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Acetobacter diazotrophicus, an indoleacetic acid producing bacterium isolated from sugarcane cultivars of México

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Abstract

Thirteen cane cultivars grown on fields in México were sampled to assess the occurrence of *Acetobacter diazotrophicus*, a recently identified N_2-fixing bacterium. Results showed that the isolation frequencies extended over a broad range (1.1 to 67%), likely to be related to the nitrogen fertilization level. The lowest isolation frequencies (1.1 to 2.5%) were obtained from plants growing at high nitrogen doses (275–300 kg ha^{-1}) and the highest values (10–67%) from plants cultivated with 120 kg N ha^{-1}. All eighteen strains of *A. diazotrophicus* produced indoleacetic acid (IAA) in defined culture medium. Estimates obtained from HPLC analyses revealed that *A. diazotrophicus* strains produced from 0.14 to 2.42 μg IAA mL^{-1} in culture medium. Considering that *A. diazotrophicus* is found within the plant tissue, the biosynthesis of IAA suggests that the bacteria could promote rooting and improve sugarcane growth by direct effects on metabolic processes, in addition to their role in N_2 fixation.

Introduction

Nitrogen fixing bacteria are commonly found in association with the roots of diverse plants. Frequently several species of diazotrophs can be isolated from the same plant (Patriquin et al., 1983), but according to the technique used the type of bacteria differs (Balandreau, 1983). Thus, Döbereiner (1961) found *Beijerinckia* spp. in 95% of the rhizosphere soil samples of sugarcane. Prevalence of *Azotobacter chroococcum* in the rhizosphere of sugarcane was reported (Zafar et al., 1986). Rennie et al. (1982) isolated *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Erwinia herbicola* and *Bacillus polymyxa*, from inside sett and roots of sugarcane, but neither *Beijerinckia* nor *Azotobacter* spp. were found. Although several nitrogen-fixing bacteria have been isolated from sugarcane, it still remains unknown which of these bacteria are the most important in the plant-associated biological nitrogen fixation.

Recently, *Acetobacter diazotrophicus*, a N_2-fixing bacterium has been isolated from sugarcane roots and inside stems collected in various sites of Brazil (Cavalcante and Döbereiner, 1988) and of Australia (Li and Macrae, 1991). Since this bacterium is able to fix N_2 even in presence of nitrates and it seems best adapted to the sugarcane environment (Cavalcante and Döbereiner, 1988), it could have more economic importance compared with other diazotrophs associated with sugarcane.

The ability to synthesize auxins has been

described in different epiphytic bacteria (Libbert and Risch, 1969) and soil microorganisms (Brown, 1972; Prikryl et al., 1985), including several diazotrophic bacteria (Barea and Brown, 1974; Clark, 1974; Tien et al., 1979). It has also been shown that epiphytic bacteria could increase the IAA content in the plant (Libbert et al., 1966). It is possible that diazotrophs living at the plant surface or as endophytic bacteria may improve plant productivity either by the involvement of plant growth-regulating substances, by supplying nitrogen, or by both.

This work was conducted to assess the occurrence of the endophytic bacterium *A. diazotrophicus* in roots and stems of different sugarcane cultivars sown in México. Also, we report for the first time the production of indoles by *A. diazotrophicus* cultured strains.

Material and methods

Locations and sugarcane samples

Three complete plants, with various stems each one, were collected from several sugarcane varieties during the years 1990–1991 in different cultivated areas of México (Table 1). Whole plants were sampled by excavating the roots to a depth of 25–30 cm. Collection from each farm was done from cane plants growing for 7–10 months, to a distance of 25 m between plants.

Media

N-free-semisolid acetic LGI medium (SA-LGI) complemented with sugarcane juice at pH 4.5 (Cavalcante and Döbereiner, 1988) was used as an enrichment culture for *Acetobacter diazotrophicus*. For isolation and pure cultures, acetic LGI agar plates (pH 4.5) supplemented with yeast extract (50 mg L^{-1}) and cycloheximide (150 mg L^{-1}) were used. Also, potato agar plates with 10% cane sugar were used. Inocula for the determination of indole compounds were grown in glucose-yeast extract (GYC) broth (De Ley et al., 1984) without $CaCO_3$. To determine extracellular indole compounds synthesized by *A. diazotrophicus* isolates, the cultures were grown at pH 6.0 in liquid LGI medium, replacing crystallized cane sugar by 1.0% sucrose (analytical grade) and supplemented with 0.1% NH_4Cl. The medium was supplemented with 100 μg mL^{-1} filter-sterilized tryptophan. All other media were sterilized at 121 °C for 20 min.

Isolation

Sugar cane root samples (three per each cultivar) were rinsed with tap water until the liquid was clear; then, the roots were washed with sterile distilled water. Subsequently, roots were immersed in 1% Chloramine T for 5 min, followed by washing in 25 m*M* phosphate buffer and by four changes of sterile water. The surface disinfected samples were macerated in a blender at high speed for 2 min in cold sterile sugar solution (5%), to give a 1/4 (w/v) dilution. Nine stems of each cultivar were divided into basal, intermediate and apical regions, in relation to distance from the soil; stalk pieces of 10-15 cm, including the node, were cleaned with a commercial detergent and washed with tap water. Subsequently, the stalk pieces were washed in sterile distilled water and immersed in 1% Chloramine T. After the rind was discarded in sterile conditions, the stem samples were treated as mentioned for roots. 500 μL aliquots from the roots or stem macerates were inoculated in 10 mL serum vials containing 5 ml of SA-LGI (90 replicates per root or 270 per stem of each cultivar). These cultures were incubated at 30 °C for 5–7 days. Then such vials were replicated into SA-LGI and incubated at 30 °C. After 5 days, the vials were assayed for acetylene reduction activity (ARA). Nitrogenase positive vials with thick yellow surface pellicle were streaked onto acetic LGI agar plates, incubated at 30 °C. After 7 days dark orange colonies were picked into SA-LGI and incubated as mentioned above. Thereafter, vials with growth were streaked onto potato agar plates to check for purity, and transferred to acetic LGI (pH 6.5) agar slants for storage of cultures. Strains were considered to be different when they were isolated from different plants, according to the criteria of Cavalcante and Döbereiner (1988).

Identification of Acetobacter diazotrophicus

Isolates identification was based on characteristic biochemical tests, such as, N_2 fixation with and without NO_3, overoxidation of ethanol and glucose, NO_3-reduction, growth and N_2 fixation with 30% glucose or sucrose, growth and N_2 fixation at pH below 3.0, growth and acid production from different carbon sources (sucrose, galactose, fructose, glucose, maltose, mannose, arabinose, ethanol, i-inositol, mannitol and glycerol), and growth on organic acids (acetate, lactate, malate, fumarate and succinate), among other tests; colonial morphology in LGI and potato agar plates was considered also (Cavalcante and Döbereiner, 1988; Gillis et al., 1989). *A. diazotrophicus* strains PAl 5^T (Type strain, ATCC 49037), PPe 4 (ATCC 49038) and PR 2 (ATCC 49039), kindly provided by Dr J Döbereiner, were used as controls in all performed tests.

Acetylene reduction assay

Gas chromatograph and conditions to determine ARA were as we described previously (Mascarúa-Esparza et al., 1988).

Indole compounds assays

To determine indole compounds synthesis by isolates of *A. diazotrophicus*, an inoculum was prepared by growing the organisms (GYC broth) in shake culture (220 rpm) for 36 h at 30 °C and then the cultures were twice centrifuged and resuspended each time with 50 m*M* KH_2PO_4 (pH 6.0). The bacterial cultures were adjusted to 50 Klett units (green filter; 1×10^8 UFC mL^{-1}). *A. diazotrophicus* strains (1.0 mL inoculum) were grown in 125 mL flasks containing 49 mL of medium in shake culture (200 rpm) for 24 h at 30 °C. Bacterial cultures were centrifuged (13,000 g for 15 min at 4 °C); the supernatant obtained was filtered through 0.45 μm pore size Millipore filter (Harari et al., 1988). Subsequently, the supernatants were adjusted with HCl to pH 2.7. Procedure for indoles extraction, treatment of the solvents and TLC plates were done according to Iino et al. (1980). Indole compounds were extracted three times with 30 mL volumes of ethyl acetate, which was previously washed with water, passed through cotton wool and adding BHT (butylated hydroxytoluene), as antioxidant. The extracts were evaporated to dryness under vacuum with rotary evaporator at 37 °C, then the residue was dissolved in 1.0 mL of methanol. An aliquot of 50 μL in methanol was chromatographed on silica gel (HF_{254}, Merck) thin layer plates. The plate was developed using the solvent system benzene-ethyl acetate-acetic acid (70:25:5). Authentic indoles, indole-3-acetic acid (IAA), indole-3-aldehyde (IAld), indole-3-lactic acid (ILA), indole-3-butyric acid (IBA), indole-3-propionic acid (IPA) and indole-3-pyruvic acid (IPyA) were chromatographed as referenced. The separated compounds were visualized by their fluorescence under ultraviolet (UV 254 nm) light and colour developed with Van Urk-Salkowski reagent (Ehmann, 1977). The spots corresponding to IAA were scraped from the silica gel plates. Solid support was suspended in methanol, centrifuged and aliquots analyzed by high performance liquid chromatography (HPLC). HPLC analysis was performed by ion-pair reversed-phase on a Waters Associates μBondapak C_{18} Rad-Pak cartridge (10 cm × 8 mm i.d.) and a mobile phase of 27.5% methanol in 0.1 *M* KH_2PO_4 (pH 6.5) containing 10 m*M* tetrabutylammonium phosphate (Millipore, Corp.). Flow rate was 1.5 mL min^{-1} and the operating pressure was 900 lb/in^2. Detection was at 280 nm (Waters 440 Absorbance detector, mercury lamp) and quantitation was made by area integration through the Waters Data Module microprocessor. Retention times for peaks were compared to that of authentic IAA standard.

Results and discussion

Several diazotrophic endophytic isolations from diverse sugarcane cultivars grown in various areas of México (Table 1), were properly identificated as belonging to the species *Acetobacter diazotrophicus*, all the strains exhibited the same morphological, biochemical and physiological characteristics reported by Cavalcante and

Table 1. Isolation frequencies of *Acetobacter diazotrophicus* from different sugarcane cultivars sown in field

Location	Cultivar	Isolation (%)	
		Root	Stem
Atencingo, Puebla[1]	MEX 57-473	2.5	N.D.
Atencingo, Puebla[1]	CP 72-2086	2.1	N.D.
Atencingo, Puebla[1]	MY 55-14	N.D.	1.3
Atencingo, Puebla[1]	MEX 76-646	0.0	0.0
Yautepec, Morelos[1]	MEX 68-808	0.0	0.0
Yautepec, Morelos[1]	CP 29-203	0.0	0.0
Yautepec, Morelos[1]	Z MEX 55-32	0.0	1.7
Yautepec, Morelos[1]	MEX 69-749	N.D.	1.1
Córdoba, Veracruz[2]	MEX 56-476	10.0	30.0
Córdoba, Veracruz[2]	MEX 69-290	N.D.	17.0
Córdoba, Veracruz[2]	MEX 73-523	55.0	67.0
Orizaba, Veracruz[2]	MEX 79-546	65.0	N.D.
Orizaba, Veracruz[2]	MEX 80-499	10.0	N.D.

[1] N fertilizer added to the cultivar: 275–300 kg ha^{-1}.
[2] N fertilizer added to the cultivar: 120 kg ha^{-1}.
N.D. Not determined.

Döbereiner (1988), and Gillis et al. (1989). Moreover, it is known that 16S ribosomal RNA (16S rRNA) molecule is universal and conserved in functions (Young, 1992). Comparisons between the 16S rRNA molecule of strains PR 2 (ATCC 49039) and UAP 5665 (isolated in this work) revealed that they belong to the same species (P. Young, pers. comm.).

Isolation frequencies from roots or inside stems ranged from 1.1% to 67% (Table 1). Interestingly, the lowest isolation frequencies (1.1 to 2.5%) were obtained from locations where the rate of nitrogen fertilization was the highest (275–300 kg ha^{-1}). In addition, we could not obtain isolates from some sugarcane varieties (CP 29 203, MEX 68 808 and MEX 76 646) that were sampled in those locations. In contrast, we successfully isolated *A. diazotrophicus* in high frequencies (10 to 67%) from all the cultivars sampled in places where N fertilization rate was 120 kg ha^{-1} only. These results suggest that associations between nitrogen fixing bacteria and plants may be severely limited when nitrogen fertilizers are supplemented in excess. Oaks (1992) considers that interactions and associations, between microorganisms and their host plant, may be inhibited by high levels of added fertilizers, as occur with *Rhizobium* spp. and mycorrhizal fungi and their host. We do not discount other factors, such as varietal and environmental effects, that could be responsible for the results mentioned above. Unfortunately, the same sugarcane variety was not found in the different sampled localities to support our hypothesis.

It is noteworthy that the isolation frequencies of *A. diazotrophicus* from different parts of the stem varied. The higher frequencies were found in the apical stem region as compared to basal or intermediate regions (data not shown). This preferential distribution of *A. diazotrophicus* also occurs with other bacterial species found in the inner sugarcane stems. *Azotobacter vinelandii* has been found only in the apex, while *Erwinia herbicola*, was found both in apical and intermediate stem regions (Ruschel and Vose, 1984). We think that preferential distribution of *A. diazotrophicus* and probably some other endophytic sugarcane bacteria could be regulated by the quantity and quality of soluble sugars prevailing in a particular region of the plant. Nevertheless, this speculation should be demonstrated.

Interestingly, all different tested strains of *A. diazotrophicus* were able to produce indoles in defined liquid medium supplemented with tryptophan, as visualized by thin layer chromatography (TLC) plates. TLC analysis of acidic ethyl acetate extracts from eighteen *A. diazotrophicus* cultures always showed an identical color, after spraying with Van Urk-Salkowski reagent, and Rfs corresponding to standard IAA (Rf 0.546),

IAld (0.416), ILA (Rf 0.178) and IPA (Rf 0.614). HPLC analysis of IAA spots scraped of TLC plate corresponded to the retention time (6.2 min) of authentic IAA.

It is well known that many microbes and plants convert tryptophan to IPyA, an intermediate of IAA biosynthesis in higher plants. Even though we do not detect IPyA, we found ILA, which is a product of reduction of IPyA (Sheldrake, 1973). This result suggests that the biosynthesis of IAA from typtophan, by *A. diazotrophicus*, is via indole-3-pyruvic acid (Sandberg et al., 1987).

Production of IAA by 18 strains of *A. diazotrophicus* analyzed quantitatively by the colorimetric Salkowsky assay (Tang and Bonner, 1947), was as high as 19 to 65 $\mu g\ mL^{-1}$ (data not shown). These IAA levels produced by *A. diazotrophicus* are similar to those reported for *Azospirillum brasilense* (Hartmann et al., 1983; Jain and Patriquin, 1985) using the Salkowsky reaction. However, it has been demonstrated (Crozier et al., 1988) that the Salkowsky assay is not a reliable method for measuring IAA.

On the other hand, estimates obtained from HPLC analyses revealed that four *A. diazotrophicus* strains produced IAA above 1.0 $\mu g\ mL^{-1}$ (Table 2), with the highest amount (2.42 $\mu g\ mL^{-1}$) found in the culture medium from PAl 5^T strain. The other six strains yielded lower amounts at rates of 0.14 to 0.77 μg IAA mL^{-1}. This bacterium produced similar rates of IAA in culture medium, to those reported for the rhizosphere bacteria of the genus *Azospirillum* in quantitative estimates obtained by HPLC procedures (Crozier et al., 1988).

Table 2. Indoleacetic acid production by different strains of *Acetobacter diazotrophicus*

Sugarcane cultivars	PIS[a]	Strain designation	IAA conc. ($\mu g\ mL^{-1}$)
MEX 57-473	(Wr)	UAP 5701	1.07
MEX 73-523	(Ap)	UAP 7306	1.00
MEX 73-523	(Ap)	UAP 7308	1.12
CP 72-2086	(Wr)	UAP 7210	0.31
MEX 69-290	(In)	UAP 6925	0.77
Z MEX 55-32	(In)	UAP 5560	0.57
MEX 56-476	(Sr)	UAP 5665	0.22
(1)		PAl 5	2.42
(1)		PPe 4	0.14
(1)		PR 2	0.49

[a] Plant isolation site: Surface-sterilized root (Sr); Washed root (Wr); Inside stem (Intermediate [In]; Apical [Ap]); stem region in relation to distance of soil.
(1) Gillis et al., 1989.

Taking into account that *A. diazotrophicus* is found within the plant tissue and is propagated via the planting material (stalk pieces), IAA production suggests that this bacterium could promote rooting and improve sugarcane growth by direct effects on metabolic processes, in addition to a role in N_2 fixation as suggested (Boddey et al., 1991; Cavalcante and Döbereiner, 1988).

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