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Ecological distribution of *Spirillum lipoferum* Beijerinck[1,2]

J. DOBEREINER, I. E. MARRIEL, AND M. NERY

Empresa Brasileira de Pesquisa Agropecuária, Km 47, via Campo Grande, Rio de Janeiro, 20000, Brazil

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A survey in various countries revealed that the N_2-fixing *Spirillum lipoferum* Beijerinck is a very common root and soil inhabitant in the tropics. More than half of the grass root and soil samples collected in tropical countries (four African countries and Brazil) contained abundant *S. lipoferum* populations, while less than 10% of the samples collected in temperate South Brazil, Kenya, and the U.S.A. contained the organism. There is a pronounced vegetation effect. *Panicum maximum* seems the most favorable among the forage grasses, while few positive samples were found under virgin tropical forest. Legume roots contained less *S. lipoferum* than adjacent soils. More than 80% of the samples from cereal roots (maize, sorghum, wheat, and rye) grown in fields fertilized with PK and Mo, in Rio de Janeiro State, were positive. Maize and sorghum grown under similar conditions in Wisconsin contained less than 10% of positive samples, but when maize fields were inoculated 90% of the root samples contained *S. lipoferum*. Alluvial soils were more favorable than eroded hill soils. Occurrence in soil was strongly pH-dependent with a pH around 7, being optimal (correlation coefficient $r = 0.90$). Sporadic occurrence was observed even in soils with pH 4.8. Surface-sterilized *P. maximum* roots collected from soils with pH ranging from 4.8 to 7.2 contained high *S. lipoferum* numbers which did not correlate with soil pH ($r = 0.41$). Amendment with malate of acid soils was not very effective in increasing nitrogenase (N_2-ase) activity, but in two soils with pH above 6.4, high N_2-ase activity was obtained after 16 to 48 h of incubation. In two soils from a temperate climate region, inoculation with *S. lipoferum* increased N_2-ase activity produced through malate amendment.

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Une étude dans différents pays révèle que l'organisme fixant l'azote *Spirillum lipoferum* Beijerinck., est un habitant très commun des racines et du sol dans les tropiques. Plus de la moitié des échantillons de racines d'herbacées et de sol recueillis dans des pays tropicaux (quatre pays africains et le Brésil) contiennent des populations abondantes de *S. lipoferum*; alors que moins de 10% des échantillons recueillis dans les pays tempérés, le Brésil du Sud, le Kénia et les U.S.A., contient l'organisme. Il y a un effet prononcé de la végétation. *Panicum maximum* semble être la plante fourragère la plus favorable, alors que quelques échantillons positifs ont été trouvés sous les forêts tropicales vierges. Les racines des légumineuses contiennent moins de *S. lipoferum* que les sols adjacents. Plus de 80% des échantillons des racines de céréale (le maïs, le sorgho, le blé et l'orge), qui se développent dans des champs fertilisés avec PK et Mo, dans l'état de Rio de Janeiro, sont positifs. Le maïs et le sorgho qui se développent sous des conditions semblables dans le Wisconsin contiennent moins de 10% d'échantillons positifs, mais lorsque les champs de maïs ont été inoculés, 90% des échantillons des racines contiennent *S. lipoferum*. Les sols alluvios sont plus favorables que les sols des collines dénudées. L'apparition dans le sol est fortement dépendante du pH avec une valeur optimale autour de 7.0 (un coefficient de corrélation $r = 0.90$). Des apparitions sporadiques ont été observées dans des sols à pH 4.8. Des racines stérilisées en surface de *P. maximum*, recueillies dans des sols ayant des pH variant de 4.8 à 7.2, contiennent un grand nombre de *S. lipoferum*, lesquels ne sont pas en corrélation avec le pH du sol ($r = 0.41$). L'amendement des sols acides avec du malate n'a pas été très efficace pour accroître l'activité de la nitrogénase, mais dans deux sols ayant un pH au dessus de 6.4, une forte activité de nitrogénase a été observée après une incubation de 16 à 48 h. Dans deux sols d'une région climatique tempérée, l'inoculation avec *S. lipoferum* accroît l'activité de la nitrogénase produite après amendement avec le malate.

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Introduction

Nitrogen fixation of economic importance in several tropical forage grasses (Balandreau 1975; Dobereiner and Day 1975; Day *et al.* 1975) and some grain crops (Balandreau and Dommergues 1973; Dobereiner *et al.* 1975) has recently been attributed to *Spirillum lipoferum* (Bulow and Dobereiner 1975; Dobereiner and Day 1974), a N_2-fixing bacterium described in 1925 by Beijerinck. Schroder (1932) was unable to confirm N_2 fixation by this organism in pure culture and it was therefore removed from the lists of N_2-fixing bacteria (Postgate 1971; Bergey 1975; Mulder and Brontenegro 1975). Becking (1963) isolated one strain of a '*Vibrio* or *Spirillum*' from African soils, which he considered "very probably identical with Beijerinck's *S. lipoferum.*" This strain incorporated $^{15}N_2$ in pure culture when grown with yeast extract supplement. *Spirillum lipoferum* strains isolated by Beijerinck or Becking are not available. Since the identification of *S. lipoferum* as the major bacterium responsible for nitrogenase (N_2-ase) activity in *Digitaria* roots (Dobereiner and Day 1974), about 150 pure cultures of this organism have been isolated from roots and soils collected all over Brazil and Africa. All isolates, in semisolid malate or lactate medium, fix N_2 comparable to *Azotobacter*, as shown by C_2H_2 reduction assays or Kjeldahl analyses (unpublished data from our laboratory). Only some of the strains, mainly those isolated from temperate regions, need yeast extract supplements. One strain isolated from *Digitaria* roots in Rio de Janeiro was deposited as type strain (American Type Culture Collection (ATCC) 29145).

The classification of this organism within the Spirillaceae, as given in the 8th edition of Bergey's Manual (1975), is difficult although it was identified as *S. lipoferum* after the original description by Beijerinck (1925) and Bergey's Manual edited in 1957 (Dobereiner and Day 1974; Krieg, 1976). Preliminary results including deoxyribonucleic acid (DNA) base composition (Krieg, personal communication) indicate the necessity of a complete reexamination of the classification of several N_2-fixing aerobes. Until conclusive data on this are published, however, the organism with which we are dealing in this paper must remain as *Spirillum lipoferum* Beijerinck.

The only existing report in the literature on the occurrence of *S. lipoferum* is given by Schroder (1932), who found it in all but two of 76 soil samples collected all over Germany and Austria. She concluded that this organism is widely distributed and is no indicator for fertile soils. This paper confirms this organism as one of the most important N_2-fixing bacteria in tropical regions.

Materials and Methods

Soil and Root Samples

Soil and root samples were collected into new polyethylene bags with precautions to avoid cross-contamination. Either roots within the soil or soil without roots at natural humidity were transported within these bags and kept under room temperature, sometimes for several weeks. Although no proper experimental proof is available, it would seem that this kind of storage does not affect *S. lipoferum* survival because some of the most active samples, such as those from Mato Grosso (Table 5), were analyzed after more than a month. Samples collected at the University Campus at Km 47 were always processed on the same day.

For the samples of Table 1, the following specifications can be given: in Senegal they were collected in the experimental fields of ORSTOM,[3] Daker, and near the airport; samples from Gambia and Liberia came from fields near the airport; those from Nigeria came from experimental fields of IITA,[4] Ibadan; and those from Kenya came from a private property near Nairobi (1700 m high). The Brazilian samples came from the North (Advanced Experimental Fields of Universidade Federal Rural do Rio de Janeiro, in Amapá), from Central Brazil (Centro de Pesquisa Agropecuária do Cerrado near Brasilia and from farms all over Mato Grosso), from Central South (University Campus, Km 47, Rio de Janeiro State, Santa Monica Experimental Farm 100 km north west, from Instituto Agronomico of Campinas, and Departamento de Nutrição Animal, Nova Odessa, both São Paulo), and from the South (Centro Nacional de Trigo, Passo Fundo, Rio Grande do Sul). Samples from the U.S.A. came from the Snake River Valley, Washington, from the Experimental Farms in Sharmany (Madison) and Hancock, from a farm in Portage, Wisconsin, and from the Morrow Plots, University of Illinois. The forage grass root samples referred to in Table 2 were collected during May–September 1974 at various sites (10 samples each) of the University Campus, none of which had been fertilized recently. The grain crop root samples of Table 2 were collected in January–February 1975 (maize) and in April–June 1975 (sorghum, rye, and wheat) from field experiments planted with PK fertilizer and sprayed with ammonium molybdate (0.5 or 1 kg/ha). The samples of Table 3 were collected in December 1975 on various sites of the University Campus. Spontaneously growing grasses and legumes were removed from the soil with a hoe. The soil which could be shaken off was considered rhizosphere soil, and the soil layer adhering to the roots as

[3]ORSTOM, Office de la Recherche Scientifique et Technique Outre-Mer.

[4]IITA, International Institute of Tropical Agriculture.

root surface soil. The soil samples referred to in Table 4 were kindly provided by Prof. A. C. J. de Castro, Universidade Federal do Rio de Janeiro, who collected them for minor element analyses all over the State of Mato Grosso. They were air-dried and sieved. The data referred to in Tables 5 and 6 are from samples collected in December 1975 at various sites of the University Campus. *Panicum maximum* soil and root samples were always taken at adjacent sites, care being taken to eliminate roots from the soil samples. The samples referred to in Table 7 were collected in July 1975 at two experimental fields (Sharmany Farm and Hancock Experimental Station, both from University of Wisconsin), in the Madison Arboretum or on the roadside near Portage. All these data were obtained during a working visit of the first author at the Department of Biochemistry, University of Wisconsin, in cooperation with R. H. Burris, S. Albrecht and J. Okon. Determinations of pH in all samples were made in 1:1 aqueous suspensions with a Beckman potentiometer.

Culture Techniques and Identification of Spirillum lipoferum

Nitrogen-free semisolid malate medium (3 ml in 6-ml serum bottles) was used for all assays. For the earlier work (until October 1975) (Tables 2 and 7 and part of Table 1) this medium had the following composition: 5 g malic acid; 4.7 g NaOH; 0.1 g K_2HPO_4; 0.4 g KH_2PO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 0.02 g $CaCl_2$; 0.01 g $FeCl_3$; 0.002 g $NaMoO_4 \cdot 2H_2O$; 5 ml 0.5% alcoholic solution bromothymol blue; 1.75 g agar; 1000 ml H_2O; pH adjusted to 6.8.

In October 1975, after a series of experiments, the above medium was improved by the following changes: K_2HPO_4 increased to 0.5 g and KH_2PO_4 was omitted; NaOH was replaced by 4 g KOH; $FeCl_3$ was replaced by 0.5 g $FeSO_4 \cdot 7H_2O$; 0.01 g $MnSO_4 \cdot H_2O$ was added; bromothymol blue was reduced to 2 ml. This medium, called NFb in our laboratory, was then used for all remaining assays (Tables 1, 4, 5, 6, and part of Table 1).

Half-centimetre-long washed root pieces (surface-sterilized for 30 s in 10% White[5] when stated), or one loop of soil was placed into the semisolid medium and incubated for 40 to 48 h at 33 to 35 °C. The cotton wool plug was then replaced by a rubber cap and 12% of the air in the bottles was replaced by C_2H_2. Care was taken not to disturb the pellicle which forms 1 mm below the surface because this stops N_2-ase activity immediately. One-hour C_2H_2-reduction rates were determined with a Perkin–Elmer (F-11) gas chromatograph.

The identification of *S. lipoferum* in the enrichment cultures of semisolid malate medium was accomplished by determining the presence of very characteristic white, dense, and undulating fine pellicles, and high N_2-ase activity (above 100 nmol C_2H_4/h per culture). Hundreds of microscopic examinations of such cultures always showed that the dominant organisms were characteristic rods with fat droplets and active spiral movements. Enrichment cultures with lower activity either showed a slower forming pellicle or a larger proportion of the other organisms. In few cases *Azotobacter* was present, which could be identified macroscopically by more diffuse and thicker pellicles. Whenever this organism was present it became dominant in the enrichment cultures because of faster growth than *S. lipoferum* in the malate medium. Cultures from several legume root samples from Africa showed similar thick diffuse pellicles, but did not reduce acetylene. Small rods morphologically different from *S. lipoferum* were found upon microscopic examination. The high nitrogen content in legume roots could have been responsible for the growth of bacteria which do not fix N_2 in this medium (*Rhizobium*?). Other than in the case mentioned above, good growth of non-*Spirillum* microorganisms was not seen in malate medium. Turbidity and fermentative N_2 fixation was often observed when the malate was replaced by sucrose. For the differentiation between samples that did or did not contain *S. lipoferum*, the arbitrary value of 10 nmols C_2H_4/h per culture was taken as minimum for positive. All cultures with such low activities or otherwise doubtful were examined microscopically. Therefore all samples stated as positive did contain the organisms but probably many samples with low numbers have been considered negative.

Isolation of *S. lipoferum* from enrichment cultures obtained with root or soil inoculant from tropical regions, as described above, is very easy. For some reason isolation of pure cultures from samples collected in temperate regions is much more difficult. After two further enrichments in the same semisolid malate medium the cultures were streaked out on plates with media of the same composition, except that 50 mg of yeast extract/litre was added. White or pinkish dry, small, raised, and round or irregular colonies were selected after 1 week and inoculated into semisolid NFb medium in serum bottles. After subjection to C_2H_2-reduction assays, 24-h-old cultures were streaked out on potato agar (200 g of potatoes cooked with the skin in one litre of H_2O, filtered, and 0.25% potassium malate and 0.25% sucrose added). On these plates, after 1 or 2 weeks, colonies of *S. lipoferum* are very characteristic, being more or less pink, irregular or round, but always dense with umbonate elevations.

By this method about 150 pure cultures have been isolated from Africa and Brazil, but only one from the U.S.A., two from South Brazil, and one from Kenya. N_2-ase activity of these strains in pure culture in NFb medium was between 100 and 1000 nmols C_2H_4/h per culture after 40 h of incubation at 33 °C. The amount of N_2 fixed in semisolid NFb medium is 10 to 30 mg/g malate. The extremely high efficiencies (100 mg N/g malate) reported in earlier papers (Dobereiner and Day 1974) were due to an error (the ethanol added to the medium with the bromothymol blue was not taken into account as carbon substrate). Comparative studies of nitrogenase activities (C_2H_2) in semisolid malate cultures measured twice daily over a 4-day period, with Kjeldahl analyses performed on parallel cultures at the end of this period, yielded the following C_2H_2:N_2 conversion factors: 4.0 ± 0.37 (10 strains in malate); 3.3 ± 0.38 (10 strains in lactate); 2.7 ± 0.13 (1 strain at three malate concentrations). Earlier observations gave a mean conversion rate of 2.7 (Day and Dobereiner 1975).

Results and Discussion

Data on *S. lipoferum* occurrence in samples collected at locations with a wide range of climatic conditions are summarized in Table 1.

[5]A commercial household disinfectant based on quaternary ammonium compounds.

TABLE 1. Geographic distribution of *S. lipoferum* in roots and soils collected in various countries

Origin of samples	Latitude	Grass roots		Soil	
		No. of samples	% positive samples	No. of samples	% positive samples
U.S.A.					
Washington	47° N	9	11	6	17
Wisconsin	43° N	53	11	—	—
Illinois	43° N	—	—	6	17
Africa					
Senegal	15° N	20	45	20	60
Gambia	14° N	4	100	6	100
Liberia	6° N	6	50	8	100
Nigeria	6° N	15	67	19	84
Kenya	2° N	10	0	18	22
Brazil					
North	0–2° S	20	60	—	—
Central	11–20° S	162	59	75	67
Central South	23° S	744	63	69	51
South	30° S	52	30	—	—

NOTE: Washed root pieces (0.5 cm) or one loop of soil were used to inoculate 3 ml semisolid malate medium, C_2H_2 reduction being measured after 40 to 48 h incubation at 33 °C. Cultures produced at least 10 nmol C_2H_4/h, showed the typical *Spirillum* pellicle, and contained thick rods filled with lipid bodies, and spirilliform movements were considered positive.

About 60% of the samples collected in the tropical belt (15° N to 23° S) contained the organism, except in Kenya, which has an altitude of 1700 m and mean night temperatures of 13 °C. In the extreme south of Brazil and in the U.S.A., *S. lipoferum* incidence was much lower but the organism is present. The relatively higher occurrence reported by Schroder (1932) in Germany perhaps can be attributed to the different criteria used by this author. In the present survey, only samples showing N_2-ase activity after 2 days were considered positive. Some of the negative samples when incubated longer, as the ones from the Wisconsin Arboretum (Table 5), did show some activity after 72 h. Using our methods, Silvester Bradly (personal communication) observed 100% positive enrichment cultures with *Potamogeton* root samples collected in a lake in Scotland.

Differences between plant species in relation to *S. lipoferum* occurrence and N_2-ase activity can be observed in Table 2. In this table a direct comparison should be made between the forage grasses which were collected systematically (10 samples at each site for each species, on the same day). *Panicum maximum* showed the most constant high incidence of *S. lipoferum* and also the highest enrichment culture activities. Other favorable grasses were *Brachiaria* and *Pennisetum* while *Hyparrhenia rufa* seemed the poorest. The mean N_2-ase activity of the enrichment cultures, even when only the positive cultures were computed, followed the same pattern indicating that enrichment culture activity measured after a short incubation period is a reasonable estimate of numbers or at least numbers of active cells in the inoculant, these cells being able to multiply within a short incubation period. Counting methods for this organism are not yet available and we therefore, in this paper, have tentatively used enrichment culture activity as an estimate for the abundance of the organism in soil or roots.

The occurrence of *S. lipoferum* in grain roots in Table 2 might give an overestimate because selected root systems with high N_2-ase activity were used and the fields had been specifically fertilized to boost N_2 fixation. They are, however, comparable among them and it is surprising that wheat and rye roots collected in our winter (night temperatures seldom below 15 °C) contained as many *S. lipoferum* as maize and sorghum roots collected in the warmer season. Incidence of this organism in wheat furthermore was not increased by seed inoculation at two sites (with strains isolated from wheat roots). *Spirillum lipoferum* occurrence in wheat roots collected in Brasilia (15 °S) and Londrina (24° S) was also abundant (Nery and Peres, unpublished data).

TABLE 2. Effect of plant species on the occurrence of *S. lipoferum* in grass roots collected at "Km 47"

Plant species	No. of sites	No. of samples	% positive samples*	nmol C_2H_4/h per culture†
Forage grasses grown with low or no fertilizer				
Panicum maximum	6	60	92	91±8‡
Brachiaria mutica	6	60	52	66±9
Pennisetum purpureum	6	60	47	65±17
Melinis minutiflora	6	60	48	56±14
Digitaria decumbens	6	60	43	37±17
Hyparrhenia rufa	5	50	22	24±6
Grain crops fertilized with PK and Mo				
Zea mays (PVA)	1	55	93	64±4
Z. mays (GH)	1	57	100	550±60
Sorghum vulgare (GH)	2	85	96	347±42
Triticum aestivum (GH)	2	122	86	198±98
Secale cereale (GH)	1	45	98	233±40

NOTE: Forage grasses were collected from a wide range of soils mostly from spontaneous pastures while the grain crops were obtained from fields in red yellow podzolic (PVA) or gray hydromorphic (GH) soils fertilized specificially to boost N_2 fixation. The root systems used for grain samples were previously selected for high N_2-ase activity. For these reasons forage and grain crops are comparable among themselves only.

*See note in Table 1.

†Half of these samples are from plants obtained with seeds inoculated with peat inoculant containing *S. lipoferum* isolated from wheat roots. There was no significant difference due to inoculation.

‡Standard deviations of the means.

TABLE 3. Comparative N_2-ase activity of *S. lipoferum* enrichment cultures obtained from roots and soil of grasses and legumes

	nmol C_2H_4/h per culture		
	Surface-sterilized roots	Roots with root surface soil	Rhizosphere soil
Harvest I			
11 Legume samples	9±4†,‡	38±13	57±14
11 Grass samples	68±18	66±26	71±14
Harvest II			
13 Legume samples	74±18	74±25	115±22
15 Grass samples	135±33	115±25	123±44

NOTE: The legume samples contain 9 species of Papilionoideae, 9 species of Mimosoideae, and 2 of Caesalpinoideae; there was no apparent difference between subfamilies. The grass samples contain 13 species of Gramineae. All enrichment cultures showed dominant *S. lipoferum* populations except 68 cultures from legume roots, 3 from soil under legumes, 24 from grass roots (7 from *Melinis minutiflora*), and 6 from soil under grasses.

*Thirty seconds in 10% White household disinfectant based on quaternary ammonium compounds.

†Each value is the mean of three enrichment cultures each of 11 to 15 samples.

‡Standard deviations of the means.

In Table 3, differences in rhizosphere effects between grasses and legumes can be observed. Cultures inoculated with partially surface-sterilized roots were less active than those inoculated with rhizosphere soil. Several legume root samples collected in Africa (figures not given), together with the grass roots (Table 1) confirmed the negative effect of legumes. Grass rhizosphere soil yielded enrichment cultures as active as partially surface-sterilized roots.

Interesting vegetation effects can also be observed over a wide range of soil samples collected in Mato Grosso, Central Brazil (11 to 21° S) (Table 4). Virgin forests seem the poorest environment for *S. lipoferum*. Five soil samples from each of two forests in Rio de Janeiro State were also negative. It can be assumed that forests and most other ecosystems in equilibrium are not nitrogen-limited (Dobereiner and Campêlo 1976) and therefore they are not a selective habitat for N_2-fixing bacteria, or plant–bacteria associations; and even legume trees in equilibrium forests are seldom nodulated (Bonnier and Brackel 1969). The most propitious vegetation

TABLE 4. Occurrence of *S. lipoferum* in soils under various characteristic vegetations, in Mato Grosso, Central Brazil

Vegetation	No. of sites	No. of sites *Spirillum lipoferum* positive*			
		Soil horizon†			pH range of positive soils
		A	B	C	
Virgin forest	4	1	0	0	6.0
Burned forest	3	1	1	0	4.8–5.0
Natural pastures	3	2	0	0	5.5–5.8
Panicum maximum pasture (hill slopes)	10	4	1	0	5.3–6.7
Panicum maximum pasture (lowland)	18	16	2	1	5.0–7.6

*See note Table 1.
†Soil horizon according to pedological identification; two sites with virgin forest in Rio de Janeiro State were also negative; values are means from three samples from each site and horizon.

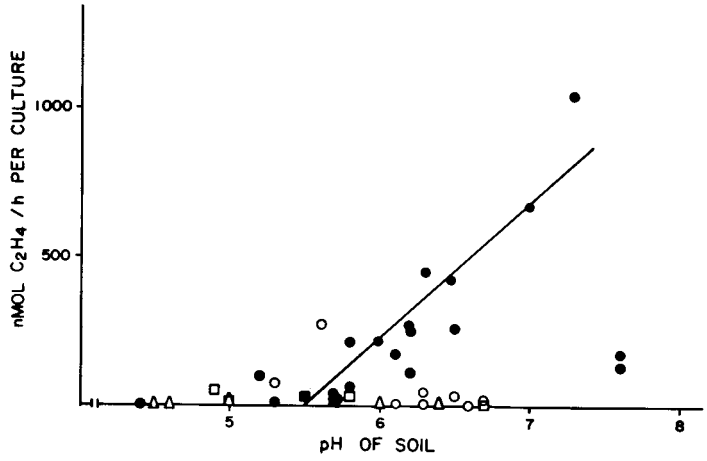

FIG. 1. Effect of soil pH on *S. lipoferum* occurrence. The regression of enrichment culture activity with the pH of the soils used as inoculum is highly significant for *Panicum maximum* lowland pastures (●), with a regression equation $Y = -2404 + 438\ X$; $r = 0.876$ (two values at pH 7.6 and one at pH 4.4 not included). No correlation with soil pH was observed for the remaining vegetation types: *P. maximum* hill-side pastures (○); natural pastures (□); virgin forests (△).

was again *P. maximum* pasture, but only in the lowland soils. This grass is routinely sown in these areas after burning the forest and shows exuberant vegetative growth yielding up to 40 tonnes (t) (dry matter) of forage per year. Breaking down of the climax vegetation which is replaced by a high-yielding and therefore nitrogen-demanding grass seems to result in specifically favorable conditions for N_2 fixation. *Panicum maximum* seeds obtained from this area contained *S. lipoferum* when they were not surface-sterilized.

Besides vegetation, the effect of soils seems relevant. When the same grass was sown into eroded hill sides, *S. lipoferum* incidence was much lower and seemed to be limited by soil factors (Table 4). As expected, lower soil horizons offer very poor conditions for *S. lipoferum* and none were found except in the *Panicum* pastures, where the deep roots might favor the occurrence of *S. lipoferum*. Soil pH seemed to be very important in all soils and when no other factors were limiting *S. lipoferum* occurrence, as in the lowland pastures, a highly significant correlation of soil pH with enrichment culture activity was obtained (Fig. 1). Maximal activity was obtained from soils with pH 7.3, while 7.6 was already too high. Similar narrow pH requirements were observed in *S. lipoferum* pure cultures grown in semisolid medium (Day and Dobereiner 1975).

A better understanding of the pH effects on *S. lipoferum* occurrence in soil and roots can be obtained from the data in Table 5. Occurrence in soil is again strongly pH dependent but *P. maximum* roots offer optimal conditions even

TABLE 5. pH dependence of N_2-ase activity of *S. lipoferum* enrichment cultures from soils and *P. maximum* roots collected at the University Campus, "Km 47"

Site No.	pH of soil	Soil inoculum	Root inoculum
1	4.8	0	499
2	5.1	0	286
3	5.2	1	593
4	5.6	365	716
5	5.8	146	559
6	5.8	166	559
7	6.4	496	509
8	6.7	852	775
9	7.0	864	691
10	7.2	639	477

NOTE: Values represent nmol C_2H_4/h per culture, after 45 h of incubation at 33 °C, means of six cultures. The correlation of the enrichment culture activity with soil pH was highly significant with soil inoculum ($r = 0.90$) and not significant with root inoculum ($r = 0.41$). Corresponding roots and soils were collected at the same site.

when grown in soil with pH 4.8. This explains the apparent paradox of the high pH requirements of the bacteria and its preferential occurrence in tropical regions where soils are usually acid. On the other hand it does not explain the failure of the organism to occur in temperate regions. The high incidence of *S. lipoferum* in *Panicum* roots grown in acid soils is also further evidence for a very close association of bacteria and grass, most probably with the bacteria being situated inside the roots as has been suggested for *Digitaria* and maize (Dobereiner and Day 1974, Bulow and Dobereiner 1975).

It is probable that the availability of carbon substrates is limiting *S. lipoferum* N_2-ase activity and growth in soils, because the most favorable soils were the lowland pastures which are relatively rich in organic matter. To check this, six soils, three acid soils with almost no *S. lipoferum* and three soils with higher pH with high enrichment culture activity, were amended with malate or sucrose and incubated under acetylene in air and under reduced oxygen tension (pO_2 0.02 atm) (Table 6). None of the soils showed any activity without amendment. N_2-ase activity after 2 to 3 h was detected in one of the soils indicating induction of N_2-ase activity in organisms already present in the soil. There was no difference between incubation under air or at pO_2 of 0.02 atm. In two of the soils with pH above 6, rapid multiplication of N_2-fixing bacteria occurred as indicated by the very high N_2-ase activity after 16 or 48 h, but in soil 8 some other major limiting factor must have inhibited bacterial growth. In soil 4, after 48 h, reasonable activities were observed in the malate-treated vials, but when the pH was adjusted with lime, high activities were observed in the sucrose-amended vials. Because of pH changes to about 9 in the malate vials and to 4 in the sucrose vials, interpretation is difficult. Microscopic examination of the most active cultures indicated that even in the sucrose-amended vials, *S. lipoferum* was abundant. In none of them was *Azotobacter* or *Clostridium* found abundantly. To confirm these results and investigate soil amendment with a temperate soil, samples were collected in the Morrow plots, in Urbana, Illinois, which have been kept for many years with or without fertilizer application (Fig. 2). Again these results show no activity in soils without amendment, but reasonable activity after 45 h of incubation with malate, and fastest growth and highest activities when a few drops of *S. lipoferum* inoculant were added. Activities in low fertility treatments were higher than in the soil from high fertility plots. In a previous enrichment culture assay (summarized in Table 1), eight *S. lipoferum* enrichment cultures from each of the six samples produced only four positive cultures, all from one sample. No *Azotobacter* was observed in these cultures. It seems therefore that the activity in the noninoculated vials was due to enrichment of organisms other than *S. lipoferum* or *Azotobacter*. The much faster increase in activity in the inoculated vials indicates that there is no major limiting factor in these temperate soils, which inhibits the growth of *S. lipoferum* at least during a few hours at optimal temperatures.

Table 7 gives some preliminary results obtained in Madison, Wisconsin. In general, the occurrence in uninoculated plots seems similar to the results shown in Table 1 except for rye grass (*Lolium perenne*) and quackgrass (*Agropyron repens*) at Sharmany farm. These showed higher frequencies and also N_2-ase activities in enrichment cultures. But these samples were taken from plots adjacent to inoculated maize plants and it is therefore possible that cross-contamination occurred. The same grasses collected in Portage and Hancock did not seem very stimulating. The clear-cut difference in frequency of positive samples and N_2-ase activity between inoculated and noninoculated plots is interesting. Although relatively large amounts of inoculant were used, which in practice would be difficult to recommend to farmers, the fact that it

TABLE 6. N_2-ase activity in soil amended with malate or sucrose

Site No.	pH of soil	Hours of incubation before C_2H_2 assay	Enrichment culture activity*	Soil	Soil with 0.5% malate	Soil with 0.5% sucrose
2	5.1	3	—	0	0	0
		16	—	0	0	0
		48	6	0	0	0
3	5.3	3	—	0	0	0
		16	—	0	0	0
		48	7	0	0	0
4	5.6	3	—	0	0	0
		16	—	0	0	0
		48	10	0	99	9
4 plus $CaCO_3$	7.7	3	—	0	0	0
		16	—	0	0	13
		48	10	0	7	6570
7	6.4	3	—	0	0	0
		16	—	0	16	34
		48	264	0	2872	614
8	6.7	3	—	0	0	0
		16	—	0	0	0
		48	335	0	0	0
9	6.9	1	—	0	0	0
		2	—	0	5	5
		3	—	0	31	62
		16	—	0	3260	19.486
		48	364	0	10	0

NOTE: Data given in nmol C_2H_4/h per 20 grams of fresh soil, means of three replicate cultures. Site nos. refer to Table 5; 20 g of freshly collected soil was sieved and placed into 120-ml vials and 1 ml of water or 10% malate or sucrose solution spread over it without mixing. The vials were incubated at 35 °C under air containing 12% C_2H_2 during the first 3 h to observe induction of N_2-ase. After assaying C_2H_4 production at 3 h, the rubber bungs were replaced by cotton wool and C_2H_2 again injected after 16 h of incubation. The same procedure was repeated for the readings after 48 h of incubation. Parallel vials, also incubated under air but assayed for C_2H_2 reduction at pO_2 0.02 atm, showed essentially the same results and are therefore not given in the table.

*In semisolid malate medium (NFb) as stated in the Material and Methods, inoculated with a loop of soil collected in the field before performing the experiment.

is possible to establish *S. lipoferum* isolated in Brazil on corn roots in Wisconsin fields is encouraging for inoculation experiments in regions where *S. lipoferum* occurrence is low. Rinaudo *et al.* (1975) recently reported highly significant N_2-ase activity increases in rice grown in pots with soil from France, with *S. lipoferum* inoculation. *Azotobacter* or *Clostridium* inoculation in the same experiment had no effect. Before striking yield increases can be expected, however, proper strain selections and better understanding of the associations formed are necessary.

The data presented in Table 7 suggest also that about 30% of the samples of a *Panicum* species collected in the Madison Arboretum were positive. This grass was the most active species among a number of prairie grasses tested (Tjepkema and Burris 1976) for N_2-ase activity in soil plant cores.

It seems difficult to understand that a N_2-fixing bacterium described in 1925 and which occurs so abundantly in a wide range of soils and climate received so little attention. The methods which led to its rediscovery seem very simple and require no specific equipment or skills. They are, however, based on modern knowledge of the key elements in the physiology of N_2-fixing microorganisms e.g. necessity of oxygen protection mechanisms and the use of C_2H_2 reduction as a method for determining N_2-ase activity. The contribution of this organism to the nitrogen economy of world and especially tropical agriculture is not yet known. The data shown in this paper indicate a wider and more abundant distribution of this organism than any other aerobic N_2-fixing bacterium except *Rhizobium*, in the tropics (Becking 1961; Dobereiner and Campelo 1971). Any substantial N_2 fixation by this organism seems restricted to the root environment (Dobereiner and Day 1975; Day *et al.* 1975) and dependent on the availability of carbon substrates. Highly significant correlations of root

TABLE 7. Occurrence of *S. lipoferum* in grass roots in Wisconsin (U.S.A.), and establishment on corn roots by inoculation

Plant species	Site	No. of samples	No. of +ve samples	Mean N_2-ase activity of +ve enrichment cultures nmol C_2H_4/h per culture
Zea mays	Madison Sharmany	30	2	19
" " inoculated*	" "	30	29	120±16‡
" "	Hancock†	20	2	16
" " inoculated*	"	20	5	16±2
" "	Portage	10	2	16
Sorghum vulgare	Madison Sharmany	10	0	—
" "	Hancock†	10	0	—
Lolium perenne	Madison Sharmany	10	7	57±11
" "	Hancock†	10	2	8
Agropyron repens	Madison Sharmany	5	5	39±8
" "	Hancock†	10	3	13
" "	Portage	10	4	15
Panicum virgatum	Madison Arboretum§	33	10	23±3

*Inoculated twice with 10 ml/plant of NH_4^+-grown liquid cultures of *S. lipoferum* (type strain ATCC 29145). Ten rhizosphere soil samples from this field did not contain *S. lipoferum*.
†Field-fertilized with 20 kg N/ha every week.
‡Standard deviations of the means.
§No growth after 48 h, data from 72-h cultures, all other cultures 40–48 h of incubation at 30 °C.

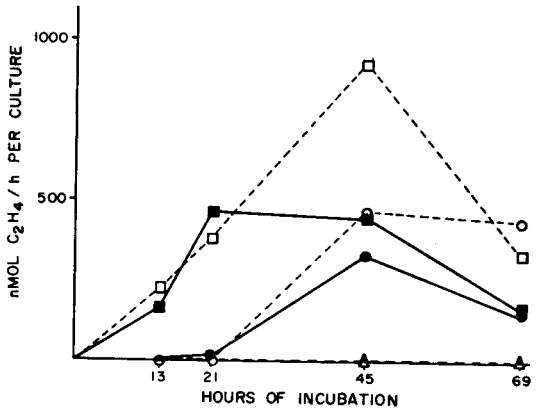

FIG. 2. N_2-ase activity of soil from temperate climate amended with malate. Three soil samples each of the low (open symbols) and high (closed symbols) fertility plots of the Morrow experiment at Urbana, University of Illinois, were used. Twenty grams of fresh soil were sieved and incubated (33 °C) in 120-ml vials without amendment (△▲), with 0.5% malate (○●), or with 0.5% malate and inoculated with 0.1 ml of potato infusion culture of *S. lipoferum* (□■). Points are means of three samples, three cultures each. All other details are like the experiment presented in Table 6.

piece N_2-ase activity with *S. lipoferum* enrichment culture activities in *Digitaria* (Dobereiner and Day 1974) and maize (Bulow and Dobereiner 1975) indicate that this organism is the major one responsible for the N_2-ase activities occurring in roots of these crops. The pronounced vegetation effect described in this paper indicates a major role also in several other tropical grasses.

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