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Short communication

A novel approach to study composition of *in situ* produced root-derived dissolved organic matter

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ABSTRACT

Carbon (C) compounds released from plant roots comprise a significant and reactive fraction of belowground C pools. These root-derived compounds modify rhizosphere soil and play a vital role in the mobility of nutrients and contaminants within ecosystems. Due to their low concentration, fast turnover, and limited spatial distribution throughout the soil, root-derived compounds are difficult to study. This study combined a ^{13}C pulse-chase technique and 1D and 2D nuclear magnetic resonance (NMR) spectroscopy techniques to analyze root-derived compounds produced in real soil. The pulsed samples displayed distinct enrichment in aliphatic and carbohydrate-type compounds indicating that pulse-chase approaches are a viable technique for isolating root-derived from background DOM. However, multiple NMR techniques may be necessary to develop a full profile of root-derived DOM. This is the first use of combined pulse-chase-NMR methodologies to analyze *in situ* produced root-derived DOM. Such a combination is applicable to various experimental designs and/or environmental scenarios, and can provide valuable information for future rhizosphere science.

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Plants substantially alter their soil environment by releasing carbon-containing compounds from their roots. Root-derived dissolved organic matter (DOM) plays an important role in the plant life cycle; mediating microbial associations and mobilizing nutrients (Fan et al., 2001). Unfortunately, three main methodological hurdles limit accurate root-derived DOM identification. First, soils contain very low concentrations of root-derived compounds, with rhizosphere organic and amino acid concentrations 1000-fold lower than inside root cells (Jones, 1998; Phillips et al., 2006). Second, microbial utilization leads to rapid DOM turnover rates (Kuzyakov and Domanski, 2000). Third, root-derived DOM is highly spatially confined, drastically decreasing in concentration farther from the root (Kuzyakov and Domanski, 2000). Studying DOM *in situ* therefore requires methods that distinguish root- from microbially-derived compounds and analytical approaches that can manage spatially heterogeneous, complex matrices and low concentrations.

Root-derived DOM can be isolated using ^{13}C pulse-labeling with an understanding of temporal dynamics for microbial exudate consumption. Pulse-labeling artificially enriches ^{13}C in the plant atmosphere, increasing ^{13}C in above- and belowground tissues and root-derived DOM (Kuzyakov and Domanski, 2000). Once released, microbial utilization of soluble exudates can start within approximately 3 h (peaking ~36 h after release), creating a small window for collection of unaltered compounds (Kuzyakov et al., 2001). Increasing ^{13}C also enables root-derived DOM detection over unlabeled background DOM via nuclear magnetic resonance (NMR), a technique sensitive to only ^{13}C. NMR was previously used to identify exudates after root washing (Fan et al., 1997, 2001). Unfortunately, root washing can alter and underestimate exudate composition compared with isotope tracer techniques (Kuzyakov and Domanski, 2000). We aimed to develop an approach to differentiate plant- from non-plant-derived DOM, and to accurately identify the complex suite of compounds present.

We grew surface sterilized *Noccaea caerulescens* seeds in 500 g of the A_p horizon of a soil collected from Pennsylvania (Barbour series: mixed, active, mesic Fluventic Dystrudept; Rosenfeld, 2013; Soil Survey Staff, 2010) in the greenhouse for 46 days. Plants were grown without any fertilizer additions. Before planting, 2 mm holes were cut in pot walls for rhizon samplers. $^{13}C–CO_2$ pulse-labeling

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was applied for 3 h before collection of soil solution for ^{13}C determination. During labeling, plants were placed in a clear 250 mL chamber (separate chamber for each pot) and soil surfaces were covered with CO_2 impermeable putty to limit soil-atmosphere gas exchange (Fig. 1A). We scrubbed ambient CO_2 from the chamber using soda lime (to < 50 ppm), after which 99 atom% ^{13}C–CO_2 was added to bring the chamber concentration to ~450 ppm, measured via LI-6200 infrared gas analyzer (LI-COR Inc.). Chamber CO_2 was maintained throughout the pulse between 350 and 450 ppm (Phillips and Fahey, 2005).

Immediately prior to pulsing, pots were fitted with rhizon samplers (0.1 μm pore-size) (Seeberg-Elverfeldt, 2005) which extract soil water into evacuated glass tubes (Fig. 1B). Unlabeled background solution was collected before pulsing and at select times following pulse-termination. For each sample, solution was collected for 30 min and immediately frozen to limit microbial degradation. After collection, a volume of distilled water equivalent to that extracted (~8 mL) was added to the soil surface to maintain volumetric water content and ensure that soil water was not depleted. Once frozen, samples were lyophilized (resulting in ~10 mg solid sample) and stored in a desiccator to await further analysis.

Subsamples of lyophilized material (~1 mg) were analyzed for ^{13}C and total C by isotope ratio mass spectrometry (EA-IRMS; Thermo Fisher Scientific Inc.). Remaining lyophilized material was reconstituted in deuterated water (D_2O) and transferred to a 5 mm NMR probe. Upon reconstitution, some material remained insoluble, even in a volume equivalent to that extracted, and was filtered out before analysis. Future studies should explore alternative reconstitution options to improve DOM dissolution (e.g. deuterated dimethyl sulfoxide (DMSO-d_6) or dialyzing/de-salting lyophilized material), or may utilize solid-state NMR if sufficient sample size is available (Simpson, 2001). NMR analyses were carried out on a Bruker AV-III 600 MHz instrument with cryoprobe to enhance signal:noise. 1D ^{1}H and 2D heteronuclear multiple quantum correlation (HMQC) NMR was performed with solvent suppression according to Simpson (2001) and Simpson et al. (2011).

Peak ^{13}C enrichment was measured four hours after pulse-termination (Fig. 2). This is consistent with observations of grasses releasing exudates within 4 h of a ^{13}C pulse (Leake et al., 2006). Samples taken 4, 6 and 8 h following pulse-termination contained slightly greater total C (1.6 ± 0.1%) compared with samples taken between 8 and 24 h (1.1 ± 0.1%). For NMR, we selected the highest ^{13}C-enrichment with least exposure to microbial degradation (i.e. 4-hours after pulse-termination). Root-derived ^{13}C-labeled DOM produced NMR chemical shifts representing aliphatic compounds (^{13}C = 10–25 ppm, ^{1}H = 0.5–1.5 ppm) and carbohydrate compounds (^{13}C = 60–80 ppm, ^{1}H = 3.2–4.3 ppm; Fig. 3B). These results are similar to those found by Leinweber et al. (2008), where aliphatic, aromatic and carbohydrate

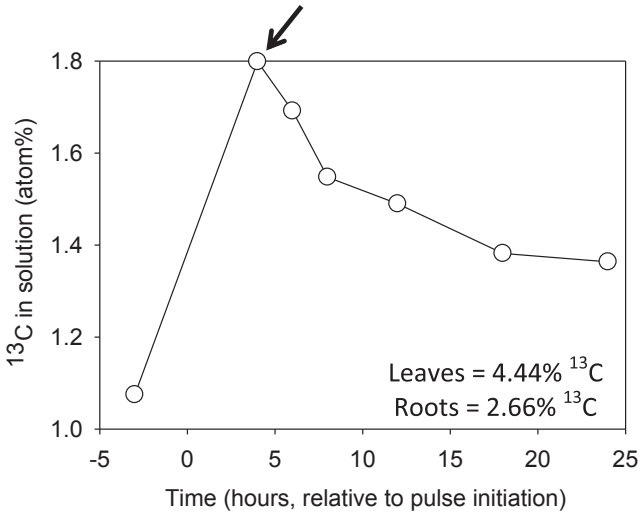

Fig. 2. ^{13}C measured in soil solution as a function of time after ^{13}C pulse-termination for a pot receiving a three-hour pulse with 99.9 atom% ^{13}C–CO_2. Measured ^{13}C in leaf and root tissue are listed inside the graph. Arrow identifies sample utilized in NMR analysis.

compounds comprised roughly 60% of maize exudates. Surprisingly, no carboxylic acid-containing compounds (^{13}C = 20–60 ppm, ^{1}H = 1.5–3.5 ppm) were detected despite their known existence in root exudates (Jones and Darrah, 1994; Jones, 1998). Several factors may have limited detection of these compounds. First, HMQC is optimized to detect direct ^{1}H–^{13}C bonds rather than long-range interactions. Using HMQC, therefore, limits detectable carboxylic acids to those containing direct ^{1}H–^{13}C–COOH linkages. For longer-range information, a separate heteronuclear multiple bond correlation (HMBC) NMR experiment can be combined with HMQC (Simpson et al., 2011). Second, organic and amino acid production may have been suppressed at our collection time. Melnitchouck et al. (2005) observed diurnal compositional differences in rhizodeposition, including increased daytime carbohydrate presence, though finer-scale temporal dynamics were not investigated. Analyzing all samples following pulse-termination may help develop a more complete root-derived DOM profile, however due to time and financial constraints compositing all post-pulse samples may be more feasible. Third, carboxylic acid adsorption to soil particles also limits their detection (van Hees et al., 2005). Finally, these compounds could be lost during sample lyophilization and/or reconstitution. This has been observed previously where amino acids were preferentially lost during drying, while carbohydrates were not affected (Fischer et al., 2007). Use of solid-state ^{13}C NMR, where no sample reconstitution is necessary, would also limit the

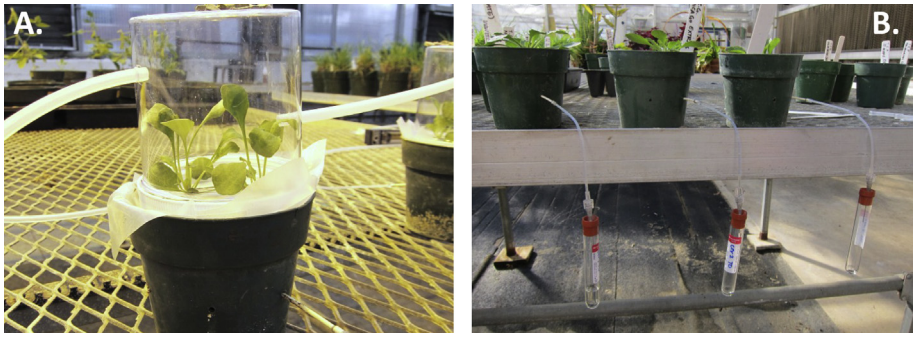

Fig. 1. Setup with 250 mL chamber for pulse-chase experiment (A), and soil solution sampling using rhizon samplers (0.1 μm pore size) and evacuated glass tubes for sample collection (B).

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