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Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited?

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Abstract

Biological N_2 fixation (BNF) by associative diazotrophic bacteria is a spontaneous process where soil N is limited and adequate C sources are available. Yet the ability of these bacteria to contribute to yields in crops is only partly a result of BNF. A range of diazotrophic plant growth-promoting rhizobacteria participate in interactions with C_3 and C_4 crop plants (e.g. rice, wheat, maize, sugarcane and cotton), significantly increasing their vegetative growth and grain yield. We review the potential of these bacteria to contribute to yield increases in a range of field crops and outline possible strategies to obtain such yield increases more reliably. The mechanisms involved have a significant plant growth-promoting potential, retaining more soil organic-N and other nutrients in the plant–soil system, thus reducing the need for fertiliser N and P. Economic and environmental benefits can include increased income from high yields, reduced fertiliser costs and reduced emission of the greenhouse gas, N_2O (with more than 300 times the global warming effect of CO_2), as well as reduced leaching of NO_3^-–N to ground water. Obtaining maximum benefits on farms from diazotrophic, plant growth promoting biofertilisers will require a systematic strategy designed to fully utilise all these beneficial factors, allowing crop yields to be maintained or even increased while fertiliser applications are reduced.

Keywords: Biological nitrogen fixation; Inoculant biofertilisers; Non-symbiotic diazotrophs; Sustainable farming systems; *Acetobacter*; *Azoarcus*; *Azorhizobium*; *Azospirillum*; *Azotobacter*; *Bacillus*; *Burkholderia*; *Citrobacter*; *Clostridium*; *Enterobacter*; *Gluconacetobacter*; *Herbaspirillum*; *Klebsiella*; *Rhizobium*

1. Introduction

Despite almost 200 yr of experience since the benefits of supplying chemical fertilisers such as superphosphate and inorganic N were realised, the full genetic potential for maximum crop yield on farms is rarely realised. Crops grown in soil, such as wheat and rice, may fail to respond to supplements of P or N because of a complex of negative factors including inadequate moisture or ineffective rates of mobilisation of nutrients required for plant growth. This result is disappointing, because the success of plant growth in hydroponics suggests that inorganic fertilisers (N, P, K, Fe, S, Mg, Ca, trace elements) supplied in adequate amounts should be capable of supporting maximum growth. For crops grown in soil, this is rarely true.

Such failures to achieve consistently high yields may reflect variable mobilisation of soil nutrients by microorganisms, but unfortunately these processes are difficult to study because of their complexity. They may be considered as an interactive system where small, sequential, differences in the supply of various nutrients will have major cumulative, non-linear effects on subsequent outputs, such as grain yield. It is impossible to isolate the effects of individual factors in such a system since these are all co-dependent and cannot be exerted in isolation. Overall, maximum crop yield represents an optimum expression of the *genotype x environment* (GxE) interaction.

We propose that inoculant biofertilisers, particularly N_2-fixing bacterial diazotrophs, can help ensure that the supply of nutrients contributing to optimised yield is maintained. Diazotrophic plant growth-promoting rhizobacteria (PGPR) may hold the key to achieving these outcomes as an evolutionary advantage because of their competitive advantages in a situation of adequate C substrates, but of N-deficiency, allowing their selective enrichment in the rhizosphere (Döbereiner and Pedrosa, 1987). Regarding both cereals and other crops, the need to supply extra N by industrial nitrogen fixation or biological N_2 fixation (BNF) to supplement N released to the available

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pool by mineralisation is expected to depend on the following:

- the amount of soil-N removed in the harvested produce,
- leaching of inorganic-N (e.g. NO_3^-–N) to groundwater,
- the magnitude of denitrification of soil-N as N_2O or N_2,
- the extent and duration of immobilisation of N and its rate of remobilisation (Angus, 2001) in the soil biomass.

In this review, we will consider the biology, potential roles and method of application of a selection of non-symbiotic diazotrophs known to be effective in well-controlled field trials. We will then describe some examples of field experiments on inoculated crops subjected to statistical analysis to test significance. This approach will allow the reasons for success or failure to be better examined with a view to improving the general standard of research in this area.

2. Mechanisms of crop yield increase

Bowen and Rovira (1999) reviewed the biology of the rhizosphere and its management to improve plant growth, summarising their interest in this area from an agronomic point of view. Their review commenced with the increases in growth when tomatoes were inoculated with *Azotobacter* (Brown et al., 1964). Similar results were obtained by Rovira (1965) for wheat following inoculation with *Azotobacter chroococcum*, *Clostridium pasteurianum* and *Bacillus polymyxa*. Bowen and Rovira likened the widespread failure of inoculation with *Azotobacter* to increase yields in field experiments in Russia (only one-third were successful) to the study by Reuter et al. (1995) of responses of crop yields to fertiliser P. In 580 field experiments conducted in southern Australia, where soil tests predicted a positive crop response to the application of superphosphate in 30% of the cases, only 10% gave a positive yield response. It was possible to improve the predictability of the test if other soil properties were considered as extra information. The lesson to be drawn from the similar failures with inoculation of field crops is the need to also consider and possibly modify other controlling factors. If this were done, it is highly probable that more inoculation trials would have given positive results.

Bowen and Rovira (1999) included a discussion on possible mechanisms for the PGPR response including plant growth regulating effects (phytohormones), both positive and negative, induced systemic resistance to microbial pathogens, siderophore production aiding plant nutrition by chelation, P solubilisation and root-associated N_2 fixation. They drew attention to the role of these PGPR microbes as yield-increasing bacteria, a term favoured by Chinese workers in the area and a particular aim for field crops in this review.

Kennedy and Islam (2001) reviewed the possible contribution by non-symbiotic bacteria to crop growth from BNF with a focus on the historical evidence as well as some justification for the mechanisms involved. Dobbelaere et al. (2003) reviewed the diazotrophic PGPR in detail, highlighting their mechanisms of action including BNF, plant growth promotion by production of auxins, cytokinins, gibberellins and ethylene, P-solubilisation, increased nutrient uptake, enhanced stress resistance, vitamin production and biocontrol. Here, we will extend our analysis of the potential for the PGPR to contribute as biofertilisers for field crops. We will advance the thesis that PGPR may promote crop yield increases by modifying soil–plant processes so that N and other nutrients are more completely retained in the plant–soil system.

Rice, wheat and maize are the three major staple food crops for the world's population. A rice crop removes around 16–17 kg N to produce 1 t dry weight of rough rice, including straw (De Datta, 1981; Ponnamperuma and Deturck, 1993; Sahrawat, 2000). A wheat crop requires about 26–28 kg N to produce 1 t of rough grain including straw (Bhuiyan, 1995; Angus, 2001). Maize plants require 9–11 kg N to produce 1 t biomass (Anuar et al., 1995). Most of the soils of the world are deficient in N and applications of fertiliser N are essential for good yields by such cereal crops. Generally, urea is the most convenient N source. But unfortunately less than 50% of the applied urea is used by plants (Garabet et al., 1998; Choudhury and Khanif, 2001; Halvorson et al., 2002). This low efficiency of use is mainly caused by NH_3 volatilisation, denitrification, and losses from leaching (De Datta and Buresh, 1989; Bijay-Singh et al., 1995). Volatilisation and denitrification pollute the atmosphere through the evolution of greenhouse gases like N_2O, NO and NH_3. Leaching of NO_3^-–N causes groundwater toxicity (Shrestha and Ladha, 1998). In addition to these environmental problems, tillage systems making long-term use of urea may deplete soil organic matter content (Wairiu and Lal, 2003).

How are these problems, resulting from the use of fertiliser-N, of such great concern to environmental scientists, to be overcome? Alternative sources of N such as the use of BNF technology may supplement or replace chemical fertiliser-N. Moreover, the effective use of organic wastes may lessen the depletion of soil organic-N content (Jeyabal and Kuppuswamy, 2001), providing an alternative solution to the problem by sustaining organic-N as a buffer for inorganic N fluxes in the soil–plant system. Thus, although the magnitude of BNF from biofertilisers may account for a fraction of total crop N requirements, the effect of reducing losses from an ecosystem may be equivalent to a much more significant contribution to the N economy of crop production.

We review current experimental evidence regarding potential inoculant biofertilisers which might be developed for field applications for individual crops, emphasising research in this area conducted since 1990.

3. Biology and other relevant characteristics of non-symbiotic diazotrophs

Apart from their common possession of genes for N_2 fixation (*nif* genes), non-symbiotic diazotrophs are diverse genetically, reflecting a range of habitats where they may be found. Some biological characteristics of these organisms are summarised in Table 1, also indicating the proposed mechanisms of PGP effects. Their diverse nature is illustrated in the main genera described below, together with evidence for their beneficial effects. This diversity will need to be carefully considered in the future design of the most efficient inoculant biofertilisers. For example, an important question is whether inoculants should be restricted to a single strain of bacterium, such as *Azospirillum*, or not. If all of the PGP mechanisms can be well expressed in a single strain of bacterium this would simplify the design of inoculant products. However, it would be unlikely that a single strain of bacterium would be capable of optimal activity. Table 1 suggests that the diversity of habitat and effectiveness might logically require more than one bacterial strain to obtain the maximum biological effects on plant growth.

3.1. Azotobacter

Azotobacter species (*Azotobacter vinelandii* and *A. chroococcum*) are free-living, aerobic heterotrophic diazotrophs that depend on an adequate supply of reduced C compounds such as sugars for energy. Their activity in rice culture can be increased by straw application (Kanungo et al., 1997), presumably as a result of microbial breakdown of cellulose into cellobiose and glucose. Yields of rice in field trials increased significantly (at 5% probability level) up to 0.9 t ha^{-1} (20% increase) with applications of *Azotobacter* (Yanni and El-Fattah, 1999). The estimated N accumulation by rice plant increased up to 15 kg ha^{-1} due to *Azotobacter* inoculation (Yanni and El-Fattah, 1999). As ^{15}N was not used as tracer, it is not possible to say how much of the accumulated N was a result of BNF.

Inoculation with *Azotobacter* replaced up to 50% of the urea-N for wheat in greenhouse trials under aseptic (gnotobiotic) conditions (Soliman et al., 1995; Hegazi et al., 1998). Strains of this genus are epiphytic colonists of the wheat rhizoplane (Kennedy et al., 1998), rather than endophytic root invaders. Inoculation with *Azotobacter* can increase cotton yield by 15–28% (Iruthayaraj, 1981) as a result of BNF, production of antibacterial and antifungal compounds, growth regulators and siderophores (Pandey and Kumar, 1989). Patil and Patil (1984) observed that seed inoculation with *A. chroococcum* plus 50–100 kg urea-N ha^{-1} gave higher cotton dry matter yield, N uptake and soil N content than those obtained with N alone (50–100 kg urea-N ha^{-1}) in greenhouse conditions using non-sterilised soils.

3.2. Azospirillum

Azospirillum species are aerobic heterotrophs that fix N_2 under microaerobic conditions (Roper and Ladha, 1995). They grow extensively in the rhizosphere of gramineous plants (Kennedy and Tchan, 1992). They can also penetrate the root to grow endophytically in intercellular crevices (Sumner, 1990) although they are usually considered as epiphytes growing close to or on root surfaces. Both *Azospirillum lipoferum* and *Azospirillum brasilense* have been isolated from roots and stems of rice plants (Ladha et al., 1982; James et al., 2000) while *Azospirillum amazonese* has been isolated from the roots

Table 1
Biology, and potential role of some diazotrophs promoting crop production

Diazotrophs	Condition for BNF	Habitat	Energy source	Mechanism of effect[a]	Reference
A. chroococcum	Aerobic	Rhizosphere	Organics in soil	BNF	Kennedy and Tchan (1992)
Clostridium spp.	Anaerobic	Soil saprophyte	Organics in soil	BNF	Kennedy and Tchan (1992)
Azospirillum spp.	Microaerobic	Rhizosphere, mildly endophytic in roots, stems and leaves	Organics in soil, root exudates and plant tissue	BNF, PGP	Reinhold and Hurek (1988), Mirza et al. (2000), Okon and Kapulnik (1986)
H. seropedicae	Microaerobic	Endophytic, rhizosphere	Root exudates	BNF, PGP	Baldani et al. (1986b, 2000)
Azoarcus sp.	Microaerobic	Endophytic	Root exudates	BNF	Hurek et al. (1994), Reinhold-Hurek et al. (1993)
B. vietnamiensis	–	Rhizosphere, endophytic	Organics in soil and root exudates	BNF, PGP	Baldani et al. (1997, 2000)
R. leguminosarum bv. *trifolii*	–	Endophytic in roots	Root exudates	PGP	Yanni et al. (1997), Biswas et al. (2000a)
R. etli bv. *phaseoli*	–	Endophytic in roots	Root exudates	PGP	Gutiérrez-Zamora and Martínez-Romero (2001)
A. caulinodans	Microaerobic	Endophytic in roots	Root exudates	PGP	Matthews et al. (2001)
A. diazotrophicus	Microaerobic	Endophytic in roots, stems and leaves	Root exudates and plant tissue	BNF	Baldani et al. (1997), Boddey et al. (1991)

[a] BNF, Biological nitrogen fixation; PGP, plant growth promotion.

(Pereira et al., 1988). Quantitative investigations at the International Rice Research Institute (IRRI) estimated that *Azospirillum* constituted about 1% of the total aerobic heterotrophs in rice soils on a numeric basis, and about 85% of the *Azospirillum* isolates were *A. lipoferum*, suggesting its preferential colonisation of rice plants (Ladha et al., 1987). Greenhouse studies using non-sterilised soil indicated that inoculation with *A. lipoferum* increased rice yield significantly (at 5% probability level) up to 6.7 g plant^{-1} (Mirza et al., 2000). Inoculation with *A. lipoferum* also can increase plant height and tiller number of rice plants (Nayak et al., 1986). In the field, Balandreau (2002) found that the estimated yield increase was around 1.8 t ha^{-1} (22% increase). Murty and Ladha (1988) showed that *Azospirillum* inoculation increased P and NH_4^+–N uptake by rice plants; however, whether this was a result of better mobilisation of these nutrients or a secondary effect of improved root growth was not demonstrated. Inoculation with *Azospirillum* can reduce bacterial leaf blight of rice with subsequent improvements in various yield components (Islam and Bora, 1998).

Inoculation with *A. brasilense* can increase wheat grain yield by up to 30% and other yield components significantly in field conditions (Okon and Labandera-Gonzalez, 1994), but only at lower rates of fertiliser-N (50–60 kg N ha^{-1}). At higher rates (110–170 kg N ha^{-1}), its effects were not statistically significant (Dobbelaere et al., 2001). However, this implies there are good prospects for supplementing a substantial amount of urea-N applied to wheat while maintaining yields by inoculating *Azospirillum*. The PGPR effects can increase N and P uptake in field trials (Galal et al., 2000; Panwar and Singh, 2000), presumably by stimulating greater plant root growth. Beneficial effects of inoculation with *Azospirillum* on wheat yields in both greenhouse and field conditions have been reported (Hegazi et al., 1998; El-Mohandes, 1999; Ganguly et al., 1999). Substantial increases in N uptake by wheat plants and grain were observed in greenhouse trials with an NH_3-excreting strain of *A. brasilense*, when the soil was initially supplemented with malate (Islam et al., 2002). The proportion of BNF versus soil N in these increases was not determined.

There were clear differences between strains of *Azospirillum* in their ability to promote growth of wheat in greenhouse trials (Han and New, 1998; Saubidet and Barneix, 1998), expressing the GxE interaction. Although *Azospirillum* promotes growth of wheat plants and grain yield, it apparently contributes little N to wheat as a direct result of BNF. It has been established by the ^{15}N tracer technique that *A. brasilense* and *A. lipoferum* contributed only 7 and 12% of wheat plant N by BNF, respectively (Malik et al., 2002). However, this contribution may be a critical component for obtaining a greater yield with less N application. The value of supplying even 10% of the N requirement of wheat should not be underestimated because it may increase its capacity to assimilate soil-N. The BNF factor should then be regarded as *catalytic* rather than the main source of N.

The inability of the wheat plant to release adequate C to the rhizosphere is likely to be a major constraint to realising the BNF potential of *Azospirillum*. Under laboratory experimental conditions, this problem can be alleviated by adding malate to the soil (Wood et al., 2001). While working with an NH_3-excreting mutant strain of *A. brasilense* (supplied by F. Pedrosa), they observed that the ^{15}N enrichment of wheat tissue was substantially increased by 48-fold, indicating that 20% of the wheat N had been derived from BNF after several days growth of seedlings. Using *nifH-lacZ* fusions, Deaker and Kennedy (2001) showed that *A. brasilense* Sp7-S, a mutant capable of more endophytic colonisation of wheat roots than the wild type Sp7, fixed more N_2 than the wild type Sp7 strain. The *nifH* nitrogenase gene was strongly expressed in the wheat rhizosphere. Apparently, the improved access to C compounds and a more favourable microaerobic O_2 concentration contributed to this effect. These results demonstrate the potential for BNF by *Azospirillum* to enhance the availability of N to wheat plants.

Inoculation with *A. brasilense* increases growth of root hairs and the number of lateral roots on cotton plants, resulting in significant increases of plant dry matter under greenhouse conditions (Bashan, 1998). Cotton roots can be inoculated by dipping the roots of seedlings in a suspension of *Azospirillum* 30 min before planting under greenhouse conditions using non-sterile soil (Fayez and Daw, 1987). Inoculation significantly increases cotton plant height and dry weight while the N content can be increased by increased N uptake by cotton plant up to 0.91 mg plant^{-1} (Fayez and Daw, 1987). *Azospirillum* is also capable of producing antifungal and antibacterial compounds, growth regulators and siderophores with cotton (Pandey and Kumar, 1989).

Both *A. brasilense* and *A. lipoferum* are found in roots, stems and leaves of the sugarcane plant while *Azospirillum amazonense* is found in roots and stems. These bacteria are facultative diazotrophs (Reis et al., 2000). Soil applications of *Azospirillum* can significantly increase cane yield in both plant and ratoon crops in the field (Shankariah and Hunsigi, 2001). They reported that the mean cane yield increases were 9 and 5 t ha^{-1} in plant and ratoon crops, respectively. Inoculation with *Azospirillum* also significantly increased the N content of sugarcane leaves in greenhouse conditions (Muthukumarasamy et al., 1999).

3.3. Acetobacter

Acetobacter (Gluconacetobacter) diazotrophicus is an acid-tolerant endophyte which grows best on sucrose-rich medium (James et al., 1994) such as sugarcane sap. A ^{15}N dilution/N balance study confirmed that up to 60–80% of sugarcane plant N (equivalent to over 200 kg N ha^{-1} yr^{-1}) was derived from BNF; *A. diazotrophicus* is apparently

responsible for much of this BNF (Boddey et al., 1991). The *Acetobacter*-sugarcane system has now become an effective experimental model. Inoculation of seedlings leads to greater growth rates while *nif*$^-$ mutants were significantly less effective in increasing plant growth, proving that the diazotrophic character (nif$^+$) is important for this system (Lee et al., 2002).

Field experimental results in India showed that application of *A. diazotrophicus* by inoculating setts increased sugarcane yield for four varieties significantly when it was applied in association with vesicular arbuscular mycorrhiza (Muthukumarasamy et al., 1999). They claimed that this practice completely substituted for the recommended dose of 275 kg urea-N ha^{-1}. This is an ambitious claim, but the result could involve a PGPR effect.

3.4. Azorhizobium

Azorhizobium caulinodans increased the dry weight and N content of wheat plants in a greenhouse experiment (Matthews et al., 2001). In a following experiment, they used a N-fixing (nif$^+$) and a non-N-fixing (nif$^-$) strains of *A. caulinodans*. Both strains increased the growth of wheat plants compared with the non-inoculated plants. This information provides clear evidence that the beneficial effect of inoculating wheat plants with *A. caulinodans* was from the production of PGP substances rather than by BNF. Its inoculation saved up to 50% of the recommended rate of urea N in greenhouse trials under gnotobiotic (or sterile) conditions (Saleh et al., 2001).

3.5. Azoarcus

Reinhold-Hurek et al. (1993) studied a strain of the endophytic Gram-negative N_2-fixing bacterium *Azoarcus* sp. BH72, originally isolated from Kallar grass (*Leptochloa fusa* Kunth) growing in the saline-sodic soils typical of Pakistan. *Azoarcus* spp. also colonise grasses, such as rice, in both the laboratory and the field (Hurek et al., 1994). In rice roots, the zone behind the meristem was most intensively colonised. The strain penetrates the rhizoplane preferentially in the zones of elongation and differentiation and subsequently colonises the root interior both inter- and intracellularly. In addition to the root cortex, these bacteria were also found in the xylem, but there was no evidence that *Azoarcus* resided intracellularly in living plant cells, which are apparently killed as bacteria penetrate the cell wall. The response of rice roots to inoculation with *Azoarcus* sp. BH72 in aseptic systems was cultivar-dependent (Reinhold-Hurek et al., 2002). Proteomic analysis to show gene expression revealed bacterial protein synthesis (Reinhold- Hurek et al., 2002). At one extreme, nitrogenase was expressed endophytically as shown by *nifH* reporter genes and at the other a defense-like response was developed without significant root colonisation by *Azoarcus*. However, because *Azoarcus* has not been applied in extensive plantings of field crops such as rice, it is difficult to assess its possible significance for crops.

3.6. Burkholderia

The genus *Burkholderia* comprises 29 species, with several of these including *Burkholderia vietnamiensis*, *Burkholderia kururiensis*, *Burkholderia tuberum* and *Burkholderia phynatum* being capable of fixing N_2 (Estrada-de los Santos et al., 2001; Vandamme et al., 2002). The species *B. vietnamiensis* described by Gillis et al. (1995), was first isolated from the rhizosphere of young rice plants cultivated on a Vietnamese soil (Trân Van et al., 1994). When used to inoculate rice in field trials it increased grain yields significantly (at 5% probability level) up to 0.8 t ha^{-1} (Trân Van et al., 2000). In these field trials, this strain was found capable of saving 25–30 kg N ha^{-1} from fertilizer. Baldani et al. (2000), using the ^{15}N tracer technique, established that *B. vietnamiensis* can fix 19% of the rice plant N (152 μg N plant^{-1}) from the atmosphere under gnotobiotic conditions. As this species was isolated from the rice roots and adhering soil, it should not be described as an endophyte (Baldani et al., 1997). Nevertheless, Baldani et al. (2000) isolated another endophytic species (*Burkholderia sp*) from the interior of roots, stems and leaves of rice in Brazil. It fixed 31% of rice plant N (372 μg N plant^{-1}) from the atmosphere, and its inoculation increased rice plant biomass by up to 22 mg plant^{-1} (69% increase) under gnotobiotic conditions (Baldani et al., 2000). The species, *Burkholderia glumae* causes grain and seedling rot of rice (Nakata, 2002). Another species, *Burkholderia cepacia*, can be hazardous to human health (Balandreau, 2002), so appropriate care and risk-reducing techniques should be employed while isolating and culturing *Burkholderia*.

Burkholderia brasilensis is an endophyte of roots, stems and leaves of sugarcane plant while *Burkholderia tropicalis* is confined to its roots and stems (Reis et al., 2000). There is also recent evidence that these organisms can produce substances antagonistic to nematodes (Meyer et al., 2000).

3.7. Clostridium

It is now almost 50 years since Parker (1953, 1954) observed that both *A. vinelandii* and *Clostridium butyricum* apparently contributed to the growth of winter wheat by providing extra soil nitrogen, in West Australian soils of light structure.

In contrast to *Azotobacter*, clostridia are obligately anaerobic heterotrophs only capable of fixing N_2 in the complete absence of oxygen (Saralov and Babanazarov, 1983; Kennedy and Tchan, 1992). Clostridia can usually be isolated from rice soils (Khamas et al., 1994; Elbadry et al., 1999) and their activity increased after returning straw to rice fields, to raise the C to N ratio. Inoculation with clostridia can increase rice yields significantly in favorable conditions when C containing compounds are incorporated

to the soil (Mishustin et al., 1983). However, *Clostridium* fixes only 5–10 mg N g^{-1} of C consumed (Mulder, 1975), equivalent to 10–20 kg N ha^{-1} from 20 t of utilisable C, demonstrating the substantial need for extra energy for anaerobic BNF supplied by fermentative metabolism of rice straw added to soil.

3.8. Enterobacteriaceae

Several genera of the enterobacteriaceae selected from soil include diazotrophs, particularly those from the rhizosphere of rice. Though a speculative idea, it seems plausible that the long use in agriculture of organic manures for crops may have developed a flora well adapted to an animal–soil–plant rhizosphere nutritional cycle. These enteric genera containing some examples of diazotrophs with PGP activity include *Klebsiella*, *Enterobacter*, *Citrobacter*, *Pseudomonas* and probably several others yet unidentified. *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Citrobacter freundii* and *Pseudomonas putida* or *Pseudomonas fluorescens* are also examples of such plant-associated bacteria.

This may pose concern for human safety, or at least a need to show that no risk is present, when inoculant bacteria even remotely related to others negatively associated with human health are produced in large quantities. This fact was recently noted for *C. freundii* (Nguyen et al., 2003), although the level of risk is probably quite slight. A similar case exists for use of *Burkholderia vietnamensis* in biofertiliser products, because its relative *B. cepacia* has been linked to cystic fibrosis. There may be a need to develop suitable protocols to ensure the safety of personnel engaged in biofertiliser production, although the production of strains commonly found in most soils and plant rhizospheres should not present a significant risk.

3.9. Herbaspirillum

Herbaspirillum is an endophyte which colonises sugarcane, rice, maize, sorghum and other cereals (James et al., 2000). It can fix 31–54% of total rice plant (30-d-old rice seedlings) N from the atmosphere (Baldani et al., 2000). The estimated N fixation by *Herbaspirillum* was 33–58 mg $tube^{-1}$ under aseptic conditions (Reis et al., 2000). In a greenhouse study, inoculation with *Herbaspirillum* increased rice yield significantly (at 5% probability level) up to 7.5 g $plant^{-1}$ (Mirza et al., 2000). Inoculation with *Herbaspirillum seropedicae* in field conditions can increase shoot and root length, 1000-grain weight and grain yield of rice (Arangarasan et al., 1998). Inoculation with *Herbaspirillum* can also enhance seed germination significantly (Pereira et al., 1988).

Mirza et al. (2000) quantified the BNF by different strains of *Herbaspirillum* in both basmati and super basmati rice. The %Ndfa (N_2 derived from the atmosphere) values were 19.5–38.7, and 38.1–58.2 in basmati and super basmati, respectively. Thus, *Herbaspirillum* can fix 19–58% of the N required by rice crop depending on *Herbaspirillum* strain and rice variety. They also quantified the N_2 fixation by *A. lipoferum* and *A. brasilense* in a rice crop using the ^{15}N isotope dilution method under greenhouse conditions. The %Ndfa values were 20.0 and 19.9 for *A. lipoferum* and *A. brasilense*, respectively, in basmati rice while values were 58.9 and 47.1, respectively, in super basmati rice. This information clearly demonstrates that BNF from inoculation with *Azospirillum* can meet at least 19.9 and 47.1% of the required N for basmati and super basmati rice, respectively.

H. seropedicae also acts as an endophytic diazotroph of wheat plants (Kennedy and Islam, 2001), colonising wheat roots internally between the cells in a fashion similar to *A. brasilense* Sp7-S. Kennedy et al. (1998) used nifA-lacZ as genetic marker for *H. seropedicae* and observed that it displayed significant endophytic colonization of 2,4-D treated wheat seedlings. Its application can increase straw and grain yields, %Ndfa and %N recovery in wheat plant under field conditions (El-Mohandes, 1999).

H. seropedicae is also found in roots and stems of sugarcane plants while *Herbaspirillum rubrisubalbicans* is an obligate endophyte of roots, stems and leaves (Reis et al., 2000). These diazotrophs can increase leaf N content and cane yield significantly, but cannot substitute for urea-N completely (Muthukumarasamy et al., 1999). The population of these bacteria is not affected by chemical N fertilisation even at applications of 300 kg urea-N ha^{-1} under field conditions (Reis et al., 2000). *Herbaspirillum* and *Azospirillum* have both been applied to sorghum (Baldani et al., 1986b; Pereira et al., 1988) with positive results.

Herbaspirilla can also colonise maize plants endophytically and fix N_2, as with sugarcane and wheat (James et al., 2000). This organism was first isolated as *H. seropedicae* from maize, rice and sorghum (Baldani et al., 1986a).

3.10. Rhizobium

Rhizobium leguminosarum bv. *trifolii* can colonise rice roots endophytically in fields where rice is grown in rotation with Egyptian berseem clover (*Trifolium alexandrinum*), replacing 25–33% of the recommended rate of N fertilizer for rice in field conditions (Yanni et al., 1997) as a result of PGPR effects. Field experiments demonstrated that the inoculation of this bacterium increased mean rice yield by 3.8 t ha^{-1} (Yanni et al., 2001). This bacterium is also able to colonise the interior of the rice roots grown under gnotobiotic conditions. It can increase shoot and root growth, grain yield and agronomic N-fertiliser efficiency significantly, although it is present in rice tissues in low numbers of the order of 10^5 cells (colony-forming units) g^{-1} dry weight, too low for significant BNF. Laboratory and greenhouse studies conducted at IRRI showed when rice is inoculated with this strain both the growth and yield

of rice, and its uptake of N, P and K increased significantly (Biswas et al., 2000a,b). ^{15}N-based studies showed that the increased N uptake was not due to BNF (Biswas et al., 2000a), emphasising that certain strains of rhizobia can enhance rice growth and yield through the changes in growth physiology or root morphology rather than BNF (Biswas et al., 2000b), improving the efficiency of utilisation of chemical N fertiliser and other soil nutrients for rice production. This beneficial effect thereby reduces the risk of environmental pollution and is just as effective as supplying newly fixed-N for the nutrition of rice plants.

3.11. Methods of application of inoculants

Table 2 summarises the methods so far used for application of diazotrophs to crop plants. In fact, the table indicates that a variety of techniques are in use, but little work has been done to optimise the process of inoculation. Where applications are made of fresh culture broths containing large numbers of viable bacteria ($>10^8$ ml^{-1}) the method of application is probably not of much consequence. In this section, we simply wish to draw attention to this factor and point out the need for critical research in this area. Experience gained with rhizobia for inoculating legumes suggests that the use of moist carriers such as peat or other granulated or encapsulated products should be beneficial in improving the survival of adequate numbers of inoculant bacteria.

Azotobacter has been applied in rice culture by various methods such as dipping seed into microbial cultures before planting, dipping seedling roots into broth cultures, soil application at nursery or main field stages, top dressing or foliar application (Kannaiyan et al., 1980; Singh et al., 1999). Cultures of *Azospirillum* can be applied to wheat plants by inoculating seed before sowing (Creus et al., 1996). Azospirilla have been applied by three methods—dipping seed in bacterial suspensions for 5 min, followed by drying in shade for 2–3 h; dipping rice seedlings roots into bacterial suspensions overnight; or by application of bacterial suspensions to the soil (Islam and Bora, 1998).

4. Validated field trials

4.1. Inoculant biofertilisers for rice

Rice is grown in both wetland and upland cultures, with about 85% of the planet's total area of rice being in flooded wetlands. In upland culture with soils drained by gravity, the roots of rice are more aerobic and N may be assimilated as NO_3^- as a result of fertiliser addition or mineralisation and nitrification of N in organic matter. In addition, aerobic diazotrophs may fix atmospheric N_2. In wetland culture involving flooded paddies, N is mainly available to rice plants from soil water as NH_4^+, requiring less energy to assimilate into amino acids than NO_3^- (Kennedy, 1992). In recent years, the cultivation of rice in aerobic soils in even in lowland situations has sometimes been promoted, because of potential benefits claimed in terms of increased N uptake and yield. Various aerobic and anaerobic bacteria can fix N_2 depending on local conditions. Thus, aerobic diazotrophs such as *Azotobacter* can live and fix atmospheric N_2 in the oxygenated rhizosphere of rice plants, presumably exercising respiratory protection for their nitrogenase (Kennedy and Tchan, 1992). Anaerobic bacteria such as *Clostridium* can simultaneously live in the reduced, anoxic soil layer, also fixing N_2 and ultimately releasing NH_3. Currently *Azotobacter* and the clostridia are not considered to have close rhizosphere associations with crop plants. This view may require reassessment as experimental tools such as

Table 2
Application methods reported for some PGP diazotrophs

Diazotrophs	Application method	References
A. chroococcum	Seed or seedling roots dipped in broth culture before sowing; soil application at nursery or main field; top dressing or foliar applications	Kannaiyan et al. (1980), Singh et al. (1999)
Azospirillum spp.	Seed inoculated before sowing, seedling roots dipped before transplanting; application of bacterial suspension to the soil	Creus et al. (1996), Sapatnekar et al. (2001), Islam and Bora (1998)
H. seropedicae	Seed inoculation	El-Mohandes (1999), Riggs et al. (2001)
B. vietnamiensis	Seed inoculation before sowing, seedling root dipping before transplanting	Trân Van et al. (2000)
Burkholderia sp.	Seed inoculation before sowing, seedling inoculation	Baldani et al. (2000)
R. leguminosarum bv. *trifolii*	Seed dipping in bacterial suspension before sowing, seed coating with inoculum strains, and application of bacterial suspension on the soil of transplanted rice field	Biswas et al. (2000a, b), Yanni et al. (1997)
R. etli bv. *phaseoli*	Seedling inoculation	Gutiérrez-Zamora and Martínez-Romero (2001)
A. caulinodans	Seed inoculation before sowing	Matthews et al. (2001)
A. diazotrophicus	Inoculating seedlings and setts of sugarcane	Muthukumarasamy et al. (1999), Lee et al. (2002)

reporter genes (e.g. *lac*Z or *gus*A) for the study of rhizosphere associations improve. Other diazotrophs are more commonly found closely associated with the rhizosphere of rice plants including *Azospirillum*, *Herbaspirillum* and *Burkholderia* (Baldani et al., 2000; Balandreau, 2002; Malik et al., 2002). In principle, all these diazotrophs including cyanobacteria can supplement urea-N by BNF, but only if conditions for expression of N_2-fixing activity and subsequent transfer of N to plants are favourable. Based on the results of many studies conducted at IRRI at Los Bãnos in the Philippines, Watanabe et al. (1987) and Roger and Ladha (1992) concluded that diazotrophs can provide 20–25% of the total N needs of rice.

Rhizobium strains can only improve the utilisation of fertiliser-N such as added urea by promoting physiological growth responses generating changes to the root morphology of the rice plant that favour its uptake (Biswas et al., 2000a,b; Yanni et al., 2001), because they can only express BNF in legume nodules where their genetic apparatus for this process is activated. However, *R. leguminosarum* bv. *trifolii*, isolated from rice, is able to nodulate a naturalised clover is nevertheless able to significantly improve the yield of this cereal (Table 3).

Because of the diversity of strains found naturally in rice rhizospheres, it has been concluded that multi-strain inoculant biofertilisers may be particularly beneficial for increased grain yields of rice, reducing its dependence on urea-N. In Vietnam the performance of a multi-strain biofertiliser (BioGro) designed for rice culture has been statistically assessed in field trials over 3 years (Nguyen et al., 2002a). This biofertiliser contained three strains of bacteria, originally selected from rice rhizospheres in the Hanoi area of Vietnam (Nguyen et al., 2003). One strain (*Pseudomonas Fluorescens/Pseudomonas putida*, 1N or 2N) was selected for its ability to reduce C_2H_2 to C_2H_4, as an indication of its potential for N_2 fixation. A second strain (*Klebsiella pneumoniae*, 4P), also a diazotroph, was selected for its ability to solubilise precipitated $Ca_3(PO_4)_2$ in an agar medium. The third strain (*Citrobacter freundii*, 3C), also a diazotroph, produces toxic extra-cellular compounds which inhibited 50% of a test group of 100 rhizosphere organisms, but to which the inoculum strains are resistant. The third strain presumably aided the establishment of the inoculum in competition with other rhizosphere organisms. Each of the three bacteria were grown in separate broth cultures, and added to separate bags of carrier, formulated by mixing clay soil (50%), rice husks (25%), sugar (1%), plus water and broth culture (24%). To avoid competition during inoculant preparation and transport these separate cultures were mixed in the field immediately before use in the carrier ratio of 10 parts of strain 2N:10 parts of strain 4P:1 part of strain 3C. Because strains 2N and 3C are difficult to count in the non-sterile carrier, only a direct count of 4P was done after inoculating the carrier (3×10^9 cfu g^{-1} carrier). The estimated numbers of 2N and 3C, based on counts of their broth cultures were approximately 1×10^8 and 1×10^7 g^{-1} carrier, respectively. Biofertiliser was applied to the field plots by spreading the carrier evenly by hand directly to the soil. To control plots, non-inoculated carrier was added at a rate equivalent to 222 kg ha^{-1}. Biofertiliser application at 111 kg ha^{-1} increased grain yield and N uptake by rice grain significantly (Table 4), although urea application was reduced by 50% to 55 kg ha^{-1}. In farmers' fields, the application of this biofertiliser also increased rice yield up to 1.1 t ha^{-1} (21% increase over non-inoculated control) although urea application was often reduced. None of the farmers agreed to reduce fertiliser inputs to 50% of their normal practice. The mean reduction was only 7.6%. Even at this reduction in fertiliser inputs, the application of biofertiliser was profitable. A separate economic analysis including a farmer survey also indicated that the use of this biofertiliser was beneficial for the rural economy in Vietnam (Barrett and Marsh, 2002).

There are prospects for utilising this multi-strain inoculant biofertiliser technology in the Australian rice industry and a potential strategy for its application on farms has been designed (Williams and Kennedy, 2002). While the Vietnamese strains may be used in this application, similar native strains of the same species should be isolated from Australian soils and evaluated to determine if they have extra advantages related to local adaptation.

Other multi-strain inoculants for rice currently being applied in the field include BioPower prepared at the National Institute for Biotechnology and Genetic Engineering in Faisalabad, Pakistan (Malik et al., 2002) and similar products in Egypt (Hegazi et al., 1998). These inoculants are claimed to give similar yield increases on rice farms of around 20%. Obviously, if such yield increases can be reliably obtained, the use of such biofertilisers for rice in Pakistan would be justified.

Table 3
Effects of *R. leguminosarum* bv. *trifolii* inoculation on rice grain yield and agronomic fertilizer-N-use efficiency under variable rates of applied fertilizer N

Rhizobium strain	Applied fertilizer-N rate (kg ha^{-1})						
	Grain yield (t ha^{-1})				Agronomic fertilizer-N-use efficiency (kg grain (kg applied fertilizer-N)$^{-1}$)		
	0	72	144	Mean	72	144	Mean
E24	4.50	6.69	7.36	6.18[a]	92.9	51.1	72.0[a]
E27	4.65	6.24	7.36	6.08[a]	86.7	51.1	68.9[a]
E37	4.95	6.79	7.04	6.26[a]	94.3	48.9	71.6[a]
E39	5.26	6.86	8.91	7.01[a]	95.2	61.9	78.6[a]
Non-inoculated	3.97	5.17	6.69	5.28	71.8	46.4	59.1

Source: adapted from Yanni et al., 2001.
[a] Mean values differ from the corresponding controls at 95% confidence level.

Table 4
Effects of farmyard manure and a multi-strain biofertiliser on the grain yield of rice and N uptake by grain

Farmyard manure (kg ha^{-1})	Biofertiliser (kg ha^{-1})				
	0	111	222	444	Mean
Grain yield (kg ha^{-1})					
5560	5476	6170	5890	5801	5834
11,120	5443	6360	6111	5979	5973
22,240	5764	5813	6116	5854	5888
Mean	5561 b	6114 a	6039 a	5878 a	
N uptake by grain (kg ha^{-1})					
5560	50.40	55.89	53.59	51.14	52.76 B
11,120	51.41	59.28	57.09	54.69	55.62 A
22,240	50.67	54.62	57.29	55.78	54.59 A
Mean	50.83 b	56.60 a	55.99 a	53.87 a	

Grain yield: LSD (0.05) for biofertiliser means = 258.1. N uptake by grain: LSD (0.05) for FYM means = 1.669, and for biofertiliser means = 2.903. Means followed by a common small letter in a row and a common capital letter in a column for a variable are not significantly different at 5% level by least significant difference (LSD). Source: adapted from Nguyen et al., 2002a.

4.2. Field trials with maize

Maize (*Zea mays*) is an important C_4 plant grown for both grain and green fodder. It requires significant amounts of N to satisfy its potential for rapid growth and biomass production. Its extensive root system allows maize to remove NO_3^-–N from deep soil layers. It is therefore often grown as a 'catch crop' to reduce NO_3^- leaching in the groundwater (Shrestha and Ladha, 1998). The N requirement of maize is normally met by fertilisation at a rate depending on soil fertility with the chemical urea (Scharf, 2001). It has been established by the ^{15}N isotope dilution technique that there is a significant BNF contribution to maize (Garcia de Salamone et al., 1996). Common diazotrophs found in the rhizosphere of maize are *Enterobacter* spp., *Rahnella aquatilis*, *Paenibacillus azotofixans*, *Azospirillum* spp., *H. seropedicae*, *Bacillus circulans* and *Klebsiella* sp. (Chelius and Triplett, 2000).

The positive effects of *Azospirillum* on maize growth are mainly derived from physiological changes of the inoculated plant roots, which enhance water and mineral nutrient uptake (Okon and Kapulnik, 1986). Both *A. brasilense* and *Azospirillum irakense* are used as inoculant biofertilisers for maize. However, Dobbelaere et al. (2001) found *A. brasilense* increased grain yield of maize by 0.7–1.0 t ha^{-1} (50–95% increase) depending on soil conditions when N was applied at low to medium (18–46 kg ha^{-1}) rates (Table 5). However, at higher rates of N, its effect on yield of grain was reduced, even to the point where there may be a decrease in yield (Dobbelaere et al., 2001).

Other species of *Azospirillum* capable of increasing the yield of maize are *A. lipoferum* and *A. indigens*, and *Azorhizobium caulinodans* was also capable of giving such beneficial effects (Riggs et al., 2001). The magnitude of this increase varied with the *Azospirillum* strain and maize cultivar. While the *A. brasilense* genotype W64a increased grain yield by about 13%, the genotype B73 increased the yield by 25% (Riggs et al., 2001). It has been established by the ^{15}N tracer technique that *Azospirillum* spp. do contribute to BNF in maize. However, the amount of fixed N varies between species and strains of *Azospirillum* and the maize cultivar. El-Komy et al. (1998) reported that %Ndfa (N derived from atmosphere) in the genotype Giza215 was 26.5–31.4% with *A. lipoferum*, but only 17.5% with *A. brasilense* under greenhouse conditions using non-sterilised soils. With genotype Giza310, the %Ndfa was 17.4–20.6% with *A. lipoferum*, but only 6.1% with *A. brasilense*. This result indicates that *A. lipoferum* can fix at least 17% of the N required by maize plant.

Riggs et al. (2001) concluded from the results of extensive greenhouse and field experiments using non-sterilised soils that there were beneficial effects of maize seed inoculation with *H. seropedicae* on maize with increased yield in greenhouse conditions by 49–82% with applied fertilizer N compared to an increase of only 16% without fertilizer N. This indicates *H. seropedicae* can improve the ability of maize plant to use fertilizer N more

Table 5
Effect of *A. brasilense* inoculation on grain yield of maize in different regions of Mexico, under different levels of applied N

Region (location)	N rate (kg ha^{-1})	Grain yield (kg ha^{-1})		Difference (%)
		Non-inoculated	Inoculated	
Oaxaca	0	2854	3419	+21
Campeche	18	1400	2100	+50
Quintana Roo	0	1502	1900	+27
	30	1234	2204	+78
Hidalgo	46	1050	2080	+95
Campeche	110	4590	5100	+10
Puebla	140	3298	3212	−3

Source: Adapted from Dobbelaere et al., 2001.

Table 6
Effects of bacterial inoculation on maize grain yield in different locations of USA where N fertilizer was applied at a level of 224 kg ha^{-1}

Year	Location	Bacteria	Grain yield (t ha^{-1})	%Increase over uninoculated control	Statistical significance level
1998	Hancock	*Klebsiella sp.*	10.80	13.9	0.05
		H. seropedicae	10.60	12.0	0.05
		G. diazotrophicus	9.50	25.3	0.05
	Arlington	*H. seropedicae*	8.96	19.5	0.05
1999	Arlington	*G. diazotrophicus*	7.88	23.4	0.05
		K. pneumoniae	5.60	30.6	0.05
		H. seropedicae	7.55	17.5	0.05
2000	Lancaster	*K. pneumoniae*	16.39	25.8	0.01
		Bacillus sp.	17.00	30.5	0.01
	Arlington	*Pantoea agglomerans*	13.56	17.8	0.01
		H. seropedicae	14.86	12.1	0.05
		Klebsiella sp.	15.90	20.0	0.01

Source: adapted from Riggs et al., 2001.

efficiently. In field experiments, the increases in yields due to *H. seropedicae* inoculation were up to 19.5% (Table 6).

Burkholderia spp. are found in the shoots, root, rhizosphere and rhizoplane of maize plants (Estrada-de los Santos et al., 2001; Estrada et al., 2002). Greenhouse trials using non-sterilised soils at the University of Wisconsin, USA showed that grain yields were increased by 36–48% by inoculating seeds with *B. cepacia* AMMDR1 (Riggs et al., 2001) at planting, depending on the maize cultivar and bacterial genotype. In the field trials, this bacterium was able to increase maize yield by 5.9–6.3% (Riggs et al., 2001).

Rhizobium etli bv. *phaseoli* can colonize maize roots, and increase plant dry weight (Gutiérrez-Zamora and Martínez-Romero, 2001). Riggs et al. (2001) had shown that inoculation of *R. leguminosarum* bv. *trifolii* increased maize yields by 34 and 11% in the greenhouse and field conditions, respectively. *Sinorhizobium sp*. can increase maize yields by 35–43% depending on the maize genotype (Riggs et al., 2001). These results emphasise the importance of evaluating combinations of different strains of *Rhizobium* and combinations of maize genotype. Because *Rhizobium* can only fix N_2 in legume nodules these must be PGPR effects.

4.3. Other field crops and diazotrophs

The N requirement for wheat is higher than that for rice, because of its higher grain protein content. Wheat yields vary widely from 1 to 7 t ha^{-1} depending on inherent soil fertility, the amount of applied fertiliser, wheat variety, diseases such as take-all, other management practices and environmental conditions (Islam, 1995; Angus, 2001). Thus, the estimated amount of N removed by wheat crops varies between 26 and 200 kg N ha^{-1}, depending on yield. To maximise wheat yields in soils that are not capable of supplying enough N, chemical N fertilisers such as urea are used to enhance N supply. The N rate applied to wheat crops ranges between 30 and 225 kg N ha^{-1} depending on soil fertility, wheat variety and targeted yields (Islam, 1995; Angus, 2001; Reeves et al., 2002).

Bacterial inoculant biofertilisers can, in principle, be used to supplement the use of urea-N. There are comparatively fewer reliable reports of the successful field applications of biofertiliser for wheat than for rice. It seems possible that in dryland production of wheat, water stress may increase the difficulty of obtaining such benefits, although this possible limitation has not yet been experimentally tested.

A range of diazotrophs including strains of *Azospirillum*, *Azotobacter*, *Azorhizobium*, *Bacillus*, *Herbaspirillum* and *Klebsiella* can supplement the use of urea-N in wheat production either by BNF or growth promotion (Okon and Labandera-Gonzalez, 1994; Hegazi et al., 1998; Kennedy et al., 1998; Kennedy and Islam, 2001). The estimated amount of BNF by such wheat–bacterial associations was between 10 and 30 kg N ha^{-1} for each crop (Kennedy and Islam, 2001), or about 10% of their total-N requirement. However, successful cases of inoculation of wheat on a continuing basis are known to the reviewers.

4.4. The special case of sugarcane

Brazil is the world leader in replacing chemical N fertilizer with BNF for sugarcane production. Sugarcane (*Saccharum officinarum*) is an important crop grown for sugar and ethanol production. It requires approximately 1.45 kg N ha^{-1} to produce 1 t of moist biomass (Bhuiyan, 1995), or about 7 kg N ha^{-1} for 1 t of dry cane (i.e. 116–274 kg N ha^{-1}). The yield of millable sugarcane biomass varies from 80 to 190 t ha^{-1} depending on soil fertility, amount of fertilizer applied, and the cultivar (Majid et al., 1995; Shankariah and Hunsigi, 2001). The large amount of N removed by sugarcane from soil and the

extended period of cultivation without replenishing N-fertiliser applications can deplete the soil-N concentration alarmingly (Hartemink, 1998). Generally 150–250 kg urea-N ha^{-1} is applied for sugarcane cultivation depending on soil fertility, genotype and the targeted yield (Majid et al., 1995; Azzazy and Elham, 2000; Shafshak et al., 2001; Shankariah and Hunsigi, 2001). Evidence from Brazil indicates fertiliser-N can be reduced to half by exploiting BNF systems, claimed to be based on diazotrophic PGPR such as *Acetobacter (Gluconacetobacter)* and *Herbaspirillum* (Boddey et al., 1995; Döbereiner, 1997; Döbereiner and Baldani, 1998). The ^{15}N natural abundance technique established that the BNF contributes up to 60% of the total assimilated N by sugarcane varieties not receiving fertiliser-N (Boddey et al., 2001). Both obligate and facultative diazotrophs live in the roots, stems and leaves of sugarcane (Reis et al., 2000), and can fix up to 150 kg N ha^{-1} of atmospheric N (Döbereiner, 1997). Because of the limits of accuracy for the ^{15}N natural abundance technique, it is possible that some of the extra N obtained is from a growth-promotion effect, which contributes to the efficient N uptake from soil.

The diazotrophs commonly present in sugarcane plants are *Acetobactor diazotrophicus*, *Azospirillum brasilense*, *A. lipoferum*, *A. amazonense*, *B. brasilensis*, *B. tropicalis*, *H. seropedicae* and *H. rubrisubalbicans* (Reis et al., 2000; Sevilla and Kennedy, 2000; Kennedy and Islam, 2001). Where fertiliser-N is applied, the numbers of these diazotrophs markedly decline in sugarcane rendering the plant more dependent on fertiliser-N.

The endophytes colonise sugarcane spontaneously, promoted by the vegetative mode of propagation of sugarcane. It seems likely that continued study of the sugar cane system will yield information of use in establishing the use of diazotrophs with other crops.

5. General discussion

5.1. PGPR effects and crop yields

Additional data can be quoted that shows significant beneficial PGPR effects improving the yields of a broad range of field crops (Table 7), although many failures to obtain such responses may have been unreported. Both greenhouse and field experiments support the ability of organisms such as *Azospirillum* to increase yield in the range 5–30% in about 70% of inoculation trials (Okon and Labandera-Gonzalez, 1994). But not all such trials are successful and there are even cases where declines in yield were associated with inoculation (Nguyen et al., 2002a); this may reflect incompatibilities between bacterial strains and plant cultivars, as well as adequate soil-N for nutrition, as noted earlier with maize (Table 5). It is also noteable that a large number of different diazotrophic as well as non-diazotrophic species may contribute to the beneficial effects on the growth and yield of cereals, including those listed in Table 7 as well as *Pseudomonas*, *Klebsiella*, *Citrobacter*, *Clostridium*, *Azoarcus*, *Azorhizobium* and others mentioned earlier. Some, such as *Acetobacter*, may be more restricted in the range of plants they can associate with because of special nutritional needs such as high sugar concentration. There is little evidence of clearly preferred combinations of plant and microbial species to obtain beneficial effects, although some studies have suggested variation in response based on genotype (Han and New, 1998).

However, there are many questions that remain to be addressed before there can be sufficient confidence in the possible agronomic role of such inoculant biofertilisers to recommend their widespread adoption. Unfortunately, no studies have yet been reported to test if re-inoculation with PGPR is needed for each successive crop. Is there any

Table 7
Beneficial effects of some diazotrophic bacteria on yield or N accumulation of different crops

Crop	Diazotroph	Experiment type	Increase in yield		Increase in N accumulation	Reference
			Amount[a]	Statistical significance level		
Rice	*Azotobacter sp.*	Field	0.9 t ha^{-1} (GY, FW)	0.05	15 kg ha^{-1}	Yanni and El-Fattah (1999)
	A. lipoferum	Greenhouse	6.7 g $plant^{-1}$ (GY, DW)	0.05	58.9%Ndfa	Mirza et al. (2000)
	Herbaspirillum spp.	Greenhouse	7.5 g $plant^{-1}$ (GY, DW)	0.05	58.2%Ndfa	Mirza et al. (2000
	Burkholderia spp.[b]	Laboratory	22 mg $plant^{-1}$ (TBM, DW)	0.05	372 μg $plant^{-1}$	Baldani et al. (2000)
	R. leguminosarum	Greenhouse	7.9 g pot^{-1} (GY, FW)	0.05	31 mg pot^{-1}	Biswas et al. (2000a)
Wheat	*Azotobacter spp.* [b]	Greenhouse	0.65 g $plant^{-1}$ (TBM, DW)	0.05	–	Hegazi et al. (1998)
	Bacillus spp.[b]	Greenhouse	0.78 g $plant^{-1}$ (TBM, DW)	0.05	–	Hegazi et al. (1998)
Maize	*A. lipoferum*	Greenhouse	0.17 g pot^{-1} (SM, DW)	0.05	19 μg pot^{-1}	El-Komy et al. (1998)
	H. seropedicae	Field	1.5 t ha^{-1} (GY, FW)	0.05	–	Riggs et al. (2001)
Sugarcane	*A. brasilense*	Field	9 t ha^{-1} (CY, FW)	0.05	–	Shankariah and Hunsigi (2001)
	A. diazotrophicus	Field	5 t ha^{-1} (CY, FW)	0.05	–	Shankariah and Hunsigi (2001)
Cotton	*A. brasilense*	Greenhouse	68 mg $plant^{-1}$ (TBM, DW)	0.05	1 mg $plant^{-1}$	Fayez and Daw (1987)

[a] GY, grain yield; FW, fresh weight; DW, dry weight; TBM, total biomass; SM, shoot mass; CY, cane yield.
[b] These trials were carried out under aseptic conditions while the rest were carried out under non-sterile conditions.

residual effect of inoculation, as often exists for *Rhizobium* with legume nodulation? Some studies have claimed advantages from using multi-strain inoculants with strains selected for different beneficial effects such as P mobilisation, phytohormone production and BNF (Nguyen et al., 2002a,b), while others prefer to use a single strain. While a logical case can easily be made for multi-strain inoculation, field studies have not yet shown that each of the strains incorporated in products was necessary for the effect. Answers to these questions can only be obtained in more critical field trials measuring yield responses from inoculation; these studies would preferably be done under conditions where sufficient extra information is obtained to allow effective risk assessment and benefit-cost analysis to be performed.

Development of the technology will also require quantitative modelling and estimation of economic gross margins such as that employed in a study of adjustment of N inputs for maize (Keating et al., 1991), to allow the potential economic benefits (Barrett and Marsh, 2002) to be adequately assessed.

5.2. Quality control

Although there is ample evidence of positive effects on yield from inoculation with diazotrophs, there is still a reluctance to accept such inoculants as legitimate agriculture. In some cases, commercial products are available with vendors possibly making false or unsupported claims. However, the main requirement in the potential application of biofertilisers must be the availability of high quality inoculant products (Kennedy and Roughley, 2002). There are many ways of preparing and applying biofertiliser products, but there are three main criteria for quality control that must be applied if the possible benefits from inoculant biofertilisers are to be achieved on farms (Nguyen et al., 2002a, 2003). These are:

5.2.1. Strain identity

The biofertiliser product must be shown to contain beneficial strains of microorganisms. Strain selection is the first step of inoculation technology. According to Nguyen et al. (2002b) criteria for successful selection of biofertiliser strains for rice are (i) the strain should be abundant in the soil, (ii) it should have high activity (e.g. BNF, phytohormone production, P solubilising activity, etc.), (iii) the strain should be as fast-growing as possible, improving the success rate if non-sterile carrier media must be used, (iv) the strain must be shown not to cause root disease and finally (v) in laboratories where freeze-drying is not available, the strains should be reselected, if necessary, to show their effectiveness has been maintained. Confirmation of the correct identity of biofertiliser strains used to prepare biofertiliser inoculants can be based on cultural characteristics, PCR methods, immunodiagnostic tests or immunoblots and DNA hybridisation techniques.

5.2.2. Inoculum potential

The biofertiliser must be verified to have adequate inoculum potential so that it can multiply in the rhizosphere sufficiently to have a beneficial effect. For this reason, starter cultures should be of good quality and allow rapid growth in the media selected. The different strains should be grown separately as far as possible and the strains mixed just before preparation of the final product for application to plants to reduce the effects of competition on growth. Carrier media should be carefully selected and tested to ensure they will support the growth of sufficient numbers of each strain. The numbers of each biofertiliser strain in the individual cultures should be estimated for each batch produced.

5.2.3. Strain effectiveness

It must be clearly demonstrated that an inoculant biofertiliser is effective in improving crop yield and in reducing the need for chemical fertilisers. While experimental field trials are the ideal in demonstrating the degree of a crop's response, a useful alternative is a series of farmer trials with full-fertiliser treatments as controls (Nguyen et al., 2002a, 2003).

It would be desirable to confirm that biofertiliser strains have survived after inoculation and have effectively colonised the root zone of crops using genetic markers, immunodiagnostic or other tests. Difficult tests such as identifying strains using PCR methods should be restricted to quality control for starter cultures used for biofertiliser products. Testing in at the field stage of application requires the simplest, but most reliable tests possible.

Success in the application of biofertiliser products will require a significant infrastructure for production and application with quality control. This would not be dissimilar to methods of quality control imposed for rhizobia, such as the inoculants research and control service maintained by manufacturers of *Rhizobium* inoculants in Australia since the 1950s. In any such system, the benefit of independent testing cannot be overemphasised to ensure standards are maintained. An infrastructure closely linked to the biofertiliser production industry allowing research to improve inoculant quality and quality control of current production as well as of stored commercial products is considered as essential.

6. Conclusion

Cocking (2002) has called for concerted action to encourage biofertiliser production associated with BNF to become a more significant feature of world agriculture. This would help to overcome chronic problems such as low farm productivity and poor returns on labour referred to by Reeves et al. (2002). International and national agencies must take note of this need and though the biofertiliser industry can be self-sustaining, there is still a need for

various agencies to support development of the infrastructure needed to develop this industry, in tune with both economic and environmental needs for sustainable international agriculture in developing countries.

The prospects for effective microbial biofertilisers for cereal crops like rice, maize and even wheat becoming available soon are bright. However, much work regarding the quality of the products still needs to be done. At the beginning decade of the third millennium the importance of this has already been signalled by evidence of the declining availability of fossil fuels. Should the price for chemical fertilisers increase, it will be essential for farmers in developing countries to have greater access to the cheaper inoculant biofertiliser technology. It is also evident that chemical fertilisers generate much more greenhouse gases such as N_2O because of their inefficient utilisation by crops and biofertiliser may reduce this effect. Inoculant biofertilisers are more environmentally sound and their use could help mitigate the onset of global warming as well as reduce the fertiliser input costs of farmers.

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