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Biological nitrogen fixation is not a major contributor to the nitrogen demand of a commercially grown South African sugarcane cultivar

Hoefsloot G[1,2], Termorshuizen A.J[2], Watt D.A.[3] & Cramer M.D[4,5]
[1]*Botany Department, University of Stellenbosch, Private Bag X1, 7602, Matieland, South Africa.* [2]*Biological Farming Systems Group, Wageningen University and Research Centre, Marijkeweg 22, 6709, PG The Netherlands.* [3]*Biotechnology Department, South African Sugar Association Experiment Station, Private Bag X02, 4300, Mount Edgecombe, South Africa.* [4]*Department of Botany, University of Cape Town, Private Bag, 7701, Rondebosch, South Africa.* [5]*Corresponding author**

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Abstract

It has previously been reported that endophytic diazotrophic bacteria contribute significantly to the nitrogen budgets of some graminaceous species. In this study the contribution of biological nitrogen fixation to the N-budget of a South African sugarcane cultivar was evaluated using ^{15}N natural abundance, acetylene reduction and ^{15}N incorporation. Plants were also screened for the presence of endophytic diazotrophic bacteria using acetylene reduction and *nifH*-gene targeted PCR with the pure bacterial strains. ^{15}N natural abundance studies on field-grown sugarcane indicated that the plants did not rely extensively on biological nitrogen fixation. Furthermore, no evidence was found for significant N_2-fixation or nitrogenase activity in field-grown or glasshouse-grown plants using ^{15}N incorporation measurements and acetylene reduction assays. Seven endophytic bacterial strains were isolated from glasshouse-grown and field-grown plants and cultured on N-free medium. The diazotrophic character of these seven strains could not be confirmed using acetylene reduction and PCR screening for *nifH*. Thus, although biological nitrogen fixation may occur in South African sugarcane varieties, the contribution of this N-source in the tested cultivar was not significant.

Abbreviations: $\Delta^{15}N$ – nitrogen isotope discrimination; $\delta^{15}N$ – nitrogen isotope composition.

Introduction

Benign communities of endophytic bacteria colonize a wide variety of plant species (Sturz et al., 2000). These bacteria were previously thought to be weakly virulent plant pathogens. However, at least part of this endophytic community is now thought to have beneficial consequences for the host plants including growth promotion and increased resistance to plant pathogens and parasites (Sturz and Nowak, 2000). Biological nitrogen fixation by endophytic diazotrophic bacteria has also been suggested to occur in various economically important graminaceous species including sugarcane, rice and forage grasses (James, 1999). Specific diazotrophic bacterial strains seem to be associated with particular cultivars of sugarcane (Muñoz-Rojas and Caballero-Mellado, 2003). These symbionts may contribute to the nitrogen economy of the crop, thus reducing

* FAX No: +27-21-650-4041.
E-mail mcramer@botzoo.uct.ac.za

the requirement for N fertilizer in agriculture. This reduced requirement for N fertilizer would be especially important in organic agriculture and for small-scale farmers who cannot afford the extensive application of fertilizers.

Some sugarcane varieties have been reported to derive significant quantities of N from biological nitrogen fixation (Döbereiner et al., 1972; Lima et al., 1987; Ruschel et al., 1974; Sevilla et al., 2001; Urquiaga et al., 1992). A ^{15}N natural abundance study by Boddey et al. (2001) showed contributions of between 25% and 60% to the N-budget of Brazilian sugarcane. The organisms held responsible for N_2-fixation in graminaceous species are diazotrophic bacteria, colonizing either the rhizosphere of the plant or xylem vessels, root cap cells, epidermal cells and lateral root junctions (Reinhold-Hurek and Hurek, 1998). Important endophytic diazotrophic bacteria found in sugarcane include *Gluconacetobacter diazotrophicus* (Gillis et al., 1989; Muñoz-Rojas and Caballero-Mellado, 2003), formerly named *Acetobacter diazotrophicus* (Cavalcante and Döbereiner, 1988); *Herbaspirillum seropedicae* (Baldani et al., 1986); *H. rubrisubalbicans* and *Burkholderia unamae* (Caballero-Mellado et al., 2004); *B. tropica* (Reis et al., 2004); *Enterobacter* sp. (Mirza et al., 2001); *Pantoe* sp. (Loiret et al., 2004). Despite the reports of significant N_2-fixation in sugarcane, N_2-fixation was not always found in sugarcane grown in other studies (Biggs et al., 2002; Yoneyama et al., 1997) using ^{15}N natural abundance measurements. Thus there is some controversy as to the contribution of diazotrophic bacteria to the nitrogen budget of sugarcane.

Fuentes-Ramírez et al. (1999) found that high rates of N-fertilizer supplementation comprising 11 mM of NH_4NO_3 per plant supplied every two weeks resulted in reduced endophytic populations of *G. diazotrophicus*. Inhibition of endophytic diazotrophic bacterial populations was also reported in sugarcane fields fertilized with 300 kg N ha^{-1} (Reis et al., 2000). The inhibition of *G. diazotrophicus* proliferation by N is particularly associated with NH_4^+ nutrition and results in morphological changes in the bacteria (Muthukumarasamy et al., 2002). Although different bacterial and cultivar associations may explain differences in the contribution of endosymbionts to N_2-fixation, the inhibitory effects of soil N levels on endophytic populations could also provide an explanation for the contradictory conclusions concerning the contribution of N_2-fixation in sugarcane since the amount of N fertilizer applied to the crop differs markedly in different regions (Baldani et al., 2002). No conclusive evidence has been reported for the existence of an efficient association between endophytic or free-living diazotrophic bacteria in South African sugarcane. Long-term low N inputs to Brazilian sugarcane cultivation might have increased the role of biological nitrogen fixation in N supply in that agricultural system. This may also be true for South African sugarcane cultivars cultivated by small-scale growers who apply little or no N fertilizer.

This study aimed to evaluate the role of N_2-fixation in a South African sugarcane cultivar. The sugarcane cv. NCo376 was selected for this study because it is mainly grown by small-scale growers. Several different methods have been developed for quantification of N_2-fixation associated with the Gramineae. Boddey (1987) concluded that the combination of different techniques is preferable since most techniques have uncertainties regarding the measurement of definitive quantification of biological nitrogen fixation. The methods used in this study were $\delta^{15}N$ natural abundance, $^{15}N_2$ incorporation, acetylene reduction and detection of nitrogenase *nifH*-genes in the endophytic bacteria isolated from sugarcane.

Materials and methods

Field material

Leaves and soil samples were collected on 28th May 2002 from commercially grown *Sacharrum* L. spp. *hybrid* . cv. NCo376 from an experimental site (Ottawa) situated near Mt. Edgecombe (Natal, South Africa). The site receives annual rainfall of 846 mm; 84% of this fell between August 2001 and May 2002 (sugarcane growth period) with 465 mm of rain precipitated in the period January to May 2002. Samples of the weedy C_4 grass *Chloris pycnothrix* (Trin.), which

was growing on the experimental plots, were collected on the 5th August 2004 to be used as a reference species. The experimental plot was established in a randomized block design in October 1995 and supplied with 0, 50, 100 and 150 kg urea-N ha^{-1} twice yearly. The sampled plots were on the top of a gentle slope on a relatively shallow (600–700 mm) clay soil (25–30% clay, on average). The form consisted of a dark melanic topsoil overlying hard rock, with pH values within the range 6.5–8.0. Organic matter was within the range 2–4%. The soil had not been subjected to any prior steaming or chemical disinfestations. Prior to sampling, the cane had last been cut on the 15th August 2001. Two guard rows were established between each treatment block and the treatment blocks comprised four rows of sugarcane plants spaced 1 m apart.

Glasshouse-grown material

Culms of plants grown on plots not supplied with nitrogen fertilizer for over 5 years were collected from the Ottawa field site. These culms were cut into 30 cm portions bearing at least four axillary buds and were planted in 1:1 mixtures of sand and vermiculite. After bud break, the plantlets were transferred to a medium of river sand and supplied with Long Ashton nutrient medium (Hewitt, 1966) modified to contain 0, 1 or 10 mM $NaNO_3$. The sand was flushed with distilled water once per week. Plants were grown in a temperature controlled (minimum 15 °C, maximum 25 °C) greenhouse at the University of Stellenbosch between November 2002 and April 2003.

$\delta^{15}N$ natural abundance study

The mid portions of the 3rd youngest leaves of sugarcane and the youngest fully expanded leaves of *Chloris pycnothrix* were sampled from the field site. Four separate replicate leaf samples were collected from two replicate blocks, which were supplied with 0, 50, 100 or 150 kg urea-N ha^{-1} twice yearly. Each sample comprised eight leaves collected from plants distributed within each of the blocks. The top 30 cm of soil was collected from close to the stem of each of the eight plants with a soil auger. The eight soil samples were bulked to constitute a single replicate. Samples were dried at 50 °C for 48 h and then milled (M20 mill, IKA Labortechnik, Germany). Between 2.10 and 2.20 mg of each sample was weighed into an 8 by 5 mm tin capsule (Elemental Microanalysis Ltd., Devon, UK) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyser (Fisons Instruments SpA, Milan, Italy). The $\delta^{15}N$ values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to the CHN analyser by a Finnigan MAT Conflo control unit. The nitrogen isotopic ratios of the samples were expressed as $\delta^{15}N = [R_{sample}/R_{standard} - 1] \times 1000$ where $\delta^{15}N$ is the isotope ratio relative to the atmospheric air standard, and R_{sample} and $R_{standard}$ (0.0036765) are the molar ratios of the heavier to the lighter isotope of the sample and standard, respectively (Evans, 2001). The isotopic fractionation was expressed as $\Delta^{15}N = \frac{\delta^{15}N_{source} - \delta^{15}N_{plant}}{1+(\delta^{15}N_{plant}/1000)}$, where $\delta^{15}N_{source}$ was taken as the $\delta^{15}N$ of the associated soil.

$^{15}N_2$ incorporation in detached plant-roots and culm segments

Roots and culm segments from five field-grown plants not supplied with N-fertilizer and four glasshouse-grown plants were pre-incubated in a 10 L container in the dark for 12 h. The pre-treatment of tissue was based on the method described by Döbereiner et al. (1972). The container was continuously flushed (36 L h^{-1}) with a gas mixture of 6% O_2 in N_2 mixed with a gas blender (GM 602; ADC Ltd., Hoddesdon, UK). After the pre-incubation, the roots were washed in 2 mM $CaSO_4$. Root or culm tissue (1 g fresh weight) was placed in 15-mL vials and sealed with a rubber septum and an aluminum cap. Coralloid roots, which are the symbiotic organ of the cycad-cyanobacterial symbiosis, were harvested from *Encephalartos altensteinii* growing in the botanical garden at the University of Stellenbosch and were used as a positive control. Ten mL of $^{15}N_2$ gas (10-atom% excess) was injected into the vials. After 12 h of exposure, plant material was dried at 80 °C and finely

ground with a mortar and pestle. Nitrogen isotopic ratios of the dried samples were determined as described previously. Nitrogen isotopic ratios of $^{15}N_2$ gas-exposed and unexposed plant material were compared using a Student's *t*-test to determine significant incorporation of ^{15}N.

Acetylene reduction in detached plant roots and culm segments

Roots and culm segments from five field-grown plants not supplied with N-fertilizer and four glasshouse-grown plants and coralloid roots were pre-treated and enclosed in a sealed vial as described above. Acetylene gas (Industrial grade, Afrox, South Africa) was introduced at a final concentration of 10%. After 5 h, a 1 mL gas-sample was taken from each vial and ethylene concentrations determined. Samples (1 mL) were injected into a Varian 3400 gas chromatograph (GC) to determine the peak areas of acetylene and ethylene. The GC was set up with the following settings: injector 100 °C; column 70 °C; detector 100 °C; Column 15 cm × 4.5 cm packed with Hayesep N 80/100; detection-limit for ethylene 0.629 nmol per mL. The data were corrected for ethylene impurities in acetylene. Concentrations of ethylene in samples were calculated using an ethylene standard (Medical grade, Matheson, USA) mixed with N_2 gas (Afrox) in a 1 L gas mixing syringe (Model 6000-01, LI-COR Inc., Lincoln, USA). Data were tested for significance using a Student's *t*-test.

Acetylene reduction in glasshouse-grown plants

For two days prior to the start of the acetylene reduction assay the plants, which had been grown on 1 mM $NaNO_3$ nutrient solution were supplied with distilled water to avoid possible negative effects of N nutrition on nitrogenase activity. Two hours before the start of the experiment, selected plants were placed in the growth room (set points growth room: 29 °C; RH 60%; PFD 1500 μmol m^{-2} s^{-1}) illuminated with 400 W sodium lights and metal halide lights (Venture, Ohio, USA) in a ratio of 3:2 and 15 bulbs per m^2. The plants were placed in airtight Perspex cylinders (total volume 40 L) provided with gas inlets and outlets. A membrane pump (AEI Technologies Inc., Pittsburgh, USA) was used for air circulation (60 L h^{-1}) and the air in the cylinders was stirred using a magnetic stirrer to promote rapid equilibration. The concentration of CO_2 in the cylinders was maintained at between 400 and 1000 μmol mol^{-1} by monitoring the CO_2 with an AEI Technologies infrared gas analyser and manually injecting pure CO_2 when necessary. Acetylene was injected in the cylinders to a final concentration of 10% (v/v) using a 1 L Licor 6000-01 gas syringe. After addition of acetylene the gas volume was sub-sampled routinely for a period of 5 h and 1 mL of sampled gas assayed for ethylene on the GC as described above.

Isolation of endophytic diazotrophic bacteria from sugarcane

The oldest internodes from glasshouse and field-grown sugarcane plants were used for isolation of endophytic bacteria according to the method described by van Antwerpen et al. (2002). The culm was cut at the bottom of the stool and the oldest three internodes and nodes were used for isolation. Both cut ends of the culm segments were sealed with hot (50 °C) candle-wax and then surface-sterilized in 10% commercial sodium hypochlorite for 10 min. The middle segment of the culm was then excised, sprayed with 70% (v/v) ethanol and flamed. Thereafter the core of the internode was removed and crushed with a sterile garlic-crusher and 50 μL of sap plated out onto solid nitrogen-free medium (Rennie, 1981) without the growth factor *p*-aminobenzoic acid and incubated at 30 °C. After two days, separate bacterial colonies differing in appearance were transferred to liquid nitrogen-free medium (Rennie, 1981) in 200 mL Erlenmeyer flasks and shaken at 150 rpm (30 °C). Bacterial strains which showed visible growth after 24 h were selected for acetylene reduction assay and *nifH*-gene PCR amplification.

Acetylene reduction assay with isolated bacterial strains

Nitrogenase activity in pure endophyte strains was determined using the acetylene reduction method described by Różycki et al. (1999). Bacterial cultures in liquid nitrogen-free medium

were sub-cultured three times on solid Luria–Bertani (LB) N-containing medium to ensure purity of the strains. Pure bacterial strains were transferred to semi-solid nitrogen-free medium (Rennie, 1981) containing 4.5 g L^{-1} agar in 15 mL vials. The diazotrophic bacterium *Klebsiella pneumoniae* wild type strain Kp5022 (Streicher et al., 1974) with genotype hisD2, hsdR1, nif^+ obtained from D Rawlings (University of Stellenbosch), was included in this experiment as a positive control. The vials were closed with cotton wool and aluminum foil and incubated for three days at 30 °C. After 3 days the vials were closed with a rubber septum and an aluminum cap. Acetylene was injected in the vials to constitute 10% (v/v) of the total gas volume. After 24 h of incubation at 30 °C 1 mL gas samples were taken from the vials and analysed for ethylene, as described previously.

nifH-gene PCR amplification

Pure endophytic strains and *K. pneumoniae* wild type strain Kp5022 were transferred from liquid nitrogen-free medium to 100 mL liquid Luria–Bertani (LB) N-containing medium and incubated at 30 °C for 48 h. Liquid cultures were centrifuged at 3500 *g* for 15 min at 4 °C. Cells were resuspended in 10 mL lysis buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA and 2 mg mL^{-1} lysozyme and incubated for 30 min at 37 °C. SDS and proteinase K were added to final concentrations of 1% (w/v) and 50 μg mL^{-1}, respectively, and incubated for 4 h at 50 °C. After incubation, DNA was extracted twice with equal volumes of chloroform:isoamylalcohol (24:1) and centrifuged at 7 800 *g* for 15 min. The aqueous phase was overlaid with 2.5 volumes of –20 °C 100% (v/v) ethanol and the high molecular weight DNA was spooled out with a glass hook. After drying, the DNA was resuspended in TE-buffer (pH 7.8) containing 10 mM Tris–HCl and 1 mM EDTA. PCR amplifications from genomic-DNA from the different strains were performed with the primer set PolF/PolR (Poly et al., 2001) synthesized by Incorporated DNA Technologies Inc. (Coralville, USA) in a total volume of 50 μl. Both primers were used at a concentration of 0.18 μM. Other reagents were at final concentrations of 0.2 mM of each dNTP, 1 U of Taq DNA Polymerase, enzyme buffer (according to manufacturer's description; Promega Madison, USA) and 1.5 mM $MgCl_2$. PCR conditions consisted of a first step at 94 °C (1 min) followed by 30 cycles with the following temperatures: 94 °C for 1 min, 55 °C (annealing step) for 1 min and 72 °C for 2 min, with a 5 min extension at 72 °C for the last cycle. All amplifications were performed in a PCR Sprint thermocycler (Hybaid Ltd., Ashford, UK). Amplification products were electrophoresed in a 2% (w/v) agarose gel and stained with ethidium bromide.

In addition to PCR with genomic-DNA, direct colony PCR with the different strains was performed. Pure endophytic strains and *K. pneumoniae* wild type strain Kp5022 were grown overnight on solid LB medium. Bacterial cells of single colonies of the different strains were transferred with sterile toothpicks to PCR tubes with 7 μl distilled water. PCR reactions were performed as described above in a total volume of 15 μl. The first step in the PCR program was extended to 3 min at 94 °C to ensure degradation of bacterial cell walls. Amplification products were submitted to electrophoresis in a 2% (w/v) agarose gel and stained with ethidium bromide.

Results

Natural abundance study

The $\delta^{15}N$ values of the soil were higher than those of leaves (Figure 1A), except at urea application rates of 150 kg N ha^{-1}. The highest $\delta^{15}N$ values for the soil were at an application rate of 50 kg urea-N ha^{-1} ($\delta^{15}N$ 5.5 ± 0.2‰, mean ± SE). For sugarcane leaves, the $\delta^{15}N$ initially decreased with increasing N fertilization and then increased to a maximum at an application rate of 150 kg urea-N ha^{-1} ($\delta^{15}N$ 5.2 ± 0.2‰). These changes in $\delta^{15}N$ resulted in an initial increase in $\Delta^{15}N$ from plots not supplied with urea to those supplied with 50 kg N ha^{-1}, decreasing to a $\Delta^{15}N$ of –0.09 ± 0.58‰ at 150 kg urea-N ha^{-1} (Figure 1B). The $\delta^{15}N$ values of the leaves of *C. pycnothrix* were lower than those of sugarcane, but followed a similar pattern increasing at high rates of N application (Figure 1A).

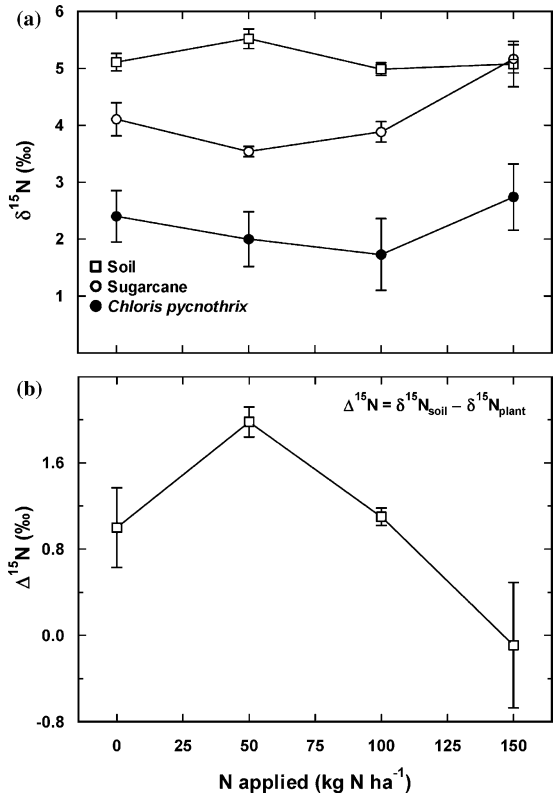

Figure 1. (A) $\delta^{15}N$ values of soil and leaf dry matter samples taken from experimental sugarcane field sites at "Ottawa" supplied with different levels of urea-N application (0, 50, 100 or 150 kg urea-N ha^{-1}); (B) $\Delta^{15}N$ for sugarcane leaves sampled at experimental sugarcane field sites at "Ottawa" supplied with different levels of urea-N application (0, 50, 100 or 150 kg urea-N ha^{-1}). A positive value indicates that the $\delta^{15}N$ value of the leaves is lower compared with the $\delta^{15}N$ value of the soil. The values are the mean ± SE (n = 4).

$^{15}N_2$ incorporation experiment

In an attempt to detect the activity of any diazotrophic bacteria in the plant, tissue was pre-incubated with 6% O_2 according to the method of Döbereiner et al. (1972). This method was used despite the fact that some endophytic diazotrophic bacteria have an O_2 optimum of 20% (Pan and Vessey, 2001) and that pre-treatment could lead to artificial enhancement of N_2-fixation (Giller and Merckx, 2003). Root and culm segments from field-grown plants not supplied with N-fertilizer and also glasshouse-grown plants supplied with 0 or 1 mM $NaNO_3$ were incubated in an atmosphere containing 10-atom% $^{15}N_2$ excess for 12 h. The $\delta^{15}N$ values of the plants supplied with 1 mM $NaNO_3$ were lower than those not supplied with $NaNO_3$ (Table 1). This was probably a consequence of the fact that the $\delta^{15}N$ value of the $NaNO_3$ supplied was low (Table 1). This did not result in significant changes in the $\delta^{15}N$ values of the root and culm segments compared to samples incubated without $^{15}N_2$ enrichment (Table 1). The $\delta^{15}N$ values of coralloid roots incubated in 10-atom% $^{15}N_2$ excess were 40.02 ± 20.20‰ compared with a $\delta^{15}N$ value of 2.45 ± 0.23‰ (n = 7) for those incubated without $^{15}N_2$ enrichment, thus demonstrating significant incorporation of N_2 in coralloid roots.

Acetylene reduction

Root and culm segments of glasshouse-grown sugarcane supplied with 0 or 1 mM $NaNO_3$ and field-grown sugarcane not supplied with N-fertilizer showed no significant acetylene-reducing activity (Table 1). Coralloid roots reduced acetylene to ethylene over the 5 h incubation period at a rate of 7.9 ± 3.9 nmol ethylene g^{-1} fresh weight (n = 4). Whole glasshouse-grown sugarcane plants incubated for 5 h showed no acetylene-reducing activity, whereas the coralloid roots of *E. altensteinii* incubated under the same circumstances showed a linear increase in ethylene concentration over time (Figure 2).

Endophytic bacteria

Seven morphologically different colonies of endophytes were obtained from culm tissue of field-grown plants that had not been supplied with N fertilizer and also from culm tissue of glasshouse-grown plants supplied with 1 or 10 mM $NaNO_3$. These colonies were selected by culture on N-free medium (Rennie, 1981). None of these seven selected strains, however, showed significant acetylene reduction activity (n = 3). In contrast, cultures of *K. pneumoniae* wild type strain Kp5022 reduced acetylene to ethylene at a rate of 20.9 ± 2.09 nmol ethylene per culture h^{-1} (n = 3). PCR of bacterial genomic DNA (data not shown) and of bacterial cells (Figure 3) from the seven selected strains with the primer set PolF/PolR (Poly et al., 2001) did not produce amplification products of the

Table 1. $\delta^{15}N$ values and ethylene production rates (mean ± SE) of dry matter total N of glasshouse-grown ($n = 4$) and field-grown ($n = 5$) sugarcane root and culm segments

Source	Sample	$\delta^{15}N$ (‰)		Ethylene (pmol g^{-1} h^{-1})
		Air	$^{15}N_2$	
Glasshouse-grown	Roots (0 mM $NaNO_3$)	4.41 ± 0.11	4.45 ± 0.05	14.5 ± 7.0
	Roots (1 mM $NaNO_3$)	3.35 ± 0.15	3.31 ± 0.86	4.9 ± 2.0
Field-grown	Culm	1.93 ± 0.16	1.85 ± 0.14	1.0 ± 0.8
	Roots	2.89 ± 0.13	2.92 ± 0.13	2.3 ± 2.2

The $\delta^{15}N$ values were determined after 12 h incubation in 15 mL vials in either 10-atom% $^{15}N_2$ excess or in air. Glasshouse-grown plants were supplied with either 0 or 1 mM $NaNO_3$, the $\delta^{15}N$ of which was −4.02 ± 0.04 ‰. There were no significant differences detected in the $\delta^{15}N$ values between the air and $^{15}N_2$ treatments, as determined using Student's *t*-tests. For ethylene production the plant material was incubated in an atmosphere containing 10% acetylene in 15 mL vials. There were no significant differences in ethylene production between the control tissue incubated in the absence of acetylene and that incubated with acetylene, as determined using Student's *t*-tests.

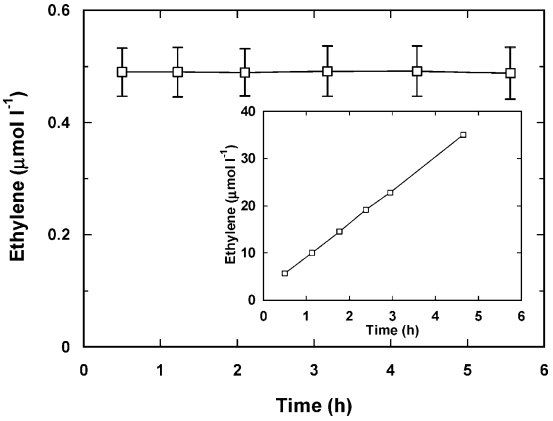

Figure 2. Ethylene production by whole glasshouse-grown sugarcane potted plants. Plants were incubated in 40 L gas tight cylinders in an atmosphere of 10% acetylene in air. CO_2 levels were kept at a concentration of between 400 and 1000 μmol mol^{-1}. The inset graph shows ethylene production by coralloid roots from *Encephalartos altensteinii* incubated in the same experimental conditions (positive control).

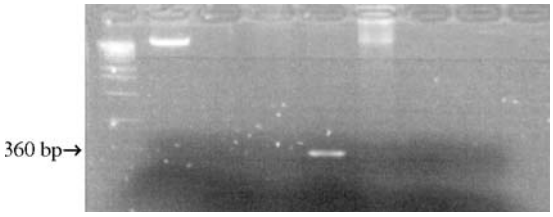

Figure 3. Results of direct colony PCR employing the primers PolF and PolR (Poly et al., 2001) to amplify *nifH*-gene fragments from cells from seven endophytic strains isolated from glasshouse-grown and field-grown sugarcane plants. The arrow indicates a DNA fragment of the correct size (ca. 360 bp) amplified from cells from *K. pneumoniae* (positive control). DNA fragments of the correct size were not obtained with any of the other endophytic strains (other lanes) indicating that these strains were not diazotrophic. Three microlitre EL molecular marker, consisting of digesting products from plasmid pEmuLuc separately digested with *BglI*, *Pvu*II and *Bam*HI, was used to indicate the size of the products. Gels consisted of 2% agarose in Tris–boric EDTA buffer (0.5%) and 3 μl of ethidium bromide was added per gel to visualize DNA products.

correct size. However, products of the correct size (ca. 360 bp) were routinely obtained with the same primer set with both the genomic DNA (data not shown) and the cells of *K. pneumoniae* (Figure 3).

Discussion

The complexity of the dynamics of soil N makes interpretation of soil $\delta^{15}N$ values extremely difficult (Högberg, 1997). The inorganic N available to plants from soils is usually a small portion of the total N of the soil, further complicating interpretation of the role of soil $\delta^{15}N$ values in determining plant $\delta^{15}N$ values. Processes that contribute to determining the soil $\delta^{15}N$ values include N ammonification, ammonia volatilization, nitrification, denitrification, ion exchange, diffusion, mass flow, plant and fungal N uptake and N_2-fixation (Högberg, 1997). It is likely that the soil $\delta^{15}N$ values are partially determined by the return of plant material to the soil, primarily in the form of root turnover, with the consequence that depression of plant $\delta^{15}N$ values due to N_2-fixation may consequently result in a reduction of soil $\delta^{15}N$ values. High application rates of N fertilizer have been shown to reduce populations

of N_2-fixing bacteria (Fuentes-Ramírez et al., 1999; Reis et al., 2000). Thus it may be expected that smaller $\delta^{15}N$ values would be found in soils where low amounts of N-fertilizer were applied. The low $\delta^{15}N$ value found at an application rate of 0 kg urea-N ha^{-1} (Figure 1A) could indicate the presence of N_2-fixation in the soil while the lower $\delta^{15}N$ values at application rates higher than 50 kg urea-N ha^{-1} could have been caused by the high supplementation of urea-N which had an $\delta^{15}N$ value of $0.06 \pm 0.19‰$ ($n = 4$). However, this speculation is undermined by the fact that soil $\delta^{15}N$ values are complex in origin and cannot be properly interpreted in a superficial study of this nature.

In common with most systems (Högberg, 1997), the leaf $\delta^{15}N$ values were generally lower than those of the soil indicating one of or a combination of the following: (a) discrimination against ^{15}N occurring during uptake and metabolism of inorganic N derived from the soil (Evans, 2001); (b) utilization of N from deeper soils with more negative $\delta^{15}N$ values (Högberg, 1997) than those of the measured surface soils; (c) variable participation of biological N_2-fixation by bacteria occurring in the rhizosphere or as endophytes (Unkovich et al., 2000). Leaves from plants grown at an application rate of 50 kg urea-N ha^{-1} had the highest $\Delta^{15}N$ values (Figure 1B), possibly indicating that these plants derived part of their nitrogen from N_2-fixation. It could be that extensive colonization of plants by diazotrophic bacteria only occurs when at least a small amount of nitrogen is available in the soil. However, the fact that the changes in $\delta^{15}N$ values of the grass *C. pycnothrix* were similar to those of sugarcane with increasing levels of applied N, indicates that the changes in sugarcane $\delta^{15}N$ values were probably not associated with N_2-fixation. The use of $\delta^{15}N$ for estimating N_2-fixation can work reasonably well in agricultural systems with few potential N sources (Robinson, 2001), but, there are other possible explanations for the changes in $\delta^{15}N$ with changes in N application rates, including increasing discrimination with increasing external concentrations of both NO_3^- and NH_4^+ (Evans, 2001). Sugarcane leaf tissue N concentration increased with increasing rates of soil urea application (data not shown). The leaf tissue $\delta^{15}N$ was linearly related to leaf N concentration (mg N g^{-1} DW, slope = 0.2, Pearson correlation coefficient = 0.73, $P < 0.001$). Analysis of the data presented by Yoneyama et al. (1997) indicated a similar relationship for a variety of sugarcane cultivars from Brazil, Philippines and Japan (slope = 0.25, Pearson correlation coefficient = 0.62, $P < 0.001$). Although it is possible that $\delta^{15}N$ values were lower at low tissue N concentrations and at low rates of N application due to increased N_2-fixation, the $\delta^{15}N$ values for the reference species (Yoneyama et al., 1997) and *C. pycnothrix* (present study) were also significantly correlated with the leaf N concentrations (data not shown). Thus the $\delta^{15}N$ data presented here provided no conclusive evidence for the presence of N_2-fixation.

Selection of "reference" species for estimation of N_2-fixation from $\delta^{15}N$ is problematic (Boddey et al., 2001) since these plants may not utilize the same inorganic and/or organic sources of N from the soil as the species being examined for N_2-fixation and may also exploit N from different depths within the soil and at different times (Dawson et al., 2002). Furthermore C_3 and C_4 photosynthetic species differ in their N requirements and considering the strong relationship between $\delta^{15}N$ and leaf N, it is likely that this could influence the accuracy of the estimations of the contribution of N_2-fixation, if based on C_3 reference species. Yoneyama et al. (1997) and Boddey et al. (2001) used an extensive set of $\delta^{15}N$ values for sugarcane and associated reference plants (including both C_4 and C_3 photosynthetic types) to indicate the contribution of biological N_2-fixation to sugarcane N. In the data presented by Yonyama et al. (1997) N_2-fixation was reported for some cultivars from one site but was not detected at other sites, indicating strong variability in the data. Furthermore, in analysis of the data, these authors constrained the contribution of N_2-fixation to never be less than 0%. While this intuitively makes sense, it does not allow for the inherent variability of the measurements, which probably results from the complexity of the interactions determining plant $\delta^{15}N$ values, as reviewed by Dawson et al. (2002). In addition it generates an erroneous impression of the magnitude of the contribution of N_2-fixation. Re-analysis of the percentage N derived from N_2-fixation (%Ndfa = 1 – (sugarcane $\delta^{15}N$/reference species

$\delta^{15}N) \times 100$, assuming $\delta^{15}N$ of fixed $N_2 = 0$, Yoneyama et al., 1997), including all the sites and cultivars sampled by these authors, revealed that N_2-fixation accounted for only ca. 3.7 ± 8.5% (mean ± SE, $n = 54$) of sugarcane N, overall. This corroborates the apparent lack of contribution of N_2-fixation to the N-budget of South African sugarcane based on $\delta^{15}N$ in the present study, although, certain sugarcane genotypes (Boddey et al., 2001; Yoneyama et al., 1997) may indeed be capable of symbiotic N_2-fixation.

The incorporation by a plant of ^{15}N-enriched N_2 gas can be regarded as strong evidence for biological N_2-fixation (Boddey, 1987). Ruschel et al. (1974) demonstrated considerable incorporation of ^{15}N in Brazilian sugarcane seedlings. In the present investigation sugarcane root and culm segments from both field-grown and glasshouse-grown plants showed no significant incorporation of $^{15}N_2$ (Table 1). The positive control (coralloid roots) indicated that the system used was capable of detecting ^{15}N incorporation. The smallest detectable significant difference in $\delta^{15}N$ means between the samples incubated in an atmosphere enriched with ^{15}N compared to those incubated in non$-^{15}N_2$ enriched atmosphere with the methods used was 0.6 as determined from power analysis ($P = 0.05$). Because the tissue N concentration of sugarcane was less than 1% N (w/w) (data not shown), a difference of 0.6 $\delta^{15}N$ corresponded with an increase in total N of the sample over the incubation period of ca. 3%. Thus if N_2-fixation did occur in the sugarcane plants tested in this study, then the contribution of N_2-fixation to the total N-pool in the excised root and culm segments would be less than 3% per 12 h.

Although the quantitative significance of the acetylene reduction assay (ARA) based on the conversion of acetylene to ethylene catalyzed by nitrogenase may be questionable (Boddey, 1987), indications of the presence of N_2-fixation can be reliably obtained with this sensitive technique (Vessey, 1994). Patriquin et al. (1980) and Döbereiner et al. (1972) showed acetylene-reducing activity in whole Brazilian sugarcane plants and excised roots, respectively. Staphorst and Strijdom (1978) showed acetylene-reducing activity in roots from South African sugarcane plants. In the current investigation, results of the acetylene reduction assay corresponded with the ^{15}N incorporation results. No significant acetylene-reducing activity was observed in excised root or culm tissue from either field-grown or glasshouse-grown plants or from whole plants cultivated in the glasshouse with 1 mM $NaNO_3$. The positive control (coralloid roots) confirmed that the system used was capable of detecting acetylene reduction in plant material. The GC analysis was adequate for detection of at least 0.63 μmol L^{-1} of ethylene. Assuming that acetylene reduction is four times more rapid than N_2-fixation (Boddey, 1987), this amount of ethylene would correspond to an increase of 0.003% in total N in the tissue over the incubation period. Thus the technique was sensitive enough to detect even small contributions of N_2-fixation to the total N pool of the tissue. It can therefore be concluded that nitrogenase activity in the plants from the field and the glasshouse was very low, if present at all. This could be due to low abundance of diazotrophic bacteria in the plants.

The suggestion that endophytic diazotrophic bacteria were only present in small amounts or absent in the plants was tested using specific primers for the *nifH*-gene. This gene encodes for the Fe-protein subunit, which is the reductase component of nitrogenase and is ubiquitous in nitrogen fixing bacteria and Archaea (Poly et al., 2001). Amplification experiments using PCR with *K. pneumoniae* genomic DNA showed that the primers amplified a product of the expected size, confirming the specificity of the primers for this gene. Seven endophytic strains were grown on N-free medium (Rennie, 1981) to select for oligonitrophilic and/or diazotrophic bacteria. This N-free medium was previously used for the isolation of diazotrophic bacteria from roots of pine, oak (Różycki et al., 1999) and Brazilian sugarcane (Rennie et al., 1982), although, in a semi-solid form rather than the solid form used in the present investigation for the initial cultivation. Amplification experiments using PCR were performed on genomic DNA of the seven selected endophytic strains, but for none of the strains were amplification products of the expected size obtained. This indicated that the bacteria isolated were not diazotrophic. This result was confirmed by the lack of acetylene-reducing activity in these seven strains. van Antwerpen

et al. (2002) reported isolation of several endophytic bacteria from the South African sugarcane cv. N12 on media with low N-content. Although several species isolated from the cane are known to be able to fix nitrogen (*Klebsiella* spp., *Pseudomonas* spp. and *Burkholderia* spp.), the N_2-fixing ability of the isolated strains has not yet been confirmed. The seven endophytic bacteria strains isolated on N-free medium in this study appeared to lack N_2-fixing ability. However, the seven strains showed visible growth in both semi-solid and liquid medium (Rennie, 1981), which contains 50 mg L^{-1} yeast extract. Possibly the isolated endophytic bacterial strains were oligonitrophilic and able to grow with very low N concentrations.

Although we were unable to detect the *nifH*-gene or nitrogenase activity in bacteria from this South African sugarcane cultivar, many bacteria isolated from sugarcane have been shown to be diazotrophic (see above). The question which remains is whether these bacteria contribute significantly to the nitrogen budget of sugarcane, or whether the diazotrophic capacity of the bacteria merely enables their own growth in the high sugar environment that the endophytes are exposed to within sugarcane. Induction of sugarcane gene expression by inoculation with *G. diazotrophicus* and *H. rubrisubalbicans* may indicate active involvement of the plant in establishment of the interaction with the bacteria (Nogueira et al., 2001). However, it remains to be conclusively determined whether the reported gene induction is specifically for enabling the symbiosis, or merely a consequence of infection.

Conclusions

Results from the natural abundance study failed to indicate significant contribution of N_2-fixation to the nitrogen budget of sugarcane. Attempts to demonstrate nitrogenase activity in field and glasshouse-grown plants using $^{15}N_2$ incorporation and acetylene reduction techniques also failed. Although endophytic bacteria were isolated and cultured on Rennie's medium, no evidence of diazotrophic activity was found in either field- or glasshouse-grown sugarcane plants. Thus it is unlikely that N_2-fixation contributes significantly to the N-budget of sugarcane cv. NCo376. Our results support the conclusion of Giller and Merckx (2003) who asserted in a review of the contribution of associative endosymbionts to N-budgets of grasslands and pastures, that these contributions are only of marginal significance. The possibility exists, however, that inoculation of South African cane with N_2-fixing bacteria may enable sugarcane to access N derived from N_2-fixation, as was reported by Oliveira et al. (2002) for micro-propagated sugarcane plants.

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