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Acetylene Reduction (Nitrogen Fixation) Associated with Corn Inoculated with *Spirillum*

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Sorghum and corn breeding lines were grown in soil in field and greenhouse experiments with and without an inoculum of N_2-fixing *Spirillum* strains from Brazil. Estimated rates of N_2 fixation associated with field-grown corn and sorghum plants were less than 4 g of N_2/ha per day. The mean estimated N_2-fixation rates determined on segments of roots from corn inoculated with *Spirillum* and grown in the greenhouse at 24 to 27°C were 15 g of N_2/ha per day (16 inbreds), 25 g of N_2/ha per day (six hybrids), and 165 g of N_2/ha per day for one hybrid which was heavily inoculated. The corresponding mean rates determined from measurements of in situ cultures of the same series of corn plants (i.e., 16 inbreds, six hybrids, and one heavily inoculated hybrid) were 0.4, 2.3, and 1.1 g of N_2/ha per day, respectively. Lower rates of C_2H_2 reduction were associated with control corn cultures which had been treated with autoclaved *Spirillum* than with cultures inoculated with live *Spirillum*. No C_2H_2 reduction was detected in plant cultures treated with ammonium nitrate. Numbers of nitrogen-fixing bacteria on excised roots of corn plants increased an average of about 30-fold during an overnight preincubation period, and as a result acetylene reduction assays of root samples after preincubation failed to serve as a valid basis for estimating N_2 fixation by corn in pot cultures. Plants grown without added nitrogen either with or without inoculum exhibited severe symptoms of nitrogen deficiency and in most cases produced significantly less dry weight than those supplied with fixed nitrogen. Although substantial rates of C_2H_2 reduction by excised corn roots were observed after preincubation under limited oxygen, the yield and nitrogen content of inoculated plants and the C_2H_2-reduction rates by inoculated pot cultures of corn, in situ, provided no evidence of appreciable N_2 fixation.

Nitrogenase activity has been observed in association with the roots of several tropical grasses (3, 7, 8) and N_2-fixing bacteria have been isolated from roots of some of these by investigators in Brazil. *Beijerinckia indica* was isolated from the root environment of sugar cane (5), *Azotobacter paspali* from the root systems of *Paspalum notatum* (4) and *Spirillum lipoferum* from roots of *Digitaria decumbens* (3a). The association of *Azotobacter paspali* with *Paspalum notatum* was estimated to fix N_2 at rates as high as 90 kg of N/ha per year (4, 6). Recently Von Bulow and Dobereiner (10) have reported that N_2 fixation may occur at rates of up to 2 kg/ha per day in association with some maize lines in Brazil. These estimated rates were based upon C_2H_2 reduction of excised roots that had been preincubated. Strains of N_2-fixing *Spirillum* were isolated from roots of maize that were active in the C_2H_2-reduction test.

We have attempted to establish N_2 fixation in the root environments of several breeding lines of corn (maize) and sorghum under field and greenhouse conditions by inoculation with two strains of *Spirillum* from Brazil.

MATERIALS AND METHODS

Sources of seeds. The field surveys of grain sorghum were conducted with 120 breeding lines furnished by M. Kolding of the Columbia Basin Agricultural Research Center, Pendleton, Ore. All corn seeds were obtained from D. Alvey, Farmers Forage Research Coop., West Lafayette, Ind. In the field survey of corn, the following 25 inbred lines were tested: W22 72:3616; W182B 72:3623; MS141 73:4459; W117 72:3621; B73 73:4451; C103 72:3558; C123 72:3559; A295 72:3501; A239 72:3593; A619 72:3505; A554 72:3504; B37 72:3553; WF9 73:4493; B14A 71:1551; W59M 73:4490; OH43 72:3601; MS100 72:3586; W153R 72:3622; W491 72:3627; W64A 72:3620; MS57 71:1742; A632 72:3506; H95 75:1247; H84 72:3572; and OH51A 72:3604. In the initial greenhouse experiment the first 16 of the above list of inbred corn lines were tested. In the second green-

house experiment the following six hybrid corn lines were tested: 72:1743 A239 Ht × OH43 Ht; 72:1789 A619 Ht × W153 R Ht; 72:1811 A632 Ht × B37 Ht; 72:2165 R53 × W153 R Ht; 72:2167 W64 A Ht × W153 R Ht; and 73:2375 B14A Ht × B37 Ht. In the third experiment, hybrid 72:2167 W64 A Ht × W153 R Ht, which showed relatively high C_2H_2-reduction rates in the second experiment, was utilized for further tests.

Field studies. The 120 sorghum lines were grown on the Carl Kaser farm at The Dalles, Ore. in plots (size of about 0.5 m by 3 m) which received 3 pounds (ca. 1.36 kg)/acre of $Na_2MoO_4 \cdot 2H_2O$, 1 pound (ca. 0.45 kg)/acre of $CoCl_2 \cdot 6H_2O$, 5 pounds (ca. 2.27 kg)/acre of Fe as sodium ferric ethylenediamine di-(*o*-hydroxyphenylacetate) (NaFeEDDHA), and 40 pounds (ca. 18.16 kg)/acre of N as $(NH_4)_2SO_4$. The 25 corn inbreds were grown on the Oregon State University Botany farm near Corvallis in plots (1 m by 3 m) which received 100 pounds (ca. 45.5 kg)/acre of P_2O_5 as superphosphate, 75 pounds (ca. 34.05 kg)/acre of K_2O, as murate of potash, 5 pounds (ca. 2.27 kg)/acre of Fe as sodium ferric ethylenediamine di-(*o*-hydroxyphenylacetate) 1 pound (ca. 0.45 kg)/acre of $CoCl_2 \cdot 6H_2O$, and 3 pounds (ca. 1.36 kg)/acre of $Na_2MoO_4 \cdot 2H_2O$.

Greenhouse growth conditions. Experiment 1 was begun on 11 June 1975, experiment 2 on 23 July, and experiment 3 on 26 September. The first two experiments were conducted in a greenhouse with natural light. In the third experiment, supplemental light (about 500 foot-candles at 3 feet [ca. 91.44 cm] above greenhouse benches) was supplied for 14 h/day. Corn plants were grown in large 21-liter pots filled with Newberg sandy loam, with a pH of 6.7 and a NO_3^--N content of 0.31 μg/g. The nitrogen treatments listed in Table 2 consisted of 4 g of NH_4NO_3 per pot in experiments 1 and 2, and 6 g of NH_4NO_3 per pot in the third experiment. All plants received applications of phosphorus, potassium, and molybdenum. In experiment 1, fertilizer applications per pot were: superphosphate (18% P_2O_5), 7.6 g; murate of potash (60% K_2O), 2.0 g; and $Na_2MoO_4 \cdot 2H_2O$, 34 mg. In experiments 2 and 3, the applications per pot were: NaH_2PO_4, 2.72 g; KCl, 2.0 g; and $Na_2MoO_4 \cdot 2H_2O$, 34 mg. The plants were supplied daily with sufficient distilled water to maintain an average soil moisture content between 17 to 22%. The soil temperature was maintained between 24 and 27°C.

Bacterial culture. Two strains of N_2-fixing *Spirillum* from J. Dobereiner in Brazil were used for inoculation of corn. *S. lipoferum* originally was isolated from *D. decumbens* (3a), and Sp 81 was isolated from maize (7). The bacterial cultures for the first two experiments and for all field experiments were grown in a modified malate medium of Dobereiner (3a) containing per liter: KH_2PO_4, 0.4 g; K_2HPO_4; 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; $FeCl_3$, 0.01 g; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g; malic acid, 3.6 g; and NH_4Cl, 0.013 g. Hereafter this is referred to as the D+N medium. In the third experiment, the KH_2PO_4 concentration in the medium was increased to 2.0 g and K_2HPO_4 to 0.5 g per liter to increase the buffering capacity of the medium.

Plant inoculation. Field-grown corn and sorghum were inoculated once with a solution containing approximately 10^8 cells of *S. lipoferum* per plant. In all experiments each control plant was treated with an equal volume of an autoclaved cell suspension. In the first greenhouse experiment, plants were inoculated twice (9 and 33 days after planting) with about 10^9 cells of *S. lipoferum* suspended in the D+N medium without malate and nitrogen. In the second experiment, each corn plant was inoculated twice with 10^9 cells of Sp 81 suspended in D+N medium without malate or nitrogen (6 and 16 days after planting) and a third time with 10^9 cells of Sp 81 in complete D+N medium (34 days after planting). In the third experiment, plants were inoculated four times (3, 13, 20, and 28 days after planting) with 10^9 cells of Sp 81 suspended in the complete malate growth medium.

In the first two greenhouse experiments, four replicate cultures of each corn line received each treatment. In an attempt to more accurately determine the effect of bacterial inoculation in the third experiment, 10 corn cultures were treated with a live suspension of Sp 81, and 10 cultures were treated with an autoclaved suspension of Sp 81 cells.

Estimation of numbers of N_2-fixing bacteria. Samples of fresh roots each weighing about 0.1 g were crushed with a sterile mortar and pestle, and suspended in 10 ml of sterile D+N medium. In some cases (see Results) the roots were surface disinfected for 1 min in 1% sodium hypochlorite and washed five times in distilled water before they were crushed. Samples of soil used for estimating N_2-fixing bacteria were suspended directly in D+N medium and serial 10-fold dilutions of these were made in D+N medium. Portions were plated on D+N agar to which was added 0.0025% bromothymol blue. The dilution tubes were flushed with a gas mixture containing 97% N_2, 1% O_2, and 2% CO_2 and were incubated for 2 days at 30°C. Acetylene (0.1 atm) was then added, and detection of C_2H_4 (greater than 1 nmol of C_2H_4/tube per 24 h) indicated the presence of N_2-fixing bacteria. The ethylene content of gas samples was determined by use of a Varian-Aerograph model 600D gas chromatograph equipped with a hydrogen flame ionization detector and a Porapak-R column. The estimated number of *Spirillum* cells was based upon observation of the morphology of colonies growing on the malate-agar plates (3a). The procedure for enumeration of N_2-fixing bacteria in our experiments only counted those bacteria capable of growing and fixing N_2 at low oxygen tensions in a malate medium. No estimate was made of the numbers of the other bacteria that may have been present in the soil or on the corn roots.

Measurement of acetylene-reduction rates associated with excised roots and intact corn plants. In the greenhouse studies, one root sample was removed from each replicate plant at each of two sampling times, 6 to 8 weeks after planting. In a modification of the preincubation technique of Von Bülow and Dobereiner (10), samples of the larger corn roots

(about 3 mm by 2 to 4 cm) were rinsed in distilled water and placed in culture tubes. Each tube was stoppered, about 0.3 ml of distilled water was added to prevent desiccation, and each tube was flushed with a gas mixture containing 97% N_2, 1% O_2, and 2% CO_2. After overnight incubation (about 16 h) at 30°C, the tubes were flushed again with the same gas mixture and 0.1 atm of C_2H_2 was added to each tube. Formation of C_2H_4 was measured by gas chromatography at periods of 2, 7, and 24 h after C_2H_2 addition.

At 6 to 7 weeks after planting, C_2H_2 reduction by the intact plants at the silking stage (10) was assayed by placing the pots in Saran bags (2) and tying the bags around the stems at a distance of about 15 cm above the soil surface. The seal between the bag and the stem was accomplished by shaping modeling clay around the stem, then gathering the bag around the clay and tying with a string. Gases were added and removed from the bags through holes punctured at points reinforced by adhesive tape. The punctured holes were sealed by use of adhesive tape. Approximately 0.1 atm of C_2H_2 was added to each bag after removing excess air from the bag with a vacuum pump, and C_2H_4 formation was measured periodically for 2 days. In field studies, soil cores (6.4-cm by 15-cm length) of corn and sorghum plants and the surrounding soil were placed in 1-quart (0.946 liter) Mason jars and assayed for C_2H_4 about 1 day after the addition to each jar of 0.1 atm C_2H_2 (J. Tjepkema and R. H. Burris, Plant Soil, in press).

A factor of 3 was used in the estimated conversion of nanomoles of C_2H_2 reduced to nanomoles of N_2 fixed by the roots and intact plants (1, 8). In the root studies, the assumption of Von Bülow and Dobereiner (9) of a root mean dry weight of 1,500 kg/ha was used to calculate the N_2 fixed per hectare per day. Calculation of N_2 fixation associated with intact plants was based on an estimate of 20,000 plants/acre.

RESULTS

Field experiments. In field studies in which 25 corn inbreds and 120 sorghum breeding lines were inoculated with *S. lipoferum*, low N_2-fixing rates of less than 4 g of N_2 per ha per day were estimated from the rates of C_2H_2 reduction by soil cores of both inoculated plants and control plants (Table 1). Excised roots of the 25 corn inbreds were also assayed for C_2H_2 reduction, and a mean N_2-fixation rate of 0.1 g of N/ha per day was estimated.

Greenhouse experiments. In a preliminary greenhouse survey of 16 of the 25 corn inbreds, the mean C_2H_2-reduction rate associated with excised roots of plants which had been inoculated with live *Spirillum* was 47 nmol/h per g of dry roots (16 g of N/ha per day), which was higher than that associated with roots of control plants (10 nmol per h per g of dry roots or about 3 g of N/ha per day). Assays of in situ corn cultures, however, showed negligible rates of N_2 fixation (C_2H_2 reduction) (Table 2).

In the second experiment where six hybrid corn lines were inoculated with *Spirillum*, higher C_2H_2-reduction rates were associated with roots of plants inoculated with live *Spirillum* (mean of 74 nmol of C_2H_4 formed/h per g of dry roots or about 25 g of N fixed/ha per day) than with roots of plants treated with autoclaved *Spirillum* (mean of 15 nmol of C_2H_4/h per g of dry roots). In most of the assays of C_2H_2 reduction by excised roots, C_2H_4 was produced at a linear rate. The effect of inoculation with *Spirillum* on rates of C_2H_2 reduction by preincubated roots was significant at the 0.01 level in this experiment. In contrast to the results with excised roots, the C_2H_2-reduction rates by the in situ corn cultures were low, with a mean of about 200 nmol of C_2H_4 formed per plant per h in each non-nitrogen treatment (about 2 g of N/ha per day). Estimated rates of N_2 fixation for excised roots and in situ cultures are presented in Table 2. There seemed to be no relation between the rates of C_2H_2 reduction associated with the excised roots of a particular plant and the rate of C_2H_2 reduction associated with that intact plant. Little or no C_2H_2 reduction was detected either by the in situ assay or by an assay of excised roots of corn plants treated with fixed nitrogen.

In the third experiment, in which a single

TABLE 1. *Estimated N_2 fixation (C_2H_2 reduction) rates by corn inbreds and sorghum breeding lines in field studies*

Material	Location	Treatment	N_2 fixation (g/ha per day)[a]	
			Mean	Range
120 sorghum lines	The Dalles, Ore.	*Spirillum lipoferum*	0.7	0.1–2.3
25 corn inbreds	Botany Farm, Corvallis	*Spirillum lipoferum*	1.3	0.3–3.5
25 corn inbreds	Botany Farm, Corvallis	Autoclaved *Spirillum lipoferum*	0.9	0.3–1.6

[a] Determined from rates of C_2H_2 reduction by soil cores containing roots as described in Materials and Methods.

TABLE 2. *Estimated rates of N_2 fixation (C_2H_2 reduction) associated with corn in greenhouse experiments*

Expt	Material[a]	Treatment	N_2 fixation (g/ha per day)				Dry weight (g/plant)[b]	Nitrogen content (g/plant)[b]
			Root assays		In situ corn cultures			
			Mean	Range	Mean	Range		
1	16 inbreds	Live *S. lipoferum*	16	0–145	0.4	0–1.0	81 ± 1.9	
	16 inbreds	Autoclaved cells + N	3	0–62	0.3	0–3.2	87 ± 2.5	
2	6 hybrids	Live Sp 81	25	0–252	2.3	0.1–14.7	41 ± 2.2	0.19 ± 0.01
	6 hybrids	Autoclaved cells	5	0–57	1.7	0.3–13.4	49 ± 1.6	0.22 ± 0.01
	6 hybrids	Autoclaved cells + N	0	0–0.2	0.3	0–0.5	77 ± 2.0	0.97 ± 0.02
3a	1 hybrid	Live Sp 81	165	9–734	1.1	0.3–2.3	48 ± 3.3	
	1 hybrid	Autoclaved cells	130	5–322	1.3	0.2–2.7	48 ± 3.1	
3b	1 hybrid	Autoclaved cells + N	1	0–5	0.1	0.07–0.13	68 ± 2.7	
	1 hybrid	Live Sp 81 "used" soil[c]	151	29–240	2.1	0.8–5.3	19 ± 1.6	

[a] The corn breeding lines tested in each experiment are listed in Materials and Methods.

[b] Mean of 10 replicate cultures in experiment 3a and 4 replicate cultures in all other experiments ± the standard error of the mean.

[c] "Used" soil was soil which had previously been inoculated and planted with the same hybrid in the second experiment.

corn hybrid was studied, substantially higher C_2H_2-reduction rates were obtained in association with roots of plants inoculated with live Sp 81 or with autoclaved Sp 81 than in the second experiment. This may have been due to stimulation of bacterial growth in the soil by the addition of large quantities of growth medium at the time of inoculation. The formation of C_2H_4 in association with root samples was linear with time. The mean rates of C_2H_4 formation by samples of roots in the first sampling at 45 days after planting (Fig. 1) were about twice the mean rates in the second sampling at 54 days. The assays of in situ plants, however, again showed low C_2H_2-reduction rates (Fig. 2) of about 100 nmol of C_2H_4 formed per h per plant (about 1 g of N/ha per day). When plants were inoculated with Sp 81 and grown in "used" soil (previously inoculated and used for growth of corn plants in the second experiment) the rates of C_2H_2 reduction associated with in situ plants were 2.0-fold higher than when plants were grown in soil inoculated only in the third experiment (Fig. 2, Table 2).

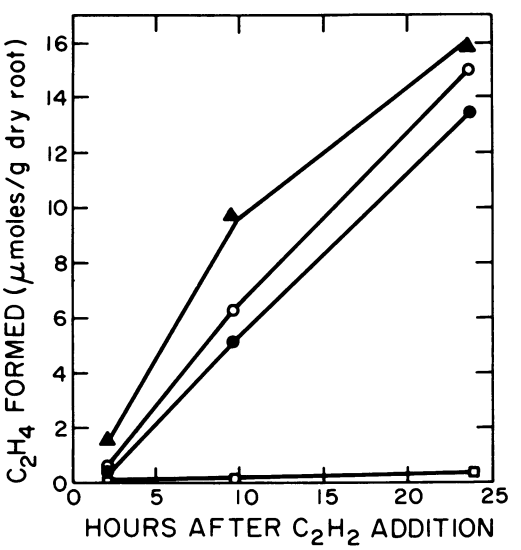

FIG. 1. *Acetylene reduction (45 days after planting) associated with excised preincubated roots of hybrid corn plants treated with live Spirillum (○), autoclaved Spirillum (●), autoclaved Spirillum plus nitrogen (□), and live Spirillum in used soil (▲).*

Dry weights and total nitrogen contents. In all three experiments, corn plants that were grown in soil to which nitrogen was added produced higher dry weights than those lacking added nitrogen (Table 2). In the second experiment, the mean dry weight (49 g) of plants treated with autoclaved *Spirillum* was significantly higher at the 99% confidence level than the mean dry weight (41 g) of plants inoculated with live *Spirillum*. However, the mean nitrogen contents of plants in the two treatments were not significantly different, and were much lower than the mean nitrogen content of plants receiving fixed nitrogen (Table 2). In the third experiment, there was no significant difference between the dry weights of plants inoculated with live *Spirillum* and those treated with au-

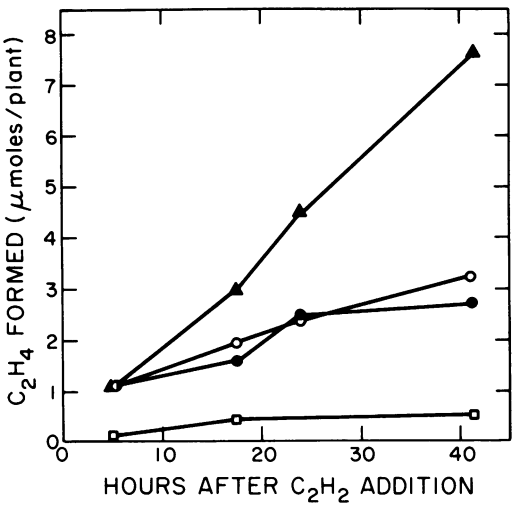

FIG. 2. *Acetylene reduction associated with in situ corn cultures treated with live Spirillum (○), autoclaved Spirillum (●), autoclaved Spirillum plus nitrogen (□), and live Spirillum in used soil (▲).*

toclaved *Spirillum*. The dry weights of the plants grown in used soil and inoculated with Sp 81 were significantly lower than those of plants receiving any of the other treatments (Table 2).

Acetylene reduction by soybeans. In a test of the validity of our in situ plant culture assay system, soybeans were grown in pots of the same soil type used for greenhouse corn experiments and were inoculated with a commercial preparation of *Rhizobium japonicum*. The C_2H_2-reduction assays were conducted with in situ cultures completely enclosed in Saran bags. After an initial lag of not more than 1 h, the rate of C_2H_2 reduction was constant for 24 h, indicating that there was no significant lag in gas diffusion in and out of the soil. Although each pot contained an average of three plants, and these were only moderately nodulated, the rates of acetylene reduction by the pots of soybeans in situ was much greater than those observed for in situ corn cultures being equivalent to 160 g of N fixed per ha per day.

Studies of numbers of N_2-fixing bacteria associated with roots and soil. An attempt was made to determine whether an increase in numbers of N_2-fixing bacteria during the preincubation of corn roots was responsible for increased C_2H_2-reduction rates by roots from greenhouse plants treated with live *Spirillum*. One root segment from each plant was crushed and diluted in D+N medium for enumeration of N_2-fixing bacteria. A second root segment was placed in a tube and preincubated overnight under 1% O_2, as described in Materials and Methods, and then assayed for N_2-fixing bacteria. After preincubation of the root segments, marked increases in numbers of N_2-fixing bacteria were found in all cases. An average of 10^7 N_2-fixing bacteria per g of wet roots was found associated with 13 preincubated root samples as compared to an average of only 3×10^5 N_2-fixing bacteria per g of freshly excised roots. The N_2-fixing bacteria associated with roots appeared to be located primarily in external areas because brief surface disinfection of five excised roots resulted in a decrease from 3×10^5 to 2×10^4 N_2-fixing bacteria per g of wet roots. In the soil treated with live *Spirillum*, about 3×10^7 N_2-fixing bacteria per g of wet soil were present at the time roots were sampled, whereas only 10^3 to 10^4 N_2-fixing bacteria were present per g of soil treated with autoclaved *Spirillum*. In the samples of roots and soils which were obtained after inoculation with live *Spirillum*, the proportion of N_2-fixing bacteria having colonial morphology similar to the *Spirillum* strains of Dobereiner varied from 0 to 100%. No *Spirillum*-like bacteria were observed in association with roots or soil that had been treated with autoclaved *Spirillum*.

DISCUSSION

In this study we observed a marked difference between the C_2H_2-reducing rates associated with excised roots of plants inoculated with live *Spirillum* and the C_2H_2-reduction rates associated with the roots of plants treated with autoclaved *Spirillum*. Highest activities were obtained with adventitious roots 3 to 4 mm in diameter. These observations are consistent with those reported by Von Bulow and Dobereiner (10).

In the second greenhouse experiment, considerable differences in nitrogenase activities were observed between two individual adventitious root samples from the same plants. Although the mean root activity of plants inoculated with live *Spirillum* was high on both sampling dates, samples of roots from about one-fourth of the plants showed no significant nitrogenase activity on either sampling date. Approximately 85% of the roots obtained from inoculated plants showed significant C_2H_2-reducing activity in the third experiment, but high C_2H_2-reduction activity was also observed with 70% of the roots obtained from plants that had been treated with autoclaved *Spirillum*.

The C_2H_2-reduction rates of excised roots of the corn hybrid studied in the third experiment were comparable to some of the C_2H_2-reduction rates associated with root samples from corn

lines investigated in Brazil. In our experiments, a maximum C_2H_2-reduction rate of 2,186 nmol of C_2H_4 formed per h per g of dry roots (734 g of N/ha per day) and a mean C_2H_2-reduction rate of 488 nmol/h per g of dry roots (165 g of N/ha per day) were observed with one hybrid corn line. Von Bulow and Dobereiner (10) reported mean nitrogenase activities of 74 to 2,167 nmol of C_2H_4 formed per h/g of dry roots in a survey of maize grown in six areas of Brazil, and in the best maize lines studied, a mean activity of 7,124 nmol of C_2H_4 formed per h per g of dry roots was reported.

The C_2H_2-reduction rates obtained with excised roots in our third experiment can be extrapolated to a mean rate of 17 kg of N_2 fixed per ha per 100-day growing season and a maximum rate of 73 kg of N_2 fixed per ha per season. Although these rates by preincubated roots are substantial, the actual amount of nitrogen fixed was not sufficient to supply the nitrogen needs of the corn plants as was indicated by the chlorotic appearance, the decreased size and weight, and the low nitrogen content of inoculated corn plants, as compared with those receiving fixed nitrogen.

The fact that there was somewhat higher C_2H_2 reduction by the intact plants which were inoculated with *Spirillum* and grown in used soil than by the other inoculated plants, indicates that with continual application of *Spirillum*, the proper microflora might be established in the soil. It is also possible that C_2H_2-reduction rates of Brazilian corn breeding lines might be considerably higher than those of corn hybrids used in our experiments. However, the temperature requirements of at least *S. lipoferum* (Dobereiner, personal communication) might preclude establishment of most corn-*Spirillum* associations under field conditions in temperate areas of the world.

The estimated rates of nitrogen fixation per hectare based on C_2H_2-reduction rates of the in situ cultures grown at 24 to 27°C were always lower than comparable N_2-fixation rates based upon activities of preincubated excised roots of the same plants. These results are in contrast to those reported by Dobereiner et al. (6) using *P. notatum*. It is significant that overnight preincubation of the corn roots in our study resulted in an approximate 30-fold increase in the numbers of N_2-fixing bacteria. These results and the observed markedly lower rates of C_2H_2 reduction of in situ corn cultures, as compared with rates by excised roots, strongly indicate that the preincubation method leads to an overestimate of the quantity of nitrogen fixed. Thus, high rates of C_2H_2 reduction after preincubation of excised roots has not proved to be a reliable index of the capability of corn to grow in a low nitrogen soil. We have been unable to demonstrate significant N_2 fixation in the root environment of corn by inoculation with *Spirillum*.

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