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A Nitrogen-Fixing Endophyte of Sugarcane Stems[1]

A New Role for the Apoplast

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The intercellular spaces of sugarcane (*Saccharum officinarum* L.) stem parenchyma are filled with solution (determined by cryo-scanning microscopy), which can be removed aseptically by centrifugation. It contained 12% sucrose (Suc; pH 5.5.) and yielded pure cultures of an acid-producing bacterium (approximately 10^4 bacteria/mL extracted fluid) on N-poor medium containing 10% Suc (pH 5.5). This bacterium was identical with the type culture of *Acetobacter diazotrophicus*, a recently discovered N_2-fixing bacterium specific to sugarcane, with respect to nine biochemical and morphological characteristics, including acetylene reduction in air. Similar bacteria were observed in situ in the intercellular spaces. This demonstrates the presence of an N_2-fixing endophyte living in apoplastic fluid of plant tissue and also that the fluid approximates the composition of the endophytes's optimal culture medium. The apoplastic fluid occupied 3% of the stem volume; this approximates 3 tons of fluid/ha of the crop. This endogenous culture broth consisting of substrate and N_2-fixing bacteria may be enough volume to account for earlier reports that some cultivars of sugarcane are independent of N fertilizers. It is suggested that genetic manipulation of apoplastic fluid composition may facilitate the establishment of similar symbioses with endophytic bacteria in other crop plants.

In some areas in Brazil, sugarcane has been grown continuously for more than 100 years without any nitrogenous fertilizer. It has long been suspected that substantial N fixation occurs in such systems (Neyra and Döbereiner, 1977). In Hawaii, experiments with ^{15}N showed that only a small proportion of fertilizer N given to sugarcane land was recovered in the crops; 70% of the N came from other sources (Takahashi, 1970). Recently, Boddey et al. (1991) and Urquiaga et al. (1992) concluded from ^{15}N and N balance studies that some sugarcane varieties in Brazil are particularly effective in obtaining their N from associative biological fixation. N fixation associated with the roots of Brazilian sugarcane in the soil has been demonstrated by acetylene reduction (Döbereiner et al., 1972) and by $^{15}N_2$ fixation (Ruschel et al., 1975, 1978; Lima et al., 1987). Although at least 11 genera of N_2-fixing bacteria have been isolated from the rhizosphere of sugarcane, none occur in large enough numbers to account for the high rates of N fixation found in these crops.

Recently, a new microaerobic N_2-fixing bacterium was isolated in large numbers from sugarcane stems, roots, leaves, and rhizosphere soil from sites in Brazil, Australia, and Mexico (Cavalcante and Döbereiner, 1988; Li and MacRae, 1991, 1992; Fuentes-Ramirez et al., 1993). This bacterium, *Acetobacter diazotrophicus* (Gillis et al., 1989), has most unusual growth requirements. It shows optimal growth with 10% Suc and pH 5.5. It will grow in medium with Suc concentrations up to 30% and rapidly acidifies its surroundings by the formation of acetic acid. It has no nitrate reductase: its N_2 fixation continued in air, was unaffected by the presence of 80 mM NO_3^-, and was barely inhibited by NH_4^+ (Fu et al., 1988; Li and MacRae, 1991). In Australia, it was isolated from sugarcane only and not from other grasses in the same location (Li and MacRae, 1991). In Brazil, it was found in the sugarcane but not in soil between rows of sugarcane nor on roots of 12 weed species in the cane fields (see Boddey et al., 1991). We have made similar isolations from a number of varieties of sugarcane in Cuba. This bacterium is a prime suspect as the contributor of fixed N to sugarcane crops, but the details of the symbiosis are unknown.

The previous isolations of *Acetobacter* from sugarcane tissues achieved by us and by all other workers have been slow and laborious. The cultures grow very slowly (15–30 d to first visibility) and need to be diluted 10^3- to 10^7-fold to ensure that a pure culture of the *Acetobacter* is obtained.

It has been suspected, but not certainly shown, that the *Acetobacter* lives within the sugarcane tissues and is not merely associated with the surface of the plant because it has often been isolated from surface-sterilized tissue (Döbereiner et al., 1988). It is difficult to prove that this bacterium is indeed an endophyte because in rugged plant material like sugarcane there are many surface refuges for bacteria, and contamination of internal tissues during excision is always a possibility. Nevertheless, our own isolations of the putative *Acetobacter*, with careful attention to sterile technique, from peeled stem pieces that had been dipped in ethanol and ignited, convinced us that the bacteria must indeed be living

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Abbreviation: SEM, scanning electron microscopy.

inside the stems in some kind of symbiotic relationship with the host tissues. There are no obvious structures in sugarcane such as the nodules of legumes that might be the basis for the exchanges of solutes between the two organisms. Nevertheless, for a successful symbiosis to occur, the same requirements must be met—a supply of carbon from the plant and N from the air, transport of amino-carbon compounds back to the plant, and a controlled supply of oxygen to allow bacterial respiration without inhibiting the nitrogenase. Therefore, we have conducted a search for the endophyte in the stem tissues to determine whether it could be found inside cells or outside them, in vascular tissues or in the ground parenchyma, in nodes or in internodes.

The stem tissues of sugarcane have been the subject of study over many years as the site of Suc storage. The parenchyma cells are in linear files, so that the spaces between them form longitudinal channels, separated from each other but open at the surface of a transverse cut. Fluid in them is accessible to diffusive exchange and can be removed by centrifuging parallel to the radius vector. Welbaum and Meinzer (1990) confirmed the older results of Hawker (1965) that there is much Suc in the apoplast of the stem parenchyma. The solution obtained by centrifuging the stem pieces contained up to 0.5 M Suc in the older internodes, and the estimates of the volume occupied by this solution are in the range of 10 to 25% of the tissue volume (Welbaum and Meinzer, 1990). These authors assumed that a first centrifugation for 10 min at 30*g* removed surface solution from the cut cells, which they discarded. Then, a second centrifugation for 10 min at 480*g* was assumed to yield solution from the apoplast. The origins of the solutions were not checked further.

We hypothesized that the Suc-requiring endophyte might be living in the Suc solution in the intercellular spaces of the sugarcane stems, and proceeded to investigate (a) whether the solution was indeed in the intercellular spaces, (b) whether the solution centrifuged from stem pieces came from the intercellular spaces, (c) whether this fluid contained the endophyte, and (d) whether the endophyte could be seen in the intercellular spaces.

Fluid in intercellular spaces of plant tissues may be seen and measured in planed frozen pieces in the cryo-SEM (Canny and Huang, 1993). We used this technique to reveal the contents of the spaces, to show the extent to which they were emptied by centrifugation, and to measure the volume fraction of the tissue they occupied.

MATERIALS AND METHODS

Plant Material

Plants of sugarcane (*Saccharum officinarum* L.), varieties Media Luna and Ja 60–5, were grown either in the fields of the Estación Biológica Docente, Facultad de Biologia, Universidad de La Habana or in pots in the greenhouses of Carleton University with supplementary lighting from Optimarc Super Metal Halide (Tungsten Products, North Bergen, NJ) lamps. At Carleton University the Cuban parent material was subcultured successively from stem node cuttings. Mature stem internodes were collected from nodes 10 to 20 proximal to the apex.

Centrifugation of the Stem Pieces

All preparatory operations up to the sealing of the centrifuge tubes were performed in a laminar flow hood. Pieces of stem internode were surface sterilized with 70% ethanol and cut transversely into lengths of approximately 3 to 4 cm with sterilized saw blades or surgical knives. Further dissection of the internode tissues into pieces that would fit into centrifuge tubes was with sterile knife blades. To test for the presence of bacteria, stem pieces were further sterilized before centrifugation by dipping them in ethanol and setting fire to them. In addition to killing any organisms on the surface of the pieces, this procedure dried the solution on the outside of the stem piece so that it was not collected in the centrifuge tube.

Small stem pieces were centrifuged in Eppendorf tubes; large pieces were centrifuged in 10- × 2.5-cm glass tubes; both types of tube were autoclaved before use. Speeds of the centrifuge were adjusted to produce accelerations from 30 to 3000*g*, and the effects of the force on the solutions in the intercellular spaces and on the surface of the piece were assessed in the cryo-SEM. An acceleration of 3000*g* was taken by Welbaum and Meinzer (1990) as the highest acceleration that did not displace fluid from the symplast. Six stem pieces of var Ja 60–5 were weighed before and after centrifuging at 3000*g* for 20 min, and the extracted solutions were pooled and weighed. The proportion of the tissue occupied by the extracted solution was expressed as a percentage (w/w). We assumed that the solution had the same density as the rest of the tissue (see below), and this percentage is also the same on a volume/volume basis.

Cryo-SEM of Stem Tissues

The sizes and shapes of liquid spaces may be examined on the flat-planed surfaces of frozen material as described by Huang et al. (1994). Briefly, pieces (approximately 5 × 2 × 2 mm) were cut from the stems and mounted on stubs with Tissue Tek (Miles, Inc., Elkhart, IN) and immediately frozen in N_2 slush. The stub was transferred under liquid N_2 to the chuck of a cryo-microtome (CR2000; Research and Manufacturing, Inc., Tucson, AZ), and the sample was planed with a glass knife at −80°C and transferred under liquid N_2 and then under vacuum to the cold block in the cryo-preparation chamber (CT1500; Oxford Instruments, Eynsham, Oxford, UK), held at −180°C. From there it was moved to the sample stage (−170°C) in the column of the SEM (JSM 6400; JEOL Ltd., Tokyo, Japan) and observed uncoated at 1 kV while the stage was warmed and the specimen was very lightly etched to reveal traces of the cell shapes. The temperature of the stage was set at −82°C for the etching, and etching was stopped when the cell walls and intercellular spaces became visible. The specimen was transferred to the preparation chamber, given a standard coating (50 nm) of Al (Hopkins et al., 1991), and returned to the sample stage at −170°C for observation. Specimens were observed in standard secondary-electron mode at 5 to 7 kV and photographed on Kodak T-Max 120 roll film, which was developed in T-Max developer (Kodak, Rochester, NY).

Measurement of Volumes of Walls and Spaces

Images of the planed frozen faces of stem parenchyma were printed at standard magnification and analyzed stereologically following the methods detailed by Steer (1981) to measure the cross-sectional areas of the cell walls and of the intercellular spaces. The spaces were sampled by intersections with a grid; wall areas were estimated from the product of length multiplied by thickness. Systematic transects of 10 images across each transverse section of the stem were made on samples before and after centrifuging. The percentage area of these spaces in transverse section equals the percentage of the volume of the tissue they occupy.

Culture of the Endophyte

Culture of the type strain (PAL ATCC 49037) of *Acetobacter diazotrophicus* and of bacteria isolated from sugarcane tissues was on the N-poor medium LG1-P (Cavalcante and Döbereiner, 1988), which contains 10% Suc and has a pH of 5.5, in liquid culture or on solid medium (1.5% agar) at 30°C. The formation of acid by the cultures was tested by adding the indicator bromothymol blue to the medium. This indicator is green at the initial pH of the medium (5.5) and changes to yellow at pH 5.0. The bacteria are unusual in accumulating this indicator and becoming yellow-orange. Because the bacteria produce acetic acid, extended culture (beyond 10 d) requires the addition of solid $CaCO_3$ to the medium to neutralize the acid.

Counting of Bacteria in Apoplastic Fluid

Very irregular counts from initial serial dilutions were obtained because the bacteria clump together in their secreted mucilage. It was necessary to vortex the extracted fluid with glass beads before making the dilutions. The dilutions were plated on solid medium, and the number of colony-forming units was determined.

Analysis of Apoplastic Fluid

The pooled extracted apoplastic solutions from the six stem pieces of Ja 60–5 were used to estimate total sugar concentrations with a refractometer. Readings were in percentage (w/v) and were corrected for temperature. The pH of the solution was measured with a hydrogen electrode.

Morphology of the Cultured Bacteria

Drops of the suspension cultures of isolates were placed on grids, negatively stained with 2% aqueous uranyl acetate at pH 3.5, washed, and examined in the transmission electron microscope (Philips 420). Micrographs were recorded on Kodak sheet film.

Nitrogenase Activity

Cultures of the bacteria growing on slopes in vials in air (26-mL gas space) were tested for nitrogenase activity by their ability to reduce acetylene. Freshly prepared acetylene (2 mL) was injected at zero time, and samples of gas (1.0 mL) were withdrawn at 10-min intervals for 1 h and analyzed in a gas chromatograph. The cultures produced no endogenous ethylene when acetylene was not present. The ethylene peaks were calibrated against a 100-ppm standard and standardized for each measurement relative to the area of the methane peak. At the conclusion of the measurements, the bacteria in each vial were washed off the agar and diluted to a standard volume. Aliquots were counted in a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA). Rates of ethylene production are expressed as nmol h^{-1} $(10^9$ cells$)^{-1}$.

In Situ Observation of Bacteria in the Apoplast

Scanning Microscopy

Small slices of stem parenchyma were freeze substituted as described by Canny and McCully (1985) except that acetone was used instead of ether. Specimens were then warmed to room temperature, rinsed in acetone, and cleaved longitudinally with a sharp razor blade. The pieces were critical point dried and sputter coated with gold, and the newly exposed faces were observed in the scanning electron microscope at 20 kV.

Transmission EM

Small longitudinal slices of stem parenchyma were fixed and embedded by standard procedures (3% glutaraldehyde in phosphate buffer at 4°C, postfixation in 1% osmium tetroxide, dehydration in acetone, and embedding in Spurr's resin; see O'Brien and McCully [1981] for details).

RESULTS

By conventional light microscopy and EM, we have failed to find any cells of the endophyte in any of the living cells of the stem nodes or internodes or within the xylem elements of the vascular strands. Furthermore, when the endophyte was deliberately introduced to the xylem in the transpiration stream, a vigorous reaction of the xylem parenchyma filled the vessels with red gums. Thus, it is most unlikely that the endophyte inhabits the xylem vessels.

Contents of the Intercellular Spaces

The images of the frozen faces of sugarcane parenchyma showed the intercellular spaces filled with solution. Within the cells of Figure 1A, the vacuoles are filled with dense patterns of lines. This is the characteristic appearance of a rapidly frozen solution containing considerable solute (Canny and Huang, 1993). During freezing, the solutes are sequestered from the ice and form sheets of roughly parallel arrays. Because the vacuoles of sugarcane stem parenchyma are known to contain Suc (10–20%), we are confident that the appearance of these vacuoles is characteristic of frozen Suc solution. It is clear from Figure 1, A and B, that the material in the intercellular spaces has frozen to give the same solute pattern as is found in the vacuoles. The image is fully consistent with the presence in the spaces of a Suc solution of similar strength to that in the vacuoles. In all of the fresh internodes examined from both varieties of sugarcane, nearly all of the intercellular spaces were filled. A count of full and empty spaces along a transect across a section of tissue

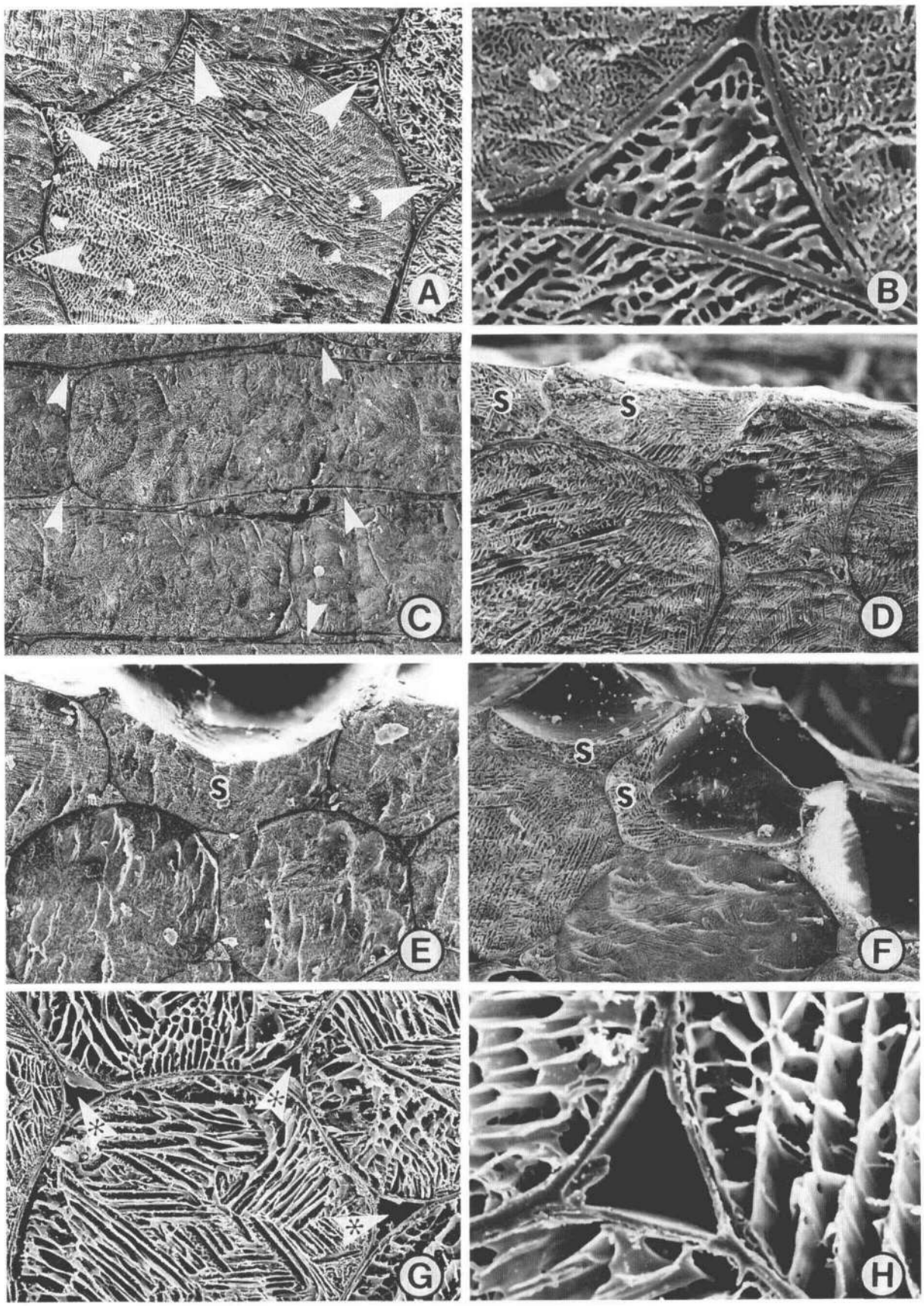

Figure 1. (Legend appears on facing page.)

showed that 4% of the spaces contained air. In longitudinal section (Fig. 1C), it can be seen that the spaces extend past many cells and that the whole space is filled with fluid. None of the common inorganic ions could be detected in vacuoles or spaces with the x-ray microanalyzer attached to the SEM (limit of sensitivity approximately 10 mM).

Sources of the Solution Obtained by Centrifugation

The procedures used by Welbaum and Meinzer (1990) to separate intercellular space fluid—centrifuging for 10 min at 30*g* and at 480*g*—were shown to be unsatisfactory. The vacuolar solutions retained in the cut cells on the surface of the uncentrifuged tissue pieces could be seen filling the remaining cell walls (Fig. 1D). Centrifuging at 30*g* removed only a small part of this surface solution (Fig. 1E). Even after centrifuging for 10 min at 30*g* and an additional 10 min at 500*g*, the surface retained substantial volumes of sugar solution still held in pockets of the cut cells by surface tension (Fig. 1F). Moreover, counts of the empty and filled intercellular spaces along a transect showed that the proportion of empty spaces was still 4%, as in the uncentrifuged tissue. The assumption of Welbaum and Meinzer (1990), that centrifuging at 30*g* clears away the contamination of the cut vacuoles, is therefore not valid. Their further assumption that subsequent centrifugation at 480*g* yields only solution from the apoplast is also invalid. Such a sample would contain almost no solution from the intercellular spaces but much released vacuolar solution, forced from the surface cells by the greater acceleration. Thus, the solution collected by Welbaum and Meinzer at this acceleration may in fact have contained mostly the surface vacuolar solution. If the intercellular spaces in the tissues used by Welbaum and Meinzer were larger than in our tissues (see "Discussion"), smaller accelerations may have been sufficient to dislodge them from the internal spaces, and the extract may have had a larger admixture of solution from the intercellular space apoplast.

To remove most of the solution from the intercellular space apoplast, it was necessary to centrifuge at 2000 to 3000*g* for 15 to 20 min. Figure 1G shows the appearance of the stem parenchyma frozen after such a treatment. All of the cells are intact, but almost all the intercellular spaces are now filled with air (Fig. 1H). This is consistent with the graph of volume extracted versus speed of centrifugation given by Welbaum and Meinzer (1990, fig. 1), which continues to increase up to 3000*g*. An approach other than differential centrifugation was devised to immobilize the sugar solution on the cut surfaces and at the same time to sterilize the surface and avoid contaminating the internal sap, as described in "Materials and Methods," namely dipping the piece in ethanol and igniting it.

Table I. *Comparison of volumes of cell walls and intercellular spaces of sugarcane stem parenchyma with the volume of solution extracted from the stems by centrifugation at 3000g*

Percentage volumes of the spaces and walls were estimated stereologically from SEM images of the frozen, planed stem faces. The mass of the extracted solution is expressed as a percentage of the mass of the stem pieces. The percentage of mass equals the percentage of volume, assuming that both solution and tissue have the same density. Means ± SD (n = 27).

Estimated Volume Percentage of		Extracted Solution	
Intercellular spaces	Cell walls	Percentage mass	Percentage volume
2.8 ± 0.5	2.6 ± 0.3	3.1	3.1

The results of the stereological analysis of the volumes of the cell walls and of the intercellular spaces are given in Table I. The spaces occupied about 3% of the tissue volume. Comparison with the weight (volume) of the extracted solution shows that its volume corresponds to that of the intercellular spaces.

Measurements in the light microscope of the number and sizes of vessels in the vascular bundles of the central parenchyma showed that their cross-sectional area occupied only 0.6% of the central stem tissue. Therefore, their contribution of xylem sap to the centrifuged solution cannot exceed 20% of its volume and may have reduced its concentration to this extent.

Concentration of the Apoplastic Fluid

The refractometer readings made on the extracted intercellular fluid indicated a Suc content of 11 to 13% (w/v). This is approximately the same as the reported concentrations for the vacuolar sap of the surrounding parenchyma cells. The

Figure 1. Scanning electron micrographs of planed frozen faces of tissue from the internodes of sugarcane stems. Filled intercellular spaces are indicated by plain arrowheads; empty intercellular spaces are indicated by arrowheads with asterisks. A, Stem parenchyma of sugarcane var Ja 60–5 planed transversely, showing the intercellular spaces filled with a solution whose freezing pattern is the same as that of the vacuoles of the surrounding cells. ×350. B, Similar preparation to that in A. Single intercellular space filled with frozen solution. ×1530. C, Longitudinally planed stem parenchyma of sugarcane var Ja 60-5 showing the long intercellular spaces filled with solution. ×190. D, The cut edge of a transversely planed piece of stem parenchyma of var Ja 60–5 showing solution lying on the surface in the cells damaged by the cut(s) and undamaged cells below. ×280. E, The cut edge of a piece of tissue similar to that in D but var Media Luna and after centrifuging at 30g for 10 min. A small proportion of the solution had been removed from the cut cells on the surface, but much vacuolar solution(s) remained held in the open cell walls by surface tension. ×280. F, The cut edge of a piece of tissue similar to that in D but after centrifuging at 30g for 10 min and then at 500g for 10 min. Some vacuolar solution(s) still remained held in the open cell walls by surface tension. ×280. G, Stem parenchyma of var Media Luna after centrifuging at 3000g for 20 min. All of the intercellular spaces have been emptied of solution, but the cells are intact. ×550. H, Preparation similar to that in G, showing a single empty intercellular space. ×2160.

similarity of composition and concentration of the two solutions was suggested also by their similar freezing patterns, as pointed out above. The pH of the fluid was 5.5.

Endophyte in the Apoplastic Fluid

Following the protocol of centrifugation for the extraction of the fluid from the intercellular space apoplast worked out above, the fluid was extracted under sterile conditions as described in "Materials and Methods." The flaming of the surface of the stem piece not only killed any contaminating microbes but dried up the vacuolar solution from the cut cells and ensured that the fluid extracted by centrifuging at 3000*g* was indeed the contents of the intercellular spaces. When this fluid was inoculated onto the *Acetobacter* growth medium, vigorous growth of the bacteria was seen after only 3 d. No dilution was necessary to produce uncontaminated cultures of the one organism. Counts of colony-forming units indicated a concentration of 1.1×10^4 bacteria/mL of apoplastic fluid.

Colonies, like those of the type strain of *A. diazotrophicus*, developed a yellow-orange color after about 3 d because of accumulation of the indicator by individual bacteria. They produced acid, changing the green color of the indicator added to the medium to yellow and later decolorizing it. If left beyond 10 d in the absence of $CaCO_3$, the colonies formed lethal concentrations of acid. The colonies, like those of the type strain, are thickly mucoid, which makes it difficult to separate and subculture individual bacteria. Further comparisons between the type strain and our isolates confirmed their similarity with respect to Gram negativity, ability to grow on 30% Suc, and catalase positivity. A summary of the similarities between our isolates and the type strain is presented in Table II.

Bacteria in the isolates were compared with those of the type strain in the electron microscope (Fig. 2), and they agreed in form, size, and the possession of three to five peritrichous flagellae. These figures may be compared with the illustration of the type strain of *Acetobacter diazotrophicus* in Gillis et al. (1989, fig. 2).

This source of the supposed *Acetobacter* is much more reliable and abundant than any of the others previously known. Successful cultures from the intercellular fluid were made from three stems of var Media Luna grown in Cuba (in six separate internodes from each) and from var Ja 60-5 grown at Carleton. Furthermore, in this latter material the endophyte was found in both the original plants grown for 3 years from cuttings from Cuba and in the second and third generations of plants cultured successively by rooted stem cuttings. Thus, the endophyte was transferred through two successive sets of cuttings into the new plants. The ability to make this transition must be a very important property for the maintenance of natural N_2 fixation in the crop.

Table II. *Comparison of characters of the bacteria isolated from the intercellular space fluid of sugarcane stems with those of the type strain of A. diazotrophicus*

Character	Isolates	Type Strain
Flagellae peritrichous	+	+
N fixation	+	+
Growth on 30% Suc	+	+
Growth on LGI-P medium	+	+
Mucoid colonies	+	+
Acid production from Suc	+	+
Yellow colonies on LGI-P	+	+
Catalase	+	+
Gram stain	−	−

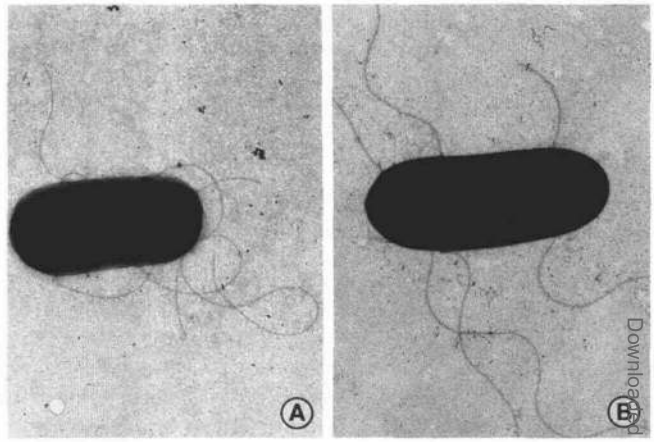

Figure 2. Transmission electron micrographs of negatively stained bacteria. ×27,200. A, Type strain of *A. diazotrophicus*. B, Isolate MC-1 from sugarcane var Media Luna.

In Situ Localization of Bacteria

Observation of bacteria in the apoplast is technically difficult. The parenchyma cell walls are heavily suberized and lignified, and they do not permit free access of fixatives or resins to uncut intercellular spaces. Any bacteria that were free in the apoplast fluid would wash out of cut spaces during the normal preparation for transmission EM. However, some bacteria do remain close to, or against the walls in corners of the intercellular spaces (Fig. 3A). Although these bacteria are often not well fixed, they do resemble morphologically the isolates we have obtained. These bacteria have almost certainly been retained because they were within a mucilaginous colony adhering to the walls lining the intercellular spaces. The aqueous fixation procedure would not preserve the mucilage, but such mucilage clumps were clearly seen in the anhydrously prepared tissue observed with the SEM. Groups of these colonies adhered to the otherwise smooth walls of the spaces, particularly at corners where a longitudinally oriented space joined a radial space (Fig. 3B). Individual bacteria could sometimes be detected at the surface of the mucilage (Fig. 3B, inset).

Nitrogenase Activity

Results of the acetylene-reduction test for nitrogenase activity are shown in Table III. Isolates from var Media Luna showed somewhat greater activity than the type strain, and those from var Ja 60–5 showed slightly less. Both varieties

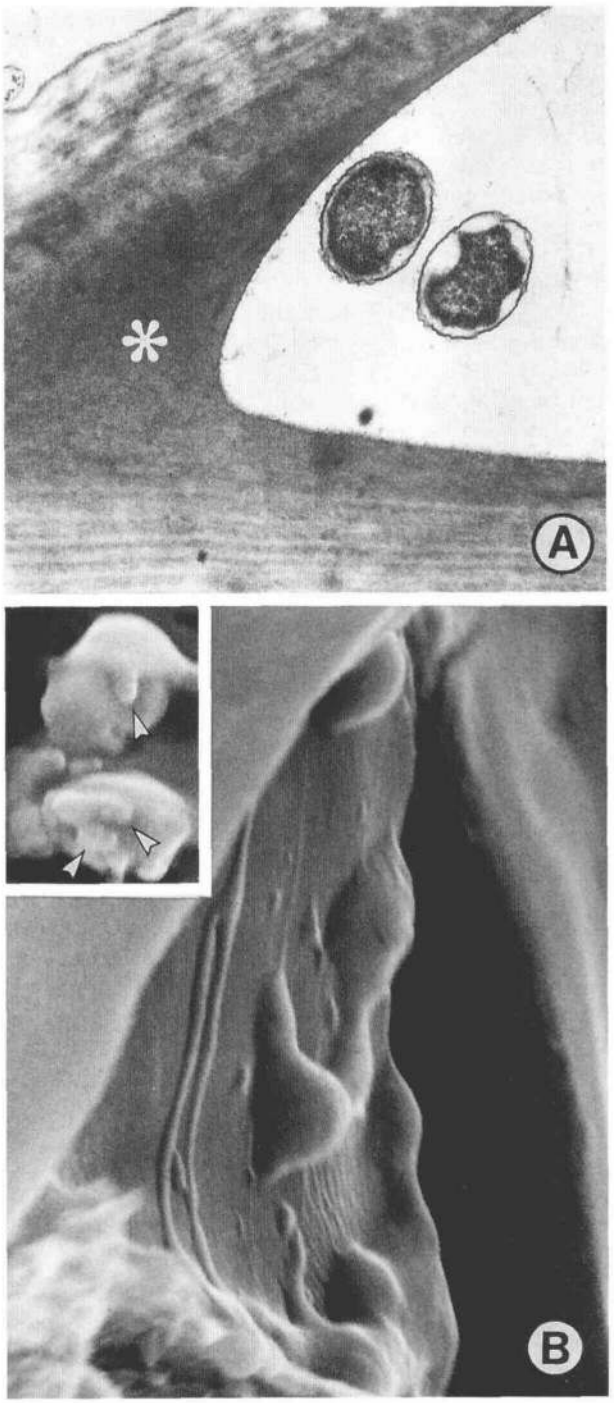

Figure 3. A transverse section through a portion of an intercellular space including two bacteria that have also been sectioned transversely. The asterisk marks the intact middle lamella between the two cells that have partially separated to form the spaces. Transmission electron micrograph; ×22,900. B, A tangential view into an intercellular space showing part of the surface of a cell wall that lines the space. A group of mucoid colonies is attached to this wall. ×10, 500. The inset shows outlines of bacteria (arrows) embedded in such colonies. Scanning electron micrograph. ×7,500.

resembled the type strain in the unusual ability to reduce acetylene in the presence of 21% oxygen.

DISCUSSION

The presence of fluid in intercellular spaces, which have usually been thought of as containing air in other plants, has been suspected. Freshly cut sugarcane stems have a translucent appearance that is inconsistent with many air-filled spaces. Hawker (1965), on the basis of studies of exchange between the tissue and solutions, was confident that the spaces contained sugar solution, although he had no way of directly rendering the space contents visible. He used diffusive exchange of [^{14}C]Suc to estimate the volume of the apparent free space. He gave the volume of this space as 14% of the tissue and concluded that it "is located in the aqueous medium permeating the cell walls and intercellular spaces" (Hawker, 1965). Welbaum and Meinzer (1990) confirmed the presence of a high level of apoplastic Suc by measurements of water potentials. Welbaum et al. (1992) went on to show that this apoplastic sugar in the parenchyma is isolated by semipermeable barriers from the tissues of the vascular bundles and develops a hydrostatic pressure in the stalks when fully hydrated. Their measurements of the properties of the solution extracted from the stem pieces by centrifuging are less convincing for the reasons given above. Their apoplastic fluid was probably mostly the contents of cut cells. The demonstration by Canny and Huang (1993) that the intercellular spaces of corn roots were more or less filled with fluid for much of the time was surprising in isolation, but gains support from the new findings. The suspicion that the space fluid might be an artifact of freezing is groundless in the current work because the solution could be centrifuged from the fresh stem and because its volume matched that of the spaces. Also, the spaces were empty in centrifuged material that was subsequently frozen. A further correspondence between the corn-root and sugarcane-stem space fluids is their similarity in composition and concentration to the contents of vacuoles of the surrounding cells. In corn, the similarity is of ions; in sugarcane, it is of Suc.

Table III. *Rates of acetylene reduction by isolates from intercellular spaces of sugarcane stems and by the type strain of A. diazotrophicus*

Provenance denotes the variety and where the sugarcane was grown. Rates were measured by the formation of ethylene from added actetylene in air by 7-d-old cultures on LGI-P medium without added $CaCO_3$. Rates of reduction as nmol ethylene produced h^{-1} (10^9 cells)$^{-1}$. For all the regression lines of ethylene versus time, $P < 0.0001$.

Strain	Provenance	Reduction Rate
		nmol h^{-1} (10^9 cells)$^{-1}$
Isolate MC-1	Media Luna, Cuba	7.0
Isolate MC-2	Media Luna, Cuba	5.4
Isolate JO-1	Ja 60-5, Ottawa[a]	4.5
Isolate JO-2	Ja 60-5, Ottawa[b]	4.1
Type	Brazil	5.2

[a] First generation of vegetative propagation. [b] Second generation of vegetative propagation.

Hawker's (1965) statement about the solution permeating the cell walls and intercellular spaces illustrates a common view that cell walls are open and porous enough to contain mobile solutions, i.e. solutions can flow in cell walls and may be flushed out of them by centrifugation. This view has been shown to be incorrect (Canny, 1990, 1993; Canny and Huang, 1994). Nearly all cell walls are spaces in which movement in the plane of the wall is by diffusion and at rates 1/100 to 1/10,000 the rate of diffusion in water. Centrifuging the pieces of sugarcane stem extracted volumes of solution equal to the volume of the spaces, not to the volume of spaces plus walls. It should be possible to extract what sugar is present in the walls by refilling the spaces with water and allowing time for diffusive equilibration before recovering the fluid in another centrifugation.

There is unanimity in the published reports of the relative volume of the apparent free space (walls plus fluid in spaces), in finding values considerably larger than ours. Bieleski (1960) reported values in the range 10 to 21%, Hawker (1965) reported 14 plus 4% of air space; Oworu et al. (1977) found 10 to 58%; Welbaum and Meinzer (1990) recorded from 4 to 27%. These authors agree in finding that older internodes have larger proportions of free space. All used indirect methods of estimating the free space—exchanges of solutes or calculations from measurements of water potentials. Although our estimates are direct geometrical measurements, our sampling is based on much more limited collections of material, and most of it was grown under glass at a latitude far outside the range of the species. Otherwise, we cannot explain the discrepancy.

Absolute identity of the bacterial isolates will depend on further work, using the appropriate techniques of molecular genetics. Nevertheless, the collected attributes of *A. diazotrophicus* are so unusual that the probability of confusion with another species is small. No other species is known to live on 30% Suc, to produce acid, to reduce N in air, to form orange mucoid colonies on media containing the indicator, and to have the form and flagellar complement illustrated in Figure 2.

The concentration of bacteria in the apoplastic fluid (10^4/mL) is not large compared with the numbers (10^6-10^7/g dry weight) of *A. diazotrophicus* obtained from homogenized tissues (Li and MacRae, 1992). On the other hand, because of the mucilage that restricts the movement and fluidity of the colonies, the centrifugation may have extracted only part of the total endophyte population present.

We believe that the finding of cultures of the N_2-fixing endophyte (presumed *Acetobacter*) living in 12% Suc solution in the intercellular spaces of sugarcane provides the long-sought answer to the question of how this plant satisfies most of its own needs for N. There is a large amount of the fluid. A harvested crop of sugarcane is approximately 100 tons/ha. If the apoplastic fluid occupies only the 3% of the stems we have measured (and not the larger volumes commonly reported in the literature), there are still 3 tons/ha of the endophyte/sugar broth. This may well be enough to contribute a large part of the approximately 180 kg of N required by the crop each season. The answer could be the basis for extensive programs of further investigation—agricultural, physiological, and microbiological. Testing for the presence, amount, and activity of the endophyte becomes easy and enables the selection of varieties that have it in abundance and the exploration of conditions that encourage it to flourish. The mechanism of the propagation of the endophyte from one generation to the next through the vegetative "sette" (the rooted stem node) must be explored and treatments devised to facilitate transfer through the apoplast.

The symbiosis revealed shows novel features. The endophyte appears to live in an internal, extracellular culture broth supplied by the host plant and closely matching its optimum growth requirements (10% Suc, pH 5.5). The details of how this culture is maintained, supplied with controlled amounts of N_2 and O_2 through the structures of internode and node, and has its pH adjusted to compensate for the acetic acid formed by the bacteria, become a priority for physiological investigation. The N-fixing activities of this system are more accessible to investigation and experiment than those of the legume nodule and provide possibilities for studies of all phases of the process: access of N_2, metabolic transformations by the endophyte, effects and control of O_2 concentration, release of the fixed N to the apoplastic solution, uptake of the fixed N by the parenchyma cells, and transport to other parts of the plant.

If one plant can harbor an endophyte in this fashion, it is possible that others can. The discovery should prompt the investigation of apoplastic solutions in other plants, especially those in which sugar may be free in the apoplast. If one grass can arrange to harbor an N_2-fixing endophyte, it is possible that others can. It may be fanciful to think of corn, wheat, and rice being encouraged to leak Suc into their intercellular spaces so that they might act as hosts to *A. diazotrophicus* but probably less fanciful than thinking that they can be induced to make and operate nodules containing *Rhizobium*.

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