Plant and Soil **137**: 111–117, 1991.

PLSO NL17

Biological nitrogen fixation associated with sugar cane

R.M. BODDEY, S. URQUIAGA, V. REIS and J. DÖBEREINER
EMBRAPA, National Centre for Soil Biology Research (CNPBS), Km 47, Seropédica, 23851, Rio de Janeiro, Brazil

Key words: *Acetobacter diazotrophicus*, Glomus clarum, N balance, N_2 fixation, ^{15}N isotope dilution, sugar cane, VA mycorrhiza

Abstract

A recent ^{15}N dilution/N balance study confirmed that certain sugar cane varieties are capable of obtaining large contributions of nitrogen from plant-associated N_2 fixation. It was estimated that up to 60 to 80% of plant N could be derived from this source, and under good conditions of water and mineral nutrient supply, it may be possible to dispense with N fertilization of these varieties altogether. The recently discovered bacterium, *Acetobacter diazotrophicus*, apparently responsible for this N_2 fixation associated with the plants, has unique physiological properties for a diazotroph, such as tolerance to low pH, and high sugar and salt concentrations, lack of nitrate reductase, and nitrogenase activity which tolerates short-term exposure to ammonium. Furthermore, it also behaves as an endophyte, in that it is unable to infect sugar cane plants unless through damaged tissue or by means of VA mycorrhizae and is propagated via the planting material (stem pieces).

Introduction

Four million motor vehicles in Brazil run on hydrated (95%) ethanol and all other cars run on gasohol containing 10 to 20% ethanol. At present this represents the largest biomass fuel program in the World, and this alcohol is produced entirely from sugar cane grown on approximately 4 million hectares, 8% of the total cropped area of the country. There are several advantages of the substitution of gasoline by ethanol: Firstly it reduces the dependence of Brazil (a country with limited petroleum reserves) on imported oil. Secondly it is less polluting than gasoline in terms of nitrogen oxides and carbon monoxide, contains no hexamethyl lead, and, most important for the rest of the planet, the growth of the crop assimilates as much (or more) carbon dioxide than is released during alcohol production and consumption. The main disadvantage of the program is that the overall cost of the ethanol produced is more expensive than gasoline (albeit in national currency not in dollars for imported oil) until the price of a barrel of oil reaches approximately US$40. The program was planned in the early 1970s when this price seemed to be an imminent possibility.

The technology adopted for sugar cane production in Brazil is most favourable for a high positive energy balance. Most of the crop is cut by hand, and employs over half a million workers, thus economizing fuel for harvesters, and the fertilizer inputs, especially nitrogen, are modest. Breeding of sugar cane has been traditionally carried out under low nitrogen inputs and responses of these varieties to N fertilizer are small. In 135 NPK experiments carried out all over Brazil on the plant crop, only 19% of the studies showed significant yield increases (Azeredo et al., 1986). Average cane yield is between 65 to 70 t ha^{-1}, and N fertilizer inputs are rarely more than 60 and 120 kg N ha^{-1} for the plant crop and ratoon crops, respectively. The crop typically accumulates between 100 and

200 kg N ha^{-1} per season (Orlando Filho et al., 1981; Sampaio et al., 1984) and virtually all of this nitrogen is removed from the field at harvest as the trash is almost always burned off before cutting. There are many areas of the country where sugar cane has been grown for decades or even centuries, and neither cane yields nor soil N reserves appear to fall with time despite this apparent deficit in N supply. These observations have led several researchers to suggest that this crop may benefit from plant-associated biological nitrogen fixation (BNF).

Quantification of biological nitrogen fixation

Experiments using ^{15}N-labelled N_2 gas performed at the Centro de Energia Nuclear na Agricultura (Piracicaba, São Paulo), showed that sugar cane incorporated some N from this source, but 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; Ruschel et al., 1975). In a ^{15}N-aided N balance experiment performed in pots containing 64 kg of soil, Lima et al. (1987) showed that the sugar cane variety CB 47-89 was able to obtain a large contribution of biologically fixed nitrogen, which they estimated to be in excess of 60% of the total N incorporated.

More recently our group at EMBRAPA-CNPBS have completed a three year ^{15}N isotope dilution and N balance study on 10 sugar cane varieties grown in a concrete tank ($20 \times 6 \times 0.8$ m) 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., 1991). The soil was fertilized with phosphorus, potassium and micronutrients and well irrigated throughout the experiment but no type of 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 high and stable over the three years (Table 1).

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 *B. arrecta* control, indicating large contributions of plant associated BNF (Table 2). At the second and third annual harvests (first and second ratoon

Table 1. Total N accumulation in aerial and root tissue of sugar cane and *Brachiaria arrecta* at three annual harvests (g N m^{-2}). Means of 4 replicates. After Urquiaga et al. (1991)

Variety/ Species	Harvest			Total N all harvests + roots.
	1987	1988	1989 (+ roots)	
CB 47-89	26.5ab[1]	20.9bc	14.0bc	61.4bc
CB 45-3	27.6ab	27.2b	29.5a	84.3ab
NA 56-79	24.6ab	18.9bc	14.4bc	57.8c
IAC 52-150	27.1ab	17.3bc	15.2b	59.6bc
P 70-1143	24.5abc	21.6bc	31.4a	77.5bc
SP 71-799	24.4abc	20.0bc	12.5bcd	56.9c
SP 79-2312	24.0abc	27.7b	11.8bcd	63.6c
Chunee	15.3c	11.9cd	5.8cde	33.0d
Caiana	5.6d	2.8d	4.3de	12.8d
Krakatau	29.4a	45.2a	28.2a	102.8a
B. arrecta	18.9bc	4.3d	1.7e	24.9d
CV (%)	28.9	36.3	36.9	25.0

[1] Means in the same column followed by the same letter are not significantly different at $p = 0.05$ (Duncan's multiple range test).

Table 2. ^{15}N enrichment of aerial and root tissue of sugar cane and *Brachiaria arrecta* at three annual harvests (atom% ^{15}N excess). Means of 4 replicates. After Urquiaga et al. (1991)

Variety/ Species	Harvest				Weighted mean all harvests + roots.
	1987	1988	1989	Roots	
CB 47-89	0.316bc[1]	0.108de	0.079	0.094	0.191bcd
CB 45-3	0.301bcd	0.120bcd	0.079	0.104	0.166cde
NA 56-79	0.310bc	0.126abc	0.081	0.132	0.198bc
IAC 52-150	0.292bcd	0.115cd	0.082	0.096	0.188bcd
Sp 70-1143	0.258cd	0.109d	0.079	0.086	0.146de
SP 71-799	0.292bcd	0.109d	0.076	0.104	0.183bcd
SP 79-2312	0.330b	0.127abc	0.085	0.090	0.198bc
Chunee	0.341b	0.128ab	0.078	0.099	0.227b
Caiana	0.310bc	0.123abc	0.079	0.097	0.190bcd
Krakatau	0.240d	0.097e	0.076	0.090	0.133e
B. arrecta	0.546a	0.134a	0.077	0.078	0.443a
CV (%)	12.6***	6.3***	4.9ns	24.0ns	13.6***

[1] Means in the same column followed by the same letter are not significantly different at $p = 0.05$ (Duncan's multiple range test).
ns no significant differences between means at $p = 0.05$.
*** differences between means significant at $p < 0.001$.

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

Table 3. 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 ($g\,N\,m^{-1}$). means of 4 replicates. After Urquiaga et al. (1991)

Variety/ Species	Final N content of soil	N accum. whole plant 3 years	Estimates of BNF contribution			
			All three years		Annual mean	
			N balance[1]	^{15}N[2]	N balance	^{15}N
CB 47-89	835	61.4bc	39.7	34.8c	13.2	11.6
CB 45-3	864	84.3ab	62.6	52.6b	20.9	17.5
NA 56-79	884	57.8c	36.1	32.6c	12.0	10.9
IAC 52-150	924	59.6bc	37.9	33.8c	12.6	11.3
SP 70-1143	852	77.5bc	55.8	51.9b	18.6	17.3
SP 71-799	860	56.9c	35.2	33.3c	11.7	11.1
SP 79-2312	845	63.6c	41.9	35.4c	14.0	11.8
Chunee	826	33.0d	11.3	16.9d	3.8	5.6
Caiana	857	11.6d	−10.1	6.7d	− 3.4	2.2
Krakatau	857	102.8a	81.1	71.8a	27.0	23.9
B. arrecta	830	24.9d	3.2	—	1.1	—
CV (%)	5.1ns	25.0***	—	29.2***	—	29.2

[1] N balance estimate of BNF contribution = total N accumulated by crop + mean total N content of soil in tank at emergence − mean total N content of soil in tank at final harvest. 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 $37.3\,g\,N\,m^{-2}$ significantly greater than zero ($p = 0.05$, Student t test).
[2] ^{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*).

somewhat higher ^{15}N enrichment in the control crop than otherwise would have occurred. These difficulties are fully discussed in the original paper (Boddey et al., 1991), 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 show 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 3).

Nitrogen fixing bacteria associated with sugar cane

Diazotrophs isolated from sugar cane

In the 1950s Döbereiner (1959, 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 (Purchase, 1980; Graciolli and Ruschel, 1981; Graciolli et al., 1983) 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*. The species *Azospirillum amazonense*, the only member of the genus which utilizes sucrose, was isolated from sugar cane in Hawaii (Döbereiner, 1987, unpublished data). None of these bacteria seemed to occur in large enough numbers to account for the extremely high rates of N_2 fixation reported above.

Acetobacter diazotrophicus

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}. Improved counting and isolation procedures were developed using N-free mineral medium containing 10% cane sugar and 0.5% cane juice acidified with acetic acid to pH 5.5.

The bacterium is a small, Gram-negative, aerobic rod showing pellicle formation (microaerobic aerotaxis) and acetylene reduction activity (ARA) also in N-free semi-solid medium with 10% sucrose but without cane juice, forming a thick surface pellicle after 5 days. Best growth occurs with high sucrose or glucose concentrations (10%) 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 (Fig. 1, Stephan et al., 1988). 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 10% sucrose, and dark orange colonies on N-poor (0.005% yeast extract) mineral agar medium with 10% sucrose and bromothymol blue. The bacterium possesses no nitrate reductase and N_2 fixation is not affected by high levels (25 m*M*) of NO_3. Also NH_4^+ causes only partial inhibition of nitrogenase (Fu et al., 1988; Teixeira et al., 1987). This type of NH_4^+ regulation of nitrogenase, which was also observed in *Azospirillum amazonense* and *Herbaspirillum seropedicae*, suggests that there are at least two general categories of NH_4^+ inhibition of nitrogenase: a. via direct covalent modification of the Fe protein, and b. with no direct effect on the Fe protein where NH_4^+ may affect the electron donation pathway to nitrogenase (Fu and Burris, 1989).

Recent results of Reis et al. (1990) confirm that the nitrogenase activity (ARA) of *A. diazotrophicus* growing in 10% sucrose with a final pH of 2.3, is less inhibited by 5 m*M* NH_4Cl than when growing in medium with 1% sucrose (Fig. 2). Under these same conditions the $^{15}NH_4^+$ assimilation by whole cells was slower with 10% sucrose than in cells growing on 1% sucrose (Table 4). This could be interpreted as osmotolerance because cells growing on 10% sucrose were more tolerant to 1% NaCl in semi-solid medium (V. Reis, unpublished data).

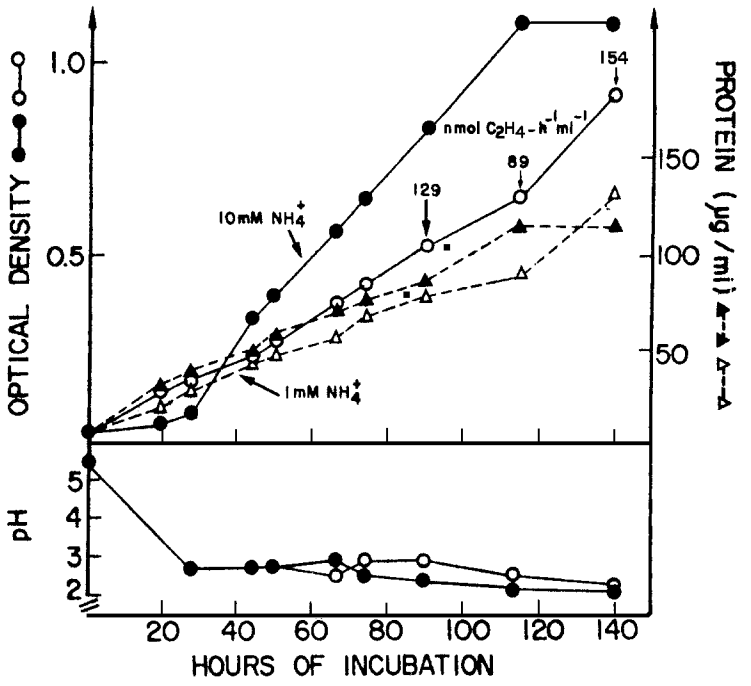

Fig. 1. Acid production and growth of *Acetobacter diazotrophicus* at two NH_4^+ concentrations in agitated liquid medium. With 1 m*M* NH_4^+ the cultures continued growth after 24 h using N_2 as sole N source (no NH_4^+ was detected in the medium and C_2H_2 reduction activity was high. (after Teixeira 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).

The incomplete inhibition of N_2 fixation by NH_4^+ in these organisms, as well as the lack of nitrate reductase mentioned above, are of considerable ecological and agronomic importance because they may permit the complementation of plant-associated BNF with N fertilization.

Taxonomic studies based on DNA and rRNA

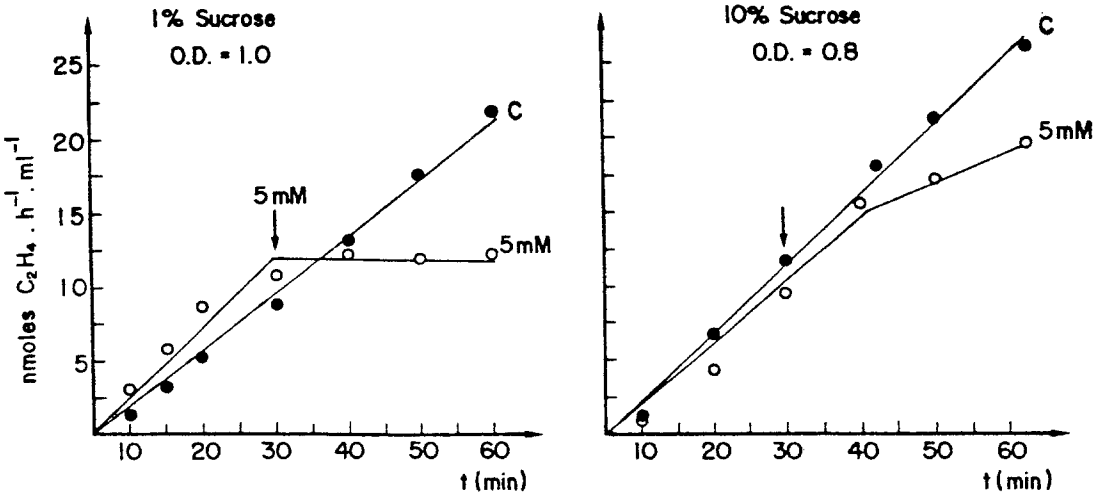

Fig. 2. Effect of 5 m*M* NH_4^+ on the nitrogenase activity (C_2H_2 reduction) of cells of *Acetobacter diazotrophicus* (PAL-5) growing in LGI medium containing 1% or 10% sucrose.

Table 4. Incorporation of ^{15}N during 30 minute exposure to $(^{15}NH_4)SO_4$ (21.3 atom % ^{15}N) by cells of *Acetobacter diazotrophicus* (PAL-5) grown for 48 h in LGI medium

$(^{15}NH_4)SO_4$	(Atom % ^{15}N excess)	
	1% sucrose O.D. = 0.8	10% sucrose O.D. = 1.0
2 m*M*	0.760	0.265
5 m*M*	0.660	0.284
10 m*M*	0.667	0.289

analyses showed that the bacterium belongs to the *Acetobacter* rRNA cystron (Gillis et al., 1989) and is most closely related to *A. liquefaciens*. This latter species, however, does not fix N_2, does not form pigmented colonies on potato media and shows several other physiological differences. DNA/DNA binding experiments confirmed it to be a new species (Gillis et al., 1989), so that the name originally proposed, (*Saccarobacter nitrocaptans*, Cavalcante and Döbereiner, 1988) had to be changed to *Acetobacter diazotrophicus*.

The bacterium has been found in many sugar cane varieties in several regions of Brazil, and numbers were in the range of 10^3 to 10^5 in rhizosphere soil, 10^3 to 10^7 in washed roots, 10^3 to 10^5 in surface sterilized roots, 10^3 to 10^6 in basal and apical stems and 10^4 to 10^7 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; Paula et al., 1989).

These observations indicate an N_2-fixing association very different from those known so far between plants and diazotrophs of the rhizosphere. The bacterium must be considered to be an endophyte which is propagated within stem cuttings. The propagation of N_2-fixing bacteria from stem cuttings into the developing sugar cane plant was first reported by Patriquin et al. (1980) although the microorganisms involved were not identified.

Sterile micropropagated sugar cane seedlings were not infected by *A. diazotrophicus* by traditional root inoculation methods, except in vitro in sugar-rich medium (V. Reis, unpublished) or in soil containing VA mycorrhizal fungi. In addition *Glomas clarum* spores containing *A. diazotrophicus* were most effective in introducing this diazotroph into roots and aerial parts of such sugar cane seedlings (Paula et al., 1991).

Concluding remarks

The results of the $^{15}N/N$ balance study show that some sugar cane varieties can obtain large contributions of plant-associated BNF, ranging from 60 to 80% of total plant N, equivalent to over 200 kg N ha^{-1} $year^{-1}$. The immediate practical application of these results in Brazil is to recommend the adoption, where possible, of the planting of these varieties and, in cases where water and P, K and micronutrient supply are optimized, it may be possible to dispense with N fertilization of these varieties altogether.

The bacterium apparently responsible for the plant-associated BNF has unique physiological properties for a diazotroph such as tolerance to low pH, and high sugar and salt concentrations, lack of nitrate reductase and nitrogenase activity which tolerates short term exposure to ammonium. Furthermore, it also behaves as an endophyte, in that it is unable to infect sugar cane plants unless the bacterium enters through damaged tissue or by means of VA mycorrhizae and is propagated via the planting material (stem pieces). Further studies of this fascinating association may not only lead to significant benefits for sugar cane and alcohol fuel production in Brazil and elsewhere, but also yield valuable information which may permit the development of viable N_2-fixing associations with other grasses or cereals.

Acknowledgements

Financial support for this research was provided by the U.S. National Academy of Sciences, National Research Council, through a grant from the U.S. Agency for International Development.

References

Azeredo D F, Bolsanello J, Weber M and Vieira J R 1986, Nitrogênio em cana-planta, doses e fracionamento. STAB 4, 26–32.

Boddey R M, Urquiaga S, Suhet A R, Peres J R and Neves M C P 1990 Quantification of the contribution of N_2 fixation to field-grown legumes: A strategy for the practical application of the ^{15}N isotope dilution technique. Soil Biol. Biochem. 22, 649–655.

Cavalcante V A and Döbereiner J 1988 A new acid-tolerant nitrogen-fixing bacterium associated with sugar cane. Plant and Soil 108, 23–31.

Döbereiner J 1959 Influência da cana-de-açúcar na população de *Beijerinckia* do solo. R. Bras. Biol. 19, 251–258.

Döbereiner J 1961 Nitrogen-fixing bacteria of the genus *Beijerinckia* Derx in the rhizosphere of sugar cane. Plant and Soil 15, 211–216.

Döbereiner J, Reis V and Lazarine A C 1988 A new N_2 fixing bacteria in association with cereals and sugar cane. *In* Nitrogen Fixation: Hundred Years After. Eds. H Bothe, F J de Bruijn and W E Newton. pp. 717–722. Gustav Fischer, Stuttgart.

Döbereiner J and Ruschel A P 1958 Uma nova espécie de *Beijerinckia*. R. Biol. 1, 261–272.

Fu H-A and Burris R H 1989 Ammonium inhibition of nitrogenase activity in *Herbaspirillum seropedicae* J. Bacteriol. 171, 3168–3175.

Fu H-A, FitzMaurice W P, Lehman L J, Roberts G P and Burris R H 1988 Regulation of nitrogenase activity in azospirilla, herbaspirilla and acetobacter and cloning of drag$^-$ and drat$^-$ homologous genes of *A. lipoferum* Sp Br 17. *In* Nitrogen Fixation: Hundred Years After. Eds. H Bothe, F J de Bruijn and W E Newton. p 336. Gustav Fischer, Stuttgart.

Gillis M, Kerters B, Hoste D J, Kroppenstedt R M, Stephan M P, Teixeira K R S, Döbereiner J and De Ley J 1989 *Acetobacter diazotrophicus* sp. nov. a nitrogen fixing acetic acid bacterium associated with sugar cane. Int. J. Syst. Bacteriol. 39, 361–364.

Graciolli L A, Freitas J R de and Ruschel A P 1983 Bactérias fixadoras de nitrogênio nas raízes, caules e folhas de cana-de-açúcar (*Saccharum* sp.). R. Microbiol. 14, 191–196.

Graciolli L A and Ruschel A P 1981 Microorganisms in the phyllosphere and rhizosphere of sugar cane. *In* Associative N_2 Fixation. Eds. P B Vose and A P Ruschel. Vol. 2. pp 91–101. CRC Press, Boca Raton, FL.

Lima E, Boddey R M and Döbereiner J 1987 Quantification of biological nitrogen fixation associated with sugar cane using a ^{15}N aided nitrogen balance. Soil Biol. Biochem. 19, 165–170.

Matsui E, Vose P B, Rodrigues N S R and Ruschel A P 1981 Use of ^{15}N enriched gas to determine N_2-fixation by undisturbed sugar cane plant in the field. *In* Associative N_2-Fixation. Eds. P B Vose and A P Ruschel. pp 153–161. CRC Press, Boca Raton FL.

Orlando Filho J, Haag H P and Zambello Jr. E 1980 Crescimento e absorção de macronutrientes pela cana-de-açúcar, variedade CB 41-76, em função da idade, em solos do Estado de São Paulo. Bol. Tec. Planalsucar 2, 1–128.

Patriquin D G, Ruschel A P and Graciolli L A 1980 Nitrogenase activity of sugar cane propagated from stem cuttings in sterile vermiculite. Soil Biol. Biochem 12, 413–417.

Paula M A de, Döbereiner J and Siqueira J O 1989 'Efeito da inoculação com fungo micorrízico VA e bactérias diazotróficas no crescimento e produção de batata-doce', in Congresso Brasileiro de Ciência do Solo, 22, Recife, SBCS, Programa e resumos, p. 109.

Paula M A, Reis V M and Döbereiner J 1991 Interactions of *Glomus clarum* with *Acetobacter diazotrophicus* in infection of sweet potato (*Ipomoea batatas*) sugar cane (*Saccharum* spp.) and sweet sorghum (*Sorghum vulgare*) Biol. Fert. Soils (*In press*).

Purchase B S 1980 Nitrogen fixation associated with sugarcane. Proc. S. Afr. Sugar Technol. Assoc., June, 173–176.

Reis V M, Zang Y and Burris R H 1990 Regulation of nitrogenase activity by ammonium and oxygen in *Acetobacter diazotrophicus*. An. Acad. Bras. Ci. 62, 317.

Ruschel A P, Henis Y and Salati E 1975 Nitrogen-15 tracing of N-fixation with soil-grown sugar cane seedlings. Soil Biol. Biochem. 7, 181–182.

Ruschel A P, Victoria R L, Salati E and Henis Y 1978 Nitrogen fixation in sugar cane (*Saccharum officinarum*). *In* Environmental Role of Nitrogen-Fixing Blue-green Algae and Asymbiotic Bacteria. Ed. V Grantall. pp 297–303. NFR, Uppsala (Ecological Bulletins, 26).

Sampaio E V S B, Salgado I H and Bettany J 1984 Dinâmica de nutrientes em cana-de acúcar. I. Eficiência da utilização de uréia (^{15}N) em aplicação única ou parcelada. Pesq. Agropec. Bras. 19, 943–949.

Stephan M P, Teixeira K R S and Döbereiner J 1988 Nitrogen fixation physiology of *Acetobacter nitrocaptans*: Effect of oxygen, pH and carbon source on respiration and nitrogenase activity. *In* Nitrogen Fixation: Hundred Years After. Eds. H Bothe, F J de Bruÿn and W E Newton. p 207. Gustav Fischer, Stuttgart.

Teixeira K R S, Stephan M P and Döbereiner J 1987 Physiological studies of *Saccharobacter nitrocaptans* a new acid tolerant N_2-fixing bacterium. *In* Poster presented at 4th International Symposium on Nitrogen Fixation with non-legumes, Rio de Janeiro, Final program abstracts, p. 149.

Urquiaga S, Cruz K H S and Boddey R M 1990 Estimation of the contribution of biological nitrogen fixation to sugar cane using ^{15}N and nitrogen balance techniques. Submitted. Soil Sci. Soc. Am. J.