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Several newly discovered grass-bacteria associations seem to have considerable potential as nitrogen-fixing systems in tropical areas. Their exploitation in agriculture might possibly result in decreased dependence on mineral fertilizers, but first the mechanisms involved need to be better understood.

The use of mineral fertilizers as a source of nitrogen for crops is limited by a number of serious drawbacks. The most important of these is their high cost, especially in developing countries, which usually have to import either the fertilizers or the fuel to make them. It is also difficult to apply amounts of nitrogen fertilizer adequate for maximal crop production without risking serious damage to the environment, because plants tend to use high fertilizer applications inefficiently and the excess nitrates and nitrites are often washed into water supplies as pollutants. This leaching process is increased by heavy rain and intensive erosion, especially in the tropics where the potential for increasing crop yields by adding nitrogen is still so enormous.

In contrast, biological systems fix atmospheric nitrogen at no economic cost and at constant low rates which permit its immediate incorporation into plant proteins. Therefore there is little accumulation of mineral nitrogen in the soil. The nitrogen-fixing enzyme nitrogenase is repressed by its end product, ammonia, and also indirectly by nitrate. Thus these systems are self-regulating and environmental hazards such as leaching of nitrates are unlikely.

The legume symbiosis is the best-known nitrogen-fixing system, but several newly discovered grass-bacteria associations also seem to have considerable agricultural potential (1, 2, 3). A bacterium known for 50 years has now been shown to be the major one responsible for nitrogen fixation in several forage grasses and grain crops. Better understanding of

such associations and their limiting factors is necessary before partial replacement of nitrogen fertilizers and substantial increases in crop yields can be expected.

WHAT USE ARE NITROGEN-FIXING GRASSES?

Most of the crops important for animal and human nutrition are in the Gramineae family. A number of grasses and grain crops have adapted to warm, sunny climates and developed an ability to use high light intensities efficiently at high ambient temperatures. Their distinct photosynthetic pathway, the so-called C_4 dicarboxylic acid pathway, enables them to fix carbon dioxide into malate with enzymes capable of working at much lower CO_2 concentrations than those of other plants. This system permits photosynthesis with almost closed stomata during part of the day and is therefore more economical in terms of water use. Under optimum temperatures these C_4 grasses can convert light intensities twice as high using half of the amount of water necessary for temperate plants.

The C_4 grasses and grain crops therefore suggest an attractive possibility of using larger amounts of light energy for biological N_2 fixation. Yields of 40 metric tonnes per ha of the dry matter are not unusual for grasses such as sugar cane or elephant grass. If only ten percent of the dry matter incorporated by these plants were used as an energy substrate for N_2 fixation and if a substrate conversion efficiency half that of legumes is assumed,

200 kg of nitrogen per ha could be fixed—supplying half the amount necessary for such a crop. Fortunately climatic conditions which permit such high yields are found in the tropics, the very regions where substantial increases in yields are still possible and also most needed.

THE NEW GRASS-BACTERIA ASSOCIATIONS

The fixation of atmospheric nitrogen under grass cover was suggested many years ago in Australia, Nigeria and Brazil (4, 5, 6). A number of nitrogen-fixing bacteria were found in association with tropical grasses—a relationship unknown in temperate regions. One of them, *Azotobacter paspali*, occurred in 98 percent of root surface soil samples from the grass *Paspalum notatum* cultivar "batatais", collected from all over Brazil and several other tropical countries, but not with other grasses or legumes (7). In 1971 (1) the indirect C_2H_2 reduction method confirmed that washed roots of this grass associated with *A paspali* fixed atmospheric nitrogen. At the same time reports came from the Philippines and the Ivory Coast (8, 9) showing nitrogen fixation in a number of other forage grasses as well as rice. Since then the list of N_2 -fixing tropical grasses has increased rapidly and it now includes some of the most important cereals, maize, sorghum and millets (10, 11, 12).

The acetylene reduction method, however, measures only the activity of the nitrogen-fixing enzyme. Actual amounts of isotopic nitrogen fixed and incorpo-

rated into the plant *P notatum* are shown in Figure 1. A number of additional experiments confirmed considerably higher $^{15}\text{N}_2$ incorporation (up to 0.70 atom percent excess over the natural isotope content in air after 72 hours) with this grass and with digit grass, an important tropical forage plant (13).

A bacterium known for 50 years. Although the microorganism responsible for N_2 fixation in *P notatum* roots had been identified, there was little information about those on the other grasses. New methods had to be devised which duplicated conditions in the root-soil habitat. Small root pieces were inserted into a nitrogen-free, semisolid nutrient medium containing malate as the sole carbon source. Characteristic curved rods containing many fat inclusions, which at first could not be identified, were prominent in most of these cultures (Figure 2). The bacteria concentrated 2 mm below the surface of the medium, where the oxygen concentration seemed to be optimal. Isolation of pure cultures was not difficult, but upon microscopic examination quite variable forms were found in common peptone media. In the search for identification, a paper written in 1925 by M W Beijerinck (14) was found which showed the delightful drawings reproduced in Figure 3. His description fit all observations of the organism and it was identified as *Spirillum lipoferum*. Since it was impossible to show N_2 fixation in pure cultures by methods available in 1925, the numerous list of nitrogen-fixing bacteria published since then make no mention of this bacterium.

A constant low oxygen supply which corresponds exactly to the amount consumed by the organisms is necessary for N_2 fixation. In the semisolid medium an oxygen gradient forms and the bacteria find the right oxygen concentrations by moving actively towards the most suitable distance from the surface. More sophisticated studies with chemostat cultures confirm that the optimum free oxygen concentration in solution corresponds to that of a liquid in equilibrium with a gas phase which contains only one hundredth the oxygen of air.

Once the necessary methods had been developed, it was demonstrated that *S lipoferum* is a very common soil and root inhabitant, especially in the tropics (Table 1). Counts indicate several millions per g roots of sorghum or maize. There is also a good correlation of root nitrogen-fixing activity (C_2H_2) and *S lipoferum* enrichment culture activity after short induction periods. Assuming that initial enrichment culture activity depends on the amount of N_2 -fixing bacteria on the roots, it was concluded that *S lipoferum* has the major responsibility for nitrogen fixation in maize, sorghum, millet and many forage grasses.

What is different in these new grass-bacteria associations? It is tempting to compare the new grass associations with the well-known legume-*Rhizobium* symbiosis, which seems to be the most sophisticated and perfect association of this kind. There, nodules with a specialized structure are the site of nitrogen fixation.

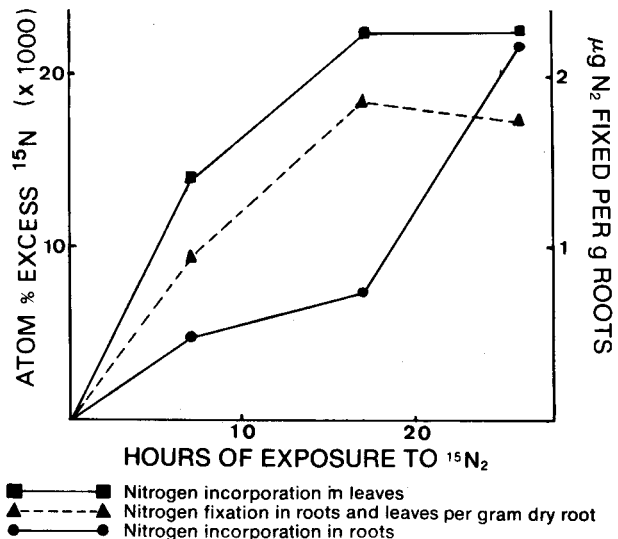


Figure 1. Nitrogen fixation and incorporation in intact soil plant cores of *Paspalum notatum* exposed to a gas mixture of 50 percent labeled nitrogen ($^{15}\text{N}_2$), 20 percent oxygen and 30 percent argon during 9-hour light, 16-hour dark and 11-hour light period. Full lines represent the amount of labeled nitrogen incorporated into

plant tissue (^{15}N atom percent excess over the natural enrichment). Broken line shows amount of nitrogen fixed and incorporated into roots and leaves per g dry weight of roots. Note rapid translocation from roots into leaves during the first hours. (Redrawn from reference 13).

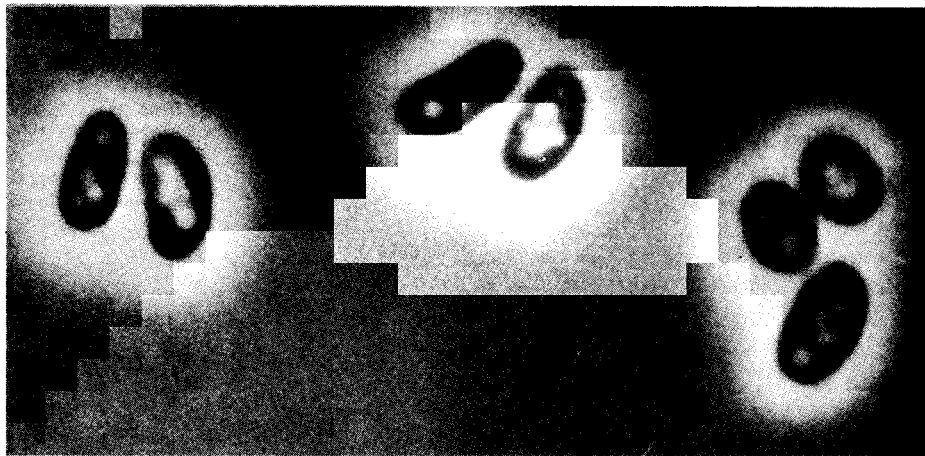


Figure 2. *Spirillum lipoferum* grown in nitrogen-free malate medium, showing lipid body inclusions consisting of polyhydroxybutyrate (courtesy Dr P J Dart, Rothamsted Experimental Station, England).

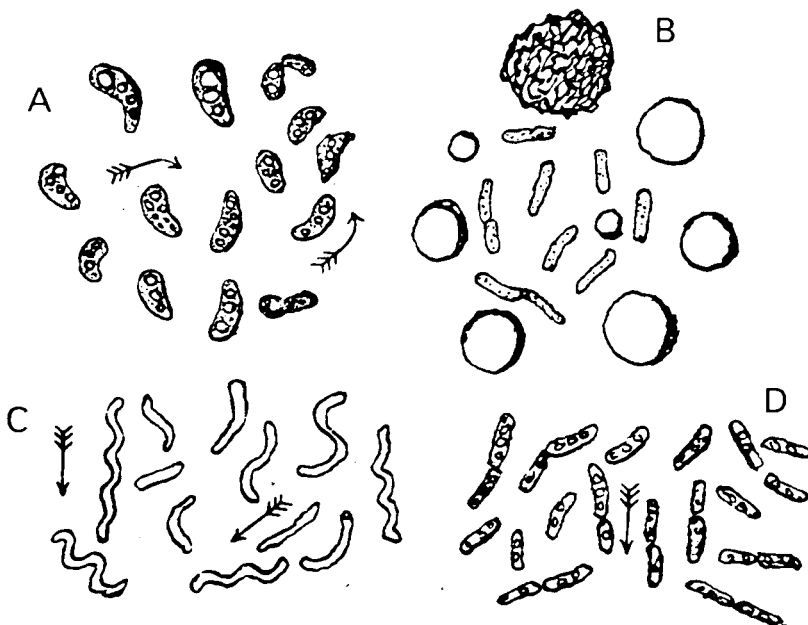


Figure 3. *Spirillum lipoferum* grown on different media: A. liquid sugar medium; B. calcium malate plates; C. diluted nutrient broth; D. liquid lactate medium. (Reproduction of original figure from Beijerinck, reference 14.)

The bacteria are enclosed by membrane envelopes containing leg-hemoglobin which is responsible for the rapid transport of oxygen to the N_2 -fixing sites and maintains the oxygen concentration in solution at a very low level. Legume nodules can therefore continue fixing nitrogen after they have been removed from the soil. Although preliminary evidence indicates that *S. lipoferum* also can be localized within root cells of grasses (2), there seem to be no specific host structures. Thus, once the roots are removed from the soil, nitrogen fixation stops and only prolonged incubation under proper oxygen tensions re-establishes it. This makes measurement of nitrogen fixation in such systems very difficult, and also explains the extreme sensitivity to all kinds of environmental influences shown even by intact systems.

As expected, temperature requirements are high. *S. lipoferum* strains isolated from roots of digit grass at 22°C remained inactive while the maize strains seemed more tolerant to cooler temperatures. Maize roots excised from field-grown plants showed similar temperature requirements. Balandreau (12), using as data a large number of observations on intact soil-plant cores with maize, has proposed a model consisting of four equations which seem to describe 70 percent of the diurnal variations in nitrogen fixation. Soil temperatures below 20°C at 18 cm depth permitted no nitrogen fixing activity. Above this limit either air temperature, soil surface temperature, or light energy input were the limiting factors. Soil humidity became limiting only when plant turgescence was affected. Rates of nitrogen fixation in grain crops are closely related to a pronounced pattern in the growth cycle. Peak activities were reached at silk emergence in maize and sorghum (Figure 4). A second peak appeared in maize at about 50 percent grain filling. The length of these peaks and the amount of nitrogen fixation during grain filling varied with cultivars (16).

Little is yet known about a possible specificity of strains of *S. lipoferum* for certain plant species. A strain isolated from digit grass in Brazil could be established on roots of field-grown maize in Wisconsin, and maize plants grown in sand cultures showed nitrogen-fixing activities (C_2H_2) in inoculated pots, but none in uninoculated blanks (17). Rice seedlings grown in test tubes with soil and inoculated with the same *S. lipoferum* strain showed three times higher activities than seedlings inoculated with either *Azotobacter* or *Beijerinckia*, two classical nitrogen-fixing bacteria which fixed as lit-

tle as the uninoculated blanks (18). In all these experiments effects on plant growth and nitrogen incorporation were small or nonexistent. Much more research is necessary to clarify possible host-bacteria specificities and interactions with other soil microorganisms.

AGRICULTURAL USE OF NITROGEN-FIXING GRASSES

The discovery of tropical grass-bacteria associations which, under conditions not yet identified, can obtain part of the nitrogen necessary for growth through biological fixation, does not mean that all nitrogen problems in grasses are solved. Although the potential has been established, its exploitation in agriculture will require better understanding of the mechanisms involved as well as identification of the limiting factors.

Climate. Optimum temperatures can prevail during most of the year in the tropics, so most efforts should be concentrated in these regions. Fortunately it is here that the greatest benefits can be expected from grass nitrogen fixation. Agricultural practices for less favored regions could include mulching, for example. Straw or plastic covers have been used for this and their effect on soil temperature can be pronounced. Soil humidity and its interaction with oxygen supply can be manipulated by irrigation and soil tillage practices.

Plant Genotype. Although environmental factors seem so important, plant genotype manipulation may be a more promising way to exploit grass nitrogen fixation because it is easier and can be expected to give more reliable results. Significant differences between genotypes have been reported for maize, rice and several forage grasses (3, 10, 11, 19). Activities on excised roots of self-pollenized maize lines were more than ten times higher than activities on the original cultivar (10). So far there is little indication as to whether these differences are due to a root anatomy which might favor sites for nitrogen fixation or make infection by the bacteria easier. Mature thick roots with many laterals showed activities eight times higher than either younger roots without laterals or the laterals themselves.

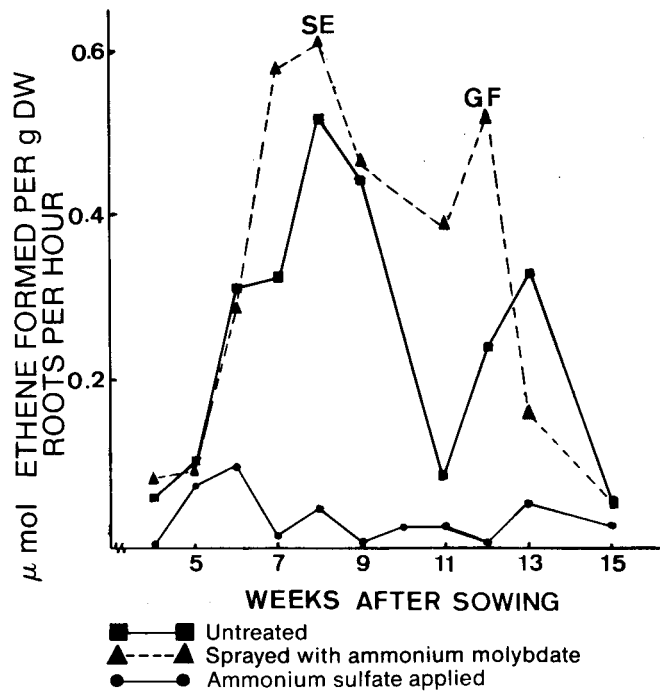


Figure 4. Nitrogen fixation during the growth cycle of field-grown maize. Note the two peaks, one at silk emergence (SE) and one at 50 percent grain filling state (GF). Nitrogen fixation activity was higher when 0.5 kg/ha of ammonium molybdate was sprayed and almost nil when 200 kg N/ha as ammonium sulfate was applied. Excised roots preincubated overnight at low oxygen tensions, and nitrogen fixation measured as rates of acetylene (C_2H_2) reduction to ethene (C_2H_4) performed by the nitrogenase enzyme system (redrawn from reference 16).

Table 1. Geographic distribution of *Spirillum lipoferum* in roots and soils collected in various countries (summarized from Reference 15).

Origin of samples	Latitude	Grass roots		Soil	
		No. of samples	% positive samples	No. of samples	% positive samples
USA	43-47°N	62	11	6	17
Brazil	0-23°S	926	62	144	59
Brazil	30°S	52	30	-	-
Africa*	6-15°N	45	58	47	89

* Gambia, Liberia, Nigeria and Senegal

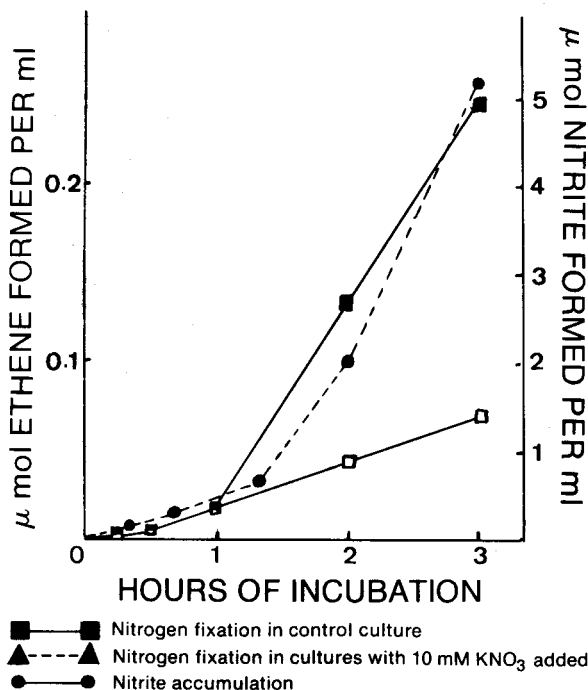


Figure 5. Nitrate-dependent anaerobic nitrogen fixation in *Spirillum lipoferum* pure cultures. Note that after a one-hour lag the cultures with 10 mM KNO₃ more than tripled their nitrogen fixation rate while the control cultures continued linearly. Nitrate reduction as measured by the accumulation of nitrite follows the same pattern, indicating that the nitrate is used for respiration to generate ATP for nitrogen fixation. The low rate of nitrogen fixation in the controls is probably explained by small amounts of oxygen in the medium (Redrawn from reference 20).

Differences may also be related to plant physiology in general, photosynthetic efficiency, or nitrogen metabolism.

Mineral Nitrogen. From what is known today it does not appear probable that high crop yields of grasses can be attained by relying entirely on biological nitrogen fixation. Although high nitrogen-fertilizer applications usually inhibit nitrogen fixation, repeated applications of 20 kg nitrogen per hectare did not affect fixation (3). *S. lipoferum* in pure culture also shows peculiar behavior with mineral nitrogen (20). In contrast to its strictly microaerophilic growth on atmospheric nitrogen, that on nitrate or ammonia is aerobic. Low concentrations of mineral nitrogen enhance growth and result in very active nitrogen-fixing cultures within two days, when the mineral nitrogen is used up. However, high concentrations (10 mM) of ammonium nitrate inhibit nitrogen fixation and induce a dissimilatory nitrate reductase in some of the strains. This enzyme enables the bacteria to use nitrate instead of oxygen for respiration. By using this pathway, nitrate can be reduced to either nitrite which is excreted or gaseous nitrous oxide or nitrogen which return to the atmosphere. There is no other nitrogen-fixing bacterium yet known which can perform all the possible transformations in the nitrogen cycle. Thus this bacterium shows an apparent paradox in the absence of oxygen: nitrate replaces

oxygen for respiration and nitrogen fixation becomes nitrate-dependent (Figure 5). What does this mean in terms of the role of this organism in plant nitrogen metabolism? We know that it can fix nitrogen from the air. But it can also take the nitrate liberated from the soil or added in fertilizers and return it to the atmosphere. Another more attractive possibility is the excretion of nitrite which could still be used by the plant. There are strains of *S. lipoferum* which have no dissimilatory nitrate reductase. Should we select these and try to establish them in the roots?

The use of *S. lipoferum* inoculants for grasses might seem a logical approach. Forage yield increases of almost 20 percent have been obtained in pearl millet in Florida (21) when inoculants were used in combination with nitrogen fertilizers. However, preliminary inoculation experiments carried out in our laboratory in Brazil have been less promising.

Although much progress has been made in this field in the last few years, there are more problems to be solved now than there seemed to be before. Even a partial solution to these questions would lead to uncalculable savings in nitrogen fertilizers and increases in the yields of tropical grasses and grain crops. Careful use of low levels of fertilizers which complement biological nitrogen fixation instead of repressing it will no doubt help avoid many environmental hazards.

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