A tripartite microbial reporter gene system for real-time assays of soil nutrient status

Dominic Standing *, Andy A. Meharg, Ken Killham

Department of Plant and Soil Science, School of Biological Sciences, University of Aberdeen, Cruickshank Building, St. Machar Drive, Aberdeen AB24 3UU, Scotland, UK

Received 11 October 2002; accepted 10 January 2003
First published online 31 January 2003

Abstract

Plant-derived carbon is the substrate which drives the rate of microbial assimilation and turnover of nutrients, in particular N and P, within the rhizosphere. To develop a better understanding of rhizosphere dynamics, a tripartite reporter gene system has been developed. We used three lux-marked Pseudomonas fluorescens strains to report on soil (1) assimilable carbon, (2) N-status, and (3) P-status. In vivo studies using soil water, spiked with C, N and P to simulate rhizosphere conditions, showed that the tripartite reporter system can provide real-time assessment of carbon and nutrient status. Good quantitative agreement for bioluminescence output between reference material and soil water samples was found for the C and P reporters. With regard to soil nitrate, the minimum bioavailable concentration was found to be greater than that analytically detectable in soil water. This is the first time that bioavailable soil C, N and P have been quantified using a tripartite reporter gene system.

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Keywords: Pseudomonas fluorescens DF57 N3; P. fluorescens DF57 P9; Reporter gene; Bio-available nutrients

1. Introduction

The rhizosphere is a dynamic and spatio-temporally complex environment in which plant roots and soil biota co-exist. The rhizosphere and bulk soil vary chemically, physically and biologically due to two key processes. These are nutrient uptake by the root and a simultaneous deposition of C compounds into the soil. It has been estimated that up to 10% of plants' fixed C is secreted into the rhizosphere [1] as soluble exudates and in young, unstressed cereals the main components of the exudates are sugars, amino acids and organic acids. The flux of C from the root drives the growth of the rhizosphere microbial community, and it has been estimated that the rhizosphere microbial community may be as much as 20-fold greater than that of the bulk soil [2,3]. To sustain balanced growth, the rhizosphere microbial community must utilise other nutrients, particularly N and P, and so affect C, N and P cycling [4]. The rhizosphere then, can be conceptualised as a gradient of C decreasing distally from the root mirrored by decreasing gradients of N and P proximal to the root. The decreasing gradients of N and P are a function of microbial uptake as well as plant root uptake [5,6]. The rhizosphere microbial population may be N and P limited on and near the rhizoplane and progressively C limited with increasing distance from the areas of rhizodeposition. However, the relationships between rhizosphere C, N and P remain elusive and one of the key limitations to our understanding is a current lack of knowledge of how N and P drive or limit microbial growth. No one has yet quantified microbial dynamics with regard to bioavailable C, N and P.

Pseudomonas fluorescens is a common soil bacterium and lux-marked strains are available where light output is linked to cellular content of reduced flavin mononucleotide where oxygen and aldehyde are in surplus [7]. These strains have been used to report on various aspects of rhizosphere-nutrient fluxes, for example, carbon flow from young roots [8], N limitation [9,10] and P limitation [9,11]. All three constructs report on metabolic activity. However, simultaneous measurements of rhizosphere C, N and P have never been made. Here, we present a real-time, in situ system to report on bioavailable C, N and P.
This is the first time that these have been simultaneously quantified in soil using a tripartite bacterial reporter gene system.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The metabolic carbon reporter, *P. fluorescens* 10586 pUCD607 (lux CDABE from *Vibrio fischeri*, kanr, ampr) [12], was grown in 100 ml Luria–Bertani (LB) broth at 25°C, 200 rpm in 250 ml Erlenmeyer flasks. Kanamycin was added (50 µg ml⁻¹) to the growth medium. The N- and P-starvation reporters, *P. fluorescens* DF57 N3 and *P. fluorescens* DF57 P9 [9] (both lux AB, kanr, smr, nmr) were also grown in 100 ml LB broth at 25°C, 200 rpm in Erlenmeyer flasks.

2.2. *P. fluorescens* 10586 pUCD607 starvation conditions

Cells, at maximum luminescence per cell (100 ml in LB at late-exponential phase), were harvested by centrifugation (5 min, 3700 × g), the supernatant discarded and the cells washed once in an equal volume of C-free M9 medium (Difco). This process was repeated and the cell suspension was shaken in C-free M9 for 2 h at 25°C, 200 rpm. Kanamycin was added to all media at 50 µg ml⁻¹. The activity of the cells during the starvation period was monitored by removing samples at the start and end of this period for dilution plate counts of colony forming units.

2.3. *P. fluorescens* DF57 N3 and *P. fluorescens* DF57 P9 starvation conditions

Cells, at maximum luminescence per cell (100 ml in LB at mid-exponential phase), were harvested by centrifugation (5 min, 3700 × g), the supernatant decanted and the cells resuspended in an equal volume N-free (for *P. fluorescens* DF57 N3) or P-free (for *P. fluorescens* DF57 P9) Davis Minimal Media (DMM; Difco). The cell suspension was then shaken (200 rpm) for 2 h at 25°C. The activity of the cells during the starvation period was monitored as above.

2.4. C, N and P substrates

Reference solutions were prepared in sterile distilled water. The C-source was 10 mM glucose-C prepared in distilled water and filter-sterilised by passage through a 0.2 µm filter (Nalgene). The N- and P-sources were ammonium nitrate (NH₄NO₃) and mono-sodium phosphate (NaH₂PO₄) prepared to 5 mM N and 0.5 mM P, respectively. A similar set of soil water solutions was prepared by adding glucose and N and P salts to filter-sterilised (0.2 µm filter) soil water. Ten-times serial dilutions into either sterile distilled water or soil water were prepared.

2.5. Soil characteristics

The soil used was a freely draining, agricultural loamy sand (Boyndie association/Corby Series, Scotland, UK) with a sand:silt:clay ratio of approximately 80:15:5. The soil characteristics are as follows; pH (H₂O) 5.49; total C, 1.85%; total N, 0.12%; cation exchange capacity, 2.11 cmol kg⁻¹. The soil was collected fresh for the experiments and used immediately.

2.6. Chemical analysis

The soil solution was extracted from equilibrated soils (3 days at ca 120% gravimetric water content) with rhizon samplers (Rhizosphere Research Products, Wageningen, The Netherlands). The collected solution was filter-sterilised by passage through a 0.2 µm filter and the nitrate-N, ammonium-N and phosphate-P concentrations determined colorimetrically by FIAstar flow injection analysis using standard EPA methods [13]. Total organic carbon was measured by UV oxidation and IR gas analysis using a Labtoc analyser (Pollution and Process Monitoring, Sevenoaks, UK). The pH (H₂O) of the soil solution was 7.0. Due to sample size constraints, the chemical analyses (flame ionisation analysis (FIA) and total organic carbon (TOC)) were performed on single samples.

2.7. Bioassay protocol

Aliquots (100 µl) of the reference and soil solutions (Tables 1 and 2) were added to 900 µl aliquots of the starved cells in luminometer cuvettes. 5 µl of 5% n-decyl

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Table 1
Bioluminescence response of *P. fluorescens* 10586 pUCD607 (late-exponential phase cells starved in C-free M9 for 2 h) to a 30 min exposure to varying concentrations of C, N and P either in distilled water (reference values) or soil water at room temperature

<table>
<thead>
<tr>
<th></th>
<th>Reference values</th>
<th>Soil water values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expected C (µg ml⁻¹)</strong></td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>Observed C (µg ml⁻¹)</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Percentage incl. bioluminescence over control</td>
<td>24</td>
<td>28</td>
</tr>
<tr>
<td>± st.dev.</td>
<td>564</td>
<td>335</td>
</tr>
</tbody>
</table>

No significant differences were found between reference and soil water values, n=3.
aldehyde (in 70% ethanol) was immediately added to the cuvettes as the luciferase substrate for the N- and P-starvation cultures. The mixtures were promptly vortexed for several seconds to ensure adequate aeration and mixing. Light output was recorded. All assays were carried out in triplicate.

2.8. Luminometry

Cuvettes containing the samples were placed in a Bio-Orbit 1251 (Labtech International, Uckfield, UK) with Multiuse® software. Light output from the samples was integrated over a 10 s period and the means of triplicate samples recorded after 30 min incubation at room temperature for the C-reporter cultures and after 120 min incubation at room temperature for the N- and P-starvation cultures. Luminescence was expressed as relative light units (RLU) with 1 RLU being equivalent to 1 mV per 10 s.

2.9. Statistical analyses

All treatments were replicated three times and the results expressed as means ± standard deviation. Student’s t-tests and linear regression (Excel 97 for Windows®) were performed where appropriate to test for statistical differences. Significant differences are reported at the P ≤ 0.05 level.

3. Results

3.1. Soil solution characterisation

TOC was determined for both distilled water (reference) and soil water (Table 1). In the distilled water controls, the C concentration was equivalent to the expected values whereas the soil water values contained up to three times C of the expected values. The ammonium, nitrate and phosphorus concentrations were determined by FIA in both distilled water (reference values) and in soil water (Table 2). In the distilled water controls, the ammonium and nitrate ratios were approximately 1:1, as expected. In soil solution, the ammonium and nitrate ratios were not 1:1. The 0.05 and 0.50 mM N applications were associated with negligible ammonium concentrations. At the highest N application, the ammonium concentrations were similar to those of the distilled water treatments. Soil water nitrate levels were also affected, with increases in the 0.0 and 0.05 mM N treatments compared with the distilled water controls. The 0.50 treatment, however, contained roughly a tenth of the expected nitrate. The highest nitrate-N treatment concentration had similar values in both the control treatments and the soil water. The total N concentrations, therefore, were different between the distilled water controls and the soil solutions. Total P concentrations were also different, although these differences were less pronounced.

3.2. Carbon reporting

The percentage increase in bioluminescence over zero-C controls in both the reference treatments and soil water treatments showed increases with increasing availability of C (Table 1) at the higher concentrations. Student’s t-tests between the reference values and soil water values returned no significant differences. The lowest application of C (1.6 µg ml⁻¹), however, did not elicit a significant response over the controls.

3.3. N and P reporting

Both P. fluorescens DF57 N3 and P. fluorescens DF57 P9 expressed decreased light output with increasing concentrations of N or P, respectively. Both the reference values and the soil water values for the N-starvation reporter were in close agreement (Fig. 1), although the lower two soil water treatments were not significantly different from (Student’s t-test) each other despite the nearly 10-fold increase in total N concentration (Table 2).

The P-starvation reporter showed a strongly linear decline in light output with increasing P availability (Fig. 2) (regression of RLU on all P data: \( R^2 = 0.99, F_{(1,11)} = 76.69, P < 0.05 \); regression of RLU on soil water values P data: \( R^2 = 0.81, F_{(1,11)} = 87.81, P < 0.05 \). Close agreement between the reference values and soil water values was observed and this relationship was par-

<table>
<thead>
<tr>
<th>N, P added, final molarity (mM) (P in brackets)</th>
<th>NH₄</th>
<th>NO₃</th>
<th>Total N</th>
<th>Total P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference values</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00 (0.000)</td>
<td>0.002</td>
<td>0.002</td>
<td>0.004</td>
<td>0.001</td>
</tr>
<tr>
<td>0.05 (0.005)</td>
<td>0.027</td>
<td>0.024</td>
<td>0.051</td>
<td>0.006</td>
</tr>
<tr>
<td>0.50 (0.050)</td>
<td>0.260</td>
<td>0.265</td>
<td>0.526</td>
<td>0.052</td>
</tr>
<tr>
<td>5.00 (0.500)</td>
<td>2.655</td>
<td>2.854</td>
<td>5.509</td>
<td>0.561</td>
</tr>
<tr>
<td>Soil water values</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00 (0.000)</td>
<td>0.000</td>
<td>0.018</td>
<td>0.018</td>
<td>0.007</td>
</tr>
<tr>
<td>0.05 (0.005)</td>
<td>0.001</td>
<td>0.044</td>
<td>0.045</td>
<td>0.012</td>
</tr>
<tr>
<td>0.50 (0.050)</td>
<td>0.007</td>
<td>0.305</td>
<td>0.311</td>
<td>0.061</td>
</tr>
<tr>
<td>5.00 (0.500)</td>
<td>2.726</td>
<td>2.842</td>
<td>5.568</td>
<td>0.561</td>
</tr>
</tbody>
</table>
particularly strong at the higher end of the scale, matching the chemical data (Table 2) for P concentration.

4. Discussion

A soil solution was spiked with C, N and P to provide a realistic approximation of a rhizosphere solution (C, N, P levels determined from a previous experiment, data not shown). A dilution series of the C, N, P spike, to three orders of magnitude, ensured the response range would include a lowest detection limit as well as biologically relevant, high nutrient concentrations. This is analogous to three sampling points moving from the rhizoplane distally to the bulk soil.

The lux-marked tripartite of _P. fluorescens_ reporter strains showed a suite of characteristic responses to C (glucose), N (ammonium nitrate) and P (sodium phosphate). While other carbon forms are found in the rhizosphere, glucose forms the major component of root-carbon flow from young, rapidly growing cereals [8] and so was an appropriate choice as a carbon spike. The light response of _P. fluorescens_ 10586 pUCD607 to C (glucose) was directly proportional, although not linear, to the concentration of substrate. This is in accordance with previous findings [8]. As the lowest concentration of glucose added did not elicit a significant response in either the reference values or soil water values, it can be assumed that the lowest detection limit of _P. fluorescens_ 10586 pUCD607 falls between this and the next lowest concentration (i.e. between 3 and 10 µg ml\(^{-1}\)). At the highest concentration (100–120 µg ml\(^{-1}\)), the percentage increase in bioluminescence was in direct agreement with other authors’ findings and corresponds to the end of the linear phase of a Michaelis–Menten first-order kinetic curve [8].

Here, the in vitro and in vivo responses of _P. fluorescens_ 10586 pUCD607 to C were similar and thus allowed a quantitative assay of a given medium.

The differential response of the N-starvation reporter, _P. fluorescens_ DF57 N3, may indicate sensitivity to N source (Fig. 1). As the ammonium in the soil water was no longer available at the two lower N additions (Table 2), the low-level response of _P. fluorescens_ DF57 N3 may well have been driven primarily by ammonium. The mechanism(s) of ammonium removal was not investigated. Roca and Olsen [14] report that while the DF57 N3 strain responded to both ammonium and nitrate, it was more sensitive to ammonium, and was unable to respond to nitrate-N below a residual concentration of 2 mM, whereas ammonium concentrations in the same medium were reduced to below the detection limit. The similarity of biosensor response to N additions was probably due to the N pools being composed primarily of nitrate and below 2 mM (Table 2). This has implications for the use of this strain for reporting in natural soils as the bioavailable N levels were found to be different from the analytically determined chemical levels. This response may be unique to _P. fluorescens_ DF57 N3, and it would be interesting to ascertain the lowest levels of assimilable nitrate for a range of soil bacteria.

The response of _P. fluorescens_ DF57 P9 to P was similar in both the reference controls and the soil solution. Chemical analysis of the samples showed that P concentrations in the controls and soil solutions were similar, indicating that the availability of P was not affected by matrix. While this may be common for sandy soils such as the Boyndie used here, it does not reflect the conditions found in more buffered soil systems and further work on a variety of different soils would be helpful in order to fully characterise this strain.
These results show that application of the tripartite lux-reporter system offers, with appropriate sampling, the potential for multi-dimensional investigations of rhizosphere nutrient dynamics. Importantly, these studies can be carried out in situ and close to real-time. The simultaneous use of these reporter strains, backed up by chemical analyses, will allow for quantified descriptions of bioavailable C, N and P fluxes, in turn leading to a greater understanding of rhizosphere processes.

Acknowledgements

The authors wish to thank Dot McKinnon and Claire Deacon for their excellent technical assistance. We thank Rebekka Artz for her helpful comments and suggestions. This work was funded by a BBSRC grant under the BIRE initiative (Grant No. 5/BRE13626).

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