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# Biological nitrogen fixation associated with sugar cane and rice: Contributions and prospects for improvement

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## Abstract

$^{15}N$ isotope and N balance studies performed over the last few years have shown that several Brazilian varieties of sugarcane are capable of obtaining over 60% of their nitrogen (>150 kg N $ha^{-1}$ $year^{-1}$) from biological nitrogen fixation (BNF). This may be due to the fact that this crop in Brazil has been systematically bred for high yields with low fertilizer N inputs. In the case of wetland rice, N balance experiments performed both in the field and in pots suggest that 30 to 60 N $ha^{-1}$ $crop^{-1}$ may be obtained from plant-associated BNF and that different varieties have different capacities to obtain N from this source. $^{15}N_2$ incorporation studies have proved that wetland rice can obtain at least some N from BNF and acetylene reduction (AR) assays also indicate differences in $N_2$-fixing ability between different rice varieties. However in situ AR field estimates suggest plant-associated BNF inputs to be less than 8 kg N $ha^{-1}$ $crop^{-1}$. The problems associated with the use of the $^{15}N$ dilution technique for BNF quantification are discussed and illustrated with data from a recent study performed at EMBRAPA–CNPAB. Although many species of diazotrophs have been isolated from the rhizosphere of both sugarcane and wetland rice, the recent discovery of endophytic $N_2$-fixing bacteria within roots, shoots and leaves of both crops suggests, at least in the case of sugarcane, that these bacteria may be the most important contributors to the observed BNF

contributions. In sugarcane both *Acetobacter diazotrophicus* and *Herbaspirillum* spp. have been found within roots and aerial tissues and these microorganisms, unlike *Azospirillum* spp. and other rhizospheric diazotrophs, have been shown to survive poorly in soil. *Herbaspirillum* spp. are found in many graminaceous crops, including rice (in roots and aerial tissue), and are able to survive and pass from crop to crop in the seeds. The physiology, ecology and infection of plants by these endophytes are fully discussed in this paper. The sugarcane/endophytic diazotroph association is the first efficient $N_2$-fixing system to be discovered associated with any member of the gramineae. As yet the individual roles of the different diazotrophs in this system have not been elucidated and far more work on the physiology and anatomy of this system is required. However, the understanding gained in these studies should serve as a foundation for the improvement/development of similar $N_2$-fixing systems in wetland rice and other cereal crops.

## Introduction

The "green revolution" in agriculture of the developing world which resulted in large increases in cereal grain production since the 1960s, has been a result of the development of plant genotypes highly responsive to chemical fertilizers, particularly nitrogen. It requires approximately 18.5 Mcal of fossil energy to produce one kg of fertilizer nitrogen and even though, unlike other fertilizers, there is an unlimited supply of this element in the air, this is more than 6 times the energy required to produce either phosphate or potassium fertilizers (Da Silva et al., 1978). With the inevitable price rises of fossil fuels (not to mention proposed carbon taxes) that must occur over the next few decades due to the depletion of petroleum reserves and increased production costs of other fuels, now is the time that alternative strategies for nitrogen supply should be developed before these increased costs force farmers to cut N inputs which will result in drastic yield reductions in the staple cereal crops which feed the burgeoning human population of the Third World.

In traditional wetland rice culture yields of 2 to 3 t grain $ha^{-1}$ (either one or two crops $year^{-1}$) seem to be sustainable indefinitely, even where no N fertilizer is applied, if flood water is well controlled. For such yields to be sustained between 60 and 80 kg of nitrogen are required for each crop (Bennett and Ladha, 1992) and while some of this input may be supplied in rainfall and irrigation water, several field N balance studies suggest that N is supplied in part by nitrogen-fixing organisms (Firth et al., 1973; Koyama and App, 1979; Walcott et al., 1977).

Virtually all of the varieties of sugar cane planted in Brazil were bred under conditions of low N fertilizer inputs. Probably for this reason, the plant-crop rarely responds to nitrogen fertilizer (Azeredo et al., 1981), and while ratoon crops do often respond to N application, quantities applied rarely exceed 100 kg N $ha^{-1}$ and fertilizer use efficency is usually less than 35% (Oliveira et al., 1994; Sampaio et al., 1984). A sugar cane crop yielding 100 t cane $ha^{-1}$ accumulates between 180 and 250 kg N $ha^{-1}$ (Orlando-Filho et al., 1980; Stanford and Ayres, 1964). The mean Brazilian yield is 65 to 70 t cane $ha^{-1}$ and average whole crop N accumulation is between 100 to 120 kg N. Of this approximately two thirds is transported to the mill in the cane stems and a further 25% is in the senescent leaves (trash), which in Brazil, as in most countries, is burned off before harvest (Oliveira et al., 1994). Less than 10% of the N in the form of flag leaves remains in the field. It is apparent from these data that continuous cropping of sugar cane should deplete soil N reserves such that cane yields eventually decline. However, such decline in yields or soil N reserves are not normally observed even after many decades, or even centuries, of cane cropping. Such observations have led several authors to suggest that sugar cane may benefit significantly from inputs from biological nitrogen fixation (BNF) (Döbereiner, 1961; Purchase, 1980; Ruschel et al., 1978).

## Quantification of biological nitrogen fixation

### *Sugar cane*

Only a few studies have been published on the quantification of the BNF contribution to sugar cane and all of them were performed in Brazil. Experiments using $^{15}N$-labelled $N_2$ gas conducted at the Centro de Energia Nuclear na Agricultura (CENA) in Piracicaba (São Paulo) showed that 90 day-old sugar cane plants obtained considerable N from BNF (Ruschel et al., 1975). However, because of the difficulties of exposing plants grown in the field to controlled atmospheres, the agronomic significance of these N inputs could not be evaluated (Matsui et al., 1981). In a subsequent

$^{15}$N-aided N balance study performed at CNPAB, sugar cane was grown in pots containing 64 kg soil (Lima et al., 1987). Both the N balance and $^{15}$N enrichment data indicated that between 40 and 60% of plant N was derived from plant-associated BNF and extrapolation to the field (15,000 plants ha$^{-1}$) suggested inputs of over 150 kg N ha$^{-1}$ year$^{-1}$.

Our group has recently completed a three-year $^{15}$N isotope dilution and N balance study on 10 sugar cane varieties grown in a concrete tank (20 × 6 × 0.8m) filled with soil amended with $^{15}$N-labelled organic matter, and using *Brachiaria arrecta* as a non-$N_2$-fixing control plant (Urquiaga et al., 1992). The soil had a low N content (0.108% N) and was fertilized with phosphorus, potassium and micronutrients and well irrigated throughout the experiment, but no N fertilizer was added. In the first year yields of fresh cane of the commercial varieties were high, ranging from the equivalent of 175 to 230 t ha$^{-1}$, and in the varieties CB 45–3 and SP 70–1143 these high yields were maintained during the subsequent two ratoon crops. In these same varieties and the *Saccharum spontaneum* variety, Krakatau, the nitrogen accumulation also continued to be high and stable over the three years. However, other varieties (e.g., CB 47–89, NA 56–79, SP 71–799, Chunee) showed a decline in total N content after the first year as would be expected from the observed decline in the availability of soil N. Over the whole three years, the weighted mean $^{15}$N enrichments of all of the sugar cane varieties were much lower than that of the non-$N_2$-fixing *B. arrecta* control, indicating large contributions of plant associated BNF (Table 1).

At the second and third annual harvests (first and second ratoon crops) there were only small difference in the $^{15}$N enrichments between the different varieties and that of the control crop, which was due to the carry-over of labelled nitrogen from one harvest to the next in the stem bases and roots of cane varieties, which did not occur in the case of the *B. arrecta*. The interpretation of the $^{15}$N data was further complicated by the fact that the uptake of soil N by the *B. arrecta* was almost certainly inhibited towards the end of each growing season due to shading of this crop by the tall sugar cane plants, and this probably resulted in a somewhat higher $^{15}$N enrichment in the control crop than otherwise would have occurred.

These difficulties are fully discussed in the original paper (Urquiaga et al., 1992), and because of them it was decided to perform a total N balance on the whole tank by the careful analysis of the N content of soil samples taken at plant emergence in the first year in comparison with samples taken at the final harvest. These data showed that there were significantly ($p<0.05$) positive N balances associated with the varieties CB 45–3, SP 70–1143, SP 79–2312 and Krakatau, and that there was a good agreement between the $^{15}$N dilution and the total N balance estimates of the contributions of BNF to the sugar cane varieties (Table 1).

These results were recently confirmed in a long-term nitrogen balance experiment conducted on a sugar cane plantation in Pernambuco, NE Brazil (Oliveira et al., 1994). In this experiment the effect of pre-harvest burning of the cane (to remove the senescent leaves) on the yield and N accumulation of the crop, and N balance of the cropping system, were investigated. At the end of the 9 year study the total N accumulated in the system was found to be between 300 and 620 kg ha$^{-1}$ greater than the initial N (Table 2). This extra N was attributed to a mean annual BNF input to the crop of between 38 and 77 kg N ha$^{-1}$, this being a minimum estimate as gaseous or leaching losses were not quantified.

*Wetland rice*

Several field N balance studies on lowland rice have been reported from studies in Thailand (Firth et al., 1973; Walcott et al., 1977), Japan (Koyama and App, 1979) and at the experimental fields of the International Rice Research Institute (IRRI) in the Philippines (App et al., 1984; Ventura et al., 1986). All studies report a positive balance even when N from rainfall and irrigation water were discounted indicating inputs of between 30 and 60 kg N ha$^{-1}$ crop$^{-1}$, but in these studies no data are available to determine what proportion of this N may be derived from free-living $N_2$-fixing cyanobacteria in the flood water, heterotrophic $N_2$ fixers in the soil or those associated with the plant.

Various nitrogen balance experiments have been performed in pots which indicate that the plant/soil system can benefit from biological $N_2$ fixation (BNF) even when the activity of cyanobacteria on the soil surface is inhibited by shading (De and Sulaiman, 1950; Willis and Green, 1948). In a very careful N balance study performed in pots by App et al. (1980) on 4 to 6 consecutive crops, the contribution of plant-associated BNF was estimated to be equivalent to 18% of plant N. In a further N balance study on 83 wild and cultivated rice cultivars (in 6 separate experiments each with 3 consecutive crops) reported by App et al. (1986), large and significant differences between cultivars were found.

*Table 1.* $^{15}N$ enrichment and total nitrogen accumulation of sugar cane and *Brachiaria arrecta* and estimates of nitrogen derived from BNF using N balance and $^{15}N$ isotope dilution techniques (g N $m^{-2}$). Means of 4 replicates. After Urquiaga et al. (1992)

| Variety / Species | Weighted mean atom % $^{15}N$ excess | Final N content of soil | N accum. whole plant 3 years | Estimates of BNF contribution | | | |
|---|---|---|---|---|---|---|---|
| | | | | All three years | | Annual mean | |
| | | | | N balance[z] | $^{15}N$[y] | N balance | $^{15}N$ |
| | | (g N $m^{-2}$) | | | | | |
| CB 47–89 | 0.191bcd | 835 | 61.4bc | 39.7 | 34.8c | 13.2 | 11.6 |
| CB 45–3 | 0.166cde | 864 | 84.3ab | 62.6 | 52.6b | 20.9 | 17.5 |
| NA 56–79 | 0.198bc | 884 | 57.8c | 36.1 | 32.6c | 12.0 | 10.9 |
| IAC 52–150 | 0.188bcd | 924 | 59.6bc | 37.9 | 33.8c | 12.6 | 11.3 |
| SP 70–1143 | 0.146de | 852 | 77.5bc | 55.8 | 51.9b | 18.6 | 17.3 |
| SP 71–799 | 0.183bcd | 860 | 56.9c | 35.2 | 33.3c | 11.7 | 11.1 |
| SP 79–2312 | 0.198bc | 845 | 63.6c | 41.9 | 35.4c | 14.0 | 11.8 |
| Chunee | 0.227b | 826 | 33.0d | 11.3 | 16.9d | 3.8 | 5.6 |
| Caiana | 0.190bcd | 857 | 11.6d | -10.1 | 6.7d | - 3.4 | 2.2 |
| Krakatau | 0.133e | 857 | 102.8a | 81.1 | 71.8a | 27.0 | 23.9 |
| *B. arrecta* | 0.443a | 830 | 24.9 | 3.2 | - | 1.1 | - |
| CV (%) | 13.6 | 5.1 | 25.0 | 29.2 | | 29.2 | |

[z] N balance estimate of BNF contribution = total N accumulated by crop + mean total N content of soil in tank at final harvest - mean total N content of soil in tank at emergence. Mean change in soil N content from emergence until final harvest = 27.1 g N $m^{-2}$ with a standard error of the difference between the means of 22.0 g N $m^{-2}$. N balances greater than 37.7 g N $m^{-2}$ (12.4 g N $m^{-2}$ $year^{-1}$) were significantly greater than zero ($p$=0.05, Student t test).

[y] $^{15}N$ isotope dilution estimate of BNF contribution = (total N accumulated by the crop) × (1 - (weighted mean atom % $^{15}N$ excess of sugar cane)/(weighted mean atom % $^{15}N$ excess of *B. arrecta*).

The positive N balances were equivalent to between 16 and 70 kg N $ha^{-1}$ $crop^{-1}$ assuming 25 plants $m^{-2}$ and, although in all 6 experiments there were significant correlations between N balance and plant N uptake, because of the nature of this technique it cannot necessarily be assumed that the N was fixed and immediately incorporated into the plants.

Direct evidence that heterotrophic diazotrophs can contribute significant quantities of N to rice plants was obtained by the short-term exposure of individual plants to $^{15}N$ enriched $N_2$ gas (Ito et al., 1980; Yoshida and Yoneyama, 1980; Eskew et al., 1981; Nayak et al., 1986), but most of the labelled nitrogen fixed remained in the rhizosphere soil. However, these data do not permit estimation of BNF contributions over the entire plant growth cycle.

There are many studies which have used the acetylene reduction (AR) assay to study BNF associated with rice. The early studies (e.g. Rinaudo and Dommergues, 1971; Yoshida and Ancajas, 1970, 1973) utilized an assay on excised roots. Later studies on rice and other grasses and cereals suggested that these techniques were unreliable and perhaps overestimated actual $N_2$ fixing activity (Barber et al., 1976; Koch, 1977; Tjepkema and Van Berkum, 1977), and subsequently in situ assays were developed (Balandreau and Dommergues, 1971; Boddey et al., 1978; Lee et al., 1977). The use of these in situ techniques in the field showed considerable AR activity associated with field grown plants (Watanabe et al., 1978a, 1981) but this technique suffers from several disadvantages for the estimation of actual BNF contributions to the plants: Firstly, the AR technique measures nitrogenase activity and not incorporation of fixed N into the plant, secondly much of the evolved ethylene may be retained in the waterlogged soil and not diffuse into the atmosphere which is sampled, and finally the measure is instantaneous and requires many assays thoughout the growing season if overall contributions of BNF to the crop are to be assessed (Boddey, 1987; Roger and Watan-

*Table 2.* Effect of pre-harvest burning on total nitrogen balance (g N $m^{-2}$) of the soil/plant system of field grown sugar cane over a sequence of the plant crop followed by 7 ratoon crops. Means of 16 replicates

| Treatment | N accumulated by crop in over 8 cuts 1983–1992 | Total N in soil/plant system Considering soil N content in the layer: 0–20cm | | | 0–60cm | | |
|---|---|---|---|---|---|---|---|
| | | $N^a$ Initial | $N^b$ Final | Balance | $N^a$ Initial | $N^b$ Final | Balance |
| | | (g N $m^{-2}$) | | | | | |
| Burned | 58.3 | 365.9 | 354.1 | -11.8 | 789.0 | 744.6 | -44.4 |
| Unburned | 73.6 | 369.1 | 400.6 | +31.5 | 774.3 | 828.7 | +54.4 |
| HSD[c] $p$=0.05 | 7.0 | 24.2 | 29.7 | 30.6 | 74.5 | 64.1 | 61.9 |
| CV (%)[d] | 14.2 | 8.9 | 10.6 | *(10.3)*[e] | 12.8 | 11.0 | *(20.9)*[e] |

[a] Initial N in soil plant/system = total N in soil at planting + added fertilizer N.
[b] Final N in soil/plant system = total N in soil at final harvest + N accumulated by crop over 8 harvests (1983 to 1992).
[c] Honest significant difference (Tukey).
[d] Coefficient of variation.
[e] Value in *italics* = Standard error of mean.

abe, 1986). In studies where many in situ AR assays were taken, the estimates of total "acetylene reduced" thoughout the whole crop cycle were approximately 40 to 60 m mol ethylene $m^{-2}$ (Boddey and Ahmad, 1981; Watanabe et al., 1978b) which extrapolate to only 5 to 8 kg $N_2$ fixed $ha^{-1}$. Results from the excised root and in situ AR assays on wetland rice were of similar magnitude (Boddey et al., 1978; Boddey, 1981) and it is generally considered that this technique over-estimates $N_2$-fixing activity (Berkum and Bohlool, 1980; Giller, 1987).

It seems therefore that there is a considerable disparity between the N balance and AR estimates of plant-associated BNF to wetland rice. Some of the field and pot N balance studies suggest contributions of more than 30 kg N $ha^{-1}$ $crop^{-1}$ whereas the acetylene reduction studies suggest inputs not higher than 8 kg $ha^{-1}$.

The $^{15}N$ isotope dilution technique has the potential to estimate contributions of BNF to the plants over the whole growth season and unlike the N balance and acetylene reduction techniques, it estimates fixed N actually incorporated into the plant tissue (Chalk, 1985; McAuliffe et al., 1958). The main problem with this technique lies in labelling the soil with $^{15}N$. If the enrichment varies with area, depth or time, different plants (the control and different rice varieties) may have different N uptake patterns and do not obtain the same $^{15}N$ enrichment in the soil derived N, an assumption essential to the application of the technique (Boddey, 1987; Witty, 1983). In the studies reported so far the soil N was not stable with time and no suitable non-$N_2$-fixing control plant was found that would grow in waterlogged soil (Nayak et al., 1986; Ventura and Watanabe, 1983).

A recent study was conducted at our institute (CNPAB) near Rio de Janeiro (Oliveira, 1994) and at the first planting 40 rice varieties were planted in a tank (20 × 6 × 0.6m) filled with waterlogged soil amended with $^{15}N$-labelled compost (Urquiaga et al., 1992) and inoculated with soil taken from a long-established rice paddy in the Paraíba valley of São Paulo State. Analyses of leaf samples showed that there was a considerable decline in $^{15}N$ enrichment in the plant tissue during plant growth and earlier maturing varieties showed higher $^{15}N$ enrichments than later maturing varieties (Table 3). There were considerable differences in total N accumulation and $^{15}N$ enrichment between different varieties but higher N accumulation was not well correlated with lower $^{15}N$ enrichment even within each maturity group (Table 4).

Subsequently 20 of these rice cultivars were replanted in the same tank. Again $^{15}N$ enrichment in plant tissue decreased with time and the varieties

*Table 3.* Grain production, N accumulation and $^{15}N$ enrichment of leaf samples at 40 days after emergence (DAE) and of whole plant at final harvest of 5 rice varieties from each of 3 maturity groups. Plants grown in tank of soil labelled with $^{15}N$. Means of 4 replicates. After Oliveira (1994)

| Rice variety | Grain yield[z] ($g\ m^{-2}$) | N accumulation ($g\ N\ m^{-2}$) | $^{15}N$ enrichment Atom % $^{15}N$ exc. Final[y] harvest |
|---|---|---|---|
| *Maturity group 1 (60–85 DAE)* | | | |
| Labelle | 396 d | 7.37 d | 0.2074a |
| CNA 6837 | 814 ab | 9.28 bc | 0.2305a |
| Bluebelle | 633 c | 8.55 cd | 0.1984a |
| BR-IRGA-410 | 766 ab | 9.25 bc | 0.2301a |
| BR-IRGA-409 | 759 ab | 10.54 ab | 0.2134a |
| C.V. (%) | 10.3 | 9.0 | 11.7 |
| Mean for whole group 7 varieties | 711 | 94.9 | 0.2160 |
| *Maturity group 2 (80–110 DAE)* | | | |
| IR 4432–28–5 | 942 b | 17.82 a | 0.1475 c |
| MG-1 | 1097 a | 15.83 ab | 0.1559 bc |
| IR-841 | 701 c | 11.46 c | 0.1586 bc |
| CICA-9 | 930 b | 14.43 b | 0.1618 bc |
| CNA 4215 | 698 c | 8.87 d | 0.1822 ab |
| C.V. (%) | 11.0 | 11.0 | 11.2 |
| Mean for whole group 18 varieties | 906 | 13.3 | 0.1608 |
| *Maturity group 3 (110–140 DAE)* | | | |
| Metica-1 | 1130 ab | 16.19 b | 0.1557 cd |
| De-Abril | 1070 ab | 22.18 a | 0.1421 d |
| IAC-4440 | 1100 ab | 15.30 b | 0.1973 a |
| CICA-8 | 1070 ab | 14.32 b | 0.1758 abc |
| IR-42 | 799 c | 15.68 b | 0.1388 d |
| C.V. (%) | 10.8 | 12.0 | 10.5 |
| Mean for whole group 15 varieties | 1060 | 15.0 | 0.1621 |

[z] Grain at 14% humidity.
[y] Weighted mean $^{15}N$ enrichment of whole plant.

*Table 4.* Regressions of total nitrogen accumulation and $^{15}N$ enrichment at final harvest of 40 rice varieties divided into 3 maturity groups planted in waterlogged $^{15}N$-labelled soil. First crop (1989/90). After Oliveira (1994)

| Maturity group | Days after emergence | Correlation coefficient | Probability | No. of data points |
|---|---|---|---|---|
| 1 | 60–85 | + 0.281 | 0.147 | 28 |
| 2 | 85–110 | - 0.320 | 0.006 | 72 |
| 3 | 110–140 | - 0. 201 | 0.124 | 60 |

IR 42 and IR 4432–28–5 showed significantly lower $^{15}N$ enrichment and higher N accumulation than the variety IAC 4440 and the non-$N_2$-fixing control plant, *Brachiaria arrecta* (data not shown). Data from the N balance study of App et al. (1986) as well as acetylene reduction assays and a natural abundance (delta) $^{15}N$ study both performed at IRRI in the Philippines also suggest that the variety IR 42 is able to obtain significant contributions from plant associated BNF (Barraquio et al., 1986; Ladha et al., 1987a, b; Watanabe et al., 1987a).

Results from the third planting of this $^{15}N$ experiment were lost due to a fire in the drying oven but at the fourth planting just these 3 varieties were planted with the same control plant and harvests were taken at six times during plant growth (Table 5). The acetylene reduction activity of the 4 crops was evaluated by incubating the plant/soil system at constant temperature in the dark as described by Barraquio et al,. (1986). No significant differences were found between varieties but the rice varieties were far higher in AR activity than the *B. arrecta* control (data not shown). After the 3rd harvest (86 DAE) the $^{15}N$ enrichment of the *B. arrecta* control was lower than that of the rice varieties (significantly so at the final harvest) but this result could not be due to a soil N uptake pattern different from the rice varieties as the data indicate that the $^{15}N$ enrichment of the soil mineral N was virtually stable during crop growth. Furthermore, while the variety IR 4432–28–5 had a lower $^{15}N$ enrichment than the other two rice varieties the total N accumulation of this cultivar showed a tendency to be lower.

Hence, the data obtained in this study do not confirm significant BNF contributions to any of the 3 varieties of wetland rice even though two of them were pre-selected for high N accumulation and low $^{15}N$ enrichment. Whether this is due to adverse soil fertility factors or indicates that BNF inputs are generally very low requires further investigation. The results illustrate the difficulties involved in the application of this technique for quantifying BNF contributions to wetland rice and the necessity to use soil with a uniform and stable $^{15}N$ enrichment.

## Plant-associated $N_2$-fixing bacteria

### *Sugar cane*

In the 1950s Döbereiner (1961) found $N_2$-fixing bacteria of the genus *Beijerinckia* in high numbers in sugar cane fields, with selective enrichment in the rhizosphere and especially on the root surface. At the same time a new species of *Beijerinckia* was discovered (*B. fluminense*) associated with this crop (Döbereiner and Ruschel, 1958). Subsequently, other authors (Graciolli et al., 1983; Purchase, 1980) isolated a wide range of $N_2$-fixing bacteria from the roots, stems and even leaves of sugar cane including species of *Erwinia, Azotobacter, Derxia, Azospirillum* and *Enterobacter*. None of these bacteria seemed to occur in large enough numbers to account for the extremely high rates of $N_2$ fixation reported above.

More recently, a new species of $N_2$-fixing bacteria, *Acetobacter diazotrophicus*, was found to occur in large numbers in the roots and stems of sugar cane (Cavalcante and Döbereiner, 1988; Gillis et al., 1989). This most extraordinary diazotroph was originally isolated from semi-solid sugar cane juice inoculated with dilutions of sugar cane roots and stems which showed acetylene reduction (nitrogenase) activity in dilutions up to $10^{-6}$ to $10^{-7}$ (fresh weight). A more specific medium (LGIP) has now been developed (Reis et al., 1994).

The bacteria is a small, Gram-negative, aerobic rod showing pellicle formation in N-free semi-solid medi-

*Table 5.* Total nitrogen accumulation and $^{15}N$ enrichment of 3 rice varieties and *Brachiaria arrecta* planted in waterlogged $^{15}N$ labelled soil during the plant growth cycle. Fourth crop (1992/3). Harvested area 0.5 $m^2$. Means of 4 replicates. After Oliveira (1994)

| Variety | Days after emergence of rice | | | | | |
|---|---|---|---|---|---|---|
| | 36 | 52 | 86 | 94 | 108 | 130 |
| *Total N accumulation (g N $m^{-2}$)* | | | | | | |
| IR 42 | 0.728ab | 0.692ab | 1.951ab | 2.389a | 3.837a | 4.449a |
| IAC 4440 | 0.902a | 0.767a | 3.101a | 3.299a | 4.093a | 4.799a |
| IR 4432–28–5 | 0.792a | 0.774a | 2.013ab | 2.476a | 3.757a | 4.055a |
| *B. arrecta* | 0.166b | 0.321b | 0.867b | 0.601b | 1.609b | 1.009b |
| C.V. (%) | 41.37 | 27.61 | 44.61 | 54.5 | 27.01 | 18.63 |
| *$^{15}N$ enrichment (Atom % $^{15}N$ excess)* | | | | | | |
| IR 42 | 0.0549c | 0.0558a | 0.0552a | 0.0527a | 0.0536a | 0.0553ab |
| IAC 4440 | 0.0680a | 0.0558a | 0.0482a | 0.0553a | 0.0497a | 0.0606a |
| IR 4432–28–5 | 0.0643ab | 0.0561a | 0.0552a | 0.0553a | 0.0484a | 0.0484bc |
| *B. arrecta* | 0.0582bc | 0.0549a | 0.0517a | 0.0482a | 0.0428a | 0.0419c |
| C.V. (%) | 5.69 | 9.36 | 10.9 | 8.38 | 16.11 | 8.65 |

Means in the same column followed by the same letter are not significantly different at $p$=0.05 (Tukey).

um with 100 g $L^{-1}$ sucrose but without cane juice, forming a thick surface pellicle after 7 to 10 days. Best growth occurs with high sucrose or glucose concentrations (100 g $L^{-1}$) and strong acid production results in a final pH of 3.0 or less. Growth and $N_2$ fixation (more than 100 n moles $C_2H_2$ $mL^{-1}$ $h^{-1}$) continues at this pH for several days (Stephan et al., 1991). Ethanol is also used as a C source for growth and is oxidized to $CO_2$ and $H_2O$. Dark brown colonies form on potato agar with 100 g $L^{-1}$ sucrose, and dark orange colonies on N-poor (0.02 g $L^{-1}$ yeast extract) mineral agar medium with 100 g $L^{-1}$ sucrose and bromothymol blue. The bacterium possesses no nitrate reductase and $N_2$ fixation is not affected by high levels (25m*M*) of $NO_3^-$. Also $NH_4^+$ causes only partial inhibition of nitrogenase, especially when grown on 100 g $L^{-1}$ sucrose (Boddey et al., 1991; Teixeira et al., 1987).

Another interesting aspect is that *A. diazotrophicus* growing in 10% sucrose showed an optimum dissolved oxygen concentration for acetylene reduction in equilibrium with 0.2 kPa $O_2$ in the atmosphere, but continued to fix $N_2$ up to 4.0 kPa, showing a much higher $O_2$ tolerance than *Azospirillum* spp. (Reis et al., 1990).

Experiments on mixed cultures of *A. diazotrophicus* with an amylolytic yeast (*Lypomyces kononenkoae*), used as a model system for plant/bacteria interactions, showed that 48% of the total nitrogen fixed by the bacteria was transferred to the yeast, starting right from the beginning of the culture (Cojho et al., 1993). These results are important in that until now the lack of evidence for efficient transfer of fixed N from diazotrophs to plants has been a source of scepticism that such associations could be of agronomic importance.

This bacterium has been found in many sugar cane varieties in several regions of Brazil as well as in Mexico, Cuba and Australia (Fuentes-Martinez et al., 1993, Li and Macrae, 1992) and numbers were in the range of $10^3$ to $10^7$ in roots, basal and apical stems, leaves and in sugar cane trash (Döbereiner et al., 1988). It was not found in soil between rows of sugar cane plants or roots from 12 different weed species taken from cane fields. It was also not found in grain or sugar sorghum, but was isolated from a few samples of washed roots and aerial parts of *Pennisetum purpureum* cv Cameroon, and from sweet potatoes (Döbereiner et al., 1988, 1994; Paula et al., 1989).

Sterile micropropagated sugar cane seedlings were not infected by *A. diazotrophicus* by traditional root inoculation methods, and generally infection of cane

plants by this bacterium is rare except when inoculated "in vitro". However, under these conditions *A. diazotrophicus* was found to colonize extensively the exterior and interior of the shoot and root (James et al., 1994). This study was performed using immuno-gold labelling with both optical and electron microscopic techniques. On the root surface the bacteria was found especially in cavities in lateral root junctions and these junctions and the root tips appeared to be preferred sites of bacterial entry. Within the roots *A. diazotrophicus* was observed in apparently intact, enlarged epidermal cells, and at the base of the stem within xylem vessels through which the bacteria appear to migrate upwards in the transpiration stream so that all shoot tissues become infected. The difficulty of infection of plants grown in soil or vermiculite can be overcome by co-inoculation with VA mycorrhizal fungi, especially originating from fungal spores infected by the bacteria (Paula et al., 1991). This technique of introduction of a $N_2$-fixing bacteria into sugar cane plants may be important for introducing selected, or genetically improved, strains into plants for further propagation in the field via stem cuttings.

Bacterial taxonomists working in Belgium found that the bacteria known as *Pseudomonas rubrisubalbicans*, a sugar cane endophyte which causes mottled stripe disease in some varieties from the USA and other countries, but not in Brazilian varieties, was closely related genetically to a $N_2$-fixing bacterium called *Herbaspirillum seropedicae* (Gillis et al., 1991). *Herbaspirillum* was first isolated from the roots of maize and other cereals at our Centre (Baldani et al., 1986). Most of the isolates of *P. rubrisubalbicans* were found to be able to fix nitrogen and were identical in most other respects to *Herbaspirillum* (Pimentel et al., 1991). Recently results from DNA/rRNA hybridization and computer-assisted auxanographic tests have established that this generically-misnamed plant endophyte, *"Pseudomonas" rubrisubalbicans*, must now be included in the genus *Herbaspirillum* (Gillis et al., 1991).

Recently a more specific culture medium (JNFb) for this organism has been developed and $^{15}N_2$ gas incorporation confirmed, not only in strains of the original *H. seropedicae*, but also in isolates from different culture collections of *H. rubrisubalbicans* identified as the causitive organism of mottled stripe disease (Table 6). *Herbaspirillum* spp. have been isolated from sugarcane leaves, stems and roots and are other $N_2$-fixing bacteria which do not survive well in the soil but only within plants (Baldani et al., 1992a).

*Table 6.* $^{15}N_2$ incorporation into cells of *P. rubrisubalbicans* and *H. seropedicae* strains grown in semi-solid JNFb medium. Means of three replicates. After Baldani et al. (1992)

| Strains | Atom % $^{15}N$ excess |
|---|---|
| *P. rubrisubalbicans* | |
| M1 (LMG[a] 1278) | 0.5271 |
| M4 (ATCC[b] 19308) | 0.4891 |
| M5 (LMG 6415) | 0.5681 |
| M6 (LMG 6420) | 0.5172 |
| IBSBF 175 (LMG 10462) | 0.3881 |
| *H. seropedicae* | |
| Z67 (ATCC 35892) | 0.5881 |
| Z78 (ATCC 35893) | 0.4405 |
| ZM 176 | 0.4891 |
| Controls | |
| M4 + 20m*M* $NH_4^+$ | 0.0002 |
| Z67 + 20m*M* $NH_4^+$ | 0.0000 |

[a] LMG Belgian type culture collection.
[b] ATCC American type culture collection.

When non-sterile soil was inoculated with $10^8$ cells $g^{-1}$ of either species of *Herbaspirillum*, the number of viable cells decreased until the bacteria was undetectable after 21 days with *H. rubrisubalbicans* and 28 days with *H. seropedicae* (Olivares et al., 1993). However, 50 days after *Herbaspirillum* spp. were undetectable, surface-sterilized, sorghum seeds were planted in these pots and *Herbaspirillum* spp. were detected in the roots and rhizosphere soil when the plants were 30 days old.

In both monoxenic sugarcane and sorghum plants inoculated with *Herbaspirillum* spp. the bacteria have been localized, using the immunogold technique and both electron and optical microscopy, within the meta and protoxylem (Olivares et al., in preparation). In the case of a sugar cane variety (B–4362), susceptible to mottled stripe disease, *H. rubrisubalbicans* was found to completely block some of the xylem vessels, whereas in a resistant variety the bacteria were encapsulated by membranes probably of plant origin.

*Wetland rice*

As long ago as 1929, an Indian research worker suggested that wetland rice plants were able to obtain some contribution of nitrogen from $N_2$-fixing bacte-

ria associated with the plant roots (Sen, 1929). His evidence was based on the isolation of *Azotobacter* spp. from rice roots. Since this time many diazotrophs have been isolated from the rhizosphere and roots of rice including species of *Beijerinckia* (Dobereiner and Ruschel, 1962), *Azospirillum* (Baldani and Dobereiner, 1980; Baldani et al., 1981; Ladha et al., 1982), *Alicagenes* (Qui et al., 1981), *Pseudomonas*, (Barraquio et al., 1983), *Klebsiella* and *Enterobacter* (Ladha et al., 1983); *Flavobacterium* (Bally et al., 1983), and *Agromonas* (Ohta and Hattori, 1983). However, the presence of $N_2$ fixing bacteria associated with rice roots does not necessarily mean that the plants obtain significant contributions from biological nitrogen fixation (BNF). For example, in a study of the inoculation of wheat plant grown in $^{15}N$-labelled soil numbers of *Azospirillum brasilense* above $10^6$ cells g fresh root$^{-1}$ were counted on washed/surface sterilized roots and plant N uptake was significantly increased by *Azospirillum* inoculation, but $^{15}N$ enrichment data showed that the response was not due to BNF inputs (Boddey et al., 1986).

*Azospirillum* spp. have been isolated in considerable numbers from the rhizosphere and histosphere of wetland rice (Baldani et al., 1981; Ladha et al., 1982, 1987b; Omar et al., 1989) and a new species of *Pseudomonas* (*P. diazotrophicus*) was reported to dominate the rhizosphere bacterial population (Barraquio et al., 1982; Watanabe et al., 1987b). However, as has been pointed out by several authors, $N_2$-fixing bacteria are distant from the main sources of carbon substrates in the root (the vascular tissue) and are in competition with other soil microorganisms for these substrates (Barber and Lynch, 1977; Berkum and Bohlool, 1980; Kennedy and Tchan, 1992). On the other hand $N_2$-fixing bacteria found within rice roots or aerial tissue are unlikely to suffer from these disadvantages, and in view of the discovery of endophytic diazotrophs in sugar cane, research at our Centre has focussed on the search for such bacteria in lowland rice.

In the first report of the discovery of *Herbaspirillum seropedicae*, this $N_2$-fixing bacteria was isolated from washed roots of upland rice as well as from those of wheat, maize and sorghum (Baldani et al., 1986). Further studies have shown that this bacteria can be found in seeds, stems and leaves of rice as well as roots. Roots, stems and leaves of rice plants grown from seeds which were surface sterilized using hydrogen peroxide followed by acidified hypochlorite, were found to be infected with *H. seropedicae*, and only careful surface sterilization of dehulled seeds prevented this (Baldani et al., 1992b; V L D Baldani, unpubl. data). In the experiment described above to quantify BNF contributions to rice plants grown in the tank of $^{15}N$-labelled soil (Oliveira, 1994) counts of *Herbaspirillum* spp. were made using the selective medium described by Baldani et al. (1992). The results showed that numbers of *Herbaspirillum* in washed roots, shoots and leaves were as high as $10^7$, $10^5$ and $10^4$ cells g fresh weight$^{-1}$, respectively, and the ontogenic variation in numbers varied in a similar manner to the acetylene reduction activity associated with the plants (Fig. 1).

A further $N_2$-fixing bacteria has been found to be present in rice roots, shoots and leaves in numbers similar to those reported in Figure 1. As was suggested before, for the first attempts to isolate $N_2$-fixing bacteria from plants it is desirable to base isolation media on the carbon substrates known to be available within the plants (Boddey and Döbereiner, 1988). This was why malate was chosen for the semi-solid media first used to isolate *Azospirillum* as it was known to be an important constituent of maize sap (Döbereiner and Day, 1976). For the same reason cane juice was used for the first attempts to isolate diazotrophs from sugar cane (Cavalcante and Döbereiner, 1988). Boreau (1977) investigated the root exudates of 20 day-old sterile rice plants and discovered that glucose was the single most important carbon source and that in the organic acid fraction oxalate and citrate were quantitatively most important. Based on these results, a N-free semi-solid medium containing glucose, oxalate and citrate (medium 'M') was inoculated with dilutions of washed rice roots and rice stems (Oliveira, 1992). Slow but significant growth with initial acid production was observed, indicating the consumption of glucose. The medium was later alkalinized, indicating subsequent use of the dicarboxylic acids. Maximal AR activity was observed after 10 days incubation in N-free medium and AR activity continued until the 18th day of growth. The bacteria are small motile rods, but have not yet been identified as any of the known diazotrophs. The isolates grow best at pH between 5 and 6 and growth is very slow at pH 7. They use glucose, mannitol, celobiose, maltose, sucrose or trehalose as sole carbon sources and will hydrolyse Tween 80. This bacteria is most closely related phenotypically to *Herbaspirillum seropedicae* and *H. rubrisubalbicans* but whether it is a member of this genus awaits further investigation using DNA/rRNA homology tests etc.

A further possible candidate for an endophytic diazotroph which will infect rice plants are bacteria of the newly denominated genus *Azoarcus* (Reinhold-Hurek

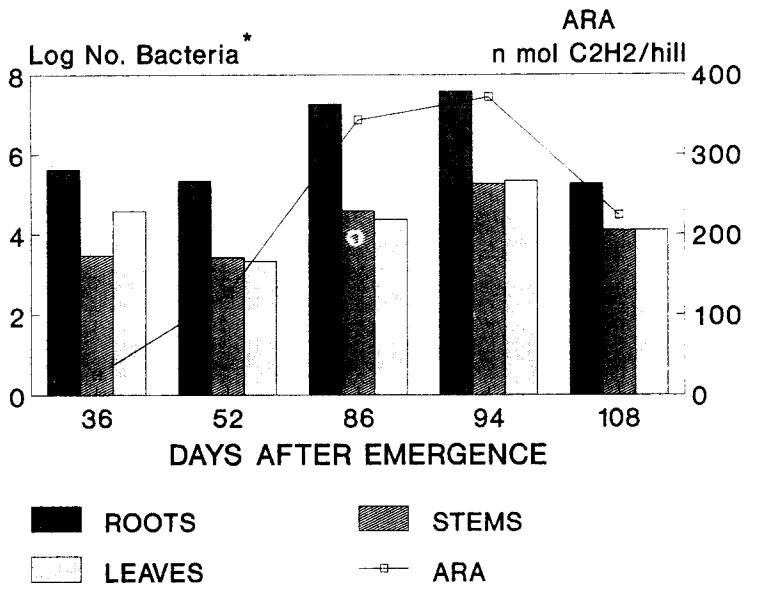


*Fig. 1.* Counts of *Herbaspirillum* spp. in roots, stems and leaves, and acetylene reduction activity (plant soil system, Barraquio et al., 1986 of the wetland rice variety IR 42 grown in a tank of $^{15}N$-labelled waterlogged soil. * Bacterial numbers expressed per g fresh weight plant tissue (after Oliveira et al., 1994).

et al., 1993). The bacteria (labelled with the beta-glucuronidase reporter gene) were found to be able to penetrate rice roots, forming large inter- and intracellular colonies in the root cortex and just occasionally within the stele and were also found within the stem bases and shoots (Desomer et al., 1992; Hurek et al., 1991).

## Prospects for the future

Brazilian sugar cane varieties are known to be capable of obtaining very considerable contributions of biologically fixed N under field conditions. Recent data suggest that water supply is critical to the maintenance of high BNF activity. A recent trial (16 areas totalling 900 ha) at a sugar cane plantation in Campos (NE Rio de Janeiro State) showed that where year round irrigation was used there was no response of ratoon cane to 200 kg $ha^{-1}$ of urea fertilizer and yields of ratoon crop cane averaged 95 t $ha^{-1}$. As a result of this trial the plantation managers abandoned N fertilization on 4000 ha of irrigated cane making an annual economy of US $ 250,000 (Boddey, 1995). All attempts to isolate *Acetobacter diazotrophicus* from sugar cane from anywhere in the world have been successful except where high N fertilizer additions have been made (J Caballero Mellado, pers. commun.). Apart from Brazil no data are yet available for the occurrence of *Herbaspirillum* spp. in this crop.

The complete absence of *A. diazotrophicus* in soil and the restricted occurrence of *Herbaspirillum* spp., suggest that once selected (or even genetically manipulated) strains of these bacteria are established in cane plants in the field, the chances are slight that wild type strains will contaminate the plants to compete with them. For phytosanitary reasons the use of direct planting of monoxenic micropropagated cane plantlets is now being tested at several cane plantations in São Paulo state and this may soon offer an economically viable opportunity to propagate cane plants infected by superior strains of endophytic diazotrophs.

With regard to wetland rice it is evident that for BNF to contribute to high rice yields a great improvement in its efficiency is required. A meeting held at IRRI (Philippines) in 1992 was dedicated solely to this subject. Three possible strategies to increase BNF contributions to wetland rice were discussed (Bennett and Ladha, 1992):

1. Induction of "nodulation" of rice using hydrolytic enzymes (Al-Mallah et al., 1989), 2,4-D (Kennedy and Tchan, 1992) or other means (Rolfe and Bender, 1990) and subsequent infection with *Rhizobium, Azospirillum* or other diazotrophs. True

$N_2$-fixing legume nodules are complicated structures equipped with vascular tissue to supply C substrate and export fixed N. They possess a sophisticated oxygen protection mechanism with leghaemoglobin and both fixed and variable physical barriers to $O_2$ diffusion, and an array of specific enzyme systems and feedback controls. The induction of deformations on the root to house bacteria only constitutes a tiny fraction of the symbiotic system and the remaining parameters are dictated pricipally by the plant genome, the *Rhizobium* being mainly responsible for "switching on" the plant nodulation program (Dénarié and Roche, 1991). It thus seems that the induced nodulation strategy has little chance of success especially as true legume nodules serve to protect the nitrogenase system from external oxygen flux from the soil and in wetland rice the soil is anaerobic and oxygen flow to the root is via the aerenchyma (expanded cortex) of the root.

2. Direct integration of *nif* genes into the plant genome. Attempts to introduce just 2 of these genes into tobacco chloroplasts has met with some success although expression was found to be at extremely low levels (Dowson-Day et al., 1991). So far it is not known exactly how many, or which, *Rhizobium* genes will be necessary to make an active $N_2$-fixing system nor what levels of activity could be achieved.

3. Improvement/modification of existing associations of $N_2$-fixing bacteria with rice plants. Little enthusiasm has been expressed for this strategy as almost all attention has been focussed on diazotrophs found in the rice rhizosphere (Kennedy and Tchan, 1992). However, the recent discovery that some sugarcane varieties can obtain very large contributions of BNF under field conditions, and the existence of abundant populations of endophytic diazotrophs (*A. diazotrophicus* and *Herbaspirillum* spp.) in this crop which are probably responsible for this activity, opens up entirely new avenues for developing a similar system for rice or other cereal crops. Already one of these endophytic diazotrophs, *Herbaspirillum* spp., has been isolated in moderately high numbers from within roots and aerial tissue of rice, although evidence is lacking that these organisms contribute any significant quantities of fixed N to the plants. However, when more knowledge is accumulated concerning how the $N_2$-fixing system in sugarcane functions, it should be a much smaller step to try to introduce this into a plant which already can be infected by similar diazotrophs than trying to build a whole $N_2$-fixing system from scratch.

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