

Effect of plant photosynthesis, carbon sources and ammonium availability on nitrogen fixation rates in the rhizosphere of *Zostera noltii*

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ABSTRACT: Rates of nitrogen fixation (measured as acetylene reduction) in the rhizosphere of the sea-grass *Zostera noltii* were highly dependent upon plant photosynthetic activity being significantly stimulated at elevated CO₂ concentrations and by light, both in the short-term and over diurnal cycles. Stimulation by light became insignificant when 5 mM sucrose was added to the sediment porewater, indicating that in the absence of added carbon sources, light stimulation was due to direct inputs of plant photosynthate to the rhizosphere. Addition of a range of carbon sources to the rhizosphere sediment stimulated rates of acetylene reduction, with this stimulation being significant for sucrose and lactate. Surprisingly, whilst low additions of ammonium to the sediment porewater (10 to 50 µM) inhibited 50% of acetylene reduction activity, approximately 30% of this activity persisted in the presence of 1 mM ammonium chloride; this indicating that in at least a proportion of the N-fixing community, nitrogenase activity was not regulated in the short term by the availability of alternative nitrogen sources.

KEY WORDS: Acetylene reduction · Nitrogen fixation · Rhizosphere · *Zostera noltii* · Plant photosynthesis · Root exudates · Carbon sources · Ammonium

INTRODUCTION

Primary productivity in coastal marine environments is generally considered to be limited by the availability of fixed nitrogen (Eppley et al. 1979). However, sea-grass meadows often exhibit high levels of primary production and therefore a high demand for fixed nitrogen (Patriquin 1972, Moriarty et al. 1990). Whilst a significant proportion of this requirement can be sustained by efficient recycling of organic nitrogen within the sediment (Dennison et al. 1987, Caffrey & Kemp 1992), heterotrophic nitrogen fixation by bacteria in the rhizosphere represents a potentially significant source of nitrogen for plant growth. Nitrogen fixation rates ranging from 0.02 to 140 mg N m⁻² d⁻¹ have been measured in seagrass ecosystems and estimated to supply up to 50%

of the fixed nitrogen requirement for plant growth (Carpone 1988, O'Donohue et al. 1991, Welsh et al. 1996a).

Rates of nitrogen fixation in the rhizosphere of both sea and salt marsh grasses have been demonstrated to be influenced by the photosynthetic activity of the plants (Boyle & Patriquin 1981, Whiting et al. 1986, O'Donohue et al. 1991, Welsh et al. 1996a) and are thought to be fuelled by the exudation of photosynthates via the plant roots. We have previously measured rates of nitrogen fixation in the seagrass meadows of the Bassin d'Arcachon, southwest France, ranging between 0.1 and 7.3 mg N m⁻² d⁻¹ (dependent upon growth season) and nitrogen fixation was calculated to supply between 6.3 and 12% of the annual fixed nitrogen requirement of the plant community (Welsh et al. 1996a).

In this study we have investigated the influence of plant photosynthesis and the availability of fixed nitrogen and carbon sources on nitrogen fixation activity in the rhizosphere of *Zostera noltii*, the dominant sea-grass species in the Bassin d'Arcachon.

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MATERIALS AND METHODS

Sampling site. The sampling station was situated in an extensive seagrass meadow on the Ile aux Oiseaux, a large intertidal mudflat located in the central region of the Bassin d'Arcachon (see Welsh et al. 1996a); a description of the seasonal dynamics of *Zostera noltii*, the dominant seagrass species, can be found in Auby & Labourg (1996). Samples were collected between June and August 1995, with the exception of those for the diurnal cycle which were collected in October 1994, by inserting 5 or 10 × 25 cm [internal diameter (i.d.)] grey plastic core tubes into the sediment until the sediment surface was level with the rims of the core tubes. The surrounding sediment was removed and the core transferred onto a perspex sheet for transportation to the laboratory. At the laboratory, cores were stored at *in situ* temperature ($\pm 2^\circ\text{C}$), under semi-natural light conditions in a 50 cm (water depth) × 3 m tank circulated with aerated natural seawater from the Bassin (dilution rate, $D \sim 0.05 \text{ h}^{-1}$). Cores were stored maximally for 3 d before use.

Determination of nitrogen fixation (acetylene reduction) rates. Nitrogen fixation rates were assessed using the acetylene reduction technique. Acetylene and ethylene concentrations were determined by gas chromatography (Welsh et al. 1996a) and data corrected for the trace quantities of ethylene present in the acetylene used.

Time course experiments. Time course experiments were performed using a modification of the whole core lacunal diffusion technique (Welsh et al. 1996a). Sub-cores of 5 cm length were taken using 20 × 4.4 cm (i.d.) plexiglass core tubes fitted with a Suba-sealed (W. Freeman, Barnsley, England) sampling port and 2 sets of silicon rubber sealed injection ports at 1 cm intervals. The water column was decanted, cores were sealed with rubber bungs, 10% of the headspace gas was replaced with acetylene via the sampling port and the cores incubated in a water bath at *in situ* temperature ($\pm 2^\circ\text{C}$) under semi-natural light conditions over a 10 h period. Cores for dark incubations were double wrapped in aluminium foil and incubated under the same conditions. Aliquot volumes (1 cm³) of the headspace gas were collected at 2 h time intervals and analysed for acetylene and ethylene concentrations.

Additions of sucrose and ammonium chloride to the sediment were made by 2 injections at 90° at each 1 cm depth interval of 100× stock solutions prepared in filter-sterilised seawater to give final porewater concentrations of 5 mM for sucrose and 10, 50, 100 and 1000 μM for ammonium; nominal porewater concentrations were based on a mean sediment porosity of 70.5 ± 5.0% ($n = 5$). Additions of CO₂ to the headspace gas were made by injection via the Suba-sealed sampling port.

To determine diurnal cycles of acetylene reduction, sediment cores (5 × 25 cm i.d.) were transferred to small water baths and the water level adjusted to be level with the sediment surface. Plexiglass core tubes (20 × 5 cm i.d.) were inserted into the sediment, sealed with a rubber bung, 10% of the headspace gas was replaced with acetylene and the cores incubated at ambient temperature (ca 25°C) in the dark for 6 h to allow equilibration of acetylene and ethylene gradients between the sediment and the headspace. Following equilibration, the cores were illuminated by tungsten light (200 $\mu\text{E m}^{-2} \text{ s}^{-1}$) for 12 h and then placed in the dark for a further 12 h. At zero time and after subsequent 3 h time intervals triplicate 1 cm³ samples of the headspace gas were analysed and acetylene reduction rates calculated by subtraction of the value for the preceding 3 h period.

Effect of carbon sources. Sub-cores of ca 5 cm length were collected in 20 × 4.4 cm (i.d.) plexiglass core tubes and sealed with rubber bungs. Carbon source additions were made by 2 injections at 90° at each 1 cm depth horizon of 100× stock solutions prepared in seawater to give a final nominal porewater concentration of 5 mM. Two injections at 90° to each other of 0.5 cm³ acetylene-saturated seawater were made at each 1 cm depth interval and 10% of the water column replaced by injection of acetylene saturated seawater; excess water was allowed to escape via a hypodermic needle inserted through the sampling port. Cores were incubated under semi-natural light conditions at *in situ* temperature ($\pm 2^\circ\text{C}$) for 3 h.

Following incubation, a 50 ml aliquot of the water column was transferred to a partially evacuated serum bottle containing 10 ml of 10% v/v trichloroacetic acid and the bottles shaken to stop further activity. The remainder of the water column was discarded and the sediment transferred to a 360 ml glass bottle containing 80 ml of 10% v/v trichloroacetic acid. Bottles were sealed with screw tops fitted with rubber-sealed injection ports and vigorously shaken to inhibit further activity and equilibrate the dissolved gases. Following equilibration, aliquot volumes (1 cm³) of the headspace gas were analysed for acetylene and ethylene concentrations.

Statistical analysis. All experiments were performed in triplicate and treatment effects tested by 1-way ANOVA (Sokal & Rohlf 1995). A *posteriori* comparison of the means was performed using the T-method.

RESULTS

Influence of carbon sources

Acetylene reduction rates (ARR) measured in control cores of *Zostera noltii* during both light and dark incubations were linear over the whole 10 h incubation

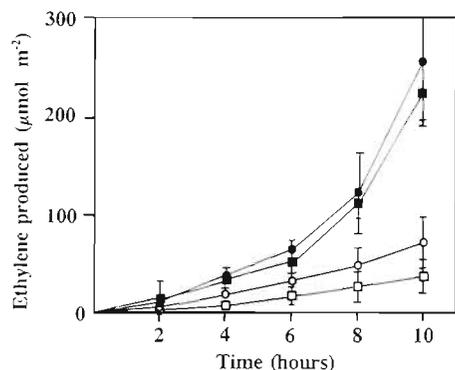


Fig. 1. Time course of ethylene production in *Zostera noltii*-colonised sediments during light incubations (\circ), dark incubations (\square), light incubations of sucrose-amended cores (\bullet) and dark incubations of sucrose-amended cores (\blacksquare). Sucrose was added to give a final nominal porewater concentration of 5 mM as described in the 'Materials and methods'. Data points represent means for triplicate cores \pm 1 standard deviation, with the exception of the dark control data which are means of duplicate cores

period ($r > 0.99$), with mean ARR of 7.66 and 4.17 $\mu\text{mol m}^{-2} \text{h}^{-1}$ for light and dark incubations, respectively (Fig. 1), being significantly different ($p < 0.05$; Student's t -test). ARR in sucrose amended cores were linear over the first 6 h of incubation ($r > 0.99$), but thereafter accelerated, with overall measured rates of 29.92 and 30.89 $\mu\text{mol m}^{-2} \text{h}^{-1}$ for light and dark incubations, respectively, being significantly different from those of control cores ($p < 0.01$; 1-way ANOVA), but the effect of light was no longer significant ($p = 0.887$; 1-way ANOVA). Similarly, additions of a range of carbon sources to the sediment porewater, including ethanol, sucrose, propionate, acetate and lactate to a final nominal concentration of 5 mM stimulated ARR by between 144 and 354% compared to those recorded for unamended control cores (data not shown). However, whilst all the tested carbon sources tended to enhance ARR, due to inter-core variations this stimulation was only significant for lactate and sucrose ($p < 0.05$; 1-way ANOVA), with recorded ARR of 21.56 ± 6.57 and $34.74 \pm 5.96 \mu\text{mol m}^{-2} \text{h}^{-1}$ for sucrose and lactate treated cores, respectively, as compared to $9.82 \pm 1.74 \mu\text{mol m}^{-2} \text{h}^{-1}$ for controls.

Influence of plant photosynthesis

ARR during light incubations were approximately double and significantly different ($p < 0.05$; Student's t -test) from those recorded during dark incubations (Fig. 1). Similarly, during artificial diurnal cycles, ARR were highly dependent on the light conditions, with ARR increasing over the first 6 h of incubation to a rate of 54.5 $\mu\text{mol m}^{-2} \text{h}^{-1}$ during the 3 to 6 h time interval,

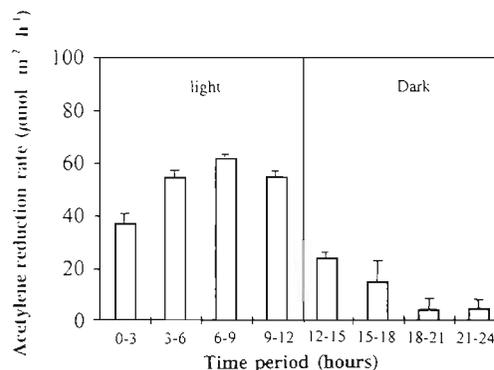


Fig. 2. Artificial diurnal cycle of acetylene reduction rates in *Zostera noltii*-colonised sediments. Data points represent the means of triplicate determinations and error bars signify 1 standard deviation

which was maintained throughout the remainder of the light period. On transfer to dark conditions ARR rates rapidly declined with time to below 5 $\mu\text{mol m}^{-2} \text{h}^{-1}$ after 6 h in the dark (Fig. 2).

Additionally, increasing the CO_2 concentration to 0.6% v/v (approx. 20 \times background concentrations), which would be expected to increase photosynthetic efficiency, also stimulated ARR in the rhizosphere (Fig. 3). ARR at the elevated CO_2 concentration were $22.96 \pm 6.83 \mu\text{mol m}^{-2} \text{h}^{-1}$ compared to $10.79 \pm 3.69 \mu\text{mol m}^{-2} \text{h}^{-1}$ for controls ($p < 0.01$; Student's t -test), demonstrating a clear association of ARR with plant photosynthesis.

Effect of ammonium

Additions of low concentrations of ammonium chloride to the sediment porewater rapidly inhibited ARR by 42 and 55% for nominal porewater ammonium concentrations of 10 and 50 μM respectively (Fig. 4). How-

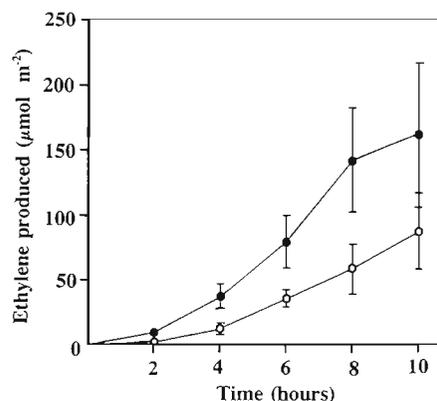


Fig. 3. Effect of headspace gas CO_2 concentration on acetylene reduction activity in *Zostera noltii*-colonised sediments. Control (\circ) and 0.6% v/v CO_2 added (\bullet). Data points represent mean values for triplicate cores \pm 1 standard deviation

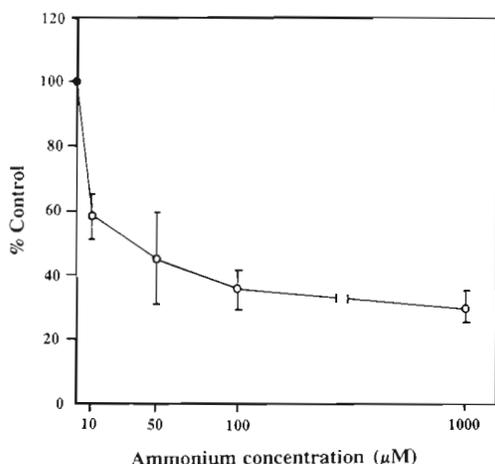


Fig. 4. Effect of ammonium additions to the sediment porewater on acetylene reduction rates in the rhizosphere of *Zostera noltii*. Data points represent mean values for 3 replicate cores \pm 1 standard deviation. The acetylene reduction rate in control cores (100%) was $14.7 \mu\text{mol m}^{-2} \text{h}^{-1}$

ever, surprisingly, further increases in ammonium concentration had little effect on the residual ARR, with rates following addition of 1 mM ammonium chloride being equivalent to $29.6 \pm 4.9\%$ of the rates recorded in control cores.

DISCUSSION

Rates of heterotrophic nitrogen fixation in coastal marine sediments are considered to be limited by the availability of suitable organic carbon sources (Nedwell & Aziz 1980, Jones 1982). However, nitrogen fixation rates in the rhizosphere of seagrasses are often high and are tightly coupled to plant photosynthesis (Whiting et al. 1986, O'Donohue et al. 1991, Welsh et al. 1996a). Similarly, in this study ARR were significantly ($p < 0.05$) stimulated in the light (Figs. 1 & 2) and under elevated atmospheric CO_2 concentrations which would be expected to enhance photosynthetic efficiency by stimulating the carboxylase activity of Rubisco over the wasteful oxygenase activity (Fig. 3). Whilst these effects could be explained by a stimulation of phototrophic nitrogen fixers such as cyanobacteria, this would appear to be unlikely. Since epiphytic cyanobacteria are generally absent or present only in low numbers and although benthic cyanobacteria (*Oscillatoria* sp.) are present mainly in areas where the seagrass cover is sparse or absent during diurnal cycles, these bacteria fixed nitrogen primarily during the dark period (authors' unpubl. data), as has been observed for non-heterocystous cyanobacteria in other coastal marine ecosystems (Bebout et al. 1994). Additionally, in previous studies we have demonstra-

ted that the bulk of nitrogen fixation in these seagrass beds occurs at depths below the photic zone and molybdate inhibition studies indicate that sulfate-reducing bacteria are the predominant nitrogen fixing micro-organisms in the rhizosphere (Welsh et al. 1996a, b).

Thus the observed stimulation of ARR by light and CO_2 in these sediments would appear to be primarily due to inputs of photosynthate to the rhizosphere via the *Zostera noltii* roots. This hypothesis is supported by the fact that when an excess of organic carbon was added to the sediment porewater the effect of light on ARR was no longer significant (Fig. 1). Similarly, in other seagrass ecosystems, bacterial activities in the rhizosphere, such as bacterial secondary productivity and sulfate reduction, have been shown to be influenced by the photosynthetic activity of the plants (Moriarty et al. 1986, 1990, Pollard & Moriarty 1991, Blackburn et al. 1994).

In order to elucidate which carbon sources may be significant in the rhizosphere, we tested the effect of a range of carbon sources on ARR. Whilst all added carbon sources stimulated ARR over those of control cores, this stimulation was only significant for sucrose and lactose ($p < 0.05$). Additions of sucrose and other sugars such as glucose, fructose and manose have previously been shown to enhance ARR in the rhizosphere of the salt marsh grass *Spartina alterniflora* (Boyle & Patriquin 1981, Yoch & Whiting 1986). However, sucrose may not be used directly by the N-fixing bacteria, as in time course experiments with sucrose-amended cores ARR increased with time (Fig. 1), perhaps indicating that it may be a product of sucrose metabolism rather than sucrose itself which stimulated acetylene reduction activity. Lactate would be expected to stimulate ARR due to the dominance of N-fixing *Desulfovibrio* species in the rhizosphere (Bourguès 1996), as lactate has been shown to support high rates of N-fixation in *Desulfovibrio* sp. (Riederer-Henderson & Wilson 1970). In a previous study, porewater concentrations of lactate in these rhizosphere sediments of *Zostera noltii* were undetectable ($< 0.8 \mu\text{M}$) and acetate was the predominant volatile fatty acid accounting for greater than 90% of the total pool (Welsh et al. 1996b). However, these data do not exclude the possibility that lactate may be an important carbon source for sulfate-reducing bacteria in the rhizosphere, as lactate production and utilisation may be closely coupled resulting in a small but highly dynamic lactate pool. In fact the large acetate pools present may be at least partially due to incomplete oxidation of lactate by *Desulfovibrio* sp. in the rhizosphere (Welsh et al. 1996b).

Nitrogen fixation activity in pure culture studies has been shown to be regulated by the availability of fixed

nitrogen, due to repression of nitrogenase synthesis (Dixon 1984) and a rapid and reversible 'switch-off' of nitrogenase activity by ammonium (Zumft & Castillo 1978, Postgate & Kent 1984). Similarly, *in situ* studies in salt marsh sediments have shown that ARR were inhibited by ammonium additions both in the long and short term (Yoch & Whiting 1986, Capone 1988), although a threshold concentration of 50 to 200 μM ammonium appears to be required (Capone 1988). In this study additions of low concentrations of ammonium (10 to 50 μM) inhibited 40 to 60% of acetylene reduction activity in the rhizosphere sediments, although little further inhibition was observed at higher ammonium concentrations, with 30% of the control activity persisting in the presence of 1 mM ammonium (Fig. 4). These data indicate that the nitrogenase activity of a substantial proportion of the N-fixing population was not inhibited by ammonium, as has been observed in cultures of *Klebsiella pneumoniae* and *Clostridium pasteurianum* (Tubb & Postgate 1973, Gordon et al. 1981) and, indeed, we have previously isolated a *Desulfovibrio* sp. from the rhizosphere of *Zostera noltii* in which nitrogenase activity is not inhibited by ammonium or organic N-sources at a concentration of 1 mM at least in the short term (Bourguès 1996). The marked inhibition of ARR by low concentrations of ammonium is also surprising, as we have previously measured porewater concentrations of 65 to 105 μM in these sediments (Welsh et al. 1996b). However, these values may be overestimates, since porewater harvesting by centrifugation in *Z. marina* sediments can overestimate porewater ammonium concentrations (J. Hansen & B. Lomstein pers. comm.) and it is probable that this effect would be greater for *Z. noltii* due to the finer nature of the root system. Additionally, the ammonium concentration in the vicinity of the plant roots may be substantially lower than the porewater average due to assimilation of ammonium by the roots themselves. Thus it is likely that the true ammonium concentration at the root surface is very much lower than our previously measured values.

In conclusion, data presented in this study indicate that photosynthetically driven exudation of carbon by the plant roots is the dominant factor which regulates nitrogen fixation in the rhizosphere of *Zostera noltii*. In contrast, whilst small additions of ammonium rapidly inhibited 50% of acetylene reduction activity, 30% of this activity persisted in the presence of 1 mM ammonium. These data indicate that the nitrogenase activity in a significant proportion of the N-fixing microflora is not regulated by ammonium in the short term, although it is probable that ammonium does regulate activity in the long term due to repression of nitrogenase synthesis.

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