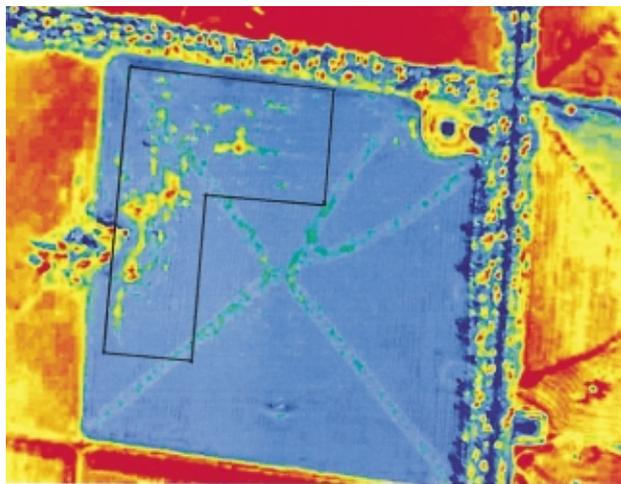


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The current and potential contribution of asymbiotic nitrogen fixation to nitrogen requirements on farms: a review

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Abstract. Significant levels of biological nitrogen fixation from sources other than nodulated legumes have become a tantalizing prospect for decades. Since the benefit to agriculture of nitrogen fixation from nodulated legumes was established, there have been widespread efforts to promote the use of various asymbiotic diazotrophic bacteria to fix extra nitrogen in soil. Despite much optimism by scientists and farmers, this prospect remains to be realised. Recently, the prospect has been pursued with renewed enthusiasm and several commercialised products have appeared. What are the reasons for this fresh enthusiasm? Are the new products based on realistic assessments of their biological potential? Why has it taken so long to advance to a stage where there is still only limited evidence that verifies hope becoming reality?

This review assesses the current contribution from asymbiotic nitrogen fixation and re-assesses the prospects for greater contributions from this source. Among the many aspects of this multi-faceted subject that will be considered are: (i) the range of free-living microbial strains currently contributing to significant asymbiotic nitrogen fixation; (ii) the significance of nitrogen-fixing microbes naturally associated with plants; (iii) the significance of endophytic systems and their role in sugarcane and other Gramineae; (iv) the possibility of extending this range by introducing new strains or discovering new systems capable of contributing additional nitrogen fixation.

The case will be made that conditions providing a sustainable contribution for more than a short time are usually missing in such systems so that spontaneous biological nitrogen fixation is usually transient. It will be argued further that if all the positive factors controlling spontaneity at the biothermodynamic level are exploited, significant biological nitrogen fixation may soon be achieved in some of these systems on farms.

Additional keywords: diazotrophs, saprophytes, endophytes, biofertilisers, cereals, sugarcane.

Introduction

Since biological nitrogen fixation (BNF) was discovered in the late 19th century, there have been positive reports of the potential benefits from asymbiotic nitrogen fixation (ANF). Because of the greater ease of management, far more attention has been devoted to the symbiotic systems, based on the *Rhizobium*–legume interaction. In natural ecosystems, *Frankia*-based systems between plants and microbes are also recognised as reliable and important in habitat colonisation (Dawson 1990; Diem and Dommergues 1990). The highly organised nature of these symbiotic systems as controlled genotype × environment interactions is in strong contrast to the disorganised nature of the asymbiotic systems. The significance of these interactions in achieving the full potential of ANF is a major theme of this review.

In an agricultural context the ‘asymbiotic’ systems are generally insignificant. This is partly because there can be no doubt of the much lower magnitude of the BNF involved in contrast to symbiotic nitrogen fixation (SNF), but also

because the lack of obvious association with the plant means that the utilisation of ANF is less direct. The affinity with which fixed nitrogen is accumulated by plants from many sources ensures that tracing their use is very difficult.

For the purposes of this review, the meaning of the term ‘asymbiotic’ must be clearly understood, particularly as it might now be considered a term less used than formerly and perhaps dated. In this article, asymbiotic will be used to include all BNF not occurring in a genetically defined system where there is a specific interaction between the diazotroph and the host plant resulting in recognisable morphological structures of nodules in the host plant, typical of SNF. Thus, this definition of asymbiotic includes any nitrogen fixation by microbial cells growing independently (free-living) in soil and in terrestrial and aquatic wetland environments as saprophytes, BNF occurring in loose or close association with the plant rhizosphere, and endophytically, but not requiring morphologically defined nodules. Although ANF in marine mangrove ecosystems is beyond the topic of

our discussion, a substantial contribution of biologically fixed nitrogen from the mangroves ecosystem to farming systems has also been observed (Zuberer and Silver 1978; Van der Valk and Attiwill 1984; Fiona and Steinke 1992).

Previous research on free-living diazotrophs

The potential of soil to fix atmospheric nitrogen (N_2) was clearly indicated by Berthelot (1888), when he found that unsterilised soil gained in nitrogen, whereas sterile soil did not. Based on logical speculation, Berthelot reasoned that certain free-living soil microorganisms were responsible. As quoted by Tchan (1988) 100 years later, Berthelot suggested this fixation process in soil was necessary to compensate for losses of nitrogen from soil to the atmosphere. It is probable that Jodin (cited by Tchan 1988 and Waksman 1932) had observed BNF earlier, in 1862, when he recorded the vigorous growth of a 'mycoderm' in a solution containing phosphate, sugar, tartaric acid or glycerol but no nitrogen. However, Berthelot's contribution was more intellectually perceptive of the important principle involved. Since Berthelot used washed sand, the gain in nitrogen observed (22–38 mg per flask) would have required far more reduced carbon substrates than were likely to be present. It seems definite, in retrospect, that photoautotrophs capable of providing about 2–4 g of sugar equivalent, would have enabled this amount of nitrogen fixation.

These claims of Berthelot were disputed. Schloesing (1888) failed to demonstrate the uptake of N_2 using gasometric methods and claimed that analytical errors were involved. This dispute demonstrates a common problem in this kind of research, i.e. the biological nature of the system introduces variables that may contribute to inconsistent results compared with purely chemical or physical systems. In order to function, biological systems involve satisfying the needs of a complex genotype \times environment interaction (Kennedy 2000). It is quite possible that a sufficient number of soil microorganisms was present in the first case to provide infection of a suitable genotype, but not in the second. Reflecting on this experiment much later, Winogradsky (1927) was also sceptical of Berthelot's results, but on the grounds of insufficient moisture being present, rather than errors of analysis. Berthelot's contribution remains his clear recognition of the logical need for a process such as BNF. Now that the cyclic nature of the nitrogen metabolism is better understood, this need is even more obvious since SNF is only responsible for part of the nitrogen fixed biologically.

Winogradsky (1893) isolated the first heterotrophic diazotroph, *Clostridium pasteurianum*, using his method 'elective', now designated as using 'selective' conditions (see Tchan 1988). This involved adjusting the environmental conditions, to predispose the system to the growth of organisms able to supply the missing nitrogen, favouring the growth of diazotrophs. In the case of diazotrophs, this involves the use of nitrogen-free media, or more importantly,

media with a high carbon:nitrogen ratio with ample reduced carbon substrates, such as sugars, but low inorganic nitrogen content. Winogradsky said:

"In nature there is an enormous reserve of organic matter poor in nitrogen. One should ask how all this organic carbon can be circulated without the existence of organisms capable of fixing free nitrogen."

While true in a qualitative sense, this conclusion also has a direct basis in thermodynamic principle since intermolecular exchange forces are directly involved in the selective process by which the growth of N_2 -fixing organisms will be favoured. Action thermodynamics (Kennedy 2000), a new approach to the study of non-equilibrium forces in nature, indicates that genotypes able to exploit a thermodynamic potential introduced into an ecosystem (e.g. such as a high carbon:nitrogen ratio) will be forcefully and inevitably selected to utilise this potential, provided other essential nutritional requirements are met. A fuller understanding of this principle and the means to take benefits from it could have important implications for improving the nitrogen status of soils and the optimum growth of crops.

As Winogradsky recognised later and Tchan (1988) emphasised, it was surprising that Winogradsky isolated first the more difficult to grow anaerobic organisms from the bottom of the tube containing mannitol, overlooking the fairly obvious surface pellicle (Jodin's mycoderm) formed by the aerobic *Azotobacter*. Indeed, without the growth of the latter, which removed the oxygen, the obligately anaerobic clostridia could not have grown at all. It was left to Beijerinck (1901) to isolate and identify *Azotobacter*. The stringent conditions for isolating organisms capable of BNF are now taken for granted but at a time when the nature of microorganisms was just beginning to be understood, none of these discoveries can be regarded as easily-gained knowledge. An additional feature of BNF is its adaptive nature, for example, all known systems are subject to repression whenever fixed nitrogen is present in amounts adequate to support sustained growth. Tchan and Kennedy (1987) pointed to the strong moisture dependence of asymbiotic diazotrophs.

Since these early discoveries, a large range of microbial genera have been shown to be capable of fixing N_2 . These include *Beijerinckia*, *Derrxia*, *Pseudomonas*, *Alcaligenes*, *Klebsiella*, *Enterobacter*, *Rhodopseudomonas*, *Azospirillum*, *Acetobacter*, (see Kennedy and Tchan 1992; Watanabe *et al.* 1992), *Azoarcus* (Reinhold-Hurek *et al.* 1993), a range of cyanobacteria (*Anabaena*, *Calothrix*, *Nostoc*, *Fischerella*, *Microcoleus*, *Nodularia*, *Gloeotrichia*, *Scytonema*, *Tolypothrix*) (Watanabe *et al.* 1992; Toledo *et al.* 1995), *Burkholderia* (Baldani *et al.* 1986a), *Herbaspirillum* (Pereira *et al.* 1988) and many others. These genera include saprophytes growing on plant residues, associative rhizosphere organisms and a number of endophytes (see Table 1). The contribution of each class to

Table 1. Asymbiotic diazotrophs

Bacteria	Host	Class	Reference
<i>Acetobacter diazotrophicus</i>	Sugarcane	Endophytic	Cavalcante and Döbereiner (1988); Lee <i>et al.</i> (1996)
<i>Alcaligenes faecalis</i>	Rice	Endophytic	Vermeiren <i>et al.</i> (1996)
<i>Aquaspirillum fasciculus</i>	Water	Associative	Giller and Wilson (1991)
<i>Aquaspirillum perigrenum</i>	Water	Associative	Giller and Wilson (1991)
<i>Azoarcus</i>	Kallar grass	Endophytic	Reinhold-Hurek <i>et al.</i> (1993)
<i>Azospirillum brasilense</i>	C ₃ plants	Associative	Baldani <i>et al.</i> (1986b)
<i>Azospirillum lipoferum</i>	Maize	Associative	Tarrand <i>et al.</i> (1978); El-komy <i>et al.</i> (1996)
<i>Azospirillum amazonense</i>		Associative	Falk <i>et al.</i> (1985)
<i>Azospirillum halopraeferens</i>	Salty habitat	Associative	Reinhold <i>et al.</i> (1987)
<i>Azorhizobium caulinodans</i>	Wheat	Endophytic	Kennedy <i>et al.</i> (1997)
<i>Azospirillum caulinodans</i>	Rice	Associative	Van Holm <i>et al.</i> (1993)
<i>Azotobacter vinelandii</i>	Soil	Saprophytic	Parker (1954); Kennedy and Toudarina (1996)
<i>Azotobacter chroococcum</i>	Soil	Saprophytic	Döbereiner and Pedrosa (1987)
<i>Azotobacter paspali</i>	<i>Paspalum</i>	Associative	Döbereiner (1970)
<i>Beijerinckia</i>	Associative	Acid-tolerant saprophyte	Becking (1961)
<i>Beijerinckia indica</i>	Associative	Acid-tolerant saprophyte	Derx (1950)
<i>Beijerinckia mobilis</i>	Associative	Acid-tolerant saprophyte	Derx (1950)
<i>Beijerinckia derxii</i>	Associative	Acid-tolerant saprophyte	Tchan (1957)
<i>Beijerinckia fluminensis</i>	Associative	Acid-tolerant saprophyte	Döbereiner and Ruschel (1958)
<i>Burkholderia brasilense</i>	Sugarcane etc.	Endophytic, epiphytic	Baldani <i>et al.</i> (1986a)
<i>Campylobacter nitrofigilis</i>	Salt-bush	Associative	McClung <i>et al.</i> (1983)
<i>Clostridium pasteurianum</i>	Soil	Saprophytic	Winogradsky (1893); Parker (1953)
Cyanobacteria (<i>Anabaena</i> , <i>Calothrix</i> , <i>Nostoc</i> , <i>Fischerella</i> , <i>Nodularia</i> , <i>Microcoleus</i> , <i>Gloeotrichia</i> , <i>Scytonema</i> , <i>Tolypothrix</i>)	Azolla	Saprophytic, associative and endophytic	Watanabe <i>et al.</i> (1992); Toledo <i>et al.</i> (1995)
<i>Enterobacter</i>	Rice	Epiphytic	Ladha <i>et al.</i> (1983)
<i>Enterobacter agglomerans</i>	Rice	Epiphytic	Papen and Werner (1979)
<i>Herbaspirillum seropedicae</i>	Sugarcane	Endophytic	Baldani <i>et al.</i> (1986a)
	Sorghum	Endophytic	Döbereiner <i>et al.</i> (1993)
	Wheat	Endophytic	Pereira <i>et al.</i> (1988); Kennedy <i>et al.</i> (1997)
<i>Klebsiella pneumoniae</i>	Rice	Associative	Ladha <i>et al.</i> (1983)
<i>Pseudomonas diazotrophicus</i>			Chan <i>et al.</i> (1994)
<i>Pseudomonas</i> spp.	Rice	Associative, endophytic	Bally <i>et al.</i> (1983); Barraquio <i>et al.</i> (1983)
<i>Rhizobium</i>	Rice		Euan <i>et al.</i> (2000)
<i>Rhodopseudomonas</i>			
<i>Serratia marcescens</i>	Rice	Associative	Rosales <i>et al.</i> (1993)

nitrogen requirements on farms will be considered in the following sections.

Current contribution of asymbiotic diazotrophs to nitrogen requirements in agricultural ecosystems

One of the primary features of asymbiotic microorganisms is that they usually fix N₂ to satisfy their own needs for nitrogen for cell growth. As indicated above from early research, conditions with high carbon:nitrogen ratios and good moisture status favour the growth of diazotrophs. Therefore, we can confidently anticipate that significant quantities of nitrogen will be fixed whenever these conditions exist naturally.

Saprophytic biological nitrogen fixation — inoculation with *Azotobacter*

Field experience has amply demonstrated the variable nature of benefits from ANF. Caron (1895, 1900) reported favourably on the role of soil inoculation with saprophytic microorganisms capable of BNF on the yield of various non-legumes, using isolates from soil and composts. However, Gerlach and Vogel (1902) and Lipman and Brown (1907) could not confirm that inoculation with *Azotobacter* was beneficial to crops.

In Russia, Kostychev *et al.* (1926) reported strong positive effects of soil inoculation with *Azotobacter* on the growth of tobacco and accumulation of soil nitrogen. This

led to a strong tradition of inoculation with this genus in Russia extending over decades, with many apparent confirmations of positive ANF. However, Mishustin (1970) claimed that only one-third of the Russian experiments had actually given positive results. In other countries, the results were inconsistent and less impressive (Kyle and Eisenstark 1951; Rubenchik 1963; Dart 1986). In Australia, Rovira (1963) and Ridge and Rovira (1968) obtained inconsistent responses of wheat to inoculation. Gainey (1949) not only obtained no response to inoculation with *Azotobacter* but was unable to recover the organism from the rhizosphere after inoculation.

Jensen (1940) carried out a major study on the potential contribution of *Azotobacter* to the nitrogen economy of Australian wheat soils. He established that $5\text{--}10 \times 10^6$ *Azotobacter* cells/g of soil were needed to fix 1 mg/kg (1 ppm). However, in some soils, even when straw was added, no net nitrogen was added; only in water-saturated conditions did significant nitrogen fixation occur, indicating that restriction of oxygen access was required for significant rates. Jensen found that only in artificial soil 0.5–1.0 mg of N was fixed/g of straw, increasing to 3–6 mg of N/g of straw in water-saturated media. On Western Australian red-brown earths used for growing wheat, Parker (1954) established a correlation between the presence of *A. vinelandii* and significant increases in soil nitrogen. He was unable to confirm that significant ANF occurred every growing season or to rule out movements of soil N from lower soil horizons as an explanation for the increases in the surface soil. This study highlights the difficulty in establishing ANF as a factor in crop growth in the field, but *Clostridium butyricum* was also found (Parker 1953) associated with these unexplained increases in soil nitrogen content. As Parker pointed out, the simultaneous occurrence of these aerobic and anaerobic diazotrophs is by no means inconsistent, because soil crumbs may exhibit a range of oxygen potentials varying from aerobic on their external surfaces to anaerobic in the interior of such aggregates because of the consumption of oxygen.

Overall, these results were not convincing of a significant and reliable contribution to ANF, but they bear closer examination. The original concept dating from the time of Berthelot and Winogradsky suggested that a high quantity of decomposing carbohydrates, for example following soil incorporation of residues from growth of cereal crops, would promote BNF. There is no evidence of consistent attention being paid to the complex set of conditions needed in the plant and microbial environment to promote BNF in these studies. In particular, the necessary high carbon:nitrogen ratio was not confirmed as given in these studies. In addition, the standard of significance is difficult to establish in such trials. To be convincing, yield increases greater than 10–15% would be required. Furthermore, the field conditions (e.g. carbon:nitrogen ratio) might dictate that 5–10% would be the correct response. Even such a low increase in yield

might be sufficient to justify inoculation if consistently obtained. It is doubtful if experimental trials could show significant results with such low yield increases but farmers would be satisfied.

Effect of phytopolymer decomposers as sources of energy

The use of plant residues to promote nitrogen fixation has been a concept understood since the first isolations of free-living diazotrophs. More deliberate attempts to increase BNF rates by cooperative systems involving co-inoculation with phytopolymer decomposers to accelerate breakdown of organic residues have demonstrated the potential of such systems (Jensen and Swaby 1941a, 1941b; Pochon and Tchan 1948; Jensen 1965; Lynch and Harper 1983). Jensen (1940) estimated that to compensate for the nitrogen exported from soil in wheat grain the system would need to fix nitrogen at the rate of 10 parts of nitrogen by weight to 1000 parts of total cereal residue, a relative rate at the lower end of the range observed in laboratory experiments using a cooperative phytopolymer decomposer and a diazotroph. This 10–20 mg/g is less than one-tenth the efficiency of nitrogen fixation observed in legume nodules of 100–200 mg of N₂ fixed/g of carbon substrate consumed (Tchan and Kennedy 1987).

For wheat, the ratio of dry straw produced to grain exported is considered as 0.8 (Staniforth 1979), comprising about 43% carbon. Thus, an annual wheat yield in Australia of 30×10^6 tonnes of grain produces almost 25×10^6 tonnes of straw, with a high carbon:nitrogen ratio of about 85:1 and potentially a huge resource for ANF. Wheat stubble contains about 0.5% nitrogen and therefore an average yield of straw (2.0 t/ha) contributes only about 10 kg nitrogen/ha to the soil nitrogen pool. Were the carbon in straw to be metabolised substantially to support ANF, additional contributions of 50–150 kg of nitrogen fixation/ha are theoretically possible.

More recently, Halsall and Gibson (1985) showed the potential benefit of mixed cultures of organisms (*Cellulomonas gelida* — *Azospirillum brasilense* or *A. lipoferum* with *Bacillus macerans* or *B. polymyxa*) capable of decomposing straw and fixing nitrogen. Co-inoculation yielded as much as 72 kg N/t of straw consumed in laboratory simulations of field conditions. Their results approached the theoretical limit and the efficiency of legume nodules (Kennedy and Cocking 1997, p. 15). Halsall and Gibson's treatments were similar to that employed by Pochon and Tchan years earlier (1948), using *Azotobacter* and *Cytophaga*. Such high rates represent a theoretical maximum in the absence of competition from the growth of other organisms, or of losses of carbon by processes such as leaching. Attempts to measure ANF supported by straw under field conditions at Gunnedah, in northern New South Wales, using acetylene reduction to measure nitrogenase activity have shown that under conditions in which soil water content was maintained at about –10 kPa, nitrogen fixation was highest and continued

Table 2. Estimates of N₂ fixed by different biological systems and availability of inoculation technologyCollated from Bohlool *et al.* (1992) and Peoples *et al.* (1995)

Biofertiliser	Amount of N ₂ fixed (kg/ha)	Availability of inoculation technology
Rice–Azolla	20–100/crop	Yes
Legume–Rhizobium		
<i>Glycine max</i>	0–450/crop	Yes
<i>Vigna radiata</i>	9–112/crop	
<i>Cajanus cajan</i>	7–235/crop	Yes
<i>Arachis hypogaea</i>	37–206/crop	Yes
<i>Cicer arietinum</i>	3–141/crop	Yes
<i>Pisum sativum</i>	17–244/crop	Yes
<i>Vicia faba</i>	53–330/crop	Yes
<i>Sesbania rostrata</i>	11–458/crop	Yes
<i>Leucaena leucocephala</i>	100–300/year	
Non-legume– <i>Frankia</i>		
<i>Casuarina sp.</i>	40–60/year	?
<i>Free-living/associative asymbiotic</i>		
Rice–blue green algae	10–80/crop	Yes
Rice–bacterial association	10–30/crop	No
Sugarcane–bacterial association	20–160/crop	No
Wheat–bacterial association	10–30/crop	Yes/no

the longest in soil containing highest amendments with straw (4500–700 kg/ha) (Roper 1985, 1987).

In studies on sugarcane waste Patriquin (1982) showed significant rates of nitrogen fixation. Total nitrogen fixed was similar with either mulched or incorporated trash, but mulching tended to delay the maximum rate of nitrogen fixation. Considering that sugarcane has a total potential to accumulate 100 tonnes of dry material per crop, the potential for nitrogen fixation with this crop from residues is unusually high.

From data obtained in the Broadbalk experiment carried out at Rothamsted, Day *et al.* (1975) estimated that gains of 34–49 kg N/ha.year were obtained, presumably from asymbiotic BNF.

Nitrogen fixation by associative diazotrophs

Since the 1970s, the focus has shifted from saprophytes to diazotrophs more directly associated with Gramineaeous plants during their vegetative phase. The rhizosphere organisms known as associative include *Azospirillum* spp. Other associative diazotrophs are *Derxia*, *Pseudomonas*, *Alcaligenes*, *Klebsiella*, *Enterobacter* and others (Table 1). Some of these associative organisms are considered to colonise the roots of grasses internally, in which case they are called endophytes, and these are considered in the next section.

The promising pioneering work of Johanna Döbereiner's group in Brazil (Day and Döbereiner 1976) was followed by the realisation that the azospirilla could only partly live up to

early hopes of providing substantial benefits on farms. In general, their nitrogen fixation appears to proceed at rates much too low to contribute more than a small fraction of the nitrogen requirements of crops (reviewed by Baldani *et al.* 1983; Bashan and Levanony 1990; Okon and Labandera-Gonzalez 1994). In his most recent review, Bashan (1998) again emphasised that the benefits to plants grown in association with azospirilla are likely to result from the PGPR (plant-growth promoting rhizobacteria) effect as a result of production of phytohormones modifying root development. Other benefits from microbial association can include biocontrol effects on pathogens and mobilisation of soil nutrients including nitrogen. Estimates of microbial contribution of nitrogen from BNF are given in Table 2, compared with systems involving SNF. As there have been a number of recent comprehensive reviews in this area, this topic will not be dealt with here (Kloepper *et al.* 1989; Bohlool *et al.* 1992; Peoples *et al.* 1995; Hallmann *et al.* 1997).

Biological nitrogen fixation by diazotrophs as endophytes — sugarcane

The positive experience with sugarcane in Brazil presents the newest set of data, indicating substantial contributions of nitrogen as ANF on farms. Using ¹⁵N₂ gas (Ruschel *et al.* 1975), sugarcane has been shown to derive nitrogen from BNF. This conclusion is supported by nitrogen balance and ¹⁵N dilution techniques (Lima *et al.* 1987; Urquiaga *et al.* 1992). This work has been extended and summarised by Boddey *et al.* (1995). Up to 200 kg/ha of nitrogen from ANF may be contributed to sugarcane crops, based on these experiments. This benefit from BNF may be reduced where fertiliser-nitrogen is provided, as would be anticipated from the known properties of nitrogenase genes which are repressed by fixed nitrogen. Furthermore, growth of the endophytic and epiphytic diazotrophs involved may also be repressed under these conditions, so that BNF is no longer possible with fertiliser nitrogen application.

Biological nitrogen fixation in sugarcane is suggested to result from the activity of endophytic bacteria. Endophytic diazotrophs isolated from sugarcane include, *Acetobacter diazotrophicus* (Cavalcante and Döbereiner 1988), *Herbaspirillum seropedicae* (Baldani *et al.* 1986a) and *H. rubrisubalbicans* and *Burkholderia brasilense* (Baldani *et al.* 1996). All these bacteria were isolated from the interior of sugarcane plants, probably occurring intercellularly, although a location in xylem cells has sometimes been suggested (Dong *et al.* 1994; James *et al.* 1994).

Acetobacter diazotrophicus is an obligate endophyte that will only infect intact inoculated sugarcane plants with great difficulty (see Reis *et al.* 1994). Its infection may be aided by association with arbuscular mycorrhizae. It is transferred vegetatively in sugarcane sets. It occurs in roots, shoots, apoplast and the xylem of cane plants (Dong *et al.* 1994; James *et al.* 1994). The organism requires high sucrose

concentration (10–15%) and can grow and fix nitrogen at pH <3.0 (optimum pH 5.6) (Cavalcante and Döbereiner 1988). Because it does not possess nitrate reductase, even 20 mmol NO₃⁻/L does not inhibit nitrogenase activity. In addition to sugarcane, *A. diazotrophicus* has been isolated from Cameroon grass (*Pennisetum purpureum*) (Reis *et al.* 1994).

Herbaspirillum seropedicae has a wider plant host range than *A. diazotrophicus*. It was initially isolated from maize, rice and sorghum (Baldani *et al.* 1986a) and only subsequently identified in sugarcane (Baldani *et al.* 1992). Its endophytic nature in sugarcane was very clear in this crop as it was extremely closely related genetically to 'Pseudomonas' *rubrisubalbicans* which was known to be the causative organism of mottled stripe disease of cane and other grasses (sorghum and *Pennisetum*). Plant pathologists never had any doubt that this bacteria (now reclassified as *Herbaspirillum rubrisubalbicans*, Baldani *et al.* 1996) was an endophyte. Other species of diazotrophs are expected to be found associated with sugarcane.

A number of outstanding questions in relation to ANF in sugarcane remain, including which species are mainly responsible for the observed ANF. Only work on field-grown plants using techniques that specifically identify the source and location of the nitrogenase activity within plants, such as isotopic labelling, can resolve this question. The mechanism of nitrogen transfer to the host plant also remains uncertain. There is also an unresolved question in the mind of some critics regarding the extent to which the endophytic bacteria are responsible for the ANF observed. We can speculate that associative and even saprophytic microorganisms may also make significant contributions to the BNF observed in trials. However, from the farmer's point of view, BNF in sugarcane is of sufficient magnitude to influence the economics of production. In this sense, the actual site of nitrogen fixation is immaterial. However, there is an important task ahead to make this system a practical technology that can be applied more widely by farmers. Questions related to ensuring sufficiently high numbers of diazotrophs in sugarcane plants, optimising inoculation technology and the associated agronomic practices to optimise BNF all remain to be answered.

Kallar grass and Azoarcus

A strain of the Gram-negative nitrogen-fixing bacterium *Azoarcus* sp. BH72, originally isolated from Kallar grass (*Leptochloa fusa* Kunth) growing in the saline-sodic soils typical of Pakistan, has been described (Reinhold-Hurek *et al.* 1993). *Azoarcus* spp. also colonise grasses such as rice under laboratory experiments (Hurek *et al.* 1994a). Basic characteristics of this bacteria are available elsewhere (Hurek *et al.* 1994b, 1995). The apical region of the root behind the root meristem was most intensively colonised, the strain penetrating the rhizoplane preferentially in the zones of elongation and differentiation and colonising the root interior both inter- and intracellularly. In addition to the

root cortex, bacteria were also found in the xylem, but no evidence for *Azoarcus* residing intracellularly in living plant cells has been found; the plant cells being apparently destroyed after bacteria penetrate the cell wall. This genus, as a potential endophytic nitrogen-fixing microsymbiont for cereals, merits continued study to confirm and extend these results in other laboratories.

Compared with the legume-*Rhizobium* symbiotic systems, the current contribution from ANF is generally relatively small (Table 2). Indeed, in some cases where there are high levels of nitrogen fertiliser applied, it is probably completely absent. In other cases, there is now strong evidence of a significant contribution. However, the imperfect knowledge of the extent of ANF and of its integration with the needs of growing cereal crops and the effects of farming practices on the availability of nitrogen fixed by ANF is a serious disadvantage. To improve this situation, not only will better conditions for higher rates of ANF be required, but monitoring of its contribution to meeting the needs for nitrogen of other species in farming systems is also essential.

Potential new contributions

There is little historical data regarding current on-farm accession of N from ANF. However, there are too many reports of significant potential of ANF to ignore. There are also indications of the reasons why ANF should so often fail. Kennedy *et al.* (2000) have pointed to the need to carefully define these limitations, such as the degree of colonisation, access to carbon substrates, the need for microaerobic conditions to prevent inhibition of nitrogenase activity and for nitrogen transfer from asymbiotic diazotrophs to crop, and to take corrective measures. It will be essential to ensure that the biothermodynamic requirements implied by these factors as part of the genotype × environment interaction are met. In all probability, all of the apparent failures can be traced to deficiencies in one or more of these factors.

In considering these requirements, several laboratories have instituted research programs which take a more proactive approach to achieving significant BNF with grasses.

Modified and engineered systems

The modern era of scientific study of nitrogen fixation now has the benefit of genetic tools to investigate the processes in more depth. Using gene fusion marker techniques, it has been possible to examine more closely processes such as root colonisation. Evidence is coming from studies at the Centre for Crop Nitrogen Fixation at Nottingham on the endophytic establishment of *Azorhizobium caulinodans* in wheat (Sabry *et al.* 1997), and evidence for crack entry and its stimulation by specific flavonoids (Webster *et al.* 1998). Nodule-like structures (probably inactive in nitrogen fixation) that are produced on rice are similar in superficial origin in the cortex of rice (and

wheat) to those produced at Nottingham on rice and wheat by the action of cellulases and pectinases together with rhizobia (reviewed by de Bruijn *et al.* 1995). The fact that the internal colonisation and crack entry of wheat and rice (and *Arabidopsis*) are not under the control of the usual *nod* genes suggests that nitrogen fixation is more likely to be endosymbiotic than nodular (Gough *et al.* 1996). The opportunity now exists to carry out a similar investigation in wheat since *Azorhizobium caulinodans* has been isolated from short lateral roots of wheat and shown to re-invade wheat (by crack entry) and also when nodulating its legume host, *Sesbania rostrata* (O'Callaghan *et al.* 1997). Labelling studies have also shown clear evidence of substantial xylem colonisation in the case of oilseed rape (O'Callaghan *et al.* 2000), raising the question of the extent to which xylem flow may be impeded. These findings regarding successful endophytic colonisation of non-legumes by diazotrophs may provide useful background to establishing novel systems for ANF in future.

We have exploited (Kennedy *et al.* 2000) the ability of synthetic plant hormones such as 2,4-D to enhance access of azospirilla to protected niches such as the base of modified lateral roots (*para*-nodules), and in channels between cortical cells of wheat roots. Quantitative measurement of the pattern of colonisation by using reporter gene (*lacZ*) fusion techniques has now allowed the recognition of important features in diazotroph:wheat associations (Tchan *et al.* 1991; Kennedy and Tchan 1992; Kennedy *et al.* 1997), based on the pioneering work of Y. F. Nie at Shandong University, Jinan, China, in the late 1980s. Related research on *para*-nodulation has been carried out in Belgium (Christian-Weniger and Van Veen 1991; Christian-Weniger and Vanderleyden 1994) indicating colonisation patterns can be strongly modified by the use of synthetic auxins such as 2,4-D and that ammonia-excreting mutants of *A. brasilense* may eventually prove beneficial in agriculture. This area of study has benefited from the application of *lacZ* markers such as *nifH* (Vande Broek *et al.* 1996; Kennedy *et al.* 1997) which has allowed definition of the degree of microaerobic conditions where nitrogenase is being expressed. The studies have revealed BNF is likely to be successful where there is adequate colonisation under endophytic or intimately epiphytic conditions (see comment by Barry Rolfe in Kennedy and Cocking (1997) for a critique of the term endophytic).

Azospirillum brasilense Sp7-S, a spontaneous mutant of wild-type Sp7 which performed better than the parent strain in infection and in BNF in the laboratory model known as the *para*-nodule, produced less exopolysaccharide with consequent impaired flocculation (Katupitiya *et al.* 1995). In refining the knowledge of the relationship between exopolysaccharide production and the pattern of colonisation, the *flcA* gene was isolated and sequenced (Pereg-Gerk *et al.* 1998). The genetic tools developed

allowed the preparation of transposon mutants with altered colonisation patterns. Recognition of variability in flocculation was based on the altered pattern of colonisation in *para*-nodulated wheat roots. This pattern was typified by concentration of the inoculant bacteria around *para*-nodules and less colonisation of the root epidermal surface, the main site colonised by wild-type *A. brasilense* Sp7. Such a pattern was predicted to have significant advantages with respect to both carbon or energy supply and improved protection from atmospheric oxygen.

The relationship between colonisation pattern, oxygen concentration and nitrogenase expression was examined using *nifH-lacZ* as a reporter (see also Vande Broek *et al.* 1996; Kennedy *et al.* 1997, 2000). As part of this work, Wood (1999) showed highly significant transfer of label (20% of the total nitrogen content) from $^{15}\text{N}_2$ to wheat shoots, when the roots of these plants were colonised by an ammonia-excreting mutant of *A. brasilense* supplied with organic acid (malate). Interestingly, no ammonia could be detected in the rhizosphere itself and the expression of nitrogenase activity was higher than in nitrogen-free broth cultures. Apparently, the ability of the wheat roots to scavenge ammonia allowed full derepression of the *nif* genes. These results confirm that immediate benefit is possible from BNF by azospirilla under these conditions. Previously, enrichment with ^{15}N had been restricted to the root system, indicating that the BNF occurring is bound to the growth of the bacterial cell and its subsequent decomposition to release nitrogen to the host. In this experiment, no ^{15}N was transferred to the shoots using the wild-type parent.

Direct benefits on plant growth were observed using *flcA*⁻ mutants of this ammonia-excreting mutant, engineered to prevent exopolysaccharide production and encystment. Such transposon-induced mutants colonise in a more endophytic mode (Pereg-Gerk *et al.* 1998). When coupled with ammonia excretion, plant dry weight was increased several-fold compared with wheat plants grown either in nitrogen-free sand or agar (Kennedy *et al.* 2000).

Inoculant biofertilisers — a prescription for success

The following sequence is considered (Kennedy *et al.* 2000) as providing a prescription for the successful evolution of a nitrogen-fixing system for cereals.

(i) *Chemical recognition by diazotrophs of the rhizosphere habitat.* This will involve the appropriate chemotactic response, shown to be under genetic control *in vitro* (see Zhulin and Armitage 1992; Zhulin *et al.* 1996) and probably also in soil. These chemical gradients also provide energy substrates for the bacterium.

(ii) *Binding interactions with the root surface and entry of endophytes.* Binding of azospirilla to the root surface (Jain and Patriquin 1984) may involve flagellae, exopolysaccharides, hydrolytic enzymes like proteinase and cellulase (Michiels *et al.* 1990, 1991; Croes *et al.* 1993; Vande Broek and

Vanderleyden 1996; Hallmann *et al.* 1997) but this binding may be inimical to colonisation elsewhere in the system. Endophytes will require entry through an appropriate portal, possibly through cracks or during epidermal extension. The process of entry also involves dispersive forces exerted by the bacterium (e.g. flagellar action etc.), thus separating cells and may even require the dispersive forces involved in bacterial and plant cell division. Such a process may involve the action of enzymes such as cellulases or polygalacturonases, allowing bonds between cells to be broken and entry of bacteria. In *para*-nodulation, the synthetic auxin 2,4-D provides the stimulus for such activity. Companion bacteria producing phytohormones may perform a similar task.

(iii) *Access to carbon substrate from the plant.* This is essential on a continuous basis to allow adequate colonisation and the establishment of an adequate density of bacterial respiratory activity to reduce oxygen activity to a point where the *nif* genes may be expressed through the mode of expression of the *nifA* regulatory codon.

(iv) *Ammonia release from diazotroph cells.* A regulated (or deregulated) release of ammonia from N₂-fixing bacterial cells into plant tissue will be required, leading to maximal expression of nitrogenase activity. This must involve a quantitative understanding of the bioenergetics of the bacterial cell membrane (Wood *et al.* 1998) and the movement of ammonia across these membranes [see Wood (1999) for a specific model].

(v) *Ammonia assimilation as organic nitrogen products* (Schubert 1986). Since ammonia is a suitable source of nitrogen for plant roots, no special apparatus should be necessary for its transport and assimilation.

(vi) *Sustained symbiosis during the plant growth cycle.* This requires extended colonisation with diazotrophs as the plant grows to maturity. The sugarcane model where colonisation of the xylem seems to be involved would seem to have advantages with respect to proliferation of colonised tissue. Improved establishment and development of cacti inoculated with diazotrophs has also been reported (Bashan *et al.* 1998). The application of synthetic auxin to induce *para*-nodules at one stage of the growth cycle seems inadequate in this respect.

Identification of these and other relevant processes can provide the logical basis for selection of plant and microbial genotypes and their genetic selection or modification to meet the needs of critical parameters for symbiosis. Genetic manipulation will only succeed if it leads to gene products compatible with the environment and provided the outcome sought can be thermodynamically spontaneous in that environment. This may seem to be a burdensome constraint on the range of possibilities, but action biothermodynamics (Kennedy 2000) can also serve as a means of drastically reducing the range of approaches worth trying, eliminating those bound to fail. Applying a strategy governed by action resonance theory would result in much greater economy in

the use of resources, compared with the common method of rather speculative hypothesis (hunches) followed by experimental trial and error.

Ideally, the developing model system, as it optimised to make a transition from ANF to SNF or some amalgam of both would be given a role in this selection process. This could be achieved by colonisation using competing strains and mutants, followed by performance testing using yield criteria. Alternatively, natural genetic variation in seed of cereals or other non-legume crops, as well as in bacterial strains, could be exploited. Since the most successful associations would yield the most vigorous systems (i.e. the greenest), these better performing systems could be used for re-isolation of the most successful candidates.

Conclusion

This review has attempted to establish a more logical basis for the possible utilisation of ANF by microbes. Based on this analysis, the authors have an optimistic view of this possibility, considering that the conditions for success may now be better defined. Many of the early findings dating back to the 19th century are relevant to identifying this basis. The analysis suggests that the reasons for past failures and the means to prevent them are now much better understood. The entire complex of essential factors needs to be dealt with as a matrix, initially ameliorating all but one of these factors to obtain an optimum solution for each, to allow the entire matrix to be optimised. Despite the expense, the importance of using field trials as early as possible in this process cannot be overemphasised, since this can help ensure that laboratory or greenhouse results can be translated to the field environment. If this viewpoint regarding the potential to optimise a matrix of factors is correct, up to half the nitrogen requirements for some cereal crops might be met from ANF within the next 10 years.

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