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Evaluation of Nitrogen Fixation by Bacteria in Association with Roots of Tropical Grasses

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INTRODUCTION

Life on earth depends on nitrogen, which is abundant in the atmosphere, sea, and rocks, but is not always present in forms which are available to plants and animals. Plants assimilate inorganic nitrogen compounds, which are transformed into cell constituents. Animals are dependent on proteins originally synthesized by plants as their major source of nitrogenous substances. The inorganic forms of nitrogen used by plants were originally returned to the environment by the decay of dead organisms and the eventual production of ammonium salts and nitrates by microorganisms. In this manner nitrogen cycles between the chemical environment and the biosphere, but during this process losses may occur through denitrification, leaching, and crop removal. Especially under agricultural conditions the amount of nitrogen removed is usually greater than the nitrogen input. Since the availability of nitrogen to plants limits agricultural production, soil fertility must be maintained. Nitrogen can be returned to the soil through biological nitrogen fixation or by fertilizer nitrogen application.

The manufacture of fertilizer nitrogen by the Haber-Bosch process requires high capital and energy costs. During 1976 and 1977 developing countries produced 21% and consumed 31% of the world's fertilizer nitrogen (77). Also, an imbalance between the availability of raw materials for ammonia production and the need to develop agriculture exists among these countries (74). The high costs of natural gas-based ammonia plants are additional financial burdens to developing countries (177).

The rising cost of fertilizer nitrogen production in developed countries has also caused concern. Uneconomical production of fertilizer nitrogen in Europe and the United States has forced many plants to close down. It has been estimated that the United States will import 25% of its total ammonia requirement by 1983 (68). Food production would be greatly reduced if fertilizer nitrogen were not used, and crop yields are frequently low in developing countries because fertilizer nitrogen is not usually available (17).

Legumes have played a major role in food production throughout history. The unique ability of these plants to enrich the soil was well recognized as early as Roman times. In 1813 Sir Humphrey Davey proposed that legumes derive their nitrogen requirements from the atmosphere. In 1888 Hellriegel and Wilfarth demonstrated that legumes fix nitrogen through the active participation of microorganisms in root nodules (35, 79). The importance of legumes to agriculture in tropical (62) as well as temperate regions (131) has been discussed previously.

Although most legumes have the ability to fix all of the nitrogen that they require, the total world area cultivated with these plants has been approximately 10% of the area used for cereal grasses (76). The production of meat and related foodstuffs depends in part on the availability of cereal and forage grasses. Sugar cane, cassava, and sorghum are also important for alcohol production to meet the energy requirements of less-developed countries (89). Biomass production of wood and bagasse can be used directly as a source of combustibles.

If biological nitrogen fixation could replace the application of fertilizer nitrogen for the production of grass crops even in part, both developed and developing countries could benefit. The production of food and in part of energy could be possible without the application of high rates of fertilizer nitrogen. Nitrogen fixation in association with grasses is not a new concept; interest in this process developed early this century (119, 120, 198, 199). Recently, high potential rates of nitrogen fixation have been reported to be associated with forage and grain grasses (47, 208). The aim of this review is to evaluate the literature on associative nitrogen fixation in grasses and to indicate the areas needing further investigation.

NITROGEN BALANCE STUDIES: A MOTIVE FOR EXAMINING THE POSSIBILITY OF NITROGEN FIXATION IN ASSOCIATION WITH GRASSES

It has been suggested that levels of combined nitrogen in soil-plant systems increase because of nitrogen fertilizer application, accretion from the atmosphere, photochemical fixation, and plant-microbe symbiotic fixation (128). The reported data on nitrogen accumulation in soils are difficult to interpret; nevertheless, it has been concluded that nonsymbiotic nitrogen fixation occurs at magnitudes of agronomic significance (128).

The long-term nitrogen balance studies at Rothamsted, England, have provided convincing evidence for nitrogen accumulation due to nonsymbiotic nitrogen fixation. A part of the total nitrogen for the annual wheat crop in the permanent wheat experiment on Broadbalk is supplied by biological nitrogen fixation (106, 213). Some evidence for an association between nitrogen-fixing bacteria and wheat is available (46, 59, 158). Part of the continuous wheat experiment was fenced off in 1883, and the natural vegetation was allowed to return. This area, the wilderness, has gained nitrogen; it has been estimated that biological fixation has contributed 34 kg of N per ha per year (104–106). Nitrogenase activity in the rhizosphere of roots of plants common to the site has been reported (99), and estimates of nitrogen fixation by assays of acetylene reduction with soil cores of plants correlated well with the nitrogen accumulation data (46). Day et al. (46) reported that nitrogen fixation by legumes and blue-green algae was negligible and did not contribute significantly to the nitrogen accumulation at this site.

Nitrogen balance studies in the tropics involving forests and plant fallows have also provided evidence for nitrogen accumulation through nonsymbiotic nitrogen fixation. Accumulations of nitrogen in the range of 38 to 165 kg of N per ha per year in standing vegetation of plant fallows have been measured (20, 141). Nitrogen fixation in the rhizosphere of the roots of these grasses has been suggested as a mechanism to explain the substantial nitrogen gains observed under plant fallows (19, 91, 142). In many parts of the tropics sugar cane has been grown for centuries without additions of nitrogen fertilizers, and it has been suggested that nitrogen fixation occurs in association with this crop (136).

Salt marshes are among the most productive ecosystems in the world (103), even though nitrogen input into salt marshes is not sufficient to support the levels of production observed (92). Biological nitrogen fixation associated with roots is considered to be the major source of nitrogen for the salt marsh grass *Spartina alterniflora* (154).

Salt marshes and rice paddies are similar because both are regularly cropped aquatic systems (157). Paddy rice has been grown for centuries in the Far East without application of nitrogen fertilizers, and a long-term nitrogen balance study at the International Rice Research Institute in the Philippines has revealed constant yields of rice without nitrogen fertilizers (211). Although blue-green algae and photosynthetic bacteria are thought to be important in the nitrogen economy of paddies, nitrogenase activity associated with the roots of rice has been measured (11, 100, 107, 211, 215; P. van Berkum and C. Sloger, Plant Physiol. **63**:S479, 1979).

Therefore, it seems that a search for nitrogen fixation in grasses growing under pioneering conditions and/or in soil where nitrogen is known to accumulate is justifiable.

METHODOLOGY AND PROBLEMS ASSOCIATED WITH MEASUREMENT OF NITROGEN FIXATION IN GRASSES

The limitations of the methods used to measure nitrogen fixation in grasses have not been fully recognized. For this reason reports of evidence for and determinations of rates of nitrogen fixation in association with grass roots can be seriously criticized. It is crucial to realize that the backbone of a scientific subject is sound methodology. This allows objective interpretation of data and also allows intelligent, rational hypotheses to be formulated.

There are only a few reports of attempts to measure directly nitrogen fixation in field-grown grasses by using incorporation of $^{15}N_2$ (51, 64). Almost all of the existing literature deals with the measurement of nitrogenase activity by the indirect acetylene reduction assay procedure. In many cases the data can be criticized because of a delay between the removal of the tissues to be examined from their natural environment and the measurement for nitrogen fixation. This concern is relevant with all of the methods available for assessing nitrogen fixation. Therefore, it is imperative that nitrogen fixation be determined immediately after samples are removed from their ecosystems. Also, measurements with undisturbed intact plants are desirable.

Nitrogenase is able to reduce a wide range of substrates besides atmospheric nitrogen (35). The reduction of acetylene to ethylene specifically was proposed as an indirect method to assay for nitrogenase and has been used widely (94, 97, 98). Acetylene reduction by grasses has been measured by using in situ assays (8, 197), soil cores removed from the field (46, 197), pots containing greenhouse-grown plants (99, 100), and excised roots (1, 47, 64, 208). The soil surrounding the roots is usually included in the assay chamber when the in situ, intact soil core, and flower pot methods are used.

The in situ assay involves placing a metal cylinder around one or more field-grown plants and gently driving the cylinder into the ground to a depth of 5 cm (8) to 20 cm (197). Either the leaves of the plants are enclosed (8), or a cover is sealed to the protruding stem with caulking compound (197). Acetylene can be generated inside the device with CaC_2 and water (8). Since the bottoms of the cylinders are not sealed, gas may leak out. The loss of gas from assay chambers has been monitored by using propane or propylene as an internal standard (8) or by following the concentration of acetylene (197). Formulas for calculating acetylene reduction have been devised to take the gas loss into account (8).

In situ assays in which metal cylinders are not used to contain acetylene around the roots have been described for aquatic plants. This method takes advantage of the ability of these plants to transport air to the roots via lacunae (156, 204; van Berkum and Sloger, Plant Physiol. **63**:S479, 1979). The leaves and stem of a plant are enclosed in a suitable container (clear plastic cylinder or bag), which is closed at the top with a seal and sampling port and at the bottom by submerging the end below water level (204; van Berkum and Sloger, Plant Physiol. **63**:S479, 1979). Injected acetylene moves rapidly into the plant, and ethylene production can be monitored in the assay chamber or by sampling the hollow stems below the water level (204; van Berkum and Sloger, Plant Physiol. **63**:S479, 1979).

Measurements with soil cores removed from the field or with greenhouse-grown plants in pots or in containers with sediment or artificial soil are made by enclosing the entire sample in suitable sealed containers and injecting acetylene. The leaves of the plants are cut off (46), enclosed (7), or allowed to protrude from sealed assay chambers (197, 203).

There are several serious problems with the measurement of acetylene reduction by the in situ and intact assay methods. The in situ soil core assays are cumbersome, and the measurements are subject to variable interpretation, especially if soil is included in the incubation vessels. It is important to comprehend fully the limitations of these methods before measurements of acetylene reduction are extrapolated to nitrogen fixation. Because soil cores of grasses apparently failed to reduce acetylene immediately after removal from Brazilian fields, Abrantes et al. (1) suggested that samples should be allowed to "equilibrate" for 1 to 3 days after collection from the field before they are assayed. Other workers have also advocated this practice. Recently, van Berkum and Day (203) suggested that if soil cores of grasses were kept and watered for several days before they were assayed for nitrogenase activity, erroneously high estimates of in situ nitrogen fixation could result. The observed doubling of the rate of acetylene reduction by soil cores of *Paspalum notatum* after 1 week (47) supports this view.

By design, the in situ and intact assay methods entail the exposure of both plant and soil to acetylene. The possible interference of soil with the measurement of acetylene reduction should not be ignored. It is well known that soil microorganisms produce ethylene under anaerobic conditions (180). The subsequent oxidation of ethylene by other soil microorganisms at potentially 50 times the rate of ethylene production (40) prevents a significant accumulation of this gas in most soils. However, the inhibition of ethylene oxidation by acetylene has been reported (48). Therefore, it is evident that the incubations of soil cores without acetylene as controls to monitor endogenous ethylene production are valueless. Ethylene production and nitrogen fixation have similar requirements, and therefore both can be expected to occur in the root region. Measurements with $^{14}C_2H_2$ have indicated that substantial errors in estimates of nitrogen fixation are possible when the total observed ethylene accumulation is interpreted as acetylene reduction (213). Witty (213) also indicated that the degree of overestimation may vary, so labeled controls should always be used.

Interference with the measurement of nitrogenase activity in soil cores may also be caused by poor penetration of acetylene into soil (46, 203). The slow diffusion of ethylene through soil from sites of nitrogenase activity to sampling ports has also been reported. Profiles of acetylene and ethylene diffusion through cores of heavy moist soil indicated that long incubation times were required to saturate the entire samples with acetylene (203). However, acetylene is a competitive inhibitor of nitrogen fixation (170), and its ability to prevent synthesis of ammonia from nitrogen may be disadvantageous. It has been suggested that the depletion of nitrogen in nitrogen-fixing systems causes further induction of nitrogenase (176). David and Fay (43) concluded that, although the biochemical effects of prolonged incubations of nitrogen-fixing systems with acetylene are complex, the result is a marked enhancement of the rate of acetylene reduction. This is liable to cause an overestimation of nitrogen fixation in the field, indicating the necessity for short-term assays and the standardization of experimental conditions for comparative studies.

Several workers have attempted to modify the intact assay method to eliminate the problems caused by gas diffusion through soil. Studies of acetylene and ethylene movement through steel cylinders containing various types of soil have demonstrated that the rate of diffusion is primarily dependent on soil texture (203). Coarse sandy soil is least resistant to gas diffusion, and the possibility of monitoring immediate linear rates of acetylene reduction with grasses from such a soil type was demonstrated (203). Hirota et al. (100) overcame the resistance of gas diffusion through soil with intact plants in desiccators by displacement of air with a mixture of helium and acetylene, with rigorous evacuation. Using this technique, they were able to detect immediate reduction of acetylene with rice.

An alternative approach has been to remove the soil. Immediate linear rates of acetylene reduction occurred when intact salt marsh grasses and rice were removed from organic sediment without exposing the roots to air (van Berkum and Sloger, Plant Physiol. **63:**S479, 1979). However, the resistance of plant tissues to the diffusion of these gases has also been suggested. Profiles of the concentrations of acetylene and ethylene with time in the containers surrounding the leaves of these aquatic grasses indicated movement of the gases into the tissues before nitrogenase activity could be detected (van Berkum and Sloger, Plant Physiol. **63:** S479, 1979). A requirement for these gases to equilibrate between the sampling port and the nitrogen-fixing sites before linear rates of acetylene reduction are detected has also been demonstrated in soybeans (van Berkum and Sloger, Plant Physiol. **63:**S479, 1979) and in bacterial cultures (van Berkum, unpublished data). Therefore, if the aim of an investigation is to calculate rates of acetylene reduction, we recommend that results be based on short-term time course assays to ascertain whether linearity can be detected.

A major problem associated with the measurement of nitrogenase activity in cores of soil is determining the location of nitrogen fixation (197). Almost all of the evidence that nitrogen fixation is associated with the roots of grasses is based on studies in which the rates of acetylene reduction by preincubated excised washed roots and by soil were compared (47, 64, 65, 208). Initially, a correlation between the rate of acetylene reduction by soil cores and by preincubated excised roots from the grasses of the core samples was suggested (59).

The foremost enigma of the excised root assay has been the reported initial 8- to 18-h period before acetylene reduction begins (136). The delay before nitrogenase activity by excised roots commences is inconsistent with the kinetics of acetylene reduction by all other nitrogen-fixing systems (202). The picture is further complicated by observations that the duration of this period of inactivity varies between replicates (202) with the ontogeny of the plants (202), with the season (154, 202), and with nitrogen fertilizer treatment (202). Because it is difficult to interpret measurements of acetylene reduction after a long delay, the excised root assay was modified. An overnight incubation of roots without acetylene at a low partial pressure of oxygen (pO_2) (the preincubation period) permitted immediate detection of acetylene reduction (63). Preincubation of excised roots has been adopted as a routine method to prepare samples for the measurement of nitrogen fixation by grasses (1). The enormous variability among samples of excised roots subjected to this form of fixed-time assay (71, 208) may be explained by the large variation in time before nitrogenase activity commences between replicates and/or treatments.

Döbereiner et al. (65) suspected that inactivation of nitrogenase by entering O_2 when roots were excised from the plants caused the long delay before the detection of acetylene reduction. However, this hypothesis is inconsistent with several reported observations. *P. notatum* roots which were excised and prepared for measurement of nitrogenase activity in nitrogen did not reduce acetylene for the initial 12 h of assay (65). Furthermore, sorghum roots exposed to air after the preincubation period reduced acetylene within 2 h of this treatment upon assay (202). Also, it is becoming evident that excised or intact roots from some grasses are able to reduce acetylene in air (204; van Berkum and Sloger, Plant Physiol. **63:**S479, 1979). A transitory effect of entering O_2 on the nitrogenase activity of *Scirpus* roots which causes initial accelerated rates of acetylene reduction has been compared with similar observations in soybeans (van Berkum and Sloger, Plant Physiol. **63:**S479, 1979).

It has been suggested that excised roots assayed after an overnight preincubation period indicate the potential rate of nitrogen fixation in grasses (47, 208). However, the authors who made this suggestion provided no evidence that overnight is the length of time required for the preincubation of excised roots in order to reestablish nitrogenase activity at rates which reflect the potential of nitrogen fixation by the plants in the field. On the other hand, it has been suggested that the potential rate of nitrogen fixation in grasses may not be realized by the preincubation of excised roots for any length of time. Excised sorghum roots preincubated for longer times had higher rates of acetylene reduction than samples subjected to the usual overnight preincubation (202). Therefore, the overnight preincubation period of excised grass roots and the determination of acetylene reduction probably is not a measurement of the potential rate of nitrogen fixation by plants.

Studies comparing acetylene reduction measurements with excised roots and with soil cores have indicated that estimates of nitrogen fixation vary greatly between the two methods (Table 1). The use of the excised root assay results in higher estimates of nitrogen fixation (15, 145, 197, 202). Furthermore, the rates of acetylene reduction with soil cores of grasses and with excised roots from these samples did not correlate (197, 203). Subsequently, it was suggested that the excised root assay causes an overestimation of nitrogen fixation in grasses, because nitrogen-fixing bacteria proliferated substantially during the incubation time (Table 2). Therefore, measurements should be made with methods preventing microbial proliferation.

Abrantes et al. (1) showed that dipping excised roots in distilled water after they were excised allowed higher rates of acetylene reduction to be detected. They speculated that this treatment prevented complete inactivation of nitrogenase by entering oxygen. However, they reported a delay of 8 to 18 h before initiation of nitrogenase activity by roots treated in this way. For this reason, their hypothesis concerning the relationship between washing and enzyme protection is inconsistent with reports of immediate and linear rates of acetylene reduction by active nitrogenase (98).

The proliferation of nitrogen-fixing bacteria during preincubation of sorghum roots was prevented by not washing the samples or by incubation at a low temperature (4°C) (202). However, these treatments also prevented the development of nitrogenase activity during the preincubation period (1, 202). Substantial rates of acetylene reduction were associated with the wash water which remained in the assay bottles,

TABLE 1. *Comparison of determinations of nitrogen fixation by excised preincubated roots and soil cores of intact grasses*

No. of cores	Rate of nitrogen fixation with:		Reference
	Core assay	Preincubated root assay	
2[a]	271.0[b]	205.0[b]	65
10[c]	275.0[d]	67.0[d]	1
12[e]	14.5[d]	74.5[d]	114
23[f]	1.6[g]	68.0[g]	15
31[h]	2.6[d]	154.0[d]	71
33[i]	200.0[b]	2100.0[b]	197
24[j]	27.0[g]	337.0[g]	203

[a] *P. notatum*. Soil cores were flown from Brazil to England and kept for 1 to 4 weeks in a greenhouse before assay.

[b] Units: nanomoles of C_2H_4 per core per hour.

[c] *D. decumbens* cv. Transvala. Soil cores were assayed from 1 to 3 days after sampling in Brazil.

[d] Units: nanomoles of C_2H_4 per gram (dry weight) of roots per hour.

[e] Intact assays with *C. dactylon*, *D. decumbens*, *Paspalum orbiculare*, and *Chloris truncata* in Hawaii.

[f] Corn was inoculated with *Azospirillum* Sp81 in the United States (greenhouse-grown plants).

[g] Units: grams of N_2 per hectare per day.

[h] Intact assays with the following species of greenhouse-grown plants inoculated with *Azospirillum* in the United States: *Pennisetum clandestinum*, *C. dactylon*, *Zoysia japonica*, *Paspalum virgatum*, *Stenotaphrum secundatum*, and *Distichilis stricta*.

[i] Field-grown corn and sorghum at reproductive growth stages in Brazil. Cores were assayed immediately.

[j] Field-grown sorghum, *D. decumbens* cv. Transvala, *P. notatum*, and *B. mutica* in Brazil. Cores were assayed immediately after collection.

and large increases in the numbers of nitrogen-fixing bacteria were observed in this component after the preincubation period of excised roots (202). Vigorous growth of nitrogen-fixing bacteria was supported by the production of organic acids through anaerobic metabolism during preincubation of excised maize roots (143). No growth occurred with unwashed roots, and the proliferation of nitrogen-fixing bacteria during the preincubation period of excised roots resulted from the washing procedure (202). Therefore, it may be concluded that water from the washing procedure which adheres to the root samples enables anaerobic metabolism and the reported production of organic acids (145) to take place. The liberation of these substrates into the water component is responsible for the reported proliferation of the microflora and the induction of nitrogenase activity when combined nitrogen is sufficiently depleted to lift repression (202). Therefore, acetylene reduction by excised roots after a preincubation period does not reflect the ability of grasses to fix atmospheric nitrogen.

An immediate reduction of acetylene by excised roots from several grasses has been reported (204). No preincubation period was necessary to induce nitrogenase activity, no proliferation of the microflora during the incubation time was detected, and assays were done at atmospheric pO_2. Because of these observations it has been suggested that the ability of collected roots to reduce acetylene immediately upon assay in air indicates whether nitrogen fixation is associated with these roots in the field (202, 204; van Berkum and Sloger, Plant Physiol. **63**:S89, 1979; van Berkum and Sloger, Plant Physiol. **63**: S479, 1979). The inability of excised roots to reduce acetylene immediately upon collection would indicate that nitrogen fixation is not associated with them. As a result, a search for immediate acetylene reduction by excised grass roots has been proposed, and this procedure can be recommended as a method to screen for nitrogen-fixing plants.

CURRENT CONCEPTS OF NITROGEN FIXATION IN GRASSES

Many different types of diazotrophic bacteria have been isolated from natural and cultivated ecosystems (107, 108, 128). Evidence for the significance of these bacteria in the nitrogen economy of these environments and their possible benefit to higher plants is not convincing. Before 1972, the role of nitrogen fixation and its importance in the nitrogen economy of different habitats were usually inferred from the incidence of nitrogen-fixing bacteria. Moore (128) concluded that estimates of nitrogen fixation based on counts of nitrogen-fixing bacteria or determinations of their efficiency in pure cultures are not valid. It has been suggested that nitrogen fixation by free-living bacteria in cultivated land is widespread but generally at low rates (175). Measurements of acetylene reduction or incorporation of $^{15}N_2$ into carbohydrate- or cellulose-amended soils have demonstrated potentials for nitrogen fixation, which could occur periodically in nature and account for appreciable gains in nitrogen (18, 37, 49, 69, 75, 93, 122, 124, 151, 165, 185).

Findings of nitrogen accumulation under grasses (19, 91) and colonization of roots by bacteria (53, 108) have stimulated interest in monitoring nitrogen fixation associated with grass roots. The reported link between photo-

TABLE 2. *Most-probable numbers of nitrogen-fixing bacteria associated with excised grass roots before and after the preincubation period*

Plant	Initial no. of bacteria	No. of bacteria after the preincubation period	Reported increase in no. of bacteria (-fold)	Reference
Corn[a]	3.0×10^5	1.0×10^7	30	15
Forage grasses[a]	1.3×10^5–2.5×10^5	1.0×10^6–1.7×10^6	8	71
Corn[a]	0.15×10^4–1.3×10^4	0.19×10^6–3.2×10^6	665[b]	145
Sorghum[c]	0.1×10^5–8.3×10^5	0.24×10^7–3.3×10^7	152	202
B. mutica[c]	1.1×10^4	3.5×10^6	320	202
D. decumbens[c]	0.6×10^4	1.1×10^5	18	202

[a] Inoculated with *Azospirillum* sp. (greenhouse-grown plants).

[b] The increase was determined from the data presented by Okon et al. (145) (mean of five determinations).

[c] Field-grown plants at Km 47, Rio de Janeiro, Brazil.

synthate supply to legume nodules and nitrogen fixation (85), coupled with the realization that nonsymbiotic nitrogen fixation in soils is limited by available energy, has prompted investigations of the interactions between photosynthesis and nitrogenase activity in grasses.

Evidence for a Link Between Photosynthesis and Nitrogenase Activity

Photosynthesis supplies reductants, energy, and carbon skeletons for the assimilation of nitrogen into plants (25). Nitrogen fixation in legumes is dependent on photosynthate supply from the host to the bacteroids inside the nodules (6, 101, 179). However, the site of nitrogen fixation is far removed from the site of photosynthesis, and, therefore, a link between the two must be established by translocation of photosynthetic products (135). Observations of diurnal cycles of nitrogenase activity in legumes with acetylene reduction measurements (22, 98, 178) have suggested that the rate of nitrogen fixation is directly related to photosynthesis (23). In legumes this suggestion is supported by measurements of $^{15}N_2$ incorporation after periods of darkness (205), of the distribution of ^{14}C-labeled photosynthate in nodules (6), and of the incorporation of $^{14}CO_2$ into amino acids produced in the nodules (152, 153) and by observed increases in nitrogen fixation and biomass production through enrichment of the canopy with CO_2 (96).

A link between photosynthate supply to the roots and its effect on nitrogen fixation in grasses has been inferred from diurnal cycle studies of acetylene reduction and prolonged incubation of plants in the dark before measurement of nitrogenase activity (63, 65, 135). Döbereiner et al. (65) reported that nitrogen fixation by *P. notatum* was indirectly related to photosynthesis because no diurnal cycle of nitrogenase activity was observed, but prolonged incubations of plants in the dark reduced the rate of acetylene reduction. In contrast, Döbereiner and Day (63) reported a pronounced diurnal cycle of acetylene reduction by *P. notatum*, with peak activities at midday and at night. These authors suggested that solubilization of starch grains in the chloroplasts during the night was responsible for the peak activity at night (63). The dependence of nitrogenase activity on plant photosynthesis was also studied in test tube-grown sorghum seedlings. Döbereiner and Day (63) suggested that a marked diurnal cycle in the rate of acetylene reduction confirmed the dependence of nitrogenase activity on plant photosynthesis. Diurnal variations in the rate of acetylene reduction by *Panicum maximum* and *Lolium perenne* have also been reported (7). The interpretation of these data has been complicated by reports of no signficant diurnal variation in acetylene reduction by natural grasslands of *Hyparrhenia* sp., *Andropogon* sp., *Loudetia simplex*, and *P. maximum* (9). Contradictory data are also available for rice (9, 210), and no significant diurnal variation in acetylene reduction has been reported for some Wisconsin prairie grasses (194).

The possibility exists that other environmental factors influence nitrogenase activity, causing fluctuations in the rate of acetylene reduction, which may be coincident with the diurnal cycle. An examination of the parameters influencing the rate of acetylene reduction is essential for the interpretation of investigations suggesting a link between photosynthate supply and the rate of nitrogenase activity. This view is especially relevant since many environmental parameters have been shown to influence the rate of acetylene reduction in grasses (10). The diurnal cycle of nitrogenase activity in *Zea mays* is influenced significantly by temperature, as well as by the light energy received by the plants (10). A positive correlation between soil temperature during the day/night cycle and the diurnal cycle of nitrogenase activity in *Sorghum vulgare* and

Brachiaria mutica has also been reported (203). Also, Gibson (86) has suggested that nitrogen fixation in legumes responds quickly to changes in temperature. The effects of temperature on the rate of acetylene reduction by soil cores (113), by *Azospirillum* sp. in pure culture (44, 134), and by preparations of cell-free extracts of nitrogenase (35) have also been demonstrated. Clearly, the measurements of acetylene reduction rates during the day/night cycle which have been reported do not indicate that photosynthesis directly drives nitrogen fixation in the roots of grasses. Measurements of nitrogenase activity with experiments designed so that light intensity is the only variable during the diurnal cycle are needed. Variations in the rate of nitrogenase activity under these conditions will probably be due only to changes in illumination.

There have been attempts to show a relationship between nitrogenase activity and photosynthesis in grasses by using $^{14}CO_2$ feeding (157). A correlation between the rate of acetylene reduction after prolonged incubation of excised roots and total ^{14}C (respired CO_2 and alcohol extracted) was demonstrated. The label was probably released by the proliferating microbial population during the 35-h incubation of the excised roots to determine acetylene reduction. *S. alterniflora* has been reported to divert major portions of its net photosynthate into vegetative reproduction (R. Lytle, Jr., and R. Hull, Plant Physiol. **63**:S189, 1979). Therefore, the adenosine 5′-triphosphate driving nitrogenase activity by bacteria was probably formed during respiration and growth on stored carbohydrates, which derived their label from photosynthesis. The labeling of stored carbohydrates by $^{14}CO_2$ feeding may also account for the presence of alcohol-extractable ^{14}C. Respiration of stored carbohydrates by the excised roots themselves during the incubation may also have contributed to the accumulated $^{14}CO_2$. Nevertheless, the idea of using isotopic labels to investigate the possible interrelationship between photosynthesis and nitrogen fixation in grasses is a worthwhile approach. Photosynthate provides the carbon skeleton for the assimilation of nitrogen into plants (25); therefore, both $^{14}CO_2$ and $^{15}N_2$ feeding of plants should be used to link photosynthesis and nitrogen fixation.

C-3 and C-4 Photosynthetic Plants

Photosynthesis is a process in which carbon compounds are manufactured from CO_2 and water in the green tissues of plants, using light energy. Research into the biochemistry of photosynthesis has revealed that apart from the reductive pentose phosphate pathway cycle (C-3), many grasses possess the C-4 dicarboxylic acid cycle (25). However, some plants have also been characterized as intermediate between the C-3 and C-4 pathways (33, 34). Grasses possessing the C-4 dicarboxylic acid pathway of photosynthesis utilize their available nitrogen more efficiently in producing dry matter and fixing atmospheric CO_2 than grasses possessing only the C-3 pathway (25). Because of this, Döbereiner et al. (65) suggested that the C-4 dicarboxylic acid pathway of photosynthesis in grasses is important for the ability of these plants to stimulate nitrogen fixation by bacteria in their root zones. As the only evidence for their hypothesis, these authors stated that with the exception of rice, all of the plants which had been tested possessed the C-4 dicarboxylic acid pathway. The importance of the C-4 dicarboxylic acid in tropical grasses has been generally accepted (44, 47, 63, 64, 136). This hypothesis is certainly attractive if the following reasoning is considered. *Azospirillum* is the microorganism which is thought to be mainly responsible for the observed nitrogen fixation in grasses (135). The preference of these bacteria for malate and other organic acids as carbon sources has been documented (45, 64, 143). In fact, *Azospirillum brasilense* is not able to utilize glucose and sucrose as sole carbon sources for growth and nitrogen fixation (45, 64, 143, 189). Malate is the primary product of photosynthesis in some (but not all) of the C-4 grasses (e.g., *Z. mays, Digitaria*) (38). Therefore, it has been suggested that malate accumulation in the plants and transport of the excess malate to the roots, in which the bacteria have been suggested to reside (64, 155), favor nitrogen fixation (58, 64, 135). There are indeed some indications that malate may accumulate in the roots of *Z. mays* (138). Also, the oxidation of malate apparently does not stimulate nitrate uptake by the roots (138). However, all physiological studies of the role of malate in nitrogen assimilation in grasses have involved studies with leaf tissue (135, 139). As yet, there is no evidence for the hypothesis that malate accumulation in the roots of C-4 grasses drives nitrogen fixation by *Azospirillum* sp.

Döbereiner et al. (65) suggested that the possession of the C-4 dicarboxylic acid pathway of photosynthesis by tropical grasses favors the establishment of nitrogen fixation in the roots because of the ability of these plants to use the intense radiation in the tropics efficiently. However, not all C-4 grasses are restricted to tropical areas (25), and studies of nitrogen fixation with C-4 grasses in temperate areas have been reported (154, 156, 157, 204). Furthermore, nitrogenase activity has been recorded in grasses which do not possess the C-4 dicarboxylic acid pathway of photosynthesis; these include rice (100, 118, 210; van Berkum and Sloger, Plant Physiol. **63**:S479, 1979), wheat (46, 59, 158), and various other grasses (181).

Many of the reports indicating the importance of the C-4 dicarboxylic acid pathway and a tropical environment as significant factors for nitrogenase activity associated with the roots of grasses are from studies with preincubated excised roots (47, 63, 64; P. van Berkum and C. A. Neyra, Plant Physiol. **57**:S533, 1976). Studies involving assays with intact C-4 grasses have indicated low rates of nitrogenase activity or no activity (3, 70, 197, 203). Therefore, no significant information is available to indicate whether more efficient photosynthesis supports higher rates of nitrogen fixation in grasses. Studies comparing nitrogen fixation by plants representative of the different photosynthetic pathways under identical conditions will be necessary before this hypothesis can be confirmed.

Ontogenetic and Seasonal Profiles of Nitrogen Fixation in Grasses

In legumes useful parameters have been suggested for comparing cycles in different symbiotic systems (93). Apparently, great variability exists among different species of legumes, but the parameters appear to be sufficiently constant to enable the prediction of the yield or fertilizer requirements of a cultivar when the acetylene reduction assay is used. For this reason there has been interest in monitoring nitrogenase activity in grasses throughout the life cycles of the plants, probably to investigate interrelationships between fertilizer nitrogen application and nitrogen fixation. These studies have been done with both the excised root and intact soil core assay techniques.

Excised roots. Seasonal variations in the nitrogenase activities of excised roots of *Pennisetum purpureum* and *Digitaria decumbens* cv. Transvala have been reported (63). The roots of *P. purpureum* were most active in the hot summer months during rapid growth of the plant and decreased after flowering. *D. decumbens* apparently had to become established before maximal rates of nitrogenase activity were observed with excised roots (63). The application of fertilizer nitrogen did not affect the nitrogenase activities of the excised roots from these two grasses (63). This observation is contrary to reports of inhibition of nitrogenase activity by combined nitrogen in legumes (82–84), pure cultures of *A. brasilense* (144, 145), and other grass systems (47, 160; P. van Berkum, Ph.D. thesis, University of London, London, England, 1978). A possible reason for the apparent lack of inhibition of nitrogenase activity by combined nitrogen may have been the mode of fertilizer nitrogen application (20 kg of N per ha every 2 weeks) in combination with the assay method (excised roots 2 weeks after nitrogen application). The excised root assay in relation to plant ontogeny and fertilizer application is discussed below.

A variation in the nitrogenase activity of excised corn roots during the life cycle of the plants has also been reported (208). Maximal activities were recorded at "the 75% silking stage." Döbereiner (58) suggested that the foliar application of ammonium molybdate increased the nitrogenase activity, although no statistical difference between treated and control plants was reported (160, 208). Pereira et al. (160) were able to confirm that the maximal rates of nitrogenase activity by excised corn roots occur during the reproductive stage of plant growth. Similar observations have been made with sorghum, but there were significant increases in the nitrogenase activity of excised roots at anthesis after foliar applications of molybdenum (van Berkum, Ph.D. thesis).

The nitrogenase activities of excised roots of corn and sorghum were reduced significantly throughout the life cycles of these plants by fertilizer nitrogen application (160; van Berkum, Ph.D. thesis). Although the rates of acetylene reduction by excised roots of sorghum plants which received fertilizer nitrogen were significantly lower during the life cycle, the profiles of the nitrogenase activities by excised roots of the plants receiving high and low levels of nitrogen were parallel (van Berkum, Ph.D. thesis).

The rates of acetylene reduction by legumes are maximal during flowering and reproductive growth (94, 153). Therefore, the profiles of nitrogenase activity in grasses during their life cycles, measured with preincubated excised roots, have been used as evidence to support this method of assay (59). The variations in the nitrogenase activities of grasses during their life cycles may also be explained if preincubation of excised roots is necessary to induce nitrogenase activity in a proliferating microbial population. The period of inactivity in excised roots of sorghum varied with the ontogeny of the plant and was shortest at anthesis, when maximal rates of nitrogenase activity as measured by fixed-time assays with preincubated excised roots were recorded (202). The variation in the inactive period between replicates (202), coupled with nonlinear rates of acetylene reduction upon induction of nitrogenase (1, 202), probably causes differences in the mean rates of acetylene reduction to be recorded with fixed-time assays when roots from plants at different stages of development are investigated. When roots from low-nitrogen plants were treated with combined nitrogen and fertilizer nitrogen was applied to plants in the field, the period of no acetylene reduction by excised sorghum roots was extended (202). Therefore, the variation before activity between replicate root samples commences and the nonlinear rate of acetylene reduction upon induction of nitrogenase activity may also explain the apparent inhibitory effect of combined nitrogen on the nitrogenase activity which was observed with fixed-time assays with excised roots. Since growth of the nitrogen-fixing microflora during the preincubation period of excised roots has been recorded (145, 202), the observed seasonal variation in nitrogenase activity in grasses may be explained by a change in the ability of the roots to support growth of the microbial population and nitrogenase activity. Growth of the bacteria, coupled with a variation in the relative availability of combined nitrogen from the decaying excised roots, may explain the observed profiles of nitrogenase activity with plant ontogeny and the recorded inhibition of nitrogen fertilizer application.

Further evidence that combined nitrogen may play a causative role in the seasonal profile of nitrogenase activity in sorghum and corn may be sought from studies interrelating excised root acetylene reduction and leaf nitrate reductase activity. Nitrate is both the inducer and the substrate for nitrate reductase in plants, and factors affecting uptake and transport of nitrate have been shown to alter nitrate reductase activity levels and the rate of supply of reduced nitrogen to plants (127). The levels of leaf nitrate reductase activity in corn (160) and sorghum (70; van Berkum, Ph.D. thesis) vary with plant age. The maximal rates of nitrogenase activity determined with excised preincubated roots coincided with the lower levels of leaf nitrate reductase activity (160; van Berkum, Ph.D. thesis). Therefore, it may be possible to extrapolate that the reduced level of leaf nitrate reductase activity is indicative of lower levels of combined nitrogen in the plants, including the roots, which in turn allows a shorter delay before induction of nitrogenase activity when excised roots are assayed for acetylene reduction.

Although the excised root assay clearly indicates variations of nitrogenase activity during plant life cycles, the patterns observed most probably do not reflect a variation in the nitrogen input to the plants through biological nitrogen fixation. Indeed, it seems more probable that the variations in the rate of acetylene reduction during the life cycles of the plants reflect a change with plant age in the ability of excised roots to support both microbial growth and nitrogenase activity upon preincubation.

Soil cores. Seasonal variations in nitrogenase activity determined with acetylene reduction by soil cores of grasses have also been studied, but there are few examples relating these variations to plant ontogeny. Balandreau (7) used an in situ assay system to measure the rate of acetylene reduction during the growth cycle of *Z. mays*. The rate of nitrogenase activity reportedly increased during plant development and after vegetative growth stabilized. Balandreau (7) hypothesized that at the late vegetative stage of plant growth the maximum photosynthetic capacity of the plant is reached, on which the development of maximal rates of nitrogen fixation is dependent. No further variation in the rate of nitrogenase activity during the reproductive phase of plant growth and no relationship to plant ontogeny was reported (7).

Seasonal variations in the nitrogenase activities of seven Wisconsin prairie grasses have also been reported (194). Nitrogenase activity increased in parallel with the growth of the leaves in two of the grasses tested and declined with leaf senescence. Tjepkema and Burris (194) sugested that this observation showed that the reduction of acetylene is effected by bacteria which depend on the grass for their energy supply. However, in five other species tested they were not able to demonstrate that the reduction of acetylene was specifically plant associated. There was no indication of how the seasonal profile of nitrogenase activity was related to plant ontogeny.

A seasonal variation in the nitrogenase activity of rice has been related to plant ontogeny. Assays with intact plants have indicated that maximal rates of acetylene reduction occur during the reproductive growth stages (118). Interestingly, excised root assays with rice have also been reported to show a seasonal variation in nitrogenase activity, but a relationship between maximal activity and ontogeny was not reported (87). The nitrogenase activity of the salt marsh grass *S. alterniflora* also varies seasonally (157).

The increase in the rate of nitrogen fixation in legumes coincides with the onset of the reproductive growth stages (153). Hardy et al. (98) suggested that the developing pods are a sink for combined nitrogen which stimulates higher rates of nitrogen fixation. The maximal rates of acetylene reduction by grasses may also be caused by flowering, with a mechanism similar to that suggested for legumes. The limitation to this hypothesis is the indirect method of assay that has been used to study the seasonal profiles of nitrogenase activity in these grasses. At flowering, corn and sorghum root systems are fully developed, which has been suggested to be a prerequisite for support of maximal rates of nitrogenase activity (136). It has also been reported that at flowering the predominant root type on corn and sorghum supports the highest rates of acetylene reduction after the preincubation period (van Berkum, Ph.D. thesis). This may also be a contributing factor to the observed seasonal profiles of nitrogenase activity in these grasses. It has been suggested that the highest rates of nitrogenase activity coincide with the maximum rates of photosynthesis (7, 194) and that the decline in nitrogenase activity after the maximal rates are reached is due to competition between the ovule and roots for photosynthate (136). On the other hand, von Bülow and Döbereiner (208) have suggested that nitrogenase activity in corn roots does not compete for photosynthate with other plant parts.

Very little is known about the cycles of nitrogen fixation in grasses, and even less is known about the interrelationship between this process and others within the plants. Many interpretations and extrapolations have been made with only scanty evidence. Future research should be more carefully controlled, and only the measured parameters should be interpreted.

Effect of Soil Moisture on Nitrogenase Activity

During their studies of nitrogen gains on Broadbalk at Rothamsted, Day et al. (46) noticed that high rates of nitrogenase activity were associated with soil cores from wet areas. They reported that the rate of nitrogenase activity by the soil cores was positively correlated with soil moisture and that the rate of acetylene reduction increased exponentially with linear increases in soil moisture. Vlassak et al. (206) reported a similar correlation between soil moisture and nitrogen fixation in soil cores of grasslands. Day et al. (46) hypothesized that the level of anaerobiosis in soil crumbs and the rhizosphere increases with higher soil moisture and that the pO_2 affects nitrogenase activity. Their hypothesis is supported by observed higher rates of acetylene reduction by soil cores incubated under N_2 compared with soil cores incubated under air (196).

The relationship between soil moisture and rate of nitrogenase activity is contradicted by a report of no correlation between the rate of acetylene reduction by *D. decumbens* roots and the soil water content (1). However, this observation was based on assays with preincubated excised roots to determine the rate of nitrogenase activity. As explained, this method of assay includes the washing of roots in distilled water before the preincubation period. The washing of roots is required for bacterial proliferation and the induction of nitrogenase activity during the preincubation period. Therefore, by washing the roots, Abrantes et al. (1) were assaying for nitrogenase activity under optimal moisture conditions.

The reported positive correlation between soil moisture and nitrogenase activity supports the hypothesis that nitrogen fixation associated with nonnodular plants may be enhanced in wet soils (196). Nitrogenase activity associated with wetland plants has been reported to be widespread (196) and to be much higher than the nitrogenase activity associated with plants growing in mesic or dry soils (109). These observations suggest that the growth habitat of grasses is an important factor for the development of nitrogen fixation in association with the roots. The importance of a moist environment for nitrogen fixation in grasses may also explain why the rates of acetylene reduction with agriculturally important cereal grasses assayed with soil cores (46, 197) are generally low and those with salt marsh plants and paddy rice (100, 204) are much higher. It has been suggested that these aquatic environments and high-moisture soils are low in available nitrogen due to denitrification and leaching of nitrates (194). If the effect of excess water is to deplete the environment of levels of combined nitrogen inhibitory to nitrogen fixation, it might be possible to manipulate associations with dry soil plants to overcome this problem.

Plant Breeding

The success with which grass lines are selected for improved ability to support nitrogenase activity and benefit from nitrogen fixation will ultimately depend on the reliability of the measurements of these processes. Without a technique which unequivocally distinguishes the rate of nitrogen fixation in grasses from other processes, the selection of improved varieties is certainly doomed. Clearly, no reliable method for measuring nitrogen fixation in grasses for the selection of desired genotypes has yet been used. Practical experience with legumes has indicated that the acetylene reduction assay is not a reliable tool for plant selection and measurements of final yield fail to distinguish between lines with improved nitrate reductase and lines with enhanced nitrogen fixation.

Nevertheless, the possibility of plant breeding for enhancing nitrogen fixation in grasses has been suggested. Investigations with *P. notatum* have revealed that the nitrogen-fixing bacterium *Azotobacter paspali* specifically associates with five tetraploid ecotypes (55, 57, 61). Only the 5 tetraploid ecotypes out of 33 stimulated *A. pas-*

pali growth in the rhizosphere of the roots. It has been suggested that other microorganisms (e.g., mycorrhizal fungi) may be involved in the establishment of this association (136). These reports are significant because the conclusions are based not on measurements of acetylene reduction, but on investigations of the ability of the plants to stimulate growth of specific bacteria. Unfortunately, further studies of the microbial ecology of this association have not been reported. It would be interesting to determine whether the preferential stimulation of specific bacteria by roots is at the expense of other populations within the microbial ecosystem of the soil. In the case of *A. paspali*, this knowledge would be of interest because these bacteria reportedly reside outside the roots and are subject to competition for space and nutrients. Also, why do the five tetraploid ecotypes specifically stimulate this nitrogen-fixing microbe, and what is the mode of action? An understanding of this specific association may enable the development of plants which also stimulate growth of desirable bacteria in the rhizosphere of their roots. If this achievement is coupled with the development of an infection process, true symbioses between grasses and nitrogen-fixing bacteria may become a reality.

By using existing techniques to determine nitrogen fixation, differences in the ability to stimulate nitrogenase activity in roots have been found among different varieties and lines of grasses. Using the excised root assay, von Bülow and Döbereiner (208) demonstrated that S1 lines of corn derived from UR-1 had consistently higher rates of nitrogenase activity. Genotypic differences have also been found in *P. notatum*, *P. purpureum*, and wheat (47, 59, 63, 64). The major limitation of these data is the excised root assay, which was used to determine the rates of acetylene reduction. As discussed, the higher values of acetylene reduction by excised roots after the preincubation period probably reflect the greater ability of some roots to support growth and subsequently nitrogenase activity by nitrogen-fixing bacteria during the assay.

$^{15}N_2$ Measurements of Nitrogen Fixation in Grasses

Only data obtained with measurements of the incorporation of ^{15}N from $^{15}N_2$ can be used to support claims that grasses benefit immediately from root-associated nitrogen fixation. Ideally, the plants should be exposed to $^{15}N_2$ under normal growing conditions in the field. The major limitations to measuring ^{15}N incorporation are the sizes of most of the grasses of interest. To expose these large plants to $^{15}N_2$ would require large volumes, so that the expense of these experiments could be prohibitive. Therefore, we recommend that the acetylene reduction assay be used to establish the reproducibility of $^{15}N_2$ investigations and to assess the repeatibility of detecting nitrogen-fixation in plants.

De-Polli et al. (51) suggested that the incorporation of $^{15}N_2$ into *P. notatum* and *D. decumbens* confirmed nitrogen fixation by these two grasses. These data have also been used to suggest that observations of acetylene reduction with other grasses demonstrate that they benefit from nitrogen fixation (58, 60, 136). Although the incorporation of $^{15}N_2$ was demonstrated with these two grasses, there was a long time between the collection of samples from the field and experimentation (between 4 and 16 days). Furthermore, the enrichment obtained was not extrapolated to rates of nitrogen fixation, and no comparisons between $^{15}N_2$ incorporation and acetylene reduction were presented.

Studies with sugar cane seedlings have also indicated some $^{15}N_2$ incorporation, but no details of rates of nitrogen fixation have been presented (167). Although they reported little ^{15}N enrichment in the leaves, Ruschel et al. (168) calculated that sugar cane seedlings fixed between 0.2 and 23.8 g of N per ha per day. Studies with undisturbed sugar cane plants in the field showed no ^{15}N enrichment in the leaves or stems (125).

ECOLOGY OF THE NITROGEN-FIXING BACTERIA THAT ASSOCIATE WITH THE ROOTS OF TROPICAL GRASSES

Nitrogen-fixing bacteria are probably among the most extensively studied soil microorganisms. Despite this, very little is known about their biology in nature and their contribution to the structure and function of the habitat that they occupy. Of all the diazotrophs, only the symbionts of leguminous plants, *Rhizobium*, have been studied in any detail. However, even with these organisms, most of our knowledge comes from the study of *Rhizobium* in its symbiotic niche, the root nodule. Very little has been learned regarding its ecology as a free-living member of the soil and rhizosphere microflora (26).

Nitrogen-fixing bacteria of many diverse genera occur in high numbers in the rhizosphere of the roots of a variety of grasses. Most notable among them are members of the genera *Azospirillum*, *Azotobacter*, *Beijerinckia*, and *Derxia*. However, the mere presence of these bacteria, even in high numbers, is not accepted as being important to the nitrogen economy of the system.

The problem has been one of methodology, for it is difficult to separate the multitude of factors involved in the interaction between two organisms within a highly complex environment, such as soil. It is essential that the ecological interactions between nitrogen-fixing bacteria in the soil and the rhizosphere be understood before progress can be made in manipulating the system for maximal economic return. Test tubes do not provide a good model of the natural habitat of the organisms, because it is impossible to simulate the plethora of interactions that operate in nature. Superior strains and improved mutants manufactured in laboratories may have little ecological relevance and even less agronomic value. New approaches and methodologies are needed for the study of the organisms directly in their natural environment.

Several recent reviews have covered the asymbiotic and associative nitrogen-fixing bacteria exhaustively. Here we only consider some of the important ecological features of the microorganisms in general and focus specifically on *Azospirillum* in tropical soils and its relationship to the roots of tropical grasses. For further details the recent reviews by Day (44), Knowles (113), and Neyra and Döbereiner (136) should be consulted.

Distribution and Biogeography

Diazotrophs are widely distributed in nature. They have been found in a wide variety of terrestrial and aquatic habitats, ranging from the polar regions to the tropics. Some of them have quite narrow ecological distributions. For example, *Beijerinckia* is considered to be restricted to the tropics (21, 56), although its presence in temperate soils and even in the polar regions has been demonstrated (4, 21, 27). *A. paspali*, although capable of free-living nitrogen fixation, has been shown to be restricted ecologically to the rhizosphere of the roots of some tetraploid varieties of *P. notatum* (136). *Azospirillum* sp. has been isolated from soils and the roots of a wide variety of plants from temperate and tropical regions (67, 145, 208). Most investigators consider this organism to be of special significance only in the tropics (44, 136). Schröder (171) found nitrogen-fixing spirilla in all but 2 of 76 soil samples from Germany and Austria. Döbereiner et al. (67) reported that less than 10% of the soils and roots from temperate regions contained *Azospirillum*, whereas more than 50% of tropical samples were positive. Alluvial soils were more favorable for *Azospirillum* than eroded hill soils. Although the pH of the soil was shown to have a strong influence on the distribution of *Azospirillum* (pH 7 is optimal), sporadic occurrence could be demonstrated in soils with pH values as low as 4.8.

Taxonomy of *Azospirillum*

The group of gram-negative nitrogen-fixing spirilla which was originally named *Spirillum lipoferum* by Beijerinck has been reclassified into at least two species in the new genus *Azospirillum*, *A. brasilense* and *Azospirillum lipoferum*. The species are differentiated by physiological criteria and deoxyribonucleic acid homology (189), but immunofluorescence and immunodiffusion studies have recently indicated antigenic differences as well (50, 170). *A. brasilense* contains two subgroups, nir$^+$ and nir$^-$, based on the ability to denitrify nitrite (137). Both groups possess assimilatory and dissimilatory nitrate reductase, but *A. brasilense* nir$^-$ does not dissimilate nitrite (123). *A. lipoferum* is also differentiated from *A. brasilense* by its ability to utilize glucose as a sole carbon source and by its requirement for biotin (189).

Serological analyses have revealed differences between the species and subgroups of *Azospirillum* (50, 170). Immunodiffusion showed that at least one heat-labile gel-diffusable antigen was common to 27 strains belonging to the different subgroups of *Azospirillum*, whereas heat-stable somatic antigens were more specific (50). Strain and species differences have also been demonstrated by immunofluorescence, but as cultures age, their degree of cross-reaction increases (50, 171).

Autecology

Important questions concerning the autecology of *Azospirillum* in soils and rhizospheres remain unanswered. These questions relate directly to the nature of the *Azospirillum* niche in roots, the ecological parameters involved in the establishment of this niche, the performance of the bacteria in the soil away from its natural niche, and the fate of specific strains of *Azospirillum* inoculated into new environments.

The difficulty is again one of methodology. Immunofluorescence is perhaps the only direct method for studying specific groups (even strains) of microorganisms directly in nature (26) and may provide the only opportunity for the study of *Azospirillum* in relation to its niche.

Information already available on the serology of *Azospirillum* spp. (50, 170) could be valuable in asking relevant questions which could be approached experimentally and would be ecologically meaningful. Preliminary attempts by Schank et al. (170) to detect a strain of *A. brasilense* in root and rhizosphere preparations from inoculated field-grown plants have been encouraging. However, more quantitative studies are needed to clarify the role of these bacteria in the rhizosphere of grasses.

Rhizosphere Competence and the Specificity of Association

The rhizosphere is an ideal habitat for heterotrophic nitrogen-fixing bacteria. The organic materials available from root cell debris and soluble root exudates are perhaps sufficient to satisfy the energy and carbon requirement of the heterotrophic free-living nitrogen fixers. However, to be of much significance to plants, these bacteria must reach very high population densities. In legume systems, a highly integrated association between the partners has evolved, whereby the microsymbionts occupy a specialized niche inside plant-derived nodules. In the grass associations the intimacy of the interaction is not as obvious.

Free-living heterotrophic nitrogen fixers are undoubtedly quite competent rhizosphere organisms. They occur in the rhizospheres of a variety of plants (108, 113, 136). However, the extent to which nitrogen fixation by the associated organisms benefits the plants has not been determined.

Asymbiotic nitrogen fixation is probably closely tied to the growth of the nitrogen-fixing microorganisms. Thus the amounts of nitrogen fixed are directly proportional to the growth responses of the bacteria to particular environmental stimuli.

Very little information is available concerning the population ecology of asymbiotic nitrogen fixers in the rhizosphere. The study of this problem requires a direct approach, whereby the organism of interest is identified in situ. Preliminary attempts at using immunofluorescence for autecological studies of asymbiotic nitrogen fixers directly in soil and rhizospheres have been promising (52, 54, 169, 191), but more detailed and quantitative studies are needed to understand the population ecology of asymbiotic nitrogen fixers in rhizospheres (26).

There would be a great competitive advantage for both the nitrogen-fixing bacteria and the plants if a more intimate internal association could be established. The ideal relationship is that between rhizobia and their leguminous hosts, whereby the bacteria multiply to high populations inside their specialized root nodule niche on legume roots. Other microorganisms, such as mycorrhizal fungi and the symbiotic nitrogen-fixing actinomycete *Frankia*, may also occupy inter- and intracellular spaces inside roots and establish highly integrated mutualistic associations with their plant hosts. Of special interest are the unidentified "helically lobed" bacteria that Old et al. (147) have reported inside collapsed epidermal cells of *Ammophila arenaria* and within roots of *Z. mays*. Entry of bacteria into epidermal and cortical cells seems to occur by intercellular penetration of the epidermal cells (78, 148).

Evidence for the occurrence of asymbiotic nitrogen-fixing bacteria inside roots has been indirect. *Azospirillum* has been isolated from "surface-sterilized" roots of field-grown grasses (67). This organism has been shown to be capable of penetrating roots of *P. maximum* grown axenically in Fahraeus slide chambers (201). Diem et al. (53) reported that nitrogen-fixing bacteria can be isolated from surface-sterilized rice roots, but only if the roots are crushed to release the bacteria.

Döbereiner and Day (64) observed that root pieces exhibiting high acetylene reduction activity after the preincubation period had cells which were packed with tetrazolium-reducing bacteria-like particles. Likewise, Patriquin and Döbereiner (155) found tetrazolium-reducing bacteria-like structures in inter- and intracellular spaces of some plant cells in the cortex, endodermis, xylem, and stele.

With the exception of the *Rhizobium*-legume system, very little has been learned about the specificity of the associations between nitrogen-fixing bacteria and plants. *A. paspali* seems to be restricted ecologically to the rhizosphere of some varieties of *P. notatum* (see above). On the other hand, *Beijerinckia* associates with the rhizospheres of a variety of tropical plants, including sugar cane, rice, and *Cyperus* (56). *Azospirillum* is known to colonize the rhizosphere of the roots of a wide variety of plants. Baldani and Döbereiner (Soil Biol. Biochem., in press) have suggested that only specific species are capable of endosymbiotic associations with particular host plants. *Azospirillum* isolates from field-grown corn roots were always *A. lipoferum*, and those from wheat and rice roots were mainly *A. brasilense* nir$^-$. Knowledge of the specificity of association between *Azospirillum* and various grasses and cereals may have significant implications in establishing nitrogen-fixing associations with selected or genetically manipulated strains of bacteria.

INOCULATION WITH NITROGEN-FIXING BACTERIA

The legume-*Rhizobium* symbiosis is a good example of accumulated knowledge being applied to agriculture. Research has led to the development of techniques for inoculating plants with nitrogen-fixing bacteria and for the assessment of the performance of the resulting symbioses. The inoculation of legumes with *Rhizobium* was found to be necessary because many soils were shown to be devoid of particular strains which were able to nodulate effectively the crops to be planted (28). *Azospirillum* reportedly is common in tropical soils, but its incidence in soils of temperate countries has been shown to be much lower (67). The possibility of establishing *Azospirillum* on roots of corn (where the incidence of these bacteria is low) through inoculation has been suggested (67). It is anticipated that inoculating grain and forage grasses with these bacteria would result in yield increases through nitrogen fixation (2, 67). The high rates of acetylene reduction with excised preincubated corn roots (208) and the reports that *Azospirillum* spp. are primarily responsible for this activity (44, 64, 208) have lended support to this prospect. We wish to emphasize that *Azospirillum* spp. have been isolated from the roots of grasses which reduce acetylene only after a long delay, during which time the bacteria proliferate and induce nitrogenase activity. Whether *Azospirillum* spp. are responsible for the reported measurements of in situ nitrogenase activity in grasses has not been demonstrated. Furthermore, there is no evidence that nitrogenase activity by *Azospirillum* is closely coupled to nitrogen assimilation by grasses. Therefore, inoculating grasses with *Azospirillum* to obtain agriculturally significant yield increases through nitrogen fixation is based on speculation of the importance of these microorganisms for nitrogen fixation in grasses, and in actual practice might have little or no effect.

Initial observations in Florida indicated that 2 of 40 genotypes responded to an inoculation of *Azospirillum* (182, 183). One genotype of *D. decumbens* and one of *P. maximum* yielded at 163 and 150%, respectively, of the uninoculated controls in the presence of these bacteria. No response to inoculation was reported with the other 38 genotypes tested, which included *D. decumbens*, *P. maximum*, *Paspalum* sp., *Cynodon* sp., and *Cenchrus* sp. (183). Fertilizer nitrogen application was necessary to induce increases in dry matter in *P. maximum*, *Pennisetum americanum*, and *Cenchrus ciliaris* when inoculated with *Azospirillum*, but no total nitrogen increase was reported (182–184, 190). On reexamination, *D. decumbens* did not respond to the inoculation of *Azospirillum* (184). Nevertheless, Smith et al. (182) concluded that up to 40 kg of N per ha can be provided to grasses through nitrogen fixation if plants are inoculated with *Azospirillum*. However, acetylene reduction assays did not correlate well with the yield responses (184), which could indicate that the reported dry matter increases because of *Azospirillum* inoculation may have been the result of processes other than nitrogen fixation. Total nitrogen increases with *Cynodon dactylon* grown in pots have also been reported when inoculated with *Azospirillum* and *A. paspali* (12). No genotype-inoculation interaction was found, inoculated plants responded well to high fertility, and no measurements for nitrogen fixation were reported (12). *C. dactylon* inoculated with *Azospirillum* and grown in pots reduced little or no acetylene when intact plants were assayed (71). The inoculation of corn with *Azospirillum* did not cause yield increases (3; C. Sloger and L. D. Owens, Plant Physiol. **61**:S7, 1978), nor were higher rates of acetylene reduction with intact plant assays detected (3, 14, 36; Sloger and Owens, Plant Physiol. **61**:S7, 1978). Similarly, *P. americanum* and *Sorghum* sp. did not benefit from the inoculation of *Azospirillum* (Sloger and Owens, Plant Physiol. **61**:S7, 1978).

Plant Growth Response to Inoculation Through Processes Other than Nitrogen Fixation

The treatment of seeds or seedling roots with cultures of bacteria to improve plant performance and crop yield is known as seed or root bacterization. The value of *Rhizobium* as an inoculant for seeds of legumes is well known, but *Azotobacter* inoculation as the bacterial fertilizer azotobactrin has also been developed. The bacterium *Azotobacter chroococcum* has been used as an inoculant because of its ability to fix atmospheric nitrogen. Large areas of the Soviet Union have been treated with this preparation because it was thought that crops would benefit and yield increases would result from this practice (39). Brown (30) indicated that bacterization of corn, wheat, barley, oats, and rice in Eastern European countries caused yield increases and that scientists from other countries confirmed many of the Soviet observations. Changes in the rhizosphere microbial population, disease suppression by the inoculants, and production of plant growth-promoting substances by the inoculum are alternative modes of action that have been suggested as explanations for the observed yield increases when these bacteria are applied to crops (30). It has been suggested that the inoculation of *Azotobacter* effectively removes bacteria which produce metabolites with the potential to retard seedling development (29).

Only limited success of disease suppression through inoculation has been shown (30).

Azotobacter cultures used as inoculants have been reported to produce gibberellic acid, indole 3-acetic acid, and cytokinin, which promote seedling and plant growth (16, 32). Gibberellic acid also increases the levels of endogenous indole 3-acetic acid in plants (150), and root exudation of tryptophan and related substances may cause indole 3-acetic acid synthesis by the inoculum in the root zone (30). The production of plant growth hormones by *Azotobacter* in pure cultures (used as inoculant) and by these bacteria in the root zone has been shown to cause significant plant growth responses (30). *A. paspali* inoculated onto tomato, lettuce, *Centrosema pubescens*, and *P. notatum* has also been shown to significantly improve plant growth (16). Brown (31) suggested that improvement in *P. notatum* pasture growth could be the result of plant growth-regulating substances produced by *A. paspali* rather than nitrogen fixation.

Azospirillum also produces plant growth hormones in pure culture (194). Also, plant growth responses observed after inoculation of *Azospirillum* have been explained by hormone production by these bacteria (88, 102, 193).

Therefore, we may conclude that the methodology used to study increased plant performance resulting from the inoculation of nitrogen-fixing bacteria is complicated and that deductions concerning improvements resulting from nitrogen fixation depend primarily upon the quantitative detection of this process. The application of nitrogen-free bacterial growth media to control plots (182–184) adds carbon to the soil and may tie up part of the available nitrogen, causing an apparent response in the inoculated plots. The inoculation of *Azospirillum* to soils which contain these bacteria (184) may hide the detection of any small plant growth response to inoculation. The production of plant hormones by the inoculant could also explain yield increases, especially if acetylene reduction measurements do not reflect the response. Ideally, inoculation studies should involve the application of nitrogen-fixing bacteria to experimental plots, along with application of nitrogenase-less mutant counterparts to control plots. Plant growth hormone production by these mutants must be identical to hormone production by the parent strains.

PHYSIOLOGY OF NITROGEN-FIXING BACTERIA THAT ASSOCIATE WITH THE ROOTS OF GRASSES

All bacteria which have been isolated from the roots of grasses able to express nitrogenase are also capable of fixing nitrogen for growth in pure culture (136). It is for this reason that this group has been classified as free-living nitrogen-fixing bacteria (130). By contrast, *Rhizobium*, although capable of expressing nitrogenase activity in pure cultures, requires a source of combined nitrogen for growth (110, 111, 115, 126, 195). Therefore, the major difference between *Rhizobium* and other free-living nitrogen-fixing bacteria is the ability of the latter group to grow in pure culture with atmospheric N_2 as the sole nitrogen source.

There are many reports of associations between free-living nitrogen-fixing bacteria and roots of plants (108, 136). Since this review is concerned with aspects of associative nitrogen fixation in grasses, descriptions of the physiology of nitrogen-fixing bacteria are restricted to those bacteria for which nitrogen-fixing associations with grass roots have been suggested.

In some cases roots of grasses have been shown to support acetylene reduction, and nitrogen-fixing bacteria have been cultured from these samples (13, 53, 64, 72, 99, 208, 209). However, the isolation of nitrogen-fixing bacteria in pure culture does not prove that an isolate fixes nitrogen or is solely responsible for nitrogenase activity in grass roots. If these were the criteria for demonstrating the causative bacteria for acetylene reduction by roots, then *Rhizobium* would not have been discovered until a few years ago. Therefore, it is quite possible that nitrogen-fixing bacteria which associate with grass roots but require a combined nitrogen source for nitrogenase activity in pure culture have been missed and are still to be discovered. The complication with associative nitrogen fixation in grasses is the absence of any obvious structure housing the bacteria responsible for nitrogen fixation.

Because of problems with the identification of bacteria responsible for nitrogen fixation in association with grasses, isolations have usually been made with enrichment cultures of roots. By comparing the rates of acetylene reduction by roots and the numbers of bacteria, Döbereiner et al. (65) concluded that *A. paspali* is responsible for associative nitrogen fixation in *P. notatum*. Studies of nitrogenase activity, dry matter, and total nitrogen accumulation related to numbers of bacteria with several *P. notatum* genotypes demonstrated that a potential for nitrogen fixation in this grass depended on the presence of *A. paspali* (47).

Döbereiner and Day (64) and von Bülow and Döbereiner (208) demonstrated correlations between surface-sterilized excised roots and enrichment culture acetylene-reducing activity.

Using this method, they concluded that *Azospirillum* are the bacteria responsible for most of the nitrogenase activity inside grass roots (136). Since it has been shown that the bacteria must proliferate for nitrogenase activity to be established in excised roots (202), it is obvious that the success with which root surfaces are sterilized is absolutely critical. In none of the descriptions reporting the isolation of nitrogen-fixing bacteria from inside roots has the surface sterility of the roots been demonstrated unequivocally. Nevertheless, *Azospirillum* spp. have attracted most of the attention as the bacteria responsible for nitrogen fixation in grasses because of their suggested location in relation to roots (64, 159) and because of their wide distribution (67). Therefore, our discussion on the physiology of nitrogen-fixing bacteria is confined to *A. paspali*, *A. brasilense*, and *A. lipoferum*.

Carbon Metabolism and Oxygen Supply

As discussed before, it is thought that nitrogen fixation by bacteria in the region of the roots derives energy from translocated photosynthate. Photosynthate may also provide carbon skeletons which serve a purpose in the assimilation of fixed nitrogen into bacteria and therefore may be precursors of the nitrogenous compounds released to the plants. Salts of malic, succinic, lactic, and pyruvic acids have been shown to support vigorous growth and acetylene reduction in pure cultures by all of the bacteria under discussion (45, 64, 144). *A. paspali* and *A. lipoferum* are also able to utilize glucose and sucrose as sole carbon sources for growth and nitrogen fixation (64, 189). By contrast, glucose and sucrose reportedly are poor substrates for *A. brasilense* (45, 64, 189). The correlation between organic acids as potentially good substrates for *Azospirillum* and their accumulation in the roots of C-4 grasses has been emphasized in support of the suggestion of a symbiotic association between these bacteria and grasses (64).

Azospirillum spp. are aerobic bacteria which require low pO_2 for the expression of nitrogenase activity (189). Studies of oxygen uptake by cell-free extracts of *A. brasilense* with malate, succinate, lactate, and pyruvate have indicated that the tricarboxylic acid cycle is operative in these organisms (143). The failure to detect enhanced levels of O_2 uptake with substances that support poor growth and nitrogen fixation (hexoses and their phosphate derivatives) has shown that the glycolytic and pentose phosphate pathways are of minor significance in *A. brasilense* (143). The tricarboxylic acid cycle is the major route for adenosine 6′-triphosphate generation in aerobes (186); respiration by *A. brasilense* generates adenosine 5′-triphosphate (140), and adenosine 5′-triphosphate is required for operation of the nitrogenase in this organism (36, 121). The tricarboxylic acid cycle has also been shown to be important for generating reducing power (186). Electrons from the tricarboxylic acid cycle intermediates in *A. brasilense* are transferred to oxidized nicotinamide adenine dinucleotide (143). Reduced nicotinamide adenine dinucleotide has been shown to be one of many possible sources of electrons for reduction of N_2 to NH_3 by nitrogenase (214). Therefore, these studies suggest that, provided photosynthate and oxygen are available to *A. brasilense* in the roots, the bacteria may be able to manufacture the prerequisites for nitrogenase activity. It has been suggested that the pO_2 is low and that substrate carbon levels are limiting at the sites of nitrogen fixation in the roots (64). The efficiency of nitrogen fixation by *A. brasilense* increased with decreased carbon supply (45, 64) and with low pO_2 (144). Using these observations, Döbereiner and Day (64) speculated that the symbiotic grass system has a high efficiency for nitrogen fixation, similar to legume-*Rhizobium* symbioses.

The bacteroids in the nodules of legumes generally do not grow (23). Considerable increases in nitrogen fixation occur as the life cycles of legume symbioses progress (153). This implies that high rates of nitrogen fixation in legumes are possible with predominantly nongrowing bacteroids in the nodules. Little is known about the interaction between growth and nitrogen fixation by *Azospirillum* in association with grass roots. However, an oxygen deficiency limits the growth of *A. brasilense* when N_2 is the sole nitrogen source (144). Neyra and Döbereiner (136) noted that under a limiting O_2 supply the reduction in the growth rate of the bacteria was far greater than the decrease in the rate of nitrogenase activity. Higher efficiency of nitrogen fixation by *A. brasilense* was explained by an increase in nitrogenase activity per bacterial cell, with a concomitant decrease in growth rate (136). However, all of these inferences are based on the results of studies with pure cultures, and, therefore, the relationship between growth and efficient nitrogen fixation by *Azospirillum* in roots of grasses is unknown.

High Oxygen Partial Pressure and Nitrogenase Activity

Because nitrogenase is sensitive to oxygen, the process of nitrogen fixation under fully aerobic conditions is not possible unless this enzyme is protected. Most studies involving nitrogen fixation by bacteria under conditions of high pO_2 have been done with *A. chroococcum* and *Azo-*

tobacter vinelandii. *Azotobacter* can fix nitrogen in air because of respiratory and conformational protection of its nitrogenase and because of the location of this enzyme within the cell (214). None of these mechanisms has been demonstrated in *A. paspali*, but they undoubtedly exist because these bacteria are able to fix nitrogen and grow in air (63). Nevertheless, the nitrogenase activity associated with the roots of *P. notatum* is sensitive to changes in pO_2 (65). The optimum pO_2 for nitrogenase activity by *P. notatum* roots was shown to be similar to that reported for *A. chroococcum* in pure culture (66).

Microaerophily has been reported to be another mechanism by which certain bacteria are capable of expressing nitrogenase (214). No respiratory protection can be afforded by these microorganisms, but there is evidence of a degree of conformational protection for their nitrogenase (214). *Azospirillum* spp. belong to this group of organisms. The necessity for microaerophilic conditions for nitrogen fixation by *Azospirillum* is supported by reports that *A. brasilense* does not grow with N_2 as the sole nitrogen source under aerobic conditions (144). Also, Tarrand et al. (189) have discussed the microaerophilic nature of *Azospirillum* spp. for nitrogen fixation, and they included this characteristic as part of the taxonomy of these bacteria. The optimum rates of nitrogenase activity by *Azospirillum* were detected between 0.005 and 0.007 atmospheres (0.507 and 0.709 kPa) of dissolved O_2 (36, 133, 144). The observed sensitivity of nitrogen fixation by *Azospirillum* to oxygen has been suggested as a reason why nitrogenase activity in grass roots can be detected only at low pO_2 (1, 136). It has been suggested that the apparent oxygen sensitivity of the nitrogenase in bacteria associated with grass roots is mediated by poor oxygen protection mechanisms under in situ conditions (136). Nevertheless, van Berkum and Sloger (204; van Berkum and Sloger, Plant Physiol. **63:**S479, 1979) demonstrated that immediate reduction of acetylene by excised and intact roots of salt marsh grasses and rice in air. The immediate reduction of acetylene by preincubated sorghum roots exposed to air before assay has also been reported (202). Therefore, the suggestion that no nitrogenase activity is associated with excised roots which fail to reduce acetylene immediately upon assay (202, 204) is an alternative to suggestions of inactivation of nitrogenase by oxygen in roots of grasses.

Effects of Temperature and pH

The optimum temperature for N_2-dependent growth by *Azospirillum* has been reported to be between 32 and 40°C and is similar to the optima reported for other nitrogen-fixing bacteria from tropical environments (44). The nitrogenase activity of *Azospirillum* is sensitive to temperatures below 18°C (44). The higher incidence of *Azospirillum* in tropical areas has been attributed to the high temperature requirements of these bacteria (136). Although all *Azospirillum* strains reportedly have similar temperature optima for nitrogen fixation, differences in cold tolerance have been reported (134). However, the sensitivity of nitrogenase activity in roots of grasses to low night temperatures has been attributed to effects upon plant growth (136).

Day and Döbereiner (45) reported that the optimal pH for N_2-dependent growth by *Azospirillum* lies between 6.8 and 7.8. They speculated that nitrogen fixation by *Azospirillum* occurs at the surface or within the cells of the roots, where these specific pH requirements can be met (45). Furthermore, the occurrence of *Azospirillum* in soils has been shown to be pH dependent, but it has been suggested that roots of grasses offer optimal conditions to the bacteria, even at low soil pH's (67).

The effects of temperature and pH on nitrogenase activity by *A. paspali* or in roots of *P. notatum* have not been documented.

Interaction of Combined Nitrogen and Nitrogenase

Ammonia, the product of the reduction of N_2 by nitrogenase (162), is preferred by all nitrogen-fixing bacteria as the nitrogen source which provides more rapid growth than any other substrate (117). *A. paspali* (63) and *A. brasilense* (143, 144) also grow well on ammonia without fixing nitrogen. *Azospirillum* promptly converts to nitrogen fixation, and growth slows when the cells are transferred from ammonia to nitrogen-free media (145). Furthermore, a low initial concentration of combined nitrogen in large semisolid batch cultures improved growth and nitrogen fixation by *Azospirillum* when small inocula were used (van Berkum, Ph.D. thesis). Ammonia-grown cells of *A. paspali* also induced nitrogenase activity upon transfer of the cells to nitrogen-free media (63). These observations indicate that ammonia controls nitrogenase activity and synthesis in nitrogen-fixing bacteria which are suggested to associate with the roots of grasses.

Regulation of nitrogenase activity and synthesis by ammonia has been studied in other free-living nitrogen-fixing bacteria. Mortenson (129) has shown that the study of the inhibition of synthesized nitrogenase is complicated because synthesis of this enzyme is immediately repressed upon addition of ammonia to the bacteria. The regulation of the synthesis of nitrogenase has been investigated mostly with *Klebsiella pneumoniae* (174, 188, 200) and *A. vinelandii* (90). The first product of the incorporation of ammonia into bacteria is glutamate, and this reaction is accomplished by two alternative pathways. Glutamate dehydrogenase provides glutamate to the cells when the concentration of ammonia in the medium is high (>1 mM) (132). When the level of ammonia is low (<1 mM) or under nitrogen-fixing conditions, reactions catalyzed by glutamine synthetase coupled to glutamate synthase provide glutamate to the cells (188, 192), and glutamate dehydrogenase is inoperative (174). L-Methionine-DL-sulfoximine and L-methionine sulfone are inhibitors of these two enzymes involved in ammonia assimilation (90). The simultaneous addition of L-methionine-DL-sulfoximine or L-methionine sulfone with an inhibitory level of NH_4^+ to nitrogen-fixing cultures of *K. pneumoniae* or *A. vinelandii* allowed nitrogenase activity to be detected, but in the absence of these glutamate analogs nitrogen fixation ceased (24, 90). The addition of ammonia to nitrogen-fixing cultures of *A. brasilense* also represses synthesis of nitrogenase (36). In the presence of excess ammonia, L-methionine-DL-sulfoximine and L-methionine sulfone also lifted repression of nitrogenase synthesis in *A. brasilense*, and acetylene reduction could be detected (143). Ammonia repressed glutamine synthetase and increased adenylylation of this enzyme in *K. pneumoniae* and *A. brasilense* (24, 143). L-Methionine-DL-sulfoximine and L-methionine sulfone suppressed adenylylation of glutamine synthetase in both microorganisms (24, 143), and primarily deadenylylated forms were present under nitrogen-fixing conditions (143). The popular current hypothesis is that the active, deadenylylated form of glutamine synthetase induces nitrogenase synthesis and that respiration or conversion of this enzyme into its inactive adenylylated form by ammonia causes repression of nitrogenase synthesis (214). The best evidence implicating glutamine synthetase in nitrogenase regulation comes from observations that nitrogenase-derepressed mutants are the result of structural alterations in glutamine synthetase (174, 175, 200). The role of glutamine synthetase in the regulation of nitrogenase in *A. brasilense* has also been supported by the isolation of mutants similar to *K. pneumoniae* (81). However, a direct negative control by an ammonia-sensing repressor at the *nif* operon in *K. pneumoniae* has also been suggested (5). The control mechanisms for nitrogenase synthesis in *A. paspali* are not known.

Assimilatory and dissimilatory nitrate reduction and denitrification by *A. brasilense* have also been demonstrated (72, 133, 137, 140), and Neyra et al. (137) concluded that studies of methods to optimize nitrogen fixation and minimize denitrification by *Azospirillum* are important. Nelson and Knowles (133) observed that nitrate inhibited the nitrogenase activity of *A. brasilense*, which has been shown to be mediated through active nitrate reduction (123). Magalhaes et al. (123) were able to isolate nitrate reductase-negative mutants of *Azospirillum*, which were able to fix nitrogen in the presence of 10 mM nitrate. These authors advocated that inoculation of these mutants onto grasses could lead to systems capable of nitrogen fixation in the presence of high nitrate levels in soils.

Döbereiner and Day (63) reported that nitrogenase activity in *A. paspali* was not affected by nitrate and suggested the lack of nitrate reductase in this organism. However, these workers did observe inhibition of *P. notatum* root nitrogenase activity by nitrate. They suggested that the roots reduced nitrate to nitrite, which effluxed out of the roots together with nitrate, causing the inhibition of nitrogenase activity (63).

Nitrogenase of *Azospirillum brasilense*

Studies of the nitrogenase from *A. brasilense* have been possible because this enzyme is released by disruption of the cells with a French pressure cell and purification methods have been described (121). *A. brasilense* has been shown to have the following three-component nitrogenase system: an Mo-Fe protein, an Fe protein, and an activating factor for the Fe protein similar to that found in *Rhodospirillum rubrum* (145). The activity of the nitrogenase of *A. brasilense* is dependent on the presence of Mn^{2+} and Mg^{2+} (36), and the activating factor for *R. rubrum* activates the Fe protein from *A. brasilense* and vice versa (121). The Mo-Fe protein from *A. brasilense* did not require activation because it was active when crossed with the Fe protein from *K. pneumoniae* (121).

H_2 Evolution and Uptake

The reutilization of evolved hydrogen by hydrogenase, producing adenosine 5′-triphosphate and reducing power, for more efficient nitrogen fixation in legumes has been suggested (172, 173). The hydrogenase activity in *A. brasilense* has also been studied. The levels of activity were suggested to be sufficient to recycle all of the hydrogen produced by nitrogenase (159), but the importance of this function for efficient nitrogen fixation in grass roots remains to be determined.

Also, the role of hydrogenase as an oxygen protection mechanism of nitrogenase in *A. lipoferum* has been suggested (207).

CONCLUSION

Undoubtedly, nitrogen fixation in association with grasses occurs. Grasses may derive nitrogen from the atmosphere through the participation of free-living or associated microorganisms by three mechanisms. First, carbohydrates released by the decay of plant material may support growth on N_2 by free-living bacteria. The nitrogen retained in the biomass of this fraction of the microflora may have long-term enrichment properties, especially in undisturbed ecosystems. Second, nitrogen fixation in the root region of grasses may occur through the release of carbon compounds from the plants into the soil. Long-term nitrogen enrichment of soil through this mechanism may occur and may be adequate to support labor-intensive and subsistence farming under tropical conditions. And third, root-associated nitrogen fixation may be closely coupled to plant metabolism. The highly mechanized farming practices of developed countries would be dependent on this form of nitrogen fixation for maximal economic return if it is to partly or completely replace nitrogen fertilizer application. The *Azospirillum*-grass associative symbiosis has been classified in the third category, but no evidence in support of this notion has been forthcoming. Nitrogen fixation in well-aerated environments has been considered to be sporadic, difficult, or impossible to reproduce and to occur at rates insufficient to meet the requirements of intensive crops (73). However, it is our opinion that the process of nitrogen fixation by grasses should be explored in full and not dismissed at a stage when very little is known.

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LITERATURE CITED

1. **Abrantes, G. T. V., J. M. Day, and J. Döbereiner.** 1975. Methods for the study of nitrogenase activity in field grown grasses. Bull. Int. Inf. Biol. Sol **21:**1–7.
2. **Albrecht, S., Y. Okon, J. Lonnquist, and R. H. Burris.** 1976. Corn roots fix nitrogen. Not much yet, but a beginning. Crops Soils **28:**16.
3. **Albrecht, S. L., Y. Okon, and R. H. Burris.** 1978. Effect of light and temperature on the association between *Zea mays* and *Spirillum lipoferum*. Plant Physiol. **60:**528–531.
4. **Anderson, G. R.** 1966. Identification of *Beijerinckia* from Pacific Northwest soils. J. Bacteriol. **91:**2105–2106.
5. **Ausubel, F. M., R. F. Margolskee, and N. Maizels.** 1977. Mutants of *Klebsiella pneumoniae* in which expression of nitrogenase is independent of glutamine synthetase control, p. 347–356. *In* W. Newton, J. R. Postgate, and C. Rodriguez-Barrucco (ed.), Recent developments in nitrogen fixation. Academic Press, Inc., London.
6. **Bach, M. K., W. E. Magee, and R. H. Burris.** 1958. Translocation of photo-synthetic products to soybean nodules and their role in nitrogen fixation. Plant Physiol. **33:**118–124.
7. **Balandreau, J.** 1975. Mesure de l'activite nitrogénasique des microorganismes fixateurs libres d'azote de la rhizosphère de quelques graminées. Rev. Ecol. Biol. Sol **12:**273–290.
8. **Balandreau, J., and Y. Dommergues.** 1973. Assaying nitrogenase (C_2H_2) activity in the field. Bull. Ecol. Res. Comm. NFR (Statens Naturvetensk. Forskningsrad) **17:**246–254.
9. **Balandreau, J., C. R. Millier, and Y. Dommergues.** 1974. Diurnal variations of nitrogenase activity in the field. Appl. Microbiol. **27:** 662–665.
10. **Balandreau, J., C. R. Millier, P. Weinhard, P. Ducerf, and Y. Dommergues.** 1977. A modelling approach of acetylene reducing activity of plant rhizosphere diazotroph systems, p. 523–529. *In* W. Newton, J. R. Postgate, and C. Rodriguez-Barrucco (ed.), Recent developments in nitrogen fixation. Academic Press, Inc., London.
11. **Balandreau, J., G. Rinaudo, I. Fares-Hamad, and Y. Dommergues.** 1975. N_2 fixation in paddy soils, p. 57–70. *In* W. D. P. Stewart (ed.), Nitrogen fixation by free-living microorganisms. International Biological Programme Series, vol. 6. Cambridge University Press, Cambridge, England.
12. **Baltensperger, A. A., S. C. Schank, R. L. Smith, R. C. Littell, J. H. Bouton, and A. E. Dudeck.** 1978. Effect of inoculation with *Azospirillum* and *Azotobacter* on turf-type Bermuda genotypes. Crop Sci. **18:**1043–1045.
13. **Barber, L. E., and H. J. Evans.** 1976. Characterization of a nitrogen-fixing bacterial strain from the roots of *Digitaria sanguinalis*. Can. J. Microbiol. **22:**254–260.
14. **Barber, L. E., J. D. Tjepkema, and H. J. Evans.** 1978. Acetylene reduction in the root environment of some grasses and other plants in Oregon. Ecol. Bull. (Stockholm) **26:**366–372.
15. **Barber, L. E., J. D. Tjepkema, S. A. Russell, and H. J. Evans.** 1976. Acetylene reduction

(nitrogen fixation) associated with corn inoculated with *Spirillum*. Appl. Environ. Microbiol. **32:**108–113.

16. **Barea, J. M., and M. E. Brown.** 1974. Effects of plant growth produced by *Azotobacter paspali* related to synthesis of plant growth regulating substances. J. Appl. Bacteriol. **37:**583–593.
17. **Barker, S. A.** 1976. Efficient fertilizer use, p. 13–30. *In* F. L. Patterson, M. Stelly, D. M. Kral, and L. C. Eisele (ed.), Agronomic research for food. American Society of Agronomy Special Publication 26. American Society of Agronomy, Madison, Wis.
18. **Barrow, N. J., and D. S. Jenkinson.** 1962. The effect of waterlogging on fixation of nitrogen by soil incubated with straw. Plant Soil **16:**258–262.
19. **Bartholomew, W. V.** 1977. Soil nitrogen changes in farming systems in the humid tropics, p. 27–42. *In* A. Ayanabe and P. J. Dart (ed.), Biological nitrogen fixation in farming systems of the tropics. John Wiley & Sons, Inc., New York.
20. **Bartholomew, W. V., J. Meyer, and H. Laudelout.** 1953. Mineral nutrient immobilisation under forest and grass fallow in the Yangambi region. INEAC Serie Scientifique No. 57. Imprimerie M. Hayez, Brussels.
21. **Becking, J. H.** 1961. Studies on nitrogen-fixing bacteria of the genus *Beijerinckia*. I. Geographical and ecological distribution. Plant Soil **14:**49–81.
22. **Bergersen, F. J.** 1970. The quantitative relationship between nitrogen fixation and the acetylene reduction assay. Aust. J. Biol. Sci. **23:**1015–1025.
23. **Bergersen, F. J.** 1977. Physiological chemistry of dinitrogen fixation by legumes, p. 519–556. *In* R. W. F. Hardy and W. S. Silver (ed.), A treatise on dinitrogen fixation, section 3. John Wiley & Sons, Inc., New York.
24. **Bishop, P. E., R. H. McParland, and C. J. Evans.** 1975. Inhibition of the adenylylation of glutamine synthetase by methionine sulfone during nitrogenase derepression. Biochem. Biophys. Res. Commun. **67:**774–781.
25. **Black, C. C., R. H. Brown, and R. C. Moore.** 1978. Plant photosynthesis. Basic Life Sci. **10:**95–110.
26. **Bohlool, B. B., and E. L. Schmidt.** 1980. The immunofluorescence approach in microbial ecology. *In* M. Alexander (ed.), Advances in microbial ecology, vol. 4. Plenum Publishing Corp., New York, in press.
27. **Boyd, W. L., and J. W. Boyd.** 1962. Presence of *Azotobacter* species in polar regions. J. Bacteriol. **83:**429–430.
28. **Brockwell, J.** 1977. Application of legume seed inoculants, p. 277–310. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on dinitrogen fixation, section 4. John Wiley & Sons, Inc., New York.
29. **Brown, M. E.** 1972. Plant growth substances produced by micro-organisms of soil and rhizosphere. J. Appl. Bacteriol. **35:**443–451.
30. **Brown, M. E.** 1974. Seed and root bacterization. Annu. Rev. Phytopathol. **12:**181–197.
31. **Brown, M. E.** 1976. Role of *Azotobacter paspali* in association with *Paspalum notatum*. J. Appl. Bacteriol. **40:**341–348.
32. **Brown, M. E., and S. K. Burlingham.** 1968. Production of plant growth substances by *Azotobacter chroococcum*. J. Gen. Microbiol. **53:**135–144.
33. **Brown, R. H.** 1976. Characteristics related to photosynthesis and photorespiration of *Panicum milloides*, p. 311–325. *In* R. H. Burris and C. C. Black (ed.), CO_2 metabolism and plant productivity. University Park Press, Baltimore.
34. **Brown, R. H., and W. V. Brown.** 1975. Photosynthetic characteristics of *Panicum milloides*, a species with reduced photorespiration. Crop Sci. **15:**681–685.
35. **Burns, R. C., and R. W. F. Hardy.** 1975. Nitrogen fixation in bacteria and higher plants. Mol. Biol. Biochem. Biophys. **21:**189.
36. **Burris, R. H., Y. Okon, and S. L. Albrecht.** 1978. Properties and reactions of *Spirillum lipoferum*. Ecol. Bull. (Stockholm) **26:**353–364.
37. **Chang, P. C., and R. Knowles.** 1965. Non-symbiotic nitrogen fixation in some Quebec soils. Can. J. Microbiol. **11:**29–38.
38. **Chollet, R., and W. L. Ogren.** 1975. Regulation of photorespiration in C-3 and C-4 species. Bot. Rev. **41:**147–179.
39. **Cooper, R.** 1959. Bacterial fertilizers in the Soviet Union. Soils Fert. **22:**327–333.
40. **Cornforth, I. S.** 1975. The persistence of ethylene in aerobic soils. Plant Soil **42:**85–96.
41. **Dart, P. J.** 1978. Cereal microbiology programme at ICRISAT, quinquennial review.
42. **Dart, P. J., and R. V. Subha-Rao.** 1979. Nitrogen fixation associated with sorghum and millet. *In* Proceedings of the International Workshop on Associative N_2 Fixation. CENA, Piracicaba, Brazil.
43. **David, K. A. V., and P. Fay.** 1977. Effects of long-term treatment with acetylene on nitrogen-fixing microorganisms. Appl. Environ. Microbiol. **34:**640–646.
44. **Day, J. M.** 1977. Nitrogen-fixing associations between bacteria and tropical grass roots, p. 271–288. *In* A. Ayanaba and P. J. Dart (ed.), Biological nitrogen fixation in farming systems of the tropics. John Wiley & Sons, Inc., New York.
45. **Day, J. M., and J. Döbereiner.** 1976. Physiological aspects of N_2-fixation by a *Spirillum* from *Digitaria* roots. Soil Biol. Biochem. **8:**45–50.
46. **Day, J. M., D. Harris, P. J. Dart, and P. van Berkum.** 1975. The Broadbalk experiment. An investigation of nitrogen gains from non-symbiotic fixation, p. 71–84. *In* W. D. P. Stewart (ed.), Nitrogen fixation by free-living microorganisms. International Biological Programme Series, vol. 6. Cambridge University Press,

Cambridge, England.

47. **Day, J. M., M. C. P. Neves, and J. Döbereiner.** 1975. Nitrogenase activity on roots of tropical grasses. Soil Biol. Biochem. **7:**107–112.
48. **de Bont, J. A. M.** 1976. Bacterial degradation of ethylene and the acetylene reduction test. Can. J. Microbiol. **22:**1060–1062.
49. **Delwiche, C. C., and J. Wijler.** 1956. Non-symbiotic nitrogen fixation in soil. Plant Soil **7:** 113–129.
50. **De-Polli, H., B. B. Bohlool, and J. Döbereiner.** 1980. Immunofluorescence differentiation of *Azospirillum* species belonging to different host-plant specificity groups. Arch. Microbiol., in press.
51. **De-Polli, H., E. Matsui, J. Döbereiner, and E. Salati.** 1977. Confirmation of nitrogen fixation in two tropical grasses by $^{15}N_2$ incorporation. Soil Biol. Biochem. **9:**119–123.
52. **De Ville, R., and Y. T. Tchan.** 1970. Etude quantitative de la population azotobacterienne du sol par la methode d'immunofluorescence. Ann. Inst. Pasteur Paris **119:**492–497.
53. **Diem, G., M. Rougier, I. Hamad-Faras, J. P. Balandreau, and Y. R. Dommergues.** 1978. Colonization of rice roots by diazotroph bacteria. Ecol. Bull. (Stockholm) **26:**305–311.
54. **Diem, H. G., G. Godbillon, and E. L. Schmidt.** 1977. Application of the fluorescent antibody technique to the study of an isolate of *Beijerinckia* in soil. Can. J. Microbiol. **23:**161–165.
55. **Döbereiner, J.** 1966. *Azotobacter paspali* n. sp. uma bacteria fixadora de nitrogênio na rhizosphèra de *Paspalum*. Pesqui. Agropecu. Bras. **1:**357–365.
56. **Döbereiner, J.** 1968. Non-symbiotic nitrogen fixation in tropical soils. Pesqui. Agropecu. Bras. **3:**1–6.
57. **Döbereiner, J.** 1970. Further research on *Azotobacter paspali* and its variety specific occurrence in the rhizosphere of *Paspalum notatum* Flugge. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 **124:**224–230.
58. **Döbereiner, J.** 1977. Physiological aspects of N_2-fixation in grass-bacteria associations, p. 513–522. *In* W. Newton, J. R. Postgate, and C. Rodriguez-Barrueco (ed.), Recent developments in nitrogen fixation. Academic Press, Inc., London.
59. **Döbereiner, J.** 1977. Plant genotype effects on nitrogen fixation in grasses, p. 325–334. *In* A. Muhammed, R. Aksel, and R. C. von Borstel (ed.), Genetic diversity in plants. Plenum Publishing Corp., New York.
60. **Döbereiner, J.** 1978. Potential for nitrogen fixation in tropical legumes and grasses, p. 13–24. *In* J. Dobereiner, R. H. Burris, and A. Hollaender (ed.), Limitations and potentials for biological nitrogen fixation in the tropics. Plenum Publishing Corp., New York.
61. **Döbereiner, J., and A. B. Campelo.** 1971. Non-symbiotic nitrogen-fixing bacteria in tropical soils, p. 457–470. Plant Soil Special Volume.
62. **Döbereiner, J., and A. B. Campelo.** 1977. Importance of legumes and their contribution to tropical agriculture, p. 191–220. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on dinitrogen fixation, section 4. John Wiley & Sons, Inc., New York.
63. **Döbereiner, J., and J. M. Day.** 1975. Nitrogen fixation in the rhizosphere of tropical grasses, p. 39–56. *In* W. D. P. Stewart (ed.), Nitrogen fixation by free-living microorganisms. International Biological Programme Series, vol. 6. Cambridge University Press, Cambridge, England.
64. **Döbereiner, J., and J. M. Day.** 1976. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen fixing sites, p. 518–538. *In* W. E. Newton and C. J. Nyman (ed.), Proceedings of the First International Symposium on Nitrogen Fixation. Washington State University Press, Pullman.
65. **Döbereiner, J., J. M. Day, and P. J. Dart.** 1972. Nitrogenase activity and oxygen sensitivity of the *Paspalum notatum-Azotobacter paspali* association. J. Gen. Microbiol. **71:**103–116.
66. **Döbereiner, J., J. M. Day, and P. J. Dart.** 1972. Rhizosphere associations between grasses and nitrogen-fixing bacteria: effect of O_2 on nitrogenase activity in the rhizosphere of *Paspalum notatum*. Soil Biol. Biochem. **5:**157–159.
67. **Döbereiner, J., I. E. Marriel, and M. Nery.** 1976. Ecological distribution of *Spirillum lipoferum* Beijerinck. Can. J. Microbiol. **22:** 1464–1473.
68. **Douglas, J.** 1979. Plenty of challenges and opportunities. Farm Chem. **142**(1):13–21.
69. **Dubber, H. J.** 1965. Untersuchungen zur N_2-bindung bei der Verrotung von Waldgraserwurzeln unter Mikroaeroben Bedingungen. Plant Soil **23:**247–256.
70. **Eck, H. V., G. C. Wilson, and T. Martinez.** 1975. Nitrate reductase activity of grain sorghum leaves as related to yields of grain, dry matter and nitrogen. Crop Sci. **15:**557–561.
71. **Eskew, D. L., and I. P. Ting.** 1977. Comparison of intact plant and excised root assays for acetylene reduction in grass rhizospheres. Plant Sci. Lett. **8:**327–331.
72. **Eskew, D. O., 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.
73. **Evans, H. J., and L. E. Barber.** 1977. Biological nitrogen fixation for food and fiber production. Science **197:**332–339.
74. **Farm Chemicals.** 1979. Brighter days ahead in ammonia? Farm Chem. **142**(3):13–27.
75. **Fehr, P. I., P. C. Pang, R. A. Hedlin, and C. M. Cho.** 1972. Some factors affecting asymbiotic nitrogen fixation in soils as measured by ^{15}N enrichment. Agron. J. **64:**251–254.
76. **Food and Agricultural Organization of the United Nations.** 1977. Annual fertilizer review for 1977. Food and Agriculture Organization of the United Nations, Rome.
77. **Food and Agricultural Organization of the United Nations.** 1978. 1977 Production year-

book, vol. 31. Food and Agricultural Organization of the United Nations, Rome.

78. **Foster, R. C., and A. D. Rouira.** 1976. Ultrastructure of wheat rhizosphere. New Phytol. **76**:343–352.
79. **Fred, E. B., I. L. Baldwin, and E. McCoy.** 1932. Root nodule bacteria and leguminous plants. University of Wisconsin, Madison.
80. **Galton, M. M., G. K. Morris, and W. T. Martin.** 1968. Salmonellae in food and feeds. Review of isolation methods and recommended procedures. U.S. Department of Health and Welfare, Public Health Service, Bureau of Disease Prevention and Environmental Control, National Communicable Disease Center, Washington, D.C.
81. **Gauthier, D., and C. Elmerich.** 1977. Relationship between glutamine synthetase and nitrogenase in *Spirillum lipoferum*. FEMS Microbiol. Lett. **2**:101–104.
82. **Gibson, A. H.** 1974. Consideration of the growing legume as a symbiotic association. Proc. Indian Natl. Sci. Acad. Part B **40**:741–767.
83. **Gibson, A. H.** 1974. The control of dinitrogen assimilation by nodulated legumes, p. 13–22. *In* R. L. Bieleski, A. R. Ferguson, and M. M. Cresswell (ed.), Mechanisms of regulation of plant growth. Bulletin 12. Royal Society of New Zealand, Wellington.
84. **Gibson, A. H.** 1975. Recovery and compensation by nodulated legumes to environmental stress, p. 385–403. *In* P. S. Nutman (ed.), Symbiotic nitrogen fixation in plants. International Biological Programme Series, vol. 7. Cambridge University Press, Cambridge, England.
85. **Gibson, A. H.** 1976. Limitation to dinitrogen fixation by legumes, p. 400–428. *In* W. E. Newton and C. J. Nyman (ed.), Proceedings of the First International Symposium on Nitrogen Fixation. Washington State University Press, Pullman.
86. **Gibson, A. H.** 1977. The influence of the environment and managerial practices on the legume-*Rhizobium* symbiosis, p. 393–450. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on dinitrogen fixation, section 4. John Wiley & Sons, Inc., New York.
87. **Gilmour, J. T., C. M. Gilmour, and T. H. Johnson.** 1978. Nitrogenase activity of rice plant root systems. Soil Biol. Biochem. **10**:261–264.
88. **Glatzle, A., and P. Martin.** 1979. Some interactions between *Azospirillum* spp. and grass seedlings. *In* Proceedings of the International Workshop on Associative N_2 Fixation. CENA, Piracicaba, Brazil.
89. **Goldemberg, J.** 1978. Brazil: energy options and current outlook. Science **200**:158–167.
90. **Gordon, J. K., and W. J. Brill.** 1974. Depression of nitrogenase synthesis in the presence of excess NH_4^+. Biochem. Biophys. Res. Commun. **59**:967–971.
91. **Greenland, D. J.** 1977. Contribution of microorganisms to the nitrogen status of tropical soils, p. 13–26. *In* A. Ayanabe and P. J. Dart (ed.), Biological nitrogen fixation in farming systems of the tropics. John Wiley & Sons, Inc., New York.
92. **Hanson, R. B.** 1977. Comparison of nitrogen fixation activity in tall and short *Spartina alterniflora* salt marsh soils. Appl. Environ. Microbiol. **33**:596–602.
93. **Hardy, R. W. F., R. C. Burns, R. R. Hebert, R. D. Holsten, and E. K. Jackson.** 1971. Biological nitrogen fixation: a key to world protein, p. 561–590. Plant Soil Special Volume.
94. **Hardy, R. W. F., R. C. Burns, and R. D. Holsten.** 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. **5**:47–81.
95. **Hardy, R. W. F., and U. D. Havelka.** 1975. Nitrogen fixation research: a key to world food? Science **188**:633–643.
96. **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, p. 421–439. *In* P. S. Nutman (ed.), Symbiotic nitrogen fixation in plants. International Biological Programme Series, vol. 7. Cambridge University Press, Cambridge, England.
97. **Hardy, R. W. F., and R. D. Holsten.** 1977. Methods for measurement of dinitrogen fixation, p. 451–486. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on dinitrogen fixation, section 4. John Wiley & Sons, Inc., New York.
98. **Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns.** 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol. **43**:1185–1207.
99. **Harris, D., and P. J. Dart.** 1973. Nitrogenase activity in the rhizosphere of *Stachys sylvatica* and some other dicotyledenous plants. Soil Biol. Biochem. **5**:277–279.
100. **Hirota, Y., T. Fujii, Y. Sano, and S. Iyama.** 1978. Nitrogen fixation in the rhizosphere of rice. Nature (London) **276**:416–417.
101. **Hoshino, M., S. Nishimura, and T. Okubo.** 1964. Studies on the assimilation and translocation of $^{14}CO_2$ in Ladino clover. Proc. Crop Sci. Soc. Jpn. **33**:130–134.
102. **Hubbell, D. H., T. M. Tien, M. H. Gaskins, and J. K. Lee.** 1979. Physiological interaction in the *Azospirillum*-grass root interaction. *In* Proceedings of the International Workshop on Associative N_2 Fixation. CENA, Piracicaba, Brazil.
103. **Jefferies, R. L.** 1972. Aspects of salt-marsh ecology with particular reference to inorganic plant nutrition, p. 61–85. *In* R. S. K. Barnes and J. Green (ed.), the estuarine environment. Applied Science Publishers, London.
104. **Jenkinson, D. S.** 1971. The accumulation of organic matter in soil left uncultivated, p. 113–137. *In* Rothamsted report for 1970, part 2. Bartholomew Press, Dorking, England.
105. **Jenkinson, D. S.** 1973. Organic matter and nitrogen in soils of the Rothamsted classical experiments. J. Sci. Food Agric. **24**:1149–1150.
106. **Jenkinson, D. S.** 1977. The nitrogen economy

of the Broadbalk experiments. I. Nitrogen balance in the experiments, p. 103–109. *In* Rothamsted report for 1976, part 2. Bartholomew Press, Dorking, England.

107. **Jensen, H. L.** 1965. Nonsymbiotic nitrogen fixation, p. 436–480. *In* W. V. Bartholomew and F. E. Clark (ed.), Soil nitrogen. American Society of Agronomy, Madison, Wis.

108. **Jurgensen, M. F., and C. B. Davey.** 1970. Nonsymbiotic nitrogen-fixing microorganisms in acid soils and the rhizosphere. Soils Fert. **33**: 435–446.

109. **Kana, T. M., and J. D. Tjepkema.** 1978. Nitrogen fixation associated with *Scirpus atrovirens* and other nonnodulated plants in Massachusetts. Can. J. Bot. **56**:2636–2640.

110. **Keister, D. L.** 1975. Acetylene reduction by pure cultures of rhizobia. J. Bacteriol. **123**:1265–1268.

111. **Keister, D. L., and V. Ranga-Rao.** 1977. The physiology of acetylene reduction in pure cultures of rhizobia, p. 419–430. *In* W. Newton, J. R. Postgate, and C. Rodriguez-Barrueco (ed.), Recent developments in nitrogen fixation. Academic Press, Inc., London.

112. **Klucas, R. V., W. Pedersen, R. C. Shearman, and L. V. Wood.** 1979. Nitrogen fixation associated with winter wheat, sorghum and Kentucky bluegrass. *In* Proceedings of the International Workshop on Associative N_2 Fixation. CENA, Piracicaba, Brazil.

113. **Knowles, R.** 1977. The significance of asymbiotic dinitrogen fixation by bacteria, p. 33–83. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on dinitrogen fixation, section 4. John Wiley & Sons, Inc., New York.

114. **Koch, B. L.** 1977. Associative nitrogenase activity by some Hawaiian grass roots. Plant Soil **47**:703–706.

115. **Kurz, W. G. W., and T. A. La Rue.** 1975. Nitrogenase activity in rhizobia in absence of plant host. Nature (London) **256**:407–408.

116. **Larson, A. I., and J. L. Neal.** 1978. Selective colonization of the rhizosphere of wheat by nitrogen-fixing bacteria. Ecol. Bull. (Stockholm) **26**:331–342.

117. **La Rue, T. A.** 1977. The bacteria, p. 19–62. *In* R. W. F. Hardy and W. S. Silver (ed.), A treatise on dinitrogen fixation, section 3. John Wiley & Sons, Inc., New York.

118. **Lee, K. K., B. Alimagno, and T. Yoshida.** 1977. Field technique using the acetylene reduction method to assay nitrogenase activity and its association with the rice rhizosphere. Plant Soil **47**:519–526.

119. **Lipman, C. B., and J. K. Taylor.** 1922. Proof of the power of the wheat plant to fix atmospheric nitrogen. Science **56**:605–606.

120. **Lipman, C. B., and J. K. Taylor.** 1923. Proof of the power of the wheat plant to fix atmospheric nitrogen. Agric. J. India **18**:155–157.

121. **Ludden, P. W., Y. Okon, and R. H. Burris.** 1978. The nitrogenase system of *Spirillum lipoferum*. Biochem. J. **173**:1001–1003.

122. **Macrae, I. C., and T. F. Castro.** 1967. Root exudates of the rice plant in relation to akagare, a physiological disorder of rice. Plant Soil **26**: 317–323.

123. **Magalhaes, L. M. S., C. A. Neyra, and J. Döbereiner.** 1978. Nitrate and nitrite reductase negative mutants of N_2-fixing *Azospirillum* spp. Arch. Microbiol. **117**:247–252.

124. **Magdoff, F. R., and D. R. Bouldin.** 1970. Nitrogen fixation in submerged soil-sand-energy material media and the aerobic-anaerobic interface. Plant Soil **33**:49–61.

125. **Matsui, E., P. B. Vose, N. S. Rodriguez, and A. P. Ruschel.** 1979. Use of ^{15}N enriched gas to determine N_2 fixation by undisturbed sugarcane plants in the field, p. 47. *In* Proceedings of the International Workshop on Associative N_2 Fixation. CENA, Piracicaba, Brazil.

126. **McComb, J. A., J. Elliot, and M. J. Dilworth.** 1975. Acetylene reduction by *Rhizobium* in pure culture. Nature (London) **256**:409–410.

127. **Meeker, G. B., A. C. Purvis, C. A. Neyra, and R. H. Hageman.** 1974. Uptake and accumulation of nitrate as a major factor in the regulation of nitrate reductase activity in corn (*Zea mays* L.) leaves: effect of high ambient CO_2 and malate, p. 49–58. *In* R. L. Bieleski, A. R. Ferguson, and M. M. Cresswell (ed.), Mechanisms of regulation of plant growth. Bulletin 12. Royal Society of New Zealand, Wellington.

128. **Moore, A. W.** 1966. Non-symbiotic nitrogen fixation in soil and soil-plant systems. Soils Fert. **29**:1185–1207.

129. **Mortenson, L. E.** 1978. Regulation of nitrogen fixation. Curr. Top. Cell. Regul. **13**:179–232.

130. **Mulder, E. G., and S. Brotonegoro.** 1974. Free-living heterotrophic nitrogen-fixing bacteria, p. 37–85. *In* A. Quispel (ed.), The biology of nitrogen fixation. North Holland Publishing Co., Amsterdam.

131. **Mulder, E. G., T. A. Lie, and A. Houwers.** 1977. The importance of legumes under temperate conditions, p. 221–242. *In* R. W. F. Hardy and A. H. Gibson (ed.), A treatise on dinitrogen fixation, section 4. John Wiley & Sons, Inc., New York.

132. **Nagatani, H., M. Shimizu, and R. C. Valentine.** 1971. The mechanism of ammonia assimilation in nitrogen fixing bacteria. Arch. Mikrobiol. **79**:164–175.

133. **Nelson, L. M., and R. Knowles.** 1978. Effect of oxygen and nitrate on nitrogen fixation and denitrification by *Azospirillum brasilense* grown in continuous culture. Can. J. Microbiol. **24**:1395–1403.

134. **Neves, M. C. P., M. Nery, and J. M. Day.** 1976. Efeito da temperatura na fixação de nitrogênio de estirpes de *Spirillum* sp. isoladas de *Digitaria* sp. e milho, p. 143–149. *In* Anais do XV Congresso Brasileiro de Ciencia do Solo. Brazilian Society of Soil Science, Campiras.

135. **Neyra, C. A.** 1978. Interactions of plant photosynthesis with dinitrogen fixation and nitrate assimilation. Basic Life Sci. **10**:111–120.

136. **Neyra, C. A., and J. Döbereiner.** 1977. Nitrogen fixation in grasses. Adv. Agron. **29**:1–38.

137. **Neyra, C. A., J. Döbereiner, R. Lalande, and R. Knowles.** 1977. Denitrification by N_2-fixing *Spirillum lipoferum*. Can. J. Microbiol. **23:** 300–305.

138. **Neyra, C. A., and R. H. Hageman.** 1976. Relationships between carbon dioxide, malate, and nitrate accumulation and reduction in corn (*Zea mays* L.) seedlings. Plant Physiol. **58:**726–730.

139. **Neyra, C. A., and R. H. Hageman.** 1978. Pathway for nitrate assimilation in corn (*Zea mays* L.) leaves: cellular distribution of enzymes and energy sources for nitrate reduction. Plant Physiol. **62:**618–621.

140. **Neyra, C. A., and P. van Berkum.** 1977. Nitrate reduction and nitrogenase activity in *Spirillum lipoferum*. Can. J. Microbiol. **23:**306–310.

141. **Nye, P. H.** 1958. The relative importance of fallows and soils in storing plant nutrients in Ghana. J. West Afr. Sci. Assoc. **4:**31–39.

142. **Nye, P. H., and D. J. Greenland.** 1960. The soil under shifting cultivation. Technical Communication 51. Commonwealth Bureau of Soils, Commonwealth Agricultural Bureau, Farnham Royal, England.

143. **Okon, Y., S. L. Albrecht, and R. H. Burris.** 1976. Carbon and ammonia metabolism of *Spirillum lipoferum*. J. Bacteriol. **128:**592–597.

144. **Okon, Y., S. L. Albrecht, and R. H. Burris.** 1976. Factors affecting growth and nitrogen fixation of *Spirillum lipoferum*. J. Bacteriol. **127:**1248–1254.

145. **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:**85–88.

146. **Okon, Y., J. P. Houchins, S. L. Albrecht, and R. H. Burris.** 1977. Growth of *Spirillum lipoferum* at constant partial pressures of oxygen, and the properties of its nitrogenase in cell-free extracts. J. Gen. Microbiol. **98:**87–93.

147. **Old, K. M., S. Hallam, and T. N. Nicolson.** 1975. Helically lobed bacteria in plant roots. Soil Biol. Biochem. **7:**73–75.

148. **Old, K. M., and T. H. Nicolson.** 1975. Electron microscopical studies of the microflora of roots of sand dune grasses. New Phytol. **74:**51–58.

149. **Pagan, J. D., J. J. Child, W. R. Scowcroft, and A. H. Gibson.** 1975. Nitrogen fixation by *Rhizobium* cultured on a defined medium. Nature (London) **256:**406–407.

150. **Paleg, L. G., and C. A. West.** 1972. The gibberellins, p. 146–181. *In* F. C. Steward (ed.), Plant physiology, vol. 6B. Physiology of development: the hormones. Academic Press, Inc., New York.

151. **Parr, F. F., S. Smith, and C. H. Willis.** 1970. Soil anaerobiosis. I. Effect of selected environments and energy sources on respiratory activity of soil microorganisms. Soil Sci. **110:**37–43.

152. **Pate, J. S.** 1962. Root-exudation studies on the exchange of C^{14}-labelled organic substances between the roots and shoots of the nodulated legume. Plant Soil **17:**333–356.

153. **Pate, J. S.** 1977. Functional biology of dinitrogen fixation by legumes, p. 473–518. *In* R. W. F. Hardy and W. S. Silver (ed.), A treatise on dinitrogen fixation, section 3. John Wiley & Sons, Inc., New York.

154. **Patriquin, D. G.** 1978. Factors affecting nitrogenase activity (acetylene reducing activity) associated with excised roots of the emergent halophyte *Spartina alterniflora* Loisel. Aquat. Bot. **4:**193–210.

155. **Patriquin, D. G., and J. Döbereiner.** 1978. Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. Can. J. Microbiol. **24:**734–742.

156. **Patriquin, D. G., and D. Denike.** 1978. *In situ* acetylene reduction assays of nitrogenase activity associated with the emergent halophyte *Spartina alterniflora* Loisel: methodological problems. Aquat. Bot. **4:**211–226.

157. **Patriquin, D. G., and C. R. McClung.** 1978. Nitrogen accretion, and the nature and possible significance of N_2-fixation (acetylene reduction) in a Nova Scotian *Spartina alterniflora* stand. Mar. Biol. **47:**227–242.

158. **Pedersen, W. L., K. Chakrabarty, R. V. Klucas, and A. K. Vidaver.** 1978. Nitrogen fixation (acetylene reduction) associated with roots of winter wheat and sorghum in Nebraska. Appl. Environ. Microbiol. **35:**129–135.

159. **Pedrosa, F. O., M. S. P. Stephan, and J. Döbereiner.** 1979. Interaction of nitrogenase and uptake hydrogenase activities in *Azospirillum brasilense*, p. 3. *In* Proceedings of the International Workshop on Associative N_2 Fixation. CENA, Piracicaba, Brazil.

160. **Pereira, P. A. A., J. F. W. von Bulow, and C. A. Neyra.** 1978. Atividade da nitrogenase, nitrato reductase e acumulacae de nitrogenio em milho praquitico *Zea mays* L. (cv. Piranao) em dois niveis de adubacao nitrogenada. R. Bras. Ci Solo **2:**28–33.

161. **Postgate, J. R.** 1969. Viable counts and viability, p. 611–628. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 1. Academic Press, Inc., New York.

162. **Postgate, J. R.** 1978. Nitrogen fixation. The Institute of Biology's Studies in Biology No. 92. Edward Arnold, Publishers, Ltd., London.

163. **Quesenberry, K. H., R. L. Smith, and S. C. Schank.** 1978. N_2-fixation research with tropical grasses, p. 33–34. *In* Proceedings of the 35th South Pasture Forage Crop Imp. Conference. Publisher, City.

164. **Rennie, R. J., and R. I. Larson.** 1979. Dinitrogen fixation associated with disomic chromosome substitution lines of spring wheat in the phytotron and in the field. *In* Proceedings of the International Workshop on Associative N_2 Fixation. CENA, Piracicaba, Brazil.

165. **Rice, W. Q., E. A. Paul, and L. R. Wetter.** 1967. The role of anaerobiosis in asymbiotic nitrogen fixation. Can. J. Microbiol. **13:**829–836.

166. **Rinaudo, G., I. Fares-Hamad, and Y. R. Dommergues.** 1977. Nitrogen fixation in the rice rhizosphere: methods of measurement and practices suggested to enhance the process, p. 313–322. *In* A. Ayanaba and P. J. Dart (ed.), Biological nitrogen fixation in farming systems of the tropics. John Wiley & Sons, Inc., New York.

167. **Ruschel, A. P., Y. Henis, and E. Salati.** 1975. Nitrogen-15 tracing of N-fixation with soil-grown sugarcane seedlings. Soil Biol. Biochem. **7**:181–182.

168. **Ruschel, A. P., R. L. Victoria, E. Salati, and Y. Henis.** 1978. Nitrogen fixation in sugarcane. Ecol. Bull. (Stockholm) **26**:297–303.

169. **Schank, S. C., R. L. Smith, G. C. Weiser, D. A. Zuberer, J. H. Bouton, K. H. Quesenberry, M. E. Tyler, J. R. Milam, and R. Littell.** 1979. Fluorescent antibody technique to identify *Azospirillum brasilense* associated with roots of grasses. Soil Biol. Biochem. **11**: 287–297.

170. **Schöllhorn, R., and R. H. Burris.** 1967. Acetylene as a competitive inhibitor of N_2 fixation. Proc. Natl. Acad. Sci. U.S.A. **58**:213–216.

171. **Schröder, M.** 1932. Die Assimilation des Luftstickstoffs durch einige Bakterien. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 **85**:178–212.

172. **Schubert, K. R., J. A. Engelke, S. A. Russell, and H. J. Evans.** 1977. Hydrogen reactions of nodulated leguminous plants. Plant Physiol. **60**:651–654.

173. **Schubert, K. R., and H. J. Evans.** 1976. Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc. Natl. Acad. Sci. U.S.A. **73**: 1207–1211.

174. **Shanugam, K. T., I. Chan, and C. Morandi.** 1975. Regulation of nitrogen fixation. Nitrogenase derepressed mutants of *Klebsiella pneumoniae*. Biochim. Biophys. Acta **408**:101–111.

175. **Shanmugam, K. T., F. O'Gara, K. Andersen, C. Morandi, and R. C. Valentine.** 1977. Genetic control of nitrogen fixation (*nif*), p. 321–330. *In* W. Newton, J. R. Postgate, and C. Rodriguez-Barrueco (ed.), Recent developments in nitrogen fixation. Academic Press, Inc., London.

176. **Shanmugam, K. T., and R. C. Valentine.** 1975. Molecular biology of nitrogen fixation. Science **187**:919–924.

177. **Skinner, K. J.** 1976. Nitrogen fixation. Key to a brighter future for agriculture? Chem. Eng. News **55**:22–35.

178. **Sloger, C., D. Bezdicek, R. Milberg, and N. Boonkerd.** 1975. Seasonal and diurnal variations in N_2 (C_2H_2)-fixing activity in field soybeans, p. 271–284. *In* W. D. P. Stewart (ed.), Nitrogen fixation by free-living microorganisms. International Biological Programme Series, vol. 6. Cambridge University Press, Cambridge, England.

179. **Small, J. G. C., and O. A. Leonard.** 1969. Translocation of C^{14}-labeled photosynthate in nodulated legumes as influenced by nitrate nitrogen. Am. J. Bot. **56**:187–194.

180. **Smith, A. M.** 1976. Ethylene production by bacteria in reduced microsites in soil and some implications to agriculture. Soil Biol. Biochem. **8**:293–298.

181. **Smith, D., and D. G. Patriquin.** 1978. A survey of angiosperms in Nova Scotia for rhizosphere nitrogenase (acetylene-reduction) activity. Can. J. Bot. **56**:2218–2223.

182. **Smith, R. L., J. H. Bouton, S. C. Schank, and K. H. Quesenberry.** 1977. Yield increases of tropical grain and forage grasses after inoculation with *Spirillum lipoferum* in Florida, p. 307–311. *In* A. Ayanaba and P. J. Dart (ed.), Biological nitrogen fixation in farming systems of the tropics. John Wiley & Sons, Inc., New York.

183. **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.

184. **Smith, R. L., S. C. Schank, J. H. Bouton, and K. H. Quesenberry.** 1978. Yield increases of tropical grasses after inoculation with *Spirillum lipoferum*. Ecol. Bull. (Stockholm) **26**: 380–385.

185. **Spiff, E. D., and C. T. I. Odu.** 1972. An assessment of non-symbiotic nitrogen in some Nigerian soils by the acetylene reduction technique. Soil Biol. Biochem. **4**:71–77.

186. **Stanier, R. Y., E. A. Adelberg, and J. Ingraham.** 1976. The microbial world. Prentice-Hall, Inc., Englewood Cliffs, N.J.

187. **Steyn, P. L., and C. C. Delwiche.** 1970. Nitrogen fixation by non-symbiotic microorganisms in some California soils. Environ. Sci. Technol. **4**:1122–1128.

188. **Streicher, S. L., K. T. Shanmugam, F. Ausubel, C. Morandi, and R. B. Goldberg.** 1974. Regulation of nitrogen fixation in *Klebsiella pneumoniae*: evidence for a role of nitrogenase synthesis. J. Bacteriol. **120**:815–821.

189. **Tarrand, J. J., N. R. Krieg, and J. Dobereiner.** 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions 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.

190. **Taylor, R. W.** 1979. Response of two grasses to inoculation with *Azospirillum* spp. in a Bahamian soil. Trop Agric. (Trinidad) **56**:361–366.

191. **Tchan, Y. T., and R. De Ville.** 1970. Application de l'immunofluorescence a l'etude des azotobacter du sol. Ann. Inst. Pasteur Paris **118**: 665–673.

192. **Tempest, D. W., J. L. Meers, and C. M. Brown.** 1970. Synthesis of glutamate in *Aerobacter aerogenes* by a hitherto unknown route. Biochem. J. **117**:405–407.

193. **Tien, T. M., M. H. Gaskins, and D. H. Hubbell.** 1979. Plant growth substances produced by

Azospirillum brasilense and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl. Environ. Microbiol. **37**:1016–1024.

194. **Tjepkema, J. D., and R. H. Burris.** 1976. Nitrogenase activity associated with some Wisconsin prairie grasses. Plant Soil **45**:81–94.

195. **Tjepkema, J. D., and H. J. Evans.** 1975. Nitrogen fixation by free-living *Rhizobium* in a defined liquid medium. Biochem. Biophys. Res. Commun. **65**:625–628.

196. **Tjepkema, J. D., and H. J. Evans.** 1976. Nitrogen fixation associated with *Juncus balticus* and other plants of Oregon wetlands. Soil Biol. Biochem. **8**:505–509.

197. **Tjepkema, J. D., and P. van Berkum.** 1977. Acetylene reduction by soil cores of maize and sorghum in Brazil. Appl. Environ. Microbiol. **33**:626–639.

198. **Truffant, G., and N. Bezssonoff.** 1923. Sur les conditions qui permettent la cooperation entre les bacteries fixatrices d'azote et le mais. C. R. Acad. Sci. **183**:1065–1067.

199. **Truffant, G., and N. Bezssonoff.** 1927. Sur l'utilisation de l'azote atmospherique per les plantes vertes. Rev. Gen. Sci. Pures Appl. **38**: 389–394.

200. **Tubb, R. S.** 1974. Glutamine synthetase and ammonium regulation of nitrogenase synthesis in *Klebsiella*. Nature (London) **251**:481–484.

201. **Umali-Garcia, M., D. H. Hubbell, and M. H. Gaskin.** 1978. Process of infection of *Panicum maximum* by *Spirillum lipoferum*. Ecol. Bull. (Stockholm) **26**:373–379.

202. **van Berkum, P.** 1980. Evaluation of acetylene reduction by excised roots for the determination of nitrogen fixation in grasses. Soil Biol. Biochem. **12**:141–145.

203. **van Berkum, P., and J. M. Day.** 1980. Nitrogenase activity associated with soil cores of grasses in Brazil. Soil Biol. Biochem. **12**:137–140.

204. **van Berkum, P., and C. Sloger.** 1979. Immediate acetylene reduction by excised grass roots not previously preincubated at low oxygen tensions. Plant Physiol. **64**:739–745.

205. **Vitranen, I. A., T. Moisio, and R. H. Burris.** 1955. Fixation of nitrogen by nodules excised from illuminated and darkened pea plants. Acta Chem. Scand. **9**:184–186.

206. **Vlassak, K., E. A. Paul, and R. E. Harris.** 1973. Assessment of biological nitrogen fixation in grassland and associated sites. Plant Soil **38**: 637–649.

207. **Volpon, A. G. T., H. De-Polli, and J. Döbereiner.** 1979. Changes in the efficiency of nitrogen fixation in various growth stages of batch cultures of *Azospirillum lipoferum*, p. 12. *In* Proceedings of the International Workshop on Associative N_2 Fixation. CENA, Piracicaba, Brazil.

208. **von Bülow, J. W. F., and J. Döbereiner.** 1975. Potential for nitrogen fixation in maize genotypes in Brazil. Proc. Natl. Acad. Sci. U.S.A. **72**:2389–2393.

209. **Watanabe, I., W. L. Barraquio, M. R. de Guzman, and D. A. Cabrera.** 1979. Nitrogen-fixing (acetylene reduction) activity and population of aerobic heterotrophic nitrogen-fixing bacteria associated with wetland rice. Appl. Environ. Microbiol. **37**:813–819.

210. **Watanabe, I., and D. R. Cabrera.** 1979. Nitrogen fixation associated with the rice plant grown in water culture. Appl. Environ. Microbiol. **37**:373–378.

211. **Watanabe, I., and K. K. Lee.** 1977. Non-symbiotic nitrogen fixation in rice and rice fields, p. 289–305. *In* A. Ayanaba and P. J. Dart (ed.), Biological nitrogen fixation in farming systems of the tropics. John Wiley & Sons, Inc., New York.

212. **Witty, J. F.** 1979. Acetylene reduction assay can overestimate nitrogen-fixation in soil. Soil Biol. Biochem. **11**:209–210.

213. **Witty, J. F., J. M. Day, and P. J. Dart.** 1977. The nitrogen economy of the Broadbalk experiments. II. Biological nitrogen fixation, p. 111–118. *In* Rothamsted report for 1976, part 2. Bartholomew Press, Dorking.

214. **Yates, M. G.** 1977. Physiological aspects of nitrogen fixation, p. 219–270. *In* W. Newton, J. R. Postgate, and C. Rodriguez-Barrueco (ed.), Recent developments in nitrogen fixation. Academic Press, Inc., London.

215. **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.