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.

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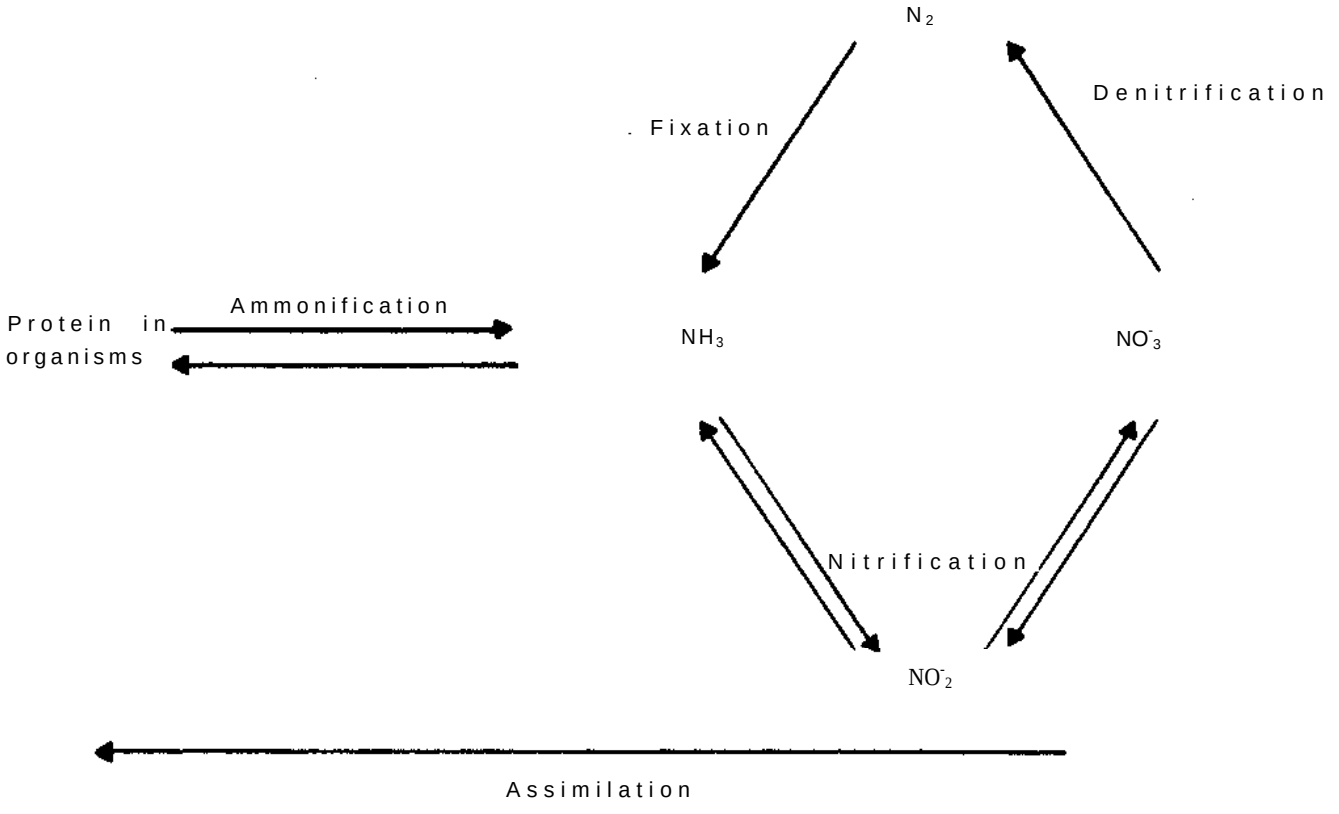

Figure 1. The nitrogen cycle.

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.

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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.