Symbiosis, 35 (2003) 209–229
Balaban, Philadelphia/Rehovot

Review article

Use of Nitrogen Fixing Bacteria Inoculants as a Substitute for Nitrogen Fertiliser for Dryland Graminaceous Crops: Progress Made, Mechanisms of Action and Future Potential

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Received October 7, 2002; Accepted December 30, 2002

Abstract

A range of free-living and endophytic N_2 fixing bacteria have been isolated and used as inoculants on non-legume plants in attempts to maintain or increase yield while reducing the need for fertiliser N. Here, the literature on inoculation of dryland graminaceous crops with N_2 fixing bacteria in temperate and tropical agricultural systems is reviewed and the progress made, mechanisms of action of the bacteria and future potential of this approach, assessed. Firstly, we consider the use of *Azotobacter* spp. in Russia in the 1940s and 1950s and *Azospirillum* spp. worldwide in the 1970s and 1980s. In both cases, effects on yield were inconsistent.

Presented at the 9th International Symposium on Nitrogen Fixation with Non-Legumes, Leuven, Belgium, September 1–5, 2002

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Secondly, factors affecting yield responses to inoculation with *Azotobacter* and *Azospirillum* are discussed. A major weakness of using these bacteria as a substitute for N fertiliser is related to their mechanism of action. Evidence indicates that any additional N taken up by plants inoculated with *Azotobacter* and *Azospirillum* was primarily derived from the soil and not N_2 fixation. It is stressed that the general N deficiency of soil cannot be countered consistently by a procedure that does not add substantial N to the system. Thirdly, we focus on recent projects which have sought N_2 fixing bacterial inoculants for graminaceous crops which do add substantial N to the system. Effects of *Agrobacterium radiobacter* on wheat and barley, *Azorhizobium caulinodans* on wheat and *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp. and mixed endophytes on sugarcane and maize are considered. It is concluded that currently, no N_2 fixing bacterial inoculant is available which can match N fertiliser in consistency to counter soil N deficiency. Endophytic bacteria may have potential as inoculants but substantial experimentation is required before this can be adequately assessed.

Keywords: Gramineae, bacterial inoculants, nitrogen fixation, *Azotobacter*, *Azospirillum*, *Agrobacterium radiobacter*, *Azorhizobium caulinodans*, endophytic bacteria, *Gluconacetobacter diazotrophicus*, *Herbaspirillum*, N fertiliser

1. Introduction

Low nitrogen (N) availability is usually the main soil nutrient factor limiting growth and yield of crop plants. As the response to additional N is usually substantial, the strategic application of fertiliser N is frequently an important management tool used to increase crop yields. However, overuse of fertiliser N has contributed to a range of environmental problems including rapid eutrophication of fresh waters and increased atmospheric ammonia and nitrogen oxide concentrations. Because of this, alternative strategies to fertiliser N have been sought to combat limiting soil N levels. Often, these make use of N_2 fixing legumes in rotations. However, many free-living and endophytic N_2 fixing bacteria can interact positively with non-legume crops in ways which increase yields. A range of these bacteria have been isolated and used as inoculants of non-legume crops, in particular cereals, in attempts to maintain or increase productivity while reducing the need for N fertiliser. Here we review the literature on inoculation of dryland graminaceous crops with N_2 fixing bacteria in temperate and tropical agricultural systems. We assess the progress made, the mechanisms of action of the bacteria and the future potential of this approach as a substitute for N fertiliser for dryland graminaceous crops.

2. Early Work Using *Azotobacter*

The first large scale programme using N_2 fixing bacterial inoculants on non-legume crops appears to have been carried out in Russia in the 1930s–1950s (Allison, 1947; Cooper, 1959; Brown, 1974; Bashan, 1998). In the 1950s, inoculum was used on a range of non-legume crops over an area of about ten million hectares (Cooper, 1959). The primary inoculant used was *Azotobacter chroococcum* and the main graminaceous crops treated were wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). In several areas, local strains of *Azotobacter* were used and it was recommended that bacterial inoculants should supplement mineral fertilisers rather than replace them. It was variously estimated that *Azotobacter* preparations benefited 30–70% of the field crops treated, with an average yield increase of 10–20% (Cooper, 1959; Brown, 1974). Due to the inconsistency of effects, the practice was abandoned in the 1960s.

In several countries, over the past twenty five years, strains of *Azotobacter* spp. have been included in studies focusing on the effects of *Azospirillum* spp. inoculants on yields of cereals and grasses. In general, effects were as great or greater with *Azospirillum* spp. (e.g. Yahalom et al., 1984; Zambre et al., 1984; Wani et al., 1985; Lee et al., 1994). Also, in 1993, a long term programme was initiated in Russia, to select N_2 fixing bacteria for use as inoculants on cereals (Zavalin et al., 2001). To date, best results have been obtained with *Agrobacterium radiobacter* and this work is discussed below (Section 5).

3. *Azospirillum* spp. as Inoculants

From 1976 to the late 1980s, field trials were carried out in many countries to test the effects of *Azospirillum* inoculants on N accumulation, growth and yield of a range of graminaceous crops. This work was reviewed in detail by Sumner (1990) and is summarised in relation to bacteria and crop species tested, associated N treatment and effect on yield in Table 1. In all experiments where tested, except those of Baldani et al. (1987), additional N gave increased yields. This emphasises the general N limitation of soil for plant growth and the reliability of fertiliser N in overcoming this limitation and increasing yield. If N_2 fixing bacteria inoculants are to substitute for N fertilisation of graminaceous crops then they also must be consistent in their effect. This was not the case with *Azospirillum*, as although in most studies, positive effects on yield were reported, often there were no effects and in a few cases, inoculation caused decreases in yield (Table 1); see also Albrecht et al. (1981) and Okon et al. (1988). Across experiments, yield was dependent on plant species and cultivar, bacterial species and strain, field site, sowing date, the amount of N

Table 1. Effect of inoculation with *Azospirillum* spp. on yield of a range of graminaceous crops. Summary of trials carried out between 1976–1988.

Bacterium	Crop	Yield	Comments	Reference
A. brasilense	*Panicum miliaceum* L.	+13.2%	± *A. brasilense* (mixed strains (2)) only variable tested; no N added	Kapulnik et al. (1981)
	Pennisetum americanum (L.) Leeke	0–31.7%	Autoclaved and live bacteria compared. Dependent on cultivar, but only 1 of 21 tested showed response; 60 kg N ha^{-1} added throughout. Effect did not repeat in year 2	Bouton et al. (1979)
		0–26%	Mixed strains (2) used. Dependent on N applied and field site. Generally effect decreased with increased applied N 0–40 kg ha^{-1}	Tilak and Subba Rao (1987)
		0%	Killed and live inoculum compared. 30 kg N ha^{-1} added	Smith et al. (1984)
		0%	Different cultivars tested over 2 experiments, no N added	Wani et al. (1985)
	Pennisetum sp.	0–24.4%	Killed and live inoculum compared. Dependent on bacterial strain and N supply; occurred at 30 and 60 kg N ha^{-1} but not 0 or 120 kg N ha^{-1}	Smith et al. (1984)
	Setaria italica (L.) Pal.	45.1%	± *A. brasilense* (mixed strains (2)) only variable tested; no N added	Kapulnik et al. (1981)
		0–57.9%	± *A. brasilense* only variable tested, dependent on bacterial strain. 70 kg N ha^{-1} added	Yahalom et al. (1984)
	Sorghum bicolor (L.) Moench	0–80.5%	Dependent on N applied, method of application and year. Effect occurred with 40 kg N ha^{-1} as basal dressing but not 0 N or 40 kg N applied as top dressing. Effect one out of two years	Pal and Malik (1981)
		0–35.1%	± *A. brasilense* only variable tested, no N added, dependent on bacterial strain	Kapulnik et al. (1981)

Table 1. Continued

Bacterium	Crop	Yield	Comments	Reference
		10.5%	Compared killed v live inoculum. 30 kg N ha^{-1} added	Smith et al. (1984)
		17.8%	± inoculum (mixed strains (2)) only variable tested. No N added	Sarig et al. (1984)
		0%	± inoculum only variable tested, no N added. No effect on yield over three years	Sarig et al. (1988)
	Sorghum sudanense (Piper) Stapf.	0%	No effect at 0, 30, 60 or 120 kg N ha^{-1}	Smith et al. (1984)
	Triticum aestivum L.	12.0%	Average value over 10 winter cultivars, 2 bacterial strains and 3 N levels (10, 80 and 160 kg N ha^{-1})	Reynders and Vlassak (1982)
		0–2.6%	Average values over 4 spring cultivars and 4 N levels (0–80 kg N ha^{-1}) in 2 experiments, dependent on bacterial strain	Reynders and Vlassak (1982)
		0–8.0%	Dependent on bacterial strain and N supply. Effect at 0 and 40 kg N ha^{-1} but not 80 kg N ha^{-1} for one cultivar but occurred at 120 kg N ha^{-1} with another cultivar	Kapulnik et al. (1983)
		0%	Different bacterial strains tested over 2 years, 30 kg N ha^{-1} added. At heading stage, dry matter and N accumulation greater with 2 out of 5 inoculant treatments but grain yield unaffected	Baldani et al. (1983)
		0–31.5%	Mixed strains (3) used. 67 kg N ha^{-1} added, dependent on cultivar	Hegazi and Saleh (1985)
		0–6.7%	Dependent on cultivar and location but NB only one positive result in study with 2 cultivars and 5 experiments 80–158 kg N ha^{-1} added	Millet et al. (1985)

Table 1. Continued

Bacterium	Crop	Yield	Comments	Reference
		0–78.9%	Compared dead v live inoculum, 30 kg N ha^{-1} added. Dependent on bacterial strain	Boddey et al. (1986)
		0%	Different bacterial strains (5) tested + 15–100 kg N ha^{-1} added. Experiments over 3 years	Baldani et al. (1987)
		18–25.8%	Mixed strains (4) used. Dependent on N supply (0–120 kg N ha^{-1}) but no obvious pattern with amount added	Kapulnik et al. (1987)
	Triticum durum L.	21.6%	Average increase over five N treatments 0–120 kg N ha^{-1}	Zambre et al. (1984)
	Triticum turgidum L.	–7.8–0%	Dependent on location but only one –ve value across study with 5 experiments. 80–158 kg N ha^{-1} added	Millet et al. (1985)
		0–10.9%	Dependent on bacterial strain. No N added	Kapulnik et al. (1983)
		0	Mixed strains (4) used. No effect at 40 or 120 kg N ha^{-1} with two cultivars	Kapulnik et al. (1987)
	Zea mays L.	0–13.7%	Mixed strains (2) used. Dependent on N supply. Effect occurred at 120 kg N ha^{-1} but not 0, 60 or 240 kg N ha^{-1}	Kapulnik et al. (1981)
		18.4%	Effect on top weight, ear yield not affected. No N added	Kapulnik et al. (1981)
		0–10.5%	± *A. brasilense* only variable tested, dependent on bacterial strain; no N added	Kapulnik et al. (1981)
A. lipoferum	*Panicum maximum* Jacq.	0–16.2%	Effects at 30–60 kg N ha^{-1}, not 0–20 or 80–120 kg N ha^{-1}	Smith et al. (1976)
		0–13.8%	Dependent on N supply. Effect occurred at 20–40 kg Na ha^{-1} but not 0 or 80 kg N ha^{-1}	Smith et al. (1977)

Table 1. Continued

Bacterium	Crop	Yield	Comments	Reference
	Pennisetum americanum	0–20%	Dependent on N supply. Effect occurred at 40 and 80 kg N ha^{-1} not 0–20 kg N ha^{-1}	Smith et al. (1976)
		0–20%	Dependent on N supply. Effect occurred at 40 and 80 kg N ha^{-1} not 0–20 kg N ha^{-1}	Smith et al. (1977)
		0%	Different cultivars tested over 4 experiments with 2 bacterial strains, 16–20 kg N ha^{-1} added in two experiments	Wani et al. (1985)
	Triticum aestivum	31.2–64.3%	Positive effect of inoculum at 0–80 kg N ha^{-1}	Rai and Gaur (1982)
	Zea mays	0–154%	Mixed strains (3) used. Dependent on cultivar. For one cultivar response to inoculum to that with 200 kg N ha^{-1} in a separate treatment	Hegazi et al. (1983)
	Triticum aestivum	0–37.6%	Effect occurred at 0 but not 75 or 150 kg N ha^{-1}	Ishac et al. (1986)
Azospirillum sp.	*Oryza sativa*	43.5, 24.7%	Values obtained at 90 and 120 kg N ha^{-1}, respectively	Kannaiyan et al. (1983)
		0–15.5%	Effect occurred at 30–60 kg N ha^{-1} in 2 out of 3 years but not 0 N	Rao et al. (1983)
	Panicum maximum	0%	*Azospirillum brasilense* and *Azospirillum* sp. mixed strains used. No effect at 44.8–134.4 kg N ha^{-1}	Taylor (1979)
	Pennisetum americanum	0–29%	*Azospirillum brasilense* and *Azospirillum* sp. mixed strains used. Effect occurred at 89.6 kg N ha^{-1} but not 44.8 or 134.4 kg N ha^{-1}	Taylor (1979)
A. amazonense	*Triticum aestivum*	37.5%	Comparison live *A. amonense* v dead *A. brasilense*, 30 kg N ha^{-1} added	Boddey et al. (1986)

applied. and the method of N application. Also, in several studies quoted with positive effects, many genotypes tested negative in preliminary studies (e.g. Smith et al., 1976, 1977). Generally, when crops responded positively to inoculation, yield increases were in the range 5–30% but they could be substantially greater than this. In some cases this may have been primarily a maturity effect as inoculation can increase the rate of crop development. This was highlighted as the case in the work of Yahalom et al. (1984) on *Setaria italica* (L.) Pal where inoculation with *Azospirillum brasilense*-Cd gave an increased seed yield of 57.9%, but the increase in forage yield was only 18.5%. Generally, where yield increases were substantial, findings were not verified over different years.

In early work on *Azotobacter* spp. it was recommended that inoculants should supplement rather than replace N fertiliser as it was thought that benefits of inoculation were greatest in moderately fertile soil (Cooper, 1959). Similarly, it has been suggested that *Azospirillum* gives best results with moderate N application (Okon and Labandera-Gonzalez, 1994). However, across experiments, there is little consistency in the relationship between yield response to inoculum and amount of nitrogen applied (Table 1) and it is difficult to argue that this is the case.

Further studies in addition to these considered in Table 1 have been carried out to test the effects of *Azospirillum* inoculant on yield of graminaceous species. Generally these involved workers from the earlier trials but again the results were negative or inconsistent. For example, Lee et al. (1994) reported no effect of *Azospirillum lipoferum* inoculation on yields of *Pennisetum glaucum* (L.) R. Br. or sorghum (*Sorghum bicolor* (L.) Moench) with 0 or 20 kg N ha^{-1} added. Also, Garcia de Salomone and Döbereiner (1996) had variable results in two experiments with a range of maize (*Zea mays* L.) genotypes. In the first experiment, the effects of a mixture of four *Azospirillum brasilense* strains and three *Azospirillum lipoferum* strains on yield of 15 maize genotypes were tested. Significant effects ranged from a 34% reduction to a 94% increase in yield depending on cultivar. In the second experiment which used seven of the cultivars, there were again significant reductions or increases in yield depending on cultivar but only four out of the seven cultivars showed the same significant response as in the first experiment. It was concluded that more detailed plant genotype-*Azospirillum* spp. strain interaction studies, taking the entire N metabolism in the plant into account, are needed to allow better inoculation results of cereal crops (Garcia de Salomone and Döbereiner, 1996).

Dobbelaere et al. (2001) reported on field experiments carried out with *Azospirillum* spp. during 1994–2000 in Israel, Belgium, Uruguay and Mexico. Work carried out in Israel examined the effects of *Azospirillum brasilense* on early growth of maize and did not consider yield. Two field experiments were carried out in Belgium in 1999–2000 to test the effects of inoculation with

Azospirillum brasilense and *Azospirillum irakense* on yield of winter wheat. In both experiments, additional N in the range 50–170 kg N ha^{-1} was applied. Inoculation resulted in significant increases in plant dry weight early in the growing season especially in non-fertilised plots, however, these effects did not result in greater yields. Also, in an earlier experiment in 1997, application of a commercial inoculum of *Azospirillum lipoferum* did not significantly affect yield of maize supplied 0–250 kg N ha^{-1}. Similar results were obtained in Uruguay, where the effects of various strains of *Azospirillum brasilense* on yields of maize, sorghum and oat (*Avena sativa* L.) were tested. Here, inoculation often resulted in increases in biomass of maize and sorghum but in most cases, differences were not statistically significant. With oat, a significant increase in yield was found at the first but not second harvest. It was concluded that effects of *Azospirillum* on crop yield are not consistent. In Mexico, a large field programme was carried out in 1999, in which approximately 450,000 ha of maize and 150,000 ha of sorghum, wheat and barley were inoculated with a mixture of *Azospirillum brasilense* strains. Grain yields of various cultivars of all species were evaluated at 171 sites and in 678 ha with diverse soil and climatic conditions and different levels of N fertilisation. Here under low N conditions, there were consistent, generally with greater than 30% increases in crop yield. The greatest response was found with domestic maize cultivars in light-sandy soils. If these results repeat in different years, then they could be of commercial importance. However, the data presented indicate that inoculation is unlikely to result in yields as great as those obtainable with optimum fertiliser N application.

4. Reasons for Variability in Response to *Azotobacter* and *Azospirillum*: Mechanism(s) of Action of Bacteria

The effects of *Azotobacter* and *Azospirillum* inoculants on growth and yield of graminaceous crops have been tested in many experiments carried out in numerous countries. Despite this, the effects of inoculation are still inconsistent. Partial cause of this inconsistency in response may be that in some cases, bacteria do not build up a high enough population in the soil to have an effect. This could be caused by several factors including an unfavourable soil environment (e.g. water or temperature stress), competition with better adapted native soil bacteria or predation by protozoans (Jjemba and Alexander, 1999; Bashan, 1999). Also, 'inappropriate' *Azospirillum* strains may have been used in experiments. Specifically, there is evidence that, especially if native *Azospirillum* populations in soil are high, strains isolated from roots of the crops they are subsequently used on, give best results (Boddey et al., 1986; Sumner, 1990; Okon and Labandera-Gonzalez, 1994). Inconsistency of response

could also be related to the extent of colonisation of inner root tissues by *Azospirillum* which is dependent on the strain used (Schloter and Hartmann, 1998).

Changes to agronomic practice or improvements to inoculation formulations may improve bacterial survival in the soil (Bashan, 1998) and careful matching of bacterial strain and crop is certainly possible. However, a major weakness of using *Azotobacter* or *Azospirillum* as a substitute for N fertiliser is related to their mechanism of action. While there is no doubt that in many cases where inoculation resulted in increased yield, this increase was at least in part due to increased N uptake and assimilation by the crop, the evidence indicates that this additional N was obtained primarily from the soil and not through N_2 fixation (e.g. Cooper, 1959; Barea and Brown, 1974; Lethridge and Davidson, 1983; Kapulnik et al., 1985; Boddey et al., 1986; Sarig et al., 1990; Lee et al., 1994; Merbach et al., 1998). The general opinion is that *Azotobacter* and *Azospirillum* act via changes in root morphology and physiology (probably hormone induced) which result in increased mineral nutrient and water uptake from the soil, especially during early growth; these effects result in greater crop growth and subsequently greater yield (e.g. Sumner, 1990; Giller and Wilson, 1991; Dobbelaere et al., 2001; Mathews et al., 2001; Riggs et al., 2001; Sevilla et al., 2001).

However, these effects can only occur if nutrients and water are available in the soil for uptake (e.g. at deeper levels): this will not always be the case. The general N deficiency of soils cannot be countered consistently by a procedure which does not add N to the system. Similarly, in dry soils, where the inoculation effect on yield has been related to increased water uptake (e.g. Sarig et al., 1988), inoculation will not be as reliable as irrigation in overcoming water deficiency.

5. Future Potential of Bacterial Inoculants

Low N availability is usually the main soil nutrient factor limiting growth and yield of graminaceous crops and the strategic application of fertiliser N is a commonly used practice to overcome this deficiency. Alternative strategies often make use of N_2 fixing legume plants in rotations to increase soil N levels. Generally, legumes as with N fertiliser, add substantial N to the system. In contrast, inoculation with *Azotobacter* or *Azospirillum*, in general, appears to add little N to the system which limits their usefulness. Over the past 10 years research has looked for alternative N_2 fixing bacterial inoculants for cereals which do add substantial N to the system. Much of this work is ongoing and its potential is assessed.

Recent studies in Russia: 'Agrobacterium radiobacter'

Since 1993, studies have been carried out in different soil types and climatic zones in Russia, to test the effects of N_2 fixing bacteria inoculants on yields of spring and winter wheat and spring barley (Zavalin et al., 2001). Several bacteria species which showed acetylene reduction activity have been tested including *'Agrobacterium radiobacter'*, *Flavobacterium* sp. and *Klebsiella mobilis* and results were correlated with environmental factors. A strain of *'Agrobacterium radiobacter'* (now *Rhizobium radiobacter*; Young et al., 2001) isolated from the rhizosphere of a rice (*Oryza sativa* L.) crop grown in Russia was the most consistent in its effects and gave the greatest increases in yield but its effects were strongly dependent on soil pH and water availability.

Generally, the greatest response to *'Agrobacterium radiobacter'* occurred without addition of N fertiliser. At suitable pH and adequate water supply, yield increases with the bacterium were generally equivalent to that obtained with the addition of 30 kg N ha^{-1} but it was ineffective at soil pH less than 5.5, and in dry years. For example, in the study of Bairamov et al. (2001), which examined the effects of different inocula on spring barley, *'Agrobacterium radiobacter'* caused increases in yield in 1997 and 1998 but not 1999 (Table 2).

In 1999, rainfall was much lower than average over the first four months of crop growth. Nevertheless, addition of 60 kg N ha, resulted in substantial increases in yield in all years. It was estimated using the ^{15}N dilution method in the zero N treatments that, depending on year, a maximum of 23–32% of N assimilated by barley inoculated with *'Agrobacterium radiobacter'* was derived from N_2 fixation. Currently, we are carrying out studies to fully characterise this bacterium and deduce the mechanism(s) of its effect on cereal yields.

Azorhizobium caulinodans and wheat

Azorhizobium caulinodans was isolated from the tropical legume species *Sesbania rostrata* (Bremek. & Oberm.) which has root and stem nodules. This bacterium is unusual in that its nitrogenase enzyme is more tolerant of oxygen than that of other rhizobia studied so far (Dreyfus et al., 1983). Sabry et al. (1997) reported that inoculation of aseptically grown wheat with *Azorhizobium caulinodans* resulted in increased plant N content and dry weight. *Azorhizobium caulinodans* entered the roots of wheat via gaps formed by emerging lateral roots and established within the intercellular spaces of the cortex, xylem and root meristems. The plants showed acetylene reduction activity, and increases in plant growth and N content were attributed to nitrogen fixation by the bacterium.

Table 2. Effect of different bacterial inoculants and additional N on yield of barley. Taken from Bairamov et al. (2001).

Inoculant	Additional N (kg ha^{-1})	Extrapolated yield (t ha^{-1}) 1997	1998	1999
0	0	0.31	0.72	0.44
Agrobacterium radiobacter	0	0.54	1.00	0.45
Flavobacterium sp.	0	0.44	0.86	0.54
Klebsiella mobilis	0	0.37	0.79	0.53
0	30	0.62	0.93	0.47
Agrobacterium radiobacter	30	0.67	1.07	0.49
Flavobacterium sp.	30	0.71	0.95	0.47
Klebsiella mobilis	30	0.57	0.77	0.60
0	60	0.82	0.93	0.70
LSD		0.118	0.185	0.175

Mathews et al. (2001) extended the study of *Azorhizobium caulinodans* effects on wheat. Here, plants were grown at two N levels (10.8 or 92 mg N kg^{-1} soil) in non-sterile soil in pots under 'temperate' controlled environment conditions. As in the study of Sabry et al. (1997), inoculation of wheat with *Azorhizobium caulinodans* resulted in increases in plant dry weight and N content: effects were independent of N supply. However, increases in growth were similar with a non-N_2-fixing strain of *A. caulinodans* or a filter-sterilised supernatant of the bacterial culture. It was concluded that the response of wheat to *Azorhizobium caulinodans* was not due to N_2 fixation by the bacteria but probably related to plant growth substances produced by the bacteria in culture. Also, it was questioned if the growth promoting effects of *Azorhizobium caulinodans* could be of practical benefit in the field as the growth effects were small and the work only studied pre-anthesis growth.

Endophytic bacteria

Nitrogen balance, ^{15}N isotope dilution and ^{15}N natural abundance studies provide strong evidence that some economically important tropical grasses including sugarcane (*Saccharum* spp.) (Urquiaga et al., 1992; Yoneyama et al., 1997; Boddey et al., 2001), kallar grass (*Leptochloa fusca* (L.) Kunth.) (Malik et al., 1997) and wetland rice (Boddey et al., 1995; Shrestha and Ladha, 1996; Malarvizhi and Ladha, 1999) can obtain a substantial proportion of their N requirements from N_2 fixation. There is also strong evidence that endophytic

N_2 fixing bacteria play a major role in this process (James, 2000 and references therein) and that within plant species, the amount of N_2 fixed is at least partly dependent on plant genotype and geographical location. For example, in the case of sugarcane, so far only a few Brazilian varieties have been shown to definitely fix N (Urquiaga et al., 1992), with the few studies from other countries producing equivocal (Japan, Philippines) or negative (Australia) results using ^{15}N natural abundance (Yoneyama et al., 1997; Biggs et al., 2002). It has been speculated that the very low application of N-fertilisers in Brazil over the last 100 years has inadvertently selected varieties for low N use and this may explain why Brazilian varieties are better able to benefit from N_2 fixation (Baldani et al., 2002). Although the specific micro-organisms responsible for this N_2 fixation have not been exclusively determined, it appears that *Azoarcus* spp. play the major role in N_2 fixation in kallar grass (Reinhold-Hurek and Hurek, 1998; Hurek et al., 2002) while *Gluconacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*) and *Herbaspirillum* spp. could be important in N_2 fixation in sugarcane (Boddey et al., 1995; Baldani et al., 1997), although many other as yet undiscovered bacteria may be involved (James and Olivares, 1998; James et al., 2001). It has been proposed that endophytic N_2 fixing bacteria have considerable potential for use in agriculture as the interior of the plant could provide a low O_2 environment which is necessary for the expression of the oxygen sensitive enzyme, nitrogenase (Baldani et al., 1997; James and Olivares, 1998). In addition, endophytic bacteria will not have to compete with rhizosphere bacteria for resources, and within the plant, transfer of C from the host to bacteria and N from the bacteria to the host is likely to be more efficient (James et al., 2001). However, if endophytic N_2 fixing bacteria are to be of use as inoculants, they must provide the plant with substantial N from N_2 fixation.

Studies on the effects of inoculation of crops with N_2 fixing endophytic bacteria are at a preliminary stage but there is strong evidence that some sugarcane varieties can obtain fixed N from bacteria applied as inoculant: the recent studies of Sevilla et al. (2001) and Oliveira et al. (2002) are highlighted. Sevilla et al. (1998) showed that inoculation of sterile sugarcane plantlets with *Gluconacetobacter diazotrophicus* resulted in increased plant height under N-deficient conditions. Sevilla et al. (2001) assessed the mechanisms of the effects of the bacterium by comparing a wild type with a nitrogenase deficient (Nif^-) mutant in two experiments. In the first experiment, plants were grown in a glasshouse for 60 days after inoculation and in the second, they were grown in the field for four months from 60 days after planting. In both experiments, the wild-type and the Nif^- mutant strains colonised the plants equally and persisted throughout growth. Under N deficient conditions, 60 days after planting in experiment 1, dry weight and total N were greater with the wild-type strain than with the Nif^- strain or

uninoculated control. In a separate small laboratory experiment inoculated plantlets were exposed to $^{15}N_2$ in an enclosed atmosphere for 24 h, and the small, albeit significant, ^{15}N incorporation showed that the wild-type strain actively fixed N_2 inside the plants whereas the Nif$^-$ mutant did not. Taken together, although these results indicate that the transfer of fixed N from *Gluconacetobacter diazotrophicus* to sugarcane could be a factor resulting in increased plant growth, it should be noted that in the greenhouse experiment the uninoculated control plants that received N fertiliser were considerably larger than the wild type-inoculated plants that received no mineral N (Sevilla et al., 2001), thus showing that *G. diazotrophicus* inoculation alone was not sufficient to replace N fertiliser. Furthermore, when N was not limiting, plant dry weight and N content were greater in plants inoculated with either the wild-type or Nif$^-$ mutant strains. This indicates that there may be a hormonal component to plant growth promotion by the bacterium. In the second experiment, with plants transferred to the field, fresh cane weight at harvest was 30–60% greater with the wild type strain than with the Nif$^-$ mutant strain or for uninoculated plants. These results show that the early benefits of inoculation on growth can be maintained for at least three months in the field, but as ^{15}N was not used in the field experiments, it is not possible to say if the effects of inoculation on the plant were primarily via N_2 fixation or by other processes.

The study of Oliveira et al. (2002) placed greater emphasis on the application of endophytic inoculants in a commercial situation and evaluated their effect on the development of a micropropagated Brazilian sugarcane variety SP70-1143, which has been shown to fix N under field conditions (Urquiaga et al., 1992), and is the most widely used variety in laboratory and greenhouse studies (Reis et al., 1999; James et al., 2001; Sevilla et al., 2001). The micropropagation technique routinely used has the advantage that it results in a decrease in pathogenic endophytes but it also has the negative effect of eliminating beneficial endophytes that could promote plant growth. Inoculation of micropropagated sugarcane could be an efficient way to reintroduce selected strains of N_2 fixing endophytes into the plant (Reis et al., 1999; James et al., 2001). Seven different combinations of inoculum using five endophytic N_2 fixing species (*Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans*, *Azospirillum amazonense* and *Burkholderia* sp.) were tested. These species were isolated from sugarcane, and the combinations used were based on their occurrence in field grown plants, priorizing the *Gluconacetobacter diazotrophicus*/*Herbaspirillum* spp. combination. After micropropagation and acclimatization in a glasshouse (45 days), plants were transferred to pots containing ^{15}N labelled soil and maintained outdoors for 6 or 12 months. Use of the ^{15}N label added approximately 20 kg N ha^{-1} and in the pots harvested at 400 days,

Brachiaria trash was added to immobilise N and limit N availability.

In some respects, the results obtained by Oliveira et al. (2002) are promising. All inoculated N_2 fixing species could be reisolated in high numbers from the rhizomes of the inoculated plants after 12 months outdoors thus indicating that the bacteria had established within the plants. Also, use of the ^{15}N dilution technique showed that inoculation increased N_2 fixation in all cases. Greatest values for the proportion of N obtained from N_2 fixation were obtained with plants inoculated with all five species (23.7–33.7%) and with *Herbaspirillum seropedicae* plus *Herbaspirillum rubrisubalbicans* (11.9–17%). At final harvest, these treatments gave an average of 30% increase in plant dry weight over controls. However, the shoot to root dry weight ratio was substantially less with inoculated plants than uninoculated plants and thus the magnitude of effects on shoot dry weight (and fresh weight) were less than this. Also, it is important to note that the mixture of all five endophytic N_2 fixing species as inoculum had a negative effect on plant survival after acclimatisation.

The studies of Sevilla et al. (2001) and Oliveira et al. (2002) leave little doubt that plants can obtain fixed N from N_2 fixing endophytes and that inoculation of plants with these endophytes can result in increased growth. Similarly, a recent study from India has shown that yield of some Indian varieties may also benefit greatly from inoculation with *Azospirillum lipoferum*, *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp., especially in combination with vesicular arbuscular mycorrhiza (Muthukumarasamy and Revathi, 1999). However, substantial experimentation is required before the potential of N_2 fixing bacteria endophytes as inoculants can be adequately assessed. The work of Riggs et al. (2001) on maize highlights this. Here the objectives were to identify maize-endophyte associations with increased plant productivity and, if possible, which also showed little or no N deficiency symptoms (chlorosis) in the absence of N fertiliser. Initially, 23 bacteria genotypes including strains of *Azospirillum brasilense*, *Azorhizobium caulinodans*, *Azoarcus indigens*, *Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae* and eight maize genotypes were tested. Glasshouse experiments were carried out in 1997 and 1998 and field trials in 1998–2000. In the field trials there were two N treatments, 0 and 234 kg N ha^{-1}, and each year the maize-bacterium combinations which gave the greatest increases in yield were selected for study the next year. In 1998, without additional N, only one of 56 bacterial strain × maize genotype combinations resulted in a significant yield increase. However, with additional N, 19 of 56 strain × genotype combinations showed a significant yield increase compared with the uninoculated control: the average increase was 11%. In 1999, again effects were more consistent with additional N and here 19 of 68 strain-genotype combinations resulted in a significant yield increase that averaged 16%. In no case in 1998 or 1999, did inoculation relieve

nitrogen stress symptoms. All field plants were fertilised with N in 2000 and of the 16 strain × genotype combinations planted, six gave a significant increase that averaged 18.9%. Positive effects were obtained from different bacterial species including *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae, Klebsiella pneumoniae* and *Pantoeae agglomerans*. Therefore, with selection over the three years of study, greater consistency and benefit of inoculation were achieved but only in plots with substantial fertiliser N added: inoculation had little effect in non-fertilised soil.

6. Conclusions

The literature on inoculation of dryland graminaceous crops with N_2 fixing bacteria in temperate and tropical agricultural systems is reviewed to assess the progress made, mechanisms of action of the bacteria and future potential of this approach as a substitute for N fertiliser. Across these studies in almost all experiments tested, additional N gave increased yield. This emphasises the general N limitation of soil for plant growth and the reliability of fertiliser N in overcoming this limitation and increasing yields. If N_2 fixing bacteria inoculants are to substitute for N fertilisation of graminaceous crops, they also must be consistent in their effect. This is not the case. No individual or mixed strain N_2 fixing bacterial inoculant is available which can match N fertiliser in its consistency to counter soil N deficiency. Also, the data indicate that inoculation is unlikely to result in yields as great as that obtainable with optimum N fertiliser. Endophytic bacteria may have potential as inoculants but substantial experimentation is required before this can be adequately assessed.

Acknowledgements

We thank Fabio Olivares and Gyaneshwar Prasad for helpful discussions.

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