Review article
Exploring the Boundaries of N_2-Fixation in Cereals and Grasses: An Hypothetical and Experimental Framework

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Abstract

Despite more than 40 years of research on free-living and endophytic bacteria associated with cereals and grasses, conclusive examples of impacts of non-symbiotic N_2-fixation in agriculture are lacking. All available methods for measurement of N_2-fixation associated with cereals and grasses have been employed, and N_2-fixation has been demonstrated to occur under controlled conditions, but this is insufficient evidence to prove a significant role for N_2-fixation by heterotrophic bacteria in the field. Recently attention has focused on endophytic N_2-fixation with claims of major inputs of N from the atmosphere, particularly in sugar cane for which the largest body of information exists, although some methodological concerns remain unanswered. Here we analyse the evidence for N_2-fixation in the rhizosphere and within graminaceous plants.

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We provide guidelines suggesting the type of information required to allow a critical analysis of the amounts of non-symbiotic N_2-fixation contributed in agriculture. Given the substantial availability of C as a substrate for N_2-fixation in sugar cane it remains the most likely candidate plant to benefit from non-symbiotic N_2-fixation. Measurements of elevated respiration in zones of high bacterial colonization may give an indication of whether these really are focal points of the rapid metabolic activity required to support significant N_2-fixation activity. The only conclusive evidence for a major role of non-symbiotic N_2-fixation in the field will come from long-term N balance studies in which ALL processes of potential gain and loss are measured. We conclude that evidence for a large contribution from heterotrophic, non-symbiotic N_2-fixation in both natural and agricultural systems is weak. Although amounts of non-symbiotic N_2-fixation in natural systems may be small (<5 kg N ha^{-1}) they could be significant in the long term over many years.

Keywords: Nitrogen fixation, Cereals, Grasses

1. Introduction

A brief 'cyclical' history

Research in this field appears to have followed a series of repeated and overlapping cycles. The pattern that appears to repeat itself follows a phase during which claims have been made for a significant role of N_2-fixation by heterotrophic bacteria associated with graminaceous and other non-legume plants, linked to new excitement as further groups of plant associated bacteria are described with further conclusions on other roles of the bacteria, most notably in production of various plant growth promoting hormones and other effects.

The earliest cycle followed the widespread use of "Azotobacterin" inoculants in Russia and the discovery of *Azotobacter paspali* associated with the roots of *Paspalum* (Döbereiner, 1961, 1966). Detailed experimental assessments (Brown and Burlingham, 1968; Brown, 1976) and review of the available evidence led Brown (1974, 1982) working at Rothamsted Experimental Station in the UK to conclude that the observed inoculation responses were due to hormonal effects of the bacteria, namely production of indole acetic acid (IAA) and related effects on root growth and stimulation of plant growth.

The advent of the acetylene-reduction assay (Dilworth, 1966) led to new opportunities for the investigation of activity of N_2-fixation. Among the first reports of application of this assay to study soil and root-associated N_2-

fixation was an influential paper published by Döbereiner, Day and Dart in 1972 working in Brazil and at Rothamsted in the same department as Brown. Cores of soil were flown to the UK from Brazil and analysed intact or dissected into various bulk soil and rhizosphere fractions resulting in demonstration of acetylene-reduction activity (ARA) closely associated with roots (Döbereiner et al., 1972). Microbiological investigations led to the discovery of *Azospirillum* spp. and other heterotrophic N_2-fixing bacteria during a period of very intense research in this area. Large responses in growth and yields of tropical cereal crops to inoculation with *Azospirillum* in the USA (Smith et al., 1976) led to widespread excitement and claims of the next 'Green Revolution'. However, the excitement was quickly tempered by further discoveries; firstly problems associated with the acetylene-reduction assay applied to soils and rhizospheres and secondly the attribution of inoculation responses to production of IAA and other hormones by *Azospirillum* (Tien et al., 1979; Okon, 1982; Lin et al., 1983).

In the late 1980's we witnessed new excitement and invigoration of research activity due to the description of heterotrophic N_2-fixing bacteria occurring endophytically in the tissues of sugar cane and other grasses. The best-described and studied cases are *Gluconacetobacter* and *Herbaspirillum* in sugar cane (Baldani et al., 1986; Cavalcante and Döbereiner, 1988) and *Azoarcus* spp. in Kallar grass (*Leptochloa fusca*) (Reinhold-Hurek et al., 1993). When coupled with detailed studies of Bob Boddey, Segundo Urquiaga and colleagues that indicated that sugar cane could derive large amounts of N from N_2-fixation (Lima et al., 1987; Urquiaga et al., 1992) led to a resurgence of interest and research activity in this area during the 1990's. Many papers have since been published on the genetics and microbial ecology of N_2-fixing endophytes (see other papers in this volume) and these led to the discovery that endophytic bacteria such as *Gluconacetobacter* could also produce IAA that gives a stimulation of growth in sugar cane (Sevilla et al., 1998).

Thus we can trace three overlapping cycles of discovery and realization concerning the role of various types of free-living and endophytic N_2-fixing bacteria associated with non-legumes and most notably with tropical grasses and cereal crops. In each case the initial premise that the bacteria increased plant growth due to N_2-fixation has been revised to include other growth-stimulating effects of the bacteria, most notably the production of the plant growth hormone IAA.

2. Methods for Estimating N_2-Fixation

Much has been written about the various methods for estimating N_2-fixation and it is not necessary to repeat all of this here, save to refer to a series of

reviews (e.g. Chalk, 1985; Boddey, 1987; Chalk and Ladha, 1999; Giller, 2001). Here we will simply describe some of the principal concerns with some of the methods.

Acetylene-reduction assay

This highly sensitive assay for nitrogenase activity was shown to have serious errors when applied to rhizosphere-associated or free-living N_2-fixation measurements in soil. These related to the blocking effect of acetylene on 'endogenous' ethylene produced in soil (de Bont, 1976; Witty, 1979) and strong lag effects in development of acetylene reduction activity associated with long incubation times used (van Berkum, 1980). The long lag effect under oxygen-restricted conditions was largely due to the roots being fermented and used as a C source for N_2-fixation (Okon et al., 1977). Despite these very critical discoveries, this method continued to be misapplied widely through the 1980's (Giller, 1987) and even still today!

N balance methods – The ultimate test?

The ultimate test of the contribution from N_2-fixation is to measure the net inputs from N_2-fixation over long periods (>10 years) in the field. Unfortunately, although this is an inherently simple idea, it is hard to control and measure all of the processes. The different inputs and outputs that need to be considered are summarized in Table 1. The difficulty in measuring many of the transfers and flows within the N cycle relate to their dynamic nature, with large diurnal and seasonal variability depending on prevailing environmental conditions. This means that calculation of reliable estimates of each individual process (and hence of reliable overall N balances), is dependant on integration of multiple measurements. These consist of process rates over relatively short time periods in the case of gaseous fluxes, or on interpolation from simple measurements of system states such as mineral-N concentrations with depth in the case of estimates of NO_3^- leaching. In all cases a fairly detailed understanding of the water balance within the experiment is necessary. In many nutrient balance studies simple equations or 'transfer functions' are used to estimate processes that are difficult to measure (e.g. Smaling, 1998; Vlaming et al., 2001), although use of these transfer functions inevitably introduces further uncertainty. Without consideration of all the various flows and transfers, the N balances are essentially 'partial' balances that cannot be considered to be an appropriate methodology for measurement of contributions from specific processes such as root associated or endophytic N_2-fixation.

Table 1. The inputs and outputs in the N cycle that need to be measured when constructing complete N balances. The flows that are difficult to estimate are shown in italics.

Inputs	Outputs
Organic manures or mineral fertilizers	Crop/animal removal
Wet N deposition	*Gaseous losses (NH3, NOx, N2)*
Dry N deposition	*Soil erosion and runoff*
Run-on	*N leaching*
Uptake from lateral flow	
N2-fixation	
Legume	
Cyanobacteria	
Heterotrophic	

Earlier claims for a significant role of N_2-fixation from heterotrophic bacteria (e.g. Firth et al., 1973; Dart and Day, 1975) put emphasis on this type of data, but examples where positive N balances over long periods in the field were described were later attributed to inputs from N_2-fixation by *Cyanobacteria* (blue-green algae) (Witty et al., 1979; Giller and Day, 1985). None of the N balance studies which have been used to support claims for substantial inputs from root associated or endophytic N_2-fixation have studied or excluded potential inputs due to N uptake from deep soil horizons or from cyanobacterial N_2-fixation.

3. Stable Isotope ^{15}N-Based Methods

Direct $^{15}N_2$ gas methods

If plant tissues are incubated in atmospheres enriched with $^{15}N_2$ gas, incorporation of N_2 by N_2-fixing bacteria and transfer of the fixed N to the plant tissues can readily be detected. This method is used to provide the ultimate proof of the presence of N_2-fixation, and has been used to demonstrate N_2-fixation in many cereals and grasses such as sugar cane (Ruschel et al., 1975), rice (Eskew et al., 1981), sorghum and millet (Giller et al., 1988). The method is highly sensitive and not subject to many errors and assumptions as long as the $^{15}N_2$ gas is thoroughly cleaned of any contamination of $^{15}NH_3$. This means that N_2-fixation can be demonstrated even when activities are fairly small, but the expense of the $^{15}N_2$ gas and problems of enclosing large plants

and controlling environmental conditions means that this method can only be used with fairly small plants. Results from experiments in laboratory incubations cannot be extrapolated to calculate integrated rates of N_2-fixation with full-grown field plants. A rare attempt to measure N_2-fixation associated with field-grown sugarcane using $^{15}N_2$ exposed the problems of using such results in the field and resulted in a detectable increase in ^{15}N-enrichment in the soil, but not in the plant (Matsui et al., 1981).

^{15}N isotope dilution and natural abundance methods

The principle on which both ^{15}N isotope dilution and natural abundance methods are based is that atmospheric N has a different isotopic signature than other sources of N available for plant uptake, namely plant-available soil and fertilizer N (for further details see the detailed review of Boddey, 1987). A non-N_2-fixing reference plant is normally used to give an estimate of the ^{15}N-enrichment of plant-available soil (and fertilizer) N. It is essential therefore that the sources of soil and fertilizer N are identical for both the N_2-fixing test plant and the reference plant as a completely uniform background ^{15}N-enrichment in space and time cannot be guaranteed. A common example where this basic assumption of the method is violated arises under strongly N-limited conditions where growth of shallow-rooting species may be highly restricted relative to more deeply rooting species. Experimental results from one of the early IAEA programmes where Sudan grass (*Sorghum sudanense*) was used as a reference plant for tropical legumes indicated *negative* N_2-fixation in many legumes which were well-nodulated and clearly fixing N in the field. This was due to the Sudan grass accessing unlabelled N from deep in the soil that was not accessible to the legumes. This example demonstrates the potential pitfalls of using different species of grasses or cereals with restricted rooting patterns as reference plants for measuring N_2-fixation in the field. Other examples in which the ^{15}N-isotope dilution method has been poorly applied to the measurement of N_2-fixation in legume crops are discussed by Chalk and Ladha (1999).

Even under controlled conditions in glasshouse experiments, treatment-dependent, sources of N have confounded results. For example, many early experiments were conducted using vermiculite as a rooting medium (e.g. Rennie, 1980). It was later shown that substantial amounts of plant-available unlabelled N were released from vermiculite when incubated under warm, moist conditions for several weeks in glasshouse experiments (Giller et al., 1986). More vigorous cultivars, or inoculated cereals with larger root systems have greater potential to exploit this extra source of unlabelled N, leading to highly misleading, treatment-dependent isotope dilution. Experiments at

ICRISAT comparing genotypes of sorghum and millet for potential N_2-fixation using ^{15}N-isotope dilution with vermiculite as a medium indicated differences between genotypes. When soil was collected from fields where ^{15}N-labelling experiments had been conducted three years earlier, so that a reasonably stable isotopic labelling could be guaranteed, these differences disappeared. Sorghum genotypes that had been shown to be 'best' and 'worst' in terms of acetylene reduction activity were compared in highly-replicated glasshouse experiments in large pots each containing 17 kg soil (Table 2). Although there were substantial differences in growth and N accumulation between the genotypes, the ^{15}N-enrichments differed by less than 4% and no significant differences in isotope dilution were detected giving no evidence for associated N_2-fixation. It is notable that a book entitled "Cereal Nitrogen Fixation" was published from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) (Wani, 1986) in the same year that the results of the experiment in Table 2 were widely publicised at the research institute, and despite an accumulated body of information that strongly indicated that most of the earlier experimental results were strongly flawed!

With the increasing availability of mass spectrometers with sufficient precision, some recent investigations have been based on variation in the natural abundance of ^{15}N. This is a method which is notoriously problematic if the relative proportions of N_2-fixed are small, not so much because the method is limited by precision of isotope analysis, but that the natural abundance of ^{15}N is influenced by many more factors than simply N_2-fixation (Shearer and Kohl, 1988; Boddey et al., 2000). Not least among the problems is what to use as a non-N_2-fixing control plant. The ^{15}N isotope dilution method and the natural abundance methods have been used to study N_2-fixation in rice plants in flooded soils under glasshouse conditions. The two methods gave similar results, against a strong decline in ^{15}N enrichment of the available soil N, indicating that the rice genotypes derived between 0 and 32% of their N from N_2-fixation (Malarvizhi and Ladha, 1996; Shrestha and Ladha, 1996).

4. ^{15}N-aided N Balances

The investigations of Boddey, Urquiaga and colleagues in Brazil using ^{15}N-aided N balances remain some of the best and most closely-controlled studies. A method often employed by this group has been to use large cylinders or tanks placed outside and to label soil with ^{15}N over long periods to achieve a 'stable' background enrichment. Different genotypes of various grass species are grown with their root systems limited to exploring the volume of soil of the container. Both ^{15}N-isotope dilution and N balance measurements are then used to cross-check the potential contributions from N_2-fixation (Lima et al., 1987; Urquiaga

Table 2.   Growth, N accumulation and ^{15}N-enrichment of sorghum genotypes grown in a glasshouse at ICRISAT, Hyderabad in large pots of soil (see text for further explanation).

Genotype	Shoot weight (g/pot)	Shoot N (mg/pot)	Atom %^{15}N excess
IS84	14.3	213	0.096
IS801	15.0	234	0.096
IS1398	25.8	215	0.096
CSV5	17.8	190	0.097
CSH5	22.0	229	0.098
IS5218	18.8	200	0.100
IS3003	21.9	233	0.100
SE	0.90	9.23	0.0021 (NS)
CV%	14.7	13.5	7.0

Twelve replicate pots (17 kg soil per pot) were sown with each sorghum genotype.

et al., 1992; Reis et al., 2001). But even these studies cannot provide unequivocal proof of N_2-fixation as not all processes can be controlled. As root systems are restricted to the containers, the plants must be irrigated with water, and given the huge quantities of water required by plants of vigourous grasses such as sugar cane, this has to be done with tap-water.

Based on climatic data from Brazil, and using the CROPWAT Model (FAO, 1995), a conservative estimate is that sugar cane transpires 1700–1850 mm water per year (equivalent to $17–18.5 \times 10^6$ l/ha/yr). Based on such estimates, and assuming only very small concentrations of NO_3^--N in the irrigation water, inputs of N of more than 50 kg N ha^{-1} could easily occur (Fig. 1). Given the fact that smaller grass or sugarcane genotypes are often used as non-N_2-fixing reference plants, and that these will have a smaller evaotranspirative demand, this could also account for differences in both ^{15}N-isotope dilution and N balance of some 20–30 kg N ha^{-1} y^{-1}. We do not claim that these effects are the underlying cause of treatment differences in isotope dilution or N balance, but simply indicate that they could contribute to treatment-dependent differences in availability of unlabelled N. In effect all estimates carry a degree of uncertainty in relation to their interpretation. Unequivocal measurements of N_2-fixed under 'real world' conditions are in fact extremely difficult, and the difficulty increases disproportionately as the potential amounts of N_2-fixation are smaller!
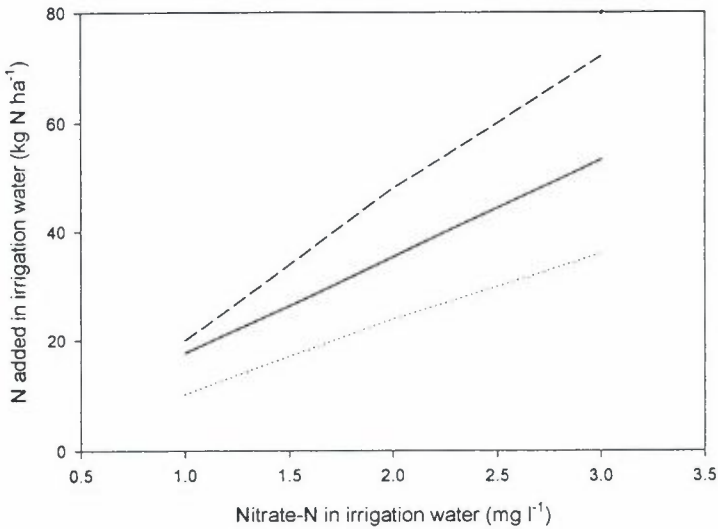
Figure 1. The amount of N added (kg N ha^{-1}) in irrigation water to sugar cane as influenced by the concentration of NO_3^--N in the water. The central line is based on a fairly conservative estimate of evapotranspiration in sugar cane of between 1700 and 1850 mm a year (17–18.5×10^6 l ha^{-1} y^{-1}) made using the CROPWAT model. The lower and upper lines are for annual evapotranspiration of 1000 mm and 2000 mm, respectively.
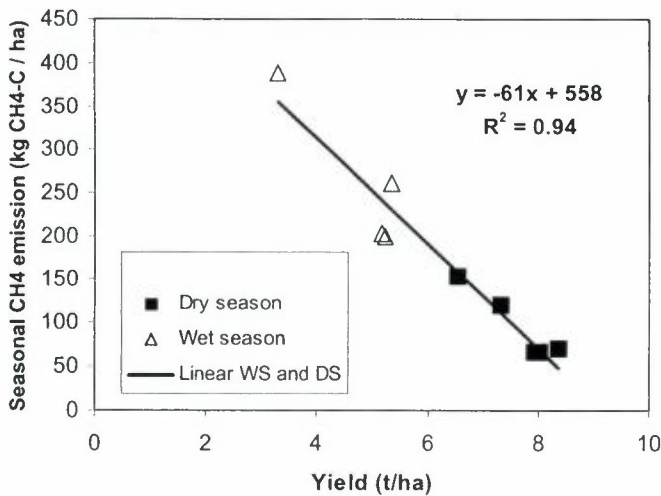

Figure 2. Seasonal methane emissions from rice soils (kg CH4-C ha^{-1}) as a function of yield of rice (t ha^{-1}) (from Denier van der Gon et al., 2002).

5. What Constitute 'Significant' Contributions from N_2-Fixation?

In the short-term, contributions of non-symbiotic N_2-fixation for cereals or pastures under agricultural conditions need to be >20 kg N ha^{-1} yr^{-1} assimilated by the crop to make a useful difference in productivity. Of all the candidate crops, sugar cane remains the most likely candidate, not least because of the abundance of C in a readily utilizable form. Indeed, heterotrophic N_2-fixation on litter in sugarcane systems where residues are not burned prior to harvest is also possible (Hill and Patriquin, 1990).

Long-term contributions of N_2-fixation to (semi) natural grasslands and/or pastures of 2–3 kg N ha^{-1} could be significant when longer periods of decades or centuries are considered (i.e. 5 kg N ha^{-1} yr^{-1} is equivalent to 500 kg N ha^{-1} in 100 yr). Such inputs are perhaps of marginal importance to the functioning of all but the most oligotrophic ecosystems. One potential guideline that could be used is to compare the inputs from N_2-fixation with those from atmospheric N deposition, with inputs of N_2-fixation equal to, or more than, inputs from rainfall and dry deposition being considered as significant in terms of ecosystem functioning. Atmospheric inputs from wet and dry deposition range from amounts in the order of only 3–5 kg N ha^{-1} in much of Africa to as much as 50 kg N ha^{-1} in some densely populated countries in Europe.

6. Missing Links – How to Convince the Sceptics?

Despite the unequivocal demonstration of the presence of free-living, root associated and endophytic N_2-fixing bacteria in abundance with many cereals and grasses, their ecological role is still uncertain. Simply demonstration of the presence of bacteria that actively express nitrogenase genes within a graminaceous plant does not mean that the amounts of N_2-fixation are of importance. Indeed in the search for a better quantitative understanding, laboratory studies are of very limited use for a number of reasons. If plants are grown under strong N limitation, conclusions that a high proportion of plant N is derived from N_2-fixation can be highly misleading as this can represent what is essentially a high proportion of nothing!

An example of such experiments is the recent study of N_2-fixation in Kallar grass (*Leptochloa fusca*) of Hurek et al. (2002). The interpretation of data based on ^{15}N natural abundance analyses is obscure and appears, on the information presented in the paper, to be fundamentally flawed. The plants were clearly growing under stressed conditions as a weight of only 1 g was reached after 8 months. The logic used to translate the amount from a figure expressed in g N plant^{-1} to 34 kg N ha^{-1} is not presented but is presumably an extrapolation based on either a typical plant density found in the field, or a

typical plant biomass. This study presents elegant and detailed molecular methodology but the conclusions of the paper are truly outrageous in a quantitative sense. A huge leap of faith is required to multiply from such growth experiments with N-starved plants to conclude that *"endophytes play an important role in N_2-fixation in natural grass ecosystems"*.

Insights into the strange results that can be derived from studies of weak plants in which the nutrient relations and physiology can be fundamentally altered can be derived from an interesting new study of methane emissions from the soil under rice plants (Denier van der Gon et al., 2002). When rice plants grow poorly, the rate of methane production is larger, indicating that a greater loss of C to the rhizosphere occurs than when plants grow and yield well (Fig. 2), raising the question whether N_2-fixation is possible without significant trade-offs in terms of growth and yield of the plants. This study indicates that sick plants behave in an unusual way and that when the plants grow strongly there is less C leakage from the roots as there is strong sink competition for C within the plant. The role of N_2-fixing endophytes remains open to question, although their presence within grasses and cereals is clearly demonstrated by many studies (James, 2000; McCully, 2001), as we lack a good quantitative understanding of C allocation to roots and rhizospheres and to endophytic bacteria.

Some suggestions for future research that may help in determining the potential for N_2-fixation in graminaceous plants are:

- By analogy with the model developed by Jones et al. (2003), a key point likely to determine the amount of N derived from N_2-fixation by endophytes is the relative abundance of non-N_2-fixing bacteria and N_2-fixers within the plant. This is clearly not a trivial exercise and may demand the development of new methods.
- In relation to rhizosphere bacteria, a question remains on whether epidermal cell junctions, where concentrations of microbes are generally found, are foci for exudation or simply protected indentations suitable for colonization (Jones et al., 2003).
- If large amounts of N_2-fixation were supported in the tissues of sugarcane, then by analogy to the legume-rhizobial symbiosis a coupling of high rates of respiration would be expected to support intense microbial activity in N_2-fixation. To our knowledge no such studies have been performed, but should be fairly simple to execute.
- If root-associated or endophytic N_2-fixation is able to provide amounts of N in the range of 40–60 kg N ha^{-1} then benefits in N accumulation should be readily demonstrated in glasshouse experiments under N-free conditions, coupled to strong plant growth as is the case with N_2-fixing legumes. Only results based on experiments where the plants grow well and accumulate a

reasonable biomass of several g plant^{-1} within a few weeks should be considered to avoid artefacts.
- The real 'acid-test' or proof of a significant role for N_2-fixation associated with graminaceous plants can only be derived from long-term N balances in which detailed assessment of all inputs (e.g. wet and dry N deposition, N from deep sources, irrigation water, etc) and losses (due to leaching of NO_3^--N and of N to the atmosphere) are included.

A detailed and robust quantitative understanding of both the C and N budgets of grasses and their associated rhizosphere and endophytic bacteria is required if we are to be able to properly assess the potential of N_2-fixation in these plants. The continuing lack of persuasive quantitative information is perhaps a reflection that the amounts of N_2-fixed are so small that it is difficult to measure!

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