakash 1977). The organic-matter application was also found to be beneficial to the *Azotobacter* inoculation. To investigate this aspect further, *Azotobacter* was inoculated at three levels of glyricidia application at two levels of nitrogen (Nagarajan 1978). The results revealed the possibility of reducing the rate of fertilizer N to 60 kg N ha^{-1} from 90 kg N ha^{-1}, if supplemented with glyricida application at 7.5 t ha^{-1} and inoculated with *Azoto-*

1. Professor and Head, Department of Microbiology, Annamalai University, Annamalai Nagar, Tamil Nadu 608 002, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

Table 1. Effect of organic amendments (6 t ha^{-1}) and *Azotobacter* inoculation on grain yield of rice.

Treatment	Grain yield (kg ha^{-1})	Increase over control (%)	Increase over respective control (%)
Control	3136	-	-
Azotobacter	3526	12.5	12.5
Sesbania	3835	22.3	-
Sesbania + *Azotobacter*	4124	31.7	9.4
Glyricidia	3526	12.5	-
Glyricidia + *Azotobacter*	4109	31.1	18.6
Sunhemp	3467	10.6	-
Sunhemp + *Azotobacter*	3860	23.1	12.5
Paddy straw	3089	-1.4	-
Paddy straw + *Azotobacter*	3610	15.1	16.8
CD (P<0.05)	0.48		

bacter (Table 2). At the 90 kg N ha^{-1} level, addition of 7.5 t ha^{-1} glyricidia gave a 32.1% increase in grain yield, while with 60 kg N ha^{-1} + 7.51 ha^{-1} glyricidia, grain yield increased by 27.8%.

The effect of neem cake on bacterization of rice (var. *white ponni*) with *Azotobacter* during the *samba* (northeast monsoon) season (Oct 1979-Feb 1980) was studied (Sahul Hameed 1980). Neem cake was applied at 0, 6, 8.5, 12.5, and 25 t ha^{-1} with *Azotobacter* inoculation. Nitrogen application at 120 kg N ha^{-1}, without *Azotobacter* inoculation or neem cake, served as the control.

The results have shown that neem cake application in combination with *Azotobacter* inoculation on rice can give grain yield increases of 12.1-15.2% and straw yield increases of 15.9-18.7% (Table 3).

Table 2. Effect of glyricidia and N with *Azotobacter chroococcum* inoculation on grain and straw yields of rice.

Treatment		Grain yield	Straw yield
Glyricidia (t ha^{-1})	Nitrogen (kg ha^{-1})	(kg ha^{-1})	(kg ha^{-1})
0	90	5029	14275
0	60	4611	13618
2.5	90	5325	15702
2.5	60	4942	13672
5.0	90	6019	16197
5.0	60	5710	15043
7.5	90	6642	17460
7.5	60	6425	16583

Table 3. Effect of neem cake and *Azotobacter* inoculation on grain yield of rice.

Treatment			Grain yield	Change over
Neem cake (t ha^{-1})	Nitrogen (kg ha^{-1})	*Azotobacter*[1]	(kg ha^{-1})	control[2]
0	90		4650	+9.4
6	90		4764	+12.1
8.5	90		4816	+13.3
12.5	90		4848	+14.1
25.0	90		4895	+15.2
0	90	NI	3884	-8.6
0	120	NI	4250	-

1.1 = Inoculated, NI = Not inoculated.
2. Control = 120 kg N ha^{-1} only.

The neem cake application markedly augmented the *Azotobacter* population in the rhizosphere at 75 DAS. The increase in population of this organism was up to 41.8 x 10^3 at 25 t ha^{-1} neem cake, as compared to 22.3 x 10^3 with no neem cake application (Table 4).

We studied the effect of graded levels of potassium application on the azotobacterization of rice (var. *white ponni*). Potassium application increased grain and straw yields of rice as also the 1000-grain mass. The application of K with *Azotobacter* inoculation augmented the *Azotobacter* population in the rhizosphere of rice. The populations increased to 20.46 x 10^3 at 200 kg K_2O ha^{-1} as compared to 12.1 x 10^3 without K application (Table 5).

Table 4. Effect of neem cake and *Azotobacter* inoculation on rhizosphere population of *Azotobacter* (x 10^3 g^{-1} oven-dry soil).

Treatments			Days after transplantation			
Neem cake (t ha^{-1})	Nitrogen (kg ha^{-1})	*Azatobacter*[1]	15	35	55	75
0	90	I	13.8	17.7	19.6	22.3
6	90	I	16.3	19.5	23.2	26.1
8.5	90	I	14.0	21.9	26.5	29.3
12.5	90	I	20.1	23.9	28.5	31.9
25.0	90	I	26.4	29.9	34.8	41.8
0	90	NI	12.6	15.1	17.2	18.5
0	120	NI	11.2	13.2	15.7	17.8

1. 1 = Inoculated, NI = Not inoculated.

Table 5. Effect *of* potassium application with *Azotobacter chroococcum* inoculation on the rhizosphere population of *Azotobacter* (x 10^3 g^{-1} oven-dry soil).

Potassium ($kg\ K_2O\ ha^{-1}$)	Days after transplantation		
	15	30	45
0	8.48	10.28	12.12
50	10.68	12.84	14.86
100	12.82	14.28	16.24
150	13.14	16.14	18.45
200	15.25	19.28	20.46

References

Nagarajan, R. 1978. Studies on certain aspects of bacterization of rice *(Oryza sativa* L.) with *Azotobacter chroococcum* Beij. M.Sc. thesis, Annamalai University, Annamalainagar, Tamil Nadu, India.

Prakash, R. 1977. Studies on the effect of bacterization of rice (*Oryza sativa* L.) and certain factors influencing the growth of *Azotobacter in vitro* and in soil. M.Sc. thesis, Annamalai University, Annamalainagar, Tamil Nadu, India.

Prakash, R, and Prasad, N.N. 1980. Effect of certain organic amendments on the efficiency of inoculation with *Azotobacter.* Pages 90-94 *in* Aspects of biological nitrogen fixation: proceedings of the Third Southern Regional Conference on Microbial Inoculants in Crop Production, 1-2 Apr 1977, Dharwad, Karnataka, India. Bangalore, Karnataka: University of Agricultural Sciences.

Sahul Hameed, M. 1980. Studies on the effect of certain organic matter amendments and crop variety on the bacterization of rice *(Oryza sativa* L.) with *Azotobacter chroococcum* Beij. M.Sc. thesis, Annamalai University, Annamalainagar, Tamil Nadu, India.

Studies on Vesicular Arbuscular Mycorrhiza in Cereals at ICRISAT Center

K.R. Krishna[1]

Abstract

The vesicular arbuscular mycorrhizal symbiosis is widespread on both sorghum and pearl millet grown in SAT regions. Pearl millet genotypes tested across three field locations showed a large variation in mean mycorrhizal colonization, which ranged between 25 and 56%. Root colonization percentage varied significantly ($P<0.05$) between male-sterile lines, restorer lines, and their derived crosses, indicating further that mycorrhizal colonization is plant-genotype dependent and that it could be heritable.

Fungal isolates differed in their ability to stimulate growth and phosphorus uptake on both sorghum and pearl millet, indicating a need to screen and select fungi for improved response. A technique based on phosphorus estimation in bleeding sap, which could be used to select fungi with improved performance on sorghum, is described.

1. Microbiologist, Pearl Millet Improvement Program, ICRISAT, Patancheru, A.P. 502 324, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics), 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502324, India: ICRISAT.

The use of ELISA (Enzyme-Linked Immunosorbent Assay) for Quality Assessment of Bacterial Inoculants

P.T.C. Nambiar[1]

Abstract

One of the major reasons for the failure of BNF technology informers' fields is the poor quality of inoculants. The conventional plant-infection technique is often used to enumerate rhizobia in carrier-based inoculants. At ICRISAT Center, we have standardized ELISA for the quality control of Rhizobium inoculants. The plant-infection technique is time consuming and laborious. The test plant has to be grown for 25-30 days for the plant-infection technique assessment, while ELISA results can be obtained within 2 days. One hundred and sixty-eight siratro plants are necessary to test the quality of eight inoculum packets by the plant-infection technique, while only a single ELISA plate is needed for the same number of peat packets. This technique can be modified for the quality assessment of other bacterial inoculants.

1. Microbiologist, Groundnut Improvement Program, ICRISAT, Patancheru, A.P. 502 324, India.

ICRISAT (International Crops Research Institute for the Semi-Arid Tropics). 1986. Cereal nitrogen fixation. Proceedings of the Working Group Meeting, 9-12 Oct 1984, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

Recommendations

The need to have fairly uniform procedures for measuring the amount of nitrogen fixed by sorghum and millets under *in situ* conditions was recognized as a prerequisite for embarking on more intensive research on the BNF technology of these crops. The acetylene-reduction method (ARA), employing whole plants either by axenic plant cultures inoculated with N_2-fixing bacteria or by nondisturbed soil cores or intact plant assay, was considered to be appropriate for qualitatively estimating the nitrogenase activity and, hence, the N_2-fixing ability of these plants. The planted soil core method, standardized by ICRISAT scientists, was endorsed to meet the assay requirements for this purpose.

Since the time of incubation with acetylene gas can be a variable factor in such assays, it is recommended that the time course assay be terminated when the linearity of the reaction begins to cease. Whenever quantitative measurements are needed, the ^{15}N dilution technique has to be resorted to, choosing a suitable nonfixing control plant.

Insofar as pot culture or field experiments are concerned, the total biomass of the plant (root, shoot, and grain), individually or collectively, may be taken as the yardstick to measure the overall benefits from inoculation with bacterial cultures. Such a measurement would take into account the benefits derived by N_2 fixation as well as by other factors, such as growth-promoting substances or enhanced nutrient uptake concommitant with improved root growth. The design and conduct of the field experiments should be left to the agronomists, but microbiologists have to supervise inoculation procedures and watch the progress of experiments to record outbreak of pests and diseases, which may limit or mask benefits from BNF. In these experiments, fertilizer nitrogen should be kept within 30-40 kg N ha^{-1}.

There can be both long-term and short-term approaches to future research on BNF technology for sorghum and millets.

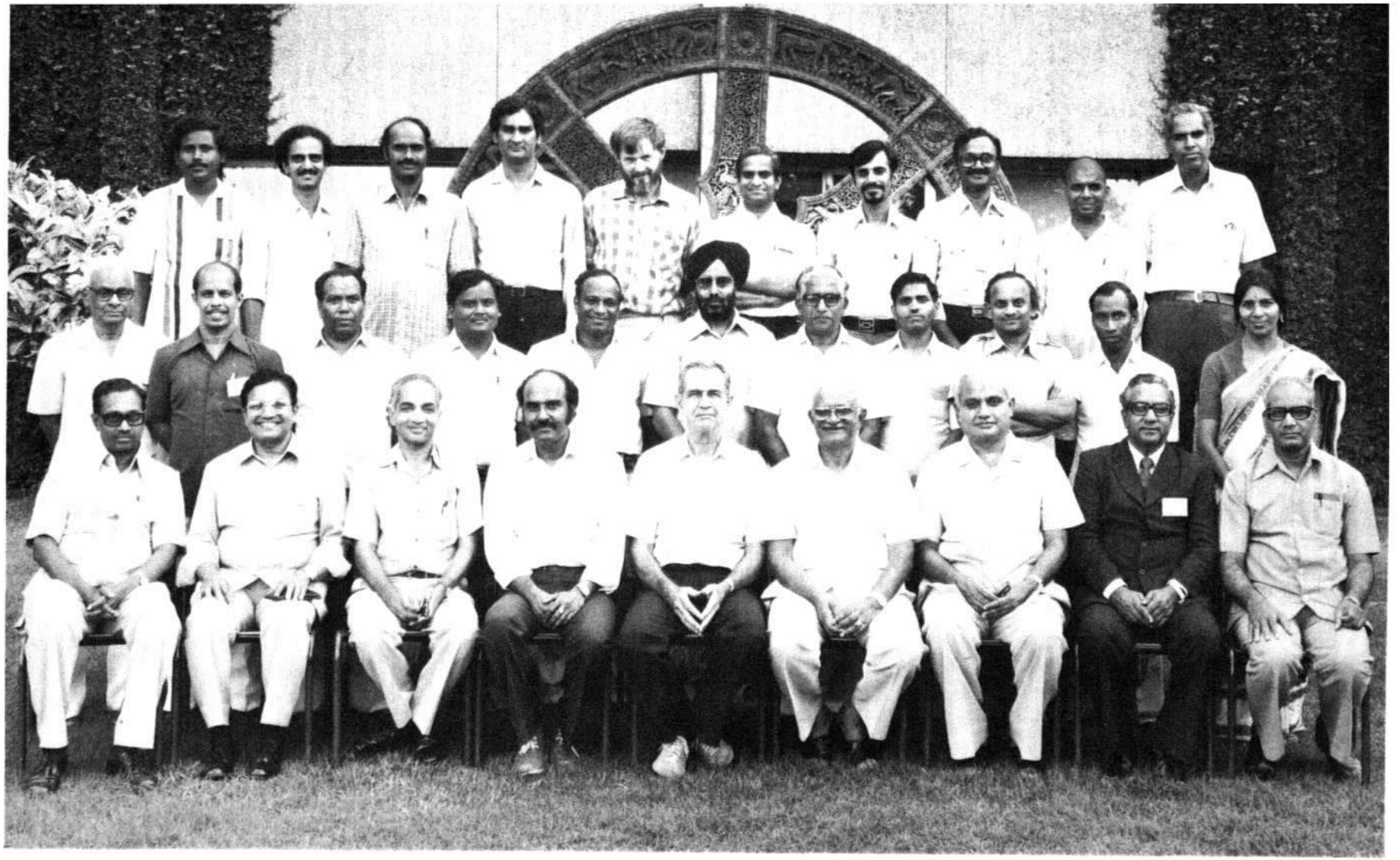
The long-term approaches may include the following:

1. To search extensively for cultivars that exhibit consistently high nitrogenase activity and plant biomass output under diverse stress conditions in the semi-arid tropics, with a view to eventually transfer the beneficial traits to cultivated varieties through intensive plant-breeding methods. This line of work may also include designing of breeding procedures to find out if the high production attribute of plants are inheritable.
2. To explore varietal interaction with specific bacterial species or strains.
3. To exchange bacterial germplasm and maintain them at different centers.

The short-term strategies may include the following:

1. To test the efficiency of superior bacterial cultures in enhancing the yield potential of sorghum and millets under field conditions in the semi-arid tropics.

2. To test various methods of inoculation under field conditions to evolve a suitable method.
3. To qualitatively and quantitatively define the extent of growth-promoting substances produced by efficient strains of bacteria in pure cultures and in the rhizosphere of crops.
4. To intensify work on identification of hitherto undefined N_2-fixing bacteria in sorghum and millets.
5. To quantitatively estimate the preponderance of several types of N_2-fixing bacteria in the root region, by standardizing the enumeration procedures.



Standing, back row, left to right: S. Chandrapaliah, K.R. Krishna, S.V. Hegde, B.S. Kundu, J.H. Williams, S.P. Wani, O.P. Rupela, J.V.D.K. Kumar Rao, K.C. Mohan Kumar, K.V.B.R. Tilak.
Standing, front row, left to right: G.S. Murthy, D. Purushothaman, S.T. Shende, G.S. Jadhav, H. Polasa, H.L.S. Tandon, P.A. Shinde, B.K. Konde, V. Rajaramamohan Rao, A.V. Rao, G. Satyakumari.
Seated, left to right: G. Oblisami, P.V. Rai, P. Tauro, Joseph Thomas, C.R. Jackson, J.S. Kanwar, N.S. Subba Rao, N.N. Prasad, C.P. Ghonsikar.

Meeting Organization

Organizing Committee

F.R. Bidinger, K.K.Lee, S.P. Wani

Session Chairpersons	*Rapporteurs*
P.V. Rai	P.T.C. Nambiar
P. Tauro	R.P. Thakur
Joseph Thomas	J.V.D.K. Kumar Rao
K.K. Lee	S.B. Chavan
G. Oblisami	O.P. Rupela
N.N. Prasad	S. Chandrapal
C. Johansen	J.V.D.K. Kumar Rao
N.S. Subba Rao	S. Pande and P. Soman

Participants

C.P. Ghonsikar
Associate Dean, PG School
Department of Microbiology
Marathwada Agricultural University
Parbhani
Maharashtra 431 402
India

S.V. Hegde
Senior Microbiologist
Department of Agricultural Microbiology
University of Agricultural Sciences
Gandhi Krishi Vignana Kendra (GKVK)
Bangalore
Karnataka 560 *065*
India

G .S.Jadhav
Agronomist
Bajra Research Station
Marathwada Agricultural University
Paithan Road
Aurangabad
Maharashtra
India

B.K. Konde
Associate Professor
Department of Microbiology and Plant Pathology
Mahatma Phule Agricultural University
Rahuri
Maharashtra 413 722
India

B.S. Kundu
Associate Professor
Department of Microbiology
College of Basic Sciences and Humanities
Haryana Agricultural University
Hisar
Haryana 125 004
India

G.S. Murthy
Chief Agronomist
Coromandel Fertilizers Ltd
126, Sarojini Devi Road
P.B.No.1589
Secunderabad
Andhra Pradesh 500 003
India

K.C. Mohan Kumar
Department of Microbiology
Andhra Pradesh Agricultural University
Rajendranagar
Hyderabad
Andhra Pradesh 500 030
India

G. Oblisami
Head
Centre of Advanced Studies
in Agricultural Microbiology
Tamil Nadu Agricultural University
Coimbatore
Tamil Nadu 641 003
India

H. Polasa
Professor and Head
Department of Microbiology
Osmania University
Hyderabad
Andhra Pradesh 500 007
India

N.N. Prasad
Professor and Head
Department of Microbiology
Annamalai University
Annamalai Nagar
Tamil Nadu 608 002
India

D. Purushothaman
Associate Professor
Center of Advanced Studies in
Agricultural Microbiology
Tamil Nadu Agricultural University
Coimbatore
Tamil Nadu 641 003
India

P.V. Rai
Professor and Head
Department of Agricultural Microbiology
University of Agricultural Sciences
Gandhi Krishi Vignana Kendra (GKVK)
Bangalore
Karnataka 560 065
India

A.V. Rao
Microbiologist
Central Arid Zone Research Institute
Jodhpur
Rajasthan 342 003
India

V. Rajaramamohan Rao
Microbiologist
Central Rice Research Institute
Cuttack
Orissa 753 006
India

S.T. Shende
Senior Microbiologist
Division of Microbiology
IARI
New Delhi 110 012
India

P.A. Shinde
Head
Department of Agricultural Microbiology
and Plant Pathology
Mahatma Phule Agricultural University
Rahuri
Maharashtra 413 722
India

N.S. Subba Rao
Project Director
Agro-Energy Center
IARI
New Delhi 110 012
India

H.L.S. Tandon
Director
Fertilizer Development and
Consultation Organization
New Delhi 110 048
India

P. Tauro
Professor of Eminence
Department of Microbiology
College of Basic Sciences and Humanities
Haryana Agricultural University
Hisar .
Haryana 125 004
India

Joseph Thomas
Biology and Agriculture Division
Bhabha Atomic Research Centre
Trombay
Bombay
Maharashtra 400 085
India

K.V.B.R. Tilak
Senior Microbiologist
Division of Microbiology
IARI
New Delhi 110 012
India

ICRISAT Participants

J.S. Kanwar, Director of Research
C.R. Jackson, Director of International Cooperation
K.K. Lee, Principal Cereals Microbiologist
S.P. Wani, Microbiologist (Millet)
K.R. Krishna, Microbiologist (Millet)
S.B. Chavan, Plant Breeder (Millet)
C. Johansen, Principal Agronomist (Pulses)
O.P. Rupela, Agronomist (Microbiology, Pulses)
J.V.D.K. Kumar Rao, Agronomist (Microbiology, Pulses)
J.H. Williams, Principal Plant Physiologist (Groundnut)
P.T.C. Nambiar, Microbiologist (Groundnut)

Acknowledgement

The organizers express their sincere thanks to Dr H.L.S. Tandon, Fertilizer Development and Consultation Organization, New Delhi, India, for useful advice, and to G. Satyakumari, Nirmala Kumar, K.V. Hanumantha Rao, the Information Services staff, and the Sorghum and Millets Information Center staff (all ICRISAT) for their help in the editing and typing of these proceedings.

RA-00097

ICRISAT

International Crops Research Institute for the Semi-Arid Tropics
Patancheru, Andhra Pradesh 502 324, India

ISBN 92-9066-111-9 Printed at ICRISAT Center ICR 85-0043