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# The contribution of associative and symbiotic nitrogen fixation to the nitrogen nutrition of non-legumes

P.M. CHALK
*School of Agriculture and Forestry, University of Melbourne, Parkville 3052, Victoria, Australia*

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**Abstract**

During the past 10 years estimates of $N_2$ fixation associated with sugar cane, forage grasses, cereals and actinorhizal plants grown in soil with and without addition of inoculum have been obtained using the $^{15}N$ isotope dilution technique. These experiments are reviewed in this paper with the aim of determining the proportional and absolute contribution of $N_2$ fixation to the N nutrition of non-legumes, and its role as a source of N in agriculture. The review also identifies deficiencies in both the totality of data which are currently available and the experimental approaches used to quantify $N_2$ fixation associated with non-legumes.

Field data indicate that associative $N_2$ fixation can potentially contribute agronomically-significant amounts of N (>30–40 kg N $ha^{-1}$ $y^{-1}$) to the N nutrition of plants of importance in tropical agriculture, including sugar cane (*Saccharum* sp.) and forage grasses (*Panicum maximum*, *Brachiaria* sp. and *Leptochloa fusca*) when grown in uninoculated, N-deficient soils. Marked variations in proportions of plant N derived from the atmosphere have been measured between species or cultivars within species.

Limited pot-culture data indicate that rice can benefit naturally from associative $N_2$ fixation, and that inoculation responses due to $N_2$ fixation can occur. Wheat can also respond to inoculation but responses do not appear to be due to associative $N_2$ fixation. $^{15}N$ dilution studies confirm that substantial amounts of $N_2$ can be fixed by actinorhizal plants.

## Introduction

Six genera of diazotrophic ($N_2$-fixing) bacteria, including at least nine species, which associate with the roots of graminaceous plants, have been isolated and identified since 1974 (Boddey and Döbereiner, 1988). The plants are mainly cereals and forage grasses of agricultural significance. Despite considerable study over the past 15 years, the importance of associative $N_2$ fixation as a source of N in agriculture has remained a controversial issue (Michiels *et al.*, 1989), due in part to perceived limitations in methods used to obtain quantitative estimates of fixation.

Many measurements of acetylene-reduction activity associated with non-leguminous plants have been reported (Boddey, 1987). The assay is a valuable tool in demonstrating the existence of putative $N_2$ fixation, but it does not measure incorporation of fixed N into the plant, and measured rates cannot be easily or accurately extrapolated to obtain quantitative estimates of $N_2$ fixed (Boddey, 1987). It is generally accepted that the $^{15}N$ isotope dilution technique has provided the most accurate time-integrated estimates of the contribution of biologically fixed N to the N nutrition of legumes and non-legumes alike. In this review, data obtained using this technique in soil-based experiments with cereals (rice and wheat), sugar cane and forage grasses

are examined, with the aim of determining whether significant proportions of plant N can be derived from associative $N_2$ fixation, and whether this might represent an agronomically-significant input of N into agricultural systems. Soil-based studies carried out with nodulated non-leguminous actinorhizal plants are also considered.

A detailed discussion of the $^{15}N$ isotope dilution technique is beyond the scope of this review. However, several comprehensive reviews of the method and its applications are available (*e.g.* Boddey, 1987; Chalk, 1985). The method as applied to non-legumes involves the enrichment of the soil with an organic or inorganic $^{15}N$ source. Unlabelled atmospheric $N_2$ which is biologically fixed dilutes the labelled N taken up by the plant from soil sources, and the measured isotope dilution is used to estimate the proportion of plant N derived from the atmosphere. The isotopic composition of the N derived from soil sources is usually estimated by measuring the $^{15}N$ enrichment of a non-fixing reference plant. The acetylene-reduction assay has proved to be a useful technique for selecting species or cultivars for use as reference plants (Boddey and Victoria, 1986; Boddey *et al.*, 1983).

Two different approaches have been adopted in the measurement of associative $N_2$ fixation. The first involves the use of normal, biologically-active uninoculated soil. In the second type of study, inoculum containing single or mixed species or strains of diazotropic bacteria is added to the soil, which may be unsterilized, heat-sterilized or fumigated. With the first approach naturally-occurring, plant-associated $N_2$ fixation is estimated, whereas in the second type of study, response to inoculation in the presence or absence of competition from indigenous bacteria is determined. Homologous strains are frequently used as inoculum. i.e. strains isolated from the surface-sterilized roots of one crop which are subsequently inoculated onto that same crop.

Boddey (1987) stressed that evidence for associative $N_2$ fixation should include both a higher N yield and a lower $^{15}N$ enrichment of the test plant compared to the reference plant, ideally under conditions where the $^{15}N$ enrichment of the available soil N pool does not change in time or space. A significant dry matter or grain yield increase due to inoculation with diazotrophic organisms should be accompanied by such evidence, before it can be confidently concluded that the response was due to $N_2$ fixation, and not to some other factor (Boddey, 1987). The criteria of higher N yield and lower $^{15}N$ enrichment are used in this review to evaluate claims of significant associative $N_2$ fixation.

## Cereals

### *Rice* (Oryza sativa *L.*)

Studies with flooded rice (Table 1) have all been carried out in pots in the glasshouse or biotron in Asian countries. The floodwater has generally been covered to inhibit photodependent $N_2$ fixation by Cyanobacteria. Data obtained by Ventura and Watanabe (1983) in the Philippines and Zhu *et al.* (1986) in China using uninoculated soils indicated that a substantial proportion of plant N (20–35%) was derived from associative $N_2$ fixation, which confirms earlier indications from N balance and $C_2H_2$ reduction assays (Boddey, 1987). Isolation and identification of the diazotrophs were not attempted (Ventura and Watanabe, 1983; Zhu *et al.*, 1986).

Other studies conducted in the Philippines and Japan have all involved inoculation. Inoculation of unsterilized soils with *Pseudomonas* sp. (H8) and *Azospirillum lipoferum* (34H) failed to show any response due to $N_2$ fixation (Watanabe and Lin, 1984). Subsequent work by the same group with marker *Azospirillum lipoferum* (34HStrRif$^r$) resistant to streptomycin and rifampicin, again failed to show any consistent response due to $N_2$ fixation with two cultivars of rice (Nayak *et al.*, 1986). The inoculated bacteria established in the unsterilized soil, but in low numbers, and they declined progressively during the course of the experiment. This latter study illustrated the importance of determining establishment and persistence of the inoculated bacteria in the soil, by using techniques such as intrinsic antibiotic resistance or immunofluorescence.

Inoculation studies in Japan with strains of *Klebsiella oxytoca* and *Enterobacter cloacae* have produced more encouraging results (Fujii *et al.*,

*Table 1.* $^{15}N$ dilution estimates of putative $N_2$ fixation associated with flooded rice[a]

| Soil treatment[b] | Test plant | | Reference plant | | $P^e$ (%) | Reference |
|---|---|---|---|---|---|---|
| | Cultivar | Inoculum | Cultivar[c] | Inoculum[d] | | |
| U, C | IR26 | *Pseudomonas* sp (H8), *Azospirillum lipoferum* (34H) | IR26 | Nil | 0 | Watanabe and Lin, 1984 |
| U, C | Hua, OS4 | *Azospirillum lipoferum* (34HStrRif$^r$) | Hua, OS4 | HK | 0 | Nayak *et al.*, 1986 |
| U | GXZ | Nil | Nil | Nil | 20–23 | Zhu *et al.*, 1986 |
| U, C | IR42 | Nil | IR42 | Nil | 32–35 | Ventura and Watanabe, 1983 |
| H, C | C5444 | *Klebsiella oxytoca* (NG13, NG1302) | C5444 | Nil | 11–19 | Yoo *et al.*, 1986 |
| U, C | C5444, T65 | *Klebsiella oxytoca* (NG13, NG13/pMC71A), *Enterobacter cloacae* (E26, E26/pMC71A) | C5444, T65 | *K. oxytoca* (NG1389) | 10–18 | Fujii *et al.*, 1987 |

[a] All experiments were in pots.
[b] U, unsterilized; H, heat sterilized; C, covered with lid, black cloth or Al foil.
[c] Nil, soil mineralizable N was used as the reference; IR42 reference was not flooded.
[d] HK, heat-killed inoculum as used on the test plant.
[e] *P*, the percentage of plant N derived from the atmosphere.

1987; Yoo *et al.*, 1986). In a study using heat-sterilized soil, Yoo *et al.* (1986) were able to show a significant contribution by *K. oxytoca* (NG13) and a non-mucoid mutant (NG1302) to the N nutrition of an Indica-type rice (C5444). The NG13 strain was originally isolated from the rhizosphere of C5444. In a subsequent experiment with non-sterilized soil, Fujii *et al.* (1987) were unable to duplicate this result, but did show a significant plant response due to $N_2$ fixation with Japonica-type rice (T65) inoculated with NG13 and *E. cloacae* (E26) as well as genetically-altered strains of NG13 and E26 (NG13/pMC71A and E26/pMC71A) capable of fixing $N_2$ in the presence of $NH_4^+$. The genetically-altered strains were not superior to the wild types in their ability to contribute to plant associated $N_2$ fixation in T65 rice.

*Wheat* (Triticum aestivum *L.*)

Studies with wheat have been carried out both in the glasshouse and in the field, and all have involved inoculation (Table 2). In one-half of the studies *Bacillus* sp. (C-11-25) was used, while one or more strains of *Azospirillum brasilense* were used in all but one of the studies (Table 2). Significant responses to inoculation in terms of dry matter yield (Kapulnik *et al.*, 1985; Kucey, 1988a; Li *et al.*, 1989) or N yield of dry matter (Kapulnik *et al.*, 1985) or grain (Boddey *et al.*, 1986) have been reported. Neither Kapulnik *et al.* (1985) nor Boddey *et al.* (1986) attributed the N response to $N_2$ fixed by inoculated diazotrophs, because there was no isotopic dilution of the $^{15}N$ enriched harvested material compared to the uninoculated control. Plant responses to inoculation with Azospirillum and other diazotrophs have been attributed to plant growth altering substances produced by the bacteria. The substances include auxins, gibberellins and cytokinins, which have been reported to influence root system and root hair morphology and the permeability of the root to certain ions. Boddey *et al.* (1986) also suggested a role of bacterial nitrate reductase in enhancing N assimilation by the host plant.

Inoculation failed to elicit a significant N yield response in several studies (Kucey, 1988a; b; Li *et al.*, 1989; Rennie and Thomas, 1987; Rennie *et al.*, 1983), although the $^{15}N$ enrichments of inoculated treatments were significantly less than controls. Rennie *et al.* (1983) and Rennie and Thomas (1987) attributed the isotope dilution to associative $N_2$ fixation, and explained the lack of an N response in harvested material to a compensatory mechanism, *i.e.* when fixed N was assimilated less N was taken up from soil sources

*Table 2.* $^{15}N$ dilution estimates of putative $N_2$ fixation associated with wheat

| Soil treatment[a] | Test plant | | Reference plant[b] | $P^c$ (%) | Reference |
|---|---|---|---|---|---|
| | Species/cultivar | Inoculum | Inoculum[d] | | |
| U | Fielder | *Azospirillum brasilense* (Cd), *Bacillus* sp. (C-11-25) | Nil | 5–11 | Kucey, 1988b |
| U | Fielder, Chester | *Azospirillum brasilense* (Cd), *Bacillus* sp (C-11-25) | Nil | 0–8 | Kucey, 1988a |
| U | 9 cultivars[e], T. durum cv. hercules | *Azospirillum brasilense* (ATCC29145), *Bacillus* sp. (C-11-25) | Nil | 0–32 | Rennie *et al.*, 1983 |
| U | 9 cultivars[f], T. durum cv. hercules | *Azospirillum brasilense* (Cd, 245) *Bacillus* sp. (C-11-25) | Nil | 0–47 | Rennie and Thomas, 1987 |
| H | Nongda 162 | *Klebsiella pneumoniae* (43) | Nil | 15–22 | Li *et al.*, 1989 |
| F | BH1146 | *Azospirillum brasilense* (Sp245, Sp107, Sp7) | A (Sp245) | 0 | Boddey *et al.*, 1986 |
| U | Miriam | *Azospirillum brasilense* (mixed strains)[g] | Nil | 0 | Kapulnik *et al.*, 1985 |
| U | Cadet, Rescue, CR5D, RC5D | *Azospirillum brasilense* (Sp107) | A | 0[h] | Lethbridge and Davidson, 1983 |

[a] U, unsterilized; H, heat sterilized; F, fumigated with methyl bromide.
[b] Reference plants and test plants were identical.
[c] *P*, the percentage of plant N derived from the atmosphere.
[d] A, autoclaved inoculum as used on the test plant.
[e] Cadet (C), Rescue (R), CR2A, CR2D, CR5B, CR5D, Park, Ticena, Fielder.
[f] Rescue, Cadet, Thatcher, Park, Neepawa, Columbus, Owens, Fielder, HY320.
[g] Cd + Sp7 + Cd1 + Sp51e + T1 + T2.
[h] $P < 5\%$ could not be detected by method and is indicated as zero.

and *vice versa*. On the basis of the isotope dilution measured, Kucey (1988a, b) calculated modest contributions of associative $N_2$ fixation (0–8% of plant N) for pot-grown Chester and Fielder wheats, and 5–11% for field-grown Fielder wheat due to inoculation with *Bacillus* sp. and *A. brasilense* (Cd). On the other hand, Rennie *et al.* (1983) and Rennie and Thomas (1987) calculated that associative $N_2$ fixation due to inoculation provided agronomically-significant quantities of N to the N nutrition of several cultivars of pot and field-grown wheat. For example, the proportion of N in Cadet wheat grown in the field in 1984 which was attributed to $N_2$ fixed by inoculated *Bacillus* sp. was 47.4% (52.4 kg N $ha^{-1}$ fixed), which is the same order of magnitude as reported for some annual grain legumes (Peoples and Herridge, 1991).

Kucey (1988a, b) was careful to point out that his estimates of $N_2$ fixed may have been in error due problems with methodology. For example, Kucey (1988b) found that the roots of inoculated plants occupied only 50–80% of the soil volume occupied by uninoculated plant roots. Because the isotope was not uniformly distributed with depth, the N derived from the soil by test and control plants could differ in isotropic composition, by virtue of their different rooting volumes. Kucey (1988b) suggested that the reduced rooting volume provided an explanation for the lack of an N response due to inoculation observed in his own work and that of Rennie and coworkers, and that it also lent support to the compensation theory proposed by Rennie *et al.* (1983).

The accuracy of the estimates of associative $N_2$ fixation due to inoculation obtained by Rennie *et al.* (1983) were questioned by Boddey *et al.* (1986) on the basis of the methodology used and a lack of correlation between total N yield and isotope dilution. The same doubt surrounds the high estimates obtained by Rennie and Thomas (1987). All of the studies reported for wheat can be criticised on the basis that no attempt was made to measure establishment or persistence of the inoculated bacteria in the rhizosphere.

## C4 grasses

### *Sugar cane* (Saccharum *sp.*)

Experiments with sugar cane have been carried out in Brazil in large pots containing 60–120 kg soil (de Freitas *et al.*, 1984; Lima *et al.*, 1987) or a concrete tank containing 80 t soil (Urquiaga *et al.*, 1989) located in the field (Table 3). Biologically-active, uninoculated soils were used.

Using non-nodulating soybean as a reference plant, de Freitas *et al.* (1984) estimated that two commercial sugar cane cultivars obtained 22–26% of plant N (shoots + roots) from associative $N_2$ fixation during 60 d after emergence. However, differences in N yields between the reference and test plants were small, which was not consistent with significant inputs of N via $N_2$ fixation. In contrast, Lima *et al.* (1987) found that the cultivar CB 4789 had more than twice the N yield and about one-half of the $^{15}N$-enrichment when compared to three other commercial cultivars grown in a labelled soil for 12 months. Using the mean $^{15}N$ enrichment of other cultivars, it was estimated that 44% of the N in CB 4789 was derived from associative $N_2$ fixation. Lima *et al.* (1987) suggested that fixation

*Table 3.* $^{15}N$ dilution estimates of putative $N_2$ fixation associated with sugar cane[a]

| Test plant | Reference plant | $P^b$ | Reference |
|---|---|---|---|
| Species/cultivar | Species/cultivar | (%) | |
| NA5679, CB4176 | *Glycine max* (non-nodulating) | 22–26 | de Freitas *et al.*, 1984 |
| CB4789 | *Saccharum* sp (3 cultivars)[c] | 44 | Lima *et al.*, 1987 |
| 7 cultivars[d], 3 species[e] | *Brachiaria radicans* (IRI442), | 37–56 | Urquiaga *et al.*, 1989 |
| | *Saccharum* sp. (SP792312) | 2–31 | |

[a] All soils were unsterilized and test reference plants were uninoculated.
[b] *P*, the percentage of plant N derived from the atmosphere.
[c] CB47355, IAC52150, NA5679 (mean values).
[d] CB4789, CB453, NA5679, IAC52150, SP701143, SP71799, SP792312.
[e] *S. barberi* cv. chunnee, *S. officinarum* cv. caiana, *S. spontaneum* cv. krakatau.

may have been underestimasted, since N balance data indicated that the reference cultivars also obtained some N from this source. While recognising the error involved in extrapolating fixation in pots to the field situation, Lima *et al.* (1987) calculated that associative $N_2$ fixation could potentially contribute 200 kg N $ha^{-1}$ to the N nutrition of CB 4789.

Work with seven commercial cultivars grown in the field for 250 days (Urquiaga *et al.*, 1989), including several cultivars used by de Freitas *et al.* (1984) and Lima *et al.* (1987), indicated that all had a greater N yield and a lower $^{15}N$ enrichment compared to *Brachiaria radicans*, the non-fixing reference plant. The percentage of plant N estimated to be derived from $N_2$ fixation ranged from 42–52% (84–128 kg N $ha^{-1}$). Conservative estimates based on the use of the commercial cultivar SP 792312 as the reference plant, ranged from 9–26% (24–64 kg N $ha^{-1}$) for the remaining six cultivars. Of the commercial cultivars tested, SP 701143, which is known to produce well under low soil fertility, derived most benefit from associative $N_2$ fixation. Of three non-commercial species of *Saccharum* included in the study, *S. spontaneum* cv. krakatau, a low sugar-producing cultivar, gave estimates of $N_2$ fixation higher than any commercial cultivar tested.

The evidence for substantial inputs of N via $N_2$ fixation associated with sugar cane, which has been provided by isotope dilution measurements, is consistent with observations made in the field. Although 100–200 kg N $ha^{-1}$ is removed in every cane harvest, the N fertility of soils is generally maintained despite continuous cropping with low fertilizer N inputs (Lima *et al.*, 1987). While no microbiological investigations were carried out in conjunction with the isotope dilution studies, Urquiaga *et al.* (1989) suggested that the novel $N_2$ fixing bacterium, *Acetobacter diazotrophicus*, isolated from the stems and roots of sugar cane at various sites in Brazil (Cavalcante and Döbereiner, 1988), would be a strong candidate.

*Forage grasses*

Measurements of $N_2$ fixation associated with several genera of C4 forage grasses have been carried out in Brazil in the field, generally in small concrete cylinders, using biologically-active, uninoculated soils (Table 4). Inoculation studies have been conducted in Pakistan by Malik and co-workers in the glasshouse and field with salt-tolerant Kallar grass (*Leptochloa fusca*) using mixed inoculum containing homologous strains of several genera of bacteria (Table 4).

Experiments in Brazil have provided convincing evidence for significant natural inputs of biologically fixed $N_2$ to the N nutrition of forage grasses. Estimates ranged from a modest contribution of 8–13% of plant N for *Paspalum notatum* cv. batatais (Boddey *et al.*, 1983; Boddey and Victoria, 1986), to a substantial input of 40% in the case of some cultivars of the economically-important *Brachiaria decumbens* (Boddey and Victoria, 1986) and *Panicum maximum* (Miranda and Boddey, 1987; Miranda *et al.*, 1990). Extrapolation of the small-plot data (Boddey and Victoria, 1986; Miranda and Boddey, 1987) indicated potential N inputs from associative $N_2$ fixation of 5–10 kg N $ha^{-1}$ 30 $d^{-1}$ during the summer months or 30–40 kg N $ha^{-1}$ $y^{-1}$. Such inputs are consistent with field observations that pure stands of *Brachiaria decumbens* in Brazil can remain productive under grazing for many years in the absence of legume $N_2$ fixation.

In an initial short-term experiment using fumigated saline-sodic soil in pots, Malik *et al.* (1987) found a large N yield response to inoculation of Kallar grass, with the measured isotope dilution indicating that 8% of plant N overall was derived from $N_2$ fixed by the inoculated diazotrophs. In subsequent pot work using the same soil, but without fumigation, N yield responses to inoculation were small or non-significant (Malik and Bilal, 1988; Malik *et al.*, 1988). Nevertheless, isotope dilution measurements indicated that putative $N_2$ fixation due to inoculation could potentially contribute 20–30% of the plant N assimilated over 8 weeks, depending on the inoculum used. Using the immunofluorescence technique, Malik *et al.* (1988) showed that the natural population of the inoculated diazotrophs was low, and that inoculated bacteria survived and proliferated in planted but not unplanted pots. However, the lack of an N response to inoculation does not support the case for significant associative $N_2$ fixation in these experiments,

*Table 4.* $^{15}N$ dilution estimates of putative $N_2$ fixation associated with C4 forage grasses

| Soil treatment[a] | Test plant | | Reference plant | | $P$[b] (%) | Reference |
|---|---|---|---|---|---|---|
| | Species/cultivar | Inoculum | Species/cultivar | Inoculum[c] | | |
| U | *Panicum maximum* (11 ecotypes)[d] | Mixed[f] | *Brachiaria radicans* (IRI442) | Nil | 24–39 | Miranda and Boddey, 1987 |
| | (24 ecotypes)[e] | Nil | *Panicum maximum* (KK16) | Nil | 16–39 | Miranda *et al.*, 1990 |
| U | *Sorghum sudanense* | Nil | *Triticum aestivum* (Ticena 2) | Nil | 35 | Vose *et al.*, 1982 |
| U | *Paspalum notatum* (batatais) | Nil | *Paspalum notatum* (pensacola) | Nil | 8–13 | Boddey *et al.*, 1983<br>Boddey and Victoria, 1986 |
| U | *Brachiaria humidicola* (IRI409), *Brachiaria decumbens* (IRI700) | Nil | *Brachiaria radicans* (IRI442) | Nil | 30–40 | Boddey and Victoria, 1986 |
| F | *Leptochloa fusca* | Mixed[g] | *Leptochloa fusca* | A[c] | 8 | Malik *et al.*, 1987 |
| U | *Leptochloa fusca* | Mixed[h] | *Leptochloa fusca* | Nil | 20–26 (P)[i]<br>9–22 (F)[i] | Malik *et al.*, 1988 |
| | | Nil | (+60 kg N $ha^{-1}$) | | 2–41 (F)[i] | |
| U | *Leptochloa fusca* | Mixed[j] | *Leptochloa fusca* + 90 kg N $ha^{-1}$ | Nil | 24–30 | Malik and Bilal, 1988 |
| | | | *Polypogon monspeliensis* | | 7–16 | |

[a] U, unsterilized; F, fumigated with chloroform.
[b] *P*, the percentage of plant N derived from the atmosphere.
[c] A, autoclaved mixed inocumum as used on the test plant.
[d] T71, T62, K190B, K187B, K249, T3, T45, Coloniao, T58, K70, KK29.
[e] T45, T84, T91, T58, T110, T68, T74, Coloniao, K51, K193, K190A, K219, KK08, K214, T29, K248, T95, K213, T60, K55, T72, T67, K191, K190B.
[f] *Azospirillum lipoferum* + *Azospirillum brasilense* + *Azospirillum amozonense*.
[g] *Klebsiella* sp. (NIAB1) + *Beijerinckia* sp. (Iso 2) + C2.
[h] *Klebsiella* sp. (NIAB1) + *Beijerinckia* sp. (Iso 2) + AH6 + St16.
[i] P, pot experiment; F, field experiment.
[j] *Klebsiella* sp. (NIAB1) + *Beijerinckia* sp. (Iso 2) + *Enterobacter* sp. (AH6) + *E. agglomerans* (11).

particularly in view of the fact that the grass responded well to N fertilizer addition to the pots (Malik and Bilal, 1988).

In a longer-term field experiment over a period of 8 months (Malik *et al.*, 1988), inoculation increased N uptake by 36%, which was equivalent to the response obtained by an addition of 30 kg N $ha^{-1}$ of ammonium sulphate. Two non-fixing reference criteria were used in the experiment, the uninoculated grass and uninoculated grass fertilized with 60 kg N $ha^{-1}$ to suppress natural $N_2$ fixation, which permitted estimation of associative $N_2$ fixation due to natural and inoculated sources. Measured isotope dilution indicated that natural fixation contributed 19 kg N $ha^{-1}$, with an approximately equal amount coming from the inoculated diazotrophs. Three harvests were taken, and the data clearly show that the proportion and amount of biologically fixed $N_2$ peaked during the hot monsoonal summer months (Malik *et al.*, 1988).

## Actinorhizal plants

Of 161 species of non-legumes which bear $N_2$-fixing nodules, those species which belong to the genus Casuarina and form a symbiotic relationship with the actinomycete, *Frankia*, appear to have the most potential for agriculture (Dart, 1986). Casuarinas play a role in rotational agriculture in less-developed countries, being used to stabilize eroding land surfaces and to improve the N status of impoverished soils, while also providing timber, firewood or charcoal (Dart, 1986).

Very few attempts have been made to quantify $N_2$ fixation by actinorhizal plants grown in soil using the $^{15}N$ dilution method. Two field studies with *Casuarina equisetifolia* conducted in Senegal in containers (1 $m^3$) of N-deficient, fumigated soil (Gauthier *et al.*, 1985; Sougoufara *et al.*, 1990), showed large N yield responses to inoculation with *Frankia*, with fixed N accounting for 53 to 65% of the N in 11 to 24-month-old plants. Extrapolation of the plot (1 $m^2$) data to a hectare basis (10,000 $m^2$) gave estimates of fixation as high as 400 kg N $ha^{-1}$ in the first year and 440 kg N $ha^{-1}$ in the second year of one study (Sougoufara *et al.*, 1990).

Gauthier *et al.* (1985) suggested that fixation by Casuarina would increase up to a plant age of 5 to 10 y. Using 5- to 6-y old potted plants of alder (*Alnus glutinosa*), a temperate species nodulated by *Frankia*, and poplar (*Populus alba*) as the non-fixing reference plant, Domenach and Kurdali (1989) estimated that 87% of the N in new leaf growth was derived from $N_2$ fixation. The results of these studies support estimates of fixation obtained with plants grown in artificial media, or in soil using non-isotopic techniques, and show that amounts of $N_2$ fixed by effectively nodulated Casuarina may sometimes exceed values reported for perennial herbaceous legumes in tropical or subtropical agriculture (Peoples and Herridge, 1991).

## Conclusions

It is only during the past 10 years that quantitative estimates of nitrogen fixation associated with non-legumes have been obtained using the $^{15}N$ isotope dilution technique. The evidence that agriculturally-important tropical C4 grasses, including sugar cane and the forage grasses Brachiaria and Panicum, can obtain significant proportions of plant N from associative $N_2$ fixation under natural conditions is unequivocal. Less certain are the estimates of amounts of $N_2$ fixed when confined small-plot data are extrapolated to an annual hectare basis, but it appears that agronomically-significant amounts of N (>30–40 kg N $ha^{-1}$ $y^{-1}$) can be fixed by some species or cultivars of these grasses. Because microbiological studies were not undertaken with measurements of $N_2$ fixation, the identity of the diazotrophs is open to speculation.

Quantitative data on $N_2$ fixation associated with cereals under natural conditions is very limited. Only two pot experiments indicate that fixation may be significant for flooded rice grown in biologically-active, uninoculated soils, and equivalent $^{15}N$ dilution data could not be found for wheat, maize, grain sorghum or millets grown in soil. There is an obvious need for natural levels of fixation associated with field-grown cereals to be quantified. The difficulty has been in obtaining a non-fixing reference plant. The approach adopted by Boddey and co-work-

ers for C4 grasses (Boddey and Victoria, 1986; Boddey *et al.*, 1983; Lima *et al.*, 1987; Miranda and Boddey, 1987; Miranda *et al.*, 1990; Urquiaga *et al.*, 1989) can be recommended, where cultivars exhibiting little or no associative $N_2$ fixation were identified and used as reference plants.

All of the measurements of $N_2$ fixation associated with wheat and the majority of those with rice have involved inoculation with cultures of diazotrophic bacteria. However, it was only in two pot studies with rice (Fujii *et al.*, 1987; Yoo *et al.*, 1986) that it could be confidently concluded that the response to inoculation was due, at least in part, to $N_2$ fixation. This conclusion was based on the dual requirement of a significant N yield response coupled with $^{15}N$ dilution. Similarly, it was concluded that none of the claims of associative $N_2$ fixation due to inoculation of wheat could be sustained due to a lack of significant N yield response coupled with $^{15}N$ dilution. Although infrequently attempted, it was noted that the measurement of establishment and proliferation of inoculated bacteria was of considerable value in interpreting the results of inoculation studies. Inclusion of an N fertilizer treatment independently of the inoculation treatment was also shown to be of value in indicating the N responsiveness of the site selected for inoculation studies (Malik *et al.*, 1988).

In all of the inoculation studies with cereals, the reference plant was the same cultivar which was left uninoculated or treated with heat-killed inoculum or a *nif-* mutant. In this situation, naturally-occurring $N_2$ fixation associated with the test plant was ignored, and it was not possible to determine what influence, if any, inoculation had on natural levels of fixation. This constraint reduced the value of such studies, which could be overcome if an independent non-fixing reference plant were available for inclusion. There is a need not only to determine if associative $N_2$ fixation may occur under natural conditions, but also to discover if it can be established or enhanced through inoculation. This need was fulfilled in only one study (Malik *et al.*, 1988), where it was shown that $N_2$ fixation naturally associated with field-grown Kallar grass was approximately doubled through inoculation. In several of the inoculation studies reported for rice, wheat, forage grasses and actinorhizal plants, heat sterilized or fumigated soils were used. This experimental approach should be questioned on the basis that both natural contributions of associative $N_2$ fixation to plant nutrition and competition from indigenous microorganisms are both removed from consideration.

Now that it has been demonstrated that associative $N_2$ fixation can contribute significant proportions of N to the nutrition of some agricultural plants, it is appropriate to consider how N inputs into agriculture via this pathway might be enhanced. Manipulation of the bacteria, the host or environmental factors are all possible strategies. So far the most popular approach has been through inoculation, but responses have been quite variable. Response to inoculation is complex because the plant may respond not only to fixed N but also to other substances produced by the bacteria. A degree of specificity between different genera or species of bacteria and plants has been observed, and generally, homologous strains of bacteria have been most successful in eliciting a positive plant response to inoculation (Boddey and Dobereiner, 1988).

Consistent responses to inoculation will only be achievable when our understanding of the interactions between the host plant, the bacteria and the environment is greatly improved. Although knowledge concerning the mechanisms of association between diazotrophs and host plants has increased over recent years, little is known about the physiology of the plant response (Michiels *et al.*, 1989). Although the seasonal nature of associative $N_2$ fixation in tropical climates has been demonstrated (Boddey and Victoria, 1986; Malik *et al.*, 1988; Miranda and Boddey, 1987), little specific information is available on environmental factors affecting fixation. However, there is general consensus that associative $N_2$ fixation is of little significance in temperate agriculture.

Little specific information is also available on edaphic factors affecting fixation. Some diazotrophs are adapted to function under extreme conditions of soil salinity and sodicity (Malik *et al.*, 1987; 1988; Malik and Bilal, 1988). However, it appears that $N_2$ fixation associated with grasses is only significant when soil N is deficient (Boddey *et al.*, 1983; Miranda and Boddey,

1987). High concentrations of available soil N at planting (Rennie and Thomas, 1987), addition of N fertilizer (Vose *et al.*, 1982) or addition of distillery waste (Lima *et al.*, 1987) to soils were reported to inhibit $N_2$ fixation associated with wheat, sudan grass and sugar cane, respectively. There appears to be scope for introducing insensitivity to soil inorganic N through genetic manipulation of associative $N_2$ fixation bacteria (Fujii *et al.*, 1987). Studies in which artificial media were used to grow plants were not included in this review because the influence of edaphic factors cannot be ignored when assessing the contribution of associative $N_2$ fixation to plant nutrition.

It has been suggested that associative $N_2$ fixation may be under the genetic control of the host plant. The ability of a plant to support and benefit from associative $N_2$ fixation has been termed *nis* (nitrogen fixation support), and it was claimed that several chromosomes are involved in the expression of *nis* in wheat (Rennie and Thomas, 1987; Rennie *et al.*, 1983). Urquiaga *et al.* (1989) suggested that it might be possible to breed for high $N_2$-fixing sugar cane varieties, perhaps using *S. spontaneum* cv. krakatau as a donor parent. The idea of breeding for enhanced associative $N_2$ fixation is an attractive one for high value crops in tropical countries where low-input agriculture is practiced. While this possibility may seem remote at the present time, adoption into tropical farming systems of ecotypes or cultivars of forage grasses, sugar cane and Casuarinas, which have already been identified as exhibiting high $N_2$ fixing potentials, should confer a long-term economic advantage, particularly on soils of low N status. The identification of cultivars or ecotypes with both high and low $N_2$ fixing potentials (Miranda *et al.*, 1990) should also prove to be very useful in comparative microbiological and physiological studies of plant-bacteria associations.

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