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Nitrogen Fixation Associated with Rinsed Roots and Rhizomes of the Eelgrass *Zostera marina*[1]

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DOUGLAS G. CAPONE AND JOHN M. BUDIN
Marine Sciences Research Center, State University of New York, Stony Brook, New York 11794

ABSTRACT

Nitrogen fixation was associated with the rinsed roots and rhizomes of the seagrass, *Zostera marina* L. Nitrogenase activity (acetylene reduction) was greater on rhizomes compared to roots, and on older roots and rhizomes relative to younger tissue. Compared to aerobic assays, anaerobic or microaerobic conditions enhanced the rate of acetylene reduction by rhizomes with attached roots, with the highest activity (100 nanomoles per gram dry weight per hour) occurring at pO_2 = 0.01 atmosphere. Addition of glucose, sucrose, or succinate also increased the rate of acetylene reduction under anaerobic conditions, with glucose providing the most stimulation. In one experiment, comparison of acetylene reduction assays with $^{15}N_2$ incorporation yielded a ratio of about 2.6:1. Seagrass communities are thought to be limited by the availability of nitrogen and, therefore, nitrogenase activity directly associated with their roots and rhizomes suggests the possibility of a N_2-fixing flora which may subsidize their nutritional demand for nitrogen.

In seagrass communities, as in many marine ecosystems, nitrogen in a combined form is often present at limiting levels (12, 13). Nonetheless, these communities are very prolific (11). Nitrogen fixation has been detected in rhizosphere sediments of several seagrasses (4, 6). The presence of a root-associated N_2-fixing flora would assist in reconciling the observed productivities of these marine angiosperms with apparent nutrient impoverishment.

The objectives of this study were 3-fold: (a) to locate within the rhizosphere the predominant sites of nitrogen fixation; (b) to assess whether the apparent age of the plant rhizosphere is correlated with the magnitude of nitrogen fixation; and (c) to determine the effect of O_2 and specific organic substrates on the rate of nitrogen fixation associated with roots and rhizomes of *Zostera marina*.

Evidence is given here indicating the existence of anaerobic and/or microaerophilic nitrogen fixing bacteria on or within the roots and rhizomes of eelgrass.

MATERIALS AND METHODS

Sample Collection and Preparation. Sediment plugs containing numerous, whole eel grass (*Zostera marina* L.) plants were collected from Bellport Bay, a shallow estuary on the south shore of Long Island, NY. Samples were collected on the morning of the experiment, and the sediment plugs were transported in buckets with bay water to the laboratory. The time span between sampling and laboratory work was about 1 to 2 h. Upon return to the laboratory, emergent leaf material was removed from the sediment surface. The remaining roots, rhizomes, and sediment were placed into flasks containing nucleopore (0.2 μm) filtered Bellport bay water. These procedures were carried out while gassing with O_2 free N_2. The bubbling action effectively rinsed the roots and rhizomes of sediment and minimized exposure to O_2 during assay set up. Approximately five 2- to 3-cm segments of roots and/or rhizomes were transferred into each of at least twelve 125-ml Erlenmeyer flasks containing 25 ml of filtered bay water. The flasks were sealed with rubber stoppers following an additional 2 min of bubbling with N_2 and then were evacuated and backflushed with N_2 four to five times to ensure anaerobic conditions.

Acetylene Reduction Assays. The acetylene reduction technique (7), as adapted for use in seagrass systems (4, 6), was employed to measure nitrogenase activity. The flasks were placed into a 25°C shaking (100 rpm, rotary) water bath followed by the addition of 12.5 ml acetylene (pC_2H_2 = 0.12 atm). Preliminary experiments indicated saturation by 0.10 atm pC_2H_2. Ethylene and acetylene concentration were measured by flame ionization gas chromatography (4, 6).

In all assays, triplicate flasks were incubated at each level of an experiment. In one experiment, rhizome sections with attached roots were categorized by apparent age. Young rhizomes were of small diameter and pale coloration, while older rhizomes were typified by their dark brown appearance, larger diameter, and relatively decrepit surface appearance. After separation of rhizomes, roots were cut from the rhizome segments and individual roots and rhizome samples of each age class were incubated either anaerobically or fully aerobically.

To determine the effect of various carbohydrates on the rate of C_2H_2 reduction, sterile solutions of D-glucose, sucrose, or succinic acid to final concentrations of 10 mM were added to the flasks containing segments of roots and rhizomes. The flasks were incubated anaerobically.

To assess the effect of O_2 on the rate of C_2H_2 reduction, flasks were sealed after gassing with O_2-free N_2, and O_2 was added to yield initial pO_2 from 0 atm (anaerobic) to 0.2 atm (aerobic).

$^{15}N_2$ Reduction Assay. The C_2H_2 reduction method was compared with concurrent assays measuring $^{15}N_2$ uptake (3). Sample and assay flask size were both scaled down, while preparative procedures were essentially the same. Samples of rhizomes with attached roots were placed in 21-ml serum bottles containing 10 ml of filtered bay water. The bottles were then sealed, evacuated, and backflushed with N_2 four times. Three flasks were injected with 0.1 ml of C_2H_2 and monitored for C_2H_4 production. One h later, the gas phase volume of a second set of three flasks was replaced with $^{15}N_2$ which had previously been generated from $^{15}NH_4Cl$ (3). Subsamples of the gas were saved and analyzed for percent enrichment. Comparisons were made of C_2H_4 production and $^{15}N_2$ incorporation over the next 8 h (*i.e.* 1–8 h). At the

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termination of the experiment, root and rhizome material was removed from the bottles, rinsed in filtered seawater, rapidly frozen, and subsequently freeze-dried. Isotope ratio analyses were performed by MS after on-line combustion in a nitrogen analyzer. Total particulate nitrogen content was determined on a Hewlett-Packard model 214 CHN analyzer.

In general, reported C_2H_2 reduction rates were derived from the mean of linear regression analysis of replicates over periods of constant C_2H_4 production. Rates are expressed in terms of root and/or rhizomes dry weight as nmol C_2H_4/g dry wt·h.

RESULTS

Preliminary experiments examined the effect of C_2H_2 concentration on *Z. marina* roots and rhizomes. Nitrogenase activity plateaued between pC_2H_2 of 0.10 and 0.20 atm. Also, no C_2H_4 production was noted in the absence of C_2H_2.

All subsequent assays utilized pC_2H_2 of 0.12. In most assays, C_2H_4 production was detected within 1 to 2 h after the addition of C_2H_2, with rates becoming linear within 2 to 6 h. In experiments not amended with organic additions, rates generally remained linear for at least 24 h, and often as long as 3 d. A typical time course is presented in Figure 1. In this experiment, C_2H_4 production was evident by 1.5 h and was constant from 5.5 to 42 h. Nitrogenase activity by attached roots and rhizomes was far greater in the absence of O_2.

Separation of root from rhizome material revealed the bulk of C_2H_2 reduction associated with rhizomes (Table I). As for rhizomes with attached roots, nitrogenase activity was greater under anaerobic, rather than aerobic, conditions. Glucose stimulated root-associated C_2H_2 reduction under both aerobic and anaerobic conditions, while only increasing rhizome-associated activity under anaerobic conditions (Table I).

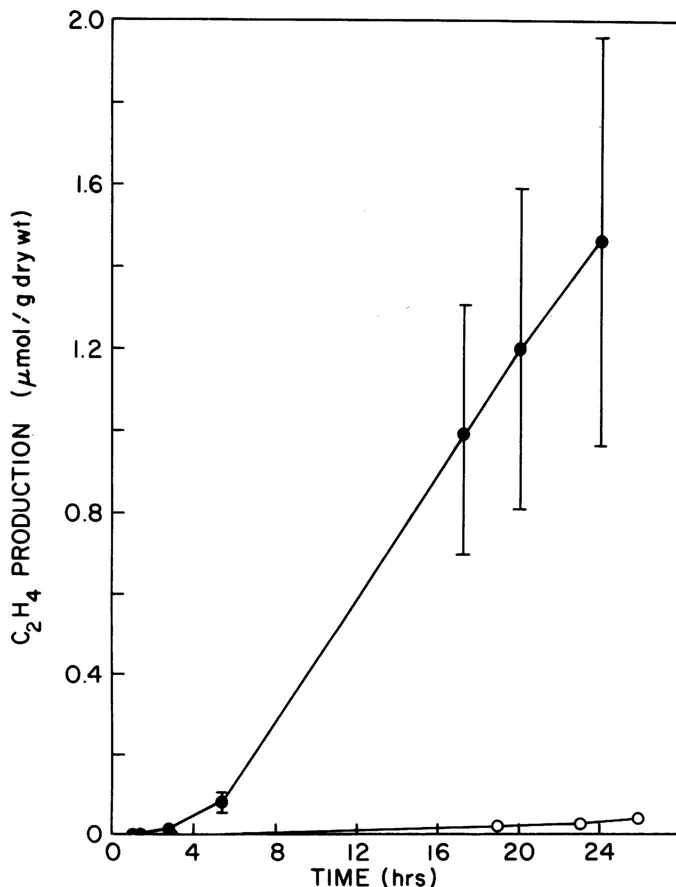

FIG. 1. Time course of C_2H_4 production by roots and rhizomes of the sea grass *Zostera marina* under aerobic (○) and anaerobic (●) conditions. Results are means of three replicates ± SE.

Table I. *Nitrogenase Activity Associated with Roots or Rhizomes of Zostera marina*

In both experiments, samples were collected, rinsed, and separated into root and rhizome fractions and incubated as indicated. For Experiment I (July 26, 1980), results are from regression analyses of individual flasks at each level of the experiment. Experiment II was run on July 16, 1980, and the results are the mean ± SE of regression analyses of triplicate flasks.

Experiment	Condition	C_2H_4 Production	
		Roots	Rhizomes
		nmol/g dry wt·h	
I	Aerobic	0.9	8
	Anaerobic	4.4	169
	Aerobic + glucose	11	4.4
	Anaerobic + glucose	36	438
II	Aerobic	0.3 ± 0.03	1.2 ± 0.1
	Anaerobic	6.9 ± 5.5	145 ± 23

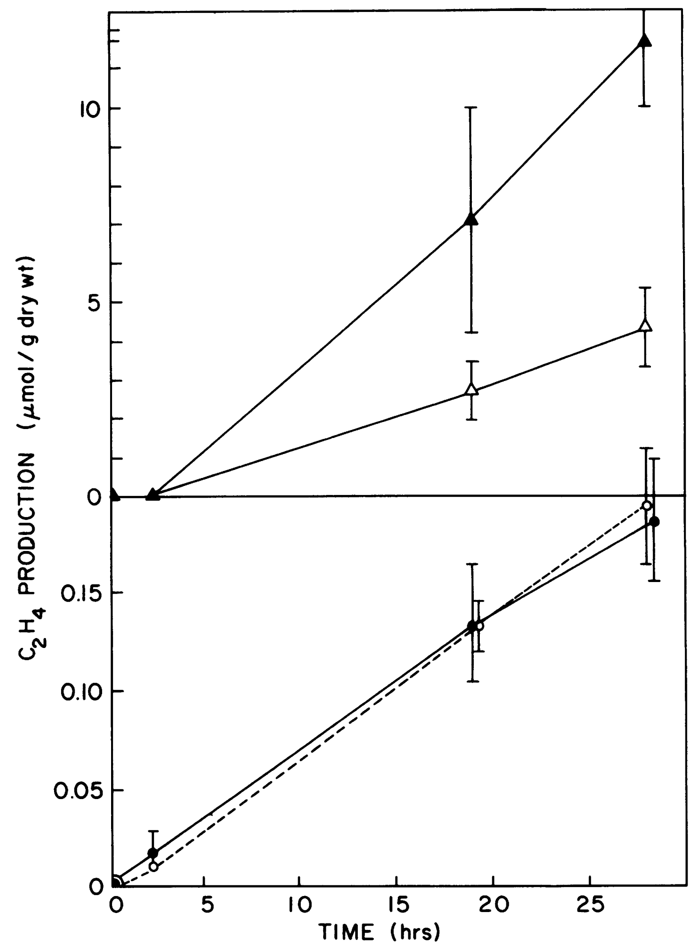

FIG. 2. Time course of C_2H_4 production by roots and rhizomes of the sea grass, *Zostera marina*. Samples were collected on October 7, 1980 and separated on the basis of apparent age (see "Materials and Methods"). Assays were run anaerobically. Results are the means of three replicates ± SE. (▲), Old rhizomes; (△), young rhizomes; (●), old roots; (○), young roots.

Rhizomes and detached roots were also examined on the basis of apparent age (Fig. 2). While older rhizomes exhibited substantially higher levels of associated nitrogenase activity than younger

Table II. *Effect of Organic Substrate Addition on Nitrogenase Activity Associated with Zostera marina*

Anaerobic assays of rhizomes with attached roots were supplemented with the indicated substrates to yield a final concentration of 10 mM. Results are means of rates over the indicated periods for each of three replicates ± SE. Samples were collected and assayed on July 28, 1980.

Addition	C_2H_4 Production		
	0–5 h	5–20 h	21–41 h
	nmol/g dry wt·h		
None	22 ± 8	74 ± 27	66 ± 32
Sucrose	33 ± 8	290 ± 90	473 ± 84
Glucose	35 ± 10	262 ± 76	627 ± 149
Succinate	36 ± 9	100 ± 45	187 ± 22

Table III. *Effect of Initial O_2 Concentration on C_2H_2 Reduction by Roots and Rhizomes of Zostera marina*

Results are the mean of three replicates ± SE. Regression coefficients (r^2) for the linear period of C_2H_4 production in each flask were greater than 0.92. Experiments were performed on July 9, 1980 (Exp. I) and August 4, 1980 (Exp. II) at the indicated initial pO_2.

Initial O_2 Concn.	C_2H_4 Production	
	Exp. I	Exp. II
atm	*nmol/g dry wt·h*	
0	22 ± 12	56 ± 11
0.001	38 ± 21	41 ± 22
0.005	ND[a]	62 ± 9
0.01	99 ± 6	86[b]
0.05	14 ± 2	ND
0.1	7.5 ± 0.6	ND
0.2	2.4 ± 0.2	ND

[a] Not determined.
[b] Single determination.

Table IV. *Comparison of Nitrogenase Activity of Rinsed Roots and Rhizomes of the Seagrass Zostera marina as Measured by C_2H_2 Reduction and $^{15}N_2$ Fixation*

Anaerobic assay were run in parallel over a 8-h period. Results are the mean of three replicates ± SE. Samples were collected and assayed on September 27, 1979.

Method	Nitrogenase Activity	
	nmol/g dry wt·h	*nmol/mg N·h*
$^{15}N_2$ fixation	21 ± 0.7	2.5 ± 0.1
C_2H_2 reduction	54 ± 3.3	6.4 ± 0.4

rhizomes, little difference was noted between roots separated from old or young rhizomes.

Under anaerobic conditions, additions of glucose, sucrose, and succinate stimulated root and rhizome C_2H_2 reduction (Table II). This effect, although not statistically significant, was somewhat evident by 5 h, with rates in amended flasks diverging rapidly and significantly ($P < 0.05$) from controls thereafter. Glucose was more effective than either sucrose or succinate in stimulating nitrogenase activity.

Several experiments examined the effect of various initial O_2 concentration on C_2H_2 reduction by rhizomes with attached roots. In two experiments, maximum activity occurred at an initial $pO_2 = 0.01$ atm with lower rates at both greater and lesser O_2 tensions. Inhibition of nitrogenase activity was clearly evident at high O_2 levels (Fig. 1; Table III). However, because of greater variances noted at the lowest O_2 levels used (initial pO_2 = 0, 0.001, 0.005 atm), no statistically significant reduction in C_2H_2 reduction is discernible at the lower levels (Table 1).

Comparison of the C_2H_2 reduction method by $^{15}N_2$ uptake yielded a C_2H_2:N_2 ratio of 2.6 (Table IV). Rates of C_2H_4 production in the scaled down assays used for this experiment were similar to those rates obtained in other experiments employing rinsed rhizomes with attached roots.

DISCUSSION

The common occurrence of root-associated N_2 fixation is well established for a variety of terrestrial grasses (18). Similar associations may also exist between diazotrophic bacteria and rooted aquatic macrophytes. Bristow (2) reported high rates of N_2 fixation by washed roots of the freshwater angiosperm *Glyceria borealis* and *Typha* sp. This activity was inhibited under aerobic conditions while being stimulated by glucose additions. Similarly, excised roots of the salt marsh grass *Spartina alterniflora* were found to exhibit high levels of nitrogenase activity after lag periods (14). This activity was directly associated with the bacterial flora on and within the roots (1). Patriquin (14) reported optimal activity (after lags) at O_2 concentrations below fully aerobic. More recently, van Berkum and Sloger (19) observed nitrogenase activity with excised *S. alterniflora* roots which, by their methods, was both immediately detectable and showed accelerating rates during the first 1 to 2 h of assay. They questioned the validity of relating nitrogenase activity in excised root assays after extended lags (*i.e.* periods of no C_2H_4 production) to *in situ* N_2 fixation.

Evidence has also been accumulating for a similar role by root-associated diazotrophs of sea grasses. Kuo *et al.* (8), using light and electron microscopy, found a characteristic bacterial flora associated with the epidermal tissues of *Posidonia australis*, while Wood and Hayaska (21) reported a dense bacterial population on the surface of *Z. marina* roots. Nitrogen-fixing bacteria have been found on roots of *Potamogeton filiformis* (17) and *Thalassia testudinum* (15). Of course, isolation or enumeration of N_2-fixing bacteria provides only presumptive evidence of a quantitative role by those bacteria. A direct demonstration for nitrogenase activity on *Z. marina* and *T. testudinum* roots was first presented by Patriquin and Knowles (15). However, in *Z. marina*, no activity was observed over the first 24 h of assay. Subsequent examinations of *Z. marina* roots and rhizomes by McRoy *et al.* (10) failed to detect any associated nitrogenase activity. They (10) suggested that the extended periods of time needed for sample transportation and assay by Patriquin and Knowles (15) may have resulted in bacterial proliferation.

In our investigations, which spanned the growing season, nitrogenase activity was consistently associated with freshly collected rinsed roots and rhizomes of *Z. marina*. Highest activities occurred under anaerobic or microaerophilic conditions, while fully aerobic conditions were clearly inhibitory (Fig. 1; Tables I and III). This observation suggests a population of facultative anaerobic and/or microaerophilic N_2-fixing bacteria. Several studies have also shown optimal nitrogenase activity by root- and rhizome-associative bacteria at low O_2 tensions (14, 18).

Organic additions produced a rapid and apparent (although not statistically significant) stimulation of nitrogenase activity within the first 5 h of assay, with significant stimulation clearly occurring thereafter. The substantial stimulation over the longer term was likely a result of bacterial proliferation. However, both observations suggest a population of substrate-limited heterotrophic diazotrophs. Plant roots are a likely source of labile organics utilized by N_2-fixing bacteria. While estimates of total root exudation have been made (20), there have only been limited attempts to characterize the nature of these exudates (21) and no studies, to our knowledge, of their suitability as bacterial substrates.

Higher activities associated with older roots and rhizomes (Fig. 2) may be a result of the relative periods of time available for bacterial colonization, as well as a presumably declining cellular

integrity with increasing root and rhizome age. The latter factor could result in greater release of organics. Patriquin and McClung (16) have also noted a relationship of apparent root age and acetylene-reducing activity for unbranched, but not branched, roots of *S. alterniflora*.

Deserving of comment are the low C_2H_2:N_2 ratio observed (Table IV), which may derive from a variety of factors. For instance, negligible endogenous H_2 production from nitrogenase or, alternatively, complete recycling of any H_2 released might account for the low ratio. It should be noted that Patriquin and Knowles (15) found very similar ratios for a variety of sea grasses, including *Z. marina*.

The root systems of *Z. marina*, which are generally found in anoxic sediments, are presumed to provide a conduit for the transport of gases and release of organic compounds into the surrounding sediments (20, 21). The plant thereby functions to create a 'rhizosphere effect' conducive to enhanced bacterial activities in the vicinity of the plant roots. At the sediment/plant root interface, one might therefore anticipate highly suitable conditions for microaerophilic N_2 fixation. In this regard, higher rates of N_2 fixation are associated with intact rhizosphere sediments from *Z. marina* communities rather than with adjacent nonvegetated areas (4). In the rhizosphere, the activity associated with roots and rhizomes rinsed free of sediments, as noted in this study, suggests that a substantial portion of the observed *in situ* activity may be directly attributed to root- and rhizome-associated bacteria.

Seagrasses such as *Z. marina* have very high productivities. Brinkhuis (personal communication) estimated above and below ground production of *Z. marina* to amount to 3 to 4 g dry wt/m^2·d at our site during the summer. Consequently, high nutrient demands have been calculated for these systems (4). The occurrence of dense seagrass beds in apparently nutrient-impoverished waters stimulated the initial investigations of N_2 fixation as a potential plant nutrient source (5, 10, 15). Capone (4) estimated that an upper limit of 20% of the calculated nitrogen demand of *Z. marina* could be satisfied by the observed rates of N_2 fixation in intact rhizosphere sediments. Given the acceleration in rate at the outset of our assays of roots and rhizomes and the high variability noted, particularly at low O_2 tensions, it is difficult to compare root-associated N_2 fixation quantitatively with that observed for intact sediments. Nonetheless, we postulate that a direct associative symbiosis involving the bidirectional exchange of materials is most likely for diazotrophic bacteria in direct contact with the plant tissue. The ability to isolate microaerophilic diazotrophs from root tissues of marine angiosperms (9, 17) including *Z. marina* (J. M. Budin and D. G. Capone, submitted), along with the detection of nitrogenase activity directly associated with roots and rhizomes as found in this report, provide further evidence for the existence of specific bacterial associations with the roots of these plants.

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