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NITROGEN FIXATION ASSOCIATED WITH CEREALS AND FORAGE GRASSES

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ABSTRACT

Nitrogen is a major factor limiting the production of cereals. The availability of this element depends on the turnover rate of the nitrogen cycle and the return of the nitrogen to the soil through biological nitrogen fixation (BNF) or by fertilizer application. Estimates of nitrogen fixation associated with cereals and grasses are not very precise, but the results indicate that nitrogen fixation in non-legumes occurs at levels which can be measured. Reliable estimates can be obtained from more difficult nitrogen balance studies which need to be continued for several years in the field. This review covers the present knowledge about the nature of the association between plant and nitrogen-fixing bacteria, types of bacteria involved and methods of isolation and screening.

The occurence of the association of nitrogen-fixing bacteria with roots of cereals and grasses is well documented (1, 2, 5, 8, 12, 57). The terms used for the study of nitrogen fixation in cereals and grasses are : diazotrophic, rhizocoenosis, associative symbiosis, and associative (di) nitrogen fixation. Associative dinitrogen fixation describes the close bacteria-plant rhizosphere associations which have been detected on grass roots (15). Associative symbiosis refers to an intracellular relationship between a dinitrogen-fixing bacterium and plant roots (14). These systems may involve any of several bacterial species and are best typified by the grass-**Azospirillum** nitrogen-fixing system first reported by Dobereiner and Day (13). These systems have been recently termed "Cryptic" associations since, they are generally macroscopically undetectable (25). For understanding these cryptic root-bacteria associations, it is essential to be aware of a general concept of soil-plant-microbe relationships that has been defined as the "rhizosphere" (48). In the rhizosphere zone, subtle root-microbe interactions may occur frequently and perhaps

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always. This paper attempts to discuss the impact of recent findings in the field of associative N_2 fixation.

MICROBIOLOGY OF THE ASSOCIATION

The list of nitrogen-fixing bacteria associated with nitrogen fixation in cereals and grasses include species of **Achromobacter, Alcaligenes, Arthrobacter, Azotobacter, Azospirillum, Azomonas, Bacillus, Beijerinckia, Clostridium, Campylobacter, Corynebacterium, Derxia, Enterobacter, Erwinia, Klebsiella, Lignobacter, Mycobacterium, Pseudomonas,** and **Xanthobacter** (2) and blue-green algae.

Enumeration and isolation of nitrogen-fixing bacteria: Counts of nitrogen-fixing bacteria are usually done following dilution plating onto a supposedly N-free selective medium. Most probable number (MPN) techniques based on the presence of nitrogenase in dilutions of the initial sample in a selective medium are also used. It is difficult to devise selective media which can support the growth of all nitrogen fixers as the nature of the carbon source used in N-free media in counting and isolating procedures favors the growth of different groups of bacteria. An added complication is the variable response between organism types to different partial pressures of oxygen in their vicinity. It has been proposed to use axenically collected root exudates media or media containing a mixture of carbon sources (45, 52) or the "spermosphere model" using a young sterile seedling growing on a mineral medium free of C and N (55).

In our laboratory, we have used three methods (i) dilution and plating using N-free sucrose and malate media (ii) MPN counts in semi-solid N-free media enriched with 50 mg yeast extract/l and (iii) MPN using aseptically grown plants. Generally higher counts are observed with dilution and plating method as compared with MPN in semi- solid media and MPN with plants. The higher counts obtained with dilution and plating might be because of the growth of non-nitrogen fixers which could scavenge the mineral N from the medium. Even though, the MPN counts were lower than those of direct plating methods, the recovery of nitrogenase positive isolates was higher from MPN tubes than from direct plating. The MPN method using plants carried on further generations for enrichment of N_2 fixers have shown increased number of positive N_2 fixers with increasing generations of enrichment in general. In this method, seed-

lings of millet or Sorghum are grown from surface sterilized seeds in C and N free Fahreus medium containing 0.3% agar. The aseptically grown seedlings are inoculated with serial dilutions of the test sample and incubated under 1% C_2H_2 in glasshouse. Nitrogenase activity is measured on 8 and 12 days and plants with positive N_2ase activity from the highest dilutions are macerated alongwith medium and serial dilutions are prepared for inoculating aseptic seedlings. In this way subsequent enrichments are carried on upto 3 or 4 generations.

Large populations of bacteria capable of growing aerobically on N-free media exist in some Alfisol fields at ICRISAT Centre. There is also a marked increase (10-fold) in number in the rhizosphere compared to the surrounding soil. A selection for particular types of bacteria also occurs in the root zone, resulting in less than half the types found in the bulk soil. The numbers of colony types and the population sizes varied with the carbon source in the isolation medium and were increased by adding a small amount of yeast extract (50 to 100 mg/l) to the media. About 50% of the bacteria isolated because of their different colony characterictics, were nitrogen-fixing bacteria. There were over a million **Enterobacteriaceae**/g soil, several species of which are known to fix nitrogen anaerobically. The macerate from roots thoroughly washed and surface sterilized with 1% chloramine T for 1 h showed 10^5 presumptive N_2 fixers/g of fresh root suggesting that some bacteria were very closely bound to the root and perhaps even in the root tissues.

A survey of 200 sites in traditional millet growing areas in North-Western India, indicated 10^6-10^7/g soil total population of organisms capable of growing on a N-free sucrose medium supplemented with 50 mg/l yeast extract. The MPN counts from these soils ranged from 10^2 to 10^5/g soil in malate and sucrose semi-solid media. Nitrogen-fixing isolates from roots included bacteria similar to **Azospirillum brasilense, A. lipoferum,** unidentified **Azospirillum** spp, **Bacillus polymyxa, Derxia gummosa, Erwinia herbicola** and **Enterobacter** spp. Soil isolates in the malate enrichment medium normally contained Enterobacteriaceae and pseudomonads. **Enterobacter cloacae** was the most common isolate, while some **Pseudomonas** types showed nitrogenase activity. Some of the soils did not contain **Azospirillum.** There is often a continuum of types overlapping in properties between named genera and species. The isolates could be classified on the basis of colony morphology into at leastt 22 diffe-

rent groups (8, 27). It is also generally agreed (24) that growth on a nitrogen-free medium is not a sufficient criterion for nitrogenase activity as some N_2-fixing bacteria can not grow on a medium completely free of combined nitrogen (66). On a large scale, use of $^{15}N_2$ incorporation to measure N_2 fixation (the definitive test) is expensive and many authors have used C_2H_2 reduction as an indirect assay technique. This technique poses some difficulties due to the short exposure period for C_2H_2 reduction and also due to oxidation of C_2H_4 by some bacteria (35). Many N_2-fixing bacteria express nitrogenase activity only when they are in sufficient numbers (21). In enumeration experiments, the threshhold level for expression of nitrogenase activity is reached only after a lag period that is dependent upon the initial population of the inoculum (60). With high dilutions this can take as long as 3 weeks. Moreover, even after establishment, nitrogenase activity can be rapidly suppressed by carbon or oxygen limitations so that the overall period of time during which nitrogenase activity occurs can be very short. To over come this problem Balandreau (2) suggested incubating the replicate tubes of rice seedlings under 1% C_2H_2 as soon as they are inoculated. However, we have observed that incubation of inoculated sorghum seedlings under 1% C_2H_2 resulted in stunted seedling growth. Before using this technique for other crops it should be checked for its applicability.

ESTIMATES OF N_2 FIXATION

There are several methods for measuring nitrogen fixation associated with field and pot grown plants; these can be broadly grouped as follows:

1. Direct: a. Chemical N determination
   b. ^{15}N based methods

2. Indirect: a. Acetylene (C_2H_2) reduction assay

N balance experiments: Nitrogen balances can be measured by standard Kjeldahl analyses for field crops, but are more reliable for plants grown in pots in a defined volume of rooting medium, enabling proper soil sampling, leaching losses to be estimated, and inputs from N_2fixation estimated. Under field conditions such experiments are difficult to conduct, as they need to run for more than one season and require rigorous sampling of the soil if they are to reliably measure soil N changes of 20 to

50 kg N/ha per annum. Also it is difficult under field conditions to estimate the losses due to denitrification, although denitrification is believed to be small (19) based on few measurements available. Small amounts of nitrogen are added to the soil through rainfall, and dust. Rainfall away from the sea contributes 1 to 4.5 kg N/ha per annum (32).

Estimates of cereal nitrogen fixation based on N balance studies vary from 11.2-148 kg/ha per annum with different crops (4, 31, 36, 39, 41, 67). For details refer the reviews (8, 53). In addition N balance studies with sorghum, millet and Napier bajra (**Pennisetum purpureum**×**P. americanum** in pot culture in vermiculite media, with and without added N fertiliser, and in soil low in nitrogen, have established that (i) there is a small but measurable N_2fixation by bacteria associated with the roots of these plants (ii) fixed N is taken up by the plant during the growing season and (iii) some of it also remains in the rooting medium (8).

Long term field experiments with sorghum, millet and forage grasses are continuing at ICRISAT to measure the N balance in these crops in an Alfisol. In the sorghum experiment the same eight cultivars with either high nitrogenase activity or high N uptake under low fertility, are grown each year on the same plots. Fertiliser N is added at the rate of 0, 20 and 40 kg N/ha. Mean initial N content of the top 0 to 15 cm of the unfertilised soil was 0.040%, 0.056% in 15-30 cm zone, and 0.053% at 30-90 cm. All above ground plant material is removed at harvest. Cumulative dry-matter yields of the cultivars varied from 17 to 30 tons/ha over 5 seasons without any nitrogen added. Highest dry matter production was observed with cv IS 15165 which is a late maturing, photosensitive African entry which does not produce grains at Hyderabad during rainy season. During seventh year of the experiment (rainy season 1984), a uniform crop of millet cv. ICMV 1 was grown. The total dry matter yield of millet cv. ICMV 1 from the plots where previously cvs CSH 5 and IS 2333 were grown across the nitrogen levels was at par with each other. The cumulative nitrogen uptake through above ground plant parts from 1978 to 1983 (except 1981) indicated that highest nitrogen uptake amongst the sorghum cultivars across the applied nitrogen levels was in case of cv. CSH 5 (230 kg/ha) and the lowest was in case of cv IS 2333 (180 kg/ha) (29). These results suggest that sorghum cultivars do vary for their N_2-fixing ability.

In a trial with tropical gnasses, during 5.5 years one of the entries (hybrid Napier bajra, NB 21) has produced 329 t/ha dry matter containing 2320 kg N. The range among species for dry matter production is wide, for instance as low as 32 t/ha including 240 kg N during 5.5 years. These results suggest that entries like NB 21 are deriving some of their requirement of N through BNF in addition to soil N pool (28).

^{15}N based methods :

The stable, heavy isotope of nitrogen is used as a tracer in biological nitrogen fixation investigations. There are two ways viz., (i) $^{15}N_2$ incorporation (ii) ^{15}N isotope dilution, to use the heavy isotope to study dinitrogen fixation.

(i) $^{15}N_2$ incorporation : A plant enclosed in a container is exposed to $^{15}N_2$, and after incubation, plant tissues are examined for above natural concentrations of the heavy isotope. This technique has been used successfully to prove N_2 fixation in association with tropical grasses (11), sugarcane (38, 50, 51) rice (16, 30, 69) and sorghum (17). An apparatus has recently been developed (17) to introduce gas mixture into the root growth chamber by purging with CO_2 followed by absorption of the carbon dioxide with soda lime as suggested by Witty and Day (68).

(ii) ^{15}N isotope dilution : The first studies with non-legumes using ^{15}N isotope dilution in the field indicated that no associated N_2 fixation occurred with maize or sorghum inoculated with **Spirillum lipoferum** (43). However, subsequently this technique has been used to estimate N_2 fixation due to inoculation and also potential of different host cultivars (18, 23, 44, 47). A possible approach is to use uniformly ^{15}N labelled soil in pots for growing the lines to screen for their potential to fix atmospheric nitrogen. The soil can be labelled by incorporating ^{15}N fertilizer with carbon source (10 : 1, C : N ratio) well in advance so that ^{15}N is incorporated into the organic form and then slowly released by mineralisation.

Acetylene Reduction Assay (ARA) : Acetylene reduction is a flexible, simple and an indirect method to test nitrogenase activity (20). Detailed assay procedures for green house and field experiments with nonlegumes have been described in detail (57, 61). An excised root assay involves overnight incubation under reduced O_2 tension (14) during which N_2-fixing bacteria proliferate

substantially due to released organic materials from the roots resulting in overestimation of N_2ase activity (57). A soil-root core assasy developed by Day **et al.** (9) has been used by several workers, however, large plant to plant variability has been observed with this assay (8, 61). The initial lag for C_2H_4 production depends on the time required for diffusion of C_2H_2 in the system and it varies according to soil type (58, 64). Wani **et al.** (63) have identified the mechanical disturbance during sampling, sampling time, time gap between cutting the plant top off and injecting C_2H_2, incubation temperature and soil moisture as some of the factors responsible for large plant to plant variability.

Soil-core assays are destructive, time consuming, somewhat variable and many factors have to be controlled which are difficult to manipulate under field conditions. To overcome some of these problems we have developed alternative intact plant assay systems—one where seedlings are grown in tubes (61, 62), and another where plants are grown in pots (64). These assay techniques enable several assays on the same plant during its growth period and also permit the experimenter to collect self-fed seed or use the plant in crossing. In these methods same plant is exposed to acetylene gas several times and it is necessary to study its effect on plant growth and N_2-fixing bacterial population in the rhizosphere. The relationship between field and greenhouse assay techniques need to be studied.

ARA is a short-term kinetic measurement, and the existence of diurnal and seasonal variations in N_2 fixation makes quantification and extrapolation to total N fixed over a growing season questionable. A further problem is that the relationship between the rate of reduction of acetylene and that of N_2 varies with the system and is seldom experimentally determined (35). Problems with the acetylene reduction assay can also be encountered, particularly when dinitrogen fixation rates measured in soils are low. Bacterial oxidation of ethylene can reduce estimates and anaerobic bacterial production of ethylene can increase estimates of dinitrogen fixation (10). In case of anaerobic dinitrogen fixation endogenous production of ethylene might lead to erroneous results. Despite these restrictions ARA, because of its ease of use and high sensitivity, will remain the technique of choice for the study of nitrogenase activity. Its use should be restricted to qualitative and comparative evaluations and the guidelines suggested by Lethbridge **et al.** (37) should be followed.

IMPROVING N_2 FIXATION

Nitrogen fixation is the result of interaction amongst host plant, bacteria and environmental factors. Nitrogen fixation associated with cereals can be improved by manipulating each of the factors involved in the association. Genotypic variation for stimulating nitrogen fixation has been observed in lines of pearl millet (6, 8, 61) wheat (45), sugarcane (49), sorghum and minor millets (8, 34, 61), and rice (3, 65). It seems that we might enhance associative N_2 fixation through genotype selection using normal methods of plant breeding; here the challenge is to introduce the capacity for N_2 fixation to a wider and more useful genetic base. However, progress in breeding area depends largely on the availability of a rapid screening method and identification of traits related to the plant character to be manipulated.

Environmental factors like temperature, radiation, soil moisture, oxygen partial pressure (pO_2), combined N and organic carbon levels in soil and other soil properties like pH, are known to affect nitrogen fixation. Acetylene reduction in sandy loam soil amended with glucose and incubated anerobically, was optimum at 37°C and maximum at 45°C (40). Similarly, higher nitrogenase activity was observed with sorghum and millet plants at 33° and 40°C than at 27°C (63). There are several reports suggesting considerably higher rates of N_2 fixation in tropics than in the temperate region (8, 12, 57).

There is a close relationship between soil moisture and di-nitrogen fixation, mainly because of pO_2 requirements of the nitrogen-fixing bacteria. With the increasing soil moisture and due to soil root and microbial respiration the oxygen partial pressure decreases resulting in high nitrogenase activity. The pO_2 in the soil can be manipulated by irrigation or by increasing the respiration by organic matter amendments, which in addition can supply energy source (carbon) to the N_2-fixing bacteria. Another possibility is by selecting mutants of the bacteria which could tolerate higher oxygen concentration in the soil. The role of carotenoid in protecting N_2ase system in bacteria from oxygen need to be studied further.

The photosynthate translocated to the roots is utilized by bacteria in the soil as the carbon source. Several workers have observed diurnal variations for N_2ase activity associated with rice, pearl millet, sorghum and forage grasses (1, 8, 13, 59, 65).

However, these studies do not show clear relationship between plant photosynthesis and root associated N_2 fixation because fluctuation of soil temperature coincides with the cycle of N_2ase activity during the day-night cycle (8, 57). 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, which suggests a strong link between N_2ase activity and radiation incidence in rice have been reported (59). However, the N_2ase activity of the above reported crops may be indirectly related to photosynthesis as is evident from studies of Dobereiner **et al.** (15) that eventhough no diurnal cycle of nitrogenase was observed in **P. notatum,** but prolonged incubations of plants in the dark reduced the activity.

Seasonal variations in the nitrogenase activities of forage grasses, maize, sorghum, pearl millet, **Setaria italica** and **Eleucine coracana** have been reported using excised root or soil core assays (13, 33, 63). With maize maximum activities were recorded at the 75% silking stage (13) and with pearl millet and sorghum at late flowering—early grain filling stage. The activity was greatly related with ontogenetic development of plant (63).

The presence of combined N affects nitrogenase activity. It has been observed under field conditions that application of N fertilizer at lower levels (20 kg N/ha) did not affect N_2ase (9) on the other hand application of N in smaller doses such as 20 kg/ha had stimulatory effect on N_2ase activity (61). By manipulating the method of application and by applying in split doses and selecting proper form of fertilizer N like slow release formulations may help to harness maximum nitrogen fixation associated with these crops without reducing the yields.

Organic matter amendments in soil result in increased nitrogenase activity (22, 64) and this aspect need to be exploited fully wherein enough crop residues are available for incorporating in the soil.

RESPONSE TO INOCULATION

There is increasing interest in attempts to enhance N_2 fixation by associative systems through artificial inoculation. A majority of recent inoculation experiments have been with **Azospirillum** spp; a large number of such experiments have been summarised by Boddey and Debereiner (5) and Wani

(61). Several experiments in Egypt, Israel, India have shown positive benefits of inoculations under field situations. In India, multilocational trials with pearl millet, sorghum and finger millet conducted over 3 years have shown significantly increased yields due to inoculation with **Azospirillum brasilense** and the effects of inoculation were more pronounced under low levels of added nitrogen (2, 53, 54, 62).

The possible contribution of nitrogen fixation to the yield of inoculated crop plants have been shown by several workers (7, 8, 23, 42, 61). Rennie (44) using ^{15}N dilution principle have shown that when sufficient carbon substrate was available to the bacteria, upto 38% of the maize plant N was derived from associated N_2 fixation by **A. brasilense**. Similarly, in glasshouse studies, it was shown that wheat cultivars grown in soil and inoculated with **Bacillus polymyxa** and **A. brasilense** derived 0-32 and 0-29% of total plant N from BNF (46). Using ^{15}N isotope dilution technique in sand culture experiments, Hegazi (22) suggested that as much as 45% of total N content of plant was derived from N_2 fixation. Tien **et al.** (56) demonstrated production of auxin, cytokinins and substances tentatively identified as gibberellins by an **Azospirillum** strain. This finding emphasizes the need to exercise caution in the interpretation of yield increases in response to bacterial inoculation. The possible role of such substances in increasing crop yields in inoculation trials has been discussed in detail by Hubbell and Gaskins in their recent review (26).

Many reports exist indicating the possible mechanisms by which the crop plants inoculated with N_2-fixing bacteria derive the benefit from such associations (7, 22, 23, 42, 46, 56). More experiments using ^{15}N studies will ensure the question that to what extent each of the various processes mentioned above contributes to increase in yield.

AREAS OF FUTURE RESEARCH

These associative nitrogen-fixing systems need to be understood in detail in order to fully harness their potential benefits. More precise estimates of the quantity of nitrogen fixed are essential; using careful nitrogen balance studies and ^{15}N-based techniques. There is a need to look at the whole bacterial systems involved in associative nitrogen-fixing system. By manipulating the culture media and cultural conditions like O_2 con-

centration, pH, temperature and concentrations of carbon and other nutrient sources, identity of many unknown organisms involved in these associations will be revealed. In particular, we need to give thought to the role of non-nitrogen fixers present in the rhizosphere as reports have shown synergism amongst nitrogen fixers and non-nitrogen fixers.

The role of bacteria in associative nitrogen-fixing systems needs to be understood more at the basic level : (ii) location of the bacteria (iii) source of energy for N_2 fixation (iii) role of plant affinity beyond that as carbohydrate supplier (iv) ecological factors governing such associations (v) types of bacteria involved and (vi) criteria to be used for selecting bacterial types for field inoculation studies.

Several reports have indicated good positive responses to crop inoculations under field conditions, however, the position in this area is highly variable. There is little agreement on several points e.g. (i) is there a host specificity for bacteria ? (ii) which bacteria to use as inoculum and whether it should be single species of bacteria or rather a mixture of bacteria ? (iii) which method of inoculation to be used ? (iv) what is suitable carrier ? and (v) which criteria to be used for checking the quality of the inoculants produced ? More emphasis need to be given on studies pertaining to establishment and survival of the added inoculum in the rhizosphere and also the factors which might affect the performance of the added inoculum. What is the exact role of inoculated bacteria in increasing the crop yields ? Are these solely due to N_2 fixation or hormonal effects or so ?

The knowledge of the agronomic practices which could help to increase N_2 fixation under normal situations and also with inoculation should go a long way to improve N_2 fixation.

It seems possible to improve plant genotypes for increased associative N_2 fixation by following routine plant breeding methods. The progress of our genetic understanding will likely move more slowly because of inadequacies in methodology under field conditions. The progress in this area will be parallel with our ability to characterise the physiology and microbiology of these associations.

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