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TECHNICAL REPORT

Roots-eye view: Using microdialysis and microCT to non-destructively map root nutrient depletion and accumulation zones

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Abstract

Improvement in fertilizer use efficiency is a key aspect for achieving sustainable agriculture in order to minimize costs, greenhouse gas emissions, and pollution from nutrient run-off. To optimize root architecture for nutrient uptake and efficiency, we need to understand what the roots encounter in their environment. Traditional methods of nutrient sampling, such as salt extractions can only be done at the end of an experiment, are impractical for sampling locations precisely and give total nutrient values that can overestimate the nutrients available to the roots. In contrast, microdialysis provides a non-invasive, continuous method for sampling available nutrients in the soil. Here, for the first time, we have used microCT imaging to position microdialysis probes at known distances from the roots and then measured the available nitrate and ammonium. We found that nitrate accumulated close to roots whereas ammonium was depleted demonstrating that this combination of complementary techniques provides a unique ability to measure root-available nutrients non-destructively and in almost real time.

KEYWORDS

accumulation zones, ammonium, depletion zones, nitrate, soil nutrients

1 | INTRODUCTION

Global food security requires an increase in food production by more than 60% by 2050 and this needs to be achieved in an environmentally sustainable manner. Along with new higher yielding crop varieties, the green revolution was driven by an increasing availability of fertilizer that could be applied to those responsive new varieties, further driving up productivity (Jones et al., 2013). As a result, high rates of fertilizer application have become central to most cropping systems. However, this practice is unsustainable because nitrogen fertilizer production is energy intensive and excess soil nitrogen is lost as nitrous oxide both contributing to greenhouse gas emissions (Butterbach-Bahl, Baggs, Dannenmann, Kiese, & Zechmeister-Boltenstern, 2013; Shcherbak, Millar, & Robertson, 2014). In addition, fertilizer run-off pollutes waterways via eutrophication creating further environmental problems (Dungait et al., 2012).

Roots are responsible for nutrient and water uptake and consequently are pivotal for crop resilience and productivity. To reduce fertilizer applications, one possible improvement is to increase the nutrient foraging ability of crop roots to maximize soil resource use (Dungait et al., 2012; Jones et al., 2013). In order to achieve this, we need to understand the physiology of intact roots within their soil environment.

Imaging techniques such as microCT (Lafond, Han, & Dutilleul, 2015; Mooney, Pridmore, Helliwell, & Bennett, 2012) and nuclear magnetic resonance imaging (Bottomley, Rogers, & Foster, 1986; Metzner, van Dusschoten, Bühler, Schurr, & Jahnke, 2014) are now available for tracking root growth in different soil conditions, which has opened up the "hidden half" of the plant. However, although these methods provide valuable insight into the physical structures in the

Abbreviations:: microCT:, X-ray micro computed tomography; KCl:, potassium chloride

soil, changes in local chemistry are a different matter. It has been much more challenging to match the root architecture to positional information about nutrient availability. Understanding the nutrient levels at known distances from roots helps us determine how fast mobile ions are drawn towards the roots via mass flow and which ions are less mobile and being depleted and potentially limiting.

2 | CURRENT METHODS FOR SAMPLING THE NUTRIENT ENVIRONMENT

Previous methods for studying depletion zones have exclusively depended on destructive methods of analysis. One example uses two soil chambers separated by a mesh preventing root penetration from the upper chamber (Gahoonia, Nielsen, Joshi, & Jahoor, 2001; Kuchenbuch & Jungk, 1982). The lower chamber is then frozen and sectioned with a microtome to create slices at increasing distances from the mesh. In this set-up, the root-soil interface is considered the mesh, and to ensure this is an accurate representation, a high density of seedlings is grown in the upper chamber. The soil slices are then analysed with extraction methods (such as $NaHCO_3$ for phosphate) to determine how much of the nutrient of interest is present (Gahoonia et al., 2001). This two-chamber method has been used to measure phosphate (Gahoonia et al., 2001) and potassium (Kuchenbuch & Jungk, 1982) depletion zones. In addition to the destructive nature of the method and the high density of seedlings required, another disadvantage is that the first slice also includes all the root hairs, resulting in a high level of nutrients in the closest slices (Kuchenbuch & Jungk, 1982).

A second method previously used to measure depletion/accumulation zones of nutrients around roots involved seedlings grown in radiolabelled soil (Hübel & Beck, 1993). This required a chamber containing three layers of soil. The middle layer of soil contained ^{33}P labelled inorganic phosphate that was sandwiched between upper and lower layers containing nonlabelled soil. The roots grew through the labelled layer, taking up the ^{33}P and resulting in patches of lower radiation. To quantify these regions of lower radiation, the chambers are frozen, sectioned, and imaged using X-ray film scanned with a densitometer (Hübel & Beck, 1993). Using this method, Hübel and Beck (1993) found two different regions: a region outside the root hair zone where phosphate was depleted whereas the zone containing root hairs accumulated phosphate.

Both of the methods described above involve destructive harvesting of both plants and soil. Radiolabelled compounds are not possible for every ion, and the production of contaminated waste means experiments need to be kept to small volumes. In contrast to these disadvantages, we present here a novel combination of technologies (microCT and microdialysis, explained below) that allows us to precisely place the probes adjacent to roots, is non-destructive, and can be repeated as many times as required, regardless of soil volume.

3 | INTRODUCTION TO MICRODIALYSIS

Microdialysis has been used extensively in neurobiology (Bungay, Morrison, & Dedrick, 1990; Saylor & Lunte, 2015). Recently, the technique has been adopted for monitoring nutrients in the soil (Figure 1a) because ions can pass across the semipermeable membrane at the end of the probe (Figure 1b) and into a sample collector while remaining sterile and without breakdown by microbes or enzymes, which are excluded by the small aperture size of the probes (20 kDa). This means organic forms of nitrogen (such as amino acids) remain intact (Brackin et al., 2015; Inselsbacher, Öhlund, Jämtgård, Huss-Danell, & Näsholm, 2011). The method is minimally invasive and removes only dissolved compounds but not soil water and provides information about available nutrient concentration, mobility, and turnover rates on site, and does so in near real time. The probes can be used at any depth, and in soil, the method has proven able to measure trace metals, chloride, nitrogen, and low molecular-weight organic anions (Cocovi-Solberg, Rosende, & Miro, 2014; Inselsbacher, Oyewole, & Nasholm, 2014; Miro & Frenzel, 2003; Miro, Jimoh, & Frenzel, 2005; Rosende, Magalhaes, Segundo, & Miro, 2013).

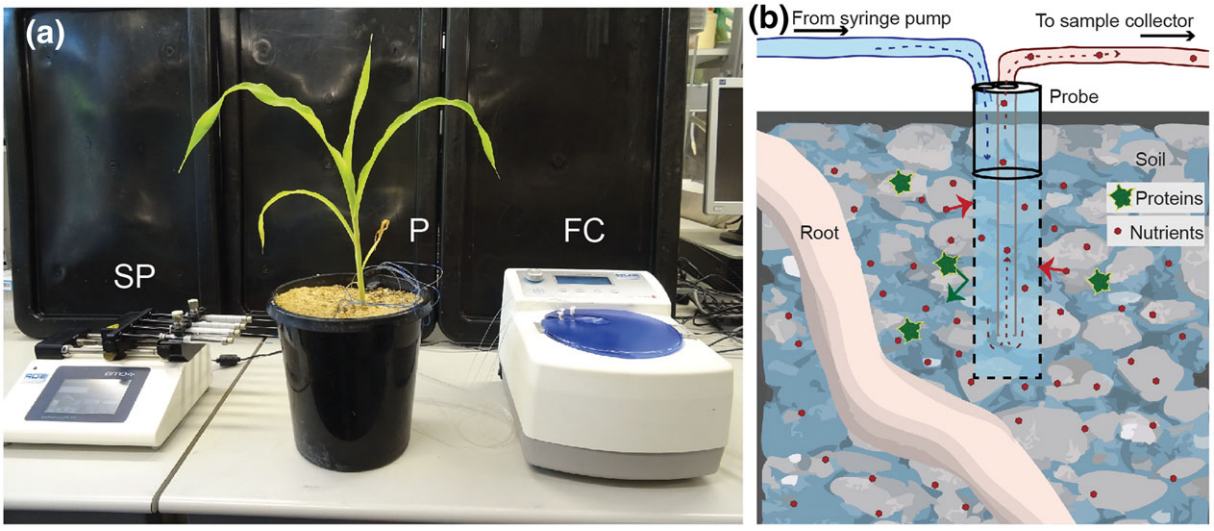

FIGURE 1 (a) Microdialysis setup and (b) probe function. The syringe pump (SP) pushes water through the tubing to the probes (P) in the soil and then onto vials within the fraction collector (FC). (b) The probes exchange ions with the soil via a semipermeable membrane so when the syringes contain pure water, the ions move into the probes and are collected in the fraction collector

TABLE 1 Soil nutrient measurement techniques and their limitations

Method	Description	Limitations
Suction cups/ lysimeters	Remove soil water through vacuum.	Requires plentiful soil water, disrupts soil environment, biased towards largest water-filled pores, can be a small sample size.
Potassium chloride extraction	Nutrients extracted using potassium chloride from homogenized soil samples	Soil structure removed, limited on how many sampling time points, lag between collection and analysis that means there can be conversion between nutrient forms, different salt extraction methods vary in results due to differences in adsorption of positively charged amino acids to negatively charged soil particles and organic matter.
Microdialysis	Semipermeable membrane	Small sample size, not widely accepted as yet.

Traditional methods for handling soil microheterogeneity (e.g., lysimeters or potassium chloride extractions) are notably limited (Table 1). In particular, it is unknown how well the measured nutrient levels match root-available nutrients. For example, suction cups (sometimes called lysimeters) are biased towards the largest water-filled pores, and salt extraction results vary depending on adsorption to soil particles. Avoiding these limitations, microdialysis samples freely available nutrients and ions in the soil at the root scale giving a more realistic roots-eye view of the soil environment. Microdialysis can be used repeatedly over time, preserves nutrients in their "native" form, and can be used to measure differences in organic or inorganic nutrient forms because microbial activity is blocked at the membrane (Inselsbacher et al., 2011; Miro & Frenzel, 2005).

Microdialysis is a technique growing in popularity for continuous and non-destructive soil nutrient sampling. Here, for the first time, we combine two cutting edge techniques—microdialysis and microCT—to obtain a "roots-eye-view" of nitrate and ammonium in the rhizosphere.

4 | MATERIALS AND METHODS

In order to get a roots-eye view of the soil environment, we used microCT to position microdialysis probes adjacent to plant roots (Figure 2a,b). To achieve this, maize seeds were germinated in columns (75 mm ∅ × 200 mm height) filled with sieved (<2 mm) sandy loam soil and grown for 19 days prior to scanning. Plants were watered from the base of the column every day, and no fertilizer was applied to these plants. Two pieces of copper strip were placed at different heights and at 90 degrees around the circumference of the columns (Figure 2b black arrows), and then the columns were initially scanned for 8 min (full scan parameters are listed below).

The copper strips on the columns in the fast scan appear as bright regions of high density in the microCT images and can be used as reference points to calculate the spatial position of the roots. Once the root positions were known relative to our copper references on each of 12 pots, we positioned two microdialysis probes (10 mm long probes with a 20 kDa cut-off-CMA 20, CMA Microdialysis AB, Solna, Sweden) parallel and as close as possible to roots of the plant and then positioned two probes further from roots in the bulk soil as controls. The two probes that we intended for close to roots we have called "adjacent," and the two probes we intended for further from roots we have called "background" for the purpose of demonstrating the repeatability of our positioning method. In the analyses, we have considered all points as a continuum based on the distances we measured from the nearest root. We used four probes per pot to increase the number of sample points given our 12 pots. Even though it is possible that the low resolution scans may have missed fine roots, our distance calculations were conducted on the high resolution scans, and because most of our background probes were further from roots than the adjacent probes, we are confident that few roots were missed in our initial scans. The membrane region of the probe is only the lower 1 cm of the probe and this is the region of the probe that we position as close and as parallel as possible to roots. When calculating the actual distance between the membrane section of the probe and the roots, we have reported distance to the nearest root from the midpoint of the 1 cm membrane. A total of 12 plants were analysed in this way (all scanned on the same day) resulting in 24 probes adjacent to roots and 24 probes in the background soil matrix. The distance between probes positioned in the background matrix and nearest root were also measured. Cannula introducers were used to create small holes in the soil matrix into which the probes were placed to avoid damaging the semipermeable membrane (Figure 2c). Although using the quick microCT scans enables the process to be iterative, we found we could accurately position the probes with a single quick scan.

All columns were scanned at a resolution of 50 μm using a v|tome|x m 240 kV X-ray microCT system (GE Sensing and Inspection Technologies GmbH, Wunstrof, Germany) at The Hounsfield Facility, University of Nottingham. The X-ray source settings were 180 kV and 200 μA with the application of a 0.1 mm copper filter to the exit window to reduce detector saturation. Two scans were required to obtain the full column length (which were digitally combined following data reconstruction). Each scan acquired 2,160 projection images over a 360° rotation of the sample using a detector exposure time of 250 ms, integrated over three averaged images resulting in a total scan time of 75 min for both scans. The preliminary probe positioning scans were collected in "fast scan" mode where no projection image integration is applied thus reducing the scan time. Reconstructed scans were analysed using VGStudioMax v2.2 (Volume Graphics GmbH, Heidelberg, Germany) to separately segment the root system and the microdialysis probes (Figure 2d) and to calculate the distance between probes and the nearest root.

Immediately after completing the full microCT scan, the probes were connected to the microdialysis pump (CMA 4004, CMA Microdialysis AB, Solna, Sweden) and collection vials (Figure 2e) to sample the soil nutrients with a flow rate of 5 μl/min for 1 hr, previously determined to be suitable for soil *N* sampling by Inselsbacher et al. (2011). In the collected samples, nitrate was measured using vanadium chloride and the Griess reaction (Miranda, Espey, & Wink, 2001) whereas ammonium was measured using the phenol-hypochlorite method (Harwood & Kühn, 1970) both adapted for 96 well plates. The colour intensities for the samples were measured at 570 nm for nitrate and at 630 nm for ammonium (MRX II plate reader, Dynex Technologies). The amount of nitrate and ammonium in the samples were converted to a flux rate (amount of *N* arriving per unit surface area of the probe per hour [nmol $N{\cdot}cm^{-2}{\cdot}hr^{-1}$]). To analyse relationships,

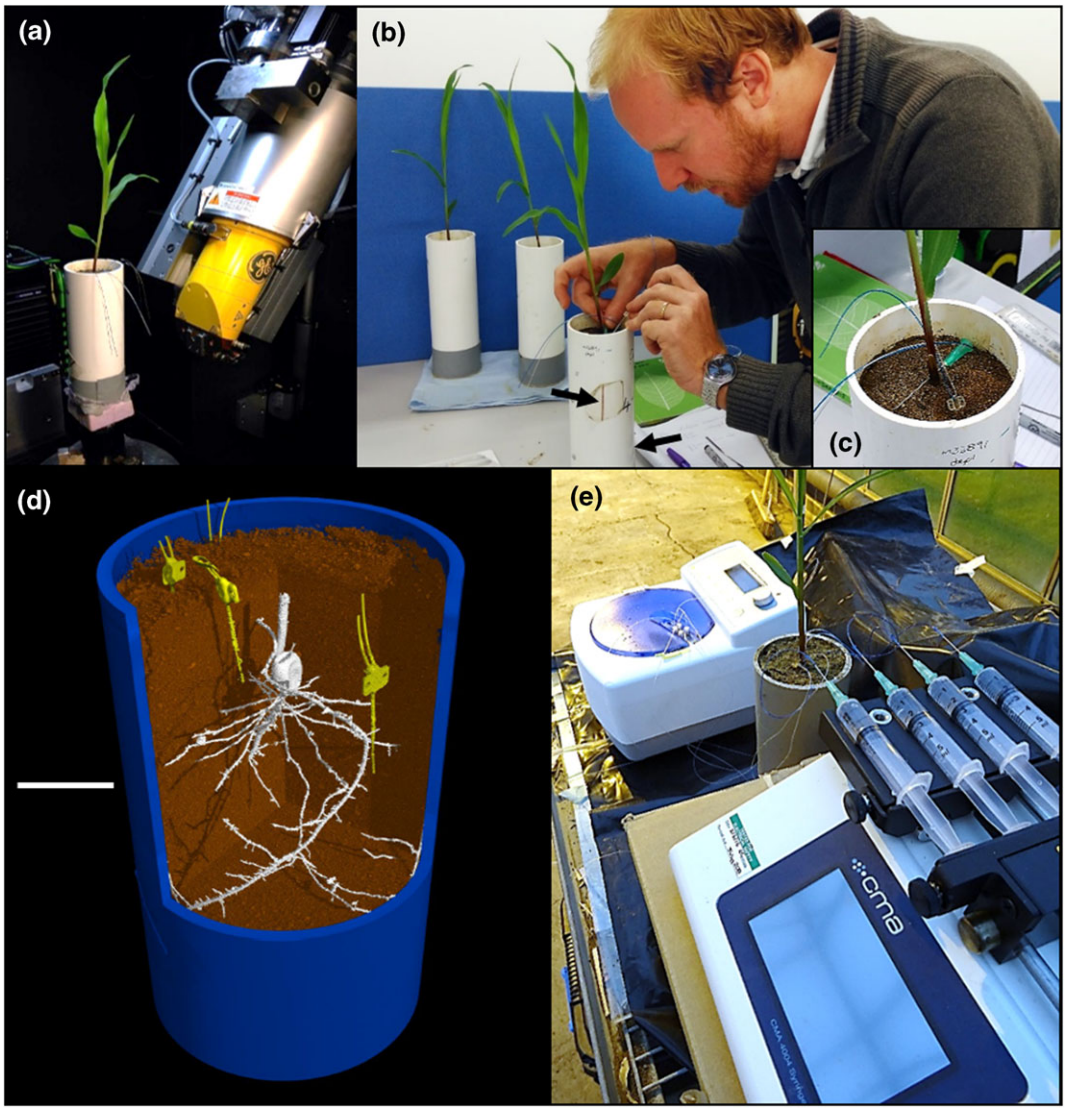

FIGURE 2 Placing microdialysis probes adjacent to roots using microCT. (a) Plants in columns were initially scanned and (black arrows in b) the position of roots were calculated using copper strips that were positioned on the outside of the pots. The copper appears as bright strips in the microCT images so can be used to measure distances both in the images and on the physical pots. (b and c) Probes were inserted either adjacent to roots or in the bulk soil and the columns were then scanned again. This process can be iterative as required. (d) The roots and probes were then separately segmented using VGStudioMax so that distances between probes and roots could be measured (scale bar = 25 mm at that depth). (e) Although images were being segmented, the microdialysis was run to collect soil nutrient samples that were then analysed using traditional colorimetric methods. (d and e) microCT imaging and microdialysis sampling can be conducted repeatedly over time to monitor changes over time

we used a non-parametric Spearman correlation test because the data were non-normally distributed. We tested nitrate and ammonium against distance from the roots and also compared nitrate to ammonium levels. The detection limit for ammonium is 0.2 nmol $N{\cdot}cm^{-2}{\cdot}hr^{-1}$ and for nitrate 0.8 nmol $N{\cdot}cm^{-2}{\cdot}hr^{-1}$. Where values fall below the detection limit, we have treated them as zero.

Figure 3a highlights that (a) some probes were placed adjacent to roots whereas (b) others were placed in the bulk soil. The microCT data can be digitally magnified (Figure 3b) to accurately calculate the distance between roots and probes (using the distance from the middle of the active section of the probe to the nearest root) and to gain information about the soil structure adjacent to the probes by measuring soil porosity, pore size, and pore connectivity (Figure 3c,d; Supporting Information Movie 1 and 2)

5 | RESULTS AND DISCUSSION

The flux rate of nitrate was higher in probes placed closest to roots (Figure 4a), whereas being lower in probes placed further from the roots (Spearman r_s = −0.431, p = .002). In contrast, ammonium was present in much lower concentrations in the soil and was lower in the probes closest to the roots compared to distances further away (Figure 4b; Spearman r_s = 0.421, p = .003). Because the samples are paired, we also tested the relationship between nitrate and ammonium levels that were inversely correlated (Spearman r_s = −0.292, p = .044) although this relationship is less than the relationship with distance. To show the relationship between distance and each of nitrate and ammonium in another way in Figure 5, we plotted the same data as the mean of all the points closer than 5 mm from the roots compared to the mean

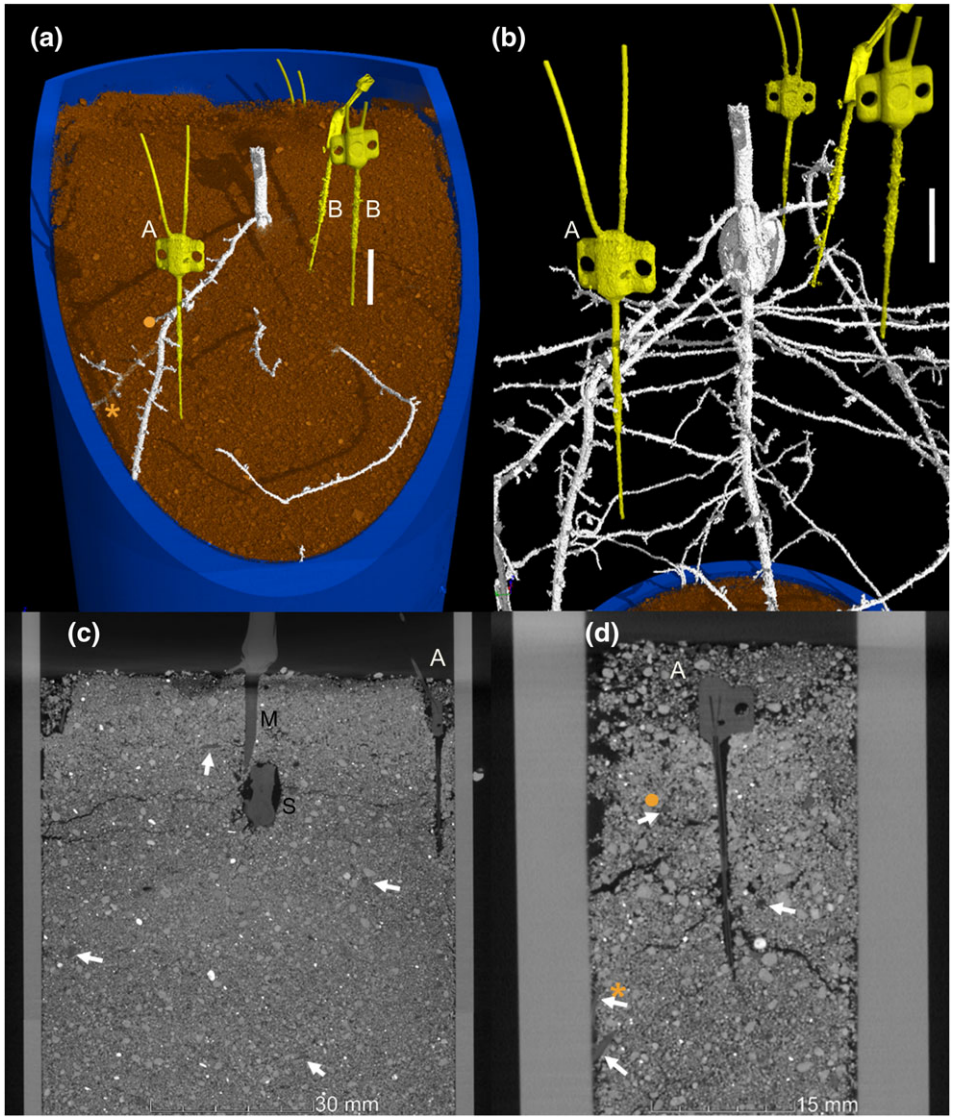

FIGURE 3 MicroCT images showing probes adjacent to roots within the soil matrix. (a) MicroCT image of microdialysis probes within the soil environment showing a probe in the foreground adjacent to a root and two probes in the midground placed away from roots in the bulk soil. The scale bar placed adjacent to Probe (B) marks 1 cm and also the active region of the probe. (b) All four probes are visible with soil removed. Scale bar = 1 cm. (c and d) Probe (A) is shown within the soil structure. (c) The seed (s) and mesocotyl (m) are visible in the centre of the image whereas Probe (A) is to the far right. (c) This view was then rotated by 90 degrees to show the image in (d). (a and d) Orange asterisk and orange dots mark points that correspond across both panels. (c and d) White arrows show roots within the soil

of all the points further than 5 mm from the roots. For nitrate, the mean of the points <5 mm is significantly higher than the mean for the points >5 mm from the roots (Figure 5a; $p < .05$) whereas for ammonium, the reverse is true (Figure 5b). This is likely to be a reflection of the high mobility of nitrate in soil (Owen & Jones, 2001). Transpiration leads to mass flow pull of negatively charged nitrate towards the roots whereas positively charged ammonium is 10 times less mobile (Owen & Jones, 2001). If soil *N* fluxes exceed root *N* uptake capacity, as has previously been observed in fertilized agricultural systems (Brackin et al., 2015), accumulation zones are a logical outcome. These are likely to be ephemeral and be depleted over time by continual uptake from the plant. In this case, it appears likely that nitrate fluxes arriving at the root surface exceed the rate of uptake, whereas ammonium fluxes do not.

Another process that may influence the local levels of nitrate and ammonium include enhanced nitrification in the rhizosphere (e.g., Li, Fan, & Shen, 2008). Although possible, the much greater increase in nitrate (which is present in very high levels) compared to the relatively modest decrease in ammonium (which is present in small levels) makes this hypothesis seem unlikely in this case. Furthermore, previous studies indicate that nitrification is decreased in the rhizosphere (except under anoxic conditions) due to decreased ammonium availability (Herman, Johnson, Jaeger, Schwartz, & Firestone, 2006; Koranda et al., 2011). Future studies could use our new technique to investigate this question in more detail.

Also visible in Figure 4a,b is the consistency with which we were able to position the probes adjacent to the roots. The black dots are probes that we deliberately placed adjacent to roots whereas the open circles are probes that we deliberately placed in the background soil matrix. There are still two probes that were further away than some of the background probes; however, they were still within 1 cm of a root.

This combination of microCT with microdialysis is unique in allowing us to measure the root architecture in three dimensions at

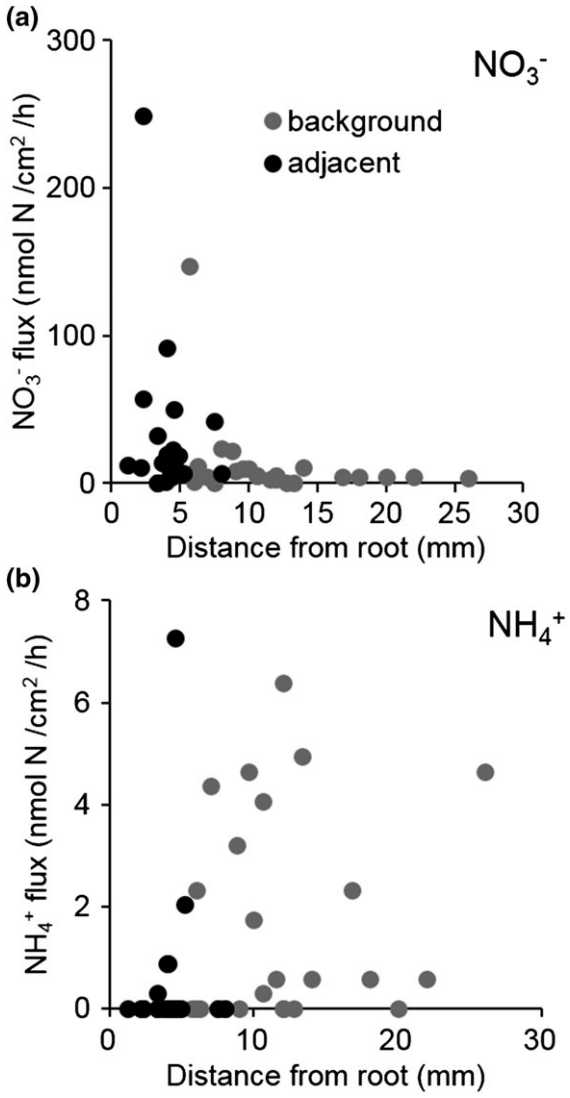

FIGURE 4 (a) Accumulation zone of nitrate and depletion zones of ammonium (b) measured as flux rate across the microdialysis membrane. Each dot represents an individual probe. Black dots are samples from probes placed deliberately close to roots whereas grey dots are from probes placed further away from roots in the bulk soil. Using a Spearman correlation, nitrate increases at positions closer to the roots (Spearman r_s = −0.431, p = .002) whereas ammonium decreases in the region immediately adjacent to roots (Spearman r_s = 0.421, p = .003, n = 48)

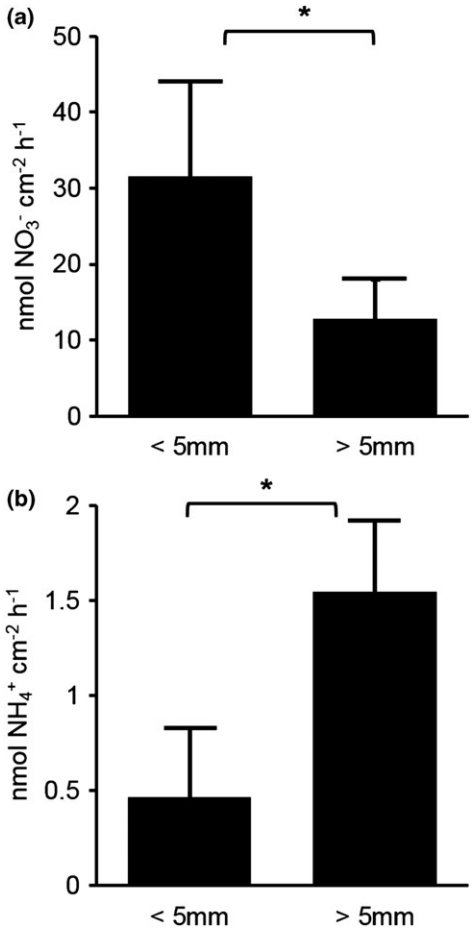

FIGURE 5 (a) Accumulation of nitrate and (b) depletion of ammonium in the 5 mm adjacent to roots measured as flux rate across the microdialysis membrane. Means are presented (with standard error bars) for points sampled closer than 5 mm (<5 mm) or further than 5 mm from the roots (same raw data as Figure 4). Asterisk represents significant differences between means at the 0.05 level (Student's t-test, n = 19) for <5 mm and (n = 27 for >5 mm)

a resolution of 50 μm while also sampling soil nutrients at known positions relative to the roots. In addition, to measure the distance from probes to roots, we can use the three dimensional reconstructions to check the surface contact between soil and probes and to understand the soil structure around roots and probes that will influence the ion exchange to both. The alternative of using rhizoboxes in combination with microdialysis may also prove useful perhaps in particular combined with other two dimensional methods newly available. Zymography, for example, is a new technique demonstrated to work in two dimensional rhizoboxes that measures enzyme activities (including at different distances to roots; Spohn, Carminati, & Kuzyakov, 2013). Microdialysis could potentially be used prior or following zymography to help explain spatial variation in enzyme activity. However, this only provides two dimensional information and the water and nutrient movement may be not representative of bulk soil due to films created on the windows. Furthermore, achieving 50 μm resolution images of the soil/probe interface is not possible using rhizoboxes. Using microCT to position the microdialysis probes gives high resolution information of both the root architecture and soil matrix whereas the probes allow the matrix to be chemically mapped.

MicroCT imaging allows us to measure root architectures under low or high nutrient regimes. However, measuring the actual nutrient availability has only been possible at the end of the experiments through destructive sampling followed by potassium chloride extractions. This method has several limitations (Table 1) such as only measuring one position and time and global nutrient content and is therefore impractical for measuring nutrients immediately adjacent to roots. Microdialysis provides a unique way to continuously measure nutrient availability and by using microCT to accurately position microdialysis probes adjacent to roots, nutrients can be non-destructively sampled over time. This is in stark contrast to traditional methods using frozen columns, which are then sliced for analysis (Gahoonia et al., 2001; Hübel & Beck, 1993; Kuchenbuch & Jungk, 1982).

Using microdialysis within the three dimensionally imaged soil environment complete with the known root architecture adds a new depth of knowledge not previously possible. This means we can measure nutrients in the soil and as they move to roots—either accumulating or depleting in regions adjacent to the roots. The images and early results demonstrate the value of combining these two novel techniques for understanding nutrient physiology in the soil–plant continuum.

Future applications of this combination of methods could involve sampling of nutrient zones at known distances from roots under different water or fertilizer application regimes to determine the dynamics of accumulation/depletion zones. In addition, using these technologies in combination under conditions that change transpiration rates such as high wind, humidity, heat, or drought could provide new insights into the effect of environmental conditions on nutrient mobility.

Integration of this novel combination of techniques with existing methods for tracking nutrients in shoots, such as stable isotopes, could greatly advance our understanding of soil-to-shoot allocation of nutrients under a range of environmental conditions. This will offer the targeted selection of crop architectures that improve nutrient use efficiency while maintaining yield production for sustainable food production.

Here we have demonstrated the value of combining microCT imaging of roots in the soil with nutrient sampling with microdialysis in order to determine the roots perspective of the physical and chemical environment through which they are growing. In this study, we showed accumulation of nitrate adjacent to the roots whereas depletion of ammonium due to the differences in mobility of the two ions in the soil.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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