

# Elevated CO<sub>2</sub> stimulates associative N<sub>2</sub> fixation in a C<sub>3</sub> plant of the Chesapeake Bay wetland

F. D. DAKORA<sup>1</sup> & B. G. DRAKE<sup>2</sup>

<sup>1</sup>Botany Department, University of Cape Town, Private Bag, Rondebosch 7701, Cape Town, South Africa and

<sup>2</sup>Smithsonian Environmental Research Center PO Box 28, Edgewater, MD 21037, USA

## ABSTRACT

In this study, the response of N<sub>2</sub> fixation to elevated CO<sub>2</sub> was measured in *Scirpus olneyi*, a C<sub>3</sub> sedge, and *Spartina patens*, a C<sub>4</sub> grass, using acetylene reduction assay and <sup>15</sup>N<sub>2</sub> gas feeding. Field plants grown in PVC tubes (25 cm long, 10 cm internal diameter) were used. Exposure to elevated CO<sub>2</sub> significantly ( $P < 0.05$ ) caused a 35% increase in nitrogenase activity and 73% increase in <sup>15</sup>N incorporated by *Scirpus olneyi*. In *Spartina patens*, elevated CO<sub>2</sub> ( $660 \pm 1 \mu\text{mol mol}^{-1}$ ) increased nitrogenase activity and <sup>15</sup>N incorporation by 13 and 23%, respectively. Estimates showed that the rate of N<sub>2</sub> fixation in *Scirpus olneyi* under elevated CO<sub>2</sub> was  $611 \pm 75 \text{ ng } ^{15}\text{N fixed plant}^{-1} \text{ h}^{-1}$  compared with  $367 \pm 46 \text{ ng } ^{15}\text{N fixed plant}^{-1} \text{ h}^{-1}$  in ambient CO<sub>2</sub> plants. In *Spartina patens*, however, the rate of N<sub>2</sub> fixation was  $12.5 \pm 1.1$  versus  $9.8 \pm 1.3 \text{ ng } ^{15}\text{N fixed plant}^{-1} \text{ h}^{-1}$  for elevated and ambient CO<sub>2</sub>, respectively. Heterotrophic non-symbiotic N<sub>2</sub> fixation in plant-free marsh sediment also increased significantly ( $P < 0.05$ ) with elevated CO<sub>2</sub>. The proportional increase in <sup>15</sup>N<sub>2</sub> fixation correlated with the relative stimulation of photosynthesis, in that N<sub>2</sub> fixation was high in the C<sub>3</sub> plant in which photosynthesis was also high, and lower in the C<sub>4</sub> plant in which photosynthesis was relatively less stimulated by growth in elevated CO<sub>2</sub>. These results are consistent with the hypothesis that carbon fixation in C<sub>3</sub> species, stimulated by rising CO<sub>2</sub>, is likely to provide additional carbon to endophytic and below-ground microbial processes.

**Key-words:** elevated CO<sub>2</sub>; C<sub>3</sub> and C<sub>4</sub> species; N<sub>2</sub> fixation; A % X (atom % <sup>15</sup>N excess).

## INTRODUCTION

With the anticipated doubling of CO<sub>2</sub> in the atmosphere, plant growth and photosynthetic activity in both natural and agricultural ecosystems are likely to increase (Kimball 1983; Long & Drake 1992; Drake, Gonzalez-Meler & Long 1997). However, long-term exposure of *Scirpus olneyi* plants to elevated CO<sub>2</sub> has resulted in decreased tissue N concentrations (Jacob, Greitner & Drake 1995; Drake *et al.*

1997). Although it might be expected that the additional carbon accumulation would occur at the expense of depletion of N reserves in the community, the fact that this does not appear to have been the case suggests that the N reserves are very large and/or that the additional carbon supplied by the stimulation of photosynthesis by elevated CO<sub>2</sub>, enhanced N<sub>2</sub> fixation as has been shown to occur in soybean and white clover (Hardy & Havelka 1975; Zanetti *et al.* 1997).

Several studies on the natural communities of *Scirpus olneyi*, *Spartina patens* and other grasses at the site where photosynthetic experiments were conducted (Curtis *et al.* 1989a, Curtis, Drake & Whigham, 1989b; Arp & Drake 1991; Long & Drake 1991; Drake & Leadley 1991; Ziska *et al.* 1991) found significant nitrogenase activity associated with roots and rhizomes of these species (van Berkum & Sloger 1979, 1981; van Berkum 1984). Those findings indicate that, in addition to N uptake by roots, these plants can potentially improve their N nutrition from biological N<sub>2</sub> fixation. In this process, N<sub>2</sub>-fixing diazotrophs localized on the root surface or within the intercellular spaces of root cortex and the aerenchyma of stems and roots (Boyle & Patriquin 1980; McClung *et al.* 1983) reduce N<sub>2</sub> to NH<sub>3</sub> as is done by root-nodule bacteria in symbiotic legumes. Through that, the plant obtains fixed N from the bacteria while in turn providing the latter with photosynthetic products. As with the legume system, associative N<sub>2</sub> fixation by bacterial diazotrophs located on or inside the roots and stems of grasses, cereals, and other non-legume species can contribute a significant amount of N to the ecosystem. Similarly, N<sub>2</sub> fixation in wetland environments such as the C-rich sediment (or 'soil') of the Chesapeake Bay can be quite considerable.

Symbiotic N<sub>2</sub> fixation is a highly energy-demanding process that constitutes a significant sink for photosynthate (Phillips 1980), requiring about 10% of recently fixed C assimilate in legumes for nitrogenase (Minchin & Pate 1973). Thus, N<sub>2</sub>-fixing plants which accumulate C with increasing CO<sub>2</sub> concentration could be expected to increase their photosynthetic rates as the atmospheric CO<sub>2</sub> concentration rises (Drake *et al.* 1987), providing increased photosynthate supply to nitrogenase and N<sub>2</sub>-fixing activity. Studies with legumes have in fact indicated that, with the exposure of nodulated plants to elevated CO<sub>2</sub>, nodule functioning was increased (Hardy & Havelka 1975; Phillips *et al.* 1976; Finn & Brun 1982) due to increased rates of photo-

Correspondence: Felix D. Dakora, Tel: +27 21 650 2964; fax: +27 21 650 4041; e-mail: dakora@botzoo.uct.ac.za

synthesis. However, there was no evidence of a direct limitation of N<sub>2</sub>-fixing activity by current photosynthate (Phillips *et al.* 1976; Finn & Brun 1982).

It is therefore likely that, as in legumes, N<sub>2</sub> fixation in plants such as the *Cyperaceae* and *Poaceae* could increase with increasing photosynthetic rates under elevated CO<sub>2</sub>. Although this type of information remains vital to our understanding of the ecological impact of the rising atmospheric CO<sub>2</sub> on the N cycle, there are so far no available data on the effect of elevated CO<sub>2</sub> on N<sub>2</sub> fixation by the bacteria/non-legume symbiosis. Equally important is the lack of information on the effect of increasing atmospheric CO<sub>2</sub> on heterotrophic non-symbiotic N<sub>2</sub> fixation in natural and agricultural ecosystems.

In this study, naturally occurring communities of a C<sub>3</sub> sedge, *Scirpus olneyi*, and of the C<sub>4</sub> grass, *Spartina patens*, were grown in two levels of atmospheric CO<sub>2</sub> concentration in open-top chambers in the field. The response of nitrogenase activity and N<sub>2</sub> fixation to elevated CO<sub>2</sub> was measured in both plants and sediment. The purpose in using these two species was to examine the relative stimulation of N<sub>2</sub> fixation in plants in which associative N<sub>2</sub> fixation has been shown to occur but which have very different photosynthetic responses to elevated CO<sub>2</sub>. We were thus able to test whether the supply of additional carbon to the ecosystem would be reflected in the response of N<sub>2</sub> fixation by those species having the capacity to respond to the elevated CO<sub>2</sub> treatment.

## MATERIALS AND METHODS

### Plant culture

Plant material used in this study came from mono-specific stands of *Scirpus olneyi* Grey and *Spartina patens* (Ait.) Muhl. which had developed as separate communities. Open-top chambers (3 m diameter, 2.5 m height) similar to those described by Drake *et al.* (1989) constructed of PVC pipe were placed over the marsh, and these provided the means to create test atmospheres of normal ambient and elevated CO<sub>2</sub> concentrations. PVC tubes (25 cm length, 10 cm internal diameter) were used to delineate cores randomly within each chamber for subsequent use in assays. From prior removal of live plants and their roots and rhizomes from these cores, the sediment in some PVC tubes was maintained plant-free for estimates of N<sub>2</sub> fixation by free-living bacteria.

### CO<sub>2</sub> treatment

Carbon dioxide treatments were applied to the emerging plants in the chambers in early June 1990. Details of chamber design, as well as CO<sub>2</sub> control and monitoring, have been described previously (Drake *et al.* 1989). Ambient air was introduced into each chamber by a high capacity blower. The elevated CO<sub>2</sub> level within a chamber was obtained by continuously injecting 100% CO<sub>2</sub> into the input blower where it was thoroughly mixed with ambient

air before entering the chamber. CO<sub>2</sub> concentrations inside the chambers were monitored daily with an infra-red gas analyser (Binos, Model 092; Leybold-Heraeus, Hanau, Germany) connected to an automatic gas sampling system. The mean seasonal CO<sub>2</sub> concentrations used were 665 ± 1 and 660 ± 1 μmol mol<sup>-1</sup>, respectively, for elevated CO<sub>2</sub> *Scirpus olneyi* and *Spartina patens*, whereas ambient plants received 364 ± 1 μmol mol<sup>-1</sup> CO<sub>2</sub>. Light was reduced by 10% and temperature increased 2 °C within the open top chambers on the marsh (Drake *et al.* 1989). Owing to the increased temperature optimum caused by the elevated CO<sub>2</sub> treatment (Long & Drake 1992), these effects of the chamber on the micro-environment of the vegetation were not considered to be significant (Drake *et al.* 1989).

### Measurement of acetylene reduction

Nitrogenase activity was measured using the open flow-through system for acetylene reduction assay as described by Dakora & Atkins (1990). Because most marsh grasses exchange gas between the external atmosphere and their roots via lacunae in the stems, and because nitrogenase activity (Boyle & Patriquin 1980) and N<sub>2</sub>-fixing bacteria (McClung *et al.* 1983) have been localized in the roots and stems of these species, whole plants were used for measuring acetylene reduction.

Assay conditions were similar to those used for the two species by van Berkum & Sloger (1981), including the maintenance of strict, near-anaerobic procedures for preventing contact of assay plants with air. Sample preparation involved washing of plant/sediment cores with water that was continuously flushed with N<sub>2</sub>. This was followed by transfer of whole washed plants to assay vessels which were maintained to near-anaerobic conditions with N<sub>2</sub> gas (van Berkum & Sloger 1981). The system consisted primarily of an assay vessel with an inlet gas line, and a clear acrylic tube (2.5 cm internal diameter) fitted with an outlet gas line at one end and, at the other, a funnel which inverted over protruding plant stems. The entire unit was sealed air-tight with Terostat VII (Terosan, Heidelberg, Germany) and plants moisturized with 10–15 mL marsh water maintained in each assay vessel. Temperature inside the vessel was monitored throughout the assay period with a thermocouple.

Sealed assay vessels were introduced into the acetylene gas stream by connecting the inlet of each vessel to the gas line, and the outlet to a sub-sealed sampling port. Flow rates of cylinder gas were controlled by valves and calibrated flow tubes to produce streams of gas containing 10% C<sub>2</sub>H<sub>2</sub> (v/v) and either 89.3% N<sub>2</sub> (v/v) and 0.067% CO<sub>2</sub> (v/v), or 89.6% N<sub>2</sub> and 0.036% CO<sub>2</sub>. Gas samples (1 mL) were collected from the sampling ports and analysed by gas liquid chromatography (Dakora & Atkins 1990). Positive controls involving the use of gas stream without acetylene were included for measuring endogenous ethylene production by the plants. However, no measurable amounts of this gas were detected.

Nitrogenase activity associated with heterotrophic, non-symbiotic N<sub>2</sub> fixation in plant-free marsh sediment was also

measured using the flow-through system of acetylene reduction assay. Plant-free sediment was obtained by physically removing all live plants together with their roots and rhizomes from sediment. PVC tubes containing such plant-free sediment cores were then put back in the field in the core holes for 3 months prior to assays. The PVC tubes, with sediment cores, were placed in tight-fitting caps each lined internally with a perforated tubing loop for upward gas distribution within the core, and connected to the exterior by an inlet gas line. A funnel fitted with an outlet gas line was inverted over the top of the PVC tube and sealed air-tight with Terostat VII. These units were then introduced into the acetylene-containing gas stream, and gas samples (1 mL) collected for analysis of C<sub>2</sub>H<sub>4</sub> content by gas chromatography (Dakora & Atkins 1990). Positive controls using the gas stream without acetylene could detect no endogenous ethylene from the sediment.

### Exposure to <sup>15</sup>N<sub>2</sub>

Two experiments were conducted with <sup>15</sup>N<sub>2</sub>, one where plants were grown in marsh sediment within PVC cores and the whole unit incubated with <sup>15</sup>N<sub>2</sub> (hereafter referred to as 'sediment material' or 'plant-sediment core material'), and the other where the plant root systems were washed free of sediment and whole plants incubated with <sup>15</sup>N<sub>2</sub> gas (now termed 'sediment-free material').

In the first experiment, each core containing plants growing in sediment was placed in a 2 L plastic container provided with a suba-sealed inlet tube at its base and 10–15 mL water for moisturizing the plants. Protruding stems were enclosed by clear acrylic tubes and kept air-tight with Terostat VII. During assembly, the incubation system was flushed with a gas stream containing 99.3% N<sub>2</sub> and 0.067% CO<sub>2</sub> for plants receiving elevated CO<sub>2</sub>, or 99.6% N<sub>2</sub> and 0.036% CO<sub>2</sub> for those from ambient CO<sub>2</sub> chambers. Because the pO<sub>2</sub> in the root environment of salt marsh grasses *in situ* is negligible (van Berkum & Sloger 1981), the N<sub>2</sub> gas was used to create near-anaerobic conditions. The tube was recapped and sealed air-tight with Terostat VII and suba-seal. About 50 mL gas was removed from the sealed atmosphere of each incubation container and replaced by a similar volume of <sup>15</sup>N<sub>2</sub> (99.8 atom % excess <sup>15</sup>N), sampled over acidified water to remove any contaminating <sup>15</sup>N-labelled NH<sub>3</sub>. Gas samples in the incubation vessel were taken immediately after introducing <sup>15</sup>N<sub>2</sub>, and at 72 h, for determination of <sup>15</sup>N enrichment in the gas phase. The plants were harvested after 72 h, and separated into shoot, root + rhizome and sediment for oven-drying (60 °C) and dry matter determination. Plants incubated with <sup>15</sup>N<sub>2</sub>, and controls without <sup>15</sup>N<sub>2</sub>, were both maintained at the CO<sub>2</sub> treatment conditions inside the field chambers under natural light/dark cycle.

The second <sup>15</sup>N<sub>2</sub> experiment involved the use of whole plants whose roots and rhizomes were washed free of sediment ('sediment-free material'). During assay, contact of the plants with air was avoided as described above. Clear acrylic tubes were used to complete the assemblies and

each system was flushed with a gas stream containing N<sub>2</sub> and the relevant concentration of CO<sub>2</sub> (0.036 or 0.067%). About 10–15 mL water was maintained for moisturizing the roots. The top of each tube was capped and sealed with Terostat VII and the inlet line was suba-sealed. The plants were then incubated with 10 times the volume of <sup>15</sup>N<sub>2</sub> indicated for plant/sediment cores. Control plants were treated the same way but incubated without <sup>15</sup>N<sub>2</sub>. The incubation time, gas sampling for <sup>15</sup>N enrichment analysis, and post-harvest separation of plants into shoot and root + rhizome were carried out as described previously.

### Plant sampling for dry matter determination

At the end of the <sup>15</sup>N<sub>2</sub>-feeding experiments, plants were removed from the PVC tubes and, where necessary, separated into sediment, shoots, and roots plus rhizomes. The bit of sediment still attached to the root system was washed off, and all organs oven-dried separately to constant weight at 65 °C. The dry matter yield of plant parts and sediment were determined through weighing, and samples finely ground for <sup>15</sup>N analysis.

### Total N and <sup>15</sup>N analysis

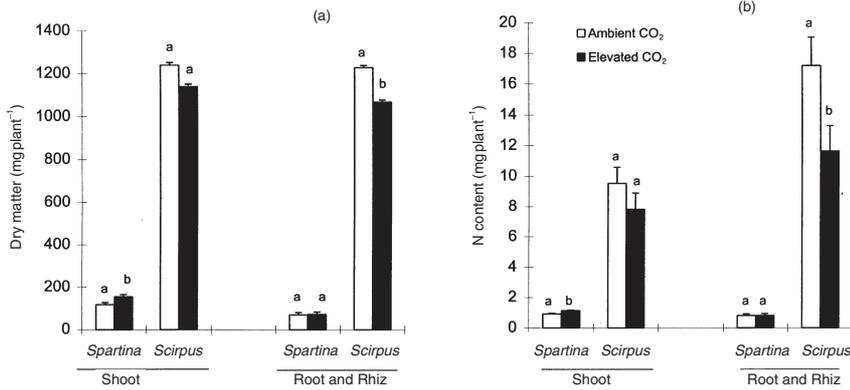
The ground plant and sediment samples together with gas samples from <sup>15</sup>N<sub>2</sub> incubation vessels were sent to Isotope Services Inc, Los Alamos, New Mexico, for <sup>15</sup>N analysis. The total N and <sup>15</sup>N content of plants exposed to <sup>15</sup>N<sub>2</sub> were determined together with unexposed controls by the Dumas combustion method followed by <sup>15</sup>N trapping and analysis using mass spectrometry (VG Isomass spectrometer, Los Alamos, New Mexico, USA).

Data generated by the mass spectrometer include % N and total atom % <sup>15</sup>N in tissue. The atom % <sup>15</sup>N (A % X) values of unexposed plants and sediment were subtracted from those of material incubated with <sup>15</sup>N<sub>2</sub>, and the amount of <sup>15</sup>N fixed estimated from that difference and the mean <sup>15</sup>N enrichment in the gas phase. Thus, <sup>15</sup>N fixed = N (sample) × A % X (sample)/A % X (gas phase). The % N values were used to determine the N content of plant samples.

## RESULTS

### Effects of elevated CO<sub>2</sub> on plant growth, N content and <sup>15</sup>N<sub>2</sub> fixation

The data presented in Figs 1 and 2 are for plant parts and Fig. 3 for whole plants in the first experiment. These are labelled 'sediment material' because they come from the experiment where whole units of plants growing in sediment material within PVC tubes were exposed to <sup>15</sup>N<sub>2</sub> gas. Figure 4 shows N<sub>2</sub>-fixing activity in the sediment itself. The results shown in Figs 5 and 6 are also for plant parts, and Fig. 7 for whole plants in the second experiment, where sediment-free plants (hence 'sediment-free material') were incubated with <sup>15</sup>N<sub>2</sub> gas.



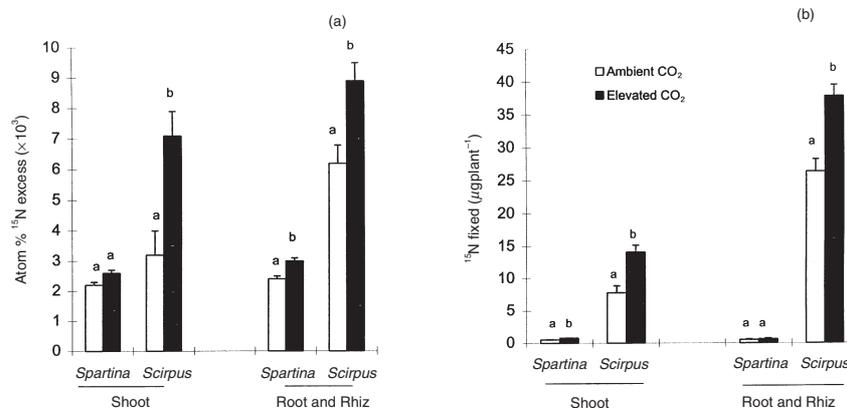
**Figure 1.** Sediment material: dry matter yield (a) and N content (b) of shoots and roots plus rhizomes of 4-month-old *Scirpus olneyi* and *Spartina patens* plants as affected by CO<sub>2</sub>. Emerging plants growing in cores of sediment were exposed to ambient or elevated CO<sub>2</sub> from June 1990 until September 1990 when they were incubated with <sup>15</sup>N<sub>2</sub> for 72 h and harvest for analysis. Vertical lines on bars represent SE ( $n = 6$  cores with plants). Different letters on bars show significant differences for each pair of ambient and elevated CO<sub>2</sub> treatment at  $P < 0.05$  using Student  $t$ -test.

The plants used in these experiments were grown in elevated or ambient CO<sub>2</sub> for 4 months before exposure to <sup>15</sup>N<sub>2</sub> gas followed by growth analysis. Although shoot dry matter was the same for ambient and elevated CO<sub>2</sub> *Scirpus* plants, the root + rhizome was significantly ( $P < 0.05$ ) different between ambient and elevated CO<sub>2</sub> treatments (Fig. 1a). In *Spartina patens*, only shoot mass was greater ( $P < 0.05$ ) with doubling of CO<sub>2</sub>. In comparison to ambient CO<sub>2</sub> plants, tissue N content of *Scirpus olneyi* was lower in both shoots and roots + rhizomes of elevated CO<sub>2</sub> plants, but this difference was significant ( $P < 0.05$ ) in only the below-ground organs (Fig. 1b). In *Spartina patens*, however, N accumulation was significantly ( $P < 0.05$ ) greater in shoots exposed to elevated CO<sub>2</sub> compared to ambient (Fig. 1b).

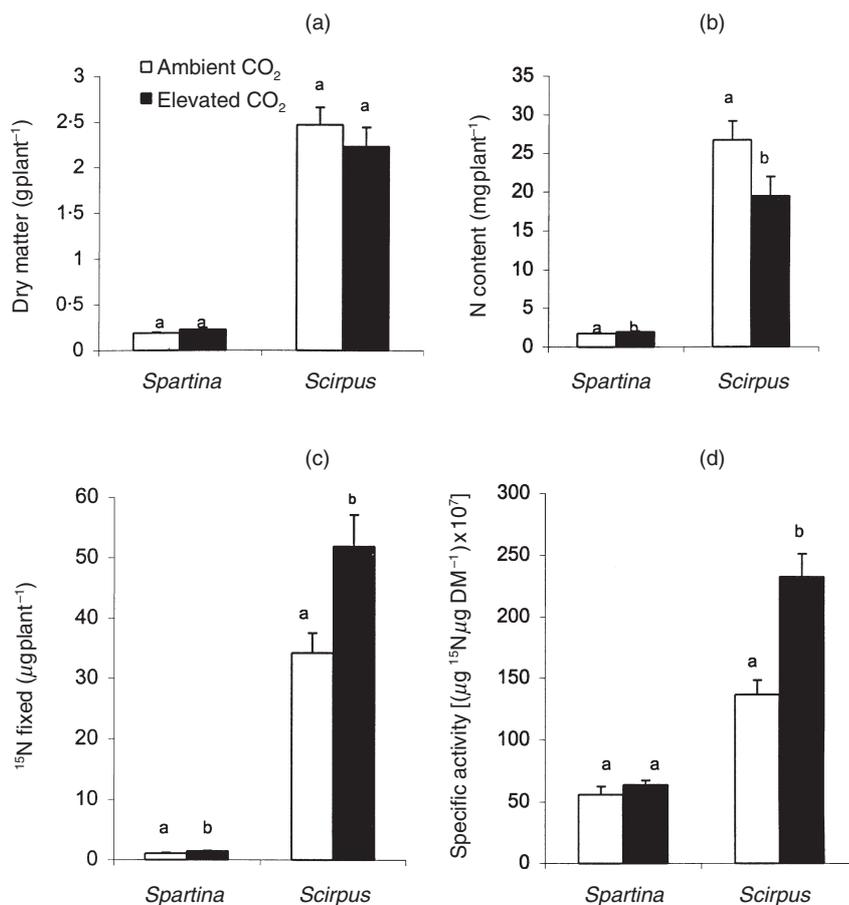
The incorporation of <sup>15</sup>N from <sup>15</sup>N<sub>2</sub> gas by shoot and subterranean organs of both test plants growing in sediment within cores was affected by elevated CO<sub>2</sub> (Fig. 2). The A % X of shoots and roots + rhizomes measured for *Scirpus* plants exposed to elevated CO<sub>2</sub> and incubated with <sup>15</sup>N<sub>2</sub> gas was significantly ( $P < 0.001$ ) higher than that of ambient

plants (Fig. 2a). As a result, the <sup>15</sup>N contents of these organs were also markedly greater in elevated CO<sub>2</sub> plants of *Scirpus olneyi* (Fig. 2b). Values of A % X for *Spartina* plants exposed to elevated CO<sub>2</sub> were also significantly greater for roots + rhizomes (Fig. 2a); however, only the shoot <sup>15</sup>N content was higher than that of ambient plants (Fig. 2b).

At whole-plant level, the experiments involving plants from sediment cores did not show any significant changes in the total dry matter of both *Spartina patens* and *Scirpus olneyi* after exposure to elevated CO<sub>2</sub> for 4 months (Fig. 3a). Although elevated CO<sub>2</sub> caused a marked ( $P < 0.05$ ) decrease in the total N of *Scirpus olneyi*, it resulted in a significant increase in whole-plant N of *Spartina patens* (Fig. 3b). Plant analysis, following <sup>15</sup>N<sub>2</sub> feeding to plant-sediment cores, showed major gains in biological N by both C<sub>3</sub> and C<sub>4</sub> plants from fixation of atmospheric N<sub>2</sub>. In comparison with ambient control, the elevated CO<sub>2</sub> plants of *Scirpus olneyi* fixed significantly ( $P < 0.001$ ) greater amounts of N (Fig. 3c) as a result of markedly ( $P < 0.001$ ) increased rates of specific N<sub>2</sub>-fixing activity



**Figure 2.** Sediment material: atom % <sup>15</sup>N excess (a) and amount of <sup>15</sup>N fixed (b) in shoots and roots plus rhizomes of 4-month-old *Scirpus olneyi* and *Spartina patens* plants as affected by CO<sub>2</sub>. Emerging plants growing in cores of sediment were exposed to ambient or elevated CO<sub>2</sub> from June 1990 until September 1990, when they were incubated with <sup>15</sup>N<sub>2</sub> for 72 h and harvested for analysis. Mean <sup>15</sup>N<sub>2</sub> gas enrichments in incubation vessels containing ambient and elevated CO<sub>2</sub> *Scirpus* and *Spartina* plants were 3.72 and 3.73 atom % <sup>15</sup>N excess, respectively. Vertical lines on bars represent SE ( $n = 6$  cores with plants). Bars followed by dissimilar letters are significantly different at  $P < 0.05$  for each pair of ambient and elevated CO<sub>2</sub> treatments.



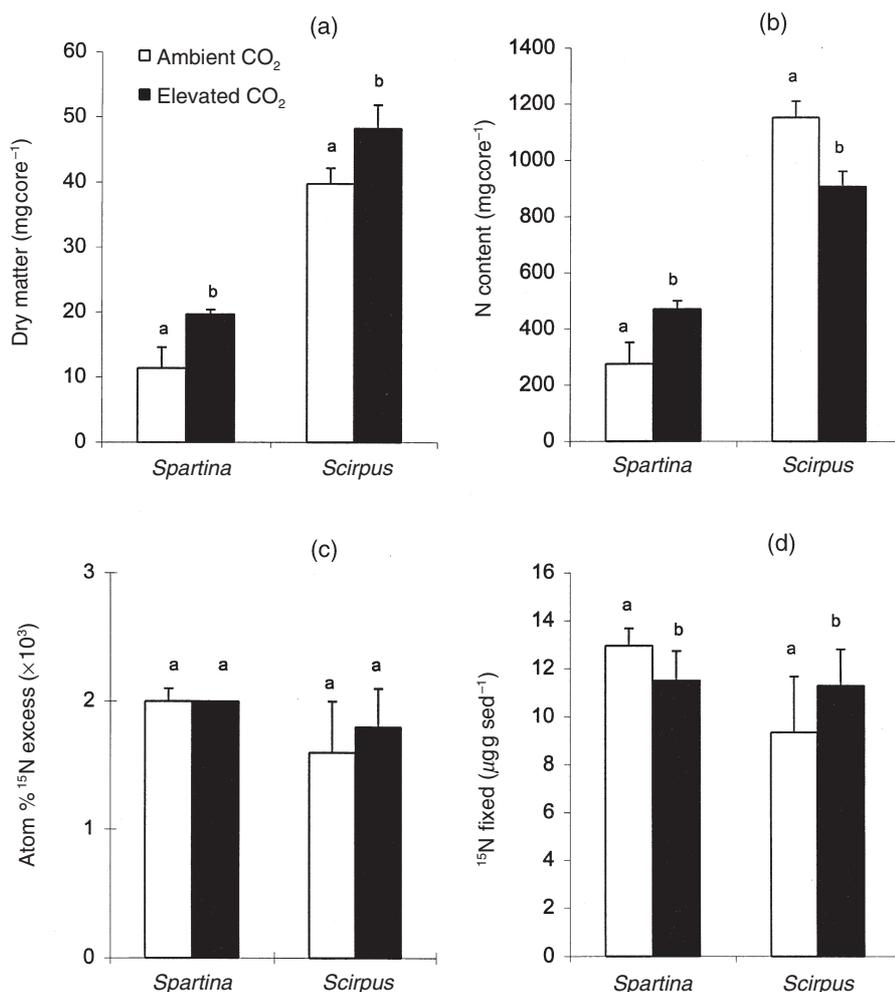
**Figure 3.** Sediment material: whole-plant growth (a), N content (b), amount of <sup>15</sup>N fixed (c), and specific N<sub>2</sub>-fixing activity (d) in 4-month-old *Scirpus olneyi* and *Spartina patens* plants grown in cores of sediment and exposed to ambient or elevated CO<sub>2</sub>. The plants were grown in cores of sediment and exposed to CO<sub>2</sub> treatments from June to September 1990, when they were incubated with <sup>15</sup>N<sub>2</sub> for 72 h and harvested for analysis. Specific activity was obtained by dividing the fixed <sup>15</sup>N content of a plant by its dry matter. Vertical lines on bars represent SE (*n* = 6 cores with plants). Bars followed by dissimilar letters are significantly different at *P* < 0.05 for each pair of ambient and elevated CO<sub>2</sub> treatment.

(Fig. 3d). Similarly, elevated CO<sub>2</sub> *Spartina patens* plants also fixed more N (*P* < 0.05) than their ambient counterparts (Fig. 3c).

The dry matter of sediment material supporting growth of the test plants was significantly greater under elevated CO<sub>2</sub> compared with ambient conditions for both species (Fig. 4a). Estimates also showed that N concentration of root-associated sediment of *Spartina patens* was 24.2 and 23.9 mg N per g sediment, respectively, for ambient and elevated CO<sub>2</sub> plants. With *Scirpus olneyi*, the N concentration was 29.0 and 18.8 mg N per g sediment, respectively, for ambient and elevated CO<sub>2</sub> plants. Thus, the elevated CO<sub>2</sub> treatment caused a highly significant (*P* < 0.01) decrease in the N content of sediment associated with *Scirpus olneyi*, and a marked (*P* < 0.05) increase in N content of sediment supporting growth of *Spartina patens* (Fig. 4b). Although there were no significant differences in A % X of the sediment material supporting growth of the two species (Fig. 4c), <sup>15</sup>N incorporation by microbes in the sediment was significantly higher under elevated CO<sub>2</sub> conditions in comparison with ambient (Fig. 4d).

In a second experiment involving ambient and elevated CO<sub>2</sub>-treated plants, the roots were washed to remove sediment, and whole plants incubated with <sup>15</sup>N<sub>2</sub> gas for 72 h under the prevailing CO<sub>2</sub> treatments described for the

plant-sediment core experiment above. With the exception of *Spartina patens*, which had greater shoot dry matter with elevated CO<sub>2</sub>, the 4 months of CO<sub>2</sub> treatments had no effect on growth of shoots and roots + rhizomes of the two test plants (Fig. 5a). However, as with the plant-sediment core experiment, shoot N content decreased significantly (*P* < 0.05) in *Scirpus olneyi* but increased markedly (*P* < 0.05) in *Spartina patens* with elevated CO<sub>2</sub> (Fig. 5b). Even in the roots and rhizomes of *Scirpus olneyi*, there was a decrease in N with elevated CO<sub>2</sub>, but this was not significant. Measures of A % X for shoots of *Scirpus olneyi* were highly significant (*P* < 0.001) in the elevated CO<sub>2</sub> treatment compared with ambient (Fig. 6a). A similar observation was made for shoots of *Spartina patens*. The two species also showed very high (*P* < 0.001) values for A % X of roots and rhizomes with elevated CO<sub>2</sub> compared with ambient conditions (Fig. 6a); and this result was similar to that obtained in the plant-sediment core experiment shown in Fig. 2a. The amount of fixed N (<sup>15</sup>N incorporation) found in shoots, roots and rhizomes of *Scirpus olneyi*, but not in *Spartina patens*, was significantly (*P* < 0.05) greater with elevated CO<sub>2</sub> (Fig. 6b), showing once again a similar pattern with the results obtained in the plant-sediment core experiment (Fig. 2b). At whole-plant level, dry matter yields were similar in each species for the CO<sub>2</sub> treatments



**Figure 4.** Sediment: dry matter (a), N content (b), atom % <sup>15</sup>N excess (c), and fixed <sup>15</sup>N (d) of the sediment associated with *Spartina* and *Scirpus* plants in the cores. Emerging plants growing in cores of sediment were exposed to ambient or elevated CO<sub>2</sub> from June to September 1990 when the sediment with plants in each core was incubated with <sup>15</sup>N<sub>2</sub> for 72 h and the sediment collected for analysis. Vertical lines on bars represent SE ( $n = 6$  cores of sediment). Bars followed by dissimilar letters are significant at  $P < 0.05$  for each pair of ambient and elevated CO<sub>2</sub> treatment.

(Fig. 7a). However, as shown in the plant-sediment core experiment, total N in *Scirpus olneyi*, but not in *Spartina patens*, decreased significantly ( $P < 0.05$ ) with elevated CO<sub>2</sub> (Fig. 7b), even though the amount of N fixed per plant increased markedly ( $P < 0.05$ ) (Fig. 7c) as a consequence of higher rates of specific N<sub>2</sub>-fixing activity of the plant (Fig. 7d). In contrast, the total N, fixed N, and specific N<sub>2</sub>-fixing activity in *Spartina patens* were not affected by the CO<sub>2</sub> treatments (see Fig. 7b–d).

#### Effects of elevated CO<sub>2</sub> on nitrogenase activity of C<sub>3</sub> and C<sub>4</sub> plants

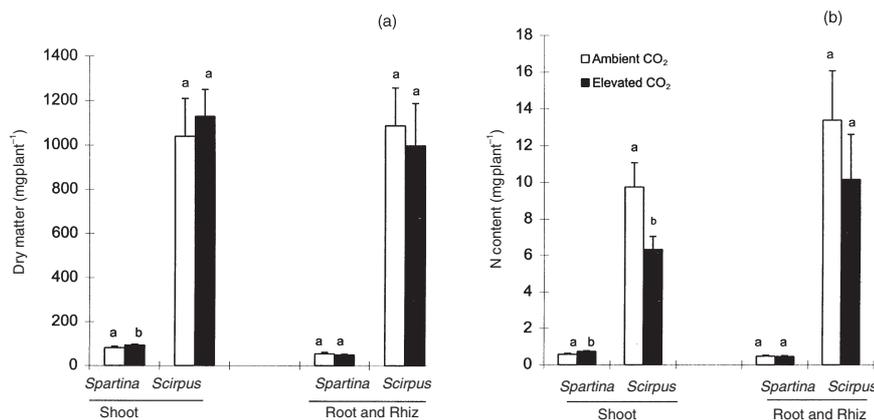
Acetylene reduction assay involving the use of sediment-free, intact, whole plants showed that in both C<sub>3</sub> and C<sub>4</sub> species, nitrogenase activity was affected by elevated CO<sub>2</sub> (Table 1). In the C<sub>3</sub> *Scirpus olneyi*, acetylene reduction increased by 35% with elevated CO<sub>2</sub>, and was significantly ( $P < 0.05$ ) higher than that of ambient (Table 1). In the case of *Spartina patens* the 13% increase obtained was not significant (Table 1).

#### Effects of elevated CO<sub>2</sub> on nitrogenase activity of sediment microbes

The effect of elevated CO<sub>2</sub> on nitrogenase activity of heterotrophic, non-symbiotic N<sub>2</sub> fixers in sediment was also measured using the open flow-through system of acetylene reduction assay. The level of nitrogenase activity in free-living diazotrophs in the marsh sediment was significantly ( $P < 0.05$ ) affected by elevated CO<sub>2</sub> (Table 2). An assay of the marsh sediment obtained from *Scirpus* and *Spartina* communities that were exposed to ambient or elevated CO<sub>2</sub>, showed significantly ( $P < 0.05$ ) higher acetylene reduction by the material from the elevated CO<sub>2</sub> compared to the ambient treatment (Table 2).

#### DISCUSSION

In this study, we have established that elevated CO<sub>2</sub> stimulates greater ( $P < 0.05$ ) N<sub>2</sub> fixation in stands of the C<sub>3</sub> sedge, *Scirpus olneyi*, than in stands of the C<sub>4</sub> grass, *Spartina patens* (Figs 3 & 7). This stimulation is in rough proportion to the

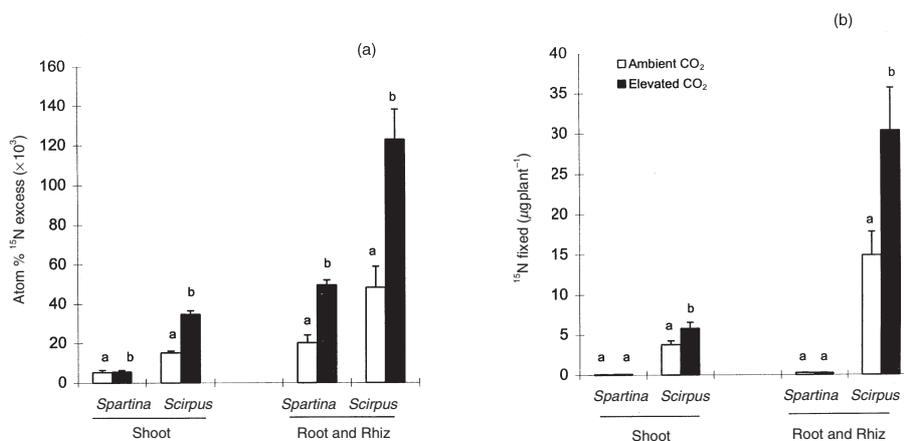


**Figure 5.** Sediment-free material: dry matter yield (a) and total N content (b) of shoots and roots plus rhizomes of 4-month-old *Scirpus olneyi* and *Spartina patens* plants as affected by CO<sub>2</sub>. Emerging plants were exposed to ambient or elevated CO<sub>2</sub> from June to September 1990, when their roots were washed free of sediment and whole plants fed <sup>15</sup>N<sub>2</sub> for 72 h and harvested for analysis. Vertical lines on bars represent SE ( $n = 6$  cores with plants). Bars followed by dissimilar letters are significantly different at  $P < 0.05$  for each pair of ambient and elevated CO<sub>2</sub> treatment.

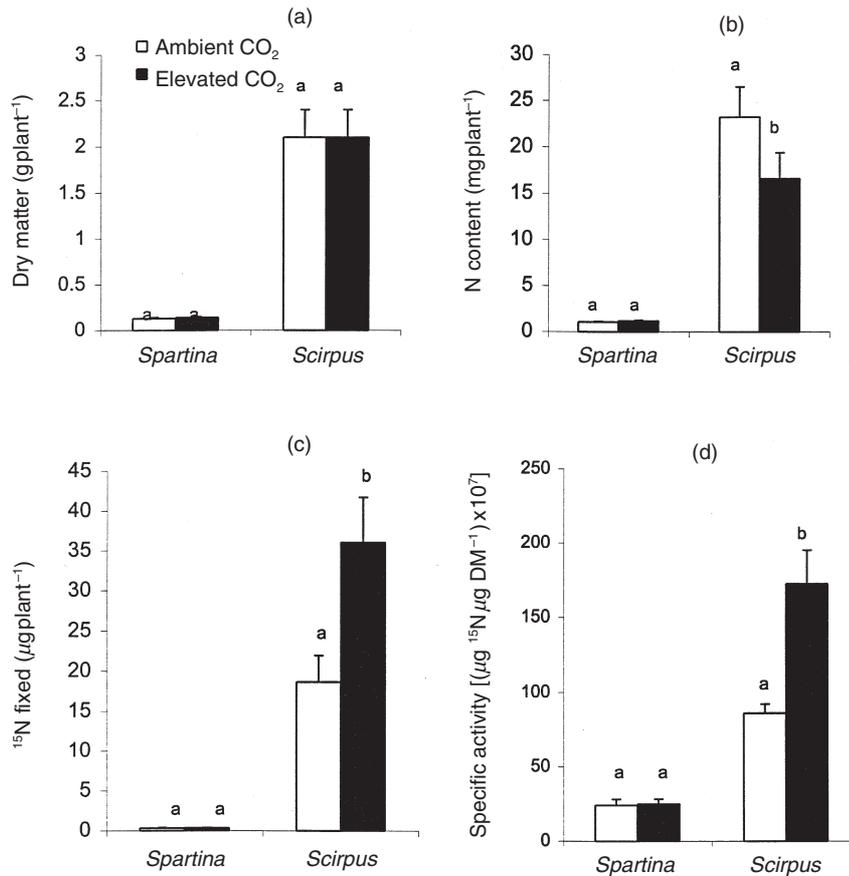
relative effect of elevated CO<sub>2</sub> on canopy photosynthesis measured throughout the day as reported by Drake & Leadley (1991). Based on measurements from acetylene reduction assays, nitrogenase activity rose by a significant ( $P < 0.05$ ) 35% in the C<sub>3</sub> sedge, and only 13% in the C<sub>4</sub> grass (Table 1) when the CO<sub>2</sub> concentration to plants was doubled. The consequence was a marked increase ( $P < 0.05$ ) of 73% in symbiotically fixed N in *Scirpus olneyi*, and 23% in *Spartina patens*. Although these results failed to confirm the suggestion by Neyra & Dobereiner (1977) that C<sub>4</sub> grasses show greater rates and efficiency in associative N<sub>2</sub> fixation than C<sub>3</sub> species, they are consistent with data reported for nodulated legumes. In those studies, shoot exposure to elevated CO<sub>2</sub> resulted in a significant stimulation of N<sub>2</sub>-fixing activity (Hardy & Havelka 1975; Phillips *et al.* 1976; Finn & Brun 1982) as a result of increased supply of photosynthetic products to root nodules. Recent studies (Zanetti *et al.* 1997; Hartwig *et al.* 1996) have also demon-

strated a significant increase in N<sub>2</sub> fixation of white clover plants growing under elevated CO<sub>2</sub>. In this case, the rise in N<sub>2</sub> fixation with elevated CO<sub>2</sub> was found to be the major source of N for the ecosystem of ryegrass/white clover mixture (Zanetti *et al.* 1997).

Even though in our study the population of heterotrophic non-symbiotic N<sub>2</sub>-fixing microbes was not assessed for the sediment, the data show a significant ( $P < 0.05$ ) increase in acetylene reduction when the marsh sediment was exposed to elevated CO<sub>2</sub> (Table 2). This increase in N<sub>2</sub> fixation was consistent with the results obtained for <sup>15</sup>N incorporation by rhizosphere microflora in the sediment associated with roots and rhizomes of *Scirpus olneyi* (Fig. 4d). The results for *Spartina patens* were however, inconsistent (Table 2 versus Fig. 4d). This probably indicates that the microbial population in plant-free sediment is functionally different from that associated with the rhizosphere of *Spartina patens* plants. Schortemeyer *et al.* (1996)



**Figure 6.** Sediment-free material: atom % <sup>15</sup>N excess (a) and amount of <sup>15</sup>N fixed (b) in shoots and roots plus rhizomes of 4-month-old *Scirpus olneyi* and *Spartina patens* plants as affected by CO<sub>2</sub>. Emerging plants were exposed to ambient or elevated CO<sub>2</sub> from June to September 1990 when roots were washed free of sediment and plants incubated with <sup>15</sup>N<sub>2</sub> for 72 h and harvested for analysis. Mean <sup>15</sup>N<sub>2</sub> gas enrichment in incubation vessels was 38.6 atom % <sup>15</sup>N excess for both elevated CO<sub>2</sub> *Scirpus* and *Spartina* plants, 38.6 atom % <sup>15</sup>N excess for ambient CO<sub>2</sub> *Scirpus*, and 38.5 atom % <sup>15</sup>N excess for ambient *Spartina*. Vertical lines on bars represent SE ( $n = 6$  cores with plants). Bars followed by dissimilar letters are significantly different at  $P < 0.05$  for each pair of ambient and elevated CO<sub>2</sub> treatment.



**Figure 7.** Sediment-free material: Whole-plant growth (a), total N content (b), amount of <sup>15</sup>N fixed (c), and specific N<sub>2</sub>-fixing activity (d) of 4-month-old *Scirpus olneyi* and *Spartina patens* plants exposed to elevated or ambient CO<sub>2</sub>, and whole plants with sediment-free roots incubated with <sup>15</sup>N<sub>2</sub>. Emerging plants were exposed to ambient or elevated CO<sub>2</sub> from June to September 1990 and their roots washed free of sediment and whole plants incubated with <sup>15</sup>N<sub>2</sub> for 72 h and harvested for analysis. Specific activity was obtained by dividing the fixed <sup>15</sup>N content of a plant by its dry matter. Vertical lines on bars represent SE ( $n = 6$  with plants). Bars followed by dissimilar letters are significant at  $P < 0.05$  for each pair of ambient and elevated CO<sub>2</sub> treatment.

found that, with elevated CO<sub>2</sub>, the population of *Rhizobium leguminosarum* bv. *trifolii* was higher in the rhizosphere of white clover, but not ryegrass, suggesting that there is selective colonization of the rhizosphere by some microbes, but not by others, with elevated CO<sub>2</sub> in the atmosphere.

Whether the stimulation of N<sub>2</sub> fixation by elevated CO<sub>2</sub> in *Scirpus olneyi* was due to current photosynthate or stored

carbohydrate is not known in this study. However, <sup>14</sup>CO<sub>2</sub> assimilation studies (Hume & Criswell 1973; Russell & Johnson 1975; Latomore, Giddens & Ashley 1977) show that N<sub>2</sub> fixation in plants is dependent on both recently formed photosynthate and stored carbohydrate (Streeter, Mederski

**Table 1.** Nitrogenase activity of whole plants of 4-month-old *Scirpus olneyi* and *Spartina patens* exposed to elevated or ambient CO<sub>2</sub>. The roots were washed free of sediment. Plants had been exposed to ambient or elevated CO<sub>2</sub> at emergence from June to September 1990, when sediment-free material was used in acetylene reduction assays

CO <sub>2</sub> level	Acetylene reduction	
	<i>Spartina</i> (nmol C <sub>2</sub> H <sub>4</sub> prod.plant <sup>-1</sup> h <sup>-1</sup> )	<i>Scirpus</i> (nmol C <sub>2</sub> H <sub>4</sub> prod.plant <sup>-1</sup> h <sup>-1</sup> )
Ambient	119 ± 1.9a	589 ± 23a
Elevated	135 ± 13a	798 ± 62b

Mean ± SE ( $n = 4$  cores of plants without sediment). Values followed by dissimilar letters are significantly different at  $P < 0.05$  for each pair of ambient and elevated CO<sub>2</sub> treatment.

**Table 2.** Nitrogenase activity of heterotrophic, nonsymbiotic N<sub>2</sub>-fixing microbes in plant-free, salt marsh sediment exposed to ambient or elevated CO<sub>2</sub>. Sediment cores, free of plants, roots or rhizomes, were maintained at the two CO<sub>2</sub> levels within *Spartina* and *Scirpus* communities from June to September 1990, when they were used for acetylene reduction assays

CO <sub>2</sub> level	Acetylene reduction	
	(nmol C <sub>2</sub> H <sub>4</sub> prod.core <sup>-1</sup> h <sup>-1</sup> )	(nmol C <sub>2</sub> H <sub>4</sub> prod.DW sediment <sup>-1</sup> h <sup>-1</sup> )
<i>Spartina</i> site		
Ambient	3562 ± 91a	19.6 ± 0.50a
Elevated	3817 ± 132b	18.2 ± 0.71b
<i>Scirpus</i> site		
Ambient	3504 ± 118a	18.0 ± 0.67a
Elevated	3849 ± 140b	19.3 ± 0.68a

Mean ± SE ( $n = 4$  plant-free sediment cores). Values followed by dissimilar letters are significantly different at  $P < 0.05$  for each pair of ambient and elevated CO<sub>2</sub> treatments.

& Ahmad 1980). The occurrence of gas leakage from stems of *Gramineae* when only the root systems are enclosed with N<sub>2</sub> (Yoshida & Yoneyama 1980; Matsui *et al.* 1981) dictate that whole plants be incubated with nitrogenase substrates during assays. However, that would require monitoring environmental factors (CO<sub>2</sub>, transpiration, temperature, light quality and intensity) which maintain normal photosynthesis (Boddey 1987). There is no doubt that the low pO<sub>2</sub> from experimental treatment affected CO<sub>2</sub> fixation in the C<sub>3</sub> species (Coombs 1987). Low pO<sub>2</sub> would affect respiration and photorespiration, both causing a relative increase in soluble C. Thus, the increased <sup>15</sup>N<sub>2</sub> fixation observed in *Scirpus olneyi* in this study was probably also influenced by the transient change in assay conditions. However such an effect from a 72 h incubation would be slight relative to the period of doubling in photosynthetic C fixation observed in *Scirpus olneyi* under elevated CO<sub>2</sub> in other studies (Drake *et al.* 1987; Curtis *et al.* 1989b; Drake & Leadley 1991). It is unlikely that the low pO<sub>2</sub> inside assay vessels affected respiratory processes in plant and bacteria tissue as van Berkum & Sloger (1981) measured nitrogenase activity in *Scirpus olneyi* using only N<sub>2</sub> gas (without O<sub>2</sub>) over a 6 h period and obtained both higher and linear rates of acetylene reduction relative to those in air. This suggests that N<sub>2</sub> fixation associated with roots and stems of marsh plant species occurs at near-anaerobic conditions.

In the legume system, N<sub>2</sub> fixation is affected by carbohydrate supply to *Rhizobium* bacteroids (Appleby 1984); it increases with increasing photosynthate availability to nodules from net photosynthesis (Hardy & Havelka 1975; Phillips 1980; Finn & Brun 1982), and indicates a direct link between N<sub>2</sub> fixation rates in root nodules and legume shoot photosynthesis. The observed increase in N<sub>2</sub> fixation by *Scirpus olneyi* under elevated CO<sub>2</sub> at whole-plant level (Figs 3 and 7), and the parallel increase in C accumulation under similar conditions (Drake *et al.* 1987; Curtis *et al.* 1989a, b; Drake & Leadley 1991) suggests that, as in legumes, a direct relationship also exists between C supply and symbiotic N yield in N<sub>2</sub>-fixation associated with the *Cyperaceae*. Clearly, enhanced photosynthate supply to root-associated diazotrophs was responsible for the increased N<sub>2</sub> fixation obtained in this study. The data from Drake & Leadley (1991) for *Scirpus olneyi* indicate that, compared with ambient conditions, the C accumulation in plants under elevated CO<sub>2</sub> increased by 53%, a value comparable to the 73% increase in fixed N per plant from elevated CO<sub>2</sub>. However, in C<sub>4</sub> *Spartina patens* where elevated CO<sub>2</sub> did not significantly enhance photosynthetic rates (Drake *et al.* 1987; Curtis *et al.* 1989b; Drake & Leadley 1991), C accumulation increased by only 38% paralleling the 23% increase in fixed N per plant obtained in this study under similar conditions.

When data on <sup>15</sup>N fixed per plant from Figs 3 and 7 were averaged and divided by 72 h, a rate of 611 ± 75 ng <sup>15</sup>N fixed.plant<sup>-1</sup> h<sup>-1</sup> was obtained for *Scirpus olneyi* in elevated CO<sub>2</sub> compared with 367 ± 46 ng <sup>15</sup>N fixed.plant<sup>-1</sup> h<sup>-1</sup> in ambient CO<sub>2</sub>. In *Spartina patens*, where photosynthetic rates and C accumulation were only slightly altered with

elevated CO<sub>2</sub> (Drake *et al.* 1987; Curtis *et al.* 1989b; Drake & Leadley 1991), the estimated rate of <sup>15</sup>N<sub>2</sub> fixation was 12.5 ± 1.1 versus 9.8 ± 1.3 ng <sup>15</sup>N fixed.plant<sup>-1</sup> h<sup>-1</sup> for elevated and ambient CO<sub>2</sub>, respectively. The ecological implications of these findings remain to be determined. However, the 10% increase in N<sub>2</sub> fixation rate of *Spartina patens* and 67% in *Scirpus olneyi* with elevated CO<sub>2</sub> suggest that a doubling in the external CO<sub>2</sub> concentration of the atmosphere is likely to increase the contribution by members of the *Cyperaceae* and *Poaceae* to the N economy of the marsh ecosystem.

Most intriguingly, however, the increase in the rate of N<sub>2</sub> fixation by *Scirpus olneyi* under elevated CO<sub>2</sub> was not apparent in the plant's total N content (Figs 3 & 7). The results of our study with *Scirpus olneyi* clearly show reduced amounts of N in elevated CO<sub>2</sub> plants which fixed significantly (*P* < 0.05) higher levels of atmospheric N<sub>2</sub> (Figs 3 & 7). This could be due to a reduction in protein concentration associated with carbon assimilation under elevated CO<sub>2</sub> conditions (Jacob *et al.* 1995), and possibly to N loss from root exudation in the marsh. In addition, estimates using the data in Figs 1, 3, 5 and 7 showed large reductions in tissue N concentrations (mg N g DW<sup>-1</sup>) of elevated CO<sub>2</sub>-grown *Scirpus olneyi* plants relative to those in ambient CO<sub>2</sub> (data not shown). A reduction in N concentration with photosynthetic carbon fixation tends to occur when the supply of N is not overabundant at elevated CO<sub>2</sub> levels. Recent studies have also shown that N concentration in tissues is almost always reduced whether or not N is provided in abundant supply (Conroy 1992; Conroy, Milham & Barlow 1992; Conroy & Hocking 1993; Zanetti *et al.* 1997). Our findings are therefore consistent with other reports in the literature (Conroy 1992; Conroy *et al.* 1992; Conroy & Hocking 1993; Curtis *et al.* 1989b; Delgado *et al.* 1994; Zanetti *et al.* 1997) which show a reduction in tissue N concentrations when plants were exposed to elevated CO<sub>2</sub>. Calculations based on plant density (see Fig. 2a,c, Curtis *et al.* 1989a) and symbiotic N yield (Figs 3 & 7) indicate that over the 3 d incubation period with <sup>15</sup>N<sub>2</sub>, an average of 0.37 kg N ha<sup>-1</sup> was fixed by *Scirpus olneyi* plants growing in elevated CO<sub>2</sub> compared with 0.18 kg N ha<sup>-1</sup> in those from ambient conditions. In *Spartina patens*, the values were 0.06 and 0.04 kg N ha<sup>-1</sup>, respectively, for elevated and ambient CO<sub>2</sub>. Evaluating these data at ecosystem level and on the basis of a whole growth season showed that up to 57 kg N ha<sup>-1</sup> were fixed by *Scirpus olneyi* plants in elevated CO<sub>2</sub> compared with only 27 kg N ha<sup>-1</sup> in ambient CO<sub>2</sub>. In *Spartina patens*, the values were 9 and 6 kg N ha<sup>-1</sup>, respectively, in elevated and ambient CO<sub>2</sub> over the same period. The data from this study (Fig. 4d; Table 2) further predict an increase in the N<sub>2</sub>-fixing activity of free-living heterotrophic diazotrophs with a doubling in atmospheric CO<sub>2</sub> concentration in the marsh ecosystem.

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