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Endorhizal and Exorhizal Acetylene-reducing Activity in a Grass (*Spartina alterniflora* Loisel.)-Diazotroph Association[1]

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ABSTRACT

Earlier studies indicated that bacteria responsible for nitrogenase activity of some grasses are located inside the roots. Those studies were conducted with excised roots in which a long, unexplained "lag phase" occurred before initiation of nitrogenase activity. When hydroponically maintained *Spartina alterniflora* Loisel. was incubated in a two-compartment system with acetylene, ethylene was produced following, at most, a 2-hour lag in both the upper (shoot) and lower (roots + water) phases. Ethylene production in the upper phase not attributable to leaf-associated acetylene-reducing activity or to diffusion of ethylene from around the roots is considered to represent "endorhizal acetylene-reducing activity," the internally produced ethylene diffusing into the upper phase via the lacunae. Ethylene produced in the lower phase is designated "exorhizal acetylene-reducing activity." The endorhizal acetylene-reducing activity, in comparison to exorhizal activity, was relatively insensitive to additions of $HgCl_2$, NH_4Cl, or carbon sources to the lower phase. Post-lag acetylene-reducing activity of roots excised from plants growing in soil responded to additions in a manner similar to that of endorhizal acetylene-reducing activity, whereas post-lag acetylene-reducing activity of rhizosphere soil responded in a manner similar to that of exorhizal acetylene-reducing activity.

A number of agriculturally important grasses exhibit nitrogenase activity which is attributable to associated diazotrophic (N_2-fixing) bacteria. The precise location of these diazotrophs may vary with the plant and bacterial species (9) as well as with the development of the plant (8). In some systems, most of the nitrogenase activity is associated with soil around the roots ("rhizosphere soil"), whereas in others it is closely associated with the roots. In the latter case, the responsible diazotrophs could be on or in the roots. Although invasion of the inner cortex and stele of roots by diazotrophs and other bacteria is probably a common phenomenon (12), there is no direct evidence for nitrogenase activity of these "endorhizal" diazotrophs *in situ*.

Several studies have provided indirect evidence that excised root nitrogenase activity is attributable to endorhizal bacteria, particularly to *Azospirillum* spp. (2, 4, 8) or to other unidentified microaerophils (10, 11, 13). However, the excised root nitrogenase activities started only after lag phases of 8 or more h. Uncertainties as to the cause of this lag phase (5, 9) make the applied significance of excised root nitrogenase activity and, hence, of factors correlated with it controversial. Active endorhizal populations would be of interest, in that they would be more likely than rhizoplane or rhizosphere soil populations to contribute fixed N_2 to the plant, they would utilize plant-derived carbon sources more efficiently, and their metabolic processes could be directly coupled to those of the plant. Such a population would represent a primitive symbiosis (4).

In this paper, evidence is presented for nitrogenase activity by both internal and external diazotroph populations of intact, hydroponically maintained *Spartina alterniflora* Loisel. This perennial, C_4-type grass, which is common in salt marshes in eastern North America, has an association with diazotrophs similar to that described for some of the domesticated tropical grasses (10). Effects of combined nitrogen or carbon source addition on nitrogenase activities of the two populations are investigated. The results of these experiments, in which there was no pronounced "lag phase" preceding nitrogenase activity, are compared to those obtained in similar experiments using excised roots and rhizosphere soils from plants grown in soil. In those experiments, nitrogenase activity occurred after a long lag phase.

MATERIALS AND METHODS

Sampling Sites and Plant Material. Plants were collected in August from a *S. alterniflora* stand close to Halifax, Nova Scotia. Plants in hydroponic culture were maintained in a greenhouse in a 600-l tank into which flowed a 1.8 l/min stream of half-strength seawater. Temperatures were generally between 10 and 17 C; 14-h days were maintained with auxiliary lighting (250 μE m^{-2} s^{-1}). Plant growth was slow but continuous.

By November, deficiency symptoms were evident, and foil packets containing 10.6 g NH_4NO_3, 0.45 g KH_2PO_4, and 1.41 g Na_2HPO_4 were added at approximately weekly intervals. Experiments with these plants were started in January. No additions were made to the tank for at least 1 week before any experiment. Sods of *Spartina*, maintained in undrained buckets in the greenhouse, were watered with distilled H_2O twice a week. Experiments with these plants were begun in March.

Assays of Nitrogenase Activity. Nitrogenase activity was measured by the C_2H_2-reduction technique (7). Plants from the hydroponic system were transferred underwater into two-phase assay systems (Fig. 1). C_2H_2 from a balloon containing 80% C_2H_2 in N_2 was introduced through the lower phase-sampling port to displace 11% of the seawater. This resulted in a gas-phase C_2H_2 concentration in equilibrium with the seawater of 8%. C_2H_2 was injected into the upper phase to provide an 8% concentration, the jars were shaken for 1 min, and, as the C_2H_2 went into solution, N_2 was introduced to eliminate the resulting vacuum. After various periods of incubation in the greenhouse, the jars were reshaken, 0.5-ml gas samples were withdrawn from each of the upper and lower phases, and these were analyzed for C_2H_4, C_2H_2, and C_3H_8 as described below.

In investigations of the effects of ammonium chloride or carbon source addition on upper and lower phase nitrogenase activities, plants were assayed on a number of successive days. C_2H_2 was added at 0800 h, and C_2H_4 production was measured between

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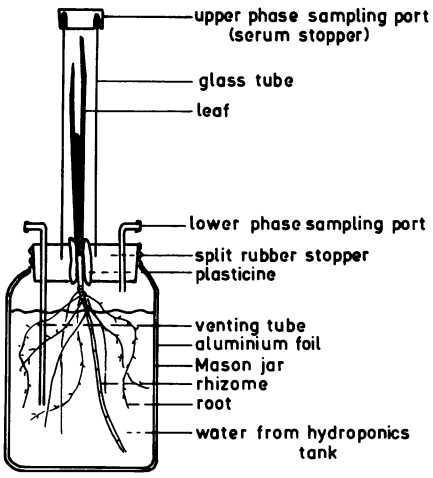

FIG. 1. *Spartina* in two-phase assay system.

1000 and 1400 h. At 1800 h, seawater was replaced with fresh seawater or with seawater containing various concentrations of NH_4Cl or with seawater containing 0.1% D-glucose and 0.1% L-malate (pH 7.2) as indicated in the Tables. Between treatments, plants were left in open jars with seawater replaced daily for at least 4 days. To determine the effect of root surface sterilization on upper- and lower-phase nitrogenase activities, $HgCl_2$ (a general nonmetabolic poison) was added to the lower phase seawater at 0.2% (w/v) concentration.

Root or rhizome samples were removed from the bucket-grown plants, washed repeatedly in seawater from the hydroponic system, and cut into 2-cm pieces, and 2 g (wet) tissue were placed into 50-ml flasks. "Young roots" were cut from new root growth at the periphery of the sod. Morphologically similar "older roots" were cut from within the sod. Rhizomes were cut from both young and older regions. "Rhizosphere soil" consisted of soil adhering to roots and rhizomes. This soil was washed through a 1-mm mesh screen to remove plant fragments, and 10-ml subsamples from the settled soil were placed in 50-ml flasks. Ten ml of hydroponic seawater were added to each sample; the flasks were closed with serum stoppers, evacuated, and backfilled with 5% O_2 in N_2; C_2H_2 was injected to provide a pC_2H_2 of 8 kPa. Gas samples were taken at specified intervals for analysis of C_2H_4 and C_2H_2.

With each set of experiments, plants were incubated without C_2H_2 to check for endogenous C_2H_4 production. Excised parts were also incubated under N_2 without C_2H_2 and with and without 3 ml 1% glucose solution, to check for C_2H_4 production in the absence of possible C_2H_4 oxidation (1). No C_2H_4 production was observed in any of these tests, so it is assumed that C_2H_4 production observed in the presence of C_2H_2 represented nitrogenase activity.

C_2H_4, C_2H_2, and propane concentrations of gas samples were determined using a Carle model 9500 basic gas chromatograph equipped with a Porapak T column (0.32 × 50 cm; 80–100 mesh) and flame ionization detection. Column temperature was 50 C; carrier gas flow rate was 30 ml/min. Retention times for C_2H_4, C_2H_2, and C_3H_8 were 15, 28, and 110 s, respectively. Solubility of C_2H_4 in the liquid phase was taken into account (6).

Counts of Bacteria. Total numbers of bacteria, and numbers of microaerophilic and of anaerobic diazotrophs in surface-sterilized and untreated roots were determined as described by Patriquin and McClung (13). Numbers of "*Azotobacter*-like" bacteria were estimated by plate counts using the medium of Clarke (3) with 15 g/l NaCl added. Subcultures from the plates were tested for C_2H_2-reducing activity in tubes containing this medium, with or without agar, and with or without yeast extract (1 g/l).

RESULTS

Exorhizal and Endorhizal Nitrogenase Activity of Plants in Hydroponic Culture. When *Spartina* plants from hydroponic culture were incubated with C_2H_2 in the two-chamber system, C_2H_4 was produced in both the upper and lower phases. No C_2H_4 production occurred in seawater alone. There was no pronounced lag, the rate of C_2H_4 production being essentially constant after 2 h (Fig. 2). For about half of the randomly selected plants, C_2H_4 production was low in comparison to initial background levels (plant 2, Fig. 2). Occasional high initial C_2H_4 values were presumably associated with incomplete flushing of gases from previous assays. Prior to subsequent experiments (below), plants were prescreened for C_2H_2-reducing activity, and only those exhibiting rates resulting in C_2H_4 levels well above background were used. Also, because the C_2H_2-reducing activity of individual plants varied irregularly and by as much as 2-fold between 2 consecutive days, the nitrogenase activity under any given set of conditions was determined on at least 2 successive days.

The upper phase C_2H_4 could have originated from one or more of three possible sources: (*a*) C_2H_2-reducing activity of diazotrophs associated with the leaves; (*b*) diffusion via the lacunae (15) from the C_2H_4 pool measured in the lower phase around the roots; or (*c*) C_2H_2-reducing activity of diazotrophs inside of the roots, the resulting C_2H_4 diffusing directly into the upper phase via the lacunae.

Production of C_2H_4 from C_2H_2 by four plants was measured on 2 successive days, and on the third day leaves were excised and their C_2H_2-reducing activities were measured separately. The excised leaf activities (1.1–1.7 nmol C_2H_4 plant^{-1} h^{-1}) were equivalent to 8 to 24% of the previously measured upper phase C_2H_4 production of the intact plants.

Two methods were utilized to estimate the flux of C_2H_4 from around the roots into the upper phase (source *b*, above). After the C_2H_2-reduction assay, seawater around the roots was renewed several times to remove traces of C_2H_4 and C_2H_2, and a small concentration of C_2H_4 was introduced into the lower phase (method 1). After 2 and 6 h, upper and lower phase gas samples were analyzed for C_2H_4. The flux of C_2H_4 ($F_{C_2H_4}$) and the mean of the differences between the upper- and lower-phase C_2H_4 concentrations at two times (ΔC_2H_4) were calculated. This procedure was repeated five times with increasing C_2H_4 concentrations. For each system, the equation best describing $F_{C_2H_4}$ as a function of ΔC_2H_4 was derived by linear regression ($r > 0.995$ in all cases). The value of $F_{C_2H_4}$ for an assay interval was then estimated by calculating

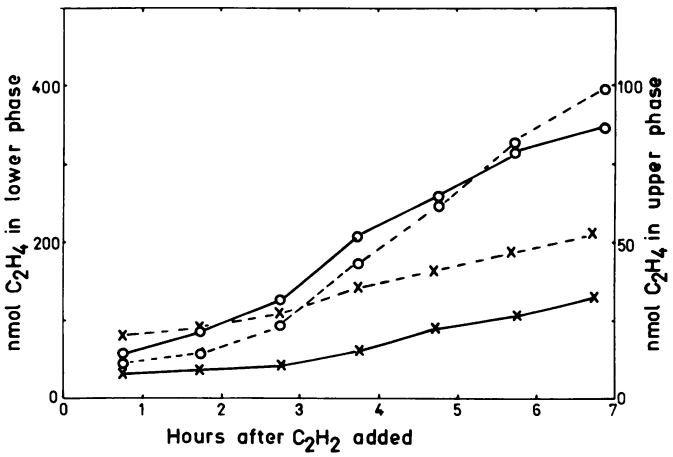

FIG. 2. Production of C_2H_4, in presence of C_2H_2, around roots (lower phase, - - -) and leaves (upper phase, ——) of *Spartina* in two-phase assay system. Each datum represents a single analysis of C_2H_4 in a single plant system. ○, plant 1; ×, plant 2.

C_2H_4 for that interval and substituting this value into the appropriate equation.

Because the resistance to C_2H_4 diffusion into the upper phase could have differed in the presence or absence of C_2H_2, $F_{C_2H_4}$ was estimated from measurements of propane flux conducted concurrently with the measurements of C_2H_2-reducing activity (method 2). Propane was injected into the lower phase to provide a concentration of approximately 0.1%, and a resistance to diffusion of this gas into the upper phase was calculated as

$$R_{C_3H_8} = \Delta\ C_3H_8/F_{C_3H_8}.$$

The expected C_2H_4 flux then was estimated as

$$F_{C_2H_4} = (\Delta\ C_2H_4/R_{C_3H_8}) \times (R_{C_3H_8}/R_{C_2H_4})$$

An empirical value for the ratio of the resistance to propane diffusion to the resistance to C_2H_4 diffusion, 1.52 ± 0.10 (mean ± range for two determinations), was determined by injecting equal concentrations of the two gases into the lower phase and measuring the resulting fluxes of the two gases into the upper phase.

The values of $F_{C_2H_4}$ calculated by either method were substantially less than the observed rates of upper phase C_2H_4 production (Table I).

The preceding results indicated that a major part of the upper phase C_2H_4 was derived from source *c* above, that is, from C_2H_2-reducing activity of diazotrophs inside of the roots. In support of this hypothesis, addition of $HgCl_2$ to seawater in the lower phase did not markedly affect upper phase C_2H_4 production, whereas it caused almost complete cessation of lower phase C_2H_4 production within 3 h (Fig. 3).

Table I. *Comparison of Calculated Rates of C_2H_4 Diffusion from around the Roots into the Upper Phase with Observed Upper-phase C_2H_4 Production*

Method of Calculation[a]	Diffusion of C_2H_4	Observed C_2H_4 Production
	nmol plant^{-1} h^{-1}	
1	2.14	4.09
1	1.48	16.8
1	0.52	26.0
1	0.63	15.6
1	0.50	3.90
2	0.65	18.3
2	2.12	14.9

[a] Methods are described in text.

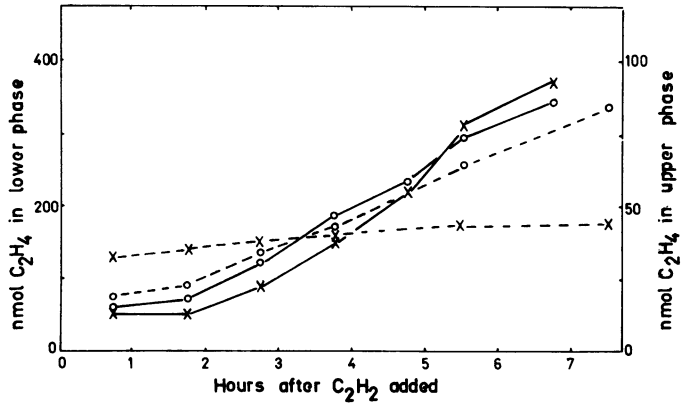

FIG. 3. Production of C_2H_4, in presence of C_2H_2, around leaves (○) and roots (×) of *Spartina* in two-phase assay system before (——) and after (– – –) addition of $HgCl_2$ to upper phase seawater. Data are for a single plant, representative of three plants tested.

Table II. *Numbers of Bacteria in Untreated and Surface-sterilized Roots from Plants Maintained in Hydroponic Culture*

	Untreated Roots	Surface-sterilized Roots	U/S[a]
	thousands/g fresh root		*ratio*
Total number (plate count)	16100	685	23.5
Azotobacter-like[b] (plate count)	590	0	high
Microaerophilic malate-utilizing diazotrophs (most probable no.)	3.5	2.8	1.25
Glucose-utilizing anaerobic diazotrophs (most probable no.)	460	46	10

[a] Untreated roots to surface-sterilized roots.

[b] Large colonies (greater than 2.5 mm diameter) which produced slime; isolates were morphologically similar to *Azotobacter*, but subcultures did not reduce C_2H_2 on solid or in liquid media, either with or without added yeast extract.

Bacteriological counts confirmed that diazotrophs were present in both the "exorhizal" (eliminated by surface sterilization) and the "endorhizal" (not eliminated) regions of roots from hydroponically grown plants (Table II). Surface sterilization reduced the numbers of both microaerophilic and anaerobic diazotrophs by factors of less than that for the total bacterial numbers, indicating a relative enrichment of these diazotrophs inside of the roots. This relative enrichment may have been greater for the microaerophils. Similar results were found for roots from field-grown plants (13). *Azotobacter*-like bacteria were found exclusively in the exorhizal region, but we were unable to demonstrate C_2H_2-reducing activity for these organisms.

Effects of Ammonium Chloride and Carbon Source Addition on C_2H_2-reducing Activity of Hydroponically-grown Plants. In the following experiments, the maximal excised leaf C_2H_2-reducing activity observed with similar plants (1.7 nmol C_2H_4 plant^{-1} h^{-1}, above) and the expected $F_{C_2H_4}$, calculated by method 1 above, were subtracted from the rate of upper phase C_2H_4 production, and the remainder, attributed to internal diazotrophs, was designated "endorhizal C_2H_2-reducing activity." Lower phase C_2H_4 production, attributed to diazotrophs around or in outer layers of the roots, was designated "exorhizal C_2H_2-reducing activity."

Endorhizal C_2H_2-reducing activity was not markedly affected by 200 μM NH_4Cl, whereas exorhizal activity was suppressed by about 64% (Table III). At 3000 μM NH_4Cl (Table III) and higher concentrations (data not shown), both the exorhizal and endorhizal C_2H_2-reducing activities were suppressed considerably. Similarly, endorhizal C_2H_2-reducing activity was less responsive to carbon source addition than was exorhizal activity (Table IV).

Effects of $HgCl_2$, NH_4Cl, or Carbon Source Addition on Excised Root and Rhizosphere Soil C_2H_2-reducing Activities. Essentially all of the C_2H_2-reducing activity of young root, rhizome, or rhizosphere soil samples from plants maintained in soil was eliminated within 3 h of adding $HgCl_2$. In contrast, a significant fraction of the C_2H_2-reducing activity associated with the older roots remained after addition of $HgCl_2$ (Table V), indicating that at least some of the responsible diazotrophs were internal. In subsequent experiments, the excised root samples were similar to the older roots used here.

Addition of carbon sources resulted in a large increase in C_2H_2-reducing activity of rhizosphere soil, but in only a relatively small increase in C_2H_2-reducing activity of excised roots (Table VI). Rhizosphere soil C_2H_2-reducing activity was very low in the absence of exogenous carbon. When carbon sources were added to the rhizosphere soils after the preincubation period, 10 h were required for a significant increase in nitrogenase activity to occur, suggesting that the pronounced response of rhizosphere soil to addition of carbon sources was related to proliferation of diazo-

Table III. *Effects of 200 and 3,000 μM NH_4Cl on Endorhizal and Exorhizal C_2H_2-reducing Activities in Intact Plants*

Plant Number and Region	C_2H_2-reducing Activity[a]		After/Before
	Before Addition of NH_4Cl	After Addition of NH_4Cl	
	nmol C_2H_4/plant·h		*ratio*
200 μM NH_4Cl			
1. Endorhizal	13.3 (2.6)	18.7 (3.0)	1.41
2. Endorhizal	24.8 (1.7)	14.5 (3.0)	0.59
3. Endorhizal	13.4 (2.2)	14.5 (1.7)	1.14
			avg. 1.05
1. Exorhizal	6.7 (0.2)	2.4 (0)	0.36
2. Exorhizal	6.1 (0.5)	1.6 (0.3)	0.26
3. Exorhizal	18.3 (1.8)	8.3 (0.2)	0.45
			avg. 0.36
3000 μM NH_4Cl			
2. Endorhizal	17.8 (5.1)	6.4 (1.6)	0.36
3. Endorhizal	12.5 (0.9)	3.4 (1.5)	0.27
4. Endorhizal	4.8 (0.1)	0	0
			avg 0.27
2. Exorhizal	10.6 (0.3)	0	0
3. Exorhizal	14.8 (1.8)	1.4 (0.5)	0.10
4. Exorhizal	9.2 (1.6)	0	0
			avg. 0.03

[a] Means and SE of values for individual plants were measured on each of 2 successive days before additions and on each of 4 successive days after additions.

Table IV. *Effects of Carbon Source Addition on Endorhizal and Exorhizal C_2H_2-reducing Activities in Intact Plants*

Plant Number and Region	C_2H_2-reducing Activity[a]		After/Before
	Before Carbon Addition	After Carbon Addition	
	nmol C_2H_4/plant·h		*ratio*
1. Endorhizal	8.2 (2.2)	72.5 (16.5)	8.9
2. Endorhizal	15.5 (3.6)	80.2 (20.2)	5.2
1. Exorhizal	24.4 (4.7)	397 (146)	16.8
2. Exorhizal	8.4 (1.7)	103 (23.9)	12.3

[a] Means and SE of values for individual plants were measured on each of 2 successive days before additions and on each of 4 successive days after additions.

Table V. *Effects of $HgCl_2$ on C_2H_2-reducing Activities of Young Roots, Older Roots, Rhizomes, and Rhizosphere Soils Taken from Plants Growing in Pots*

Sample	C_2H_2-reducing Activity[a]	
	Untreated	+0.2% $HgCl_2$[b]
Young roots	40.8 (9.6)	1.7 (0.5)
Older roots	39.0 (7.4)	10.7 (2.3)
Rhizomes	74.8	3.3
Rhizosphere soil	431 (8.2)	0.1 (0.1)

[a] Measured in nmol C_2H_4 h^{-1} per g fresh root or per 10 ml soil slurry, mean and range for 2 samples.

[b] $HgCl_2$ and C_2H_2 were added after preincubation period, and C_2H_4 was measured at 3 and 5 h after these additions.

trophs.

In experiments in which the effects of ammonium chloride on excised root and rhizosphere soil C_2H_2-reducing activities were examined (Table VI), mixed carbon sources were included in the seawater in each flask. Ammonium chloride was added prior to the preincubation period, and samples were evacuated and backfilled in order to facilitate infiltration of the roots by NH_4Cl. Addition of 200 μM NH_4Cl suppressed the excised root C_2H_2-reducing activity by an average of 17% and that of the rhizosphere soil by 69%; these reductions are similar to those observed for endorhizal and exorhizal C_2H_2-reducing activities, respectively, in the intact plants (Table III). Higher concentrations of NH_4Cl suppressed C_2H_2-reducing activities of excised roots and rhizosphere soil almost completely (Table VI).

Table VI. *Effects of Addition of Carbon Sources or of Ammonium Chloride on C_2H_2-reducing Activity of Excised Roots and Rhizosphere Soils Taken from Plants Growing in Pots*

For carbon source additions, 100 mg glucose and 100 mg malate (neutralized) were added together with 1 ml concentrated mineral salts solution (3) to 9 ml seawater in each flask prior to initial backfilling; 10 ml seawater were added to each control. All samples in the ammonium chloride addition experiments included 10 ml seawater containing 10 mg glucose and 10 mg malate; ammonium chloride was added at indicated concentrations prior to initial evacuation.

Sample and Treatment	C_2H_2-reducing Activity[a]	Sample ARA[b]/ Control Value
		ratio
Excised roots		
Control	92.5 (8.7)	1.0
+ C sources	293 (147)	3.2
Rhizosphere soil		
Control	1.4 (0.2)	1.0
+ C sources	561 (78.9)	415
Excised roots		
Control	390 (43.1)	1.0
+ 200 μM NH_4Cl	324 (32.8)	0.83
+ 3000 μM NH_4Cl	0.5 (0.1)	0.001
Rhizosphere soil		
Control	677 (73.2)	1.0
+ 200 μM NH_4Cl	212 (75.6)	0.31
+ 3000 μM NH_4Cl	0.3 (0)	0.00

[a] Measured in nmol C_2H_4 h^{-1} per g fresh root or per 10 ml soil slurry; mean and SE for four replicates; C_2H_4 was measured at 1 and 3 h after addition of C_2H_2.

[b] Acetylene-reducing activity.

DISCUSSION

The lacunal system in *Spartina* provides a low resistance pathway through the plant for the diffusion of gases from around the leaves to the inner root tissues and vice versa (15). This feature facilitated separate measurements of C_2H_2-reducing activities of laterally separated diazotrophic populations in the roots. The endorhizal region of plants in hydroponic culture probably included all tissues immediately adjacent to and internal from the midcortex lacunae. The exorhizal region must have included the epidermis and some or all of the three or four outer layers of the cortex. The relative enrichment of microaerophilic diazotrophs in the root interior of both plants in hydroponic culture (Table I) and plants in the field (13) suggests that these organisms may have been responsible for a major part of the internal nitrogenase activity. Comparison of endorhizal and exorhizal C_2H_2-reducing activities for individual plants (Table III) indicates that diazotrophs of both regions contributed substantially to the total nitrogenase activity.

Excised root C_2H_2-reducing activity responded to additions of $HgCl_2$, NH_4Cl, and carbon sources in a manner qualitatively similar to the responses of endorhizal C_2H_2-reducing activity of the plants in hydroponic culture. Responses of the rhizosphere soil

C_2H_2-reducing activity were similar to those of the exorhizal C_2H_2-reducing activity. These similarities support previous evidence (10, 11) that excised root C_2H_2-reducing activity in *Spartina* is associated in part with internally located diazotrophs and also substantiate the external nature of the exorhizal C_2H_2-reducing activity of plants in hydroponic culture. Older roots, but not the younger ones, exhibited C_2H_2-reducing activity after exposure to $HgCl_2$. This is consistent with other evidence indicating that internal infections or invasions become more pervasive with increasing age of the plants (8, 13). Preincubation of excised roots with 200 μM NH_4Cl and addition of 0.2% $HgCl_2$ after preincubation are modifications of previously employed excised root assay methodology (2, 11), which might be useful in screening plants for internally located diazotrophs.

The relative insensitivity of excised root and endorhizal C_2H_2-reducing activities to NH_4Cl, in comparison with rhizosphere soil and exorhizal C_2H_2-reducing activities, does not seem to have been due simply to protection afforded by the internal location of the diazotrophs. For both the intact plants and excised roots, additions of NH_4Cl were made long before the assays, and the excised roots were subjected to repeated evacuation to further facilitate infiltration of the tissues. Possibly this relative insensitivity reflects a physiological interaction between the roots and diazotrophs closely associated with the roots, analagous to interactions which operate between lower plants and endosymbiotic blue-green algae (14). This would support the viewpoint that the grass-diazotroph associations represent primitive symbiosis (4).

LITERATURE CITED

1. DE BONT JAM 1976 Bacterial degradation of ethylene and the acetylene reduction test. Can J Microbiol 22: 1060–1062
2. VON BULOW JFW, J DÖBEREINER 1975 Potential for nitrogen fixation in maize genotypes in Brazil. Proc Natl Acad Sci USA 72: 2389–2393
3. CLARK FE 1965 *Azotobacter*. *In* CA Black, DD Evans, JL White, LE Ensminger, and FE Clark, eds, Methods of Soil Analysis, Part 2, Chap 105. American Society of Agronomy, Madison, Wisconsin, pp 1493–1497
4. DÖBEREINER J, JM DAY 1976 Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen fixing sites. *In* WE Newton, CJ Nyman, eds, Proceedings of the First International Symposium on Nitrogen Fixation, Vol 2. Washington State University Press, Pullman, Washington, pp 518–538
5. EVANS HJ, LE BARBER 1975 Biological nitrogen fixation for food and fiber production. Science 197: 332–339
6. FLEET RJ, RD HAMILTON, NER CAMBELL 1976 Aquatic acetylene-reduction techniques: solutions to several problems. Can J Microbiol 22: 43–51
7. HARDY RWF, RD HOLSTEN, EK JACKSON, RC BURNS 1968 The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol 43: 1185–1207
8. MAGALHÃES FMM, D PATRIQUIN, J DÖBEREINER 1977 Infection of field grown maize with *Azospirillum* spp. Rev. Brasil. Biol. 39: 587–596
9. NEYRA CA, J DÖBEREINER 1977 Nitrogen fixation in grasses. Adv Agron 29: 1–38
10. PATRIQUIN DG 1978 Nitrogen fixation (acetylene reduction) associated with cord grass, *Spartina alterniflora* Loisel. Ecol Bull (Stockholm) 26: 20–27
11. PATRIQUIN DG 1978 Factors affecting nitrogenase activity (acetylene-reducing activity) associated with excised roots of the emergent halophyte *Spartina alterniflora* Loisel. Aquat Bot 4: 193–210
12. PATRIQUIN DG, 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
13. PATRIQUIN DG, CR 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
14. STEWART WDP, P ROWELL 1977 Modifications of nitrogen-giving algae in the lichen symbiosis. Nature 265: 371–372
15. TEAL JM, JW KANWISHER 1966 Gas transport in the marsh grass *Spartina alterniflora*. J Exp Bot 17: 355–361