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FLUORESCENT ANTIBODY TECHNIQUE TO IDENTIFY *AZOSPIRILLUM BRASILENSE* ASSOCIATED WITH ROOTS OF GRASSES

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Summary—Bacteria associated with roots of grasses from Florida, Ecuador and Venezuela were isolated and their N_2 -fixing ability was demonstrated by C_2H_2 reduction assay. The bacterial isolates have been classified as *Azospirillum brasilense* (formerly *Spirillum lipoferum*). These N_2 -fixing isolates have been compared with several Brazilian strains. Fluorescent antibody (FA) techniques were used to assist identifying isolates of N_2 -fixing bacteria from grass roots. Tests with antisera prepared against four strains of *Azospirillum* were used to define serological groups. Antigen-antibody specificity was demonstrated using both *Azotobacter* and *Azospirillum* antisera against known species of other soil microorganisms and numerous unidentified soil bacteria. Several applications of the FA technique are suggested to identify N_2 -fixing bacteria associated with grass roots.

INTRODUCTION

Recent work has shown that various grasses have a loose association between their roots and certain N_2 -fixing bacteria (Dobereiner *et al.*, 1972; Dobereiner and Day, 1976; Diem *et al.*, 1978). These grass-bacterial systems have been termed associative symbioses to distinguish them from the better known nodule-forming, legume symbioses (Evans and Barber, 1977). Apart from the study of *Beijerinckia* on rice roots by Diem *et al.* (1978) few of the organisms fixing N_2 in the rhizospheres of grasses have been identified by immunofluorescent techniques.

Pioneer work (Coors *et al.*, 1942) on the fluorescent antibody (FA) technique showed that it was possible to identify individual bacterial cells. Applications to microbial ecology were attempted (Hobson and Mann, 1957) with extensive work by Schmidt and his colleagues (Schmidt and Bankole, 1962; Schmidt *et al.*, 1968; Schmidt, 1973; Schmidt, 1974; Rennie and Schmidt, 1977). Work on anaerobic habitats has shown an additional application of direct counts of FA-reacting bacteria to the ecology of lake sediments (Strayer and Tiedje, 1978). The FA technique must be very carefully used, employing appropriate controls to assure that false readings are not made and Kawamura (1977), and Schmidt (1973) list the controls needed for application of FA techniques to ecological studies of microorganisms. Diem *et al.* (1978) encountered some difficulties with autofluorescence of grass roots.

Cytological studies to ascertain the site of infection of *Azospirillum* in "Transvala" digitgrass roots were

attempted with 2,3,5-triphenyltetrazolium chloride which produced bright-red formazan crystals in the cortical cells (Dobereiner and Day 1976). Later cytological examination of field grown tropical maize roots in Brazil reported the establishment of bacteria within roots (Patriquin and Dobereiner, 1978).

Serological tests were examined for possible use in the detection and specific identification of *Spirillum lipoferum* (Dazzo and Milam, 1976) now known as *Azospirillum brasilense* (Tarrand *et al.*, 1978; M. E. Tyler, unpublished).

At the University of Florida, roots of tropical grasses have been inoculated with liquid cultures of *A. brasilense* and yield increases of grain and forage grasses have been obtained in the inoculated plots (Smith *et al.*, 1978). Both specific and non-specific staining techniques for *Azospirillum* have been tested. We describe the preparation of antisera, tests for antisera specificity using known bacterial cultures and unknown soil bacteria, plant-bacterial colonization sites seen in root cross sections and on whole root segments viewed when using epifluorescence.

MATERIALS AND METHODS

The indirect fluorescent antibody (FA) technique was used. This technique involves two binding reactions: in the first, rabbit anti-bacterial immunoglobulins (Ig) are bound to the bacteria; in the second, anti-rabbit fluorescein-conjugated goat Ig are bound to the rabbit proteins. This two-stage system generally permits a brighter fluorescent label to be attached to the cells than the direct method (Kawamura, 1977). The goat fluorescein-conjugated anti-rabbit Ig (Cappel Laboratories, Inc., Cochranville, PA) supplied in lyophilized form used FITC (fluorescein isothiocyanate) as the label.

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Ig was prepared against *A. brasilense* strains 13t, 5t, 84 and JM125A2, and *Azotobacter paspali* in New Zealand rabbits by multiple injections of a live bacterial cell suspension (Freund's incomplete adjuvant and washed cells 1 l, v, v) suspended in phosphate-buffered physiological saline (pH 7.2) and containing about 10^{10} cells ml^{-1} . The *Azospirillum* cells were grown for 24 h in N-rich tryptic soy broth (Difco) with sodium malate added as an additional C source. *Azotobacter* received sucrose as the C source. Injections were administered, intradermally and in foot-pads. Normal serum was taken by cardiac puncture from each rabbit before injections were begun. Antisera were harvested at 90–120 days when the rabbits were bled by cardiac puncture. Blood was permitted to clot, and the antisera was then separated from the clot. Purification of antisera was accomplished by precipitating three times with ammonium sulfate and desalting on Sephadex column eluted with phosphate-buffered physiological saline (pH 7.2). Titer was measured using the FA system and the Ig diluted to the next lower dilution than that giving an observable reduction in fluorescence with homologous cells.

Cross sections of inoculated roots (in axenic systems and from greenhouse and field experiments) were made using a microtome-cryostat. Sections were treated with toluidine blue stain or FA. Sections for FA analysis were attached to slides using a thin layer of white glue. Fixation in anhydrous acetone for 10 min was followed by a 20 min incubation (37°C) with the primary rabbit Ig and with the secondary antiserum. Concurrent with the second stage incubation, rhodamine-conjugated horse anti-human IgG was also used. Preparations were rinsed between stages and at the end of incubation in phosphate-buffered saline (PBS), pH 7.2. A drop of buffered glycerol was used as a mounting medium and the cover slip was gently lowered over the section. Sections were viewed with a Leitz fluorescent microscope with epi-illumination, at 250 and 1000 \times magnifications. Illumination was provided by a 100W high pressure Hg lamp, using a 500 nm BG 12 primary (exciter) filter and a 522 nm secondary (barrier) filter mounted in a Ploem illuminator (Leitz Corp). Reactions of the fluorescing bacteria were visually rated 0 (none), 1 (dim), 2 (medium bright), 3 (very bright). Control slides included the normal serum from each rabbit which was used in antigen testing. Non-related bacteria were used in testing the FITC labeled antibody. Contact or soil-buried slides were prestained (Bohlool and Schmidt, 1968) with gelatin-rhodamine isothiocyanate conjugate to minimize non specific binding and soil particle autofluorescence. Both axenic and field grown roots were tested.

Controls against non-specific reactions were run by testing bacteria on roots and soil using the secondary goat anti-rabbit antisera, the normal sera collected before immunizing the rabbits, the concurrent addition of the secondary sera and the normal sera. Non-specific reactions in bacteria were not observed, but fungal spores occasionally reacted non-specifically with a 1 or 2 rating.

Control tests against autofluorescence were made by observing bacteria, roots and soil with distilled water replacing each antisera. Autofluorescence of the grass roots was particularly prominent in the stele, but since most of the bacteria were observed in the mucigel area, this autofluorescence was not a serious problem with the grasses studied.

The FA technique was also used on field-grown roots of *Panicum maximum* Jacq breeding line 199, *Pennisetum americanum* (L.) K. Shum cv "Gahi-3", and *Dycteria decumbens* Stent cv "Transvala", as well as sterile roots from the first two species. Root pieces from plants that had been inoculated in the field with either live or autoclaved *Azospirillum* 13t were collected and placed in Karnovsky's fixative (Karnovsky, 1965). In order to facilitate FA staining of whole root segments, incubation steps and rinses were accomplished in small test tubes before mounting the whole root pieces under a coverslip. The root surface was scanned and bacteria were counted without sectioning. Portions of the root showing abundant fluorescing bacteria were then excised and sectioned using the freezing microtome. Photomicrographs were made with Kodak High Speed Ektachrome (ASA 160) or with Anscochrome (ASA 64) film. Black and white plates were prepared from the colored slides.

RESULTS

Antisera

All *Azospirillum* antisera FITC conjugates exhibited fluorescence endpoint titers over 1 000. The corresponding titer of the *Azotobacter* antiserum was extremely low (1/10). For this reason, *Azotobacter* cross-reactivity studies were very limited. Each of the *Azospirillum* antisera, although prepared at different times in different rabbits, had similar cross-reactivity, and therefore the data for several of the antisera were pooled.

Testing of the staining reaction was accomplished by not using the primary antibody, but applying the FITC secondary antibody in a series of tests with related and non-related organisms (Table 1). In addition to checking for autofluorescence, this permitted the verification that the secondary antibody was not

Table 1. Autofluorescence and non-specific binding of goat anti-rabbit FITC conjugate

	Autofluorescence	Non-specific binding
20 Strains of <i>Azospirillum</i>	Neg	Neg
35 Gl- soil isolates	Neg	Neg
4 Gl+ spore formers	Neg	Neg
2 Actinomycetes	Neg	Neg
<i>Pseudomonas aeruginosa</i>	Neg	Neg

Table 2. Cross reactivity of *Azospirillum brasilense* (Str 13t) antibodies (rabbit) with heterologous non-nitrogen fixing microorganisms*

35 Gram negative soil isolates**	Neg.
4 Gram-positive spore formers**	Neg.
2 Actinomycetes**	Neg.
<i>Saccharomyces</i>	Neg.
<i>Pseudomonas aeruginosa</i>	1-2

* Indirect method
 ** Isolates from Arredondo fine sand

attaching non-specifically. These tests were all negative (no fluorescence).

Cross-reactivity tests

The specificity of the primary antisera was tested against various soil organisms (Table 2). These results were all negative. Similar tests done on known diazotrophs were also negative (Table 3). Both 1- and 7-day old cells of pure cultures of *Azotobacter chroococcum*, *Bacillus cereus* and *Pseudomonas aeruginosa* tested against JM 125A2 antiserum gave negative results, as did colonies of 50 unidentified bacteria picked from soil or root culture plates.

When specificity of the sera was tested using various strains within the species *A. brasilense*, there was variable cross-reactivity among strains (Table 4). Both 1- and 7-day old bacterial cells of strains of *A. brasilense* reacted strongly with 13t antiserum. We therefore designated these strains as the 13t serogroup. However, bacterial strains 51e, 75, 84 and 76, also from Brazil, did not give a 3 positive immunofluorescence response, when tested with 13t antisera.

Table 3. Cross reactivity of *Azospirillum brasilense* (Str 13t) antibody (rabbit) against selected diazotrophs*

Organism	Immunofluorescence
<i>Azotobacter paspali</i>	None
<i>Klebsiella pneumoniae</i>	None
<i>Enterobacter cloacae</i>	None
<i>Azospirillum lipoferum</i>	
Strain RG6XX	None
Strain 59bK	None

* Indirect method

Similarly, reactions with *Azospirillum* isolates from Florida, Venezuela and Ecuador were generally nil with the 13t antiserum. Antiserum prepared against a Florida isolate JM 125A2, was highly strain specific (Table 4) while antisera to strains 51e and 84 gave some cross-reactions with isolates from Florida, Venezuela and Ecuador.

Additional verification of strain specificity was done by mixing equal proportions of *A. brasilense*, strains 13t and JM 125A2, and observing under fluorescent and phase contrast microscopy. The fluorescing bacterial cells reacted only with their homologous antiserum.

Root sectioning

In these tests, toluidine blue served as a good general stain for bacteria associated with roots. Figure 2 shows the presence of a number of large rod-shaped bacteria in a cortical cell (refer to Fig. 1). However, since toluidine blue staining is not specific, further

Table 4. Characterization and cross reactivity of four types of *Azospirillum* antisera on *Azospirillum* strains from Florida, Venezuela and Brazil

Organism	Origin	Antisera tested* and age of culture (days)							
		13t		51e		SP84		125A2	
		1	7	1	7	1	7	1	7
13t	Brazil	3	3	1	2	0	1	1	1
SP7	Brazil	2	3	0	0	0	0	0	0
51e	Brazil	1	2	3	3	0	0	0	2
51ek	Brazil	3	3	3	3	0	0	2	3
SPL-1	Brazil	2	3	0	0	1	1	0	0
ATCC7	Brazil	3	3	nd	1	nd	0	nd	1
T60K	Brazil	2	3	1	0	0	0	1	1
4K	Brazil	1	3	1	3	0	0	1	2
75K	Brazil	0	0	1	3	0	0	0	2
SP84	Brazil	0	0	0	1	3	3	0	3
SP76	Brazil	0	0	0	0	0	0	0	0
119A4	Florida	0	0	0	0	0	1	0	1
125A2	Florida	0	0	1	0	1	3	3	3
82A'	Venezuela	0	0	0	1	1	3	1	2
52b	Venezuela	0	0	0	2	0	0	0	1
75A1	Venezuela	0	0	0	0	0	0	0	1
75C3	Venezuela	0	0	2	3	0	0	0	0
75C2B	Venezuela	0	1	0	3	0	0	0	2
24B4	Ecuador	0	0	2	3	0	2	0	2
28A2	Ecuador	0	0	0	0	0	0	0	0
6A2	Ecuador	0	0	0	2	0	0	0	0
6B2	Ecuador	0	2	0	2	0	2	0	0

* Visual ratings: 0 (no fluorescence), 1 (dim), 2 (medium bright), 3 (very bright, positive fluorescence)
 nd = not determined

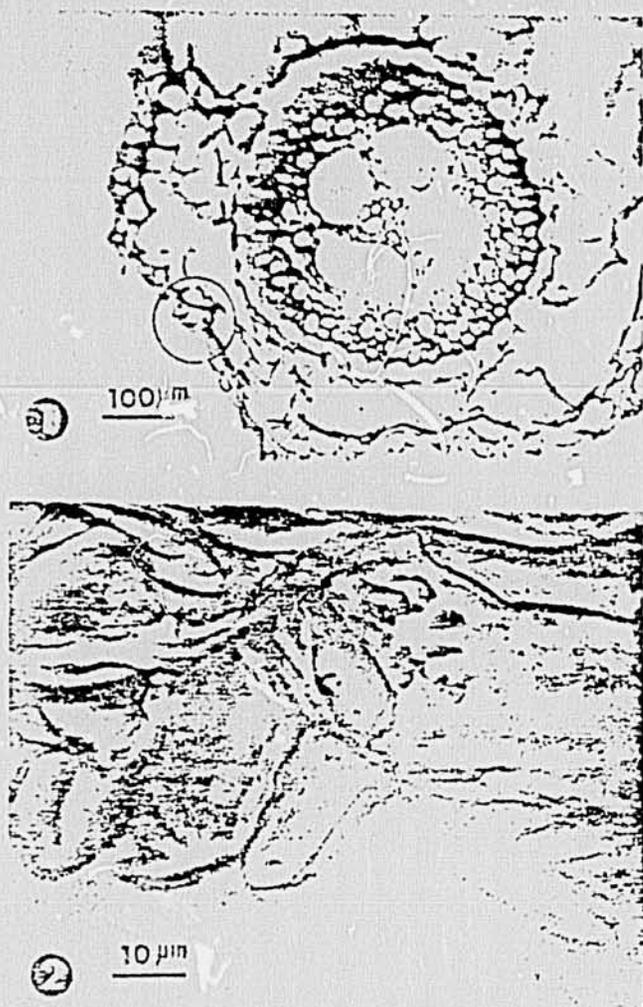


Fig. 1. Transverse section of a sorghum root inoculated with *Azospirillum brasilense* (13t) in axenic system.

Fig. 2. Portion of Fig. 1 at higher magnification. Note large rod-shaped bacteria which appear to be in the cortical cell (arrows). Toluidine blue-0 stained section.

investigation was necessary to locate and identify cells of *Azospirillum*.

The application of the FA technique to roots of buffel-grass (*Cenchrus ciliaris*) which had been inoculated with *A. brasilense* (strain 13t) are shown in Fig. 3. A high level of fluorescence associated with the mucigel layer around the periphery of the root is evident. The bacteria give rise to a bright apple-green fluorescence, while the plant tissue is red-brown due to the Rhodamine application. *Cynodon*, *Cenchrus*, *Digitaria*, and *Pennisetum* show less autofluorescence than *Paspalum* and *Panicum* species. Roots from the sterile plants, when dipped into 13t JM 125A2 bacterial inoculum for 1 min showed positive bacterial staining only with their homologous antiserum. Most of our work has been done on the tropical grasses *Cynodon dactylon*, *Pennisetum americanum* and *Panicum maximum* using either sterilized soil in axenic systems or field soil conditions.

Unsterilized field soils were also tested using contact slides treated with FA. A few fungal spores were observed to fluoresce, but morphological characteristics such as their size, round shape and thicker cell walls prevented confusing them with *A. brasilense*. Fluorescent bacteria were always observed on the contact slides from the inoculated axenic soils, and none on the contact slides from sterile soils. Contact slides were observed 1, 7 and 21 days after inoculation. Root segments from the inoculated plant sterile soil systems also had large numbers of fluorescing *A. brasilense* whereas the aseptic plant-soil systems had none.

Fluorescing *Azospirillum* cells were primarily observed in the mucigel layer, but also occurred in cortical cells of the field-grown roots. Figure 4 shows a cryostat-sectioned root of *Cynodon dactylon* treated with FA (13t antiserum), at the same magnification as the FA-treated bacteria in pure culture (Fig. 5).

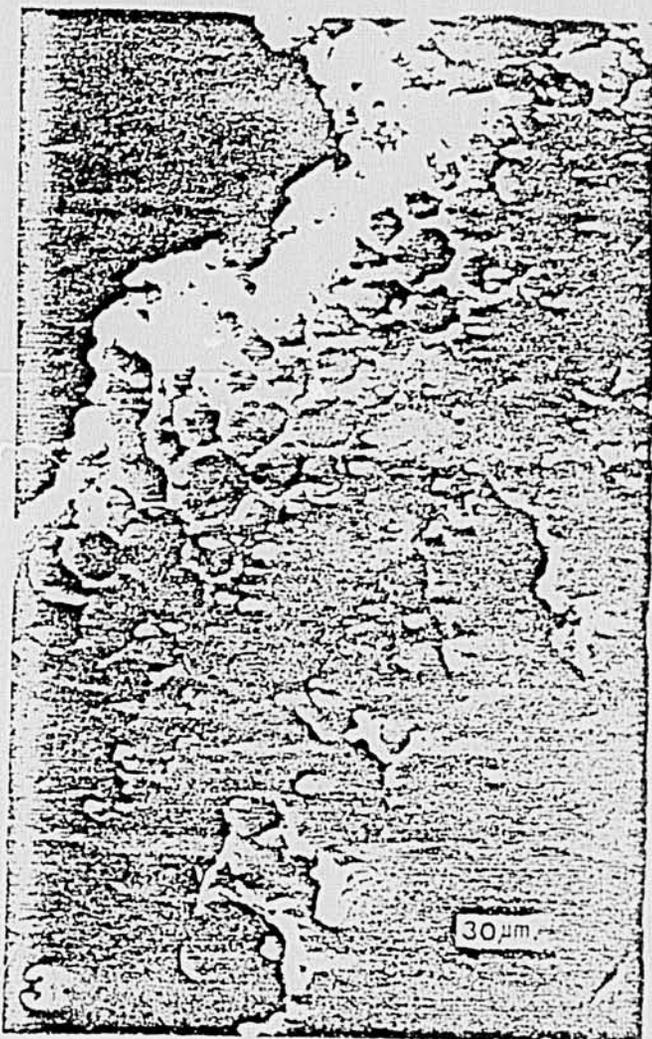


Fig. 3. Cross-section of a buffelgrass root which had been inoculated with *Azospirillum brasilense*, and stained with FA.

A. brasilense from field grown plants has been isolated on selective media and their colonies treated with FA. They reacted similarly to the homologous-inoculated strain. Control slides using the goat anti-rabbit FITC conjugate alone, did not react if the primary antibody was not applied. The specificity of the two types of *Azospirillum* antisera was demonstrated by concurrently, or individually testing the system in the axenic systems described above.

Direct observation of whole roots segments with FA

Roots from three different grass species which had been inoculated with *A. brasilense* strains 131, JM 125A2 or both, were assayed at the end of the summer, 1977, prior to harvest of the plants (Table 4). Encouraged by bacterial counting methods reported (Royra *et al.*, 1975), survival studies (Gray, 1976) and by other recent immunofluorescence work (Rennie and Schmidt, 1977) counts of *Azospirillum* on the root surfaces of *P. maximum*, *P. americanum* and *D.*

decumbens were attempted. Root samples were collected before and after C_2H_2 reduction, to measure increases of *Azospirillum* during the 24h incubation with C_2H_2 . Photographs of whole root segments are given in Figs 6 and 7. *A. brasilense* bacteria were clearly seen embedded in the mucigel, particularly in grooves of the root. Certain areas of the root were "highly infected" with *A. brasilense* as is shown in Figs 6 and 7. Figure 8, as an insert, shows an FA-stained preparation of the pure culture at the same magnification.

Table 5 summarizes the FA data and shows that *P. maximum* had a significantly higher number of *A. brasilense*, strain 131 sero-group than strain JM 125A2. These guineagrass plots were inoculated with 131 inoculum for 3 consecutive years. The Florida isolate, JM 125A2, was isolated from an adjacent field (never included), and thus it represents a native population of *Azospirillum*. The *P. americanum* and *D. decumbens* plots were inoculated in the summer of

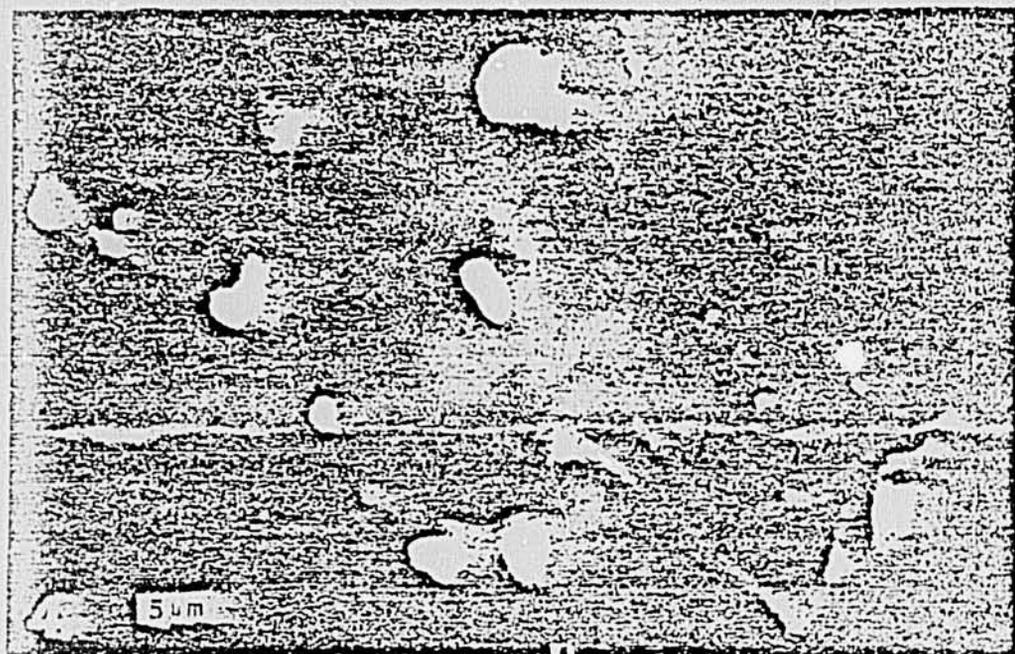


Fig. 4. Cortical cell of a grass root (*Cynodon dactylon*) showing fluorescing bacteria (13t).

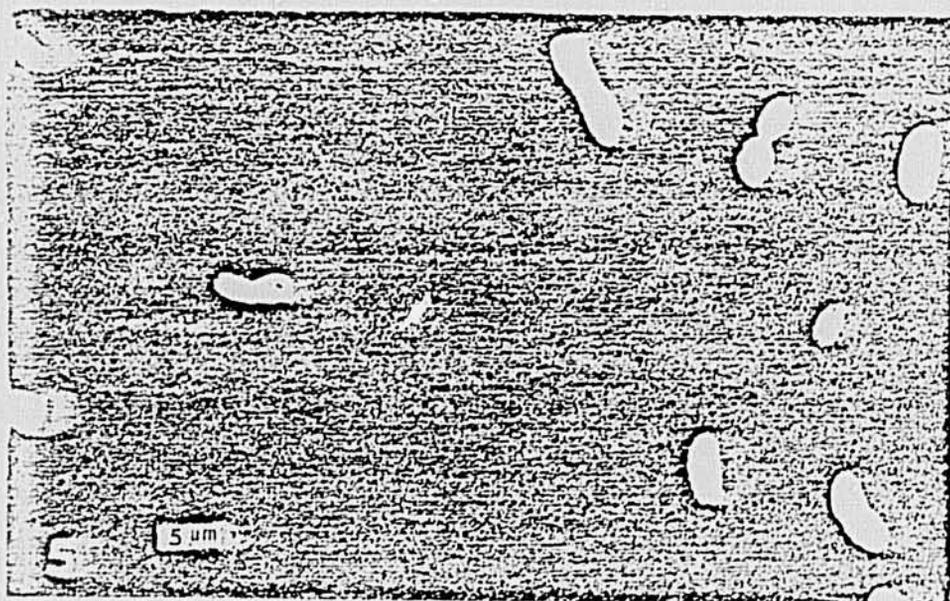


Fig. 5. Specific fluorescence of 13t bacterial cells using FA.

1977 with equal portions of strains 13t and JM 125A2.

In all cases, the bacterial populations of *A. brasilense* increased on the root surfaces during the 24 h incubation in standard C_2H_2 reduction assays. The increase is 2× to 3× as determined by direct microscopic counting by the FA technique. The physiologi-

cal factors affecting bacteria colonizing roots are important and the growth and development of various groups and types of bacteria have been found to be selective (Macura, 1967). Using a non-specific fluorescent stain (auramine O), 13t and JM 125A2 antisera on similar root sections, we observed an overwhelming preponderance of non-*Azospirillum* bacterial types



Figs 6 and 7. Direct observation of whole root segments of *P. maximum* stained with FA. Note bacteria in mucilage area.

Fig. 5 (inset). Pure culture of *A. brasilense* strain 131 stained with 131 antiserum.

on the root surfaces. Approximately 99% of the bacteria in these longitudinal sections did not belong to either of the two serogroups tested. This indicates that a wide diversity of bacteria other than *Azospirillum* are competing for energy sources at the root surface.

DISCUSSION

Since immunofluorescence techniques have not

been reported previously on the interactions of *Azospirillum* in the rhizosphere of grass roots, it was necessary to test a large number of heterologous organisms in order to ensure that our antisera was specific for *A. brasilense*. After observing that the Brazilian strain (131) was serologically different from the Florida strain (JM125A2), we proceeded to test the system by dipping sterile grass roots into inoculum

Table 5. Bacterial counts by direct observation of whole root segments using FA*

	Before Incubation		After Incubation	
	131	JM125A2	131	JM125A2
<i>Panicum maximum</i>				
Guineagrass†	9.01	5.48	15.10	7.48
<i>Pennisetum americanum</i>				
Grati 3 Pearl millet‡	0.35	0.27	0.76	1.17
<i>Digitaria decumbens</i>				
Transvala digitgrass§	1.31	2.70	2.91	3.83

* Mean number of fluorescing bacteria within a 59 μm² area. Twenty-five subsamples were scored for each value.

† Inoculated in 1975-76 with 131.

‡ Inoculated in 1977 with both 131 and JM125A2.

§ Inoculated in 1977 with both 131 and JM125A2.

All counts made at end of summer 1977, prior to harvest of the plants.

of 13t or JM 125A2. There are some problems when the root is added to the system. The stellar portion of the root exhibits autofluorescence of a color which closely matches FITC. Also there is some non-specific binding of the goat FITC conjugate to sterile roots. However, the mucigel area is devoid of the strong autofluorescence, and hence the bacteria in this region can be readily seen and counted. The non-specific binding of goat FITC conjugate to root hairs and the autofluorescence of the root are easily distinguished from fluorescing bacteria. Autofluorescence of the rice root was described as a problem by Diem *et al.* (1978), yet their photographs clearly showed fluorescing bacteria in the mucigel area.

Probably, the largest problem encountered using the FA procedure on field-grown roots was the non-random distribution of *A. brasilense* along the root. We followed the sampling suggestions of Rovira *et al.* (1975) and counted bacteria within 25 microscopic fields for each root sample in order to do the statistical analysis. The limitation imposed by this non-random bacterial distribution is one of the time required to accomplish the quantification of bacterial populations.

The FA system reported in this paper has verified the presence of the specific inoculated bacteria in the root zone of the grass plants. It is a workable system despite the large number of controls necessary. In inoculation studies, it is useful to prove that the bacterial strain applied is able to persist in the new environment. Although we showed that both the Brazilian and Floridian strains of *A. brasilense* persisted, the numbers of fluorescing bacteria in the mucigel were lower than expected. The bacteria appeared to be embedded in the mucigel and possibly this gives an advantage in energy transfer between the plant and bacterium, even in this loose association. No distinctive morphological structures were present on the grass root, even when bacteria occurred in large numbers.

Additional studies on population dynamics of *A. brasilense* and other nitrogen-fixing bacteria are necessary to clarify their role in biological nitrogen fixation associated with grasses. Use of a marked strain of 13t (bearing resistance to two antibiotics) coupled with FA tests is currently underway. Localization of bacteria in plant cells using electron microscopy with peroxidase-anti-peroxidase labelling of the bacteria may demonstrate the ultrastructural aspects of these associations.

Future studies will involve the use of lipo-polysaccharide antisera or other techniques which are purported to exhibit greater specificity. We are encouraged with the FA technique and have used it extensively over the past 2 yr to monitor and verify that bacteria applied as inoculum do persist in the soil after inoculation. Use of bacteria collected on membrane filters should also be investigated more thoroughly, but we have preferred the visualization of the specific bacteria directly on the root surface.

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REFERENCES

- COONS A. H., CHREECH H. J., JONES E. N. and BERLINER E. (1942) Demonstration of pneumococcal antigen in tissues by use of fluorescent antibody. *J. Immun.* 45, 159-170.
- DAZZO F. B. and MILAM J. R. (1976) Serological studies of *Spirillum lipoferum*. *Proc. Soil Crop Sci. Soc. Fla.* 35, 121-126.
- DIEM H. G., SCHMIDT R. L. and DOMBERGLIS Y. R. (1978) The use of the fluorescent-antibody technique to study the behaviour of a *Bijerinakia* strain in the rhizosphere and spermosphere of rice. In *Environmental Role N₂-Fixing Blue-Green Algae and Asymbiotic Bacteria* (U. Granhall, Ed.) *Ecol. Bull.* 26, 312-338.
- DOBREINER J., DAY J. M. and DART P. J. (1972) Nitrogenase activity and oxygen sensitivity of the *Paspalum notatum*-*Azotobacter paspali* association. *J. gen. Microbiol.* 71, 103-116.
- DOBREINER J. and DAY J. M. (1976) Associative symbiosis in tropical grasses. Characterization of micro-organisms and di-nitrogen fixing sites. In *Proceedings First International Symposium on Nitrogen Fixation* (W. E. Newton and C. J. Nyman, Eds), Vol. 2, pp. 518-533. University of Washington Press, Pullman.
- EVANS H. J. and BARBER L. E. (1977) Biological nitrogen fixation for food and fiber production. *Science* 197, 332-339.
- GRAY T. R. G. (1976) The survival of vegetative microbes in soil. In *The Survival of Vegetative Microbes* (T. R. G. Gray and J. R. Postgate, Eds), pp. 327-364. Cambridge University Press.
- HOBSON P. N. and MANN S. O. (1957) Some studies on the identification of rumen bacteria with fluorescent antibody. *J. gen. Microbiol.* 16, 463-471.
- KARNOVSKY M. J. (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electronic microscopy. *J. gen. Microbiol.* 16, 137A.
- KAWANURA AKIYOSHI (Ed.) (1977) *Fluorescent Antibody Techniques and Their Applications*, 2nd edn. University of Tokyo Press.
- MACLEA J. (1957) Physiological studies of rhizosphere bacteria. In *The Ecology of Soil Bacteria* (T. R. G. Gray and D. Parkinson, Eds), pp. 379-395. Liverpool University Press, Liverpool.
- PATRIQUIN D. G. and DOBREINER J. (1976) Bacteria in the endo-rhizosphere of maize in Brazil. In *International Symposium on Limitations and Potentials for Biological N₂-Fixation in the Tropics* (J. Dobreiner, R. Burris and A. Hollaender, Eds), p. 349. University of Brasilia, Brasilia.
- RENNIE R. J. and SCHMIDT E. L. (1977) Immunofluorescence studies of *Nitrobacter* populations in soils. *Can. J. Microbiol.* 23, 1011-1017.
- ROVIRA A. D., NEWMAN E. I., BOWEN H. J. and CAMPBELL R. (1975) Quantitative assessment of the rhizoplane microflora by direct microscopy. *Soil Biol. Biochem.* 6, 211-216.
- SCHMIDT E. L. (1973) Fluorescent antibody techniques for the study of microbial ecology. *Ecol. Bull.* 17, 67-76.
- SCHMIDT E. L. (1974) Quantitative autecological study of microorganisms in soil by immunofluorescence. *Soil Sci.* 118, 141-142.
- SCHMIDT E. L. and BANKOLE R. O. (1962) Detection of *Aspergillus flavus* in soil by immunofluorescent staining. *Science* 136, 776-777.
- SCHMIDT E. L., BANKOLE R. O. and BULLOOL B. B. (1968) Fluorescent-antibody approach to study of *Rhizobium* in soil. *J. Biol.* 95, 1987-1992.
- SMITH R. L., BOLTON J. H., SCHANK S. C. and QUESNBERY K. H. (1977) Yield increases on tropical grain and forage grasses after inoculation with *Spirillum lipoferum*. In *Biological Nitrogen Fixation in Farming Systems of*

- the Humid Tropics*. (A. Ayanaba and P. Dart, Eds), pp 307-311. John Wiley, Chichester.
- SMITH R. L., SCHANK S. C., BOUTON J. H. and QUENSEN-FERRY K. H. (1978) Yield increases of tropical grasses after inoculation with *Spirillum lipoferum*. In *Environmental Role N₂-fixing Blue-Green Algae and Asymbiotic Bacteria* (U. Granhall Ed) *Ecol. Bull.* 26, 380-385.
- SIRAYEH R. F. and TIFUJE J. M. (1978) Application of fluorescent antibody technique to the study of a methanogenic bacterium in lake sediments. *Appl. Envir. Microbiol.* 35, 192-198.
- TARRANT J. J., KRIEG N. R. and DOBEREINER J. (1978) A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beyerinck) comb. nov. and *Azospirillum brasiliense* sp. nov. *Can. J. Microbiol.* 24, 967-980.