EARLY EVENTS IN THE ESTABLISHMENT OF AN ASSOCIATIVE
SYMBIOSIS OF *AZOSPIRILLUM BRASILENSE* SP 7 WITH
GRASS ROOTS

BY

MERCEDES UMALI-GARCIA

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1978

ACKNOWLEDGMENT

The author wishes to extend her sincere thanks and gratitude to the following:

Dr. DAVID H. HUBBELL, for constant encouragement, patience, sincere friendship, and for giving her the chance to appreciate the value of independent work;

Dr. MURRAY H. GASKINS, for the critical evaluation of the different phases of the study, provision of laboratory space and for various forms of support;

Dr. HENRY C. ALDRICH, for useful suggestions in the ultrastructure aspects of the work, and for the challenges which helped her much in making independent decisions;

Drs. REX L. SMITH and STANLEY C. SCHANK, for the intellectual discussions, and for providing experimental plant materials;

Dr. MAX E. TYLER and JAMES R. MILAM, for constructive comments, and for providing experimental bacterial cultures;

Dr. I. VASIL, for making suggestions, and for giving her the chance to learn the tissue culture technique;

Dr. FRANK B. DAZZO, for guidance in various aspects of the work, especially the biochemical portion, and for the friendship and unsolicited concern;

Mr. STUART PANKRATZ and STANLEY FLEGLER, for the kind and warm accomodation extended her at Michigan State University;

FACULTY AND FRIENDS in the Department of Soil Science,
for their moral and material support;

Mr. VICTOR MORALES, for the interesting intellectual
discussions and friendship;

The US-AID, IFAS-University of Florida, and USDA, for
the financial support;

The UNIVERSITY OF THE PHILIPPINES at Los Banos, for
giving the chance to earn the most coveted training;

Her husband, BENJAMIN M. GARCIA, Sr., for the love
and understanding;

Her children, VENER, BENJIE, MINA, JOVEN, RUBEN and
BIEN, from whom she draws much needed strength;

TO ALL THESE PEOPLE, this manuscript is dedicated.

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENT	ii
LIST OF TABLES	v
LIST OF FIGURES	vi
ABSTRACT	x
INTRODUCTION	1
REVIEW OF LITERATURE	3
The Organism	5
Laboratory and Field Studies	6
Physiology of Interaction	8
Infection Studies	12
MATERIALS AND METHODS	14
Bacteria and Culture Methods	14
Plant Hosts	15
Plant-Bacterium Interaction	15
Aseptic Collection of Root Exudate	16
Adsorption Studies	16
Sanning (SEM) and Transmission (TEM)	
Electron Microscopy	17
Pectolytic Activity Assay	18
Enzyme Assay	18
RESULTS	21
Growth Studies	21
Host-Bacterium Interaction	27
Culture Solution and Root	
Morphology	27
Adsorption Studies	34
Binding Specificity	46
Transmission Electron Microscopy	46
Pectolytic Enzyme Activity	73
DISCUSSION AND SUMMARY	79
Growth Studies	79
Host-Bacterium Interaction	80

LIST OF TABLES

Table	Page
1. Growth of 6 week old guinea grass inoculated with <i>Azospirillum brasilense</i> Sp 7 ..	33
2. Effect of combined nitrogen (5mM KNO ₃) on adsorption of <i>A. brasilense</i> Sp 7 to root hairs of pearl millet	37
3. Adsorption of bacteria to pearl millet seedling root hairs	47
4. Effect of pearl millet root exudate on adherence of <i>A. brasilense</i> Sp 7 to pearl millet root hairs (1 h incubation).	50

LIST OF FIGURES

Figure	Page
1. Growth curve of <i>Azospirillum brasilense</i> in trypticase soy broth (TSB), Okon's succinate medium and in pectin broth	23
2. Forty eight hour old <i>A. brasilense</i> in TSB, negatively stained with PTA.....	26
3a. TEM photomicrograph of 48 h cells grown in TSB	26
3b. TEM photomicrograph of 48 h cells grown in -N malate	26
4. A 5-day old <i>A. brasilense</i> in Okon's mineral medium with pectin	26
5a. Root system of inoculated and uninoculated 1 month old guinea grass	29
5b. Root system of inoculated and uninoculated pearl millet	29
5c. A segment of a root of guinea grass inoculated and grown in -N medium	30
6. Suppressed elongation of the main root axis 2 days following inoculation with <i>A. brasilense</i>	30
7. Dark field photomicrograph of pearl millet root tip inoculated with <i>A. brasilense</i> ...	36
8. Mucigel at maturation region of the pearl millet root	36
9. Phase contrast photomicrograph of root tip of pearl millet showing boundary formed by bacteria on mucigel	36
10. Dark field photomicrograph of root tip of uninoculated root tip of pearl millet	36

Figure		Page
11a.	SEM of <i>A. brasilense</i> adsorbed to matured root hairs of pearl millet grown in -N medium	39
11b.	Higher magnification of similar root hair shown in Figure 11a	39
11c.	Higher magnification of two of adsorbed bacteria on the root hair surface showing granules common to both	39
12.	SEM of <i>A. brasilense</i> adsorbed to the epidermis with mucigel in -N medium	39
13a.	SEM photomicrograph of <i>A. brasilense</i> adsorbed to sloughed cells of pearl millet created by emerged lateral root	41
13b.	Higher magnification of marked area in Figure 13a	41
14.	SEM photomicrograph of colonization of pearl millet by <i>A. brasilense</i> in areas close to void space created by emerging lateral root	43
15.	Reduced adherence of bacteria to pearl millet root hairs grown in +N Fahraeus medium	45
16.	Adherence of azospirilla to epidermal cells of pearl millet roots grown in +N Fahraeus medium	45
17.	Phase contrast photomicrograph of pearl millet root hair inoculated with preincubated <i>A. brasilense</i> in root exudate	49
18.	SEM photomicrograph of pearl millet root hairs with adsorbed bacteria	49
20a.	TEM of x-section of a root hair from an inoculated root.....	52
20b.	TEM of a x-section of a root hair from an uninoculated root of guinea grass	52

Figure	Page
21. Root cell wall of pearl millet has staining property as that of the envelope that enclosed the rhizosphere bacteria	55
22. Two or several azospirilla in a common envelope	55
23. Dark field photomicrograph of pearl millet root showing microcolonies associated with mucigel on the root surface and root hair	57
24. Thinning of epidermal walls of guinea grass associated with microcolonies of <i>A. brasilense</i>	57
25. Fibrillar material in contact with bacterium on the surface of root cell wall of pearl millet	57
26. A x-section from a matured root of 1 month old uninoculated guinea grass ..	59
27. TEM photomicrograph of a serial thin section of a root from above the root hair region	59
28a. Serial thin section in which the root is sectioned and the bacteria are shown outside and inside the middle lamella of the cortical cells after the epidermal layer has sloughed	61
28b. A higher magnification of similar thin section showing bacteria in the middle lamella of the cortical cells	61
29. TEM photomicrograph of a lysed root hair of guinea grass showing entry of azospirilla	63
30. A thin section from a section above the root hair region of guinea grass root showing azospirilla in the void space created by emerging lateral root primordium	63
31. Serial thin section of guinea grass root showing middle lamella of cortex with invading azospirilla	63

Figure		Page
32.	Spread of infection of <i>A. brasilense</i> in the middle lamella of cortical tissue of guinea grass root	66
33a.	TEM photomicrograph of cortical cells from similar root segment as in Figure 32 from an uninoculated guinea grass grown in nitrogen-free medium	68
33b.	TEM photomicrograph of cortical cells from similar root segment as in Figure 32 and 33a from an uninoculated guinea grass grown in the presence of nitrogen	68
34a.	Azospirilla on and inside pearl millet root cell	70
34b.	A high magnification of area marked in Figure 34a	70
35.	Azospirilla with thickened walls enveloped with middle lamellar components of pearl millet cortical cells	72
36.	Clearing of areas around wells on pectin agar plates suggesting a presumptive pectolytic activity of different fractions obtained from the culture filtrate through $\text{NH}_4)_2\text{SO}_4$ precipitation ..	76
37a.	Pectin lyase activity of bacterial washing (B_3) and the fifth fraction (F_5) from the culture filtrate	78
37b.	The elution profile of the degradation products of the polygalacturonic acid by F_5	78

Abstract of Dissertation Presented to the
Graduate Council of the University of Florida in Partial
Fulfillment of the Requirements for the Degree of Doctor
of Philosophy

EARLY EVENTS IN THE ESTABLISHMENT OF AN ASSOCIATIVE
SYMBIOSIS OF *AZOSPIRILLUM BRASILENSE* SP 7 WITH
GRASS ROOTS

BY

Mercedes Unali-Garcia

December, 1978

Chairman: Dr. David H. Hubbell

Major Department: Soil Science

The objectives of this study were to document by light and electron microscopy the events leading to the successful establishment of an associative symbiosis of *Azospirillum brasilense* Sp 7 with the roots of a grass host under axenic conditions and to define physiological and biochemical interactions of the association suggested by the ultrastructural studies.

The association between grass roots and *Azospirillum brasilense* Sp 7 was investigated by the Fahraeus glass slide technique for axenic plant culture. Young inoculated roots of pearl millet and guinea grass produced more mucigel, more root hairs and more lateral roots than did the uninoculated controls. Within 12 h after inoculation cells of *A. brasilense* Sp 7 were embedded in the mucigel at the root cap and along the root axis. The bacteria were also firmly adsorbed to root hairs and epidermal cells but supplementing the medium with combined nitrogen

reduced the adherence to root hairs. Preincubation in root exudate enhanced ability of the bacteria to attach firmly to root surfaces. The active root exudate factor is non-dialyzable and is inactivated by protease.

Examination of the grass rhizosphere by electron microscopy revealed azospirilla enclosed within a slime layer with high affinity for electron dense materials normally associated with the host root cell wall. The bacteria entered the root tissue through lysed root hairs, open spaces created by sloughing of epidermal cells and lateral root emergence, and afterward invaded the middle lamellae of underlying cortical tissue. Colonization remained intercellular as long as the adjacent host cells were alive. Clear zones suggesting hydrolytic activity followed the contour of the bacterial cells in the middle lamellae. Pectin lyase and endopolygalacturonase activities were detected in pure cultures grown with purified pectin. This study shows that *Azospirillum brasilense* Sp 7 is capable of limited intercellular infection of the roots of certain grasses.

INTRODUCTION

Potential nitrogenase activity has been reported in grass roots and grass rhizosphere soil of tropical and subtropical grass land and cultivated fields (Neyra and Dobereiner, 1977). The reported nitrogen fixation may be due to *Azospirillum* spp.^{1/} However, the exact nature of the effect of this bacterium on the growth of grasses is not yet established.

Unlike the *Rhizobium*-legume symbiosis, *Azospirillum*-grass association does not produce visible structures in the roots which indicate successful infection. Studies on sugar cane and *Paspalum notatum* suggest that the majority of the N_2 -fixing organisms are closely associated with root surfaces and are probably within the mucigel layer (Dobereiner et al., 1972b). Only light microscope evidence for the physical presence of *Azospirillum* on root tissues of plants has been presented. *Azospirillum* has been reported to be detected in cortical tissues of *Digitaria* (Dobereiner and Day, 1974), in cortical and stelar cells of maize (Patriquin and Dobereiner, 1977; Patriquin et al., 1978; Scott et al., 1977), and in root hairs, epidermis and xylem vessels of sand grown plants (Lakshmi et al., 1977).

^{1/} *Azospirillum* refers to the free living N_2 -fixing bacterium that was formerly identified by Dobereiner and Day (1974) as *Spirillum lipoferum* based on Bergey's manual (1957) and on the original description by Beijerinck (1925). However, this manuscript will adopt the more recent genus description, *Azospirillum* as proposed by Tarrand et al. (1978), except on reports cited in the previous literature.

Microbial colonization of root surfaces of field grown sand dune grasses was reported by Old and Nicholson (1975) and of wheat root surfaces by Foster and Rovira (1973). No investigation has yet been carried out under controlled experimental conditions to demonstrate the sequence of events of bacterial infection of young grass roots. Studies on the ultrastructure of the infection process of legumes by *Rhizobium* have been successful in complementing physiological studies of legume infection. It seemed reasonable to apply the same approach in studying the establishment of the grass-bacteria associations.

The potential application of new N_2 -fixing systems in agronomic practice as exemplified by the grass-bacteria association is well recognized (Hubbell, 1976). This investigation was, therefore, initiated to present a detailed account of root colonization, root adherence, and infection events in the grass-*Azospirillum* association as revealed by microscopic techniques and to study the physiological and biochemical interactions of this association suggested by the studies.

REVIEW OF LITERATURE

In 1972 Dobereiner and coworkers (Dobereiner et al. 1972a) identified an association of *Azotobacter* with *Paspalum notatum* and calculated that this association could fix 90 kg N/ha/yr. However, Kass et al. (1971), after a test with diploid and tetraploid varieties of *P. notatum*, indicated that successful establishment of *A. paspali* in the rhizosphere of *P. notatum* grown in sand occurred only with tetraploid varieties, and when the soil was amended with glucose at the time of inoculation. Dobereiner et al. (1972b) reported considerable nitrogenase activity in the roots of sugar cane as well as between the rows. Labelled nitrogen ($^{15}\text{N}_2$) tracer experiments of sugar cane seedlings grown in soil gave direct evidence of nitrogen fixation associated with the roots. Variations in both the dry weight and nitrogen content among the sugar cane varieties as well as among the individual plants of the same variety were noted (Ruschel et al., 1975).

The rice rhizosphere is also reported to fix an appreciable amount of nitrogen. Yoshida and Ancajas (1971, 1973) have demonstrated that nitrogen fixing activities take place in the root zones of rice plants and in the paddy soil.

Genotypic differences has been demonstrated in several tropical grasses using the excised root assay (Dobereiner and Day, 1974; Day et al., 1975; Dobereiner and Day, 1975; Bulow and

Dobereiner, 1975). De-Polli et al. (1977) demonstrated nitrogen fixation in two tropical grasses, *Paspalum* and *Digitaria*, by $^{15}\text{N}_2$ incorporation.

Another N_2 -fixing bacterium (unidentified) was characterized by Barber et al. (1976) from roots of *Digitaria sanguinalis*.

Roots of winter wheat (*Triticum*) and sorghum (*Sorghum*) have been reported to harbor members of *Enterobacteriaceae*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Erwinia herbicola*, which fix N_2 *in vitro* (Pedersen et al., 1978).

A summary of reported rates of acetylene reduction and N_2 -fixation with the "associative systems" was prepared by Evans and Barber (1977) and Neyra et al. (1977). Neyra et al. (1977) indicated that the most common encountered bacterial species was *Azospirillum*. Balandreau and Villemin (1973) and Dobereiner et al. (1972a) pointed out that these grass species possess the C_4 dicarboxylic acid photosynthetic pathway. Rice (*Oryza*) was noted as an exception to this general observation.

Brown (1972) and Barea and Brown (1974) suggested that the growth responses of several plants inoculated with *Azotobacter paspali* were due to the synthesis of plant growth regulating substances by the soil organisms. Indole-3-acetic acid, three gibberellins and two cytokinins were detected in the culture filtrates of this organism.

The Organism

Azospirillum has been consistently isolated from soil samples collected throughout Germany (Beijerinck, 1925). Soil samples from tropical countries of Africa (Becking, 1963) and Brazil (Dobereiner et al., 1976; Bulow and Dobereiner, 1975) showed the same trend.

The N_2 -fixing organism which Day and Dobereiner (1974) isolated from *D. decumbens* cv. 'Transvala' was identified as *Spirillum lipoferum*. This identification was based on the description of this organism by Beijerinck as reported in Bergey's manual (1957).

In 1976, Dobereiner and coworkers studied the ecological distribution of *Azospirillum* and indicated that its occurrence was very much influenced by vegetation. It is isolated in lowest frequency in tropical virgin forest. *Azospirillum* could be isolated from soils with a pH range of 4.0 - 5.2.

The physiology and growth requirements of *Azospirillum* in pure culture have been well defined by several investigators (Dobereiner and Day, 1975; Okon et al., 1976a, b; 1977; Sampaio et al., 1978).

A. brasilense grew well and fixed N_2 under microaerophilic conditions with malate, succinate, lactate and pyruvate as carbon sources. Its doubling time when supplied with NH_4^+ was 1 h at $pO_2 = 0.005 - 0.007$ atm. When grown on N_2 , the levels of poly- β -hydroxybutyrate and β -hydroxybutyrate dehydrogenase increased (Okon et al., 1976a). The optimal pO_2 for acetylene reduction in

stagnant cultures was 0.006 - 0.002 atm. depending upon the cell density (Okon et al., 1976a).

Tyler et al. (1977) and Krieg (1977) studied the DNA homology of the different isolates from tropical and subtropical countries. The guanosine/cytosine ratio in these isolates were higher by 5% than the *Spirillum lipoferum* identified by Beijerinck (1925) and reported in Bergey's manual (1957) and were intermediate between *Pseudomonas* and *Spirillum* (Tyler et al., 1977). Based on the DNA homology series and physiological and growth requirements, it was proposed that the generic name of *Spirillum* be changed to *Azospirillum*. *Azospirillum* was divided into two groups differing in their requirements for glucose and vitamins and in DNA homology (Tarrand et al., 1978). Cells of group II strains were reported to be larger and more pleomorphic than cells in group I. Group I strains utilize NO_3^- to N_2 (Okon et al., 1976a; Sampaio et al., 1978). The ability of *Azospirillum* to reduce NO_3^- to N_2 was considered by Neyra et al. (1977) to be very useful for studying various aspects of nitrogen transformation in nature.

Laboratory and Field Studies

Laboratory and field studies have been carried out to assess the N_2 -fixing activity of *Azospirillum* in several places. Dobereiner (1978) considered that *Azospirillum* was most responsible for nitrogenase activity in roots of *Digitaria*, *Panicum*, *Zea*, *Triticum* and *Sorghum* in Brazil.

Nitrogen fixation was reported in a temperate maize-*Spirillum* association in low and variable amounts (Sloger and Owens, 1976). However, the amount of N_2 fixed was considered to be sufficient for appreciable benefits to uncultivated grasses (Evans and Barber, 1977).

Greenhouse experiments conducted by Gaskins et al. (1977) and Subba-Rao and coworkers (1978) demonstrated yield differences following inoculation.

Field inoculation experiments with pearl millet (*Pennisetum americanum*) and guinea grass (*Panicum maximum*) showed that in 3 out of 4 years, *Azospirillum* inoculation can induce significantly higher dry matter yields by treated plants (Smith et al., 1976; Bouton, 1977). Further inoculations of plants grown on the same sites did not exhibit marked differences in yield from the first experiment. They believed that lack of response to further inoculations may be due to the activity of the surviving *Azospirillum* as evidenced by the presence of the bacteria in the samples studied by immunofluorescence (Schank and Smith, pers. comm.). They also have observed differences in response among other species and cultivars. Gahi 3, a pearl millet hybrid, responded significantly to field inoculation. It gave 32% more dry weight and 37% more total nitrogen compared to killed inoculum controls (Bouton, 1977). The effect of genotype on response to inoculation with *Azospirillum* has also been shown for maize (Barber et al., 1976; Bulow and Dobereiner, 1975); *Paspalum notatum*, *Pennisetum purpureum* and *Triticum* (Dobereiner and Day, 1975; Smith et al., 1977 pers. comm.) and for *Brachiaria* (Pereira et al., 1977).

Physiology of Interaction

Azospirillum was isolated from soils having a wide range pH 4.8 - 7.2 (Dobereiner et al., 1976). More cells occurred on the surface of the roots than in the soil where the plant was growing. The fact that the optimum growth of *Azospirillum* in pure culture requires a pH 6.8 - 7.2 (Dobereiner and Day, 1975; Okon et al., 1976a, b) suggests that the plant host may be responsible for maintaining a favorable pH for the bacteria.

Biological nitrogen fixation, with other components not limiting, is directly related to the amount of carbohydrate available to and utilizable by the microorganisms as energy source (Graham and Halliday, 1977; Hardy and Havelka, 1976; Streeter and Bosler, 1976; Wheeler and Lawrie, 1976). The role of photosynthate in root exudate on growth of rhizosphere microorganisms has been well documented as reflected in the review of Rovira (1969) and Schroth and Hildebrand (1964). Diurnal variation in nitrogenase activity was observed in bahia grass (*Paspalum notatum*) and sorghum (*Sorghum vulgare*) by Dobereiner and Day (1975), in guinea grass (*Panicum maximum*) and maize (*Zea mays*) in contrast to rice (*Oryza sativa*) which did not exhibit any peak at night (Balandreau et al., 1974). It is believed that malate might play a role in the supply of energy for nitrogen fixation, malate being a primary product of C₄ photosynthesis (Chollet and Ogren, 1975). However, seasonal changes could have an effect on the population of *Azospirillum* in plant roots. Scott et al. (1977) demonstrated no significant change in the number of the organisms throughout the life cycle of

the plant during summer months while the number went down 10 - 100 fold during winter.

The stage of the life cycle of the plant has some effect on the variation in nitrogenase activity. In field grown maize, two peaks of nitrogenase activity were observed; the first was during silk emergence and the second was at the start of grain filling (Bulow and Dobereiner, 1975; Neyra et al., 1977). Sorghum was observed to have maximal nitrogenase activity at flowering. In all cases decline of nitrogenase activity was observed at the grain filling stage.

The presence of *Azospirillum* only on the root surface and in intercellular spaces of guinea grass and not inside the plant cells of young tissues (Umali-Garcia et al., 1978) suggests dependence of the microorganisms on the root exudate for growth. Gaskins (pers. comm.), by careful analysis of the exudate of several C_4 grasses, found that simple and complex carbohydrates constitute a major portion of the organic compounds of the root exudate. Carboxylic acids and their derivatives were present in very small amounts. He feels that if *Azospirillum* species have to depend on the exudate, the scarcity of the organic acids may limit nitrogen fixation by the bacteria. *Azospirillum* has been demonstrated to produce IAA and other plant growth regulating hormones (Tien et al., 1978). The production of growth hormones by *Azospirillum* may be a factor that contributes to the observed differences in yield and root morphology in inoculation studies. The role of IAA in affecting the growth of roots of plants has

been demonstrated in the studies by Brown (1976) and Barea and Brown (1974).

Increase in nitrogenase activity and number of *Azospirillum* in the soil treated with herbicide Alachlor and Atrazin was reported by Marriel and Cruz (1977). Nitrogenase activity was 3 times higher with Alachlor and 2 times with Atrazin at $p = 0.01$. The mechanism of action of these herbicides on the plant or on the bacteria is not known. However, it should be noted that these have an indole ring in their structure which might be metabolized to compounds having hormonal activity.

Nitrogen fixation in the grass-bacteria system has been commonly detected by measuring acetylene reduction in soil cores and excised roots. Amount of nitrogen in the plant samples and dry weight are usually determined using intact plants; intact plants are frequently used in the nitrogenase assay. Correlation of results in core assays and preincubated excised root assays is not firmly established as valid (Dobereiner, 1978) although a few reports indicated such correlation (Neyra et al., 1977). Nitrogenase activities in maize and sorghum cores under tropical and temperate conditions were much lower and did not correlate with the excised root assay (Barber et al., 1976; Tjepkema and van Berkum, 1977). Acetylene reduction as a measure of nitrogen fixation was carefully reviewed and evaluated by Gaskins and Carter (1976).

Preincubation at low pO_2 overnight prevents the lag usually observed in acetylene reduction assay period; however,

this usually resulted in increased number of bacteria. A 30-fold increase in the numbers of bacteria in maize roots when incubated at 30°C for 24 h at low oxygen pressure was observed by Barber et al. (1976) while a 100-fold increase was reported by Okon et al. (1976b). It is not yet established whether the *Azospirillum* cells localized on the root surface or inside the root tissues contribute to nitrogenase activity (Neyra and Dobereiner, 1977).

Combined nitrogen can inhibit nitrogenase activity of *Azospirillum*. Neyra et al. (1977) reported that 10mM NO_3^- inhibits nitrogenase activity but under anaerobiosis some strains could reduce NO_3^- to N_2 . Denitrification was also demonstrated in the field (Eira, 1977).

Heavy nitrogen fertilization could repress nitrogen fixation in sorghum and maize (Barber et al., 1976), *Digitaria decumbens* cv. 'Transvala' (Abrantes et al., 1975), rice seedlings (Balandreau et al., 1975), and in wheat (Barber et al., 1976); Nery et al., 1977). Nevertheless, addition of a low amount of nitrogen (40 kg N/ha) at planting time enhanced nitrogenase activity in maize roots (Neyra et al., 1977), 30 - 40 kg N/ha enhanced activity in guinea grass and pearl millet (Smith and Schank, pers. . comm.) and 50 kg N/ha in wheat (Nery et al., 1977; Barber et al., 1976). However, the enhanced nitrogenase activity indicated by excised root assays or core assays has not been shown to correlate with assays conducted with intact plants. It is believed that biological nitrogen fixation alone may not satisfy all the nitrogen requirements of grasses and therefore, studies on the

interaction of combined nitrogen and biological nitrogen fixation and assimilation need further attention. If *Azospirillum* must depend on root exudates for their energy supply, it may be necessary to give the host plants a reasonable amount of nitrogen to permit adequate initial growth.

Infection Studies

The presence of unidentified bacteria on the roots of wheat grown in sand and soil mixture under laboratory conditions was studied using TEM by Rovira and Campbell (1974). They claimed that bacteria were more abundant near root hair region and older roots than at the very tip of the root. The mucigel, when present, showed numerous adsorbed bacteria.

Scanning (SEM) and transmission (TEM) electron microscope were used by Old and Nicholson (1975) to study the microbial invasion of roots of 3 species of sand dune grasses. The bacteria gained entry through perforations in the cell walls or through exposed middle lamellae. They reported that the cortical cells of all the 3 species examined contained vesicular-arbuscular endophyte.

Electron microscopic studies of the wheat rhizosphere by Foster and Rovira (1976) revealed sparse colonization of the young roots but considerable colonization in the outer cortical cells exposed by sloughing and cell wall surfaces of the old roots.

There is light microscope evidence of the physical presence of *Azospirillum* in the cortical cells of *Digitaria decumbens* (Dobereiner and Day, 1974), *Zea mays* (Bulow and Dobereiner, 1975;

Patriquin and Dobereiner, 1977) and in *Saccharum officinarum* (Patriquin et al., 1978), based on reduction sites in the cortex of these grasses which were detected following staining with Chloramine T or tetrazolium. Lakshmi et al. (1977) reported light microscope evidence of the colonization of root hairs, epidermis and xylem vessels by *Azospirillum*. A preliminary study on the infection of *Panicum maximum* by *Azospirillum brasilense* Sp 7 using TEM in axenic cultures was conducted by Umali-Garcia et al. (1978). The bacteria entered through lysed root hairs and breaks at point of lateral root emergence and subsequently invaded the underlying cortical cells. The bacteria remained intercellular in the living cortical tissues of the root. A presumptive pectinase activity of *Azospirillum* was detected in pure culture. The role of pectinase produced by *Azospirillum* in infection of grass roots is not known.

Serological studies of *Azospirillum* and other bacteria were conducted by Dazzo and Milam (1976). They found that the age of culture has an effect on agglutination, the 7-day old culture gave the best titer. Incubated sterile roots of guinea grass in goat anti-*Azospirillum* antiserum revealed a characteristic fluorescence which suggested that guinea grass had common antigens with that of *Azospirillum*.

MATERIALS AND METHODS

Bacteria and Culture Methods

Azospirillum brasilense Sp 7 obtained from Dr. J. Dobereiner was used in adsorption and infection studies. For specificity studies the following were used: *Azospirillum brasilense* strains Sp 13t and Sp 7 (isolated by J. Dobereiner from *Digitaria*); JM 125A2 (isolated by J. Milam from *Pennisetum*), *Pseudomonas fluorescens*, *Klebsiella pneumoniae*, *Azotobacter vinelandii* UW10, *Escherichia coli*, (MSU and UF laboratory cultures), and *Rhizobium trifolii* 0403 (P. Nutman). *R. trifolii* 0403 was grown in yeast extract mannitol agar (Fred et al., 1932) while the others were grown in trypticase soy (Difco) broth or agar. Pectin broth, which was prepared by mixing 200 ml autoclaved purified pectin (Cooke et al., 1976) at 3.0 g/l to 800 ml solution containing the same amount of minerals/liter described by Okon et al. (1976) fortified with 1g NH₄Cl, was used as the growth medium when the filtrate was needed for pectinase activity analysis.

Growth curves were obtained by growing the bacteria in appropriate media in a side arm flask at 35°C, and measuring the optical density at wavelengths that gave the least absorbance for the uninoculated medium.

Cells were harvested and washed with isotonic buffer and resuspended to a desired concentration before being used as inocula.

The morphology of *Azospirillum* Sp 7 in different media was studied by examining Gram stained cells under the light microscope and negatively stained cells under the electron microscope.

For negative staining of the bacteria for electron microscope examination, the pelleted cells were fixed in 3% glutaraldehyde for 1 h and washed successively with phosphate buffer saline (Dazzo et al., 1976) and deionized water. Cells were dried on 300 mesh Formvar-coated grids and stained with phosphotungstic acid for 3 minutes.

Plant Hosts

Panicum maximum 285 (guinea grass strain selection by Dr. Rex Smith, Department of Agronomy, University of Florida) and *Pennisetum americanum* cv. Gahi 3 (pearl millet) were used as plant hosts. Both are small seeded species and therefore suitable for axenic culture of seedlings on glass slide assemblies (Fahraeus, 1957). Both have responded to field inoculation (Smith et al., 1976; Bouton, 1977) with *A. brasilense* Sp 13t.

Plant-Bacterium Interaction

The grass seeds were surface sterilized in 95% ethanol for 1 min followed by 2.6% sodium hypochlorite for 20 min, and washed in 5-6 changes of sterile deionized water. The seeds were left overnight in the last change of water and the procedure repeated the following day. The seeds were then placed aseptically on sterile agar plates and allowed to germinate with plates inverted so that the radicles would not come in contact with any solid

surface. When the radicles were about 2 cm long, they were carefully transferred to autoclaved Fahraeus assemblies (Fahraeus, 1957). In some experiments, this medium was supplemented with 5 mM $\text{Ca}(\text{NO}_3)_2$ or KNO_3 . The slide assemblies were incubated for 1 month in a growth chamber that supplied 80% of full sunlight, 14 h photoperiod at 35 -36°C.

Aseptic Collection of Root Exudate

Surface-sterilized seeds were germinated on a layer of water agar supported by a stainless steel wire in sterile quart Mason jars containing 50 ml of Fahraeus medium. After one week of incubation (conditions as described above) the sterile root exudate was centrifuged at 8000 x g for 15 min and then passed through a 0.40 μm filter (Millipore). The filtrate was either dialyzed against phosphate-buffered saline (Dazzo et al., 1976), ultracentrifuged, concentrated by dialysis against Fahraeus medium containing 20% polyethylene glycol or incubated at 22°C for 12 h with gentle shaking with protease insolubilized on carboxymethyl-cellulose (Sigma Chem. Co., St. Louis, Mo.).

Adsorption Studies

Two separate adsorption experiments were conducted. The first was to test the effect of combined N on adsorption of *A. brasilense* Sp 7 to pearl millet roots; the second was to determine whether preincubating the cells in root exudate of pearl millet would enhance adsorption of bacteria to roots of the grass host.

For studying the effect of combined N, sterile seedlings

were grown for 2 days in Fahraeus assemblies with and without N and then inoculated with cells from a 48 h old culture. For studying the effect of root exudate, the inoculum was preincubated in the collected root exudate solution for 3 h, rinsed with sterile Fahraeus medium and resuspended to a common optical density reading in a nitrogen-free medium. The inoculated seedlings were incubated with gentle shaking for 10 - 30 min in sterile beakers after which the roots were rinsed with sterile Fahraeus medium and examined by phase contrast microscope. Quantitative assessment of bacterial adsorption to the grass root hairs was performed according to a modification of the direct microscope assay of Dazzo et al., (1976). Bacterial cells in firm physical contact with the matured host root hair walls of approximately 200 μ m in length were considered adsorbed and were counted.

Scanning (SEM) and Transmission (TEM) Electron Microscopy

Rinsed inoculated and uninoculated roots, obtained from adsorption studies, were fixed with 3% glutaraldehyde in 0.05M cacodylate buffer, pH 7.0, washed, postfixed with 1% osmium tetroxide and dehydrated in an alcohol series. Use of small concentration (10 increment) changes for 15 min in each concentration prevented collapse of root hairs. Roots for SEM were subjected to critical point drying (Bomar Critical Point Dryer) and coated with 200 - 300 Å gold in a Film-Vac Sputter coater and examined on an ISI Super III SEM at 15 KV. For TEM, seedlings grown for 1 - 4 weeks in Fahraeus assemblies as previously described were harvested

and rinsed. Root materials were fixed and dehydrated as above, passed through 3 changes of dehydrated acetone, infiltrated and embedded in Spurr's plastic (Spurr, 1969). Ultrathin sections were post stained with lead citrate (Reynolds, 1963) and examined with a Hitachi HU 11E (75KV) and Philips 300 (80KV) transmission electron microscope.

For acidic polysaccharide staining, Pate and Ordal's (Pate and Ordal, 1967) technique was used while for embedding, Luft's technique was used (Luft, 1961). The roots were prefixed in a mixture containing a 0.5 ml each of 3.5% glutaraldehyde, 0.2 M cacodylate buffer and ruthenium red stock solution (1.5 mg/ml) followed by a 10 minute rinse in a 0.15 M cacodylate buffer and fixed for 3 h in a mixture containing 0.5 ml of 4% osmium tetroxide, 0.2 M cacodylate buffer and ruthenium red stock solution. Dehydration, infiltration and embedding were the same as described previously for TEM.

Pectolytic Activity Assay

Pectin broth was prepared by dissolving and autoclaving 3 grams of purified pectin (Cooke et al., 1976) in 200 ml deionized water (1.5 w/v) and combining it with 800 ml of the mineral medium described by Okon et al. (1977). The medium adjusted to pH 7.0 was inoculated with 10 ml of inoculum (10^9 cells/ml) from a 48 h old culture of *A. brasilense* Sp 7 in Okon's defined mineral medium (Okon et al., 1977). The cells were grown in pectin broth for 7 days at 37°C, centrifuged, and the supernatant subjected to fractional ammonium sulfate precipitation (0-20, 20-40, 40-60, 60-80, 80-95% saturation). The precipitated fractions were

dialyzed against a 0.1M citrate-phosphate buffer at pH 5.2 at 4°C in a Spectrapor membrane tubing with a molecular cut off of 6,000 - 8,000. The buffer and membrane tubings were changed several times until the fractions were free of sulfate. Aliquots of 0.05 ml from each of the dialyzed fractions were added to wells on pectin agar plates (Cooke et al., 1976) and incubated in an upright position at 37°C for 12 h. Tests for pectolytic activity were performed by flooding the plates with either iodide-potassium iodide solution (Dingle et al., 1953) or 1% hexadecyltrimethyl ammonium bromide (Hankin et al., 1971). Zones of clearing around the wells were considered presumptive evidence for pectolytic activity in the fractions. The bacterial pellet obtained from the same culture was washed 3 times with 5 ml citrate-phosphate buffer and the washings were individually tested for pectolytic activity by the same procedure.

Enzyme Assays

The procedure used for detection of pectin lyase was modified from that of Lisker and Retig (1974) and Albersheim (1966). A 0.9% pectin (Sigma grade I) was dissolved in 0.1M Tris-HCl with 0.002M CaCl_2 , adjusted to pH 8.3, and 8 serial dilutions were prepared. A 0.5 ml aliquot from each of the crude enzyme fractions was added to 2 ml diluted substrate and incubated at 30°C. Measurement of degradation products at 230 nm was conducted hourly.

To test for polygalacturonase activity, 0.2 ml of boiled and unboiled dialyzed 95% ammonium sulfate fraction was incubated with 1.8 ml of 0.075% polygalacturonic acid in 0.02M citrate

buffer, pH 4.8 at 30°C. After digestion for 12 h, the products were fractionated by gel filtration on a column (1.7 x 30 cm) of P-2 polyacrylamide gel (Bio Rad Labs., Richmond, Calif.), equilibrated with citrate-phosphate buffer, pH 5.2. Fractions (200 drops/tube) eluting from the column were assayed for total carbohydrate by the phenol-sulfuric acid method (Keleti and Lederer, 1974).

RESULTS

Growth Studies

Azospirillum brasilense Sp 7 was grown in TSB (a rich medium) to serve as a basis for comparison for the growth and morphology of the bacteria in defined media (e. g. -N malate, pectin broth) and in the presence of the host plant. Any morphological changes, therefore, in the rich medium were considered inherent in the organism.

A. brasilense Sp 7 exhibited a normal growth curve in TSB, Okon's medium and in pectin medium (Figure 1). Fastest growth was in TSB. Generation time in TSB was 6 h, the exponential phase began at 2 h and ended at 12 h.

It grew well in -N pectin broth and -N malate in still culture. The effect of shaking with air on growing *Azospirillum* cells in a -N medium was reported by Okon et al. (1976a). The growth curve of *A. brasilense* Sp 7, therefore, was determined only in pectin medium and succinate medium fortified with 1 g/l NH_4Cl . The growth of *A. brasilense* Sp 7 on +N pectin medium was typical of that in Okon's medium.

The cells grown in TSB were plump rods during the exponential phase and gradually flattened as the stationary phase began. Some cells were attached to each other end to end giving the appearance of a long spiral as described by Dazzo and Milam (1976).

Figure 1. Growth curve of *Azospirillum brasilense*
in trypticase soy broth (TSB), Okon's
succinate medium and in pectin broth

Flocculation and slime production were noted after 48 h in TSB while in pectin medium this was not seen until after 72 h.

In both media, the bacteria appeared as small rods at early exponential phase and gradually increased in size from mid exponential until stationary phase. It was noted that in pectin medium the size of the bacterial cells was variable at 12 - 48 h but beyond this the cells became uniform. At 72 h and later, the cells were smaller in size and tended to be more rounded than long. Negatively stained 48 h TSB grown cells revealed the bacteria to be coated by a darkly stained material presumably polysaccharide. Aggregates of 3 - 4 or more cells enclosed in a common slime coating were noted (Figure 2a-b). *A. brasilense* Sp 7 had a single polar flagellum in TSB (Figure 2b). Cells grown in TSB had very dense cytoplasm with only very few electron transparent bodies, poly- β -hydroxybutyrate (PHB) (Figure 3a). Cells grown in nitrogen free malate had numerous PHB and polyphosphate bodies. (Figure 3b).

Electron microscopic examination of 48 h old cells from +N pectin broth revealed dense cytoplasm, prominent granules and occasional polysaccharide capsules. Old cultures, as well as cells grown in -N pectin broth and -N malate, store PHB. Figure 4 shows an *Azospirillum* cell with a thin capsular coat.

Figure 2. Forty eight hour old *A. brasilense* Sp 7 grown in TSB and negatively stained with phosphotungstic acid (PTA).

a. Four cells aggregated in a common slime matrix (X 47,500)

b. An individual cell with a single polar flagellum (X 18,900)

Figure 3a. TEM photomicrograph of 48 h cells grown in TSB (X 14,400)

Figure 3b. TEM photomicrograph of 48 h old cells grown in -N malate (X 31,050)

Figure 4. A 5-day-old cell grown in Okon's mineral medium with pectin. The bacterium has a reticulated cytoplasm (c) with prominent polyphosphate body (ph). The cell wall (arrows) is irregular with thin capsular coat (double arrows). (X 15,600)

Host-Bacterium Interaction

Culture Solution and Root Morphology

The inoculated plant slide cultures became turbid a day following inoculation and remained so until the end of the experiment, indicating the ability of the bacteria to survive and grow in the grass rhizosphere. Roots of uninoculated pearl millet and guinea grass produced few lateral branches and root hairs. In contrast, plants inoculated with *A. brasilense* Sp 7 produced an abundance of lateral roots (Figure 5a-b) and root hairs (Figure 5c). The effect of inoculation on lateral root and root hair morphogenesis was suppressed by NO_3^- (Figure 5a-b).

Suppressed elongation of the main root axis of inoculated plants was obvious at 2 days, as shown by the closeness of the root hair region to the meristematic tip (Figure 6) in contrast to the much elongated meristematic tip in the uninoculated controls (Figure 10). Three weeks to 1 month old inoculated plants had more lateral roots than the uninoculated controls (Figure 5a-b). The adventitious roots and branches of the main roots were mostly forked or whorl-like (Figure 5c), as compared to less branched roots of the control (see Figure 5a, 5b). Different inoculum levels produced different morphological effects. The highest inoculum level used (10^9 cells/20 ml solution) had longer roots. Roots of plants grown in the culture supernatant of *Azospirillum* showed the same pattern of shortened lateral roots. The shoots were not as green as those that were inoculated with live inoculum.

Figure 5a. Root system of inoculated and uninoculated 1 month old guinea grass. The root system of inoculated -N plant was dense and much branched. The roots of the inoculated but grown in +N plant were comparatively long but less branched than those grown in -N culture solution. The root system of uninoculated plant was the least dense and had the least branching.

Figure 5b. Root system of inoculated and uninoculated pearl millet. The -N inoculated roots have more root hairs while the +N inoculated ones have less.

Figure 5c. A segment of a root of guinea grass inoculated and grown in -N culture solution. Note whorl-like type of branching and very profuse root hairs. (X 750)

Figure 6. Suppressed elongation of the main root axis of of pearl millet 2 days following inoculation with *A. brasilense* Sp 7 (X 764)

All the plants were stunted under the growth conditions described in the Materials and Methods, although the tops of inoculated plants and those grown in the presence of nitrogen remained green throughout the duration of the experiment except for purple coloration at the base of the leaf sheaths and on the leaf blades. Carbon dioxide may have posed a limitation on the growth of the plants in this closed system. However, the dry weight of 1 month old inoculated plants was higher than the uninoculated controls and comparable to those plants amended with fixed nitrogen (Table 1). Most of the uninoculated controls died before the termination of the experiment. Gaskins (pers. comm.) found that dry weight increases of seedling plants increased as a result of inoculation with aeration, but not comparable to those plants grown in open systems. It may not be safe to extrapolate the results obtained in this experiment to field conditions.

The inoculated roots of both +N and -N grown plants produced abundant mucigel at the root tip (Figure 7-9) which extended beyond the root hair region (Figure 8). However, the mucigel in the presence of nitrogen was much less than that in the roots grown in the absence of nitrogen. The mucigel in the root tips of +N grown plants was removed by washing. Numerous azospirilla colonized the slime on the root cap, slime along the root axis, and on the matured root hairs. Bacteria adsorbed in the mucigel formed a boundary in the mucigel around the root cap (Figure 7-9) and root hair region (Figure 8). Rinsing with sterile buffer did not remove the bacteria from the mucigel. The root tips

Table 1. Growth of 6 weeks old guinea grass inoculated with *Azospirillum brasilense* Sp 7

Number of Plants	Treatment	Root Length (cm)	Shoot Length (cm)	Number of Adventitious Roots	Branching (rating)	Dry Weight $\bar{x} \pm S. D.$ (mg)
10	-N control	8.0	4.0	3.0	1	20.2 ± 2.4
10	+N control	10.0	10.0	4.0	3	42.0 ± 2.0
20	Live inoculum	7.5	9.0	10.0	4	40.0 ± 8.0
15	Killed inoculum	8.0	6.0	6.0	2	30.5 ± 4.0

Rating : 4 is most branched; 1 is the least branched

of uninoculated roots produced very little mucigel and were intact (Figure 10), whereas those of inoculated ones had more mucigel and the root cap cells were loose or sloughed (Figure 9).

Examination of the culture solution of -N plants revealed bacterial rosettes and numerous azospirilla attached to the sloughed cells suspended in the culture solution. There was random but prominent bacterial colonization on surfaces of matured root hairs and epidermis. Numerous granules of about $0.15 - 0.20 \mu\text{m}$ were common on both root hair and bacterial cell surfaces (Figure 11a-c). There was extensive colonization of epidermal surfaces where mucigel was present (Figure 12), of sloughed tissue (Figure 13a-b), and in void spaces created by lateral root emergence (Figure 14). Very few bacteria adhered to the young undifferentiated epidermal surfaces (Figure 13a).

Adsorption Studies

The age of inoculum had a very pronounced effect on the adsorption of azospirilla to the root hair during a 12 h period. The 48 h culture adsorbed most while the 11 day old culture was the least adsorbed. Adsorption occurred in the order of $12 \text{ h} < 48 \text{ h} > 72 \text{ h} \approx 5 \text{ days} \approx 11 \text{ days}$. When interaction was allowed for a longer period, the effect of age on adsorption became insignificant.

Supplementing the medium with either NH_4^+ or NO_3^- (5 -15 mM) suppressed adsorption of bacteria to root hairs (Figure 15, Table 2) but not to the epidermis (Figure 16).

- Figure 7. Dark field photomicrograph of pearl millet root tip inoculated with *A. brasilense* Sp 7. Note mucigel (M) layer and azospirilla (arrows) embedded in it. (X 150)
- Figure 8 Mucigel (M) at maturation region of the pearl millet root. Azospirilla are apparent in the mucigel. (X 180)
- Figure 9. Phase contrast photomicrograph of root tip of pearl millet showing boundary (arrows) formed by bacteria on mucigel (M). Sloughed root cap cells (rc) are embedded in the mucigel. (X 1,200)
- Figure 10. Dark field photomicrograph of root tip of uninoculated root of pearl millet. Root cap (arrows) is intact with practically no mucigel. (X 135)

Table 2. Effect of combined nitrogen (5 mM KNO₃) on adsorption of *A. brasilense* Sp 7 to root hairs of pearl millet cv. Gahi 3

Root Hairs Counted	Adsorbed Cells ($\bar{x} \pm S. D.$)	
	+N	-N
80	3.15 $\pm$ 0.6	35.0 $\pm$ 10.5

40 root hairs, each 200 μ m in length were examined for each treatment

Figure 11a. SEM photomicrograph of *A. brasilense* Sp 7 adsorbed to matured root hairs of pearl millet grown in -N medium. (X 4,285)

Figure 11b. Higher magnification photograph of similar root hair shown in Figure 11a. Aggregation of bacterial cells and presence of granules (arrows) on both root hair and bacterial surface are obvious. (X 8,500)

Figure 11c. Higher magnification of two of the bacteria on the root hair surface showing granules common to both. (X 15,000)

Figure 12. SEM photograph of *A. brasilense* Sp 7 cells adsorbed to the epidermal cells with mucigel of pearl millet root grown in -N medium. (X 1,181)

Figure 13a. SEM photomicrograph of *A. brasilense* Sp 7 adsorbed to sloughed cells (sc) created by emerged lateral root (lr) of pearl millet. Note the absence of azospirilla on the surface of the young lateral root. Marked area shows numerous cells on sloughed off tissue. (X 333)

Figure 13b. Higher magnification of marked area in Figure 13a. The azospirilla (B) are associated with the mucigel on the root surface. (2,666)

Figure 14. SEM photomicrograph of colonization of pearl millet by *A. brasilense* Sp 7 in areas close to void space (arrows) created by emerging lateral root. (X 5,357)

Figure 15. Reduced adherence of bacteria to pearl millet root hairs (RH) grown in +N Fahraeus medium. Note the smooth surface of the root hairs. (X 3,200)

Figure 16. Adherence of azospirilla to epidermal cells of pearl millet roots grown in +N Fahraeus medium. As in Figure 12, the azospirilla are adsorbed to the root surface just as well. (X 1,625)

Binding Specificity

Forty eight-hour old azospirilla bound rapidly to root surfaces. Table 3 shows the trend in binding of the different bacterial species to pearl millet when incubated for 10 - 30 min in -N Fahraeus medium.

All three of the *A. brasilense* strains tested adsorbed strongly to pearl millet root hairs. *R. trifolii* 0403 and *P. fluorescens* adsorbed to root hairs but less firmly than *A. brasilense*. *A. vinelandii* UW10, *E. coli* and *K. pneumoniae* did not adsorb to the root hair surface.

Preincubation of *A. brasilense* cells in pearl millet root exudate promoted their firm adherence to the root hair surface (Figure 17, 18, 19; Table 4). This root exudate factor was inactivated by protease and was non-dialyzable. Bacteria which had been preincubated in root exudate previously dialyzed against 20% polyethylene glycol showed greatest adsorption to pearl millet root surfaces. Bacteria that had been preincubated in protease-treated root exudate showed very little tendency to adsorb to the roots. Clumping made it hard to quantitate observations, hence adsorption was rated on a 1 - 5 scale. Azospirilla did not bind root hairs of pearl millet which had been placed in steam bath for an hour.

Transmission Electron Microscopy

Electron dense granules approximately 0.20 μm in diameter were evident in the granular matrix of the mucigel on the root surface (Figure 20a), in contrast to the almost smooth root hair surface of the uninoculated root (Figure 20b).

Table 3. Adsorption of bacteria to pearl millet seedling root hairs

Bacteria	Root Hairs Counted	Adsorbed Cells ^{1/} $\bar{x} \pm S. D.$
<i>Azospirillum brasilense</i> Sp 13t	16	23.7 $\pm$ 2.4
<i>A. brasilense</i> Sp 7	18	33.0 $\pm$ 4.0
<i>A. brasilense</i> Sp JM125A2	19	32.5 $\pm$ 2.9
<i>Pseudomonas fluorescens</i>	15	11.4 $\pm$ 1.3
<i>Rhizobium trifolii</i> 0403	19	14.4 $\pm$ 2.0
<i>Azotobacter</i> <i>vinelandii</i> UW10	15	0.6 $\pm$ 0.3
<i>Klebsiella pneumoniae</i>	18	0.3 $\pm$ 0.2
<i>Escherichia coli</i>	16	0.06 $\pm$ 0.06

^{1/} Mean of 2 replicates

- Figure 17. Phase contrast photomicrograph of pearl millet root hair inoculated with preincubated *A. brasilense* Sp 7 in root exudate. Some bacteria are attached end to end (arrows). RH - root hair; B - azospirilla (X 1,400)
- Figure 18. SEM photomicrograph of similar root hair as in Figure 17. The undifferentiated epidermal cells are not as affected as the root hair. (X 1,200)
- Figure 19. SEM photomicrograph of pearl millet root hairs (RH) with adsorbed bacteria. Some root hairs (background) appeared lysed. (X 1,120)

Table 4. Effect of pearl millet root exudate on adherence of *A. brasilense* Sp 7 to pearl millet root hairs (1 h incubation)

Root Exudate	Specific Activity (μ g protein/ml)	Rating
Dialyzed	10.5	4
Ultracentrifuged	17.5	4
Unconcentrated	13.5	2
Dialyzed against polyethylene glycol	56.0	5
Fahraeus medium (-N)	-	1
Incubated with protease	27.5	1

5 is most adsorbed; 1 is least adsorbed

Figure 20a. TEM photomicrograph of x-section of a root hair from an inoculated root grown in nitrogen-free medium. Dark granules (arrows) are on the surface of the root hair. (X 10,514).

Figure 20b. TEM photomicrograph of x-section of a root hair from an uninoculated root of guinea grass grown in nitrogen-free medium. The dark granules seen in Figure 20a are absent. (X17,265)

Groups of 2 - 4 bacterial cells were enclosed in a slime envelope, the electron density of which was close to that of the root cell wall (Figure 21, 22). Random microcolonies were associated with the mucigel on the root surfaces (Figure 23), with thin areas in the epidermal walls and near areas where epidermal cells were beginning to slough (Figure 24). Examination of similar materials stained with ruthenium red showed bacteria embedded in a heavy mucigel on the surface of the host cell wall. Fibrillar materials were common in the slime of the root cell wall and the thin bacterial capsule (Figure 25, 34a-b). The epidermal layer of matured uninoculated roots appeared as discrete, intact, undulating surfaces, and lacked thinned walls or evidence of sloughing (Figure 26).

Examination of 1, 2, 3, and 4 weeks old inoculated roots revealed that infection does not occur at the meristematic region but does occur in older regions of the root. There was progressive sloughing of epidermal cells in older tissues. Bacteria entered spaces between sloughed epidermal layer and cortex (Figure 27) and concurrently, invaded the exposed middle lamellar regions of underlying tissue (Figure 28a-b). *Azospirilla* also gained entrance through lysed root hairs (Figure 29) and void spaces created by lateral root emergence (Figure 30).

Zones of hydrolysis in the middle lamella of invaded tissues were prominent and these zones appear to follow the contour of the bacterial cell wall (Figure 28b).

The plasmalemma drew away from the wall of cells which were in close contact with the bacteria (Figure 24). Loss of cell

Figure 21. Root cell wall of pearl millet (cw) has staining property as that of the envelope that enclosed the rhizosphere bacteria (B) in Figure 22.
(X 30,534)

Figure 22. Two or several azospirilla are enclosed in a common envelope. Note some of the azospirilla that are enclosed as compared with the other bacteria that are free.
(X 16,509)

Both stained with DAB and were not post stained.

- Figure 23. Dark field photomicrograph of pearl millet root showing microcolonies (arrows) associated with mucigel on the root surface and root hair. RH - root hair; ra - root axis (X 141)
- Figure 24. Thinning of epidermal walls (arrows) of guinea grass root associated with microcolonies of *A. brasilense* Sp 7. (X 5,636)
- Figure 25. Fibrillar materials are in contact with the bacteria in the surface of the root cell wall (cw) of pearl millet. Note dark granules (arrows) common to the cell wall and the bacteria. (X 6,272)
Stained with ruthenium red.

Figure 26. A x-section from a matured root of a 1 month old uninoculated guinea grass. The epidermal layer (arrows) is compact and undulating. (X 5,293)

Figure 27. TEM photomicrograph of a serial thin section of a guinea grass root from above the root hair region. Bacteria enter through void space (arrows) between the sloughed epidermal layer (E) and cortex (C). (X 3,600)

Figure 28a. Serial thin section in which the root is sectioned and the bacteria are shown outside and inside the middle lamella of the cortical cells after the epidermal layer has sloughed. Note the darkly stained cytoplasmic components in the vacuole (V). The specimen was 3 days old following inoculation. (X 6,400)

Figure 28b. A higher magnification of similar thin section showing the bacteria in the middle lamella of the cortical cells. v indicates vesicles in the middle lamella. (X 32,941)

The plant host is guinea grass.

Figure 29. TEM photomicrograph of a lysed root hair of guinea grass showing entry of azospirilla. (X 3,381)

Figure 30. A thin section from a section above the root hair region of guinea grass root showing azospirilla in the void space created by emerging lateral root primordium (p). Vesicles and mitochondria are seen in the cytoplasm of cells adjacent to the infection site. (X 8,662)

Figure 31. Serial thin section of guinea grass root showing middle lamella of cortex with invading azospirilla. There is apparent division of one bacterium (B). Note polysomes (arrows) and vesicles (v). (X 18,666)

wall integrity (Figure 34a-b), accumulation of degraded cytoplasmic components (Figure 28a), and presence of fibrillar materials near regions colonized by the bacteria (Figure 34a-b, 25), were early manifestations of the host response to the organism. However, after a week, very few cells showed degradation of the cytoplasmic components, although some dark staining bodies could still be seen in the cytoplasm.

Examination of roots from older cultures showed progressive infection of middle lamellae of inner cortical tissues (Figure 32) and apparent division in the middle lamellae of this tissue (Figure 31). The bacteria had very dense cytoplasm and lacked stored PHB but the bacteria were not observed inside the living cells. Fibrillar materials were not observed around the bacterial cells in the middle lamella of the roots from older cultures. The host responses to invasion were shown by vesiculation in the middle lamellae (Figure 28a) and in the cytoplasm (Figure 30, 31, 32), and production of conspicuous endoplasmic reticula, polysomes and Golgi apparatus. TEM examination of similar root segments from the uninoculated plants grown in -N medium did not exhibit such characteristics of the cytoplasm. The only instance where *A. brasilense* Sp 7 cells were observed inside the host cells was when the latter were moribund (Figure 34a). Some bacteria that were exterior to but in contact with the cell wall of the host had thin capsular coat (Figure 34a-b). The absence of encapsulated bacteria inside the host cells may be due to inability of ruthenium red to penetrate deeper in the tissue of the host. The bacteria that were completely covered with middle lamellar material appeared to have

Figure 32. Spread of infection of *A. brasilense* Sp 7 in the middle lamella of cortical tissue of guinea grass root. The middle lamella (ML) is eroded. There are numerous polysomes (arrows), mitochondria (m) with compact cristae, and vesicles (v). (X 34,285)

Figure 33a. TEM photomicrograph of cortical cells from similar root segment as in figure 32 from an uninoculated guinea grass grown in nitrogen-free medium (X 4,694)

Figure 33b. TEM photomicrograph of cortical cells from similar root segment as in Figures 32 and 33a from an uninoculated guinea grass grown in the presence of nitrogen. (X 5,526)

Figure 34a. Azospirilla on and inside pearl millet root cells. The azospirilla (marked area) on the outer region of the host cell wall are encapsulated. The cytoplasm is moribund. (X 14,400)

Figure 34b. A high magnification of the marked area in Figure 34a. The arrows point to the capsule. (X 85,105)

Figure 35. Azospirilla with thickened walls enveloped with middle lamellar components of pearl millet cortical cell. Stained with ruthenium red. (X19,800)

35

thicker walls than those outside the host cells. *A. brasilense* Sp 7 was reisolated from similar root materials used in SEM and TEM studies by use of Dobereiner and Day's technique (Dobereiner and Day, 1974).

Pectolytic Enzyme Activity

The middle lamella consists largely of pectic material (Esau, 1977; Northcote and Pickett-Heaps, 1966; Hall et al., 1976). Hydrolysis of the middle lamellae of colonized roots suggested digestion of pectin components by the invading bacteria (Guchert et al., 1975). The hydrolyzed areas observed in the middle lamellae of colonized host cells in this study may be evidence of digestion of pectin components. It was not known whether this was caused by the pectic enzymes of the bacteria or by the induction of enzymes of the host by the presence of the bacteria.

Dobereiner's mineral medium (Dobereiner, 1974) with pectin as carbon source was not highly satisfactory for growth of *A. brasilense* Sp 7 because the pH declined as the cells grew, and the lowered pH suppressed further growth. Growth was better in the mineral medium described by Okon et al. (1977) which contains much higher phosphate levels and thus is more strongly buffered (Figure 1). The importance of a high phosphate level in the media used in pectolytic enzyme assay was emphasized by Hankin et al. (1977).

Variable pectolytic activity was detected on pectin agar plates seeded with live inoculum. Presumptive pectolytic activity was detected as zones of hydrolysis on pectin agar plates surrounding wells which contained $(\text{NH}_4)_2\text{SO}_4$ precipitated fractions

from culture filtrates of *A. brasilense* Sp 7 (Figure 36). Activity was not detected if pectin was deleted from the broth culture. The hydrolytic activity exhibited by the fractions in the plate assay suggested that more than one enzyme may be present in the culture filtrate. Zones of hydrolysis also developed around wells containing washings from the pelleted cells grown on pectin broth.

Pectin lyase was detected in very small amount in the different fractions (Figure 37a). Fraction 5 (specific activity of 32.5 $\mu\text{g/ml}$) was used in the assay for polygalacturonase activity (Figure 37b). Gel filtration chromatography of polygalacturonic acid digestion products showed that oligosaccharides of various molecular weights were generated from incubation of substrate with this fraction. The degradative activity present in the fractions was destroyed by boiling.

Figure 36. Clearing of areas around wells on pectin agar plate suggesting a presumptive pectolytic activity of the different fractions obtained from the culture filtrate through $\text{NH}_4)_2\text{SO}_4$ precipitation. C - citrate-phosphate buffer; E - commercial enzyme pectinase (1 mg/ml); numbers 1 - 5 are fractions from the culture filtrate: 1 - 0-20%, 2 - 20 - 40%, 3 - 40 - 60%, 4 - 60 - 80%, and 5 - 80 - 95% $\text{NH}_4)_2\text{SO}_4$ saturation.

36

Figure 37a. Pectin lyase activity of bacterial washing (B₃) and the fifth fraction (F₅) from the culture filtrate.

Figure 37b. The elution profile of the degradation product of the polygalacturonic acid by F₅. Dotted line is boiled fraction, solid line is unboiled enzyme fraction.

DISCUSSION AND SUMMARY

Growth Studies

A. brasilense Sp 7 exhibited a normal growth curve in TSB. The irregular pattern of the growth curve in pectin broth might have been due to the effect of added NH_4Cl , the exhaustion of which may have limited cell division prior to synthesis of nitrogenase. The resumption of growth may be derived from fixed N_2 .

Negatively stained 48 h TSB grown cells revealed the bacteria to be coated by a darkly stained polysaccharide. The aggregates of 3 or more cells enclosed in a common coating perhaps constitute the flocs seen under the light microscope.

Azospirilla formed rosettes at 24 - 48 h in pectin broth. There were also cells that were aligned end to end similar to the 48 h old cells in TSB and those reported by Dazzo and Milam (1976). Chain and rosette formation observed in the pectin and TSB grown cells were also typical characteristics of cells grown in the presence of the plant host in Fahraeus assemblies.

Cells grown in TSB had very dense cytoplasm with only very few electron transparent bodies (PHB). Cells grown in -N malate had numerous PHB and polyphosphate bodies. Okon et al. (1976b) found that levels of PHB and β -hydroxybutyrate dehydrogenase in *Azospirillum* were elevated in cells grown in N_2 compared with ammonium grown cells. The occurrence of PHB in

Azotobacter vinelandii grown in -N medium had been reported by Page and Sadoff (1975). Pectin grown cells stored PHB and had prominent polyphosphate bodies, usually at the center of the cell. The prominence of polyphosphate granules stored by pectin grown cells might be caused by high rates of phosphate assimilation. The dense cytoplasm of cells in pectin broth was similar to that in cells observed in the middle lamellae of host root cells.

Host-Bacterium Interaction

The inoculated solution in the Fahraeus assembly became turbid and remained so until the end of the experiment. The turbidity of the solution indicated the ability of the bacteria to survive and grow in the grass rhizosphere. The young roots of inoculated plants appeared slimy and produced root hairs profusely. Light microscopic examination of 24 h inoculated roots revealed bacteria attached on various parts of the root system. Two weeks after inoculation the lateral roots were shorter and larger in diameter in inoculated cultures than in uninoculated controls. Forked or whorl type branching of inoculated roots were common. The roots of all the inoculated plants were well developed and the shoots remained green comparable to those grown in +N controls except for purple coloration at the base of the leaf sheaths. The appearance of purple color on the leaves was characteristic of nitrogen deficient sorghum plants as described by Doak and Miller (1968). The fact that -N inoculated plants were comparatively as green as the +N controls suggested that the bacteria were able

to fix nitrogen but this was not sufficient to maintain the growth of host plants for a long period of time. Carbon dioxide and insufficient mineral supply may have provided limitations for the growth of both plants and microorganisms.

Gaskins et al. (1977) and Tien et al. (1978) found detectable amount of IAA, gibberellin and cytokinin-like substance from the pure culture supernatant. Carbon compounds such as sugars and amino acids are exuded from the roots of plants (Povira, 1969) and are utilized by the organisms. Plants can take up indoles, and auxin can initiate a lateral root stimulation (Scott et al., 1972). Since auxin, cytokinin and gibberellins are synthesized by *Azospirillum* in pure culture (Gaskins et al., 1977; Tien et al., 1978), there is a possibility that these substances are also synthesized in the grass rhizosphere. The formation of short branched roots by grasses treated with high levels of *Azospirillum* inoculum is typical of the hormone effect described by Slankis (1973) in ectomycorrhizal roots. The production of more lateral roots and more persistent and profuse root hairs by inoculated plants is evidence of stimulation by auxin as described by Scott et al. (1977). The interaction of hormones produced by *Azospirillum* has not been thoroughly studied although characteristic root morphogenesis has been induced in axenic culture using different combinations of hormones (Tien et al., 1978). Both the hormone effect and the fixation ability of *Azospirillum* will require further investigation before the beneficial effects of inoculation can be understood. However, the increased dry weight of inoculated plants over the

uninoculated controls and the turbidity of the solution throughout the duration of the experiment suggests that both plant and bacteria benefited from the association.

Roots of the inoculated plants produced more mucigel and formed more lateral branches and more root hairs than those of uninoculated controls. The phenomenon of enhanced mucigel production by roots when in association with the bacteria has been reported in a legume (Dart and Mercer, 1964), in wheat (Rovira and Campbell, 1974), and in several plant species grown in field and garden soil (Greaves and Darbyshire, 1972). Examination of inoculated roots showed mucigel to be most abundant at the root tip, especially at the root cap and the maturation region and root hairs. Matured undifferentiated epidermal cells have some amount of mucigel but surfaces of older tissues are devoid of it. Cortical cells adjacent to the infected sites appeared to have more numerous Golgi bodies, rough endoplasmic reticula and vesicles indicating high biosynthetic activity. Similar tissues from the uninoculated roots appeared to have few of these organelles. The apparent presence of more organelles in inoculated roots may be due to the fixed nitrogen by the bacteria.

The Golgi apparatus-mediated secretion of polysaccharide by outer root cap cells of *Zea mays* has been well documented by the studies of Morre' and Mollenhauer (1967), Juniper and Roberts (1966) and Northcote and Pickett-Heaps (1966). The relative contribution of plant and bacteria to mucigel production in inoculated plant cultures is not known. Nevertheless, the possibility of complementary slime production by both bacteria and host should not

be overlooked since slimy material settles at the bottom of the flask when the bacteria were grown in pure culture and this material stained positively with polysaccharide stains, phosphotungstic acid and ruthenium red. It would seem worthwhile to follow the production of mucigel or exudation of carbon compounds such as sugars and amino acids with labelled compounds fed to the leaves of the host plant.

The presence of numerous clumps of bacteria in the mucigel may be significant to the economy of the grass-bacteria association. The mucigel may mediate ion exchange between the immediate soil environment and the root surface of the plant or the microorganism (Greaves and Darbyshire, 1972; Jenny and Grossenbacher, 1963). The mucigel seems to provide a protective niche for rapid bacterial multiplication, perhaps by accumulating root exudate or by preventing subsequent colonization by other organisms. The mucigel may promote nitrogen fixation by protecting the enzyme nitrogenase by limiting O₂ diffusion.

Bacteria outside the host cells and in close contact with the plant cell walls and/or mucigel showed irregular cell coat indicating differential synthesis or breakdown of extracellular material. Fibrillar materials and small electron dense granules on the area where the bacteria and host cell wall were associated might be a part of a recognition or binding site. The electron dense granules may be aggregation of proteins since these granules were not seen when the material was not post stained with lead citrate or uranyl acetate. The fibrillar structure have been reported in the *Rhizobium*-legume (Napoli et al., 1976) and in

broad bean-saprophytic bacterium interaction (Sing and Schroth, 1977). The absence of fibrillar material around the bacterial cells when the bacteria were in the middle lamella or inside the host cells and their ability to divide in the middle lamella has a resemblance to the *Rhizobium*-clover system in the sense that the bacteroids do not have the characteristic fibrillar structure present around the infecting rhizobia. The absence of this material around the azospirilla seen in the middle lamella of the grass host may also be due to the poor penetration of ruthenium red. However, the fibrillar structure shown in Napoli's photomicrograph of the infecting rhizobia (Napoli et al., 1975) were demonstrated in materials that were not stained with ruthenium red. It is expected that if similar structures were present around cells inside the middle lamella of the grass host, could also be demonstrated with post staining with lead citrate and uranyl acetate.

Mucigel is produced by plants grown in the +N and -N culture. However, bacteria were adsorbed to the mucigel at the root cap and epidermis in root grown in +N but not to the root hairs. The mechanism of control is not yet understood. The same phenomenon of reduced adherence of *R. trifolii* to clover root hairs grown in +N was also reported by Dazzo and Brill (1978) in the *Rhizobium*-clover symbiosis. Reduction in selective adherence of rhizobia to clover root hairs was concurrent with the decrease in the immunologically detectable levels of the recognition lectin, trifoliin.

The roots of grasses used in the adsorption studies displayed selectivity in their binding of bacteria to certain

regions of the root. Cells were firmly adsorbed to root hairs of plants grown in the absence of nitrogen. However, bacteria were associated only with the mucigel at the root cap and epidermis above the root hair region in roots grown in the presence of nitrogen and were not adsorbed to root hairs. The root hairs also displayed selectivity in their binding of certain kinds of bacteria. It is interesting to note that *R. trifolii* and *P. fluorescens* adhered to pearl millet roots since these bacteria share certain immunological (Dazzo and Milam, 1976) and biochemical (Tarrand et al., 1977) characteristics with *Azospirillum*. The mechanism of adherence, and hence the reason for these differences in binding is unknown.

TEM studies revealed random microcolonies on areas where mucigel was present and where root cell walls were thin. There was invasion of the middle lamella in areas where the mucigel was slight or absent and where the epidermis was sloughed. Bacterial colonization of epidermal cells that had no mucigel was noted by Old and Nicholson (1975) in field grown sand dune grasses. The thinning of cell walls associated with microcolonies suggests hydrolytic enzyme in those areas. Absence of mucigel on surfaces where there was invasion of epidermal tissues and underlying cortical tissues could be explained as follows: The plant tissues in those regions may already be very old, and therefore have lost the capacity to produce slime. It is also possible that the mucigel in those areas may have been degraded by the organisms, as suggested by the transparent zones surrounding the cells in the middle lamella and the mucigel layer that follow the contour of the bacteria. In field grown sand dune grasses the bacteria were inside the root tissues in areas where the mucigel was not present (Old and Nicholson, 1975). The invasion of

older parts of the root and not the very young tissues (e. g. meristematic tissue) at the root tip seems reasonable for the microorganisms if exudation sites are considered. Studies using ^{14}C -labelled assimilate have shown that diffusable exudate is released along the full length of the root while non-diffusable carbon compounds come from the root tip region (Rovira, 1969). The production of soluble exudates in the root cortex and the outward diffusion through intercellular spaces would make the niches between the epidermal cells (Tinker and Sanders, 1975) and middle lamellae of the cortical tissues favorable nutritionally for bacterial growth.

Analysis of the composition of the mucigel of maize has been reported by Northcote and Pickett-Heaps (1966) and of wheat by Dayan et al. (1977). In maize, fucose was the only sugar not present in the mucigel of plants studied. Labelling this particular sugar paved the way to a better understanding of the synthesis of polysaccharide in the roots of maize. An understanding of the effect of root exudate composition may help understand the apparent specificity of infection of grasses by *Azospirillum*. Sugars and amino acids in the root exudate may offer a selective advantage for certain microorganisms.

As seen in the TEM micrographs, the staining reaction to ruthenium red by mucigel on the outer cell wall of the plant cell and by those of the interlamellar materials suggests that pectic components were common to both parts of the host cell. The presence of transparent areas in the mucigel and in the middle lamellae where the bacteria are present suggest hydrolysis of the component material. The presence of numerous bacteria in the mucigel

mucigel suggests that this substance may be of considerable influence on the rhizoplane population (Brams, 1969).

Age of inoculum affected the rapidity with which azospirilla adhered to the root hair surface only under the short term incubation (e. g. 1 h - 12 h). Forty eight hour old cultures adsorbed more to the root hairs than did the 12 h old cultures. Napoli (pers. comm.) observed that 48 h cultures of rhizobia were more infective than younger cultures. Similar trends in attachment studies of *R. trifolii* 0403 to clover root hairs has been demonstrated by Dazzo et al. (1976). The phenomenon was believed to be a result of transient adherence of polysaccharide on the *Rhizobium* cell surface. Detected polysaccharide has been shown to undergo immunological changes as the cells enter stationary phase. These chemical changes were considered to reflect increased adherence of rhizobia to the root hair surface.

The entry of bacteria through lysed root hairs and void spaces created by sloughed epithelial cells and branching roots has been reported by Umali-Garcia et al. (1978). The bacteria remained intercellular in plant root tissue, but could enter the root cells when the cytoplasm became moribund. Bacteria were not seen in the xylem of roots grown undisturbed in Fahraeus assemblies, but were seen in xylem of roots that were severed or injured and dipped in inoculum for 5 minutes. Other workers, (Lakshmi et al., 1977; Patriquin et al., 1978) have reported *Azospirillum* in the xylem vessels of maize and sugar cane roots grown in solid medium (e. g. sand-soil mixture). The invasion of xylem vessels of grass

roots may be a consequence of mechanical injury, since the observations were made on roots grown in and removed from solid medium prior to inoculation. Invasion of inner tissues of the roots is very likely to occur in the field as has been described by Old and Nicholson (1975) and Foster and Rovira (1973).

The host cells near infected areas underwent cytoplasmic changes. The appearance of numerous endoplasmic reticula, free ribosomes, mitochondria with compact cristae, increase in the number of dictyosomes and accumulation of vesicles close to the cell wall after 1 month interaction of the bacteria and host plant suggest rapid biosynthetic activity and rapid turnover of synthesized materials. Dictyosomes are thought to be involved in formation of the cell wall (McCoy, 1932; Mollenhauer and Morre', 1966; Northcote and Pickett-Heaps, 1966). The formation of vesicles may have arisen from the demand for increased cell wall formation due to infection (Tu, 1976).

Pectin lyase and endopolygalacturonase have been detected in cultures of *A. brasilense* Sp 7. Pectolytic enzyme involvement in plant infection by pathogenic microorganisms has been well documented (Basham and Bateman, 1975; Bateman and Millar, 1966; Fisher et al., 1973; Albersheim et al., 1969; Mussell and Strand, 1976). The presence of these pectin hydrolyzing enzymes may explain the loosening of microfibrils of the host cell wall and consequent decrease in stainability of areas associated with azospirilla. Ultrastructural evidence for digestion of mucigel by bacteria was reported by Guchert et al. (1975). The effect of pectic enzymes

on solubilization of proteins bound to plant cell walls has been reported by Lund and Mapson (1971), Stephen and Wood (1974), and Strand and Mussell (1975). Peroxidase activity was reported to be associated with the proteins released from cell walls by polygalacturonase (Strand et al., 1976). The thick and predominantly dark root cell wall of inoculated plants reacted with 3, 3-diaminobenzidine (DAB), a substance for peroxidase, suggest peroxidase activity (Umali-Garcia et al., 1976, Unpub.). The presence of peroxidase on the walls of inoculated plants may be an effect of the enzyme released by endopolygalacturonase produced by the bacteria.

Thinning of epidermal cell walls associated with azospirilla suggests that pectolytic enzymes produced by the bacteria are active in the early invasion process. If related to infection and subsequent invasion, a rapid increase in enzymatic activity during the early phase of infection would be expected. The demonstration of this activity in the solution of plant cultures would be worth attempting. Identifying of the different enzyme species involved and determining which type predominates at specific phases of the infection process would contribute to the understanding of the establishment of the association.

Light microscopic examination of older cells of *Cynodon dactylon* isolates by Eskew et al. (1977) were reported to produce cell clusters which he interpreted as "cyst-like" in -N medium when the bacteria were in the stationary phase. Zoogloeia-like clusters of cells were also formed by *Beijerinokia fluminensis* (Dobereiner and Ruschel, 1958). Dobereiner (1974) believed that these might

have a role in protecting the cells from oxygen. Negatively stained azospirilla in stationary phase revealed 3 or more cells sharing a common slime coat. It should be interesting to follow up the occurrence of these structures in different culture media to relate their role to survival of azospirilla in the field and laboratory. *Azotobacter* has been shown to produce cyst in old cultures, and the cyst were related to better survival in the field (Brown et al., 1964).

Most field inoculation with azospirilla used suspension of washed bacteria in the late exponential phase. Old inoculum showing low metabolic activity has not been used. The use of 11 day old culture has been tried in glass assemblies and showed promise of good survival (Umali-Garcia et al., 1976, Unpub.). The role of the age of inoculum on survival of azospirilla in the field needs consideration.

Perforations on the walls of exposed cell lamellae as reported by Old and Nicholson (1975) were not observed in this study. Distorted and collapsed epidermal cells extensively colonized by bacteria as described by Foster and Rovira (1973), were obvious in root tissues examined. Perforations in field grown plant roots may result from the presence of vesicular-arbuscular endophyte that Old and Nicholson (1975) have noted. It may also result from entry of other soil organisms such as nematodes. These perforations were not seen in the scanning electron micrographs of inoculated pearl millet grown in axenic cultures.

The presence of azospirilla in the middle lamella of the cortical tissues and in the mucigel suggests an intimate association. The presence of active cytoplasm of the host and existence of divid-

dividing cells in the host tissues further suggests compatibility of the association.

The experiments reported here show that *Azospirillum brasilense* under axenic conditions is invasive on grass roots and that there is intimate but limited colonization of the middle lamella within the root cortex of the young roots. Limited internal colonization of the root is confined to the middle lamellar region, and may limit the agronomic exploitation of this N_2 -fixing association.

LITERATURE CITED

- Abrantes, G. T. V., J. M. Day and J. Dobereiner. 1975. Methods for the study of nitrogenase activity in field grasses. *Bul. Int. Biol. Lyon* 21.
- Albersheim, P. 1966. Pectin lyase in fungi. In: Colowick, S. P. and N. O. Kaplan (eds.). *Methods in Enzymology* 8: 628-631. Academic Press, Inc. New York.
- Albersheim, P., T. M. Jones and P. D. English. 1969. Biochemistry of the cell wall in relation to infective processes. *Ann. Rev. Phytopath.* 7: 171-194.
- Balandreau, J. P., C. R. Millier and Y. R. Dommergues. 1974. Diurnal variations of nitrogenase activity in the field. *Appl. Microbiol.* 27: 662-665.
- Balandreau, J., G. Rinaudo, I. Fares-Hamad and Y. Dommergues. 1975. Nitrogen fixation in the rhizosphere of rice plants. In: Stewart, W. D. P. (ed.). *Nitrogen Fixation by Free-living Microorganisms*. Cambridge University Press. New York. pp. 57-70.
- Balandreau, J. P. and G. Villemin. 1973. Fixation biologique dell'azote moleculaire en savane de Lamto (Basse Cote D'Ivoire). *Resultas preliminaires. Rev. Ecol. Biol. Sol.* 10: 25-33.
- Barber, L. E. and H. J. Evans. 1976. Characterization of a N_2 -fixing strain from the roots of *Digitaria sanguinalis*. *Can. J. Microbiol.* 22: 254-260.
- Barber, L. E., J. D. Tjepkema, S. A. Russell and H. J. Evans. 1976. Acetylene reduction (N_2 -fixation) associated with corn inoculated with *Spirillum*. *Appl. Environ. Microbiol.* 32: 108-113.
- Barea, J. M. and M. E. Brown. 1974. Effects on plant growth produced by *Azotobacter paspali* related to synthesis of plant growth regulating substances. *J. Appl. Bacteriol.* 37: 583-593.
- Barnett, N. M. 1974. Release of peroxidase from soybean hypocotyl cell walls by *Sclerotium rolfsii* culture filtrates. *Can. j. Bot.* 52: 265-271.

- Basham, H. G. and D. F. Bateman. 1975. Killing of plant cells by pectic enzymes: The lack of direct injurious interactions between pectic enzymes or their soluble products and plant cells. *Phytopath.* 65: 141-153.
- Bateman, D. F. and R. L. Millar. 1966. Pectic enzymes in tissue degradation. *Ann. Rev. Phytopath.* 4: 119-145.
- Becking, J. H. 1963. Fixation of molecular nitrogen by an aerobic *Vibrio* or *Spirillum* sp. *Antonie van Leeuwenhoek J. Microbiol. Serol.* 29: 326.
- Becking, J. H. 1975. Root nodules in nonlegume. In: J. G. Torrey and D. L. Clarkson (eds.). *The Development and Functions of Roots.* Acad. Press. London. pp. 507-566.
- Beijerinck, M. W. 1925. Über ein *Spirillum* welches freien stickstoff binden kann? *Zentralbl. Bakteriologie, Parasitenkunde, Infektionskrankheiten, Hygiene, Abteilung B.* 263: 353-359.
- Bergersen, J. 1970. The quantitative relationship between nitrogen fixation and the acetylene reduction assay. *Aust. J. Biol. Sci.* 23: 1015-1025.
- Bergey's Manual of Determinative Bacteriology. 1957. 7th ed. Williams and Wilkins Co. Baltimore, Maryland.
- Bouton, J. H. 1977. Response of pearl millet inbreds and hybrids to inoculation with *Spirillum lipoferum*. Doctoral dissertation. University of Florida, Gainesville. August, 1977.
- Bowen, G. D. and A. D. Rovira. 1976. Microbial colonization of plant roots. *Ann. Rev. Phytopath.* 14: 121-144.
- Brams, E. 1969. The mucilaginous layer of citrus roots: Its delineation in the rhizosphere and removal from roots. *Plant Soil* 30: 105-108.
- Brown, M. E. 1972. Plant growth substances produced by microorganisms of soil and rhizosphere. *J. Appl. Bacteriol.* 35: 443-451.
- Brown, M. E. 1976. Role of *Azotobacter paspali* in association with *Paspalum notatum*. *J. Appl. Bacteriol.* 40: 341-348.
- Brown, M. E., S. K. Burlingham and R. M. Jackson. 1964. Studies on *Azotobacter* species in soil. III. Effect of artificial inoculation on crop yields. *Plant Soil* 20: 194-214.

- Bulow, J. von and J. Dobereiner. 1975. Potential for nitrogen fixation in maize genotypes in Brazil. *Proc. Nat. Acad. Sci.* 72: 2389-2393.
- Burris, R. H. 1976. Non-leguminous N_2 -fixing system. A synthesis paper. II. *Intl. Symp. on Nitrogen Fixation*. Salamanca, Spain, 1976.
- Campbell, R. and A. D. Rovira. 1973. The study of the rhizosphere by scanning electron microscopy. *Soil Biol. Biochem.* 5: 747-752.
- Carlson, R., C. Napoli and P. Albersheim. 1977. Chemical and immunological studies of the lipopolysaccharide of *Rhizobium*. *Proc. Sixth Am. Rhizobium Conference*. August 29 - September 2, 1977. Gainesville, Florida.
- Chollet, R. and W. L. Ogren. 1975. Regulation of photorespiration in C_3 and C_4 species. *The Bot. Rev.* 41: 137-179.
- Cooke, R. D., C. E. M. Ferber and L. Kanagasabapathy. 1976. Purification and characterization of polygalacturonase from a commercial *Aspergillus niger* preparation. *Biochem. Biophys. Acta.* 452: 440-451.
- Dart, P. J. and F. V. Mercer. 1964. The legume rhizosphere. *Archiv. Mikrobiol.* 47: 344-378.
- Day, J. M., M. C. P. Neves and J. Dobereiner. 1975. Nitrogenase activity on the roots of tropical forage grass. *Soil Biol. Biochem.* 7: 107-112.
- Dayan, E., A. Banin and Y. Henis. 1977. Studies on the mucilaginous layer of barley (*Hordeum vulgare*) roots. *Plant Soil* 47: 171-192.
- Dazzo, F. B. and W. J. Brill. 1978. Regulation of fixed nitrogen of host-symbiont recognition in the *Rhizobium*-clover symbiosis. *Plant Physiol.* 62: 18-20.
- Dazzo, F. B. and J. R. Milam. 1976. Serological studies of *Spirillum lipoferum*. *Soil Crop Sci. Soc. Florida* 35: 122-126.
- Dazzo, F. B., C. Napoli and D. H. Hubbell. 1976. Adsorption of bacteria to root as related to host specificity in the *Rhizobium*-clover symbiosis. *Appl. Environ. Microbiol.* 32: 166-171.
- De-Polli, H., E. Matsui, J. Dobereiner and E. Salati, 1977. Confirmation of nitrogen fixation in two tropical grasses by ^{15}N incorporation. *Soil Biol. Biochem.* 119-123.

- Dingle, J., W. W. Reid and G. L. Solomon. 1953. The enzymatic degradation of pectin and other polysaccharides. II. Application of the "cup plate" assay to the estimation of enzyme. J. Sci. Food Agric. 3-4: 149-155.
- Doak, K. D. and P. R. Miller. 1968. Influence of mineral nutrition on pigmentation in sorghum. Agron. J. 60: 430-432.
- Dobereiner, J. 1968. Non-symbiotic nitrogen fixation in tropical soils. Perg. Agropic. Bras. 3: 1-6.
- Dobereiner, J. 1974. Nitrogen-fixing bacteria in the rhizosphere. In: Quispel, A. (ed.). The Biology of Nitrogen Fixation. North Holland Pub. Co. Amsterdam. pp. 86-120.
- Dobereiner, J. 1978. Influence of environmental factors in the occurrence of *Spirillum lipoferum* in the soil and roots. In: Granhall, U. (ed.). Environmental Role of Nitrogen-fixing Blue-green Algae and Symbiotic Bacteria. Ecol. Bull. IFR (Stockholm) 26: 343-351.
- Dobereiner, J. and J. M. Day. 1974. Associative symbiosis in tropical grasses: Characterization of microorganisms and dinitrogen fixing sites. Intl. Symp. on N_2 Fixation. Interdisciplinary Discussions. June, 1974. Washington.
- Dobereiner, J. and J. M. Day. 1975. Nitrogen fixation in the rhizosphere of tropical grasses. In: Stewart, W. D. (ed.). Nitrogen Fixation by Free-living Microorganisms. Cambridge University Press. Cambridge.
- Dobereiner, J., J. M. Day and P. J. Dart. 1972a. Nitrogenase activity of *Paspalum notatum*-*Azotobacter paspali* association. J. Gen. Microbiol. 71: 103-116.
- Dobereiner, J., J. M. Day and P. J. Dart. 1972b. Nitrogenase activity in the rhizosphere of sugar cane and some other tropical grasses. Plant Soil 37: 191-196.
- Dobereiner, J., M. Nery and I. E. Marriel. 1976. Ecological distribution of *Spirillum lipoferum* Beijerinck. Can. J. Microbiol. 22: 1464-1473.
- Eira, P. A. de. 1977. Nitrogen transformation in soils under *Digitaria decumbens* vegetation. Intl. Symp. on the Limitations and Potentials of Biological Nitrogen Fixation in the Tropics. Universidad de Brasília, Brazil, 1977.
- Esau, K. 1977. Anatomy of Seed Plants. J. Wiley and Sons. N. Y. 376 pp.

- Eskew, D. L., D. D. Focht and I. P. Ting. 1977. Nitrogen fixation, denitrification and pleomorphic growth in a highly pigmented *Spirillum lipoferum*. Appl. Environ. Microbiol. 34: 582-585.
- Evans, H. J. and L. E. Barber. 1977. Biological nitrogen fixation for food and fiber production. Science 197: 332-339.
- Fahraeus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. 16: 374-381.
- Fisher, M. L., A. J. Anderson and P. Albersheim. 1973. Host-pathogen interaction. VI. A simple plant protein efficiently inhibits endopolygalacturonase secreted by *Colletotrichum lindemuthianum* and *Aspergillus niger*. Plant Physiol. 51: 489-491.
- Foster, R. C. and A. D. Rovira. 1973. The rhizosphere of wheat studied by electron microscopy of ultrathin sections. Bull. Ecol. HFR (Stockholm) 17: 83-102.
- Foster, R. C. and A. D. Rovira. 1976. Ultrastructure of wheat rhizosphere. New Phytol. 76: 343-352.
- Fred, E. B., I. Baldwin and E. McCoy. 1932. The Root Nodule Bacteria and Leguminous Plants. University of Wisconsin Press. Madison. 342 pp.
- Gaskins, M. H. and J. L. Carter. 1976. Nitrogenase activity: A review and evaluation of assay methods. Soil Crop Sci. Soc. Florida 35: 10-15.
- Gaskins, M. H., M. U. Garcia, T. M. Tien and D. H. Hubbell. 1977. Nitrogen fixation and growth substance production by *Spirillum lipoferum*, and their effects on plant growth. Plant Physiol. Supp. 59: 128.
- Graham, P. H. and J. Halliday. 1977. Inoculation and nitrogen fixation in the genus *Phaseolus*. In: Vincent, J. M. (ed.). Exploring the Legume-*Rhizobium* Symbiosis in Tropical Agriculture. Symp. Proc. University of Hawaii Press.
- Greaves, M. P. and J. F. Darbyshire. 1972. The ultrastructure of the mucilaginous layer of plant roots. Soil Biol. Biochem. 4: 443-449.
- Guchert, A., H. Breisch and O. Reisinger. 1975. Interface sol-racine. I. Etude au microscope electronique mucigel-argile microorganismes. Soil Biol. Biochem. 7: 241.

- Hall, J. L., T. J. Flowers and R. M. Roberts. 1976. Plant Cell Structure and Metabolism. Longman. N. Y. 426 pp.
- Hankin, L., M. Zucker and D. C. Sands. 1971. Improved soil medium for the detection and enumeration of pectolytic bacteria. Appl. Microbiol. 2: 205-209.
- Hardy, R. W. F. and U. D. Havelka. 1976. Photosynthate as a major factor limiting nitrogen fixation by field grown legumes with emphasis on soybeans. In: Nutman, P. S. (ed.). Symbiotic Nitrogen Fixation in Plants. Cambridge University Press. Cambridge.
- Hubbell, D. H. 1976. Plant roots and biological nitrogen fixation. Soil Crop Sci. Soc. Florida 36: 37-40.
- Jenny, H. and K. Grossenbacher. 1963. Root-soil boundary zones as seen in the electron microscope. Soil Sci. Soc. Proc. 27: 273-277.
- Juniper, B. E. and R. M. Roberts. 1966. Polysaccharide synthesis and the fine structure of root cells. Res. Microsc. Soc. 85: 63-72.
- Kass, D. L., M. Drossdoff and M. Alexander. 1971. Nitrogen fixation by *Azotobacter paspali* in association with bahia grass (*Paspalum notatum*). Soil Sci. Soc. Am. Proc. 35: 286-289.
- Keleti, G. and W. H. Lederer. 1974. Handbook of Micromethods for the Biological Sciences. Van Nostrand Reinhold Co. N. Y. 166 pp.
- Krieg, N. R. 1977. Taxonomic studies of *Spirillum lipoferum*. Basic Life Science 9: 463-472.
- Lakshmi, V., A. Satyanarayana Rao, K. Vijayalakshmi, M. L. Kumari, K. V. B. R. Tilak and N. S. Subba-Rao. 1977. Establishment and survival of *Spirillum lipoferum*. Proc. Ind. Acad. Sci. 86B: 397-404.
- Lisker, N. and N. Retig. 1974. Detection of polygalacturonase and pectin lyase isoenzyme in polyacrylamide gel. Chrom. 7522: 245-249.
- Lowry, O. H., N. J. Rosebrough, A. L. Fair and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193: 265-275.
- Luft, J. H. 1961. Improvements of epoxy resin methods. J. Biophys. Biochim. Cytol. 9: 407-414.

- Lund, B. M. and L. W. Mapson. 1971. Stimulation by *Erwinia carotovora* of the synthesis of ethylene in cauliflower tissue. *Biochem. J.* 119: 251-263.
- Marriel, I. E. and J. C. Cruz. 1977. Increased N_2 -fixation (C_2H_2 reduction) in field grown maize by herbicide treatment. *Intl. Symp. on the Limitations and Potentials of Biological Nitrogen Fixation in the Tropics*. Universidad de Brasilia. Brazil, 1977.
- McCoy, E. 1932. Infection of *Bacterium radiclecola* in relation to the microchemistry of the hosts' cell wall. *Proc. Roy. Soc. (Lond.). B. Biol. Sci.* 110: 510-533.
- Mollenhauer, H. H. and D. J. Morre'. 1966. Golgi apparatus and plant secretion. *Ann. Rev. Plant Physiol.* 17: 27-46.
- Morre'. D. and H. Mollenhauer. 1967. Golgi apparatus mediated polysaccharide secretion by outer root cap cells of *Zea mays* L: Kinetics and secretory pathway. *Planta* 74: 286.
- Mussell, H. L. and L. Strand. 1976. Pectic enzymes: Involvement and pathogenesis and possible relevance to tolerance and specificity. In: Solheim, B. and J. Raa (eds.). *Host-Pathogen Interaction*. Universitetsforlagen. Norway. 487 pp.
- Napoli, C., F. B. Dazzo and D. H. Hubbell. 1975. Production of cellulose microfibrils by *Rhizobium*. *Appl. Microbiol.* 30: 123-132.
- Nery, M., F. T. V. Abrantes, O. dos Santos and J. Dobereiner. 1977. Nitrogen fixation (C_2H_2 reduction) in wheat. *Intl. Symp. on the Limitations and Potentials of Biological Nitrogen Fixation in the Tropics*. Universidad de Brasilia. Brazil, 1977.
- Neyra, C. A. and J. Dobereiner. 1977. Nitrogen fixation in grasses. *Advances in Agronomy* 29: 1-38.
- Neyra, C. A., J. Dobereiner, R. Lalande and R. Knowles. 1977. Denitrification by N_2 -fixing *Spirillum lipoferum*. *Can. J. Microbiol.* 23: 300-305.
- Northcote, D. H. and J. D. Pickett-Heaps. 1966. A function of the Golgi apparatus in polysaccharide synthesis and transport in the root cap cells of wheat. *Biochem. J.* 98: 159-167.

- Okon, Y., S. L. Albrecht and R. H. Burris. 1976a. Factors affecting growth and nitrogen fixation of *Spirillum lipoferum*. J. Bacteriol. 127: 1248-1254.
- Okon, Y., S. L. Albrecht and R. H. Burris. 1976b. Carbon and ammonia metabolism of *Spirillum lipoferum*. J. Bacteriol. 128: 592-597.
- Okon, Y., S. L. Albrecht and R. H. Burris. 1977. Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl. Environ. Microbiol. 33: 85-87.
- Old, K. M. and T. H. Nicholson. 1975. Electron microscopy of the microbial colonization of roots of sand dune grasses. New Phytol. 74: 51-58.
- Page, W. S. and H. L. Sadoff. 1975. Relationship between calcium and uronic acids in the encystment of *Azotobacter vinelandii*. J. Bacteriol. 122: 145-151.
- Pate, J. L. and E. J. Ordal. 1967. Fine structure of *Chondrococcus columbaris*. III. The surface layers of *Chondrococcus columbaris*. J. Cell Biol. 35: 37-51.
- Patriquin, D. G. and J. Dobereiner. 1977. Bacteria in the endorhizosphere of maize in Brazil. Intl. Symp. on the Limitations and Potentials of Biological Nitrogen Fixation in the Tropics. Universidad de Brasilia. Brazil, 1977.
- Patriquin, D. G., F. M. M. Magalhaes, C. A. Scott and J. Dobereiner. 1978. Infection of field grown maize in Rio de Janeiro by *Azospirillum*. Steenbock-Kettering Intl. Symp. on Nitrogen Fixation. University of Wisconsin-Madison. June, 1978.
- Pedersen, W. L., K. Chakrabarty, R. V. Klucas and A. K. Vidaver. 1978. Nitrogen fixation (C_2H_2 reduction) associated with roots of winter wheat and sorghum in Nebraska. Appl. Environ. Microbiol. 35: 129-135.
- Pereira, P. A., C. A. Neyra and J. Dobereiner. 1977. Nitrogenase activity, assimilation of NO_3^- and denitrification in *Brachiaria* cores. Intl. Symp. on the Limitations and Potentials of Biological Nitrogen Fixation in the Tropics. Universidad de Brasilia. Brazil, 1977.
- Reynolds, E. S. 1963. The use of lead citrate at high pH as electron opaque stain for electron microscopy. J. Cell Biol. 17: 208-212.
- Rovira, A. D. 1969. Plant root exudates. Bot. Rev. 35: 35-59.

- Rovira, A. D. and R. C. Campbell. 1974. Scanning electron microscopy of microorganisms on the roots of wheat. *Microbial. Ecol.* 1: 15-23.
- Ruschel, A. P., Y. Henis and E. Salati. 1975. Nitrogen-15 tracing of N_2 fixation with soil grown sugar cane seedlings. *Soil Biol. Biochem.* 7: 181-182.
- Sampaio, M. J. A., L. de Vasconcelos and J. Dobereiner. 1978. Characterization of three groups of *Spirillum lipoferum* Beijerinck. In: Granhall, U. (ed.). *Environmental Role of N_2 -fixing Blue-green Algae and Asymbiotic Bacteria.* *Ecol. Bull. NFR (Stockholm)* 26: 364-365.
- Schroder, M. 1932. Die assimilation des lufstickstoffs durch einige bakterien. *Zentralbl. Bacteriol. Parasitknde.* 85: 178-212.
- Schroth, M. N. and D. C. Hildebrand. 1964. Influence of plant exudates on root infecting fungi. *Ann. Rev. Phytopath.* 2: 101-132.
- Scott, C. A., F. M. M. Magalhaes, V. L. dos Santos Divan and D. B. Scott. 1977. Numbers of *Azospirillum* spp. associated with the roots of field grown maize. *Intl. Symp. on the Limitations and Potentials of the Biological Nitrogen Fixation in the Tropics.* Universidad de Brasilia, Brazil, 1977.
- Sing, V. O. and M. N. Schroth. 1977. Bacteria-plant surface interactions: Active immobilization of saprophytic bacteria in plant leaves. *Science* 197: 759-761.
- Slankis, V. 1973. Hormonal relationships in mycorrhizal development. In: Marks, G. C. and T. Kozlowski (eds.). *Ectomycorrhizae: Their Ecology and Physiology.* Acad. Press. N. Y. p. 231-298.
- Sloger, C. and L. D. Owens. 1976. Nitrogen fixation by a temperate corn-*Spirillum* association. *Intl. Symp. on N_2 Fixation.* Salamanca, Spain, 1976.
- Smith, R. L., J. H. Bouton, S. C. Schank, K. H. Quesenberry, M. E. Tyler, J. R. Milam, M. H. Gaskins and R. C. Littell. 1976. Nitrogen fixation in grasses inoculated with *Spirillum lipoferum*. *Science* 193: 1003-1005.
- Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruc. Res.* 26: 31-43.
- Stephen, G. J. and R. K. S. Wood. 1974. Release of enzymes from cell walls by an endopeptidase. *Nature* 251: 358.

- Strand, L. I., C. Rechtoris and H. Mussell. 1976. Polygalacturonase release cell wall-bound proteins. *Plant Physiol.* 58: 722-725.
- Streeter, J. G. and M. E. Bosler. 1976. Carbohydrates in soybean nodules: Identification of compounds and possible relationships to nitrogen fixation. *Plant Sci. Letters* 7: 321-329.
- Subba-Rao, N. S., K. V. B. R. Tilak, M. L. Kumari and C. S. Singh. 1978. Response of crops to *Spirillum lipoferum* inoculation. Steenbock-Kettering Intl. Symp. on Nitrogen Fixation. University of Wisconsin-Madison. June, 1978.
- Tarrand, J. J., N. R. Krieg and J. Dobereiner. 1978. Taxonomic study of *Spirillum lipoferum* group, with description of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can. J. Microbiol.* 24: 967-980.
- Taylor, G. S. and D. Parkinson. 1961. Growth of saprophytic fungi on root surfaces. *Plant Soil* 15: 261-267.
- Tien, T. M., M. H. Gaskins, D. H. Hubbell and M. Umali-Garcia. 1978. Why does inoculation with *Azospirillum brasilense* (*Spirillum lipoferum*) increase yield of grasses. Steenbock-Kettering Intl. Symp. on Nitrogen Fixation. University of Wisconsin-Madison. June, 1978.
- Tinker, P. B. H. and F. E. Sanders. 1975. Rhizosphere microorganisms and plant nutrition. *Soil Sci.* 119: 363-368.
- Tjepkema, J. and P. van Berkum. 1977. Acetylene reduction by soil cores of maize and sorghum in Brazil. *Appl. Environ. Microbiol.* 33: 626-629.
- Tu, J. C. 1976. Cytoplasmic changes during and after infection of soybean nodule cells with rhizobia. *Phytopath.* 66: 1065-1071.
- Tyler, M. E., J. R. Milam and D. A. Zuberer. 1977. Taxonomy of the diazotroph of tropical grasses called *Spirillum lipoferum*. Intl. Symp. on the Limitations and Potentials of Biological Nitrogen Fixation in the Tropics. Universidad de Brasilia. Brazil, 1977.
- Umali-Garcia, M., D. H. Hubbell and M. H. Gaskins. 1978. Process of infection of *Panicum maximum* by *Spirillum lipoferum*. In: Granhall, U. (ed.). *Environmental Role of Nitrogen-Fixing Blue-green Algae and Asymbiotic Bacteria*. *Ecol. Bull. NFR* (Stockholm) 26: 373-379.

- Wheeler, C. T. and A. C. Lawrie. 1976. Nitrogen fixation in root nodules of alder and pea in relation to the supply of photosynthate assimilates. In: Nutman, P. S. (ed.). *Symbiotic Nitrogen Fixation in Plants*. Cambridge University Press. Cambridge. 584 pp.
- Yoshida, T. and R. R. Ancajas. 1971. Nitrogen fixation by bacteria in the root zone of rice. *Proc. Soil Sci. Soc. Am.* 35: 156-157.
- Yoshida, T. and R. R. Ancajas. 1973. The fixation of atmospheric nitrogen in the rice rhizosphere. *Soil Biol. Biochem.* 5: 153-155.

BIOGRAPHICAL SKETCH

Mercedes U. Garcia (née Mercedes M. Umali) was born to Lorenza C. Maligaya and Jose C. Umali in September 24, 1936. She finished her elementary education in her home town, Calaca, Batangas, Philippines, in 1951 and her secondary education in the City of Manila in 1955. She obtained her B. S. in Agriculture degree (Honors' Curriculum), major in Plant Breeding, from the University of Philippines at Los Banos in 1960. She was awarded a Graduate Research Fellowship in 1962 - 1964 which enabled her to finish the requirements for an M. S. in Forest Genetics. She has been employed at the University of the Philippines since 1960, and currently holds a faculty position in the same institution. She is married to Benjamin M. Garcia, Sr., with whom she has six children, namely, Vener, Benjie, Mina, Joven, Ruben and Bien.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David H. Hubbell, Chairman
Professor of Soil Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Murray H. Gaskins
Professor of Agronomy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Rex L. Smith
Associate Professor of Agronomy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stanley C. Schank
Professor of Agronomy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Indra K. Vasil
Professor of Botany
Chairman of Department of Botany

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1978

Dean, College of Agriculture

Dean, Graduate School