RESEARCH PAPER

Root exudation from *Hordeum vulgare* in response to localized nitrate supply

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Abstract

Root proliferation as a response to exploit zones of nutrient enrichment in soil has been demonstrated for a wide range of plant species. However, the effectiveness of this as a strategy to acquire nutrients is also dependent on interactions with the soil microbial community. Specifically, C-flow from roots modifies microbial activity and probably the balance between nutrient mineralization and immobilization processes in the rhizosphere. In this study, near-natural abundance ¹³C-labelling and gene-reporter methods were applied to determine the effects of uneven nitrate supply to roots of *Hordeum vulgare* on assimilate partitioning and root exudation. Plants were initially grown in uniform nitrate supply in split-root, sand microcosms after which one treatment continued to receive uniform supply, and the other received nitrate to one root compartment only. At the time of imposing the treatments, the CO₂ supplied to the plants was switched to a cylinder source, providing a distinct δ¹³C-signature and allowing the fate of new assimilate within the plants to be determined. The labelling approach allowed quantification of the expected preferential allocation of new C-assimilate to roots in enriched nitrate, prior to any measurable effect on whole biomass or root architecture. Biosensor (*lux*-marked *Pseudomonas fluorescens* 10586 pUCD607) bioluminescence, quantified spatially by CCD imaging, demonstrated that root exudation was significantly increased for roots in enriched nitrate. This response of root exudation, being primarily associated with root apices and concurrent with enhanced assimilate supply, strongly suggests that C-flow from roots is an integral component of the proliferation response to nitrate.

Key words: Biosensor reporting, carbon partitioning, ¹³C-labelling, *Hordeum vulgare*, nutrient patches, rhizodeposition, root exudation, root proliferation.

Introduction

Responses of root systems to a spatially uneven supply of nutrients, such as preferential growth and spatially co-ordinated regulation of uptake kinetics, are well established (Fitter, 1994, Robinson, 1994). These responses are widely held to be evolved strategies to maximize the efficiency of root foraging for nutrients in natural soils. The responses are particularly evident in experimental systems where mineral nutrients are supplied in the absence of competition from microbial populations (Drew and Saker, 1975, 1978). However, when the natural heterogeneity and complexity of soils are introduced, although root proliferation is still generally observed, the benefits for nutrient acquisition are less apparent (Hodge *et al*., 1999).

In soil there are a host of factors that affect plant nutrient acquisition. Sites of localized nutrient enrichment (hot-spots) are most commonly associated with microbial decomposition of recently incorporated organic matter (e.g. root turnover, litter-fall, and soil animal bodies). Therefore, there is a strong temporal aspect to the availability of mineral nutrients, dependent on the activity of indigenous saprotophic microbial communities. In addition, nutrient uptake occurs from rhizosphere and mycorrhizosphere soils, which are chemically, physically, and biologically distinct from that of the soil as a whole. The availability of nutrients to roots is dependent on competition for these nutrients with rhizosphere microbial communities, which are, in turn, affected by root-mediated impacts on microbial...
physiology (e.g. through root impacts on soil water potential and C-substrate availability).

The release of organic compounds from roots (rhizodeposition) is a key factor in plant–microbe interactions through strong effects on microbial activity (Lynch and Whipp, 1990) and microbial community structure (Garland, 1996; Marschner et al., 2001) in the soil immediately surrounding the roots. Consequently, rhizodeposition affects plant acquisition of nutrients from organic matter through impacts on the activity and abundance of decomposer populations. Indeed, this effect may be disproportionate to the amount of C-input (i.e. a priming effect) as the chemical homology of certain plant inputs (e.g. phenolic secondary metabolites) with components of soil organic matter (SOM) may select for organisms that produce extracellular enzymes that act on both rhizodeposits and SOM (Kuzyakov et al., 2000). However, it has also been hypothesized that utilization of C-rich rhizodeposits will promote microbial immobilization of N and other nutrients (Robinson et al., 1989). Nutrients immobilized within microbial cells have been demonstrated to be readily acquired by plant roots (Kuikman and van Veen, 1989), as a consequence of predator-driven turnover of the microbial biomass (Clarholm, 1985; Kuikman et al., 1990). As this microbial turnover is generally much more rapid than that of root tissues, it would be expected that an increasing proportion of microbially immobilized nutrient would be acquired by the plant over time.

As a consequence of these impacts of rhizodeposition on the dynamics of nutrient partitioning in the region of soil from which root uptake occurs, it is important to understand how efflux of organic C from roots is affected by directed root growth in response to heterogeneous nutrient supply. It has been established using isotopic and gene-reporter methods that root apices are disproportionately important sites of exudation, relative to the whole root (Darwent et al., 2003; Thornton et al., 2004). This is a consequence of preferential assimilate allocation to sites of active growth and the inherent leakiness of these sites due to developmental immaturity. Therefore, increased growth and root branching in regions of nutrient enrichment may be expected to be co-incident with increased specific root exudation (per unit root length and per unit soil volume), which would strongly affect microbial activity, and therefore nutrient dynamics.

In this study, near natural abundance $^{13}$C-labelling was applied to measure assimilate allocation to roots of *Hordeum vulgare* quantitatively, during the imposition of a heterogeneous nitrate supply. Following a short period of heterogeneous nitrate supply (4 d), a *lux*-marked rhizobacterial biosensor was applied to the root systems to resolve C-availability spatially as a consequence of root exudation. The aim of the study was to assess the impacts of preferential assimilate allocation in response to heterogeneous nitrate supply on root exudation, utilizing an ecologically relevant bacterial biosensor to report on root C-flow.

### Materials and methods

#### Plant material

Seeds of winter barley (*Hordeum vulgare* L. cv. Optic) were surface-sterilized in 0.5% (v/v) peracetic acid for 15 min, then repeatedly washed with sterile deionized water, as described by Paterson and Sim (1999). A portion of the seeds was spread over sterile, non-selective agar plates (malt extract agar, Oxoid, Basingstoke, UK) and allowed to germinate in the dark at 20 °C. After 7 d, the plates were assessed for microbial colony development to verify sterility of the seeds. The remaining seeds were placed aseptically into germination vessels. These vessels consisted of a nylon mesh dish (70 mm diameter) supported on the surface of 150 cm³ of sterile deionized water, enclosed within a sterile crystallizing dish. The seeds were maintained in the dark for 3 d at 20 °C, at which point roots had started to emerge from the seeds. The germination vessels were then transferred to a light-bench (16 h day length with a PAR of 450 μmol m⁻² s⁻¹, 20 °C) for a further 4 d. At this point, of the seeds that had germinated, most had 3–5 emerging roots.

#### Microcosms and growth conditions

The microcosms were based on the design of Darwent et al. (2003); the reference includes a line diagram of the system), with an additional barrier to allow separate supply of nutrients to two portions of a root system of a single *H. vulgare* plant. In brief, each microcosm was constructed from a polystyrene box (Norlab Instruments Ltd., 28×16×10 cm). The box was inverted and the lid used to support a 0.5 cm layer of sand (0.25–0.39 mm diameter, Garside Sands, Bedfordshire, UK), to which nutrients were supplied and into which roots extended. Prior to use, the sand was heated to 800 °C (8 h) in a muffle-furnace to eliminate organic C. The layer of sand was enclosed between two outer strips (0.5×0.5×0.5 cm) of Terostat™ elastomer (Teroson, NDA Engineering Equipment Ltd., Bedford, UK) and split into two compartments with a central strip of Terostat™ (same dimensions). Each compartment was 3.25 cm wide and extended to 25 cm in length. The sand was covered by acid-washed, sterile glass microscope slides, supported and secured by the elastomer strips.

The sand was saturated with nutrient solution (see below) and then allowed to drain by slanting the assembly to 30° above horizontal. Seedlings having four emerged roots were selected and single plants were placed into plastic tubes (2 cm length×2 cm diameter) secured to the top of the central elastomer strip. Two roots were then guided into each sand compartment (designated A and B) of the microcosm. The microcosms (20 in total) were transferred to a transparent Perspex tank (200×50×50 cm), housed within a controlled environment room. The photoperiod was set to a 14/10 h light/dark cycle (with 1:1 high pressure sodium to metal halide lamps, 825 μmol m⁻² s⁻¹ PAR outside the tank) and a PAR of 350 μmol m⁻² s⁻¹ within the tank. The plants experienced a constant temperature within the tank of 20 °C, which was maintained by setting the controlled environment room to 20 °C during the dark period and 6.5 °C during the light period (to counteract the radiant heat generated by the growth lamps).

#### Nitrate and $^{15}$C treatments

The Perspex tank was constructed such that it had air-inlet and -outlet vent holes. The tank also had two access ports to facilitate manipulation of the microcosm systems. These access ports were equipped with rubber gaskets, maintaining an airtight seal when closed. Initially, the plants were supplied with air from a compressed
air supply at a rate of 24.1 m⁻¹. The air supply was delivered through the tank inlet via fluorinated ethylene propylene (FEP) tubing, the supply was split and delivery was distributed via a manifold along the length of the tank. The supply rate was regulated via Brooks 580S thermal mass flow controllers, coupled to a Brooks control unit (both Flotech Solutions Ltd., Stockport, UK). The CO₂ concentrations of the inlet supply and exhaust vent were monitored with an LCA4 infrared gas analyser (The Analytical Development Co. Ltd., Hoddesdon, UK). The compressed air supply via a self-regenerating pressure swing adsorption CO₂-scrubber unit (Texol, Dundee, UK), and the CO₂-compressed air supply was equalized by manipulation of KCl and NaCl concentrations. Also during the dark period, thus minimizing impacts on tank CO₂ concentration and δ¹³C-composition.

During the first 18 d of plant growth in the Perspex tank, root compartments A and B were supplied with identical nutrient solutions (10 cm⁻³ spread evenly across the surface of each compartment). The nutrient solution contained: 2.1 mol m⁻³ CaCl₂; 0.5 mol m⁻³ KNO₃; 0.5 mol m⁻³ NaNO₃; 0.5 mol m⁻³ KCl; 0.5 mol m⁻³ NaCl; 0.75 mol m⁻³ MgSO₄; 0.5 mol m⁻³ K₂SO₄; 0.31 mol m⁻³ NaH₂PO₄; 26.0 mol m⁻³ Na₃HPO₄; 50 mmol m⁻³ H₃BO₃; 8.6 mmol m⁻³ MnSO₄; 2.0 mmol m⁻³ ZnSO₄; 1 mmol m⁻³ CuSO₄, and 10.0 mmol m⁻³ ferric citrate.

Prior to the start of the light period, 19 d after transferring the plants to the Perspex tank, four plants were removed for harvest (see below). Eight plants continued to be supplied with identical nutrient solutions to each root compartment (‘uniform’ treatment). While a further eight plants were supplied with the nutrient solution detailed above, but containing 2 mol m⁻³ NO₃ (1 mol m⁻³ KNO₃ and 1 mol m⁻³ NaNO₃) to compartment A and 0 mol m⁻³ NO₃ to compartment B (‘split’ treatment). The total ionic strength of the solutions supplied was equalized by manipulation of KCl and NaCl concentrations. Also at this time, the δ¹³C signature of the CO₂ supplied to the Perspex tank was changed (−36.1 ± 0.2‰). This was achieved by routing the compressed air supply via a self-regenerating pressure swing adsorption CO₂-scrubber unit (Texol, Dundee, UK), and the CO₂-free air was then blended with CO₂ from a commercial gas cylinder (British Oxygen Co., Worsley, UK). The CO₂ blending was controlled precisely with a separate mass flow controller unit and set to match the CO₂ concentration delivered to the tank with that of the unscrubbed compressed air. The CO₂ concentration of the inlet and vent were monitored as described (inlet 374 ± 1 µmol mol⁻¹) and the airflow rate (24.1 min⁻¹) also matched that supplied previously.

**Harvest and δ¹³C analysis**

After 22 d growth in the Perspex tank (4 d after switching nutrient and CO₂ supplies), four plants receiving a homogeneous NO₃ supply and four receiving a heterogeneous NO₃ supply were removed and harvested. The shoot material was removed at the root-shoot junction and the root system was harvested as two samples (i.e. biomass from compartments A and B). All material was immediately frozen at −80 °C and freeze-dried, prior to milling and isotopic analysis. The δ¹³C (‰) and total C content of the milled samples were determined using a TracerMAT continuous flow mass spectrometer (Finnigan MAT, Hemel Hempstead, UK). All δ¹³C results are expressed relative to the PDB (Pee-Dee Belemnite) international standard.

**Biosensor application and imaging**

The remaining eight plants (four homogeneous and four heterogeneous NO₃ supply) were used to assess root exudation through application of a bioluminescent C-biosensor. The biosensor was *Pseudomonas fluorescens* 10586 pUCD607 (lux CDABE from *Vibrio fisheri*, kan³, amp¹) originally derived by Amin-Hanjani et al. (1993). The biosensor was prepared and applied to the microcosms as described by Darwent et al. (2003). The sole modification being that the layer of sand was thinned out by repeatedly washing out the root compartments (with the nutrient solutions corresponding to the treatments applied). An even distribution of sand (2–3 mm depth) was achieved by gently shaking the microcosm system, whilst maintaining the sand layer in a horizontal plane. This increased the visibility of the roots and increased the resolution of subsequent bioluminescence imaging.

The root compartments were imaged 24 h after application of the C-starved biosensor. Images were captured with a nitrogen-cooled, charge-coupled device (camera type EEV CCD 47–10 grade 1 CCD, Pixcellent Imaging Ltd., Cambridge, UK) located within a light-tight box. Bright- and dark-field images of the root compartments were captured and stored to record root distribution and bioluminescence, respectively. Relative light units (RLU) from each pixel within the 1029×1029 CCD array were exported to a spreadsheet. The bright- and dark-field images were superimposed as separate layers in Photoshop CS (Adobe Systems Incorporated) to view the spatial distribution of bioluminescence in relation to root structure (Figs 1, 2).

The total bioluminescence from each root compartment was determined from the dark-field images after subtracting the background level detected by the system in the absence of any input signal. The background level (dark current) was determined over the same exposure time (5 min) as used for the dark-field images, but in the absence of any light source. A spreadsheet macro was used to transform the data, setting zero values for all pixels at or below the background level. The total bioluminescence was determined by summing all pixels, and specific bioluminescence was determined by dividing the total bioluminescence by the number of pixels contributing to this total.

**Fig. 1.** CCD camera image of biosensor bioluminescence in response to root exudation from *Hordeum vulgare* grown in uniform nitrate supply (1 mol m⁻³ to sides A and B) throughout the experimental period. The image is presented on a false-colour temperature-scale of bioluminescence intensity (blue < red).
Biosensor bioluminescence in response to glucose concentration, as a function of nitrate availability

The potential effects of nitrate concentration on the bioluminescence response to C-substrate was assessed by exposing the biosensor to known concentrations of glucose, at each nitrate concentration applied to *H. vulgare* in the main experiment. Opaque micro-titre plates (96 well, flat-bottom, Bibby Sterlin Ltd., Staffordshire, UK) were prepared by adding 0.1 g of sterile, C-free sand to each well. To each well, 100 μl of nutrient solution (detailed previously, containing nitrate to give final concentrations of 0, 0.5, 1, 2, 5, 10, and 20 mol m⁻³ in the titre-wells). Each combination of glucose and nitrate concentration was replicated (n=6). Biosensor bioluminescence in each well was determined using the CCD scanner following 5 h incubation at 20 °C.

Root system analysis

Root systems from plants to which the biosensor was applied were separated into biomass from compartments A and B, and stored in 50% (v/v) ethanol at 5 °C prior to analysis. Morphological parameters (total length, surface area, number of tips, and size class distributions) were determined using a Win RHIZO™ scanner (Régent Instruments Inc., Quebec, Canada) after first spreading the roots in distilled water on a glass plate.

Statistical analysis

Student t tests (Genstat 5.3, NAG Ltd., Oxford, UK) were used to test the statistical significance of differences between pairs of means (biomass, δ¹³C, and bioluminescence). Biomass data was transformed (log₁₀ function) prior to analysis to account for standard errors proportional to the means.

Results

Recent assimilate allocation in response to nitrate treatments

The natural abundance δ¹³C signature of the CO₂ in the compressed air supplied to the plants during the first 18 d of growth was −10.5±0.1‰. The δ¹³C signatures of plant biomass fractions (shoot and roots from each partition) were found not to be significantly different from each other (whole-plant mean=−29.90±0.03‰). The assimilatory discrimination against δ¹³C can be calculated as:

\[
\Delta_{\text{assimilation}} = \left( \frac{\delta_i - \delta_s}{1000 + \delta_s} \right) \times 1000
\]

where \(\delta_i\) is the δ¹³C signature of the CO₂ source and \(\delta_s\) is the δ¹³C signature of the tissue C (Farquhar et al., 1989).

Thus, under the conditions of the experiment, \(\Delta_{\text{assimilation}}\) was 20.0‰. The depletion of CO₂ in the outflow from the Perspex tank containing the plants never exceeded 3 μmol mol⁻¹ (relative to the inflow concentration). Immediately before imposition of heterogeneous nitrate supply, the root biomass was not significantly different between root sections (Side A 31.6±7.5 mg; Side B 34.9±5.7 mg; shoot 115.6±16.7 mg).

The biomass of *H. vulgare* shoot material and partitioning of total root biomass (between each root partition) at final harvest were unaffected by nutrient treatment. However, total root biomass and total plant biomass were significantly (P < 0.05) less for plants receiving uneven nitrate supply (Table 1). No significant effects of nitrate treatment on root architectural parameters were found at harvest (results not shown).

The δ¹³C signatures of all plant tissue fractions at final harvest were significantly (P < 0.05) more depleted of recent assimilate (mg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Biomass (mg)</th>
<th>δ¹³C (%o)</th>
<th>Recent assimilate (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uniform</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root side A</td>
<td>46.0±5.7</td>
<td>−36.09±0.41</td>
<td>10.26±1.49</td>
</tr>
<tr>
<td>Root side B</td>
<td>54.5±11.0</td>
<td>−35.63±0.32</td>
<td>8.82±1.74</td>
</tr>
<tr>
<td>Shoot</td>
<td>60.8±2.7</td>
<td>−36.61±0.43</td>
<td>15.61±1.35</td>
</tr>
<tr>
<td>Split</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root side A</td>
<td>43.9±2.3</td>
<td>−36.96±0.53</td>
<td>9.27±1.26</td>
</tr>
<tr>
<td>Root side B</td>
<td>37.4±3.4</td>
<td>−34.34±0.50</td>
<td>5.29±0.50</td>
</tr>
<tr>
<td>Shoot</td>
<td>59.7±4.2</td>
<td>−37.08±0.46</td>
<td>17.28±1.16</td>
</tr>
</tbody>
</table>

Table 1. Biomass partitioning, tissue δ¹³C signature and net deposition of assimilate during the nitrate treatment period (4 d) in *Hordeum vulgare*

For uniform nitrate supply sides A and B continued to receive 1 mol m⁻³ NO₃ during the treatment period, for split nitrate supply side A received 2 mol m⁻³ and side B 0 mol m⁻³ during the treatment period. Results are means of four replicates (± SE), significant differences between means are discussed in the text.

Fig. 2. CCD camera image of biosensor bioluminescence in response to root exudation from *Hordeum vulgare* grown in split nitrate supply for the final 4 d of growth (side A 2 mol m⁻³; side B 0 mol m⁻³). The image is presented on a false-colour temperature-scale of bioluminescence intensity (blue < red).
\textsuperscript{13}C than those harvested prior to switching from the atmospheric to the bottled CO\textsubscript{2} source (Table 1, cf. preswitching whole-plant mean of $-29.90 \pm 0.03\%_o$). The $\delta^{13}C$ signature of the CO\textsubscript{2} derived from the bottled CO\textsubscript{2} source was $-36.1 \pm 0.2\%_o$. Therefore, using the \textit{A} value derived above, assimilates acquired from this source would have a $\delta^{13}C$ signature of $-55.5\%_o$. The contribution of each assimilate source (i.e. derived from atmospheric or bottled CO\textsubscript{2} sources) can be derived from the following two source mixing equation:

$$P_{\text{bot}} = \left(\frac{\delta^{13}C_{\text{atm}} - \delta^{13}C_{d}}{\delta^{13}C_{\text{atm}} - \delta^{13}C_{\text{bot}}}\right)$$

where $P_{\text{bot}}$ is the proportion of tissue C derived from the bottled source, $\delta^{13}C_{\text{atm}}$ is the $\delta^{13}C$ signature of the assimilates derived from the atmospheric CO\textsubscript{2} source, $\delta^{13}C_{d}$ is the $\delta^{13}C$ signature of the tissue, and $\delta^{13}C_{\text{bot}}$ is the $\delta^{13}C$ signature of assimilates derived from the bottled source. As the C in plant tissues has only two sources:

$$P_{\text{bot}} + P_{\text{atm}} = 1$$

The contributions of recent (i.e. post CO\textsubscript{2}-switch) assimilate to each plant tissue compartment is presented in Table 1. The heterogeneous nitrate supply was found to result in uneven partitioning of current assimilate within the root system of \textit{H. vulgare}. The net deposition of current assimilate was significantly ($P < 0.05$) greater to the portion of the root system receiving elevated NO\textsubscript{3}\textsuperscript{-} supply. However, quantitatively this allocation was not significantly different to that received by the root partitions in the uniform NO\textsubscript{3}\textsuperscript{-} supply treatment. The total whole-plant net C-assimilation from the bottled source (i.e. post CO\textsubscript{2}-switch) was marginally greater ($P < 0.05$) for those plants receiving uniform NO\textsubscript{3}\textsuperscript{-} supply. The mean root-to-shoot partitioning ratio suggested that plants in non-uniform NO\textsubscript{3}\textsuperscript{-} supply allocated less current assimilate to roots, although this was not statistically significant.

\textbf{Biosensor bioluminescence in response to root exudation}

After CCD images of biosensor bioluminescence were captured, the pH of nutrient solution at points adjacent to root axes and randomly across the sand matrix, from each root compartment of each treatment, was determined to assess potentially confounding effects on biosensor activity. The measurements, obtained by spotting solution collected by Pasteur pipette directly on to pH-reactive paper (BDH, Poole, UK), confirmed that the pH of the solution was unaffected by the proximity of roots and nitrate treatment. Nitrate concentration within the supplied nutrient solution was found not to affect the bioluminescence response to glucose concentration (0 to 20 mol m\textsuperscript{-3}) of the carbon-starved biosensor (Fig. 3). The responses at each nitrate concentration also demonstrate that biosensor activity was approximately linear over the range of pixel intensities measured in response to root exudation from \textit{H. vulgare}.

\textit{Biosensor bioluminescence} captured by the CCD camera clearly indicated sites of root exudation from \textit{H. vulgare}. Overlays of light- and dark-field images of \textit{H. vulgare} root systems and biosensor bioluminescence demonstrated that pixels recording above background luminescence were associated exclusively with the presence of roots and greatest at root apices (Figs 1, 2). The visual assessment that root exudation was affected by heterogeneous nitrate supply was examined quantitatively by an analysis of pixel intensities across the arrays captured by the CCD camera.

Total bioluminescence (side A+side B) from the biosensor was reduced significantly ($P < 0.05$) by imposition of uneven nitrate supply (Table 2). In addition, uneven nitrate supply significantly ($P < 0.05$) affected the distribution of light output derived from root-associated biosensor activity. Side A, receiving elevated nitrate supply, was found to contribute 2.7 times the bioluminescence recorded for Side B (Table 2). As root biomass and root length were not affected by nutrient treatments at the time of harvest, this effect of split nitrate supply was on specific bioluminescence (i.e. RLU per unit root biomass or root length), as opposed to being related to effects of treatments on root biomass accumulation or root extension (Table 3).

\textbf{Discussion}

\textbf{Allocation of recent assimilate}

The near natural abundance steady-state $\textsuperscript{13}C$-labelling approach was possible due to the 25.6\%\textsubscript{o} difference between
the $\delta^{13}C$ signatures of the compressed air and bottled sources. The isotopic discrimination against $^{13}C$ (A) during assimilation, and apparent in the depletion of $^{13}C$ in plant tissues (relative to the CO$_2$ source), was quantified as 20.0$\%$ (equation 1). This large $\Delta$ value for assimilation is consistent with the minimal CO$_2$ depletion in the growth chambers (<3 µmol mol$^{-1}$), achieved by the high gas flow rates through them (Evans et al., 1986). Therefore, shifts in the $\delta^{13}C$ signature of plant tissues following the switch in CO$_2$ supply can be attributed to the contribution of newly assimilated C, as opposed to chamber-effects on $\Delta$ due to CO$_2$ depletion (e.g. due to increasing assimilation with increasing biomass). The maintenance of steady-state conditions (CO$_2$ concentration and enrichment) allowed sensitive and quantitative assessment of assimilate allocation, which is not possible with more commonly applied pulse-labelling techniques. In this study, the labelling approach was applied to allow quantification of the classic assimilate allocation response to heterogeneous nitrate supply to roots, prior to development of measurable biomass effects at the level of the whole root. Prior to this study, the split nitrate supply treatment was demonstrated to induce root proliferation in terms of biomass and root length (2 week treatment period, results not shown). In the preliminary experiment, shoot nitrate concentration at harvest (mean 1.23±0.09 mg NO$_3^-$ N g$^{-1}$) was not significantly affected by the split compared with the homogeneous nitrate supply, indicating that compensatory root responses (physiological and morphological) did develop under the conditions imposed.

On harvest of the current experiment (4 d after imposition of nitrate treatments and $^{13}C$-labelling), the relative abundance of $^{13}C$ in all tissues was significantly reduced, in comparison with that of plants harvested prior to switching to the $^{13}C$-depleted bottled CO$_2$ source. The tissue fraction that was altered least during the treatment period was the root compartment receiving no nitrate supply. This indicates that net deposition of recent assimilate to this root partition was significantly less than that to either the root compartment receiving enhanced nitrate supply or those receiving equal supplies in the uniform treatment. Although this very strongly suggests that gross assimilate allocation to the root compartment receiving no nitrate supply was reduced, it is important to note that, in this experiment, no account was made of respiratory losses from plant tissues. However, several studies (e.g. Sattelmacher and Thoms, 1995) have demonstrated that roots in localized nutrient enrichment have elevated specific respiration rates, associated with physiological and growth responses to the availability of the nutrient source. Indeed, it can be argued that the increased sink-strength of roots in local nutrient sources, generated by elevated respiration associated with nutrient acquisition, is an integral component of preferential assimilate allocation and growth (Marcelis, 1996). Therefore, it is highly probable that the difference in gross allocation of recent assimilate between root compartments

| Table 2. Number of active pixels (i.e. above background) and total bioluminescence captured (RLU, 5 min CCD camera exposure) for whole root systems and for each partition, as affected by 4 d nitrate supply treatment to Hordeum vulgare |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Uniform | Split | Uniform | Split | Uniform | Split | Uniform | Split | Uniform | Split | Uniform | Split |
| Number of active pixels ($\times 10^5$) | Total | Side A | Side B | Total | Side A | Side B | Total | Side A | Side B | Total | Side A | Side B |
| Uniform | 15.27±0.86 | 8.07±0.25 | 7.20±0.51 | 1.94±0.09 | 1.02±0.07 | 0.92±0.08 | a | a | a | a | a | a |
| Split | 13.47±0.92 | 7.57±0.57 | 5.90±0.42 | 1.52±0.11 | 1.11±0.09 | 0.41±0.07 |

| Table 3. Specific biosensor bioluminescence (mg root$^{-1}$, active pixel$^{-1}$) associated with whole root partitions and root tips of Hordeum vulgare, and number of sites of intense bioluminescence ($>100$ RLU), as affected by nitrate supply treatments |  |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Specific bioluminescence (RLU) | | | | | | | | Number of distinct sites identified as sites of intense exudation |
| | Pixel$^{-1}$ | (Tip pixel)$^{-1}$ | mg root$^{-1}$ ($\times 10^5$) | |
| | Side A | Side B | Side A | Side B | Side A | Side B | Side A | Side B |
| Uniform | 12.64±1.02 | 12.74±1.15 | a | 132.5±6.1 | 127.8±5.3 | a | 1.72±0.33 | 1.62±0.35 | 21.2±3.8 | 20.6±2.1 | a |
| Split | 14.66±1.13 | 6.97±0.85 | b | 152.8±7.2 | 74.4±11.3 | b | 2.50±0.60 | 1.36±0.52 | 32.8±4.6 | 10.1±1.1 | b |
in the split nitrate supply treatment was even greater than that measured for net deposition in this instance.

**Lux-reporting of root exudation**

The bacterial biosensor (*Pseudomonas fluorescens*) used in the present study is a typical copiotrophic rhizosphere organism, able to utilize a wide range of soluble organic compounds that are components of root exudation (Lemanceau et al., 1995; Lugtenberg and Dekkers, 1999; Rainey, 1999). In previous studies (Yeomans et al., 1999; Darwent et al., 2003), it has been demonstrated that the bioluminescence in response to sugars, amino acids, carboxylic acids, and complex mixtures (root tissue extracts) is proportional to available C concentrations for each compound class. However, it is important to note that, in this study, bioluminescence is a reporter of biological activity (of an ecologically relevant organism) that would be affected by components of root exudation with specific biological actions (e.g. signal molecules). A key requirement for maintenance of the relationship between bioluminescence and C-availability (particularly in the context of nutrient manipulations) is that the activity of the biosensor is only limited by the availability of C-substrate, a condition achieved in this study by C-starvation in C-free media prior to application. Potential influences of N-form on solution pH around roots were checked, but the frequent solution replacement and the buffering capacity of the phosphate salts in the nutrient solution ensured that pH variation was less than 0.2 pH units.

The impacts of nitrate supply treatments on the relative intensity and distribution of root exudation, as reported on by biosensor bioluminescence, demonstrates considerable congruence with the partitioning of current assimilate. At the whole root-system level, total biosensor activity was significantly (*P* <0.05) reduced in the split nitrate treatment (Table 2), and as with current assimilate allocation, this was the result of a reduced contribution from the root section receiving no nitrate supply. This tight correlation between assimilate supply to roots and subsequent C-flow from roots has been observed at the whole plant level. For example, in 14C pulse-labelling studies the timing of label translocation to roots and appearance in rhizosphere soil is tightly coupled (Rattray et al., 1995). In addition, Kuzyakov and Cheng (2001) demonstrated that exudate release from roots was largely dependent on current photosynthetic rate. The results of this study illustrate that this coupling of assimilate supply and exudate release is also co-ordinated at a finer scale within root systems.

It was clearly apparent that measures of specific bioluminescence (per pixel and per mg root) were strongly affected by the split nitrate supply treatment (Table 3). Root exudation was significantly reduced on the side receiving no nitrate supply, despite the biomass not being significantly different. In addition, the proportion of the root system contributing to exudation (i.e. pixel area contributing measurable biosensor bioluminescence) was reduced (Table 2). It is important to note that, unlike pulse-labelling/autoradiography approaches (McCully and Canny, 1985), biosensor reporting is not biased in only accounting for the fate of current assimilate. Thus, it is probable that the root section receiving no nitrate supply and exhibiting reduced exudation was depleted in soluble C concentrations which drive diffusive release of exudate compounds (Henry et al., 2005). This raises interesting questions as to whether, in the longer-term, roots external to a nutrient patch would senesce and constitute additional, more complex C-input to soil through root turnover. Previous work suggests that this would be dependent on plant species (e.g. ecotype in relation to soil fertility) and longevity of the patch as a source of nutrients (Eissenstat and Yanai, 1997). Additional work is required to determine whether exposure of whole root axes to differential nitrate supply (as in this study) has comparable effects on exudation as does root exploitation of smaller, discrete patches in soil.

The mean values for total and specific exudation from the root section receiving enhanced nitrate supply were each higher than the corresponding sections receiving uniform supply (Tables 2, 3). However, clear statistical significance was not apparent, except for bioluminescence associated with root tips (*P* <0.05). The specific exudation associated with root tips was substantially greater (approximately ×10) than that of the whole root in root compartments of each treatment (Table 3). This highlights the importance of root meristems as sinks for assimilate, which combined with their developmental immaturity (inherent leakiness) results in their being sites of intense exudation (Thornton et al., 2004).

Increased specific exudation from root apices in enhanced nitrate supply suggests that assimilate allocation was greater to these sites, relative to apices in depleted and uniform nitrate supply. This is consistent with the preferential allocation of assimilate as a precursor of directed growth of roots into a region of nutrient enrichment (Sattelmacher and Thoms, 1989). In addition, grain counting analysis of the CCD images (to determine the number of discrete sources of exudation from the root systems) indicated that the number of sites exhibiting markedly elevated exudation was significantly increased in the enhanced nitrate supply and reduced in the depleted supply (Table 3). This is consistent with there being a greater number of root tips and sites of lateral root emergence that are active in the elevated nitrate supply.

**Conclusion**

The results of this study demonstrate that increased root exudation accompanies initial root responses of *H. vulgare* to localized nitrate supply. Increased exudation is primarily
localized to sites of root growth (i.e. root apices), and therefore, although whole-root exudation may not be markedly affected by nutrient enrichment, the new growth within the nutrient patch will be concurrent with increased C-deposition into the patch. It is suggested that this C-flux is an important component of root proliferation responses to nutrient patches, potentially strongly affecting plant-microbial competition and the size/timing of nutrient fluxes around roots.

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