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Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat

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Nitrogen-free, semi-solid defined medium with crystallized cane sugar (100 g/l) supplemented with cane juice (5 ml/l) was the most selective for isolating *Acetobacter diazotrophicus*. Surveys of *A. diazotrophicus* using this medium showed that > 10^3 cells/g fresh wt were present at all sites in all parts of the sugar cane plant and in all trash samples examined, reaching up to 10^7/g. Additional samples, from forage grasses and cereals and from weed species collected within the sugar cane fields, were all negative. Heat treatment (50°C for 30 min) of the sugar cane setts did not affect *A. diazotrophicus* numbers within the plant. Nitrogenase activity of intact soil–plant systems in pots planted with heat-treated setts did not respond to inoculation with *A. diazotrophicus*. The endophytic habitat of this diazotroph and its propagation within the stem cuttings was confirmed.

Key words: Acetobacter diazotrophicus, endophyte, nitrogen fixation, sugar cane.

The substantial biological N_2 fixation (BNF) associated with sugar cane has been demonstrated by N-balance and ^{15}N-dilution experiments (Lima *et al.* 1987; Urquiaga *et al.* 1992). *Saccharum spontaneum*, a wild forage sugar cane from the Philippines, as well as certain Brazilian sugar cane genotypes, can obtain > 60% of their total N from biological fixation. This has been considered as the key to economically- and energetically-viable biofuel programmes.

According to the concept of rhizosphere associations, diazotrophic bacteria have to compete with non-diazotrophs for carbon substrates (Barber & Lynch 1977) and must lyse and decompose before fixed N becomes available to the associated plant. Recent evidence for endophytic and beneficial plant/bacteria associations was discussed by Kloeper *et al.* (1992). Examples of such endophytic relationships are those of *Alcaligenes faecalis* in wetland rice (You & Zhou 1989) and a newly discovered diazotroph, named *Azoarcus*, in Kallar grass and rice (Reinhold-Hurek *et al.* 1993). In addition, the bacteria *Herbaspirillum seropedicae* and *H. rubrisubalbicans*, which in some regions is a mild plant pathogen (Pimentel *et al.* 1991), can fix N_2 and appear to be plant endophytes (Döbereiner *et al.* 1993).

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Acetobacter diazotrophicus is of special interest because, besides fixing N_2 in the presence of KNO_3 and at low pH values (< 3.0) (Stephan *et al.* 1991), it can excrete almost half of the fixed N in a form potentially available to plants (Cojho *et al.* 1993). This diazotroph has not only been isolated in Brazil, but also from sugar cane in Australia (Li & McRae 1991), Mexico (Fuentes-Ramirez *et al.* 1993), Argentina, Uruguay, and Cuba (unpublished data). Brazilian cane varieties grown in the south of Mexico need much less N-fertilizer than the US-genotypes planted in the north (Fuentes-Ramirez *et al.* 1993). Difficulties in finding this bacterium in other regions may be related to the methods used for isolation, which have not been described in detail since our first paper on this diazotroph (Cavalcante & Döbereiner 1988). In the present paper we report the most successful methods of isolation and some results on the specific occurrence of the diazotroph in sugar cane.

Materials and Methods

Basic Culture Medium

N-free semi-solid medium was inoculated with serial dilutions of various sugar cane parts in 10% sugar solution or with small, 1-mm pieces of plant material. The medium used, a modification of LGIP medium (Calvalcante & Döbereiner 1988) contained (g/l): K_2HPO_4, 0.2; KH_2PO_4, 0.6; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.02;

$Na_2MoO_4.2H_2O$, 0.002; $FeCl_3.6H_2O$, 0.01; 0.5% Bromothymol blue in 0.2 M KOH, 5; agar, 2.0; and carbon substrate, 100. The pH was adjusted to 5.5 with acetic acid.

Nitrogenase Activity in Pure Culture
The improved isolation media were evaluated in vials, each containing 5 ml semi-solid medium inoculated with 0.1 ml of a 48-h-old *A. diazotrophicus* culture in liquid, modified LGIP medium supplemented with 1 mM $(NH_4)_2SO_4$. Nitrogenase activity was determined after 1 h incubation at 30°C, with 10% acetylene in the gas phase. Ethylene accumulation was estimated on a 50-cm Poropak N column using a Perkin Elmer F-11 gas chromatograph fitted with a hydrogen flame ionization detector.

Enumeration of A. diazotrophicus *in Sugar Cane Samples*
Counts and attempted isolations of *A. diazotrophicus* were made in the semi-solid, modified LGIP medium supplemented with 100 g crystallized cane sugar/l and 5 ml cane juice/l. Vials of this medium were each inoculated with one of a series of serial dilutions of plant parts homogenized in water containing 100 g sugar/l. Vials were considered positive when they presented, after 10 days, the typical dark orange surface pellicle of *A. diazotrophicus* and clear, colourless medium below. Most Probable Numbers (MPN) were calculated according to Alexander (1982). Isolation was performed by streaking such cultures on agar plates (containing 20 g agar/l and 20 mg yeast extract/l of the same medium). Colonies on the plates become dark orange due to the assimilation of the Bromothymol blue. Orange colonies with white margins were not *A. diazotrophicus* but another, unidentified diazotroph. After 7 days the pure orange colonies were transferred into LGIP medium without cane juice and then purified on potato/agar plates with 100 g crystallized cane sugar/l (Cavalcante & Döbereiner 1988), on which dark brown colonies develop.

Nitrogenase Activity in Intact Plant Systems
Nitrogenase activity was measured in 3-month-old sugar cane plants in an intact system in pots. A plastic bag was sealed around the stem of each plant with solid agar. The atmosphere was changed to 10% acetylene in air and the plants incubated for 6 h in the greenhouse. Ethylene accumulation was estimated as described above, using *Brachiaria arrecta* (Miranda *et al.* 1990), and pots without plants were used as controls.

Results and Discussion

Of the different sources of sucrose tested as carbon sources in the N-free LGIP medium, crystallized cane sugar gave the fastest growth of *A. diazotrophicus* as evidenced by the highest rate of nitrogenase activity (Table 1). Addition of 5 ml cane juice/l enhanced growth of *A. diazotrophicus*, indicating that some unidentified impurities from sugar cane favour growth. Cane juice contains small amounts of glucose and fructose (0.2% to 0.6%), some organic nitrogen (0.02% to 0.04%) and various vitamins which can enhance bacterial growth, as did the yeast extract used by Li & MacRae (1992). The poor growth on commercial fine sugar, compared with that on the pure sucrose, may indicate the presence of residues from the industrial manufacture of the fine sugar which prejudice the growth of the bacteria.

Table 1. N_2-dependent growth, evaluated by nitrogenase activity, of two *A. diazotrophicus* strains (PAL-5 and PPE-4) in semi-solid LGIP medium with various carbon sources.

Carbon source	Nitrogenase activity (nmol acetylene reduced/h. vial)*	
	PAL-5	PPE-4
Sucrose	212	230
Sucrose + cane juice	230	220
Commercial fine sugar	110	130
Commercial sugar + cane juice	120	132
Crystallized sugar	315	310
Crystallized sugar + cane juice	350	420

*Values are means of three replicates, obtained after 144 h of incubation.

Growth of *A. diazotrophicus* strain PAL-5 (ATCC 49037) in pure culture or in a mixture (1:1 v/v) with sugar cane stem macerates diluted 100 times was also enhanced by the addition of crystallized sugar, even though this strain grew much faster than strain PPE-4 used in the first experiment (Figure 1). During the isolation of *A. diazotrophicus*, a non-N_2-fixing bacterium that formed yellow colonies on LGIP medium (AM1) was often observed. To improve the selectivity of the LGIP medium for isolating *A. diazotrophicus*, various modifications were therefore tested with mixed cultures of these two organisms. The data in Table 2 show that the addition of 1 and 10 ml of cane juice/l improved the selectivity whereas addition of more cane juice or of gluconic acid reduced it.

The N-free, semi-solid, modified LGIP medium shown to be the most selective was used to see if *A. diazotrophicus* was present in the four sugar cane cultivars used in Rio de Janeiro State (Table 3). The diazotroph was found in all samples except one, at 10^5 to 10^7 cells/g fresh roots, from both new and established cane fields, indicating the general colonization of

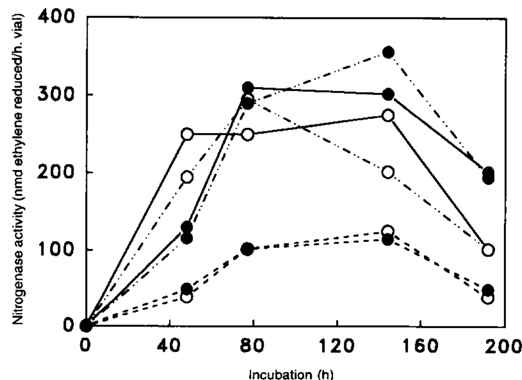

Figure 1. N_2-dependent growth of strain PAL-5 evaluated by nitrogenase activity in semi-solid LGIP medium, with (– · · –) or without (——) cane juice and with (●) or without (O) cane macerate (containing the natural sugar cane microflora) diluted 100 times. Commercial fine sugar with (- -●- -) or without (- -O- -) cane macerate was used as a control.

Table 2. Competition of *A. diazotrophicus* (PAL-5) with a non-N_2-fixing sugar cane isolate (AM1), during N_2-dependent growth in various media.

Ratio of PAL-5/AM1 in inoculum	Acetylene reduction activity (nmol ethylene/h. vial) in LGIP medium supplemented with:*				
	Nothing (control)	Gluconic acid (at 1 g/l)	Cane juice (ml/l):		
			1	10	100
1/0	208	367	437	627	362
1/1	44	97	362	203	ND
1/10	20	42	302	194	4
1/100	13	36	127	111	1
1/1000	0	1	1	0	0

*Values are means of three replicates.
ND—Not determined.

cane by this diazotroph. Similar, very high numbers were demonstrated in sugar cane in Australia by indirect ELISA (Li & MacRae 1992).

The endophytic nature of *A. diazotrophicus*, reported previously by Döbereiner *et al.* (1988) and Li & MacRae (1992), was confirmed by counts of this diazotroph in roots, stems, aerial parts and cane trash collected at two sites from three cultivars, both at early stages of growth and at maturation. Numbers in all plant parts were between 10^3 and 10^6/g fresh wt. Highest numbers (up to 8×10^6/g fresh wt) were observed in cane trash (i.e. leaves left on the ground from the last cut). The diazotroph was also isolated from xylem sap, indicating translocation of the bacterium through the plant tissues in the xylem (Reis 1991).

Using the improved isolation methodology, various samples from six different forage grass species, from roots, stems and leaves of rice, from roots of sorghum and maize and from 11 weed species collected within sugar cane fields were all found to be negative for *A. diazotrophicus.* An endophytic nature and high specificity were proposed for this bacterium on the basis of observations showing it to occur exclusively in sugar cane, sweet potatoes and Cameroon grass; all plants that are propagated vegetatively and that contain high sugar concentrations (Döbereiner *et al.* 1988). This specificity was confirmed by Li & MacRae (1992), who were unable to isolate the diazotroph from a large number of Gramineae with the exception of sugar cane.

Table 3. Occurrence of *A. diazotrophicus* in sugar cane roots in two fields in Rio de Janeiro State.

	Cell density (10^5 cells/g fresh wt) in the sugar cane cultivars:*			
	CB45-3	NA56-79	RB73-9735	RB73-9359
New area planted without N fertilizer				
First cut	2	3	4	0.2
Regrowth	20	100	70	ND
Area with monoculture of sugar cane for many years with 60 kg N/ha applied on regrowth (ratoon crop)				
First cut	9	> 100	> 100	70
Regrowth	< 0.01	70	80	2

*Values are means of two replicates.
ND—Not determined.

To confirm transmission of *A. diazotrophicus* within sugar cane cuttings, the methods used to treat setts before planting were investigated. In the São Paulo region of Brazil, for example, sugar cane setts are normally heat treated before planting to eliminate the plant pathogen *Clavibacter xyli* subsp. *xili* which causes ratoon stunting disease. The occurrence of *A. diazotrophicus* in cane from fields where such heat treatment is used was compared with its occurrence in untreated plants. All 10-day-old rootlets sprouting from sugar cane pieces of five cultivars contained 10^4 to 10^6 *A. diazotrophicus* cells/g fresh wt, whether the pieces were from areas where sugar cane setts were heat treated before planting (52°C for 30 min) or were from untreated plants. There were no clear differences between heat-treated and untreated cane setts, confirming the tolerance of the diazotroph to the temperature used, as has been observed under conditions of pure culture (Reis 1991). Table 4 shows the results of acetylene reduction assays of intact sugar cane systems using setts treated with heat shock. The uninoculated controls had similar nitrogenase activity to the inoculated setts. These observations support the hypothesis that the diazotrophs are transmitted mainly within the stem cuttings and that inoculation of the setts will not enhance N_2 fixation.

The sugar cane mealybug (*Saccharococcus sacchari*) which punctures sugar cane at the leaf sheath 'pocket' and which is common in all countries which grow sugar cane, may transmit the bacterium to uninfected cane. We have isolated the bacterium from this mealybug (data not shown) and Ashbolt & Inkerman (1990) also reported the occurrence of acetic acid bacteria in this mealybug and identified some of the isolates as *A. diazotrophicus* according to Gillis *et al.* (1989).

Overall, the present results and those of Paula *et al.* (1991), who reported the occurrence of *A. diazotrophicus* in roots, tubers and stems of sweet potato, and those of Li & MacRae (1992), confirm the endophytic nature of a diazotroph–plant association. This is in contrast to the classical rhizosphere associations which were thought to account for all BNF in non-legume crops, and is a much better explanation for the big

Table 4. Nitrogenase activity in intact soil–plant systems of heat-treated (52°C for 30 min) sugar cane setts inoculated, or not, with *A. diazotrophicus*.

	Nitrogenase activity (μmol ethylene reduced/h. pot)*		
	Cane cultivar SP70-1143	Cane cultivar SP79-2312	Control *B. arrecta*
Soil from area cultivated with *Paspalum notatum*			
Not inoculated	1.38 (0.17)	1.07 (0.30)	0
Inoculated	1.16†	1.18 (0.39)	0
Soil from area cultivated with sugar cane			
Not inoculated	2.82 (0.63)	4.40 (0.86)	0
Inoculated	2.10 (1.05)	4.59 (2.38)	0

* Values are means (and standard errors) of four replicates.
† In this treatment three replicates were lost.

contributions BNF makes to Brazilian sugar cane genotypes, varieties which have been selected for centuries with low N fertilizer levels (Lima *et al.* 1987; Urquiaga *et al.* 1992).

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