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**THE N₂-FIXING BACTERIUM FROM THE APOPLAST
OF SUGARCANE:
LOCALIZATION, ISOLATION AND CHARACTERIZATION**

ZHONGMIN DONG, B.Sc., M.Sc.

**A thesis submitted to
the Faculty of Graduate Study and Research
in partial fulfillment of
the requirements for the degree of**

Doctor of Philosophy

**Department of Biology
Carleton University**

September 1995

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localization, isolation and characterization

submitted by

ZHONGMIN DONG, B.Sc., M.Sc.

in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Biology

Thesis Supervisors

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ABSTRACT

The intercellular spaces of sugarcane (*Saccharum officinarum* L.) stem parenchyma are filled with fluid (determined by cryo-scanning microscopy), which can be removed aseptically by centrifugation. This fluid contains 12% sucrose (pH 5.5) and yielded pure cultures of an acid-producing bacterium ($\sim 10^4$ bacteria per ml of extracted fluid) on nitrogen-poor medium containing 10% sucrose (pH 5.5). This bacterium is identical to the type culture of *Acetobacter diazotrophicus* (a recently-discovered nitrogen-fixing bacterium specific to sugarcane) with respect to cellular fatty acid composition analysis and biochemical and morphological characteristics, including acetylene reduction and hydrogen evolution in air. Similar bacteria were observed *in situ* in the intercellular spaces. This is the first definitive demonstration of an N_2 -fixing endophyte living in the apoplastic fluid of plant tissue, together with the demonstration that the fluid approximates the composition of the endophyte's optimal culture medium. The apoplastic fluid occupied 3% of the stem volume, constituting about 3 tonnes per hectare of the crop. This endogenous culture broth of substrate and N_2 -fixing bacteria, may account for earlier reports that some cultivars of sugarcane are independent of nitrogen fertilizers. It is suggested that genetic manipulation of the apoplastic fluid composition may facilitate the establishment of similar symbioses with endophytic bacteria in other crop plants.

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TABLE OF CONTENTS

	Pages
Acceptance Sheet	ii
Abstract	iii
Acknowledgments	iv
Table of Contents	vi
List of Tables	x
List of Figures	xi
 Chapter 1: General introduction	 1
The sugarcane plant	1
Biological nitrogen fixation	4
N ₂ -fixing bacteria associated with sugarcane	12
Characterization of <i>Acetobacter diazotrophicus</i>	13
Requirement for a successful associative relationship	16
Specific questions to be investigated in this thesis	18
Organization of subsequent chapters	19
Chapter 2: General methods and materials	21
Plant material	22
Culture of bacteria	23
Gas chromatography	25
Measurements of nitrogenase activity	30
Microscopy and photomicrography	34
Chapter 3: Do <i>Acetobacter</i> live in the xylem of sugarcane	

stems? Anatomical and physiological data.	49
Introduction	50
Materials and methods	53
Plant material	53
Anatomy and histochemistry	54
Apoplastic tracers	55
Results	57
Anatomy of vascular bundles	57
Anatomy of stem parenchyma tissues	60
Vessel continuity	62
Extent of apoplast transport	63
The reaction of xylem to <i>Acetobacter</i>	65
Discussion	66
Isolation of bundles	66
Vessel extent and apoplast transport	67
Response to <i>Acetobacter</i> in vessels	69
Chapter 4: A nitrogen-fixing endophyte of sugarcane stem:	
A new role for the apoplast	81
Introduction	82
Materials and methods	85
Plant material	85
Centrifugation of the stem pieces	86
Cryo-scanning electron microscopy of stem tissues	87
Measurement of volumes of walls and spaces	88
Culture of the endophyte	88
Counting of bacteria in apoplastic fluid	89
Analysis of apoplastic fluid	89

Morphology of the cultured bacteria	89
Nitrogenase activity	90
<i>In situ</i> observation of bacteria in the apoplast	90
Results	91
Contents of the intercellular spaces	91
Sources of the solution obtained by centrifugation	92
Concentration of the apoplastic fluid	94
Endophyte in the apoplastic fluid	95
<i>In situ</i> localization of bacteria	96
Nitrogenase activity	97
Discussion	98
Chapter 5: Further evidence that the N ₂ -fixing endophytic bacterium from the intercellular spaces of sugarcane stems is <i>Acetobacter diazotrophicus</i>	109
Introduction	110
Materials and methods	110
Plants	111
Bacterial strains	111
Conventional morphological criteria and biochemical tests	112
CFA analysis	112
Measurements of hydrogen production	112
Results	114
Discussion	116
Chapter 6: Detection and measurement of hydrogen evolution and acetylene reduction by nitrogenase	124
Introduction	124
Ethylene production from acetylene	128

Hydrogen evolution	129
Hydrogen evolution from <i>A. diazotrophicus</i>	130
Hydrogen evolution from sugarcane stems	130
Effect of oxygen tension on hydrogen evolution	131
Effect of dinitrogen tension on hydrogen evolution	131
Effects of acetylene on hydrogen evolution	132
Speed of assay	134
Chapter 7: Nitrogenase activities and colony structures of <i>Acetobacter diazotrophicus</i> under different oxygen tensions	137
Introduction	137
Materials and methods	139
Strains and culture	139
Colony structure	139
Immobilization and staining of colony mucilaginous	
material	140
Nitrogenase activities	140
Results	141
Morphology	141
Nitrogenase activity	144
Discussion	145
Chapter 8: General conclusion	154
Location of the <i>A. diazotrophicus</i> inside the	
sugarcane stem	155
<i>A. diazotrophicus</i> passes from one generation to the next	155
Nitrogenase activity of <i>A. diazotrophicus</i> is higher than	
that suggested by the acetylene reduction assay	156

Cellular fatty acid (CFA) composition analysis provided further evidence of identification and taxonomic position of <i>A. diazotrophicus</i>	156
The colonies of <i>A. diazotrophicus</i> have the ability to control oxygen concentration at the site of nitrogen fixation	157
The preliminary study of parenchyma cell walls of sugarcane internode stems suggests a possible pathway between the apoplast and the symplast	158
The discovery suggests the possibility of finding or creating similar symbioses in other crops	158
References	159

LIST OF TABLES

Chapter 2

Table 2-1. Calibration of the GC with hydrogen	41
Table 2-2. Calibration of the GC with ethylene	42

Chapter 4

Table 4-1. Volumes of cell walls and intercellular spaces of sugarcane stem parenchyma	103
Table 4-2. Characters of isolates and <i>A. diazotrophicus</i>	104
Table 4-3. Rates of acetylene reduction by isolates	105

Chapter 5

Table 5-1. Standard morphological and biochemical characteristic of <i>A. diazotrophicus</i> type strain and isolates	120
Table 5-2. CFA composition analysis of sugarcane isolates and <i>A. diazotrophicus</i> ATCC 49037	121
Table 5-3. Relative rates of hydrogen and ethylene production by <i>A. diazotrophicus</i> and soybean nodules at different acetylene partial pressures in air	122
Table 5-4. Effect on hydrogen production by <i>A.</i> <i>diazotrophicus</i> of replacing the nitrogenase substrate, nitrogen, with argon at high and low oxygen concentrations	123

LIST OF FIGURES

Chapter 2

- Fig. 2-1. Standard curves of hydrogen gas (ppm)
vs. peak height and peak area with
standard deviations. 43
- Fig. 2-2. Interpolation curve of peak height readings of
standard H₂ samples plotted against ppm. 44
- Fig. 2-3. Double-reciprocal curve of standard hydrogen
gas samples. 45
- Fig. 2-4. Data of Fig. 2-3 plotted in the range from 100
ppm to 1000 ppm, showing the detail of the
left end of the line. 46
- Fig. 2-5. Standard curve for hydrogen in the range
0 -100 ppm, in terms of simplified calibration
equation. 47
- Fig. 2-6. Standard curve for ethylene with curve fit, ranging
from 4 to 100 ppm against peak height. 48

Chapter 3

- Fig. 3-1. Sections of sugarcane stem vascular bundles and
xylem parenchyma cells 71
- Fig. 3-2. Hand sections of stem with green latex paint 72
- Fig. 3-3. Bundle sheath cells of internode stem 73
- Fig. 3-4. Cross sections of internode parenchyma tissue 74
- Fig. 3-5. Sections of internode stems showing the
intercellular spaces and two populations
of parenchyma cells 75
- Fig. 3-6. Cross sections of internodes fed with

sulphorhodamine G	76
Fig. 3-7. Sections of stems fed with sulphorhodamine G in node region	77
Fig. 3-8. Cross sections of internode stem fed with <i>A. diazotrophicus</i>	78
Fig. 3-9. Xylem parenchyma cells responding to <i>A.</i> <i>diazotrophicus</i> in the adjacent xylem vessels	79
Fig. 3-10. Cross sections of vascular bundles of mature internode stem	80
Chapter 4	
Fig. 4-1. Scanning electron micrographs of planed frozen faces of tissue from the stem internodes	106
Fig. 4-2. Transmission electron micrographs of negatively- stained bacteria	107
Fig. 4-3. Electron micrographs of bacteria in plant tissue	108
Chapter 6	
Fig. 6-1. Hydrogen evolution by <i>A. diazotrophicus</i> with acetylene	136
Chapter 7	
Fig. 7-1. Colonies of <i>A. diazotrophicus</i> grown in 2l and 2 kPa oxygen and the bacterial mucilage on nylon membrane	149
Fig. 7-2. <i>A. diazotrophicus</i> colonies on the LGI plates	150
Fig. 7-3. Cross sections of freeze-substituted <i>A.</i> <i>diazotrophicus</i> colonies	151
Fig. 7-4. Hydrogen evolution rate of <i>A. diazotrophicus</i> colonies incubated in 2% O ₂ at different O ₂	

tension

152

Fig. 7-5. Hydrogen evolution rate of *A. diazotrophicus*
colonies incubated in 21% O₂ at different O₂
tension

153

CHAPTER ONE.
GENERAL INTRODUCTION

1.1. THE SUGARCANE PLANT

Sugarcane varieties are species and hybrids of the genus *Saccharum* (Gramineae). For centuries, *S. sinensis* has been grown in China and *S. barberi* in India. The noble sugarcane, the horticultural species *S. officinarum* L., was selected for use in gardens and adapted for survival as garden or horticultural plants (Alexander, 1973), which caused the sugar industry to spread throughout the tropics and subtropics. The clones of *S. officinarum* were for centuries the source of most of the cane sugar of international trade. Now they have been replaced in commercial production by hybrid varieties produced in cane breeding stations in dozens of countries.

The noble sugarcane was introduced to the New World by Columbus in 1493 and rapidly became the most important cash crop in the Caribbean area during the late seventeenth and the eighteenth centuries (Burr *et al.*, 1957). Today, cane sugar is produced for commerce in more than 50 countries and territories within a band around the world bounded by 30° north and south latitudes. World sugar production in 1980 was estimated at over 84 million metric tonnes of which more than 60% (or more than 51 million tonnes) came from cane (Blackburn, 1984). Besides this sugar production, in recent years sugarcane has been used to produce alcohol, with more than 10 billion litres annually available for the replacement of fossil fuels in Brazil (Boddey *et al.*, 1991).

Perhaps more research has been applied to develop sugarcane than to any other commercial crop. All major sugarcane producing

areas maintain experiment stations and publish one or more journals, annual reports, and special bulletins. This has built up a large specialized literature not found in the usual science journals. Numerous excellent books have been written about the classification, origin, growing and processing of sugarcane. The library of the Experiment Station, Hawaiian Sugar Planters' Association, has 2886 books catalogued in the sugar section. Most general references can be found in Alexander (1973) and van Dillewijn (1952).

It is well known that sugarcane fields can provide a huge biological output. In 1965, a new pathway of CO₂ assimilation was found in sugarcane (Kortschak *et al.*, 1965). In a large measure the discovery of this new pathway, the C₄ pathway, began as a sugarcane phenomenon (Hatch and Slack, 1966). Net CO₂ assimilation rates were found to exceed by 1.5 to 2 times the rates usually found in other plants (Alexander, 1973). In addition, sugarcane's habit of growth, which permits it to continue to store sucrose over long periods of time (two years or more) without coming to a definite maturity and death, and to be harvested two or more times from the same roots, makes it one of the most efficient and productive of all crops. It is reported that in field conditions sugarcane can, during a 22 month growing period, convert 1.43 percent of the incident sunlight into organic matter (Burlew, 1953). A harvest of sugarcane removes about 100 tn ha⁻¹, which contains, in its stalks and burned leaves, around 180 kg of nitrogen. Nevertheless, many cultivars show no response to nitrogen fertilizers (Ruschel *et al.*, 1978). There are also known cases in Brazil where sugarcane has been grown

continuously for more than 100 years in the same fields without any fertilizer (Döbereiner *et al.*, 1972a). These observations have led to a growing conviction that sugarcane may be obtaining considerable N from biological N₂-fixation.

Experiments with ¹⁵N with sugarcane in Hawaii showed that only a small proportion of the applied nitrogen fertilizer was recovered in the crops, the majority of the nitrogen in the crop came from other sources (Takahashi, 1970). Ruschel *et al.* (1975) using sugarcane seedlings incubated in a ¹⁵N₂ atmosphere, supplied the first direct evidence for biological N₂-fixation in sugarcane plants. Recently, using N balance and isotope dilution methods, Lima *et al.* (1987) showed that a sugarcane variety was able to fix substantial amounts of N₂, which they estimated to be over 60% of the total nitrogen incorporated. Boddey *et al.* (1991) using a similar technique did a three year experiment in a large concrete tank, filled with soil containing ¹⁵N-labelled organic matter. The results of this study showed that certain varieties of sugarcane can obtain 80 percent of their nitrogen from the associated biological N₂-fixation.

1.2. BIOLOGICAL NITROGEN FIXATION

The amount of N present on this planet as dinitrogen is vast, approximately 4×10^{15} tonnes in the atmosphere and about 20 times that bound in sedimentary and primary rocks beneath the surface (Gallon and Chaplin, 1987). None is accessible to plants until it is fixed, ie. converted to ammonia. Dinitrogen can be fixed by biological

or by industrial processes. Nitrogenous fertilizer today is mostly made from atmospheric dinitrogen by a reaction which is known as the Haber-Bosch process, essentially a catalytic reduction of dinitrogen to ammonia: $\text{N}_2 + 3 \text{H}_2 = 2 \text{NH}_3$. This process requires considerable energy to prepare hydrogen, to compress the reagents and heat them. In addition, quite a complex industrial installation is required, and a further consumption of energy is required to transport the fertilizer from the factory to where it is needed. In sharp contrast, the energy for the conversion of dinitrogen to ammonia in biological N_2 -fixation generally comes from sunlight. The input of the world's soil and water fixed nitrogen is mainly supplied by biological N_2 -fixation today. But in most agricultured lands, nitrogen available to plants mainly comes from industrial fertilizers.

The biological N_2 -fixing reaction, in chemical terms, is most unusual. The dinitrogen molecule is normally very unreactive due to the exceedingly stable triple bond which links the two nitrogen atoms. Nitrogenase, the enzyme responsible for this process, makes the dinitrogen molecule reactive at ordinary temperatures and pressures and, furthermore, in water. Nitrogenase is actually an enzyme system, as it consists of two distinct proteins. Dinitrogenase (also referred to as MoFe protein or protein I) has 4 sub-units, with a total relative molecular mass of about 240 kDa, which binds and reduces dinitrogen. Dinitrogenase reductase (also referred to as Fe protein or protein II) is a dimer of two identical subunits (Kim and Rees, 1992). This enzyme is initially reduced by ferredoxin or

flavodoxin *in vivo*, and subsequently transfers electrons to the dinitrogenase. Strong reductants will not reduce dinitrogenase directly but must function via dinitrogenase reductase. The reduction of dinitrogen is accompanied by an obligatory reduction of two protons to a hydrogen, the overall reaction becomes as follows:

As 2 MgATP are required for each electron transferred, the reaction requires a minimum of 16 MgATP under ideal conditions. Under normal physiological conditions the requirement is close to 20-30 MgATP. In terms of overall cellular energy, the reducing power is equivalent to at least a further 9 ATP per dinitrogen, assuming that, if not used in dinitrogen reduction, the electrons would be available for oxidative phosphorylation.

Nitrogenase is inefficient in that it permits electrons to bypass the nitrogen and to combine instead directly with protons to form hydrogen gas. At least one hydrogen is produced for every nitrogen reduced. When nitrogenase is not functioning optimally, then far more electrons are diverted to proton reduction. Thus the extent of hydrogen production can be used to monitor the activity of the N₂-fixing process (Hunt and Layzell, 1993; Simpson and Burris, 1984).

In addition to nitrogen and protons, nitrogenase can catalyse the reduction of many other substrates. Cyanide, methyl isocyanide, azide, acetylene, cyclopropene, cyanamid, carbon monoxide, nitrogen

monoxide and diazirine also serve as substrates (Burris, 1991). The most useful alternative substrate has been acetylene, as it furnishes a simple and highly sensitive test for nitrogenase. It is reduced to ethylene which can be detected in very low concentration by gas chromatography (Hardy *et al.*, 1968). The basis of the assay is that in atmospheres of 3 to 10 percent acetylene, virtually all electron flow through nitrogenase is used to reduce acetylene to ethylene. The production of ethylene from acetylene is presumptive evidence for the presence of nitrogenase, and provides an indirect measure of nitrogenase activity (Vessey, 1994). However, some criticisms have been raised about the accuracy of the traditional acetylene reduction assay (Denison *et al.*, 1983; Minchin *et al.*, 1983).

Both hydrogen evolution and acetylene reduction are indirect methods of measuring nitrogenase. They are simple, sensitive and inexpensive. Although they are very good for detecting nitrogenase activity, the conversion of the data obtained into absolute values for N_2 -fixation is usually quite difficult.

The only direct methods of estimating N_2 -fixation are isotopic methods. Unfortunately, the sole radioactive isotope available is ^{13}N , which has a half-life of only 10.5 min. The stable nitrogen isotope of mass 15 has been widely used. The detection of ^{15}N requires the use of a mass spectrometer or photometric emission spectrometer. The technique is less sensitive than that of acetylene reduction and hydrogen evolution. There are a number of variants of this method. The most commonly used are N-balance and ^{15}N enriched N-balance

methods. The methods involve many assumptions. An excellent account of these and other methods used for quantification and problems of N_2 -fixation by bacteria associated with grasses is given by Boddey (1987).

Nitrogenase activity can also be measured by growth in weight and/or total nitrogen of plants or microorganisms in the absence of combined nitrogen. In practice it is unwise to rely on these methods alone for cultures of microorganisms or for systems of low activity unless very strict precautions are taken.

The measurements of whole-plant N-increment, and the isotope techniques, are valuable for seasonal measurements and field studies. However, they are not appropriate for physiological studies which require dynamic measurements of nitrogenase activity. The only two methods that allow short term measurement of nitrogenase activity in undisturbed conditions are the acetylene reduction assay and measurement of hydrogen evolution. In this study, these two methods are used for the measurements of nitrogenase activity of sugarcane plants and bacteria.

The ability to fix nitrogen is restricted to the most primitive organisms, the prokaryotes, and even within this group the property is by no means universal. Only a few microorganisms have this ability and other creatures on earth exploit these nitrogen fixers.

There is a great diversity among N_2 -fixing bacteria. They may

be phototrophic or chemotrophic, autotrophic or heterotrophic, they may fix dinitrogen only under aerobic, anaerobic, or microaerobic conditions. And they may be symbiotic, free-living, or form casual associations with other organisms. From the agricultural point of view, two most important types of crop are cereals (grasses) and legumes. Some N_2 -fixing bacteria can contribute directly through symbiotic relationships to the productivity of legumes. Less direct contributions may be made to the growth of grasses by associative bacteria, free-living bacteria and cyanobacteria.

The symbiotic relationships between organisms are those in which both partners may show considerable biochemical and morphological changes in facilitating the partnership (Sprent and Sprent, 1990). They are usually quite specific and involve the evolution of mechanisms which encourage the development and maintenance of the association. There are three main types of symbioses with N_2 -fixing bacteria that are well known: those involving interactions between legumes and bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*; those between non-leguminous angiosperms and the actinomycete *Frankia* (actinorhizal association); and those between cycads and cyanobacteria. Common to the three types of symbioses is the fact that the bacteria are housed in specialized structures developed by the plants, nodules (root or stem) in the legumes and actinorhizal plants, coralloid roots in the cycads. The vast majority of nodules occur on the roots of the host plants. Because of the importance to agriculture of N_2 -fixing symbioses involving legumes, they have been most widely studied.

On the other hand, there is increasing interest in other N_2 -fixing bacteria, particularly the ones with grasses.

Although the first product of N_2 -fixation is ammonia, the ammonia is nearly always assimilated into the bacteria as rapidly as it is formed. In most circumstances the free-living microbes have to die and decompose, that is, mineralization has to take place, before the plants can gain access to the nitrogen fixed. So, the free-living N_2 -fixing microbes, at least when in the free-living state, are rarely of serious importance in the terrestrial nitrogen economy. On a global scale, it is generally agreed that free-living bacteria do not make a major contribution to the nitrogen input in agricultural systems (Gallon and Chaplin, 1987). However, it is not always easy to separate N_2 -fixation by free-living organisms from that produced by bacteria associated with plant roots. Normally, the latter are called associative symbioses.

In the associative symbioses, there is usually little or no obvious morphological adaptation by either partner. This kind of association ranges from the often quite specific occupation of the extra-cellular environment of the host to the relatively non-specific attraction between diazotrophic bacteria and the rhizosphere. In some cases, the diazotroph is capable of fixing nitrogen in the natural environment away from its host. In this respect, there is a continuous spectrum between the bacteria of these associations and those which usually occur in the free-living form. But quite often an associative symbiosis is defined by its being retained when roots are

surface sterilized (Sprent and Sprent, 1990). Thus bacteria are believed to be inside the root. The evidence for N_2 -fixation in these types of symbiosis is often obtained from acetylene reduction assays of the sterile-water washed roots. In some experiments, the roots were surface sterilized with antibacterial agents. But the rugged root surface may provide many refuges for bacteria, and the presence of bacteria living in dead cells is always a possibility. If they are indeed inside the root, how they get there is another question. Whether they obtain entry through wounds, or downwards from cut plant stubble after harvest is not clear.

Most of the associative N_2 -fixing organisms are often found on and/or in the roots, but some of them are also found with leaves and stems. Although some associations are quite casual and non-specific, some of them appear to show specificity. Mohandas (1988) found a new species of associative *Azospirillum* performing N_2 -fixation only in a tomato plant. Another example is the association between the grass *Paspalum notatum* and *Azotobacter paspali* (Döbereiner *et al.*, 1972b). In crops, less specific associative N_2 -fixations were found in wheat, corn, rice and sorghum (Rao *et al.*, 1981; Klucses and Döbereiner, 1981). Although the associative N_2 -fixing is readily demonstrable in grasses, wheat, corn, sorghum, rice and sugarcane, in most cases the importance of the process to the plant has not been adequately determined. The potential for practical applications of these interactions is significant but basic information is absolutely essential before applied research is advisable.

Numerous problems still exist in assessing the quantitative importance of the associative N₂-fixation in grasses. We still must answer such basic questions relating to the process in grasses as (1) what kinds (species) of bacteria are contributing to N₂-fixation? (2) what are the sites of N₂-fixation? (3) can the plant supply adequate levels of substrates for the bacteria? (4) what is the efficiency of the process? and (5) do existing assay methods give realistic assessments of nitrogenase activity in different situations?

1.3. N₂-FIXING BACTERIA ASSOCIATED WITH SUGARCANE

Fixation of nitrogen by some bacterial association with sugarcane has been suspected for some time. This was confirmed (as said above) by total nitrogen, ¹⁵N and nitrogen balance methods (Ruschel, 1975; Ruschel *et al.*, 1978; Lima *et al.*, 1987; Boddey *et al.*, 1991), and association of N₂-fixing microorganisms with sugarcane was demonstrated by the acetylene reduction technique (Döbereiner *et al.*, 1972a).

From the 1950's, various methods have been used in many laboratories to isolate and identify the responsible diazotrophs. Many free-living N₂-fixing bacteria have been isolated from the sugarcane fields, especially from the rhizosphere. The famous ones are *Beijerinckia* (Döbereiner, 1961), *Azotobacter* (Singh *et al.*, 1981), *Bacillus* (Seldin *et al.*, 1984) and *Azospirillum* (Döbereiner, 1988). As mentioned previously, the free-living diazotrophs, when in the free-living state, are rarely of serious importance in the terrestrial

nitrogen economy. It is very hard to be certain that free-living N_2 -fixing microbes could contribute as much as 80 percent of sugarcane's needs. It is more likely, from an ecological point of view, that the nitrogen fixers in sugarcane fields are those which fix nitrogen in association with sugarcane, because fixed nitrogen is supplied just where it is needed.

Using the acetylene reduction technique, Döbereiner *et al.* (1972a) found that sugarcane roots are more active than the rhizosphere soil, and washing them has little effect, suggesting the possibility of N_2 -fixing endophytes. Ruschel and Ruschel (1977) showed that germinated cuttings from which the roots and shoots were excised, had a higher nitrogenase activity than other parts. The internal part of the cuttings had a higher nitrogenase activity than the rind and the roots. Patriquin *et al.* (1980) found unidentified diazotrophs present within surface-sterile sugarcane stem cuttings. These bacteria multiply after the sprouting of the cuttings. All these observations suggest that N_2 -fixing endophytes maybe present within the sugarcane plants.

1.4. CHARACTERIZATION OF ACETOBACTER DIAZOTROPHICUS

Recently a new microaerobic N_2 -fixing bacterium was isolated in large numbers from sugarcane stems, roots, leaves and rhizosphere soil from various sites in Brazil, Cuba and Australia (Li and MacRae, 1991). This bacterium was first named *Saccharobacter nitrocaptans* (Cavalcante and Döbereiner, 1988) and, then renamed

as *Acetobacter diazotrophicus* (Gillis *et al.*, 1989). In Australia, this bacterium was isolated only from sugarcane but not from the 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 plants or roots from 12 different weed species taken from cane fields (see Boddey *et al.*, 1991).

Acetobacter diazotrophicus is a very unusual diazotroph. It grows best on a sucrose-rich medium and can continue to grow in up to 30% sucrose. Its optimal pH is 5.5, and it continues to reduce acetylene even at a pH lower than 3 (Cavalcante and Döbereiner, 1988). The bacterium possesses no nitrate reductase, and N₂-fixation is not affected by high levels (30 mM) of nitrate, although partial inhibition of growth was noted in the presence of 50-80 mM nitrate (Boddey *et al.*, 1991; Cavalcante and Döbereiner, 1988; Gillis *et al.*, 1989; Li and MacRae, 1991). Also, ammonium causes only partial inhibition of nitrogenase (Fu *et al.*, 1988). This incomplete inhibition of N₂-fixation by ammonium in the bacterium, as well as the lack of nitrate reductase, may make it particularly effective in N₂-fixing in sugarcane fields that have been fertilized.

It has been suggested that these microorganisms are specifically associated with sugarcane because they prefer high concentrations of sucrose for growth and N₂-fixation, and do not use common amino acids or organic acids for growth (Li and MacRae, 1991). The acetylene reduction studies showed that the internal tissues of sugarcane contained nitrogen fixers (Ruschel *et al.*, 1977).

Most of these nitrogen fixers (then unknown) were believed to exist in the peripheral nodal regions where root primordia originate, but in a quiescent state (Patriquin *et al.*, 1980). Recently, *Acetobacter diazotrophicus* was isolated from carefully surface sterilized sugarcane stems. Precise plate-counting work showed that it existed in internodes and nodes as well (Clemente, Univ. Havana, personal communication). All this work seemed to confirm the endophytic nature of this bacterium. However, almost nothing was known about this endophytic association, where the *Acetobacter* was located or how it contributed to the plant.

The nitrogenase can function only under anaerobic and microaerophilic conditions. The optimal oxygen partial pressure for acetylene reduction by this bacterium is 0.2 kPa (Reis *et al.*, 1990). However, much of the work on the nitrogenase activity of this bacteria has used cultures grown in semisolid or liquid medium, measuring acetylene reduction under air with 10% acetylene (Cavalcante and Döbereiner, 1988; Stephan *et al.*, 1991). In these conditions the bacteria will proliferate at the depth in the medium where the oxygen concentration is optimal. It is not clear how this bacterium growing exposed to air can limit the access of oxygen to the oxygen-sensitive site of nitrogenase. The production of hydrogen by this bacterium, as a very useful tool for determining nitrogenase activity (especially with the sugarcane plant) is explored for the first time in this study.

1.5. REQUIREMENT FOR A SUCCESSFUL ASSOCIATIVE

RELATIONSHIP

For the N_2 -fixation to occur, the symbiotic or associative systems need: 1) a supply of carbohydrate to N_2 -fixers; 2) a low oxygen tension for nitrogenase activity; and 3) delivery of the fixed nitrogen (ammonium from bacteria and/or amino acids from plant cells) to the growing regions of the plant. To meet these three requirements, legumes develop a special complicated structure, the nodule. In legume nodules, a vascular system has developed for delivery of the carbohydrate to the bacteria by the phloem and the return of fixed nitrogen to the plant by the xylem. Oxygen concentration is controlled by leghaemoglobin within the bacteria-containing (infected) cells and by oxygen diffusion barriers in the outer regions of the nodules. There are no obvious structures in sugarcane such as the nodules of legumes. Prior to this study, it has not been clear whether the nitrogen fixing bacteria may exist inside living plant cells (perhaps as bacteroids), or outside living cells, perhaps in the intercellular spaces and/or mature xylem lumens. The position of the bacteria in the plant was still not certain, nor was it known whether N_2 -fixation can take place principally within the root or stem as opposed to the rhizosphere.

It has been suggested that this N_2 -fixing bacterium may move through the xylem inside the sugarcane tissues (James *et al.*, 1994; Reis *et al.*, 1994), and the xylem has even been considered as a potential environment for N_2 -fixation by the *Acetobacter* (Sprent and James, 1994). The solute concentrations of apoplastic solution and of

xylem sap of sugarcane have been the subject of study over many years. Because *Acetobacter diazotrophicus* needs a high concentration of sucrose, the sugar concentration in the xylem should be high if the bacteria live there. In sugarcane stem tissue, diffusion of sucrose into the xylem would seem likely, because the apoplast of storage tissue contains up to 700 mM sucrose and is separated from the xylem by only a few cell layers (Welbaum and Meinzer, 1990). Hawker (1965) reported concentration of up to 9% sucrose in the xylem of sugarcane. However other reports have shown that xylem sap is essentially free of sucrose (Bull *et al.*, 1972; Welbaum *et al.*, 1992). Hawker (1985) found no barriers to sugar movement in the vascular bundles of sugarcane stem using histochemical techniques and light microscopy. But more recent observations by electron microscopy indicated that the xylem is separated by bundle sheath cell walls that were suberized and lignified in the mature stems, and constitute a barrier to the movement of sucrose and other relatively large molecular weight solutes but not water (Jacobsen *et al.*, 1992; Welbaum *et al.*, 1992). This isolation of the xylem is one difficulty for proposals of the xylem as a potential environment of the diazotroph.

The xylem vessels are the sites for long distance water transport. Movement of bacteria in the xylem has been studied mainly as a mode of infection of stems and seeds of plants by pathogenic bacteria. Vessel lengths range from a few centimetres to many metres. At both ends of the vessel, the only path for solution is through pits. The holes in the pit membranes (ie. primary walls) are very small (<200nm), and do not permit the passage of air

bubbles or micro-organisms. Any bacteria inside the xylem would accumulate at the vessel ends and block the pathway, inhibiting water movement. Such accumulation of bacteria at vessel end walls leads to the aptly-named wilt diseases caused by many pathogens. It is not likely that diazotrophs, as beneficial bacteria, would live inside the xylem, unless in some modified xylem specially structured for this symbiosis. In this study I examined the sugarcane plants for the continuity of xylem vessels, for the extent of the xylem apoplast in the stems, and the possibility that *Acetobacter* might live inside of xylem as an endophyte, and I also explored other possible sites for the bacteria within the stem tissues.

1.6. SPECIFIC QUESTIONS TO BE INVESTIGATED IN THIS THESIS

1. Where is the *Acetobacter* located within the sugarcane stems?

Sugarcane stems have many primary vascular bundles scattered throughout a transverse section of the stem among the ground parenchyma cells. The vacuoles of parenchyma cells contain 10-20% sucrose which could be used by *Acetobacter*. If *Acetobacter diazotrophicus* exists within the tissues and fixes nitrogen in sugarcane, what region in stem tissue is most likely to provide the best habitat for these bacteria, and what is the arrangement structurally and physiologically for this symbiotic N₂-fixation?

2. Are these bacteria passed from one generation to the next under cultural conditions in greenhouse? Are the bacteria isolated

from Cuban field-grown material the same as those isolated from plant subculture at Carleton and the same as the original type culture?

3. What are the morphological and behavioral characteristics of the isolates and the type culture which may give clues to their ability to control oxygen concentration within the plant?
4. What is the evidence that the isolates are capable of fixing nitrogen?
5. What is the evidence that the sugarcane plant can provide a suitable environment in which the bacteria can fix nitrogen?

1.7. ORGANIZATION OF SUBSEQUENT CHAPTERS

The questions will be addressed by my experimental investigations and results reported and discussed as follows.

Chapter 2 will give general methods and materials used throughout the study. Some specific methods will be included at the beginning of individual chapters.

Chapter 3 reports relevant structural and physiological features of the sugarcane stem, examines the possibility that *Acetobacter diazotrophicus* would live inside the xylem as an endophyte.

Chapter 4 reports the localization, isolation and preliminary characterization of *Acetobacter* from the apoplast of sugarcane stems (this chapter has been published as a major paper, Dong *et al.*, 1994).

Chapter 5 reports detailed comparisons of isolated *Acetobacter* from the original Cuban field-grown plants, and from three generations of plants subcultured in the greenhouse at Carleton, with the original type culture isolated in Brazil (this chapter has been published as a second major publication, Dong *et al.*, 1995).

Chapter 6 describes the experiments characterizing acetylene reduction and hydrogen production by the isolates in culture (some of these results have also been included in Dong *et al.*, 1995). The results reported in Chapter 6 are used to show that the isolates can fix dinitrogen, and to define conditions under which this can happen.

Chapter 7 describes the colony morphology of the isolates and the changes in this morphology under different conditions of oxygen tension. A correlation between colony morphology and hydrogen production was expected and verified. These results give some clues as to how *Acetobacter diazotrophicus* may protect its nitrogenase from oxygen within the plant, yet ensure a sufficient supply of oxygen for the maintenance of the high rate of sugar metabolism of the bacteria.

A specific discussion is included in each of chapters 3 to 7, chapter 8 presents a general conclusion.

CHAPTER TWO.
GENERAL METHODS AND MATERIALS

This chapter will give general methods and materials used throughout the study. Some specific methods will be included at the beginning of individual chapters.

2.1. PLANT MATERIAL

Plants of sugarcane (*Saccharum* spp. hybrid), varieties Media Luna and Ja 60-5, were grown in the fields of the Estacion Biologica Docente, Facultad de Biologia, Universidad de La Habana. The mature stems were collected and dipped into paraffin wax. The wax covered stems were packed with wax paper and transported to the laboratory in Carleton University, Ottawa within 2 to 3 days.

The Cuban parent material, stems of Ja 60-5 and Media Luna, were cut into pieces with one or two nodes. These cuttings were incubated in wet vermiculite in a dark, warm place. In 3 to 5 days adventitious roots at nodal regions emerged and the axillary bud germinated. When the roots reached 5 to 10 cm long, the cuttings were transferred to 12 inch peat pots filled with a 4:1:1 (v/v) mixture of commercial potting mix, perlite and vermiculite and kept in a greenhouse at the campus of Carleton University. Cuttings with two nodes of mature stems from the first generation plants in the greenhouse were used to produce the next generation of plants. Three generations of these plants were studied. The plants were grown in the greenhouse with supplementary lighting from Optimarc Super Metal Halide lamps (Tungsten Products, North Bergen, NJ). Greenhouse temperatures were typically 20-30°C during the day and

20°C at night. Plants were supplied with water daily and complete fertilizer monthly.

Plants ranging in age from 10 to 20 months were used. The plants chosen were 3.5 to 4 m tall with the diameter of mature stems of 30-50 mm. The reference point for determining internode number was the top visible dewlap (TVD) leaf (Meinzer and Moore 1988), the youngest leaf whose blade is fully exposed and not enclosed in the sheaths of older leaves. The first internode is the one below the TVD leaf sheath attachment. Additional internodes were numbered consecutively down the stalk. In the experiments with detached plants, the internodes were numbered upwards from the cutting point. The first internode is the bottom one which had been cut.

2.2. CULTURES OF BACTERIA

N-free LGI medium (Cavalcante and Döbereiner 1988) complemented with 5 ml sugarcane juice per litre at pH 5.5 was used as an enrichment culture for all the isolates. The sugarcane juice was obtained by squeezing the mature sugarcane stems with a press. The LGI medium consisted of (quantities per litre): K_2HPO_4 , 0.2 g; KH_2PO_4 , 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; bromothymol blue, 0.025 g; crystallized cane sugar 100 g; vitamins solution (10 mg biotine and 20 mg pyridaxol HCl in 100 ml H_2O), 1 ml; final pH 5.5 adjusted by 1% acetic acid solution. Additional 15 g agar per L was used in solid medium for

plates and slopes. Semisolid medium which was used for isolation contained 4 g agar per L. The medium was autoclaved for 15 min after all the ingredients were added (including the sugarcane juice). Slopes were obtained by using 10 ml medium in 36 ml tubes.

Acetobacter produces acids which drop the pH of the medium and eventually kill the bacteria. To buffer the acids released by the bacteria, solid autoclaved CaCO_3 (about 0.05 - 0.1 g) was added to the bottom of autoclaved medium before it solidified for the extended cultures.

Bromothymol blue was used in the medium as a pH indicator which is green at pH 5.5 and changes to yellow at pH 5.0. The yellow colour increases its tint with decrease of pH. The dye becomes orange colour when pH is below 2.5. *Acetobacter* accumulate bromothymol blue inside the cells and this gives the colonies of bacterium a dark orange colour which indicates the low pH inside the bacterial cells.

A loop was used for inoculation on solid medium. For isolation with semisolid medium, aqueous inoculum was stabbed into the medium by a micro-pipette. All the strains were incubated at 30°C. The first change that can be seen was the colour change of the media which was caused by the dropping of pH. Growth of bacteria inoculated on the solid medium can be seen in two days. Colonies can be seen in about 3 days from pure culture inoculation. Colonies had the appearance of "fried egg" with the yellow bacteria in a

central spot inside a clear periphery in five days. Maximum growth was obtained in about a week. In semi-solid medium a white grey layer of bacteria was first formed on the surface of the medium. The colour of the medium changed from pale green to yellow and colourless. The bacteria layer finally became pale yellow.

All the isolats and type strain of bacteria were grown on plates for five days and then washed off with liquid medium (without agar). The bacteria suspensions were diluted with 20% glycerine 1:1 (v/v). These mixtures were put into sterile plastic vials with autoclaved glass beads. The vials were frozen and stored in a liquid nitrogen storage locator.

2.3. GAS CHROMATOGRAPHY

Gas chromatography is essentially a physical method of separation of mixed gases. The gases to be separated are distributed between two phases. One is a stationary bed which is the fillings of the column, while the other is a flowing gas which percolates through the stationary bed. The chromatography process occurs as a result of repeated adsorption-desorption events during the movement of the sample gases along the column, and the separation is due to different distribution coefficients of the individual sample gases. In this study the samples were carried by compressed air into the column where the separation process takes place. At the end of the column, the separated individual sample gases were detected and the detector signal was recorded on a recorder.

2.3.1. Apparatus

The separation and measurement of ethylene are usually accomplished by gas chromatography using a flame ionization detector. Such equipment is expensive, its use generally restricted to the laboratory, and it is not able to detect hydrogen. Convenient, inexpensive and sensitive means of estimating hydrogen evolution and acetylene reduction are required to evaluate the nitrogenase activity. Mallard *et al.* (1977) and Holfeld *et al.* (1979) developed a portable gas chromatograph (GC), which can detect hydrogen and ethylene. In order to detect and estimate the nitrogenase activity in our study on sugarcane, we had such a portable Gas Chromatograph made in the Carleton Science and Technology Work Shop according to the design of Holfeld *et al.* (1979).

A Taguchi Gas Sensor was used as the detector in the portable chromatograph. It consists of a tin oxide semiconductor, moulded around a small filament heater. When the filament is heated in air in the presence of combustible gases, the semiconductor's resistance decreases. The sensor responds to hydrogen and ethylene and other combustible organic compounds.

The column is stainless steel tubing, 0.63 m long with 0.2 mm internal diameter, packed with Porapak N. The injector was made by holding two silicone rubber septa against a tee joint union by a nut. The other two ends of the tee union accept the column and the

incoming carrier gas.

The electronic circuit was built according to Mallard *et al.* (1977) and Holfeld *et al.* (1979). The change in sensor conductivity is exported to a computing integrator (LCI-100; Perkin-Elmer), by which the chromatograms with retention times and peak heights and areas were recorded.

The GC is powered by a 12 V DC power source, so that a cigarette lighter in an automobile may be used in the field. A compressed air supply of about 55 kPa produces a carrier gas flow of about 20 ml/min at room temperature.

2.3.2. Calibration of the instrument and sources of gases used

For calibration, commercial ethylene gas was used as a standard. The standard hydrogen gas was produced by chemical reaction between zinc metal and diluted sulphuric acid. The standard gases were diluted to different concentrations with air and a 1 ml gas sample was measured with the GC. Five repeat tests have been done for each concentration of each standard gas to determine the retention time, peak heights and peak areas. The results were used to build standard curves and standard equations.

It is important to distinguish other detectable gases in the samples from hydrogen and ethylene. Methane, carbon dioxide and acetylene were also measured with the GC under the same conditions

to get the retention times of these gases and the sensitivities of the GC for these gases. This information was used for identifying and confirming the hydrogen and ethylene peaks.

2.3.2.1. Hydrogen

The retention times, peak heights and areas of different concentrations of hydrogen gas are recorded. Table 2-1 gives the means and standard deviations of peak heights and areas of 5 samples of each concentration.

Figure 2-1 shows the two standard curves of hydrogen plotted by peak height and peak area. The standard deviations for area are bigger than those for height. The area of a peak is easily distorted by a peak nearby. Hence I used the height curve as the standard curve for hydrogen.

By plotting height reading alone and using an interpolation curve, we can get the hydrogen standard curve shown in Fig. 2-2. The sensor of the GC shows the typical hyperbolic curve relating the peak height to the hydrogen concentration, with a gradual approach to the maximum height reading (C). If we assume the hydrogen concentration is k when the reading is exactly 0.5C, we will have:

$$R = H C / (H + k) \quad (1)$$

Here R is the reading of the GC; H is the concentration of the

hydrogen gas.

To determine k and C , equation (1) can be algebraically transformed into an equivalent linear equation. We can simply take the reciprocal of both sides of equation (1) to give:

$$1/R = (k+H)/CH = (k/C) (1/H) + (1/C) \quad (2)$$

Now, we have a straight line equation, if $1/R$ is plotted against $1/H$ (Fig. 2-3). This line will have a slope of k/C , an intercept of $1/C$ on the $1/R$ axis, and an intercept of $-1/k$ on the $1/H$ axis. Because several points are so close to the zero point, they overlap. By plotting only the data from 100 to 1000 ppm, we see the left end of the line (Fig. 2-4). The double-reciprocal plot has the great advantage of allowing a more accurate determination of C , which can only be approximated from a simple plot of R vs. H , as shown in Fig. 2-2 and Fig. 2-4. The data obtained from the GC are in a straight line in the double-reciprocal plot indicating the GC performs very reliably in the tested range.

Most of the time we are dealing with measurements of samples with hydrogen concentrations below 100 ppm, and need to work out concentration from peak height. We can use a simplified equation (Fig. 2-5):

$$Y_H = 0.180 X_H^{0.995} \quad (3)$$

In this equation, Y_H is concentration of hydrogen gas in ppm, X_H is the height reading of the hydrogen peak from the GC when the sample size is 1 ml.

2.3.2.2. Ethylene

One ml samples of standard ethylene gas of different concentrations in air were measured with the GC. The means and the standard deviations of peak heights of 5 samples of each concentration were given in Table 2-2.

Fig. 2-6 shows the standard curve for ethylene, plotting peak height against concentration. This curve is very similar to that of hydrogen gas. So in the same way we can use simplified equation:

$$Y_E = 0.214 X_E^{1.117} \quad (4)$$

In this equation, Y_E is concentration of ethylene gas in ppm, X_E is the height reading of the ethylene peak from the GC when the sample size is 1 ml.

2.4. MEASUREMENTS OF NITROGENASE ACTIVITY

2.4.1. Materials

All the isolates and the type strain of *Acetobacter diazotrophicus* were inoculated on slopes of 10 ml solid LGI medium

(with 5 ml sugarcane juice per liter) in 36 ml glass tubes and incubated at 30°C for 4 or 5 days before being tested. Some cultures were grown under 2 kPa oxygen partial pressure. Oxygen tension was controlled by replacing oxygen by dinitrogen in an anaerobic jar. The growing temperature 30°C was maintained during the measurements by putting cultures in a water bath.

Sugarcane plants (Ja 60-5) from the greenhouse of Carleton University were used for nitrogenase activity tests. Plants of three generations were collected and tested. Plants were cut down at the base of the stem and transported immediately to the laboratory. A piece of sugarcane plant stem about 1.2 m long was put into a 1.3 m long plastic drain pipe with a diameter of 2 inches (from local home hardware shop). Both ends of the pipe were capped with rubber stoppers. Gas samples were taken from a specially installed injector with two silicone rubber septa. Empty pipes and stoppers alone, soybean nodules and corn plant shoots were used as controls in this test. This test was done at room temperature.

2.4.2. Acetylene reduction

The acetylene was obtained by reaction of calcium carbide (Lot no. 01107HF, Aldrich Chemical Inc., Milwaukee, WI) and water. The tubes with bacteria on media were flushed with air for 1 min, then sealed with gas-tight serum stoppers. The acetylene reduction assay was conducted by adding the calculated volume of acetylene to give 10% acetylene into these tubes and measuring the ethylene

accumulation in the tubes at about 10 min intervals over 1 to 2 h. The gas samples (1 ml) were withdrawn with 1 ml syringes. The soybean nodules with 10% acetylene in the tubes and the plastic pipes and all the strains without acetylene in tubes were tested for ethylene production as controls. The plastic pipes with stoppers alone were tested for ethylene as a control test.

The retention times of peaks were compared with that of standard ethylene gas to identify the ethylene peak. For further confirmation of the ethylene peak, the gas samples were spiked by assaying a mixture of standard ethylene gas of known concentration with the sample gas. The chromatograms were compared with those of standard ethylene gas and sample gas alone.

The ethylene peaks of sample gases were converted into concentrations and total amount in the tubes and pipes with standard equation (4) in 2.3.2.2.

2.4.3. Hydrogen evolution

Hydrogen gas production was measured to monitor the nitrogenase activity of the bacterial colonies and the detached pieces of sugarcane plants.

The tubes containing bacterial colonies on the media were flushed with air for about 1 min, and then sealed with gas-tight serum stoppers. The tubes were held in a water bath (30°C) during

the experiments. Gas samples (1 ml) were withdrawn with 1 ml syringes at about 10 min intervals. Analysis was done by immediately injecting each gas sample into the GC. Hydrogen evolution from sugarcane stems was measured by taking gas samples from sealed drain pipes at room temperature at about 8 h intervals over 2 to 3 days. Soybean nodules were used as positive controls. Corn plants were used as negative controls.

The retention times of sample gases were compared with those of standard hydrogen, methane and ethylene gases to identify the peaks. The gas samples were also spiked with hydrogen gas of known concentration and the mixture assayed. Since the sensor can detect only combustible gases, there was no possible confusion between the hydrogen peak and a carbon dioxide peak.

To confirm that the hydrogen is produced from the nitrogenase not from other sources, the hydrogen evolution rate in an oxygen/nitrogen mixture was compared with that in an oxygen/argon mixture. In the oxygen/argon mixture, without substrate dinitrogen, the nitrogenase should pass all the electrons to protons to produce 3 to 10 times as much hydrogen as in an oxygen/nitrogen mixture. The mixture of oxygen/argon was obtained by flushing the tube with argon (extra pure) for a few minutes by using two needles sticking into the tube with a gas tight serum stopper, and then removing measured volumes of argon and adding known volumes of oxygen (extra pure) by syringe. The completeness of this flushing method was proved by using a tube

with a high concentration of hydrogen, and measuring the hydrogen concentration after flushing. With a tube filled with more than 1000 ppm hydrogen, after this flushing procedure the tube contents gave no hydrogen peak at all in the machine which had a sensitivity of 0.5 ppn. for hydrogen.

To test the effect of different concentrations of acetylene on the hydrogen evolution of nitrogenase, the hydrogen evolution rate was measured for 1 h first, and then the tubes were flushed with air for 5 min and resealed with serum stoppers. Different partial pressures of acetylene, 2.5, 10, 20 and 30 kPa, were obtained in different tubes by removing measured volumes air and adding known volumes of pure acetylene by syringe. Gas samples were taken again at about 10 min intervals and analyzed for hydrogen and ethylene.

2.5. MICROSCOPY AND PHOTOMICROGRAPHY

2.5.1. Preparation for conventional microscopy

Hand-cut sections of fresh tissue were cut with surgical steel razor blades. Large longitudinal sections of nodal regions were obtained with a sliding microtome. The sugarcane stems fed with the apoplastic dye sulphorhodamine G were hand-cut in paraffin oil (O'Dowd and Canny, 1993).

For transmission electron microscopy (TEM), the mature sugarcane stem tissues were cut into small pieces and completely immersed in a drop of cold 3% (v/v) glutaraldehyde (from 8% stock

solution, Lot no. 449, JBS#013A, J. B. EM. Inc., Pointe Claire, Quebec) at pH 6.8 in 0.025 M potassium phosphate buffer. The drop was placed on dental wax. The tissues were then excised with tweezers and a razor blade into pieces 2 x 0.5 x 1 mm. These pieces were then quickly transferred to fresh 3% glutaraldehyde in 0.025 M potassium phosphate buffer at pH 6.8 overnight on ice. The next day the tissues were rinsed on ice three times with pH 6.8 0.025 M potassium phosphate buffer and then post-fixed in 1% (w/v) osmium tetroxide (Lot no. 408, JBS#016, J. B. EM.) in the same buffer for 2 h on ice. The tissues were dehydrated through an acetone series and then infiltrated with Spurr's monomer mixture following O'Brien and McCully (1967). The Spurr's resin monomer mixture contains: 10 g vinylcyclohexene dioxide (VCD; JBS#035, J. B. EM.); 6 g diglycidyl ether of polypropylene glycol (DER; JBS#036, J. B. EM.); 26 g nonenyl succinic anhydride (NSA; JBS#037-1, Lot no. 940404, J. B. EM.); and 0.4 g of the accelerator dimethylaminoethanol (DMAE; Lot no. 920821, J. B. EM.). At the beginning of the infiltration 10% resin mixture in acetone was added to the last 100% acetone change drop by drop to reach 1% resin mixture in the vial in 2 h. The tissues were infiltrated in this 1% resin overnight. The concentration of resin in the vials was increased by adding diluted resin to reach 75% by the end of the day. The half opened vials were left in the fumehood overnight to let the acetone vaporize. The resin mixture in the vials was replaced with fresh 100% resin mixture next day and then changed daily for another two days. Tissue pieces were polymerized in 100% resin mixture at 70°C overnight. Ultrathin sections were cut with either glass or diamond knives, and stained

with uranyl acetate and lead citrate and viewed with the transmission electron microscope. For the details of these techniques see O'Brien and McCully (1981).

2.5.2. Freeze-substitution

Fresh sugarcane stem tissues were cut into 2 x 0.5 mm pieces and immediately plunged into the freezing mixture with teflon tipped tweezers, isopentane:methyl cyclohexane (1:1), cooled by liquid nitrogen to its melting point. The frozen tissues were transferred to dry acetone/acrolein (10:1) and freeze-substituted at -80°C for 10 days. After a slow warm-up, the tissues were critical-point dried and gold coated for scanning electron microscopy.

The bacterial colonies on LGI plates were carefully cut off with a piece of agar and plunged with tweezers into the freezing mixture described above. Frozen colonies were freeze-substituted in methanol/acrolein (10:1) at -80°C in a deep freezer for a week. (Freeze-substitution in the dry acetone resulted in sucrose in the agar forming crystals which ruined the structures of colonies and made sectioning impossible.) After a week's freeze-substitution in methanol, sucrose crystals were seen free at the bottom of the vial and on the surface of agar block, from which they could be removed easily. The freeze-substituted colonies were kept in a dewar and transferred from the deep freezer at -80°C to a freezer at -20°C . Next day the dewar was transferred to a refrigerator at 5°C for a further 24 h. The next day colonies were rinsed three times (10 min

each) in methanol on ice. The colonies were then post-fixed (on ice) with 1% OsO_4 in methanol for 1 h, and then rinsed three times again with methanol. The methanol was gradually replaced with acetone in steps: 5, 10, 20, 30, 50, 70, 90, and 100% with two 10 min changes for each step. The colonies were then infiltrated and embedded with Spurr's resin monomer mixture. Cross sections of colonies were obtained with a microtome, stained with toluidine blue at pH 4.4, mounted in immersion oil and observed with an optical microscope.

2.5.3. Staining Methods

2.5.3.1. Toluidine blue

Both hand-cut and plastic-embedded sections were stained with 0.05% toluidine blue O (colour index 52040, Lot no. 73736, Polysciences, Inc., Warrington, PA) in benzoate buffer at pH 4.4 for 30 seconds and then rinsed three times and mounted in tap water for bright-field microscopy. The pink to red metachromatic colours of toluidine blue staining indicate the presence of carboxylated or sulphated polysaccharides such as pectin, while the blue to green colours of this staining show the presence of aromatic compounds such as lignin (O'Brien *et al.*, 1964). Nylon membranes with bacterial mucilage on it were stained with 0.05% toluidine blue at pH 4.4 in benzoate buffer and at pH 1.5 in 1% acetic acid.

2.5.3.2. Fluorescent stain for lignin/suberin

For fluorescence microscopy, sections were examined unstained in tap water with UV excitation for the autofluorescence of cell walls. Some sections were stained with rhodamine B (colour index 45170, Lot No. 16249, Allied Chemical Co., New York, NY) in 1:10,000 aqueous solution for 8 min then mounted in tap water and observed with fluorescence optics. The rhodamine B stains hydrophobic materials and the positive staining gives bright white yellow colour with UV excitation (Boerner, 1952 in Wessely *et al.* 1981).

2.5.3.3. Bacterial stains

Sections of sugarcane stem with bacteria in the xylem vessels were stained in a two step staining method using the periodic acid-Schiff's (PAS) reaction followed by diamidino-phenyl-indole (DAPI) (Lot no. 80921, Polysciences, Inc., Warrington, PA) staining. The sections were treated with 1% periodic acid solution for 10 min, washed with water for 5 min and then stained with Schiff's reagent (Lot No. 936286-12, Fisher Scientific, Fair Lawn, NJ) for 1 min. The rinsed sections (three rinses in 10 min) were mounted in 1:10,000 aqueous DAPI solution. A positive reaction with PAS gives a pink to red colour with bright-field optics. The autofluorescence of non-suberized tissue was quenched by this PAS reaction and the DNA of both bacteria and plant cells and the cell walls of bacteria fluoresce bright blue or yellow when excited by UV light (Coleman and Goff, 1985).

2.5.3.4. Sudan stain

Hand-cut sections of mature sugarcane stems were immersed in a saturated solution of Sudan III (colour index 248, Anachemia Ltd., Montreal, Quebec)) in 70% ethanol and left for 3-5 h. The sections were then quickly rinsed with 70% ethanol and mounted in a drop of melted glycerine jelly. Bright-field optics were used. Expected colour for a positive reaction with Sudan III is orange-pink colour which indicates the presence of lipid materials (O'Brien and McCully, 1981).

2.5.3.5. Phloroglucinol stain for lignin

Hand-cut cross sections of mature stems were mounted in a large drop of a saturated aqueous solution of phloroglucinol (1,3,5, trihydroxybenzene, Lot no. 792781, Fisher Scientific, Fair Lawn, NJ) in 20% HCl. Lignin will appear red-violet colour under bright-field optics (Jensen, 1962).

2.5.3.6. Neutral red test for cell wall permeability

Thick hand-cut cross sections of mature stems were stained for 5-10 min in a fresh 0.1% (w/v) solution of neutral red (Lot no. 1B469, Chroma-Gesellschaft) in 0.025 M potassium phosphate buffer, pH 7.8, mounted in buffer and observed with bright-field optics. This staining was used to detect diffusive barriers of parenchyma cell walls. Vacuoles of living cells without a diffusive barrier on walls will be stained red (Wenzel and McCully, 1991).

2.5.4. Microscopy

Optical microscopy was with an Olympus Vanox system with SPlan and UVFL series objectives, or with a Zeiss Axiophot system. Fluorescence studies were carried out on a Vanox Olympus microscope using a UV exciter filter (UG-1, 350 nm) and a "U" barrier filter (L-420 with built-in dichroic mirror, DM400).

Electron microscopy was with a Phillips 420 transmission electron microscope.

Photomicrographs were recorded on Kodak T-Max 100 and Kodak 4489 electron microscope film, and in colour on Ektachrome daylight film or Fuji Velvia using appropriate colour correction filters for bright-field images.

2.5.4. Cryo-scanning electron microscopy

Refer to Materials and Methods in Chapter 4. Details of these methods are given in Huang *et al.* 1994.

Table 2-1. Calibration of hydrogen: Height and area of the peaks produced by different concentrations of standard hydrogen gas in the gas chromatograph (means of 5 determinations)

concentration		peak height (chart units)		peak area (x 10 ⁵ chart units)	
ppm	mean	standard deviation	mean	standard deviation	
4	23	0.4	2	0.1	
10	55	3	5	0.5	
25	143	4	19	0.8	
50	292	5	39	0.8	
100	567	5	72	1	
200	1018	24	119	2	
300	1338	5	148	0.3	
400	1609	15	175	6	
600	2049	18	217	3	
1000	2594	46	271	3	

Table 2-2. Calibration of ethylene: Height of the peaks produced by different concentrations of standard ethylene gas in the gas chromatograph (means of 5 determinations)

concentration (ppm)	mean (chart units)	standard deviation
5	15	1.6
20	53	1.9
40	99	2.9
50	126	8.6
80	196	11.0
100	239	12.1
120	280	8.6
160	328	15.4
200	412	27.8

Figure 2-1

Fig. 2-1. Standard curves for concentration of hydrogen gas (ppm) vs. peak height and peak area with standard deviations.

Figure 2-2

Fig. 2-2. Interpolation curve of peak height readings of standard H₂ samples plotted against concentration in ppm.

Figure 2-3

Fig. 2-3. Double-reciprocal curve of standard hydrogen gas samples.
H, concentration of hydrogen gas sample in ppm; R, reading
of peak height.

Figure 2-4

Fig. 2-4. Data of Fig. 2-3 plotted in the range from 100 ppm to 1000 ppm, showing the detail of the left end of the line.

Figure 2-5

Fig. 2-5. Standard curve for hydrogen in the range 0 -100 ppm, in terms of simplified calibration equation (3).

Figure 2-6

Fig. 2-6. Standard curve for ethylene with curve fit, ranging from 4 to 100 ppm against peak height.

CHAPTER THREE.
DO ACETOBACTER LIVE IN THE XYLEM OF SUGARCANE
STEMS? SOME ANATOMICAL AND PHYSIOLOGICAL
DATA

3.1. INTRODUCTION

Sugarcane is normally propagated asexually from stem cuttings (setts, or "seed"-pieces), each having two or more nodes. At each node, above the leaf scar, there are 30-120 root primordia and a bud. A few days after planting, the root primordia and the bud sprout. Measurements of the acetylene reducing activity of stem cuttings suggested that the N_2 -fixing bacteria (species unknown) move from stem cuttings to roots and the new stems (Patriquin, *et al.* 1980). We found that *Acetobacter* must move into new shoots from cuttings since they can be isolated from second generation of plants in greenhouse (Dong *et al.*, 1995). It was suggested that the *Acetobacter* may move through the plant tissues in the xylem by some authors (James *et al.* 1994; Reis *et al.* 1994) and the xylem has even been considered as a potential environment for nitrogen fixation (Sprent and James, 1994). This chapter will examine the structure and function of sugarcane stems, especially the xylem system in the stems.

There are several major criteria that must be met if *Acetobacter* or any other harmless bacteria are to be able to live in the xylem and move freely into newly developing stems. 1. They must not block the xylem because the plant will wilt and die or be weakened. 2. There must be no blockage to the free movement of the bacteria along the xylem -- mechanical blocks such as vessel end walls, also physiological blocks like apoplast to symplast shunts of the transpiration stream. 3. The plant must not undergo any sort of

hypersensitive response to the bacteria in the xylem. 4. Sugar as a carbon source must be present in the xylem sap. For *Acetobacter*, there should be a reasonably high concentration of sugar in the sap.

The xylem is the route for upward movement of water in the plants and its conducting components are suited for mass flow of liquid. In most flowering plant these conducting components are vessels which are open pipes made up by dead vessel elements, each vessel element having developed from a single cell. Vessels are seldom continuous from top to bottom of a plant but several vessels can be stacked end to end along the length of the plant with a cross wall between them. The open pipe between two cross walls is called a vessel. Two vessels often lie adjacent to each other and these have thin spots along their common lateral walls. These thin spots or pits are often not open but closed by a very thin wall known as a pit "membrane". Movement of bacteria in the vessels has been indicated as a mode of infection of stems and seeds of plants by pathogenic and non-pathogenic bacteria. As beneficial endophytes, nitrogen fixers should not damage or block the vessel system but pass through the vessels or multiply and be functional inside them if the xylem is their habitat. Vessel lengths (the distance between two end walls) range from a few centimetres to many metres. The end walls of vessels are porous to solution flow, but the pores are very small, they stop colloidal suspensions, air bubbles and micro-organisms. Some sugarcane varieties, because comparatively few vessels extend continuously from one internode to another, show morphological resistance to fungal spore spread (Atkinson, 1938; Teakle *et al.* 1978).

These studies suggested that a large number of continuous vessels is an essential prerequisite of the ready migration of microorganism up or down the stalk.

The study of water movement on its way in the tracheary elements has a long history (O'Brien and McCully, 1981). Water can flow from one vessel to another vessel without leaving the apoplast by crossing pit membranes on the side walls, or passing through the end walls of the vessels. The location of the end walls of vessels and hence vessel length distribution can be revealed by finding where colloidal particles are stopped by the end walls (Zimmermann and Jeje, 1981). Until recently it was thought that all movement of water in the transpiration stream was through the apoplast only but study of the transpiration stream in leaves uncovered an associated symplastic water path (Canny, 1990a, b; 1993). In this latter study a fluorescent dye (sulphorhodamine G) was used to trace water pathways. This dye is negatively charged, travels almost as fast as the water and is confined to the apoplast as it cannot cross cell plasma membranes. The dye will accumulate at places where water enters the symplasm through these cell membranes. Such local high concentrations of dye were termed "sumps" (Canny, 1990a). The site and flux of water into the symplast is called a "flume" (Canny, 1990a). The site of a flume can be revealed by the presence of a sump, ie. an accumulation of the tracer dye. Sumps and flumes would be barriers to movement of bacteria along the xylem, so I have looked for these in the sugarcane stems. In my study, a simple method of locating sumps, and therefore flumes (O'Dowd and Canny,

1993), is used to examine the extent of the xylem apoplast and to search for movement of water between apoplast and symplast in sugarcane stem.

Because *Acetobacter diazotrophicus* shows optimal growth with 10% sucrose, it would be expected that this optimal synthetic medium should be close in composition to the natural environment of the bacteria in the sugarcane stem. Although sugarcane stem parenchyma may have 15% sucrose in the vacuoles, this sugar may not be available to support N₂-fixation by bacteria in the xylem if the xylem is isolated from the parenchyma cells as has been reported by Jacobsen *et al.*, (1992). Evidence of a solute barrier around the xylem, and the definition of the extent of the xylem apoplast are two key tests of the hypothesis of xylem-living *A. diazotrophicus*.

In the present study I examined the sugarcane plants from which the *Acetobacter* was isolated for the continuity of xylem vessels and the extent of the xylem apoplast in the stems. I also tested the possibility that *Acetobacter* would live in the xylem as an endophyte by deliberately introducing the organisms into stem xylem and following the reaction of the surrounding parenchyma cells.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

Sugarcane (*Saccharum* sp. hybrid, cultivar Ja 60-5) plants were grown in the greenhouse of Carleton University. Plants used were one year old sub-cultured from stem cuttings, 2.5 to 3 m tall. Healthy plants were excised near the soil level under water. The cut ends of the shoots were placed in clean water, recut one node above, and used for the introduction of dye, latex suspension and bacteria as described below.

3.2.2. Anatomy and histochemistry

3.2.2.1. Light Microscopy

Fresh free-hand transverse sections of sugarcane stems were made with razor blades at the midpoint of mature internodes. The sections were stained with toluidine blue pH 4.4 for structure and tested for lignin with phloroglucinol-HCl and for suberin with Sudan III as described in section 2.5 of Staining Methods.

The permeability of parenchyma cell walls was tested on fresh hand sections by staining with neutral red as described in section 2.5 of Chapter Two.

The location of suberin was examined by staining with PAS and rhodamine B, and observing under UV light as described in 2.5 of Chapter Two.

3.2.2.2. Scanning Electron Microscopy and Transmission Electron

Microscopy

As described in Materials and Methods of Chapter Four.

3.2.3. Apoplastic tracers

3.2.3.1. Latex paint

The continuity of xylem vessels was examined using a water suspension of a commercial green latex paint. The paint suspension with minimal particle agglomeration was prepared according to Zimmerman and Jeje (1981) by diluting commercial green latex paint 200 times with water. The suspension was left standing for a week while large particles settled. The particles left in the pale green supernatant are approximately 1 μm in diameter so should pass neither through xylem walls nor through pit membranes. On the other hand, they are easily carried along vessels, and through perforation plates at the ends of individual vessel elements. This test was used to find bacterial screens in the apoplast.

The sugarcane shoots cut under water were transferred quickly from water to a container filled with supernatant from the green paint suspension, and the plants were allowed to transpire in the greenhouse. After two to three days, hand sections of stems were cut and observed with an Olympus dissecting microscope.

3.2.3.2. Fluorescent dye

The extent of apoplastic transport was studied by using 0.05% sulphorhodamine G (SG) (Lot no. 6301 Aldrich Chemical Co. Inc.) water solution (w/v) as a tracer. This is a fluorescent dye which is confined to the apoplast and does not bind to cell walls but it is a small molecule which passes easily through vessel end walls and pit membranes. Because they are charged the dye molecules cannot pass through plasma membranes. This test was used to locate solute barriers where transpiration water moves from apoplast to symplast.

The transpiring shoots of sugarcane were transferred from water to the dye solution and left standing for 20 to 60 minutes. Hand sections of the stem were cut in paraffin oil. This procedure stabilizes the water-soluble dye in place in the stem and retains it *in situ* (O'Dowd and Canny, 1993). Sections were mounted under coverslips in paraffin oil and examined with fluorescence optics under UV or green light excitation.

3.2.3.3. Can *Acetobacter* live in the xylem?

3.2.3.3.1. Inoculation

Acetobacter diazotrophicus was inoculated on slopes of LGI medium as described in section 2.2. After three days incubation at 30°C, the colonies were washed off with 5% sucrose water solution from slopes into liquid LGI medium (without agar). This bacterial suspension was used to inoculate the xylem of sugarcane by

transferring cut sugarcane shoots from water into the suspension and letting them transpire in greenhouse for different times (one to five days) before the observation.

3.2.3.3.2. Observation

Hand sections were cut transversely from fresh stems tissues of the above plants. The sections were stained with PAS and DAPI and observed with a fluorescence microscope using UV excitation as described in section 2.5. Small longitudinal slices of internode tissues were also fixed and embedded in Spurr's resin. Ultrathin sections were cut, stained in both uranyl acetate and lead citrate and examined in a Phillips 420 TEM. For details of those methods see Chapter Two.

3.3. RESULTS

The sugarcane stem is solid, unbranched, roughly circular or oval in cross-section. The stem is clearly differentiated into joints, each comprising a node and an internode. Each stem has a hard, wax-covered rind (epidermis) surrounding a mass of softer tissue (parenchyma) which is interspersed with vascular bundles.

3.3.1. Anatomy of vascular bundles

Around 1000 vascular bundles are scattered throughout a sugarcane internode. The vascular bundles are more abundant

towards the periphery than in the centre, so the mechanical structure of the stem is fundamentally tube-like. Protoxylem may be absent in peripheral vascular bundles, but even when present it may lack a lacuna. Typical internal bundles have large protoxylem lacunae. Metaxylem virtually always consists of two large vessels (rarely one), separated from the protoxylem and phloem by parenchyma cells (Fig. 3-1 A).

All the vascular bundles in the internodes are parallel, and the number of vascular bundles in each internode remains constant. In contrast, in the node region there is considerable branching and merging. Two to three mm below the point of leaf sheath insertion some bundles begin to branch and merge. In the node many large bundles and bundle branches turn off the axis to supply the leaf sheath as leaf traces. Immediately above this zone there is a region of finer lateral bundles arising as branches from the vertical bundles. These fine bundles ramify through the node and lead to the root primordia.

A vascular bundle is made up of a sclerenchymatous sheath (composed of cells with thick, lignified walls) which encloses the xylem and phloem tissues. The sclerenchymatous sheath is most strongly developed on the outer and the inner facing sides of the bundle, typically forming two caps (Fig. 3-1 A). The part of this sheath adjoining the phloem consists of thicker-walled cells than those adjoining the xylem. The sclerenchyma sheath surrounding the vascular tissue may be two or more cell layers thick. All the

sclerenchyma cells are lignified and suberized in the mature stem. The sclerenchyma sheaths of peripheral bundles are particularly well developed, giving these bundles a conspicuous appearance.

Xylem and phloem are disposed collaterally in relation to each other in bundles. The xylem is composed of protoxylem and two large pitted vessels. The protoxylem which consists of a short row of annular and spiral elements is more extensive in the node than in the internodes. The internodal bundles, except those near the periphery, possess a more or less conspicuous protoxylem lacuna. The phloem consists of sieve tubes and companion cells. It is most extensive in the bundles of the node and least conspicuous in the peripheral bundles of the internode.

A single layer of parenchyma cells usually separates the xylem and phloem from the sclerenchyma sheath. The metaxylem is separated from the protoxylem and phloem by parenchyma cells as well. Electron micrographs of the vascular tissue revealed a suberised lamella developed in the walls of the innermost sclerenchymatous sheath cells (Fig. 3-3). This lamella forms a complete circle around each cell producing barriers between the sclerenchymatous sheath cells (Fig. 3-3 B & C) as well as between the sclerenchymatous sheath and storage parenchyma cells (Fig. 3-3 D). The suberised lamella appeared as a distinct bilayer with two electron-dense bands separated by a region of lower electron density (Fig. 3-3 B & C).

In the middle part of a mature internode all the late metaxylem (LMX) vessels are mature and functioning since the apoplast dye and latex paint were transported through them until near the node (see details below). Below the node, from about 5 mm beneath the leaf insertion, some LMX vessels are still alive. Thick sections stained with toluidine blue showed that some vessels have cross walls (Fig. 3-2 D) with bright-field optics and strands of cytoplasm with phase contrast microscopy (Fig. 3-10 C). Inside the vascular bundle, lignification occurs in xylem vessels and even some sieve tubes.

The images of frozen fresh stem tissues showed that the protoxylem lacunae in the internode were filled with liquid (Fig. 3-1 B). The xylem lumens were filled with thinner patterns of lines compared with the phloem cells. This is the typical characteristic appearance of a rapid frozen solution containing a low concentration of solutes (Canny and Huang, 1993). The frozen pattern of protoxylem sap is the same as that of LMX sap, suggesting that they have a similar concentration of solutes.

3.3.2. Anatomy of stem parenchyma tissues

The ground tissue or matrix of an internode is composed of parenchyma cells in which the vascular bundles are embedded. This parenchyma consists of longitudinally arranged files of thin-walled cells. These cell files are separated by long narrow intercellular spaces at their corners (Fig. 3-5 A & B). Most of the intercellular

spaces extend past many cells in longitudinal section and are triangle-shaped in cross section (Fig. 3-4 A). Because the stem parenchyma cells lie in longitudinal files, the spaces are continuous along the stem, but not across it. The size of parenchyma cells increases gradually toward the centre of the stem with the same size of cells in each file. There are some files of small cells among the files of big cells.

The positive phloroglucinol-HCl reaction of the entire stem tissue showed that most tissues are lignified in mature sugarcane stem. The majority of parenchyma cell walls are lignified, with some isolated parenchyma cells nonlignified. Some parenchyma cell walls are lignified only where they contact other cells, leaving the portions facing intercellular spaces nonlignified (Fig. 3-4 A & B).

The results of the tests for suberin with Sudan III showed that all storage parenchyma cell walls are suberized except for some isolated cells. These latter cells appear to be the same ones which are totally or partially nonlignified. Thus the pattern of suberization of these cells is similar to that of lignification. The reactions of cell walls to Sudan staining were consistent with that of fluorescent staining. With PAS and rhodamine G staining, fluorescence observation confirmed that the isolated cells are either unsuberized or suberized where they contact other suberized cells (Fig. 3-4 C), but remain unsuberized where they face intercellular spaces.

Neutral red is widely used as a vital dye. The positive reaction

of neutral red staining is accumulation of dyes in vacuole saps (Wenzel and McCully, 1991). However the premise of this reaction is that the cell walls must be permeable to the neutral red. The suberized and lignified cell walls are obviously not qualified for this prerequisite. In hand sections of mature sugarcane stems stained with neutral red some separated parenchyma cells showed neutral red accumulation, again suggesting that these cell walls are not suberized or not fully suberized (Fig. 3-5 C & D).

3.3.1. Vessel continuity

Two days after the sugarcane plants had been transpiring in the green paint suspension, the plants began to show water stress. Old leaves became wilted in three days, suggesting blockage of the water transport. The transpiration stream carries the paint particles to the vessel ends. The water passes through, leaving the particles at increasing concentration until the vessels are virtually packed with particles. This filtration process finally leads to the blockage of the vessels. At the end of the infusion time, the stems were cut into segments and transported to the laboratory. Transverse sections were made from each internode and observed under a microscope. The latex particles stayed in the vessels during the sectioning and were green with bright-field optics.

In the first internode above the cut surface, all the metaxylem vessels contain green paint particles. Especially immediately below the first node, both large metaxylem vessels in all bundles are

packed with the particles (Fig. 3-2 A). In the second internode counted from the bottom cut surface (after one node), there were about 10% of bundles with only one metaxylem vessel containing green paint and one metaxylem clear (Fig. 3-2 B). The other 90% bundles contained two clear vessels. After two nodes, in the third internode, there were about 1% of bundles with one vessel filled with green paint.

In the node region large metaxylem vessels quite often split into two or more smaller diameter vessels. These vessels could be connected through perforations resulting in distribution of green paint into all the vessel branches. However, sometimes two vessels are not connected directly, but with parenchyma cells in between which let water pass through from one vessel to another but leaving green paint behind (Fig. 3-2 C).

3.3.4. Extent of apoplast transport

The fluorescent dye sulphorhodamine G, which is restricted to the apoplast, was drawn by transpiration through the cut end of cut plants of sugarcane to study the isolation of xylem from storage tissue and the extension of the xylem apoplast.

In the internodes the dye was confined to the vascular bundles even two hours after the dye introduction (Fig. 3-6 D). However in the node region, while some bundles are still isolated in the node region, some bundles are not isolated from parenchyma tissues at all.

All walls of parenchyma cells around them are pink and fluoresce under green light and UV light (Fig. 3-7 D).

All the protoxylem elements, including lacunae, have dye inside even to the very top of the plant. All the protoxylem elements are pink and fluoresce red under green light and under UV light. No sumps were found in the protoxylem.

The pattern of continuity in the metaxylem is quite different. In the first internode above the cut surface, all the LMX vessels are pink, full of sulphorhodamine G and conducting the transpiration stream. In the second internode, only about 10% of the bundles have one LMX vessel containing the dye. All the other metaxylem vessels are clear. After two nodes, only a few LMX vessels contain the dye. In all the internodes after the first node, only one LMX vessel in a bundle carries dye.

Examination of the tissue just below the first node showed most LMX vessels were full of red dye crystals (sumps) (Fig. 3-6 A). There were about 10% of bundles having one LMX vessel with a sump inside, and one LMX vessel carrying dye solution. Longitudinal sections showed that at the node the LMX vessel elements were short and quite often split into several small vessels, and there were sumps in most of these small vessels. The sumps are first formed in the pit cavities and pit canals and gradually in the vessel lumina (Fig. 3-7 C, A & B).

In the second internode there were about 10% of the bundles having one LMX vessel containing dye solution (Fig. 3-6 B). All the protoxylem vessels were full of dye solution. After three nodes dye solution was not found in any of the LMX vessels but in all the protoxylem vessels (Fig. 3-6 C).

3.3.5. The reaction of xylem to *Acetobacter*

Hand sections of plant stems fed with *A. diazotrophicus* broth showed accumulation of the bacteria in the same places as the green paint particles. All the LMX vessels in the first internode contained many bacteria (Fig. 3-8 A). In the second internode only 10% of the vascular bundles had one LMX vessel with bacteria. All the other vessels were free from bacteria.

After introducing *A. diazotrophicus* into plants, the xylem parenchyma cells around the LMX vessels began to synthesize unidentified mucilaginous material. The mucilages showed strong metachromatic pink with toluidine blue staining. In the first day of bacterial introduction, the xylem lumen was almost free of the polymers. The mucilaginous material was deposited in the parenchyma cells between the cell membrane and the cell wall at the xylem side (Fig. 3-1 C). This mucilage material gradually filled the pits and passed to the xylem through the floor of the pits and lined the xylem side walls (Fig. 3-1 D). After three days of exposure to bacteria, the mucilage polymers accumulated in the lumen and finally occluded it (Fig. 3-9 D). The mucilage polymers turned red

after four days introduction of the bacteria (Fig. 3-8 B).

Before and during the accumulation of the mucilaginous material in the xylem lumen, adjacent xylem parenchyma cells exhibited structures suggestive of intensive cellular synthetic activity. They contained dense protoplasm, large nuclei, much rough endoplasm reticulum, many mitochondria, and a notable increase in the number of Golgi bodies (Fig. 3-9 A, B & C).

3.4. DISCUSSION

3.4.1. Isolation of bundles

Since the *A. diazotrophicus* needs a high concentration of sucrose (Cavalcante and Döbereiner, 1988), their natural site in plant would have sucrose available. In sugarcane stems, diffusion of sucrose into the xylem would seem likely, since the apoplastic fluid in the storage parenchyma tissue contains up to 13% (w/v) sucrose (Dong *et al.* 1994) and is separated from the xylem by only a few cells. Hawker (1965) reported concentrations of up to 9% sucrose in the xylem of sugarcane. Other reports have shown that xylem sap is essentially free of sucrose (Welbaum *et al.*, 1992). The apoplastic tracer dye fed into the stem of sugarcane from cut end was confined to the vascular bundles in internodes, with no dye in parenchyma tissues. This confinement of dye in the xylem apoplast suggests that a semipermeable barrier exists between the xylem and the storage tissue in sugarcane stem tissue, restricting movement of large

molecular solutes. Lignification of the cell walls may reduce the porosity of the walls. Suberin deposits throughout the cell walls and the suberised lamella in the sclerenchymatous sheath cell walls could prevent sucrose from diffusing into the xylem. Thus both structural and experimental evidence is consistent with vascular tissue being isolated from the storage parenchyma for solutes.

In mature internodes most of the storage parenchyma cells are lignified and suberized to a significant degree, except for a portion of the walls of some isolated cells. These results agree with those of Jacobsen *et al.* (1992) who demonstrated that some isolated cells are not lignified in young tissue. Then gradually in advanced stages of development, their walls become lignified where they contact other lignified cells, but remain nonlignified where they face intercellular spaces. These isolated cells remain unsuberized. These cells may have the capacity to act like transfer cells involved in transportation of sucrose and other organic molecules between symplast and apoplast (intercellular spaces among the parenchyma cells).

3.4.2. Vessel extent and apoplast transport

The results of the green paint experiment showed that sugarcane Ja 60-5 has few vessels that extend continuously from one internode to another. The studies on anatomical resistance to movement of the sugarcane pathogens showed that resistant clones mainly had vessels which were discontinuous at the node, whereas susceptible clones had many vessels which passed through the node

without interruption (Atkinson, 1939; Teakle and Appleton, 1978). With few continuous vessels in Ja 60-5, any bacteria that got into the vessels of one internode have a small chance to spread into vessels of another internode ($10\% \times 1/2 = 5\%$). Low concentration of sucrose in vessels (which is the result of isolation of bundles) and morphological resistance to longitudinal spread combine to limit the possibility that *A. diazotrophicus* might live in and spread through the xylem vessels.

Mostly two vessels contact each other directly without any living cells in between. Xylem sap can pass from one vessel to another through end walls or pits on the vessel walls. Because the green paint particles are too big to pass through the pits, the vessel fed with paint suspension will accumulate paint particles, and the next vessel contains none. This is the result of filtration by the pits. In sugarcane the vessel coming from the internode below meets the vessel coming from the internode above at the node region. These two vessels do not have direct contact with each other. There are parenchyma cells between the two vessels (Fig. 3-2 C). So, the filtration process occurs in sugarcane node when water passes from the vessel below to the vessel above not via the pits but by living cells. This hypothesis was further supported by the SG experiment (see next section).

In sugarcane stems the protoxylem lacuna seems to function as a large vessel. It is filled with xylem sap, and transported sulphorhodamine G solution from the base of the plant to leaves. All

the protoxylem lacunae disappeared in the node region, and changed into several small protoxylem vessels. The pits on the end walls of these small vessels trapped the green paint particles, any bacteria and air bubbles but allow the dye solution to pass.

Sugarcane stems showed segmentation in water transportation. Water was transported in xylem vessels in the internode. But in the node region, as indicated by the experiment with SR solution, most vessels ($90\% + 10\% \times 1/2 = 95\%$) end and water left the apoplast and entered the symplast depositing a high concentration of SG as a sump. The segmentation of the flow apoplast by living cells which will stop transport of particulate matter where there is a flume is a new finding.

In the monocotyledon leaves the sumps of SR formed in the intercellular spaces inside the parenchyma sheath (Canny, 1990a, b). In contrast to the leaf sumps the sumps of sugarcane stems were within the xylem vessels, not outside them. This is similar to what occurs in dicotyledon's leaves. The separation of the water into the symplast apparently occurred at the cell membranes of xylem parenchyma cells where they were in contact with the transpiration stream through the pits in the vessel walls.

3.4.3. Response to *Acetobacter* in vessels

Responses of the xylem to infection by *Acetobacter* were vigorous. They involved active synthesis and secretion of polymers

in the xylem parenchyma cells adjoining vessels. No mucilage deposit was found within the xylem of control plants which were fed with sterile water or sterile medium. Similar secretions of polymers from parenchyma cells into the xylem vessels have been reported as plant responses to pathogen infection (Bishop and Cooper, 1984; Schmitt and Liese, 1990; Shi *et al.*, 1991). These secretions and the microbes occlude the xylem vessels and cause wilting of the plant.

As a conclusion, it seems not possible for *A. diazotrophicus* to live in and move through the vessels in sugarcane stems.

Figure 3-1

Fig. 3-1. A. Hand-cut cross section of sugarcane stem internode stained with toluidine blue (pH 4.4), showing structure of a vascular bundle. Bright-field optics. 250x. LMX, late metaxylem; PH, phloem; PX, protoxylem lacuna.

B. Planed frozen vascular tissue from the internode of sugarcane stem, showing the xylem sap in late metaxylem (LMX) and the protoxylem lacuna (PX). Cryo-scanning electron micrograph. 340x. PH, phloem.

C. Xylem parenchyma cell (XP), and late metaxylem vessel of sugarcane. *Acetobacter diazotrophicus* was put into the xylem vessel lumen (VL). There are bacteria (B) in the vessel and mucilaginous material (MU) between the plasma membrane and wall of the parenchyma cell just against xylem pit. Transmission electron micrograph. 14280x. VW, vessel wall; M, mitochondria.

D. Similar to location as C, where some mucilaginous material has entered the xylem through a pit. The mucilage has been changed (darker). 14280x. VW, vessel wall; VL, vessel lumen; B, bacterium; XP, xylem parenchyma.

2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
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PRECISIONSM RESOLUTION TARGETS

Figure 3-2

Fig. 3-2. Hand-cut transverse sections of internodes of sugarcane stem which had been fed with green latex paint from the base of the stem piece, showing the locations of paint particles. Bright-field optics.

A. Below the first node above the feeding point, showing that all the LMX vessels were full of concentrated paint particles (dark green). Unstained. 45x.

B. Below the second node above the feeding point, showing that only one LMX vessel had paint particle inside (green). Unstained. 150x.

C. Similar to section as A., showing parenchyma cells between two LMX vessels. Unstained. 115x.

D. Similar to section as B, showing cross walls (CW) in immature LMX vessels. Toluidine blue O (pH 4.4) stained. 230x.

Figure 3-3

Fig. 3-3. Vascular bundle sheath cells (BC) of sugarcane stem internode showing the suberized lamella (arrows) in sheath cell walls. A. Bundle sheath cells. B. and C. Cell wall between bundle sheath cells. D. Cell walls between bundle sheath cell (left) and parenchyma cell (right). A. 4500x, B. 40800x, C. 23250x and D. 50000x.

Figure 3-4

Fig. 3-4. Hand-cut transverse sections of mature internode parenchyma tissue.

A. Unstained section, violet-induced fluorescence, showing the less fluorescent walls of isolated cell (red arrows). 460x.

B. Section reacted with phloroglucinol-HCl, showing lignified walls of most parenchyma cells and non-lignified walls of the isolated parenchyma cells (blue arrows). Bright field optics. 600x.

C. Hydrophobic walls (white fluorescence) of most parenchyma cells and hydrophylic walls of isolated parenchyma (blue fluorescence; red arrows) and walls facing intercellular spaces. Rhodamine B staining, violet-induced fluorescence; fluorescence optics. 460x.

Figure 3-5

Fig. 3-5. A. and B. Longitudinal sections of mature internode. Long intercellular spaces (arrows in A and * in B) are shown by partial polarized light and basic fuchsin staining. A. 230x, B. 1500x.

C. and D. Thick hand-cut sections of mature internode, neutral red stained showing two populations of parenchyma cells. 115x. C. Cross section, D. longitudinal section.

Figure 3-6

Fig. 3-6. Cross sections of internodes of sugarcane fed with 0.05% sulphorhodamine G (SG) from the base of the stem piece, A., B., and C. UV light induced fluorescence, and D. green light induced fluorescence.

A. Section just below the first node above the base, showing the accumulation of the dye, called sumps (red) in LMX vessels. 150x. PX, protoxylem.

B. Section below the second node above the base, showing dye sump (red) in one LMX vessel and white fluorescence indicating a lower concentration of SG solution in protoxylem lacuna (*). 115x.

C. Section below the fourth node above the base, showing SG fluorescence in protoxylem lacuna (*). 150x. LMX, late metaxylem.

D. Cross section of a stem fed with SG for two hours, showing the red fluorescence of SG confined in vascular bundle. 115x.

Figure 3-7

Fig. 3-7. Sections in a node region of sugarcane stem fed with SG solution. UV induced fluorescence.

A. Longitudinal section below the first node above the base of the stem piece, showing dye sump (red) inside the LMX vessel. 150x.

B. Cross section of a split bundle, showing the sump developing from the walls towards the center of the lumen inside one LMX vessel. 230x.

C. Longitudinal section of a small vessel filled with SG, showing the dye accumulation in the pits. 1500x.

D. Longitudinal section showing the yellow fluorescence of low concentration of SG solution leaked into the parenchyma tissue. 115x. VB, vascular bundle.

Figure 3-8

Fig. 3-8. Cross section of sugarcane stem internode fed with *A. diazotrophicus* broth through the cut base of the stem piece.

A. Section below the first node above the cut stem base after two hours of introduction of *A. diazotrophicus*. PAS and DAPI staining, UV-induced fluorescence, showing the bacteria inside the LMX vessels. 460x.

B. Similar section of a stem fed with *A. diazotrophicus* for four days, unstained, showing the red material produced by the xylem parenchyma plugging the LMX vessels. Bright field optics. 300x.

Figure 3-9

Fig. 3-9. Xylem parenchyma cells adjacent to xylem vessels which contain *A. diazotrophicus*. A to C show evidence of intensive cellular synthetic activity with numerous Golgi bodies with associated vesicles. And D shows dark staining mucilaginous material (MU) still within the parenchyma wall in A and moved into the xylem lumen in D. G, Golgi body; M, mitochondria; N, nucleus; RE, rough endoplasmic reticulum; VW, vessel wall; arrows, ribosomes; arrowheads, suberized lamella. A. 14280x, B. 25160x, C. 40800x, and D. 4488x.

Figure 3-10

Fig. 3-10. Cross sections of vascular bundles of mature sugarcane stem internode.

A. and B. Suberized lamella of bundle sheath cells fluoresce white. LMX, late metaxylem; PH, phloem. Fluorescence optics. A. 350x; B. 230x.

C. Cytoplasmic strands (red arrows) inside of the developing LMX vessel elements indicate that they were still alive. Nomarski DIC optics. x.460

CHAPTER FOUR
A NITROGEN-FIXING ENDOPHYTE
OF SUGARCANE STEM:
A NEW ROLE FOR THE APOPLAST

4.1. INTRODUCTION

In some areas in Brazil sugarcane has been grown continuously for more than 100 years without any nitrogenous fertilizer, and it has long been suspected that substantial nitrogen 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) have concluded from ^{15}N and nitrogen balance studies that some sugarcane varieties in Brazil are particularly effective in obtaining their nitrogen from associative biological fixation. Nitrogen fixation in the soil associated with the roots of Brazilian sugarcane has been demonstrated by acetylene reduction (Döbereiner, Day and Dart, 1972) and by $^{15}\text{N}_2$ fixation (Ruschel, Henis and Salatia, 1975; Ruschel *et al.*, 1978; Lima, Boddey and Döbereiner, 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 found in 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-Ramires *et al.*, 1993). This bacterium, *Acetobacter diazotrophicus* (Gillis *et al.*, 1989), has most unusual growth requirements. It shows optimal

growth with 10% sucrose and pH 5.5. It will grow in medium with sucrose 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. It is a prime suspect as the contributor of 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 the other workers have been slow and laborious. The cultures grow very slowly (15 to 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 been often isolated from surface-sterilized tissue (Döbereiner, Reis and Lazarine, 1988). The proof that it is indeed an endophyte is difficult, 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 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, which 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, nitrogen from the air and amino-carbon compounds back to the plant, and a controlled supply of oxygen for the bacterial respiration without inhibiting the nitrogenase. We have therefore conducted a search for the endophyte in the stem tissues, to see if it could be found inside cells or outside them, in vascular tissues or in the ground parenchyma, in nodes or internodes.

The stem tissues of sugarcane have been the subject of study over many years as the site of sucrose 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) have confirmed the older results of Hawker (1965) that there is much sucrose in the apoplast of the stem parenchyma. The solution obtained by centrifuging the stem pieces contained up to 0.5 M sucrose 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). They 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 further checked.

We hypothesized that the sucrose-requiring endophyte might be living in the sucrose 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-scanning electron microscope (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.

4.2. MATERIALS AND METHODS

4.2.1. Plant material

Plants of sugarcane (*Saccharum officinarum* L.), varieties Media Luna and Ja 60-5 were grown either in the fields of the FINCA field station of the Universidad de La Habana, or in pots in the greenhouses of Carleton University with supplementary lighting from Optimarc HID lamps. At Carleton University the Cuban parent material was sub-cultured successively from stem-node cuttings. Mature stem internodes were collected from nodes 10 to 20 proximal to the apex.

4.2.2. 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 ca. 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. In testing for the presence of bacteria, stem pieces were further sterilized before centrifugation by dipping them in ethanol and setting fire to them. Besides killing any organisms of the surface of the pieces, this procedure dried up 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 in 10 x 2.5 cm glass tubes, both of which were autoclaved before use. Speeds of the centrifuge were adjusted to produce accelerations from 30 g 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-scanning electron microscope. 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 % (w/w). Assuming that the solution had the same density as the rest of the tissue (see below), this percentage is also (v/v).

4.2.3. Cryo-scanning electron microscopy of stem tissues

The sizes and shapes of liquid spaces may be examined on the flat planed surfaces of frozen material as outlined by Huang *et al.* (1994). Briefly, pieces (ca. 5 x 2 x 2 mm) were cut from the stems and mounted on stubs with Tissue Tek and immediately frozen in N₂-slush. The stub was transferred under liquid N₂ to the chuck of a cryo-microtome (CR2000, Research and Manufacturing, Inc., Tucson, AZ, USA) and the sample was planed with a glass knife at -80°C, transferred under liquid N₂ 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 scanning electron microscope (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, Jackson and Oates, 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, photographed on Kodak T-Max 120 rollfilm developed in T-Max developer.

4.2.4. Measurement of volumes of walls and spaces

Images of the planed frozen faces of stem parenchyma were printed at standard magnification and analysed 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 volume of the tissue they occupy.

4.2.5. Culture of the endophyte

Culture of the type strain (PAL 5, ATCC49037) of *Acetobacter diazotrophicus* and of bacteria isolated from sugarcane tissues was on the nitrogen-poor medium LGI-P (Cavalcante and Döbereiner, 1988)

which contains 10% sucrose 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 bromophenol 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 a yellow-orange color. 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.

4.2.6. 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 determined.

4.2.7. 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 % (w/v) and were corrected for temperature. The pH of the solution was measured with a hydrogen electrode.

4.2.8. 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.

4.2.9. 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 over 1 h, and analysed 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, PA 19422). Rates of ethylene production are expressed as $\text{nmol h}^{-1} (10^9 \text{ cells})^{-1}$.

4.2.10. *In situ* observation of bacteria in the apoplast

a) 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, sputter-coated with gold and the newly exposed faces observed in the SEM at 20 kV.

b) Transmission electron microscopy. Small longitudinal slices of stem parenchyma were fixed and embedded by standard procedures (3% glutaraldehyde in phosphate buffer at 4°C, post-fixation in 1% osmium tetroxide, dehydration in acetone and embedment in Spurr's resin; see O'Brien and McCully, 1981 for details).

4.3. RESULTS

By conventional light and electron microscopy 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. Further, 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-colored gums. It is thus most unlikely that the endophyte inhabits the xylem vessels.

4.3.1. 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 Fig. 4-1 A, the vacuoles are filled with dense patterns of lines.

This is the characteristic appearance of a rapidly-frozen solution containing considerable solute (see 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 sucrose (10 to 20%), we are confident that the appearance of these vacuoles is characteristic of frozen sucrose solution. It is clear from Figs 4-1 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 sucrose solution of similar strength to that in the vacuoles. In all the fresh internodes examined from both varieties of sugarcane, nearly all the intercellular spaces were filled. A count of full and empty spaces along a transect across a section of tissue showed 4% of the spaces contained air. In longitudinal section (Fig. 4-1 C), it can be seen that the spaces extend past many cells, and that the whole of the space is filled with fluid. None of the common inorganic ions could be detected in vacuoles or spaces with the X-ray microanalyser attached to the SEM (limit of sensitivity ca. 10 mM).

4.3.2. 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. 4-1 D). Centrifuging at 30 g removed only a small part of this surface

solution (Fig. 4-1 E). Even after centrifuging for 10 min at 30 g and a further 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. 4-1 F). Moreover, counts of the empty and filled intercellular spaces along a transect showed 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 have contained in fact 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 4-1 G shows the appearance of the stem parenchyma frozen after such a treatment. All the cells are intact but almost all the intercellular spaces are now filled with air (Fig. 4-1 H). 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. Another approach 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 under Materials and Methods, namely, dipping the piece in ethanol and igniting it.

The results of the sterological analysis of the volumes of the cell walls and of the intercellular spaces are given in Table 4-1. Each space 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. Their contribution of xylem sap to the centrifuged solution cannot therefore exceed 20% of its volume, and may have reduced its concentration to this extent.

4.3.3. Concentration of the apoplastic fluid

The refractometer readings made on the extracted intercellular fluid indicated a sucrose 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 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.

4.3.4. 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 under 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 on to the *Acetobacter* growth medium, vigorous growth of the bacteria was seen after only three days. 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 per mL of apoplastic fluid.

Colonies, like those of the type strain of *Acetobacter diazotrophicus*, developed a yellow-orange color after about three days due to 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, contained the

bacteria embedded in a thick mucilaginous capsule which makes them difficult to separate and sub-culture. Further comparisons between the type strain and our isolates confirmed their similarity in respect of Gram negativity, ability to grow on 30% sucrose, and catalase positivity. A summary of the similarities between our isolates and the type strain is presented in Table 4-2.

Bacteria in the isolates were compared with those of the type strain in the electron microscope (Figs. 4-2 A and B), and 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. Further, in this latter material the endophyte was found in both the original plants grown for three years from cuttings from Cuba, and in the second and third generations of plants cultured successively by rooted stem cuttings. Thus, the endophyte transferred itself 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 N₂-fixation in the crop.

4.3.5. *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 electron microscopy. However, some bacteria do remain close to, or against the walls in corners of the intercellular spaces (Fig. 4-3 A). 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. 4-3 B). Individual bacteria could sometimes be detected at the surface of the mucilage (insert, Fig. 4-3 B).

4.3.6. Nitrogenase activity

Results of the acetylene-reduction test for nitrogenase activity are shown in Table 4-3. Isolates from var. Media Luna showed somewhat greater activity than the type strain, while those from var. Ja 60-5 showed slightly less. Both varieties resembled the type

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

4.4. DISCUSSION

The presence of fluid in intercellular spaces, which have usually been thought of as containing air in other plants, has been previously 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 though he had no way of directly rendering the space contents visible. He used diffusive exchange of [^{14}C]sucrose to estimate the volume of the apparent free space. He gives the volume of this space as 14% of the tissue, and concludes 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 sucrose by measurements of water potentials. Welbaum *et al.* (1992) have gone 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, that 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 artefact 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 vacuoles of the surrounding cells. In corn, the similarity was of ions, in sugarcane it is of sucrose.

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; that 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 where 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) gives values in the range 10 to 21%, Hawker (1965) gives 14% plus 4% of air space; Oworu, McDavid and MacColl (1977) found 10 to 58%; Welbaum and Meinzer (1990) record 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. While our estimates are direct geometrical measurements, our sampling is based on much more limited collections of material, and most of it 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 upon further work, using the appropriate techniques of molecular genetics. Nevertheless, the collected attributes of *Acetobacter diazotrophicus* are so unusual that the probability of confusion with another species is small. No other species is known to live on 30% sucrose, and to produce acid, and to reduce nitrogen in air, and to form yellow colonies on media containing the indicator which are covered by a capsule, and to have the form and flagellar complement illustrated in Fig. 4-2.

The concentration of bacteria in the apoplastic fluid (10^4 per mL) is not large compared with the numbers (10^6 - 10^7 / g dry wt.) of *Acetobacter diazotrophicus* obtained from homogenized tissues (Li and MacRae, 1992). On the other hand, because of the capsule 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 the N_2 -fixing endophyte (presumed *Acetobacter*) living in 12% sucrose 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 nitrogen. There is a large amount of the fluid. A harvested crop of sugarcane is approximately 100 tonnes per hectare. If the apoplastic fluid occupies only the 3% of the stems we have measured (and not the larger volumes commonplace in the literature), there are still 3 tonnes per hectare of the endophyte/sugar broth. This may well be enough to contribute a large part of the ~180 kg N required each season by the crop. 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% sucrose, 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 the acetic acid formed by the bacteria, become a priority for physiological investigation. The nitrogen-fixing activities of this system are more accessible to investigation and experiment than those of the legume nodule, and provide possibilities for studies on 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 where 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 sucrose into their intercellular spaces so that they might act as hosts to *Acetobacter diazotrophicus*, but probably less fanciful than thinking that they can be induced to make and operate nodules containing *Rhizobium*.

Table 4-1. *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 3000 g.*

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 % of the mass of the stem pieces. The % mass equals the % volume assuming that both solution and tissue have the same density. Means $\pm$ SD (N = 27).

Estimated Volume % of		Extracted Solution	
Intercellular Spaces	Cell Walls	% Mass	% Volume
2.8 $\pm$ 0.5	2.6 $\pm$ 0.3	3.1	3.1

Table 4-2. *Comparison of characters of the bacteria isolated from the intercellular space fluid of sugarcane stems with those of the type strain of Acetobacter diazotrophicus.*

Character	Isolates	Type Strain
Flagella peritrichous	+	+
Nitrogen fixation	+	+
Growth on 30% sucrose	+	+
Growth on LGI-P Medium	+	+
Mucilaginous capsule	+	+
Acid production	+	+
Yellow colonies on LGI-P	+	+
Catalase	+	+
Gram Stain	-	-

Table 4-3. *Rates of acetylene reduction by isolates from intercellular spaces of sugarcane stems, and by the type strain of *Acetobacter diazotrophicus*.*

Provenance denotes the variety and where the sugarcane was grown. Rates were measured by the formation of ethylene from added acetylene in air, by 7-d-old cultures on LGI-P medium without added CaCO_3 . Rates of reduction as $\text{nmol C}_2\text{H}_4$ produced per hour per 10^9 cells. For all the regression lines of $[\text{C}_2\text{H}_4]$ vs time, $P < 0.0001$

Strain	Provenance	Reduction rate $\text{nmol h}^{-1} 10^9 \text{ cells}^{-1}$
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

Figure 4-1

Fig. 4-1. A to H show 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, by asterisked arrowheads.

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. x 350.

B. Similar preparation to that in Fig. 1A. Single intercellular space filled with frozen solution. x 1530.

C. Longitudinally planed stem parenchyma of sugarcane var. Ja 60-5 showing the long intercellular spaces filled with solution. x 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. x 280.

E. The cut edge of a piece of tissue similar to that in D but var. Media Luna and after centrifuging at 30 g 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. x 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 500 g for 10 min. Some vacuolar solution (s) still remained held in the open cell walls by surface tension. x 280.

G. Stem parenchyma of var. Media Luna after centrifuging at 3000 g for 20 min. All the intercellular spaces have been emptied of solution, but the cells are intact. x 550.

H. Similar preparation to that in Fig. 1G. showing a single empty intercellular space. x 2160.

Figure 4-2

Fig. 4-2. Transmission electron micrographs of negatively stained bacteria. x 27 200.

A. Type strain of *Acetobacter diazotrophicus*.

B. Isolate MC-1 from sugarcane var. Media Luna.

Figure 4-3

Fig. 4-3. A. A transverse section through a portion of an intercellular space including two bacteria which have also been sectioned transversely. The asterisk marks intact middle lamella between the two cells that have partially separated to form the spaces. Transmission electron micrograph x 22 900.

B. A tangential view into an intercellular space showing part of the surface of a cell wall which lines the space. A group of mucilaginous colonies is attached to this wall. The insert shows outlines of bacteria (arrows) embedded in such colonies. SEM micrograph. Main figure x 10 500; Insert x 7 500.

CHAPTER FIVE.
FURTHER EVIDENCE THAT THE N₂-FIXING ENDOPHYTIC
BACTERIUM FROM THE INTERCELLULAR SPACES OF
SUGARCANE STEMS IS *ACETOBACTER*
DIAZOTROPHICUS

5.1. INTRODUCTION

Nitrogen-fixing, acid-producing, and acid-tolerant Gram-negative bacteria have been isolated from the fluid in intercellular spaces of sugarcane stems (Dong *et al.* 1994). These isolates grow best on a sucrose-rich medium and can continue to grow in up to 30% sucrose. Previously, we had found, by use of a small number of biochemical tests, that these bacteria were culturally, metabolically, and phenotypically similar to the N_2 -fixing species *Acetobacter diazotrophicus*, which was first described in 1989 (Cavalcante and Döbereiner 1988; Gillis *et al.* 1989). In the present report, we provide a more complete description of the phenotypic and cultural characteristics of this bacterium by use of a highly comprehensive panel of conventional biochemical tests and determination of cellular fatty acid (CFA) composition by a modern automated system.

The production of H_2 by the isolates has been compared with that of the type strain because this gas is an obligatory product of nitrogenase activity, resulting in N_2 fixation. The level of H_2 production can be used to monitor the activity of the N_2 -fixing process (Hunt and Layzell 1993; Simpson and Burris 1984). This H_2 production is suppressed to different degrees in different N_2 -fixing bacteria by the presence of acetylene (Dilworth 1987). The extent of the suppression has been used to further characterize the isolates from the sugarcane stems.

5.2. MATERIALS AND METHODS

5.2.1. Plants

Two varieties of sugarcane (*Saccharum officinarum* L.), cv. Media Luna and Ja 60-5, which had been cultivated in the field in Havana, Cuba, or were first-, second-, or third-generation subcultures of Cuban stocks propagated vegetatively in a greenhouse in Ottawa, Canada, were used (Dong *et al.* 1994).

5.2.2. Bacterial strains

Isolates were obtained by inoculating the fluid removed by aseptic centrifugation from intercellular spaces of the sugarcane stems into semisolid LGI medium (Dong *et al.* 1994). Only those eight isolates obtained from different plants which were capable of reducing acetylene were compared. The type strain of *A. diazotrophicus*, with which all isolates were compared, was PAL-5 (ATCC-49037 ex Döbereiner, from the culture collection of the Departamento de Microbiologia, Universidad de La Habana). The strains designated MC-1, -2, -3, -4, and -7 came from cv. Media Luna (field material from Havana, Cuba), and those designated JO-1, -2, and -4 came from cv. Ja 60-5 (Carleton University greenhouse). All bacteria were stored in 10% glycerol in a liquid N₂ storage apparatus. Bacteria newly subcultured from these frozen cultures on slopes of modified LGI medium (Cavalcante and Döbereiner 1988), a nitrogen-free, sucrose-rich (100 g/liter) medium supplemented with sugarcane juice (5 ml/liter), were used throughout the study.

5.2.3. Conventional morphological criteria and biochemical tests

All of the tests and morphological observations made on the isolates are listed in Table 1. These include those used in an earlier study (Dong *et al.* 1994). All tests were done at 30°C in air.

5.2.4. CFA analysis

Cells for fatty acid analysis were grown on LGI at 30°C for 6 days and saponified, methylated, and analyzed by gas-liquid chromatography with the Microbial Identification System and Library Generation System software (MIDI, Newark, Del.) as described previously (Bernard *et al.* 1991).

At the Laboratory Center for Disease Control (Ottawa, Canada), tentative peak identifications were made on the basis of use of a reference standard of fatty acids obtained from MIDI. Chromatographic profiles were created and updated for storage in an in-house library for aerobes and facultative anaerobes called LCDC1 (Bernard *et al.* 1991), whose method, LCDCAER2, was rooted to the commercial method AEROBE (MIDI), version 3.8, and validated with Library Generation System software, including principal component analyses. CFA profiles of all strains were tested against the commercial aerobe libraries TSBA and CLIN version 3.8 (MIDI), which do not contain *A. diazotrophicus*.

5.2.5. Measurements of hydrogen production

Cultures were grown on solid medium in 36-ml glass tubes containing 10 ml of LGI medium at 30°C for 5 days before the test. This temperature was maintained throughout the test by placing the tubes in a water bath. The H₂ evolution was measured as follows. The tubes were sealed with gas-tight serum stoppers. Gas samples (1 ml) were withdrawn with syringes at about 10 min intervals and analyzed. After making measurements for 1 to 2 h to obtain the rate of H₂ production at 0% acetylene, the tubes were flushed with air for 5 min and resealed with serum stoppers. Acetylene was then added to the tubes by removing measured volumes of air and adding known volumes of pure acetylene by syringe to create partial pressures of 2.5, 10, 20, and 30 kPa. Gas samples were taken again at about 10 min intervals and analyzed for both H₂ and ethylene. Values from two replicate tubes each for all of the isolates and the type strain were compared.

Detached nodulated roots of soybean were tested like the gas samples for H₂ and ethylene production under the different partial pressures of acetylene as a further control.

To test whether the H₂ was produced by nitrogenase rather than by some other enzyme system, the rates of H₂ evolution in oxygen-nitrogen mixtures by the type strain and one of the isolates (MC-1) were compared with the rates in oxygen-argon mixtures. In the absence of its substrate (N₂), H₂ evolution from the nitrogenase

should increase dramatically up to a theoretical maximum of fourfold (Eisbrenner and Evans 1983). H_2 production increases in the absence of N_2 because all electrons flow through nitrogenase are used to reduce protons to H_2 .

All analyses were done by immediately injecting each gas sample into a portable gas chromatograph which was equipped with a semiconductor sensor (Taguchi gas sensor, Figaro Engineering Co., Toyonaka City, Japan) with Porapak N (0.63 m by 0.2 mm, internal diameter) at room temperature (Holfeld *et al.* 1979). Chromatograms with retention times and peak heights and areas were recorded with a computing integrator (LCI-100; Perkin-Elmer). The gases were identified tentatively by comparing retention times with those of standard gases. The sample gas was also spiked by assaying the mixture of standard gas of known concentration with sampled gas. Chromatograms were compared with those of standard gas and sample gas alone to ensure the identification of each peak present.

There was no possible confusion between the H_2 peak and a carbon dioxide peak, since the sensor was very insensitive to carbon dioxide.

5.3. RESULTS

All of the isolates and the type strain were identical for the morphological and biochemical characteristics examined. These results are summarized in Table 5-1.

The CFA composition, expressed as percentages for the type strain and the isolates, is presented in Table 5-2. All strains were found to have no match with all entries contained in library CLIN. However, all strains had the genus *Acetobacter* as the only choice when tested against the TSBA library, with similarity index values ranging from 0.185 to 0.481. The *Acetobacter* species picked up by the system from the TSBA library were *A. xylinum*, *A. pasteurianus* and *A. liquefaciens*. The principal component 1 value for all data when averaged into a library entry with the Library Generation System software was 1.25.

In a previous paper (Dong *et al.* 1994), nitrogenase activity was estimated by the reduction of acetylene to produce ethylene. We now show that this activity can also be assayed by the production of H_2 by cultures growing in air. This H_2 production was detected from the type strain and all isolates and continued at a steady rate for several hours. In an individual culture tube (10 ml media slope) the production rate of H_2 is about 60 nmoles per hour. In the presence of acetylene (e.g., when using this test as a nitrogenase assay), both H_2 and ethylene are produced, but the H_2 is produced at a reduced rate. The reduction increases as the acetylene concentration rises, but even at 30 kPa, the rate is substantial (Table 5-3). The rates of H_2 production in the presence of acetylene remained steady over at least 2 h. This behavior is in strong contrast to that of soybean nodules, whose H_2 production is strongly suppressed by acetylene. The contrast is shown in Table 5-3, where the gas production rates

by individual cultures and nodule preparations have been normalized to 100% in air. The results for the type strain and the three isolates tested were not significantly different and have been pooled to give the means in Table 5-3. As the acetylene concentration increases, H_2 production decreases and ethylene production increases, but the effect on the bacteria is much less than that on the nodules. At the concentration of acetylene commonly used in nitrogenase assays (i.e., 10 kPa), the H_2 production by the bacteria was reduced to 65%, but that by the nodules was reduced to only 11%. At a partial pressure of acetylene of 30 kPa, the H_2 production by the bacteria was still 15%, but that by the nodules was only 0.1%.

The results of tests on whether the H_2 produced by the *Acetobacter* was indeed the result of nitrogenase activity and not by some other metabolic pathway are shown in Table 5-4. The isolate tested and the type strain gave similar results, with H_2 production rising when N_2 is replaced by argon. When the oxygen concentration in the argon mixture is 2%, the stimulation of H_2 production rises to threefold. Although this is less than the theoretical value of fourfold, it is good evidence that the H_2 is in fact produced by nitrogenase and not some other enzyme.

5.4. DISCUSSION

We found that all sugarcane isolates whether recovered from field-grown plants in Cuba or from greenhouse propagated stocks in

Ottawa, Canada, had identical phenotypic and morphological characteristics to each other and to those of the type strain of *A. diazotrophicus* for all properties listed in Table 5-1. All strains studied were consistent with each other both qualitatively and quantitatively for CFAs detected with the Microbial Identification System software (Table 5-2). All bacteria were found to exhibit low similarity index values solely towards species in the genus *Acetobacter* (*A. xylinum*, *A. pasteurianus* and *A. liquefaciens*) when compared with the commercial library TSBA. As discussed in a review by Welch (1991), only bacteria which are considered to be very closely related genetically will demonstrate highly similar CFA compositions; this supports the assertion here that the sugarcane isolates and the type strain of the species *A. diazotrophicus* have a relationship with other members of the *Acetobacter* genus or closely related genera. It was observed that the principal component 1 value for the entry created for all bacteria studied was 1.25. It has been suggested (Mukwaya and Welch 1989; Welch 1991) that such a low variance for CFA composition data among strains considered to be of the same species is suggestive of genetic homogeneity, which must be corroborated by additional molecular typing techniques.

The stability and persistence of this endophytic species under these widely different cultural conditions are in keeping with the findings of Caballero-Mellado and Martinez-Romero (Caballero-Mellado and Martinez-Romero 1994) that there is little genetic diversity among isolates of this bacterium from macerates of different varieties of sugarcane plants growing in diverse regions of

Mexico and Brazil.

The finding that *A. diazotrophicus* has unusual properties among known N_2 -fixing bacteria with respect to its continued production of H_2 in the presence of acetylene is new. Acetylene inhibition of H_2 production is well known for *Rhizobium* spp. and results from saturation of nitrogenase activity which occurs at a partial pressure of acetylene of about 10 kPa (Hardy *et al.* 1973). The remote possibility that the H_2 produced by *A. diazotrophicus* originates by another enzyme system cannot be tested simply. The nitrogenase of this bacterium is not inhibited much by the presence of combined nitrogen (Fu *et al.* 1988; Li and MacRae 1991), so that the normal expedient of testing for the H_2 evolution in the presence of fixed nitrogen cannot be adopted. However, the markedly increased production of H_2 in the absence of the N_2 substrate of this enzyme suggests strongly that the H_2 does originate from protons liberated by the nitrogenase system and not another enzyme system. *A. diazotrophicus* is not unique in continuing to reduce N_2 (and liberate H_2) in the presence of much higher acetylene concentrations. Similar results have been reported by Spiff and Odu (Spiff and Odu 1973) and MacRae (1977) for some *Beijerinckia* strains and by Dilworth (1987) for *Azotobacter* strains.

In the *Azotobacter* strains, this continuation of H_2 production in the presence of high acetylene concentrations has been shown to be due to an alternative nitrogenase, synthesized only under extreme molybdenum starvation (Bishop *et al.* 1980; Robson *et al.* 1986).

However, a similar nitrogenase is not acting in the continuing production of H_2 by *A. diazotrophicus* in the presence of high acetylene concentrations because normal levels of molybdenum (2 ppm Na_2MoO_4) were always present in the medium.

TABLE 5-1. Standard morphological and biochemical characteristics of *Acetobacter diazotrophicus*, type strain and isolates

Test	Type strain	Isolates								
		PAL-5	MC-1	JO-1	MC-2	JO-2	MC-3	MC-4	JO-4	MC-7
Gram stain ^{a*}	-	-	-	-	-	-	-	-	-	-
Endospore ^a	-	-	-	-	-	-	-	-	-	-
Motility ^a	+	+	+	+	+	+	+	+	+	+
Flagellae peritrichous ^a	+	+	+	+	+	+	+	+	+	+
Nitrate reduction ^d	-	-	-	-	-	-	-	-	-	-
Catalase ^{a*}	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis ^a	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis ^a	-	-	-	-	-	-	-	-	-	-
Oxidase ^a	-	-	-	-	-	-	-	-	-	-
Overoxidation of ethanol ^b	+	+	+	+	+	+	+	+	+	+
Overoxidation of glucose 1% ^a	+	+	+	+	+	+	+	+	+	+
Brown pigment on GYC agar ^b	+	+	+	+	+	+	+	+	+	+
Brown colonies on potato agar ^c	+	+	+	+	+	+	+	+	+	+
Growth with 30% glucose	+	+	+	+	+	+	+	+	+	+
Growth with 30% sucrose ^a	+	+	+	+	+	+	+	+	+	+
Oxidation of glycerol ^a	+	+	+	+	+	+	+	+	+	+
Formation of H ₂ S ^a	-	-	-	-	-	-	-	-	-	-
Growth on L-GI-P plates ^{c*}	+	+	+	+	+	+	+	+	+	+
Methyl Red ¹	-	-	-	-	-	-	-	-	-	-
Growth on carbon sources: ^b										
D-glucose	+	+	+	+	+	+	+	+	+	+
D-arabinose	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+
D-inositol	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+
Acid production from: ^b										
D-glucose	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+
D-inositol	-	-	-	-	-	-	-	-	-	-
D-mannitol	-	-	-	-	-	-	-	-	-	-
D-arabinose	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+
Growth on aa with D-mannitol: ^b										
L-glycine	-	-	-	-	-	-	-	-	-	-
D-L tryptophan	-	-	-	-	-	-	-	-	-	-
L-asparagine	+-	+-	+-	+-	+-	+-	+-	+-	+-	+-
L-threonine	-	-	-	-	-	-	-	-	-	-
L-glutamine	+-	+-	+-	+-	+-	+-	+-	+-	+-	+-

Legends: +: positive; -: negative; +/-: slight growth.

^a Methods in Gerhardt *et al.*

^b Methods in De Ley *et al.*

^c Methods of Cavalcante and Döbereiner.

^d Methods in Pelczar and Chen.

*. From table II of Dong *et al.*

TABLE 5-2. Cellular Fatty Acid composition analysis of sugarcane isolates and ATCC 49037, *Acetobacter diazotrophicus* (%) ^{a, b}

Strains	14:0	2OH-14:0	3OH-14:0	16:0	2OH-16:0	3OH-16:0	18:2	18:1.ω7c	18:0	19:0.cycω8c	3OH-18:0
ATCC 49037											
(PAL-5)	5	3	2	14	6	4	1	55	2	5	2
Isolates											
JO-1	5	3	2	13	6	3	1	57	3	6	2
JO-2	6	4	2	13	7	3	—	57	2	5	2
JO-4	5	3	2	14	6	3	1	56	2	6	2
MC-1	6	3	1	13	6	3	2	57	3	5	2
MC-2	6	4	2	13	7	3	—	58	2	5	2
MC-3	5	3	2	14	6	3	—	57	2	5	2
MC-4	5	3	2	13	6	3	2	56	2	5	2
MC-7	5	4	1	13	7	3	2	55	2	6	2
means	5.4	3.4	1.6	13.1	6.4	3.4	1.03	56.4	2.3	5.3	1.8
SD	0.2	0.3	0.1	0.5	0.2	0.2	0.06	0.9	0.3	0.5	0.2

^a Number to the left of the colon is the number of carbon atoms and the number to the right is the number of double bonds, ω, double bond position from the hydrocarbon end of the chain; c, cis isomer. OH, hydroxyl group at 2nd or 3rd carbon; cyc, cyclopropane form. The MIDI peak naming table (ver 3.8) more commonly refers to 3OH-14:0 as 'summed feature 3', 18:2 as part of 'summed feature 6' and to 18:1.ω7c as part of 'summed feature 7'. Values shown are percentages of total fatty acids for the nine individual isolates rounded off to the nearest percent. The last two lines give the means and standard deviations for the nine isolates — 0% to 0.29%. In Bernard et al (3) it was described that in the PNT of AEROBE ver 3.8, MIDI acknowledged that they had in error used '20:3' instead 3OH-18:0 for the CFA with equivalent chain length (ECL) 19.54^c

^b Trace to small volumes (1.9%) of peaks identified with ECL 18.588, 18.717, ECL 19.710 and ECL 20.188 were observed for all or most sugarcane isolates and are observed among CFA compositions for a number of genera of Gram negative bacilli (1). The CFA which elutes at ECL 20.188 has been characterized previously by Wallace et al (24) and is identifiable as 2OH-19:0-cyclopropane.

TABLE 5-3. Relative rates of hydrogen and ethylene production by *Acetobacter diazotrophicus* and soybean nodules at different acetylene partial pressures in air. Normalized means of three tests each comprising the type strain and isolates (Percent $\pm$ SD)

Acetylene (kPa)		0	2.5	10	20	30
Acetobacter	H ₂	100	84 $\pm$ 4	65 $\pm$ 5	26 $\pm$ 5	15 $\pm$ 3
	C ₂ H ₄	0	62 $\pm$ 13	145 $\pm$ 27	159 $\pm$ 40	179 $\pm$ 35
Nodules	H ₂	100	16 $\pm$ 5	11 $\pm$ 4	1.7 $\pm$ 1.1	0.08 $\pm$ 0.07
	C ₂ H ₄	0	108 $\pm$ 16	262 $\pm$ 34	254 $\pm$ 47	265 $\pm$ 38

TABLE 5-4. Effect on hydrogen production by *Acetobacter diazotrophicus*^a of replacing the nitrogenase substrate, nitrogen, with argon at high and low oxygen concentrations.

Oxygen (%)	H ₂ production ^b in	
	Nitrogen/oxygen	Argon/oxygen
20	100	159±1.6
2	100	300±11

^aIsolate MC-1 and type strain.

^bHydrogen production is expressed as the percentage relative to 100% in the nitrogen/oxygen mixture, as the means of four replicates each containing two tubes of the type strain and two of the isolate.

CHAPTER SIX.
DETECTION AND MEASUREMENT OF HYDROGEN
EVOLUTION AND ACETYLENE REDUCTION BY
NITROGENASE

6.1. INTRODUCTION

Nitrogen is one of the most critical elements for plants. In most fertile areas, the crop productivity is often limited by the amount of nitrogen available in the soil. An alternative to fertilizer is to find and encourage the N₂-fixing bacteria which are associated with crops. To detect nitrogenase activity and discover the nitrogen fixers from fields and isolates, a convenient method of measuring nitrogenase activity is needed.

This chapter describes the basis of detecting and estimating nitrogenase activity and discusses the relative merits of using methods detecting acetylene reduction or hydrogen evolution. It is not divided into Materials, Methods and Results, but is presented as a continuous argument.

The natural reactions catalyzed by nitrogenase are the reduction of dinitrogen and protons:

Proton reduction to hydrogen gas is an obligate reaction of nitrogenase when it reduces dinitrogen as shown in (1) and (2). The rate of hydrogen production can be used to monitor the activity of the N₂-fixing process (Hunt and Layzell, 1993; Simpson and Burris,

1984). Hydrogen evolution rate with dinitrogen reduction, in reactions both (1) and (2), provides a measurement only of apparent nitrogenase activity because only a portion of the total electron flux through nitrogenase is used for dinitrogen reduction.

In an atmosphere lacking dinitrogen, e.g. in argon/oxygen, nitrogenase will pass all the electrons to protons. The hydrogen evolution rate in this case gives a measure of total nitrogenase activity and provides the measurement of total electron flux through nitrogenase. Six electrons are needed to reduce each dinitrogen and two are needed to form one hydrogen molecule. So, if the difference between the total nitrogenase activity and the apparent nitrogenase activity is D , then electrons used to reduce dinitrogen will be $2D$. The reduction rate of dinitrogen will be $2D/6 = D/3$.

Nitrogenase is an enzyme with low specificity capable of catalyzing the reduction of a wide variety of substrates, including the reduction of acetylene to ethylene (Burris, 1991). In the presence of 3 to 10 percent acetylene all electron flux through nitrogenase is used to reduce acetylene to ethylene. The measurement of acetylene reduction therefore provides an assay for total electron flow through nitrogenase.

The closed acetylene reduction assay has been used as a measure of nitrogenase activity for more than twenty years. However, starting about ten years ago this assay has come under criticism as being inaccurate in measuring this activity in

Rhizobium/legume symbioses (Vessey, 1994). Acetylene somehow has effects on nodules which could in turn influence the activity of N_2 -fixing bacteria living with them. Ethylene, the product of this assay, is a plant hormone which has strong effects on plant cells at very low concentration. Plants under stress, such as when detached or in 10% acetylene, will produce ethylene themselves. All these indistinguishable errors make this assay inaccurate, especially when nitrogenase activity is low. Sometimes it is hard to tell whether there is any nitrogenase activity or not when plant parts were included in the system.

The acetylene reduction assay gives only total nitrogenase activity as it measures total electron flux through nitrogenase. Because hydrogen evolution is an obligate part of the N_2 -fixation reaction, and the ratio of electron flux to proton and dinitrogen is variable, the acetylene assay gives an uncertain estimate of the N_2 -fixation rate. However using hydrogen evolution analysis, measuring the total nitrogenase activity and apparent nitrogenase activity, the rate of N_2 -fixation may be calculated and the allocation of electrons by nitrogenase between protons and dinitrogen can be determined.

So while acetylene reduction is still used as a standard assay for nitrogenase activity, it has many drawbacks. In contrast, hydrogen evolution analysis gives a better measure of nitrogenase activity. It is quicker, less labor-intensive, presents no hazards to the experimenter (acetylene is explosive) and gives a more positive assessment of nitrogenase activity.

In order to detect the nitrogenase activity of the isolates and bacteria associated with sugarcane, a portable gas chromatograph was made which would measure both hydrogen evolution during N_2 -fixation, and ethylene production from acetylene reduction. The results of tests with this instrument were also used as one criterion to characterize the isolates.

6.2. ETHYLENE PRODUCTION FROM ACETYLENE

The acetylene reduction assay was conducted by adding the calibrated volume of acetylene to give 10% acetylene in the tubes, and measuring the ethylene accumulation in the tubes at 10 min intervals over 60 min. Soybean nodules in 10% acetylene, and all the strains without acetylene, were tested for ethylene production as positive and negative controls.

All the strains showed no ethylene production without acetylene. With 10% acetylene, ethylene accumulation (and therefore acetylene reduction) was observed with all isolates of *A. diazotrophicus* (Table 4-3). It should be noticed that this acetylene reduction was observed in the presence of 21 kPa oxygen partial pressure, a level of oxygen which inhibits nitrogenase activity of *Rhizobium* (Layzell and Hunt, 1990). In contrast, detached sugarcane stems incubated at the same oxygen pressure showed no acetylene reduction in five hours.

6.3. HYDROGEN EVOLUTION

Hydrogen gas production was measured to monitor the nitrogenase activity of the bacterial colonies and the detached pieces of sugarcane plants.

The tubes containing bacterial colonies on the media were air-flushed for about 1 min and then sealed with gas-tight serum stoppers. The tubes were held in a water bath (30°C) during the experiments. Gas samples (1 ml) were withdrawn with 1 ml syringes at about 10 min intervals. Each sample was analyzed by immediate injection into a gas chromatograph (GC). Soybean nodules were used as positive controls.

Sugarcane plants (Ja 60-5) from the greenhouse of Carleton University were used for nitrogenase activity tests. Plants of three generations were tested. Plants were cut at the base and transported immediately to the laboratory. A piece of sugarcane stem about 1.2 m long was put into a 1.3 m long drain pipe with a diameter of 2 inches held at room temperature. Both ends of the pipe were capped with robber stoppers. Gas samples were taken from a specially installed injector with two silicone rubber septa. Soybean nodules and corn plants were used as positive and negative controls in this test.

The peaks and areas of sample gases were compared with standard curves and converted into concentrations and total amounts

in the tubes by using standard equations.

6.3.1. Hydrogen evolution from *A. diazotrophicus*

Hydrogen evolution was successfully detected from colonies of isolates of *A. diazotrophicus*. Under 21% oxygen, 30°C, 5-day cultures of isolates on 10 ml media slopes normally accumulate 100 ppm hydrogen in 2 to 3 h. In this closed system, the hydrogen production is linear. No decline was noticed in the first several hours.

6.3.2. Hydrogen evolution from sugarcane stems

Hydrogen gas from the plant stem in the drain pipe was detected after 24 h. At 72 h, hydrogen concentration reached 15 ppm. Converted into an accumulated amount, this is 1217 nmoles hydrogen. The detached soybean nodules in the pipe showed nitrogenase activity by hydrogen evolution. The corn plants produced no detectable hydrogen.

A 1.2 m long piece of sugarcane stem produced more than 1200 n moles hydrogen at room temperature in 72 h, while the control corn plants gives no hydrogen peak at all. This suggests an active nitrogenase activity in sugarcane stems. Even with the type strain on culture medium in a tube at 30°C, it takes overnight to produce this amount of hydrogen. In the assay with isolates, the hydrogen evolution rate doubled when temperature was raised from

room temperature to 30°C. So, we would expect a higher hydrogen evolution rate from sugarcane stem at a higher temperature.

6.3.3. Effects of oxygen tension on hydrogen evolution

The hydrogen evolution of *Acetobacter* colonies is sensitive to the increase of oxygen tension. The hydrogen evolution dropped immediately and stayed low for a long time (days) after a high tension oxygen shock. This suggests that the hydrogen measured is produced by nitrogenase because nitrogenase is very sensitive to oxygen, and will be destroyed by high tension of oxygen. The details of oxygen shock to *Acetobacter* colonies will be discussed in chapter 7.

6.3.4. Effects of dinitrogen (substrate) tension on hydrogen evolution

To confirm that the hydrogen is produced from the nitrogenase and not from other sources, the hydrogen evolution rate in an oxygen/nitrogen mixture was compared with that in oxygen/argon. In the oxygen/argon mixture, without dinitrogen as a substrate the nitrogenase should pass all the electrons to protons to produce up to 3 to 10 times as much hydrogen as in an oxygen/nitrogen mixture. The mixture of oxygen/argon was obtained by flushing the tube with argon (extra pure) for a few minutes by using two needles sticking into the tube with a gas tight stopper, and then removing measured volumes of argon and adding known volumes of oxygen (extra pure) by syringe. The completeness of this flushing method was proved by

using a tube with a high concentration of hydrogen, and measuring the hydrogen concentration after flushing. When a tube was filled with more than 1000 ppm hydrogen and flushed, no hydrogen peak was detected in the GC with a sensitivity of 0.5 ppm.

The hydrogen evolution rates of bacterial colonies grown under 2 kPa oxygen increased three-fold in an atmosphere of oxygen/argon, and 1.6-fold when the colonies were grown and measured in 21 kPa oxygen (Table 5-4).

6.3.5. Effects of acetylene (competitor) on hydrogen evolution

To test the effect of acetylene concentration on the nitrogenase, after 1 to 2 h measurement of hydrogen evolution in air the tubes were flushed with air for 5 min and resealed with serum stoppers. Different partial pressures of acetylene, 2.5, 10, 20 and 30 kPa, were established in different tubes by removing measured volumes air and adding known volumes of pure acetylene by syringe. Gas samples were taken again at about 10 min intervals and analyzed for hydrogen and ethylene.

In the presence of acetylene, ethylene accumulation was detected and hydrogen evolution was decreased compared with air. With increasing acetylene level, ethylene production increased while the hydrogen production rate decreased (Table 5-3; Fig. 6-1). At an acetylene partial pressure of 10 kPa, which is the level commonly used in acetylene reduction studies, hydrogen production rate

decreased to 65% of that in air. Even under 30 kPa acetylene, hydrogen production rate was 15% of that in air.

In a similar test with detached nodulated soybean roots, hydrogen production rate was decreased to 20% of that in air at 2.5 kPa acetylene partial pressure, and to 5% at 10 kPa. Hydrogen production was almost undetectable at 20 kPa acetylene (Table 5-3).

Acetylene, which for the soybean nodules is a very effective substrate in preventing hydrogen evolution, is nowhere near as effective with the N_2 -fixing *Acetobacter* bacterium. In legume nodules, the production of hydrogen should cease in assays where acetylene reduction is being monitored at acetylene pressures of 10 kPa because such acetylene concentration should saturate the nitrogenase in whole cells of N_2 -fixing microorganisms (Hardy *et al.*, 1973). However, some organisms appear to require high partial pressures of acetylene to saturate nitrogenase activity. My preliminary results suggest that *A. diazotrophicus* is such an organism. This property resembles that of the vanadium-containing nitrogenase produced by *Azotobacter* (Robson *et al.*, 1986). It has been reported that some species of *Beijerinckia* also require high partial pressures of acetylene to saturate their nitrogenase activities (Spiff and Odu, 1973; MacRae, 1977). It is possible that these bacteria operate with an alternative nitrogenase, for which such responses are already known (Dilworth, 1987). The *Azotobacter* strains synthesize the alternative nitrogenase only under conditions of molybdenum-starvation (Bishop *et al.*, 1980). But *A.*

diazotrophicus develops this property with molybdenum in the medium. Whether this bacterium produces a different Mo-Fe nitrogenase or utilizes another metal instead of molybdenum remains to be determined.

6.4. SPEED OF ASSAYS

The retention time of a gas in a GC is the time from injection to the peak of the gas is detected by the GC. The retention time of the slowest detectable gas limits the time interval between successive tests (the slowest target gas in some advanced GCs). At room temperature, the retention times of hydrogen and acetylene in the GC I used are 9 s and 5 min, respectively. These two times will limit the shortest intervals between two hydrogen tests or two acetylene reduction tests. Furthermore, the hydrogen concentration in a sample is much lower than the concentration of acetylene (which was usually 10 percent), so the hydrogen peak is much smaller and narrower than the acetylene peak. Both the height and the width of a peak affect the time for the GC to return to zero: the higher and the wider the peak, the slower the GC returns to zero. With the GC used in this study at room temperature, hydrogen tests can be run at 1-min intervals, while the acetylene reduction tests can be run at only 5-min intervals. Thus the hydrogen test was a more efficient method.

Figure 6-1

Fig. 6-1. Hydrogen evolution in nanomoles per tube (26 ml gas space) by cultures of *Acetobacter diazotrophicus* at different partial pressures of acetylene. $\square$ —hydrogen evolution in air; $\blacksquare$ —hydrogen evolution with acetylene. A, 2.5 kPa acetylene; B, 10 kPa acetylene; C, 20 kPa acetylene and D, 30 kPa acetylene.

CHAPTER SEVEN.
NITROGENASE ACTIVITIES AND COLONY
STRUCTURES OF *ACETOBACTER DIAZOTROPHICUS*
UNDER DIFFERENT OXYGEN TENSIONS

7.1. INTRODUCTION

Nitrogenase is oxygen labile, yet the reaction it catalyses requires large amounts of ATP synthesized by aerobic respiration. The mechanisms by which nitrogen fixers protect the nitrogenase from oxygen inactivation, yet meet their ATP requirements, have been the subject of much research (Robson and Postgate, 1980; Cauwenberghe *et al.*, 1993; Oresnik and Layzell, 1994). The mechanisms of controlling oxygen concentration and supplying oxygen flux mostly depend on special structures like heterocysts and nodules. This chapter seeks to define how these requirements may be met for *A. diazotrophicus* in sugarcane which has no obvious structure to limit oxygen access.

Acetobacter diazotrophicus, the recently discovered N₂-fixing endophyte from sugarcane, is able to fix N₂ and grow on dinitrogen as sole N source in a semisolid sucrose-rich medium under 21 kPa oxygen partial pressure (Cavalcante and Döbereiner, 1988). In our laboratory, we found that this bacterium grows on dinitrogen as sole N source also on a solid medium under 21 kPa oxygen, after initial growth with starter nitrogen in the medium (Dong *et al.*, 1994). Reis *et al.* (1990) reported that the optimum oxygen concentration for this bacterium to fix N₂ is 0.2 kPa. So, when this bacterium grows on solid medium under 21 kPa oxygen, it must have a mechanism to control the oxygen concentration inside the bacterial colonies to protect the nitrogenase. Here I report comparative studies on the structure and N₂-fixing ability of the colonies on solid medium under

21 and 2 kPa oxygen partial pressures.

7.2. MATERIALS AND METHODS

7.2.1. Strain and culture

The strain used in this study, JO-2, was obtained from intercellular spaces of sugarcane stems (Dong *et al.* 1994), and identified as *Acetobacter diazotrophicus* by morphological and biochemical tests, cellular fatty acid analysis and nitrogenase activity tests (Dong *et al.* 1995).

Strain JO-2 was inoculated on LGI-P medium with 5 ml/L sugarcane juice in the medium and incubated at 30°C under either 21 kPa or 2 kPa oxygen partial pressure. Oxygen tension was controlled by replacing oxygen by dinitrogen in a standard bacteriological anaerobic jar containing the cultures. A modified LGI plate with added 0.32 g/L ammonium sulphate as extra nitrogen was used for the colony formation test. All the tests were performed after five days incubation.

7.2.2. Colony structure

Colonies grown under 2 kPa and 21 kPa oxygen were observed under a dissecting microscope. Fixation tests with standard procedures destroyed colony structures. Freeze-substitution was tried as a way to preserve the structure for microscopy, as described

in section 2.5.2. Semi-thin cross sections of colonies were stained with toluidine blue pH 4.4 and observed with an Olympus Vanox microscope using bright-field and phase contrast optics.

7.2.3. Immobilization and staining of colony mucilaginous material

The mucilaginous pellicles of colonies incubated in air were collected with a pipet into an Eppendorf centrifuge tube. An equal volume of distilled water was added and the tube was vortexed. The solution was centrifuged at 8000 g for 5 minutes to remove the bacteria. One drop of the supernatant liquid was placed on a strip of nylon transfer membrane (Hybond-N+, Lot no. 1D222, Amersham Canada, Ltd., Oakville, Ontario, L6L 5T7). The membrane strips were left to air dry for 12-16 h.

The dried strips were rehydrated with distilled water immediately prior to staining procedures. Strips were then stained with the Periodic Acid Schiff's (PAS) reaction, and toluidine blue at pH 4.4 or pH 1 as described in sections 2.5.3.1. and 2.5.3.3.

7.2.4. Nitrogenase activities

Nitrogenase activity of the cultures was monitored by measuring hydrogen gas production (see Chapter 6). A temperature of 30°C was maintained by putting the vessels containing the cultures in a water bath during the measurements. Total nitrogenase activity and apparent nitrogenase activity of *A. diazotrophicus*

cultures under 2 kPa and 20 kPa oxygen partial pressure were measured in argon/oxygen and nitrogen/oxygen atmospheres respectively.

The effect of oxygen on nitrogenase was monitored by measuring hydrogen evolution from cultures before and after changes of oxygen concentrations. The hydrogen evolution rate was measured for 1 h first under the oxygen partial pressure at which the cultures were grown. Then the cultures were exposed to different oxygen partial pressures and the nitrogenase activity was measured for a further 2 to 3 h. After this oxygen change the original oxygen pressure was restored and the hydrogen production rate of the cultures was measured for another hour. Control tubes with similar bacterial cultures were measured simultaneously at the oxygen concentration at which the cultures were grown.

7.3. RESULTS

7.3.1. Morphology

When *A. diazotrophicus* was streaked on the LGI plates and incubated under air, a few of the bacteria formed massive, glutinous colonies. These colonies had an orange mass in the middle indicating a large number of concentrated bacteria which had accumulated the pH indicator and which had a very low pH in their interior. Around this central bacterial mass there was a thick translucent, colourless, mucilaginous material containing few bacteria (Fig. 7-1 A). Such

massive colonies were normally found at the end of the inoculating streak. The colonies in the early part of the streak were small, colourless colonies, or colonies with very small orange centres, which did not develop further (Fig. 7-2 A). Under incubation in air the mucilaginous material was always formed. The orange centre indicating a dense bacterial population formed only when this mucilaginous material reached a certain size. The mucilage when immobilized on the membrane was strongly PAS positive (Fig. 7-1 D) indicating the presence of polysacchrides with 1,2 diol groups, and stained pink with toluidine blue at pH 4.4 and pH 1 indicating the presence of strong acidic polysaccharides (Fig. 7-1 C).

This colony dimorphism was very consistent and subcultures from either small or large colonies repeated the dimorphism. I reasoned that this was because on the early part of the streak there were more bacteria to forage the limited fixed N in the medium and none could reach a certain critical size quickly enough to protect the bacteria inside the colony and allow them to begin N₂-fixation.

I further reasoned that if the streak was made and the colonies grown under 2 kPa oxygen, the mucilage protection would not be necessary, and all colonies should be uniformly orange, filled with dense bacteria. When this was tested under 2 kPa oxygen tension, many more big colonies formed all over the plates. All these colonies were uniformly orange in colour without (or with very thin) mucilaginous material around (Fig. 7-1 B & 7-2 B).

The next test was to supply ample mineral N in the medium and incubated in air, when the colony size should not be limited by the bacterial numbers. Colonies on medium with added ammonium confirmed this logic. When fixed nitrogen was not limiting, more big colonies formed on the plates. The colony morphology was similar to those incubated under 21 kPa oxygen, but the number of the large colonies was similar to that under lower oxygen conditions. All these colonies showed orange centres surrounded by thick colourless mucilage. This suggested that my interpretation of the primary observation of colony size was correct.

The structure of colonies was studied to explore the nature of the protective mucilage. Cross sections of colonies prepared with the freeze-substitution technique showed the distribution of bacteria and mucilaginous material. In transverse section colonies were lens shaped. Those incubated in either 21 kPa or 2 kPa oxygen showed two populations of bacteria (Fig. 7-3), one at the bottom of the colony (adjacent to the agar) and the other close to the top of the colony with a less populated area between. The difference between colonies incubated in different oxygen tensions was the position of the top bacterial population. At 2 kPa oxygen, this population was at the surface of the colony, without any or with a very thin layer of mucilaginous material over it (Fig. 7-3 B and D). Whereas in the colony incubated in 21 kPa oxygen, the top bacterial population was located in the middle of the colony, with thick mucilaginous material around it (Fig. 7-3 A and C).

3 OF/DE 3

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

7.3.2. Nitrogenase activity

The effects of the mucilage barrier on the N_2 -fixing activity of the bacteria were explored by changing the oxygen concentration over colonies grown at either low or high oxygen.

Total nitrogenase activity (hydrogen evolution rate in an atmosphere of argon and oxygen) and apparent nitrogenase activity (hydrogen evolution rate in air) of *A. diazotrophicus* cultures on solid media at the two oxygen levels were measured by hydrogen evolution analysis. Apparent nitrogenase activity and total nitrogenase activity of cultures held continuously under 2 kPa or 21 kPa oxygen partial pressures were summarized previously in Table 5-4. As seen in that data, hydrogen evolution rates of cultures under the oxygen partial pressures at which the cultures were grown were quite stable. When the cultures were exposed to higher oxygen pressures than those to which they were accustomed, the hydrogen evolution rates dropped immediately. In cultures grown under 2 kPa oxygen (these had very thin mucilaginous pellicles around the colonies) 4, 8 and 21 kPa oxygen exposures decreased the hydrogen evolution rate dramatically (Fig. 7-4. A and B). The higher the new oxygen concentration, the greater the decreases in hydrogen evolution. Following 4 kPa oxygen exposure, the hydrogen evolution rate continued to decrease after the initial drop, whereas with 8 and 21 kPa oxygen exposures, the hydrogen evolution rates stayed at about the same level after the initial drops. When the oxygen tension was changed back to the 2 kPa the hydrogen evolution rate

showed a second drop, and eventually recovered to or rose to slightly above the rate in high oxygen tension (Fig. 7-4).

For the colonies with mucilaginous pellicles grown in 21 kPa oxygen the hydrogen evolution rate was much lower than that of colonies of similar inoculation and incubation, but grown in 2 kPa oxygen. When these colonies were exposed to higher oxygen tension the hydrogen evolution rate decreased immediately (Fig. 7-5. A, B, C, and D). Then the rate increased gradually. This subsequent increase is the opposite reaction to that of the colonies incubated in 2 kPa oxygen. The rate dropped again when the oxygen tension was reinstated and it recovered slowly (Fig. 7-5).

7.4. DISCUSSION

Nitrogen-fixing reactions cannot function in the presence of high concentrations of oxygen, and the microbes which can fix nitrogen in air use various stratagems to exclude oxygen from the N_2 -fixing site. The large quantities of extracellular polysaccharides found around the *A. diazotrophicus* growing in air are like similar pellicles frequently formed by other aerobic N_2 -fixing bacteria. The histochemical work reported here is the first on colonies of *A. diazotrophicus* and their mucilage pellicles. The results are consistent with other general reports of the chemical composition of slime and capsule components of bacteria (Sutherland, 1977). The positive staining reaction of the colony mucilage with PAS reaction indicates the presence of 1,2 diol groups (Pearse, 1968). The pink to red

metachromatic colours of the mucilage with toluidine blue staining show the presence of carboxylated or sulphated polysaccharides (Pearse, 1985). However, the pink to red colour of toluidine blue staining at pH 1 is unusual, and indicates the very acidic nature of this material.

Though the hypothesis is not firmly established, it seems likely that this slime layer impedes diffusion of oxygen to the bacteria within, and represents a crude physical barrier against excessive oxygenation. Inside the colonies, the limited diffused oxygen may sustain multiplication and ATP generation. Provided the population of bacteria is sufficiently dense inside the colony on agar, some fixation of nitrogen could occur. The determining factor is whether the cells, by their collective respiration, can lower the oxygen tension in their own vicinity to a certain level. So, in a certain range of oxygen tension, the bacteria might control the colony size and the thickness of extracellular matrix to admit sufficient oxygen for cell metabolism while not damaging nitrogenase. In the centre of the colony, the cells are oxygen-limited in the sense that they are consuming oxygen as fast as it is supplied.

This hypothesis is supported by my experiments on the growth of the colonies under different conditions. The dimorphic growth of *A. diazotrophicus* on the LGI-P plates suggests its microaerophilic nature. A few colonies scavenge the small amounts of fixed nitrogen initially present in the medium (from sugarcane juice and impurities in the chemicals used), and reach a critical size such that, at their

centres, the oxygen tension is low enough for N_2 -fixation to be initiated. These become the large orange colonies surrounded by the mucilage pellicle. The other colonies obtain insufficient fixed nitrogen to reach a critical mass quickly enough to develop into functioning colonies before the pH of the medium becomes too low for the bacteria to reproduce further. A lowered oxygen tension decreases the necessary critical mass, so that all colonies can get started. One millimolar ammonium provides sufficient available fixed nitrogen for them all to reach the size that is big enough to reduce the oxygen tension and permit the derepression of the nitrogenase system.

The results indicate that the tolerance of nitrogenase activity to oxygen in the aerobically grown *Acetobacter diazotrophicus* is due to the mucilage pellicles of colonies. The mucilage pellicles control the oxygen diffusion rate into the colony, keeping the concentration low inside the colony where the N_2 -fixation occurs. The mucilage probably is also important in the controlling of oxygen in the intercellular spaces of sugarcane stems where the bacteria were isolated from.

Figure 7-1

Fig. 7-1. A. A colony of *A. diazotrophicus* incubated at 21 kPa partial pressure of oxygen. Mucilaginous material surrounds the dense mass of orange-colored bacteria. 30x.

B. A similar colony to the one in A but incubated in 2 kPa partial pressure of oxygen. The orange bacteria reach the edge of the colony. 30x.

C. and D. Bacterial mucilage immobilized on nylon membrane. 60x. C. Toluidine blue staining, pH 4.4; D. PAS staining.

Figure 7-2

Fig. 7-2. *A. diazotrophicus* colonies on the LGI plates. 7x.

A. Incubated in 21 kPa oxygen.

B. Incubated in 2 kPa oxygen.

Figure 7-3

Fig. 7-3. Sections through freeze-substituted *A. diazotrophicus* colonies embedded in epoxy resin. me, agar medium; mu, mucilage. Toluidine blue stained.

A. Section of a colony grown in 21 kPa oxygen. Arrowheads indicate the upper surface of the colony. Yellow spots are bacteria. Note the two regions of concentrated bacteria, one at the surface of the agar medium and the other in the center of the colony. Dark field optics. 50x.

B. Cross section of a colony grown in 2 kPa oxygen. Dark field optics. 50x.

C. Magnification of the A, showing a greater amount of mucilage (mu) overlying the two concentrated regions of bacteria. Phase contrast optics. 340x.

D. Part of the colony shown in B. note that the upper concentration of bacteria is now close to the surface of the colony. Phase contrast optics. 340x.

Figure 7-4

Fig. 7-4. Hydrogen evolution rate of *A. diazotrophicus* colonies incubated in 2% O₂ at different O₂ tension. A. 4% O₂ shock and recovery; B. 8% O₂ shock and recovery.

Figure 7-5

Fig. 7-5. Hydrogen evolution rate of *A. diazotrophicus* colonies incubated in 21% O₂ at different O₂ tension.

CHAPTER EIGHT.
GENERAL CONCLUSION

In the General Introduction questions were raised about the relationship between sugarcane and *Acetobacter diazotrophicus*. The symbiotic nature of their interaction has been suggested by other authors based on the fact that *A. diazotrophicus* has often been isolated from surface sterilized sugarcane tissues. The present study provides additional evidence for this symbiosis, and opens up several lines for further investigation. The main new findings in the present study are:

8.1. Location of the *A. diazotrophicus* inside the sugarcane stem.

The N-fixing bacterium *A. diazotrophicus* has been found and isolated from the intercellular spaces of sugarcane internode stems (Chapter 4). The possibilities that this bacterium might be inside living plant cells, or in the xylem lumen have been eliminated by careful studies (Chapter 3). This finding of cultures of the N-fixing endophyte living in 12% sucrose solution in the intercellular spaces of sugarcane provides the first part of the long-sought answer to the question of how this plant could satisfy most of its own needs for N. The quantitative proof that the symbiosis does provide the necessary N remains to be tested.

8.2. *A. diazotrophicus* passes from one generation to the next.

A. diazotrophicus has been isolated from the original sugarcane

plants grown at Carleton from cuttings from Cuba and from the second and third generations of plants cultured successively by rooted stem cuttings (Chapter 4). 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 nitrogen fixation in the crop. The details of how this transfer occurs at the level of stem, bud and root tissues, remain to be elucidated.

8.3. Nitrogenase activity of *A. diazotrophicus* is higher than that suggested by the acetylene reduction assay.

The finding that *A. diazotrophicus* has unusual properties among known N-fixing bacteria with respect to its continued production of hydrogen gas in the presence of acetylene is new (Chapter 5 and 6). The acetylene reduction assay will not give total nitrogenase activity of this bacterium, because hydrogen evolution continued even under 30 kPa acetylene partial pressure. Therefore, the total nitrogenase activity must be higher than the acetylene reduction assay suggested. This study showed that measuring the hydrogen evolution rate is a better estimate of the nitrogenase activity of this bacterium. It is especially useful when plant tissue is included in the system. The nature of the nitrogenase in *A. diazotrophicus* is still unknown. Whether the bacteria operate with an alternative nitrogenase remains to be determined.

8.4. Cellular fatty acid (CFA) composition analysis provided

further evidence of identification and taxonomic position of *A. diazotrophicus*.

The results of CFA analysis showed that all the isolates are closely similar to each other and with the type strain, and are consistent with those found previously for *Acetobacter* spp. housed in the commercial data library TSBA (Chapter 5). The similarity between *A. diazotrophicus* and other *Acetobacter* shown by CFA profiles supports the taxonomic position of this bacterium given by Gillis *et al.* (1989).

8.5. Colonies of *A. diazotrophicus* have the ability to control oxygen concentration at the site of nitrogen fixation.

The results in Chapter 7 indicate a tolerance of nitrogenase activity to oxygen in the aerobically grown *Acetobacter diazotrophicus*. This tolerance increased when the organisms were grown at the higher oxygen tension. The large quantities of extracellular polysaccharides found around the *A. diazotrophicus* growing in air seem likely to impede diffusion of oxygen to the bacteria within, and provide a crude physical barrier against excessive oxygenation. The oxygen that diffused through the mucilage in the colony was used to sustain the multiplication and ATP generation. So, in the centre of the colony, the cells are oxygen-limited in the sense that they are consuming oxygen as fast as it is supplied.

8.6. The preliminary study of parenchyma cell walls of sugarcane internode stem suggests a possible pathway between the apoplast and the symplast.

In the mature internode of sugarcane stems the isolated nonlignified, nonsuberized cells in the storage compartment appear to have the capacity to act like transfer cells involved in exchanges of solutes between the apoplast and the symplast of the plant (Chapter 3). These cells may provide the supply of sugar from plant to bacteria, and the transport of fixed nitrogen from bacteria to plant.

8.7. The discovery suggests the possibility of finding or creating similar symbioses in other crops.

This is the first report of a N₂-fixing symbiont in grasses, and the first of an N₂-fixing bacterium living heterotrophically in a natural "culture broth" excreted by plant cells. It is an entirely new kind of N₂-fixing system, and one readily accessible to experimentation and manipulation. The long-desired introduction of bacteria with N-fixing ability into wheat, rice and corn might be more easily accomplished by encouraging these plants to leak sucrose into their intercellular spaces where bacteria could flourish, than by trying to induce these plants to form the nodules and associated vascular interconnections with the plants that would be necessary to accommodate *Rhizobium* in a symbiotic relationship.

REFERENCES

- Alexander AG 1973. Sugarcane Physiology. Elsevier Scientific Publishing Company, New York.
- Bernard K 1994. Personal communication.
- Bernard K, Bellefeuille M and Ewan EP 1991. Cellular fatty acid composition as an adjunct to the identification of asporogenous, aerobic Gram-positive rods. J. Clin. Microbiol. 29:83-89.
- Bernard K, Tessier S, Winstanley J, Chang D and Borczyk A 1994. Early recognition of atypical *Francisella tularensis* strains lacking a cysteine requirement J. Clin. Microbiol. 32:551-553.
- Bielecki RL 1960. The physiology of sugar-cane. II. Characteristics of sugar uptake in slices of mature and immature storage tissue. Aust. J. Biol. Sci. 13:203-220.
- Bishop CD and Cooper RM 1984. Ultrastructure of vascular colonization of vascular gel in infected banana roots. Phytopathology. 57:11-13.
- Bishop PE, Jarlenski DML and Heterington DR 1980. Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. Proc. Natl. Acad. Sci. USA. 77:7342-7346.
- Blackburn F 1984. Sugar-cane. Longman Group Limited, London.
- Boddey RM, Urquiaga S, Reis V and Döbereiner J 1991. Biological nitrogen fixation associated with sugar cane. Plant Soil 137:111-117.
- Bull TA, Gayler KR and Glasziou KT 1972. Lateral movement of water and sugar across xylem in sugarcane stalks. Plant Physiol. 49:1007-1011.
- Burlew JS 1953. Algal culture from Laboratory to Pilot Plant. Carnegie Institute of Washington, Washington, DC. p 272.

- Burr GO, Hartt CE, Brodie HW, Tanimoto T, Kortschak HP, Takahashi D, Ashton FM and Coleman RE 1957. The sugarcane plant. *Ann. Rev. Plant Physiol.* **8**:275-308.
- Burris RH 1991. Nitrogenases. *J. Biologic. Chemis.* **266**:9339-9342.
- Caballero-Mellado J and Martinez-Romero E 1994. Limited genetic diversity in the endophytic sugarcane bacterium *Acetobacter diazotrophicus*. *Appl. Environ. Microbiol.* **60**:1532-1537.
- Canny MJ 1990a. What becomes of the transpiration stream? *New Phytol.* **114**:341-368.
- Canny MJ 1990b. Rates of apoplastic diffusion in wheat leaves. *New Phytol.* **116**:263-268.
- Canny MJ 1993. The transpiration stream in the leaf apoplast water and solutes. *Philos. Trans. R. Soc. Lond. Biol. Sci.* **341**:87-100.
- Canny MJ and Huang CX 1993. What is in the intercellular spaces of roots? Evidence from the cryo-scanning electron microscope. *Physiol. Plant* **87**:561-568.
- Canny MJ and Huang CX 1994. Rates of diffusion into roots of maize. *New Phytol.* **126**:11-19.
- Canny MJ and McCully ME 1985. Locating water-soluble vital stains in plant tissues by freeze-substitution and resin-embedding. *J. Microsc.* **142**:63-70.
- Cauwenberghe OR van, Newcomb W, Canny MJ and Layzell DB 1993. Dimensions and distribution of intercellular spaces in cryo-planed soybean nodules. *Physiol. Plant.* **89**:252-261.
- Cavalcante VA and Döbereiner J 1988. A new acid-tolerant nitrogen fixing bacterium associated with sugarcane. *Plant Soil* **108**:23-31.

- Coleman AW and Goff LJ 1985. Applications of fluorochromes to pollen biology. I. Mithramycin and 4',6-diamidino-2-phenylindole (DAPI) as vital stains and for quantitation of nuclear DNA. *Stain Technol.* **60**:145-150.
- De Lay J, Swings J and Gassele F 1984. Genus I. *Acetobacter* Beijerinck 1898, 215. In NR Krieg and JGHolt eds, *Bergey's Manual of Systematic Bacteriology*. Vol. I. The Williams and Wilkins Co., Baltimore, USA, pp 268-274.
- Denison RF, Weisz PR and Sinclair TR 1983. Analysis of acetylene reduction rates of soybean nodules at low acetylene concentration. *Plant Physiol.* **73**:648-651.
- Dilworth MJ 1987. Where to now? *Phil. Trans. R. Soc. Lond. B* **317**:279-289.
- Döbereiner J 1961. Nitrogen fixing bacteria of the genus *Beijerinckia* Derx in the rhizosphere of sugarcane. *Plant and Soil.* **15**:211-216.
- Döbereiner J, Day JM and Dart PJ 1972a. Nitrogenase activity in the rhizosphere of sugar cane and some other tropical grasses. *Plant and Soil* **37**:191-196.
- Döbereiner J, Day JM and Dart PJ 1972b. Nitrogenase activity and oxygen sensitivity of the *Paspalum notatum* -*Azotobacter paspali* association. *J. Gen. Microbiol.* **71**:103-116.
- Döberiner J, Reis V and Lazarine AC 1988. A new N₂ fixing bacteria in association with cereals and sugarcane. In H Bothe, FJ de Bruijn, WE Newton, eds. *Nitrogen Fixation: Hundred Years After*. Gustav Fischer, Stuttgart, Germany, pp 717-722.
- Dong Z, Canny MJ, McCully ME, Roboredo MR, Cabadilla CF, Ortega E

- and Rodes R 1994. A nitrogen-fixing endophyte of sugarcane stems: A new role for the apoplast. *Plant Physiol.* **105**:1139-1147.
- Dong Z, Heydrich M, Bernard K and McCully ME 1995. Further evidence that N₂-fixing endophytic bacterium from the intercellular spaces of sugarcane stems is *Acetobacter diazotrophicus*. *Appl. Environ. Microbiol.* **61**:1843-1846.
- Eisbrenner G and Evans HJ 1983. Aspects of hydrogen metabolism in nitrogen-fixing legumes . *Annu. Rev. Plant Physiol.* **34**: 105-136.
- Fu HA, FitzMaurice WP, Lehman LJ, Roberts GP and Burris RH 1988. Regulation of nitrogenase activity in azospirilla, herbaspirilla and acetobacter and cloning of *drag*- and *drat*-homologous genes of *A. lipoferum* SpBr 17. In H Bothe, FJ de Bruijn, WE Newton, eds. *Nitrogen Fixation: Hundred Years After*. Gustav Fischer, Stuttgart, Germany, p 336.
- Fuentes-Ramirez LE, Jimenez-Salgado : Abarca-Ocampo IR and Caballero-Mellado J 1993. *Acetobacter diazotrophicus*, an indole acetic acid producing bacterium isolated from sugarcane cultivars of Mexico. *Plant Soil* **154**:145-150.
- Gallon JR and Chaplin AE 1987. *An Introduction to Nitrogen Fixation*. Cassel Educational Limited, London.
- Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR and Phillips GB (eds) 1981. *Manual of Methods for General Bacteriology*. American Society for Microbiology. Washington DC, USA.
- Gillis M, Kersters K, Hoste B, Janssens P, Kroppenstedt MP, Stephan

- KRS, Texeira J, Döbereiner J and deLey J 1989. *Acetobacter diazotrophicus* sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugarcane. *Int. J. Syst. Bacteriol.* **39**:361-364.
- Hardy RWF, Holsten RD, Jackson EK and Burns RC 1968. The acetylene - ethylene assay for N_2 fixation: Laboratory and field evaluation. *Plant Physiol.* **43**:1185-1207.
- Hardy RWF, Burns RC and Holsten RD 1973. Applications of the acetylene-ethylene assay for measurements of nitrogen fixation. *Soil Biol. Biochem.* **5**:47-81.
- Hatch HD and Slack CR 1966. Photosynthesis by sugarcane leaves. A new carboxylation reaction and the pathway of sugar formation. *Biochem. J.* **101**:103-111.
- Hawker JS 1965. The sugar content of cell walls and intercellular spaces in sugarcane stems and its relation to sugar transport. *Aust. J. Biol. Sci.* **18**:959-969.
- Hawker JS 1985. Sucrose. *In Biochemistry of Storage Carbohydrates in Green Plants*. Eds Dey, P. M. and Dixon, R. A. Academic Press, New York.
- Holfeld HS, Mallard CS and LaRue TA 1979. Portable gas chromatograph. *Plant Soil* **52**:595-598.
- Hopkins DM, Jackson AD. and Oates K 1991. The effect of aluminium coating on elemental standards in X-ray microanalysis. *J. Electron Microsc. Technique* **18**:176-182.
- Huang CX, Canny MJ, Oates K and McCully ME 1994. Planing frozen hydrated plant specimens for SEM observation and EDX microanalysis. *Microsc. Res. Technique* **28**:67-74.
- Hunt S and Layzell DB 1993. Gas exchange of legume nodules and the

- regulation of nitrogenase activity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**:483-511.
- Jacobsen KR, Fisher DG, Maretzki A and Moore PH 1992. Developmental changes in the anatomy of the sugarcane stem in relation to phloem unloading and sucrose storage. *Bot Acta* **105**:70-80.
- James EK, Reis VM, Olivares FL, Baldani JI and Döbereiner J 1994. Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus*. *J. Exp. Bot.* **45**:757-766.
- Kim J and Rees DC 1992. Structural models for the metal centers in the nitrogenase molybdenum-iron protein. *Science*. **257**:1677-1682.
- Klucses RV and Döbereiner J 1981 Grasses, wheat, maize and Sorghum. In PB Vose, AP Ruschel, eds. *Associative N₂-Fixation*. Vol.II. CRC Press Inc. Florida, pp 243-246.
- Kortschak HP, Hartt CE and Burr GO 1965. Carbon dioxide fixation in sugarcane leaves. *Plant Physiol.* **40**:209-213.
- Layzell DB and Hunt S 1990. Oxygen and the regulation of nitrogen fixation in legume nodules. *Physiol. Plant.* **80**:322-327.
- Li RP and MacRae IC 1991. Specific association of diazotrophic acetobacters with sugarcane. *Soil Biol. Biochem.* **23**:999-1002.
- Li RP and MacRae IC 1992. Specific identification and enumeration of *Acetobacter diazotrophicus* in sugarcane. *Soil Biol. Biochem.* **24**:413-419.
- Lima E, Boddey RM and Döbereiner J 1987. Quantification of biological nitrogen fixation associated with sugarcane using a 15-N aided nitrogen balance. *Soil Biol. Biochem.* **19**:165-170.

- MacRae IC 1977. Influence of partial pressure of acetylene and nitrogen upon nitrogenase activity of species of *Beijerinckia*. Aust. J. Biol. Sci. **30**:593-596.
- Mallard TM, Mallard CS, Holfeld HS and Larue TA 1977. Portable gas chromatograph for the acetylene reduction assay for nitrogenase. Anal. Chem. **49**:1275-1277.
- Minchin FR, Sheehy JE, Witty JF and Muller M 1983. A major error in the acetylene reduction assay: Decreases in nodular nitrogenase activity under assay conditions. J. Exp. Bot. **34**:641-649.
- Mohandas S 1988. Nitrogen fixation in tomato (*Lycopersicon esculentum* Mill "Pusa Ruby"). Pl. Soil, **107**:219-225.
- Mukwaya G. and Welch D 1989. Subgrouping of *Pseudomonas cepacia* by cellular fatty acid composition. J. Clin. Microbiol. **27**:2640-2646.
- Neyra CA and Döbereiner J 1977. Nitrogen fixation in grasses. Adv. Agron. **29**:1-38.
- O'Brien TP, Feder N and McCully ME 1964. Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma **59**:368-373.
- O'Brien TP and McCully ME 1981. The Study of Plant Structure. Principles and Selected Methods. Termarcarphi, Melbourne.
- O'Dowd NA and Canny MJ 1993. A simple method for locating the start of symplastic water flow (flumes) in leaves. New Phytol. **125**:743-748.
- Oresnik IJ and Hayzell DB 1994. Composition and distribution of adenylates in soybean (*Glycine max* L.) nodule tissue. Plant Physiol. **104**:217-225.
- Oworu OO, McDavid CR and MacColl D 1977. Comparison of rates of

- storage of sucrose in eight clones of sugar-cane as measured by sucrose uptake *in vitro*. *Ann. Bot.* **41**:393-399.
- Patriquin DG, Gracioli LA and Ruschel AP 1980. Nitrogenase activity of sugarcane propagated from stem cuttings in sterile vermiculite. *Soil Biol. Biochem.* **12**:413-417.
- Pearse AGE 1968. *Histochemistry, Theoretical and Applied*. Vol 1. JA Churchill Ltd., London.
- Pearse AGE 1985. *Histochemistry, Theoretical and Applied*. Vol 2 Churchill Livingstone, London.
- Pelczar MJ and Chen ECS 1977. *Laboratory Exercises in Microbiology*. 4th ed. McGraw-Hill Book Company.
- Rao VR, Charyala PBBN, Nayak DN and Ramakrishna C 1981. $^{15}\text{N}_2$ incorporation in rice soils and by *Azospirillum lipoferum* from rice root association. In PB Vose, AP Ruschel, eds, *Associative N_2 -Fixation*. Vol.II. CRC Press Inc. Florida, pp 197-204.
- Reis VW, Olivares FL and Döbereiner J 1994. Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. *World J. Microbiol. Biotechnol.* **10**:401-405.
- Reis VM, Zang Y and Burris RH 1990. Regulation of nitrogenase activity by ammonium and oxygen in *Acetobacter diazotrophicus*. *Anais da Academia Brasileira de Ciencias* **62**:317.
- Robson RL, Eady RR, Richardson TH, Miller RW, Hawkins M and Postgate JR 1986. The alternative nitrogenase of *Azotobacter chroococcum* is a vanadium enzyme. *Nature. Lond.* **322**:388-390.

- Robson RL and Postgate JR 1980. Oxygen and hydrogen in biological nitrogen fixation. *Annu. Rev. Microbiol.* **34**:183-207.
- Ruschel AP, Henis Y and Salati E 1975. Nitrogen-15 tracing of N-fixation with soil grown sugarcane seedlings. *Soil Biol. Biochem.* **7**:181-182.
- Ruschel AP, Victoria RL, Salatia E and Henis Y 1978. Biological nitrogen fixation in sugarcane. *Ecol. Bull.* **26**:297-303.
- Schmitt U and Liese W 1990. Wound reaction of the parenchyma in *Betyla*. *IAWA Bulletin.* **11**:413-420.
- Seldin L, Elsas JD van and Penido EGC 1984. *Bacillus azotofixans* sp. nov., a nitrogen-fixing species from Brazilian soils and grass roots. *Int. J. Syst. Bacteriol.* **34**:451-456.
- Shi J, Mueller WC and Beckman CH 1991. Ultrastructural responses of vessel contact cells in cotton plants resistant and susceptible to infection by *Fusarium oxysporum* f. sp. *vasinfectum*. *Physiological and Molecular Plant Pathology.* **38**:211-222.
- Simpson FB and Burris RH 1984. A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. *Science* **224**:1095-1097.
- Singh K, Sinha AP and Agnihotri VP 1981. Role of *Azotobacter* in sugarcane culture and the effect of pesticides on its population in soil. In PB Vose, AP Ruschel, eds, *Associative N₂-Fixation*. Vol. II. CRC Press Inc, Florida, pp 103-108.
- Spiff ED and Odu CT 1973. Acetylene reduction by *Beijerinckia* under various partial pressures of oxygen and acetylene. *J. Gen. Microbiol.* **78**:207-209.
- Sprent JI and James EK 1994. N₂-fixation by endophytic bacteria:

- questions of entry and operation. Presentation in The 6th International Workshop on *Azospirillum* and Related Microorganisms. Hungary (manuscript in press).
- Sprent JI and Sprent P 1990. Nitrogen Fixing Organisms. Pure and Applied Aspects. Chapman and Hall, London.
- Steer M 1981. Understanding Cell Structure. Cambridge University Press, Cambridge, UK.
- Sutherland IW 1977. Bacterial exopolysaccharides - their nature and production. *In* Surface Carbohydrates of the Prokaryotic Cell. Ed. IW Sutherland. Academic Press, New York.
- Takahashi DT 1970. ¹⁵N field studies with sugarcane in coral soils. Hawaii Planter Rec. **58**:13-20, 119-126.
- Urquiaga S, Cruz KHS and Boddey RM 1992. Contribution of nitrogen fixation to sugar cane: nitrogen-15 and nitrogen-balance estimates. Soil Sci. Soc. Am. J. **56**:105-114.
- van Dillewijn C 1952. Botany of Sugarcane. The Chronica Botanica Co., Waltham, Mass.
- Vessey JK 1994. Measurement of nitrogenase activity in legume root nodules: In defence of the acetylene reduction assay. Plant and Soil. **158**:151-162.
- Wallace P, Hollis D, Weaver R and Moss CW 1990. Biochemical and chemical characterization of pink-pigmented oxidative bacteria. J. Clin. Microbiol. **28**:689-693.
- Welbaum GE and Meinzer FC 1990. Compartmentation of solutes and water in developing sugarcane stalk tissue. Plant Physiol. **93**:1147-1153.
- Welbaum GE, Meinzer FC, Grayson RL and Thornham KT 1992.

- Evidence for and consequences of a barrier to solute diffusion between the apoplast and vascular bundles in sugarcane stalk tissue. *Aust. J. Plant Physiol.* **19**:611-623.
- Welch DF 1991. Applications of cellular fatty acid analysis. *Clin. Microbiol. Rev.* **4**:422-438.
- Wenzel CL and McCully ME 1991. Early senescence of cortical cells in the roots of cereals. How good is the evidence? *Amer. J. Bot.* **78**:1528-1541.
- Wessely Z, Shapiro SH, Klavins JV and Harold M 1981. Identification of Mallory bodies with rhodamine B fluorescence and other stains for keratin. *Stain Technol.* **56**:169-176.
- Zimmermann MH and Jeje AA 1981. Vessel-length distribution in stems of some American woody plants. *Can. J. Bot.* **59**:1882-1892.

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