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Demographic quantification of carbon and nitrogen dynamics associated with root turnover in white clover

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Running title: Clover root C and N dynamics

Abstract

As well as capturing resources, roots lose resources during their lives. We quantified carbon (C) and nitrogen (N) losses associated with root turnover in white clover (*Trifolium repens* L.). We grew contrasting cultivars for 18 weeks in soil microcosms. Using repeated in situ observations, destructive sampling, and demographic analysis, we measured changes in C and N concentrations in dry matter of 1st- or 2nd-order (terminal) roots to derive C and N fluxes into and out of root cohorts. C and N fluxes from roots during turnover depended on cohort age and order. 90% of losses occurred from 2nd-order cohorts younger than 18 weeks. Losses were greater from roots of the larger, faster-growing cultivar Alice than from the smaller lower-yielding cultivar S184. C:N ratios of roots and lost material were similar within each order and between cultivars, but smaller in 2nd- compared with 1st-order roots. C and N losses during root turnover could be equivalent to at least 6% of above-ground dry matter production in S184 and 12% in Alice at the field scale. C and N losses associated with root turnover will have potentially significant and previously unrecognised impacts on crop productivity, resource dynamics and long-term soil fertility.

Key words: carbon, C and N loss, root turnover, growth, nutrients/nitrogen, *Trifolium repens*

INTRODUCTION

Legumes have been included in low-input agricultural rotations for millennia. They provide significant sources of forage, protein and oils, and maintain long-term soil fertility mainly through the return to the soil of nitrogen (N)-rich crop residues at the end of the growing season (Robson *et al.* 2002). A potentially important, yet poorly understood, aspect of legume N dynamics is the loss from living plants of captured N. Such losses occur during organ senescence or when plants are damaged by pests, herbivores or extreme weather, but can also occur from healthy, living structures as part of their normal metabolism.

Whatever their origin, the loss of N and other resources and their potential impacts on productivity remain hard to quantify. This is especially true for losses from roots. Analyses of leaf nutrients of many species has revealed that about half of the N in leaves is lost from the plant during senescence, and the rest is retranslocated internally; this also applies to most other nutrients (Robinson 2016). But no comparably detailed information exists for the fate of nutrients in the roots of any species.

A root imports resources as it grows. As the root ages and eventually senesces, some or all of its contents will be lost to the soil, and an important input of new material to soil organic matter, SOM (Rasmussen *et al.* 2010). The scale of that input will depend on the absolute and relative amounts of carbon (C) and N gained and lost during a root's life (Griffiths & Robinson 1992), and on the cumulative C and N fluxes through all roots during the plant's life. The latter depend, in turn, on the dynamic distributions of sizes, ages, longevities, phenologies and growth rates among the components of the root system (Eissenstat & Yanai 1997; Guo *et al.* 2007; Goebel *et al.* 2011; McCormack *et al.* 2015). Such distributions reflect the demography of the root system.

Root demographic analyses involve repeated censuses of births, deaths, survival and growth of identifiable members of a root system, information obtained non-destructively using observation chambers, mini-rhizotrons, tomography, or magnetic resonance imaging (Vetterlein & Doussan 2016). Root 'birth' is the emergence of a new root from its parent; 'death' the disappearance of a root caused by senescence,

damage or herbivory; 'survival' is the time between root birth and death; and root 'growth' is defined here as the progressive extension of a root in length and diameter. Demographic approaches provide a wealth of information about the dynamic behaviour of root structures (Gill & Jackson 2000 and references therein). But there is scant information about how that behaviour relates to associated C and N fluxes. For example, Hendrick & Pregitzer (1993) estimated annual total N, but not C, fluxes during fine-root turnover in sugar maple (*Acer saccharum*). Pregitzer *et al*. (1997) measured C and N concentrations in roots of different order in tree (*A. saccharum* and *Fraxinus americana*) and forb (*Hydrophyllum canadense* and *Viola pubescens*) species, but reported no temporal dynamics. Ruess *et al*. (2003) measured fine-root dynamics in an Alaskan black spruce (*Picea mariana*) forest, focusing on how root turnover related to *in vitro* respiration, rather than *in situ* C and N dynamics. The conclusion reached by Ruess *et al.* that "The fate of fine-root C and N following root disappearance remains a key question in the dynamics of C and element cycling", remains valid.

Our objective here was to measure C and N fluxes associated with the production, growth and death of roots within intact root systems of white clover (*Trifolium repens* L.), one of the most important legumes of temperate managed grasslands (Abberton & Marshall 2005), and to relate these to potential impacts on crop productivity. To meet these objectives we used a novel approach that combined sequential sampling and chemical analyses of root tissues along with simultaneous root demography. We aimed to answer four questions: (1) How much C and N are present in white clover roots of different age and developmental order in intact, soil-grown root systems? (2) How do those amounts of C and N change as a root system develops and as root cohorts age? (3) How much C and N is lost from a root system when a root cohort dies? (4) What are the potential implications of such losses for crop productivity?

METHODS

Experimental requirements

To estimate C and N fluxes associated with root turnover, sequential destructive sampling is required to provide material for chemical analysis of roots alongside

demographic information obtained non-destructively. To meet these conflicting needs, we used plants grown in soil rhizotrons. This allowed direct observation and detailed tracking of individual roots within whole, intact root systems during censuses, as well as destructive harvesting for the recovery of roots of known position and developmental order for C and N analysis.

Plant material and growing conditions

Two white clover (*Trifolium repens* L.) cultivars (S184 and Alice) were compared. Both have been recommended for commercial use in the UK. Alice is a fast-growing, large-leaved, high-yielding cultivar. S184 is smaller-leaved and lower yielding. Annual aboveground dry-matter yields of Alice averaged 4.0 t ha^{-1} in field trials; those of S184 were 2.5 t ha^{-1} (Gilliland 2004). On that basis, we expected that C and N losses from the higher-yielding cultivar Alice would exceed those from S184. Perennial ryegrass swards containing Alice or S184 have similar above-ground phenologies from Spring to Autumn (Gilliland 2004).

Plants were grown individually, from seed, for 18 weeks in flat glass-walled rhizotrons. Each rhizotron was 61 cm deep × 30 cm wide × 1.5 cm thick, providing a soil volume of 2.7 L at a bulk density of about 1.5 g cm^{-3}, at the upper end of the range for heavily grazed pastures (Van Haveren 1983; Davies *et al.* 1989). Further details are in Scott *et al.* (2005).

Thirty rhizotrons, 15 for each cultivar, were packed with sieved pasture soil from Craibstone, Aberdeenshire, UK (Countesswells soil association, derived from humus-iron podzol overlying granitic rock) in a 1:1 w/w mixture with sand to improve drainage. Rhizotrons were held at an angle of 20^{o} to the vertical to encourage roots to track the rear inner surface of the glass wall. Water was initially provided at 50 mL per rhizotron every second day, sufficient to maintain field capacity. Irrigation was increased to match plant demand during the experiment. All rhizotrons were maintained in the same controlled-environment chamber (Conviron, Winnipeg, Canada) with a 14 h photoperiod with a 20^{o}C/10°C day/night regime. Fluorescent and incandescent bulbs provided PAR at 500 μmol m^{-2} s^{-1}. Each rhizotron was enclosed in a light-proof baffle to shield soil and roots.

Non-destructive root censuses

During root censuses, baffles were removed and rhizotrons scanned at 300 dpi on an A3-size flatbed scanner (Epson 836XL), calibrated for compatibility with WinRHIZOTron™ software (Régent Instruments, Québec, Canada). Twenty-four bit colour images were saved as uncompressed TIFF files. If root systems extended below 40cm, the upper 40cm and lower 20cm sections of the rhizotron were scanned separately, the images joined using Adobe Photoshop™. Sequential images of the same root system were traced using the manual tracing function of WinRHIZOTron™. When a new scanned image was analysed, the previous image of the same root system was overlaid on it. All roots were numbered uniquely as discrete 'paths' such that each new root was subsequently tracked as it extended and for as long as it survived. The position, length and diameter of each root was traced and recorded. Growth rates of existing roots were also recorded, as were root births. Roots or parts of roots that disappeared between one time point and the next were classed as dead.

Non-destructive census data were obtained weekly for each rhizotron. But, to provide sufficient root material for C and N analysis (see below), the minimum possible interval for destructive sampling was three weeks. Therefore, weekly root censuses were accumulated into 3-week intervals to match that to which the C and N data were constrained.

Following a widely used developmental ordering scheme (Rose 1983; cf. topological ordering e.g., Fitter 1986), we defined roots arising from the base of the stem as 1st-order roots, and those arising from 1st-order roots as 2nd-order roots; the latter were the finest, terminal branches as no 3rd-order roots were observed. This approach allowed us to distinguish the behaviour of roots according to their age and developmental origin. By contrast, most literature references to 'fine-roots' refer to all roots < 2 mm diameter, irrespective of their age or developmental order (Wells & Eissenstat 2001; Pregitzer, 2002; Guo *et al.* 2008). Note that some developmental ordering schemes (e.g., McCormack *et al.* 2015) define all terminal fine-roots as 1st-order irrespective of their time of appearance, a convention that re-orders roots whenever a new branching level arises.

Output was generated as spreadsheets in which each row contained data for each numbered root including its order, diameter, length, start and end positions (as 2D spatial coordinates) and whether it was alive or dead. Roots produced during the first 3-week period were classified as belonging to "cohort **3**"; roots produced between 3-6 weeks belonged to "cohort **6**"; and so on for each 3-week interval. Accordingly, there were no cohorts numbered **1, 2, 4, 5,** etc. The total root length of each cohort at each census was calculated, as were changes in length between successive censuses caused by births and deaths.

Destructive harvesting

Every three weeks, five replicate rhizotrons of each cultivar were harvested. The rear glass panel was removed. Roots were excised using scalpel and tweezers, and any adhering soil removed. Excised roots were combined into batches according to their age (cohort) and order. The age and order of roots excised at the time of harvest was determined by reference to scanned images (see above). For example, a 2^{nd}-order root born between weeks 3 and 6 was designated as "2^{nd}-order, cohort **6**"; after 18 weeks plant growth, that root would therefore be between 12 and 15 weeks old. Once identified on screen, the root was then located within the rhizotron (unless the root had died), excised and batched for analysis with other roots of similar order and cohort harvested from that plant. Oven-dry weights of root batches were recorded (± 0.1 mg) after drying (60^{o}C for 24 h). Specific root lengths (λ; m g^{-1}) of each batch were derived by dividing total length by dry weight. Total C and N concentrations (% or mg g^{-1}) in the dry matter of replicate batches were determined by isotope ratio mass spectrometry for which minimum sample dry weights of 1 mg were needed. Total C and N contents per unit root length (mg m^{-1}) were calculated by dividing concentrations by λ.

Estimating C and N fluxes demographically

The data used as inputs to the root demography calculations were, for each root cohort and order, the C and N contents per unit root length as determined from destructive sampling, and the lengths of existing, new and disappeared roots at each 3-week interval estimated from censuses.

[Table 1 here]

Root C and N dynamics were calculated by adapting standard life-table analysis from population biology (Begon *et al.* 1996, Ch. 1), but using quantities of C and N, rather than numbers of individuals, in successive cohorts. This allows 'balance sheets' for C and N in root structures to be calculated as successive cohorts are produced, grow and senesce (Table 1). The logic of this scheme is that a root can pass from one age class to the next, undergoing little physiological change, its C and N remaining within its tissues. As an existing root extends, it imports C and N internally via its vascular system or, in the case of N, by uptake from the soil, to support its growth. This constitutes a gain in resources by that root, reflected as an increase in C and N contents. When a root senesces or dies, some of its gained C and N are lost, as indicated by a reduction in the cohort's C or N content from the previous census. These steps occur simultaneously. The calculations rest on several assumptions:

(1) Roots are populations of individuals grouped into cohorts produced at discrete 3-week intervals. A root assigned to cohort **3**, for example, was produced within the first 3 weeks of plant growth.
(2) Soil contamination of small root samples was negligible. Although we did not check this directly, root samples were cleaned scrupulously and our calculations suggest that even if up to one-tenth of a sample's dry weight comprised contaminating soil, C and N determinations would still have been within 2% of those reported below.
(3) C and N losses by rhizodeposition, volatilisation or exudation (Paynel *et al.* 2001; Jones *et al.* 2004; Sierra & Desfontaines 2009) were negligible relative to those attributable directly to root turnover.
(4) C lost from roots by respiration (Ruess *et al.* 2003) was ignored, but was not negligible. The relationship between root respiration and longevity is complex, involving variable rates of consumption of recently assimilated and stored C pools (Lynch *et al.* 2013). Respiration-derived C losses will add variable, but unknown, amounts to our estimates of C losses associated with the turnover of root structures.
(5) No internal retranslocation of C or N before root death occurred. Any such retranslocation would be a net gain by (or reduced loss from) the plant. The evidence suggests that for N the amounts are negligible (Gordon & Jackson 2000).

(6) Roots visible against the glass wall were representative of the entire root system (Nagel *et al*. 2013).

(7) Root herbivory was negligible. Root-feeding nematodes would have been present in the field soil that we used, but distributed equally across rhizotrons. No other major root herbivores such as leatherjackets (Tipulidae) were observed.

(8) Plants grew normally in the rhizotrons compared with the field. This is unlikely to have been strictly true, a failing that our experiment shares with others in which roots are confined to less soil than they would have access to in the field (Poorter *et al.* 2016). It would have been impossible to obtain the information we needed using any other system. A rhizotron will always be a compromise, one that nevertheless remains an essential tool in *in situ* root studies (Nagel *et al.* 2013).

Collectively, these assumptions mean that the estimated fluxes were probably *minimum* amounts of C and N transferred within root cohorts as they aged, or that were lost from the roots to the soil when they died. These, however, are the C and N fluxes associated with the growth and replacement of root structures within the root system, the specific targets of this study.

Statistical analyses

Effects of cultivar, root age and root order on variations in total C and N concentrations and on specific root length (λ) were tested using General Linear Models (GLMs) in Minitab (Minitab Inc.). λ data were ln-transformed to homogenize variances. Interactions between cultivar, root order or root age were included in the GLMs, but none were detected. 'Rhizotron' was included as a random factor. Models were refined further based on the experiment's power to detect genuine effects given the degrees of freedom and with the false discovery rate set to 0.01 (Colquhoun 2014). This indicated that the appropriate *P*-value below which the effect of a factor should be considered statistically 'significant' was $P = 0.002$, a far more rigorous criterion than the conventional $P = 0.05$.

RESULTS

Structural detail possible with rhizotron imaging

[Fig. 1 here]

The structural detail provided by sequentially scanning entire root systems of white clover is illustrated in Fig. 1. By 18 weeks, a root system of Alice typically comprised over 2000 surviving 1^{st}- and 2^{nd}-order roots, representing a 40-fold net increase in root number since week 3. No 3^{rd}-order roots were present, despite the illusion that some can be seen in Fig. 1; these were caused by minor software artefacts generated during image overlay.

Root C and N concentrations and specific root lengths

[Table 2 here]

C and N concentrations in root dry matter were influenced most strongly by root order (Table 2). In both cultivars, C concentrations were smaller in 2^{nd}- compared with 1^{st}-order roots, averaging 31.1 ± 0.55% in 1^{st}-order and 25.2 ± 0.82% in 2^{nd}-order; mean N concentrations varied likewise: 1.79 ± 0.06% in 1^{st}-order; 1.64 ± 0.08% in 2^{nd}-order. C concentration also depended on root age, accounted for largely by the notably smaller C concentrations in most 3-week-old roots compared with those of other ages, especially in S184.

The mean root C concentrations of the two cultivars averaged over the two root orders were statistically indistinguishable: 29.2 ± 0.643% in Alice and 27.7 ± 0.702% in S184, as were the corresponding values for N concentration: 1.73 ± 0.07% and 1.71 ± 0.07%.

Root order was also the only influence on specific root length. λ averaged 97.0 ± 5.59 m g^{-1} in 1^{st}-order roots and 241.0 ± 19.1 m g^{-1} in 2^{nd}-order roots, respectively. This implies smaller diameters in 2^{nd}-order roots, as expected of terminal members of a hierarchical branching system.

The coefficients of variation of root C and N concentrations and λ were c. 25% overall. This indicates the typical variation that could be expected on the C and N fluxes derived below using the scheme outlined in Table 1.

Root C and N dynamics

[Table 3 here]

Most C turnover in the root system of the larger cultivar, Alice, during 18 weeks of plant growth occurred in the 2nd-order roots: 3.7-times as much C was lost from those roots compared with from 1st-order roots (Table 3). The amount of C accumulated in the dry matter of 2nd-order roots exceeded that in the 1st-order roots by 1.7-fold. Unsurprisingly, most C loss associated with root turnover occurred towards the end of the experiment as roots aged, but the oldest roots (cohort **3**) did not contribute most of that loss. Cohorts **6**, **9** and **12** accounted for at least 92% of the total C lost in both cultivars because those were the largest cohorts, produced when the root system was growing exponentially.

Similar temporal patterns of C gain and turnover-related loss occurred in the smaller-leaved cultivar, S184. Most C loss again occurred from the 2nd-order roots whose losses were 1.6-times greater than from the 1st-order roots (Table 3). Unlike Alice, however, S184 accumulated twice as much C in 1st- compared with 2nd-order roots: 672 and 312 mg C, respectively. Proportionally less gained C was lost from the roots of S184 than from Alice, only 8.3 and 2.4% from the 2nd- and 1st-order roots, respectively.

[Table 4 here]

Alice invested 76.7 mg N in root biomass over 18 weeks of growth; 2nd-order roots received 51.0 mg, and 1st-order 25.7 mg (Table 4). The patterns of N loss by root turnover in 2nd- and 1st-order roots of Alice were similar to those for C. Over 18 weeks, 7.2 mg N were lost from 2nd-order roots and 1.5 mg from 1st-orders. S184 invested 57.3 mg N in root biomass over 18 weeks; 2nd-order roots received 21.1 mg N, less than the 36.2 mg N invested in 1st-order roots. Although 1st-order roots contained more N than 2nd-orders, the latter lost more N.

Most investment of C and N in new root cohorts occurred during the first three weeks of a cohort's existence, with one exception: in cohort **6** of Alice, more C, 72.3 mg, was used to produce 1st-order roots between 3-6 weeks old than the 59.0 mg in the 0-3 week-old roots of 6-week-old plants (Table 3). Typically, after the initially large investment, each 1st- or 2nd-order root cohort lost more C and N by root turnover than it gained by growth during each 3-week period. The successive production of younger cohorts ensured that in the root system as a whole, C and N gains by growth exceeded C and N losses by turnover. Losses were distributed unevenly between 1st- and 2nd-order roots. Greater proportional losses occurred from 2nd-order roots than from 1st-order. Mean C and N losses were 14% from 2nd-order roots of Alice compared with about 6% from 1st orders; the corresponding figures for S184 were 8 and 2%, respectively. C and N losses from S184 were proportionally smaller than those from Alice.

[Table 5 here]

The detailed demographic information in Tables 3 and 4 was combined to estimate the C:N ratios of roots and of material gained by roots during growth and lost during turnover (Table 5). The most notable features of Table 5 are: (a) the temporal stability of the C:N ratios of roots within each order; (b) the similarity of root C:N ratio between the two cultivars for roots in the same order; and (c) the similarity between mean C:N ratios of roots and of material lost from them.

[Fig. 2 here]

The amounts of C and N gained on a whole-plant basis by the cohorts of 1st- and 2nd-order roots of Alice amounted to 1218 mg C and 76.7 mg N during 18 weeks of plant growth; the corresponding figures for S184 were 984 mg C and 57.3 mg N (Fig. 2). The corresponding C and N losses from root turnover between weeks 3 and 18 totalled 134 mg C and 8.5 mg N from the roots of Alice, and 42.2 mg C and 2.3 mg N from the roots of S184. These figures align with our expectation that losses from the higher yielding cultivar Alice would exceed those from the smaller S184.

DISCUSSION

C and N dynamics associated with root turnover

Our data show clear and considerable differences in the potential for C and N transfer to soil as a result of root turnover in white clover. Absolute and relative amounts of C and N transferred to soil during root turnover in white clover varied with respect to root age (i.e., cohort) and developmental order. Genetic differences were also apparent in that C and N fluxes were greater from the roots of larger, faster-growing cultivar Alice than from the smaller lower-yielding cultivar S184 grown under the same conditions.

Most C and N loss arose from the turnover of 2nd-order roots (Tables 3 and 4). This is strong evidence that terminal roots, the developmentally youngest and most ephemeral members of the root system, account for a disproportionately large fraction of the plant's dynamic interactions with surrounding soil, particularly the transfer into the rhizosphere of C, N and other root contents. Terminal roots have been long-suspected as having that function (Pregitzer 2002), but convincing evidence for it had previously proved elusive.

An obvious difference between the white clover plants used in our experiments and their field-grown counterparts is that the latter would be periodically cut or grazed. Defoliation increases root turnover in some pasture species, but not white clover (Reid *et al.* 2015). It is likely that the turnover rates we measured in undefoliated plants would be uninfluenced by cutting.

If the data in Tables 3 and 4 are generally applicable, genotypes with greater turnover, especially of terminal roots, will be needed for the effective management of grassland swards to increase long-term C sequestration (Rees *et al.* 2005; Marshall *et al.* 2016). Genotypes with greater root turnover, and therefore C and N deposition, at depth will also be needed to minimise the risk of plant-derived labile C being rapidly converted to CO_2 in surface soil and lost to the atmosphere. Developing white clover genotypes with beneficial root traits has considerable potential (Caradus & Woodfield 1998; Abberton & Marshall 2005) although, historically, breeding programmes have focused on maximising aboveground production and forage quality. Marshall *et al.* (2016) argue persuasively that this focus needs to encompass belowground traits to

fully realise the environmental and economic potential of managed grass-legume swards. The development of automated, non-destructive phenotyping tools for this purpose (Nagel *et al.* 2012; Marshall *et al.* 2016) is invaluable provided that they accurately quantify the finest, most ephemeral roots within even the largest root systems.

Technical issues

Like all sampling-based approaches, root demography has its strengths and weaknesses (Sturite *et al.* 2007). One of the most fundamental but neglected sources of variation is the interval between successive censuses. If the interval is too long relative to turnover rate, growth and death rates of individual roots will be under-estimated. For example, Stewart & Frank (2008) found that root growth and mortality rates in upland grassland when estimated monthly using mini-rhizotrons were less than half of those estimated when observations were separated by only 3 d, an interval short enough to detect the dynamics of the most ephemeral roots. Based on a 3-week census interval, imposed by the requirements of chemical analysis (see Methods), our data showing that 2nd-order roots made the largest contribution to the loss of root C and N from white clover root systems could be under-estimates. The scale of the contributions of such roots to root C and N dynamics could be even larger than our data indicate.

Direct estimates of the amounts of C and N lost from entire root systems of clover have been obtained using *in situ* isotope (^{14}C, ^{15}N) labelling (e.g., Rasmussen *et al.* 2007). Isotopically estimated losses and transfers to neighbouring plants reflect the net effects of all the turnover, exudation and rhizodeposition processes in the whole root system between labelling and harvest. What isotopic approaches cannot do is to distinguish the contributions of developmentally distinct parts of the root system (e.g., 1st- versus 2nd-order roots; Guo *et al.* 2008); nor can they separate the effects of root turnover *per se* from other processes (Kuzyakov & Domanski 2000). To fully appreciate how the interplay between physiology, developmental morphology and demography controls such fluxes it is necessary to sample and analyse roots according to their order in the branching hierarchy and not to assume functional homogeneity throughout the root system (Valenzuela-Estrada *et al.* 2008; Rasmussen *et al.* 2010;

399 Goebel *et al.* 2011; Vetterlein & Doussan 2016), and to then to scale up information
400 obtained at the individual-root level to that of the whole system.

401 **Scaling to seasonal effects**

402 Our 18-week experiment was sufficiently long to capture the detailed root dynamics
403 of white clover plants up to that age, a period coinciding with that of maximum rates
404 of vegetative growth and resource capture of temperate clover crops (Black 1957;
405 Silsbury 1984). Obviously, C and N fluxes associated with root turnover throughout
406 that period would be dwarfed by those occurring when legume crop residues are
407 ploughed into soil at the end of the growing season which, for white clover in
408 temperate regions, typically lasts 20-25 weeks (Rasmussen *et al*,. 2013). Even so,
409 Rasmussen *et al.* (2013) concluded that short-term N fluxes from clover roots could
410 also make significant contributions to N budgets of grass-clover swards. Our data
411 show that N loss rates are not constant across the root system nor through time during
412 the vegetative growth of white clover. Moreover, there is likely to be genetic variation
413 in N fluxes if the comparison between Alice and S184 indicates a general association
414 between root N loss and potential productivity, and if our findings can be translated to
415 field settings.

416 A possible issue that we have not investigated here is that of phenological differences
417 between cultivars, and their influences on root C and N loss. Any phenological
418 differences between cultivars would have been detected by the sequential sampling
419 (cf. experiments comprising only one final harvest: Trinder *et al.*, 2012). The data in
420 Tables 3 & 4 suggests no obvious cultivar difference in the phenology of root C or N
421 losses during the experiment. But over an entire annual cycle it is possible that
422 cultivar differences in the timing of root-derived C and N inputs to soil could occur.

423 The longevity of white clover roots is enormously variable. Estimates of mean or
424 median lifespans ranging from 1-6 (Watson *et al.* 2000), 15 (Reid *et al.* (2015), 4-37
425 (Harper *et al.* 1991) and 40 weeks (Sturite *et al.* 2007) have been reported. This
426 variation mainly reflects seasonal and geographic influences. Greater and more rapid
427 root mortality of the white clover cultivar S184 occurred at a warmer site in Italy than
428 at a colder UK site (Watson *et al.* 2000). Sturite *et al.* (2007) reported a strong linear
429 decline in median longevity of white clover roots as soil temperatures increased.

Whether warmer soil results in the loss of more or less C and N via root turnover will depend on the balance between faster root growth and more rapid mortality. If soil warming accelerates the latter more than the former, C and N losses will probably increase; if warming increases growth more than death, losses should decrease. But the temperature responses of root demographics can be transient and are influenced indirectly by temperature-related changes in nutrient availability, at least in temperate grasslands (Fitter *et al.* 1999; Edwards *et al.* 2004). It would be valuable to apply the demographic approach reported here to directly test the effects of temperature and other factors on root C and N dynamics to clarify the extent to which they are environmentally constrained.

Implications for crop productivity

N lost from a legume's root system can be equated notionally to a potential productivity 'loss' for that crop, it might also equate to a gain for the next crop in the rotation if it can take advantage of that N. Likewise, C lost from a root system cannot contribute directly to the productivity of that crop but, as SOM, might sustain the productivity of subsequent crops (Rasmussen *et al.* 2010) or contribute to long-term C sequestration (Rees *et al.* 2005; Marshall *et al.* 2016).

To scale up the C and N losses per plant (Fig. 2) to estimate potential effects on field crops, we assumed a typical planting density of 100 m^{-2} (Marshall & James 2006). The estimated mean weekly C and N losses by root turnover over 18 weeks' growth would have been equivalent to 7.5 and 0.5 kg ha^{-1} for Alice and 2.3 and 0.1 kg ha^{-1} for S184, respectively. If total above-ground dry matter production was 4.0 t ha^{-1} for Alice and 2.5 t ha^{-1} for S184 (Gilliland 2004) and mean cultivar-specific C and N concentrations in dry matter those reported in Table 2, total C and N losses from the roots of Alice would be about 134 and 8.5 kg ha^{-1}, respectively; corresponding figures for S184 are 42.2 and 2.3 kg ha^{-1}.

The C and N losses we estimated for white clover are, therefore, equivalent to about 6% of above-ground dry matter production of the slower-growing cultivar S184 and up to 12% of that of the higher-yielding cultivar Alice. The plausibility of these estimates is supported by independent evidence. Using extensive isotope labelling data, Kuzyakov & Domanski (2000) suggested that annual root-derived C fluxes (including root turnover,

exudation, rhizodeposition and other processes, but excluding respiration) into pasture soil averages about 7% of total aboveground dry matter production. The similarity of this figure to the 6-12% we estimated for C and N loss solely via root turnover hints that the bulk of such fluxes does indeed originate from root turnover, and that exudation and similar processes make negligible contributions at the field scale (see assumption (3) in Methods).

Even so, 6-12% might appear to be trivial fractions of potential crop productivity, given the much larger variations caused by unpredictable weather or heterogeneous soil conditions (Wilman *et al.* 2005; Frankow-Lindberg *et al.* 2009; Lobell *et al.* 2009). But we again emphasise that ours are conservative estimates of C and N losses associated only with root turnover and, therefore, of the potential of that process to reduce notional productivity, and are estimated for only an 18-week period. Consequently, it is likely that the constraint on potential productivity attributable to root turnover will exceed our estimates. It is more complicated than that, however, because accumulated crop-derived C and N inputs influence soil conditions that can modify future productivity (e.g., N availability, SOM composition). Therefore, it is equally possible that any potential losses in clover productivity caused by root turnover could be offset in the long-term by improved soil fertility that will benefit a subsequent crop in the rotation.

CONCLUSIONS

The detailed information reported here provides a new perspective on C and N dynamics associated with root turnover in an agriculturally important legume. Using a novel approach combining non-destructive root censuses with sequential destructive sampling, and demographic modelling, we have estimated that C and N fluxes associated with root turnover in white clover represent a potential loss in crop productivity of at least 6-12%. Those fluxes were not distributed evenly over whole root systems, but arose mainly from the turnover of relatively young, ephemeral terminal members of the root system. There is likely to be significant genetic variation in the contributions of white clover to soil fertility and potential C sequestration via root-derived C and N inputs.

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637

Figure legends

Figure 1 Sequential digital tracing of the same root system of a *Trifolium repens* cv. Alice individual at 3-week intervals over 18 weeks of plant growth. Each root path is identified uniquely (green numbers on images). Tracings have been superimposed on a black background for clarity. (a) Week 3, 58 root paths; (b) week 6, 179 paths; (c) week 9, 727 paths; (d) week 12, 1302 paths; (e) week 15, 1674 paths; (f) week 18, 2299 paths.

Figure 2 Summary of total C and N contained in root systems of two white clover cultivars after 3 and 18 weeks' growth (numbers in boxes), and the net amounts lost from the root system during 18 weeks' growth (numbers in arrows), derived from data in Tables 3 and 4. a: Cv. Alice. b: Cv. S184.

651 **Table 1** Demographic scheme to calculate C or N dynamics of two root cohorts of a single root order.

Plant age	**Root cohort**						
	1			**2**			
	Mass	**Gain**	**Loss**	**Mass**	**Gain**	**Loss**	**Total loss (mg per preceding time period)**
1	X_1	$E_1 = QR_{1n}/\lambda$	$L_1 = QR_{1d}/\lambda$				
2	$X_2 = X_1 + E_1 – L_1$	$E_2 = QR_{2n}/\lambda$	$L_2 = QR_{2d}/\lambda$	Y_2	$F_2 = QR_{2n}/\lambda$	$M_2 = QR_{2d}/\lambda$	$\mathbf{L_1}$
3	$X_3 = X_2 + E_2 – L_2$	$E_3 = QR_{3n}/\lambda$	$L_3 = QR_{3d}/\lambda$	$Y_3 = Y_2 + F_2 – M_2$	$F_3 = QR_{3n}/\lambda$	$M_3 = QR_{3d}/\lambda$	$\mathbf{L_2 + M_2}$
							Total (mg)
Loss per cohort			$\mathbf{L_1 + L_2}$			$\mathbf{M_2}$	$\mathbf{L_1 + L_2 + M_2}$
Mass per cohort	$\mathbf{X_1 + E_1 + E_2}$			$\mathbf{Y_2 + F_2}$			$\mathbf{X_1 + E_1 + E_2 + Y_2 + F_2}$

652

653 This example shows the calculations for two root cohorts (denoted as **1** and **2**, which were formed by a plant at age 1 and between ages 1 and 2,
654 respectively) of the same developmental order. Fluxes of material into or out of root dry matter associated with growth or death are indicated as
655 Gain or Loss. X = mass (mg) of C or N in cohort **1**. E = C or N flux (mg) into cohort **1** caused by new root growth. L = C or N lost (mg) from
656 cohort **1** by root death. Y, F, M = corresponding values for cohort **2**. Subscripted numbers denote the plant age at which the flux occurred or to
657 which the masses of C or N apply. Q = C or N concentration (mg g^{-1}) in root dry matter; R = root length (m); subscripted letters ‘n’ and ‘d’
658 denote newly produced and dead root lengths, respectively; λ = specific root length (m g^{-1}) calculated separately for each cohort. (In this
659 example, fluxes subscripted 3, do not feature in the calculations because these would contribute to gains by and losses only from plants of age 4

and older.) Total losses during each preceding time interval (i.e., between plant harvests), summed for all cohorts, are calculated in the final column. Total C or N masses in, and losses from, each cohort, and for all cohorts combined, are calculated in the final three rows. To accommodate data for older plants and more root cohorts, this scheme is extended accordingly. C or N fluxes were derived separately for each root order.

Table 2 Cultivar-, order- and age-dependent variations in root C and N concentrations and specific root length (λ) of white clover from which C and N fluxes were derived.

Cultivar	Root order	Plant age (wk)	C (%)		N (%)		λ (m g^{-1})	
			mean	se	mean	se	mean	se
Alice	1	3	25.1	1.54	1.76	0.41	105.0	21.0
		6	32.3	1.44	1.88	0.14	109.2	21.0
		9	34.4	1.33	2.03	0.01	126.1	16.8
		12	34.9	1.54	1.95	0.08	88.2	10.1
		15	33.8	1.33	2.02	0.09	75.6	27.7
		18	34.6	1.64	1.71	0.11	21.0	0.67
	2	3	29.2	4.10	1.84	0.30	210.1	79.8
		6	25.6	1.03	1.65	0.12	208.4	67.2
		9	22.1	1.23	1.43	0.17	264.7	29.4
		12	25.1	1.85	1.31	0.16	214.3	33.6
		15	23.6	2.05	1.46	0.25	189.1	31.1
		18	-	-	-	-	-	-
S184	1	3	19.3	4.25	1.64	0.39	134.5	21.0
		6	27.7	1.78	1.77	0.15	168.1	18.5
		9	33.3	0.40	1.90	0.004	147.1	22.7
		12	32.1	0.30	1.54	0.01	100.8	12.6
		15	32.6	0.20	1.70	0.01	75.6	10.1
		18	33.1	0.15	1.55	0.01	12.6	0.42
	2	3	20.7	3.95	1.84	0.31	210.1	33.6
		6	27.5	1.19	2.29	0.14	247.9	33.6
		9	26.0	1.38	1.74	0.17	247.9	25.2
		12	26.7	1.58	1.44	0.17	357.1	96.6
		15	25.9	2.17	1.39	0.27	260.5	54.6
		18	-	-	-	-	-	-

Summary analysis of variance [a]

	d.f.	*F*	*P*	*F*	*P*	*F*	*P*
Cultivar	1	2.38	0.125	4.3	0.04	1.16	0.287
Root order	1	**60.61**	**<0.001**	**11.82**	**<0.001**	**61.47**	**<0.001**
Plant age	4	**7.86**	**<0.001**	2.05	0.09	1.93	0.121
Error	164						

[a] Statistical effects of cultivar, root order and root age on total C and N concentrations (both symbolised as Q in Table 1) and λ, as determined by GLMs, are summarised as *F* ratios and *P* values; those in bold indicate $P \leq 0.002$, as explained in Methods. λ data were ln-transformed before analysis to homogenise variances. $n = 5$ throughout.

Table 3 Mean masses of C (mg) gained by, lost from, and contained in 1st- and 2nd-order root cohorts of two white clover cultivars of different ages.

Cultivar	Order	Plant age (weeks)	Root cohort number														
			3			6			9			12			15			
			Mass	Gain	Loss	Mass	Gain	Loss	Mass	Gain	Loss	Mass	Gain	Loss	Mass	Gain	Loss	
Alice	**1**	3	29.4	0.8	0.0													**Loss (mg per 3 wk)**
		6	30.2	0.1	0.0	59.0	72.3	0.0										**0.0**
		9	30.3	0.7	0.0	131.3	2.7	1.3	134.0	35.5	1.7							**0.0**
		12	31.0	0.0	0.0	132.7	0.5	0.5	167.8	1.1	2.6	80.8	4.4	2.2				**3.0**
		15	31.0	0.0	0.1	132.7	0.0	7.7	166.3	0.1	4.9	83.0	0.4	7.5	25.1	9.1	0.1	**5.3**
		18	30.9	-	-	125.0	-	-	161.5	-	-	75.9	-	-	34.1	-	-	**20.3**
																		Total (mg)
		Loss (mg per cohort)			**0.1**			**9.5**			**9.2**			**9.7**			**0.1**	**28.5**
		Mass (mg per cohort)	**31.0**			**135**			**171**			**85.6**			**34.2**			**456**
	2	3	7.8	0.4	0.3													**Loss (mg per 3 wk)**
		6	7.9	0.0	1.3	60.3	5.2	1.3										**0.3**
		9	6.6	0.0	1.8	64.2	0.0	12.4	238.0	34.8	3.8							**2.6**
		12	4.8	0.0	2.4	51.8	0.0	15.9	269.0	7.5	16.4	288.0	5.6	6.1				**18.0**
		15	2.4	0.0	1.1	35.9	0.0	7.7	260.1	1.6	19.2	287.5	3.1	17.1	106.0	3.4	0.1	**40.8**
		18	1.3	-	-	28.2	-	-	242.5	-	-	273.5	-	-	109.3	-	-	**45.2**
																		Total (mg)
		Loss (mg per cohort)			**6.9**			**37.3**			**39.4**			**23.2**			**0.1**	**107**
		Mass (mg per cohort)	**8.2**			**66**			**282**			**297**			**109**			**762**
S184	**1**	3	39.3	10.6	0.0													**Loss (mg per 3 wk)**
		6	49.9	0.0	0.0	140.0	54.3	0.0										**0.0**
		9	49.9	0.0	0.0	194.3	16.0	4.7	96.4	60.4	0.0							**0.0**
		12	49.9	0.0	0.6	205.6	0.0	4.8	156.8	0.0	0.0	133.0	38.8	0.0				**4.7**
		15	49.3	0.3	0.2	200.8	0.0	3.5	156.8	0.0	2.6	171.8	2.7	0.0	51.7	28.7	0.0	**5.4**
		18	49.4	-	-	197.3	-	-	154.2	-	-	174.5	-	-	80.4	-	-	**6.3**
																		Total (mg)
		Loss (mg per cohort)			**0.8**			**13**			**2.6**			**0**			**0.0**	**16.4**
		Mass (mg per cohort)	**50.2**			**210**			**157**			**175**			**80.4**			**672**
	2	3	2.6	0.0	0.0													**Loss (mg per 3 wk)**
		6	2.6	0.1	0.1	33.0	2.8	0.1										**0.0**
		9	2.6	0.0	1.0	35.7	0.2	1.9	59.4	20.4	0.4							**0.2**
		12	1.6	0.0	0.4	34.0	0.0	5.8	79.4	0.9	2.5	96.6	7.4	1.2				**3.3**
		15	1.2	0.0	0.2	28.2	0.0	4.7	77.8	0.2	5.4	102.8	0.5	1.8	72.5	14.9	0.3	**9.9**
		18	1.0	-	-	23.5	-	-	72.6	-	-	101.5	-	-	87.1	-	-	**12.4**
																		Total (mg)
		Loss (mg per cohort)			**1.7**			**12.5**			**8.3**			**3.0**			**0.3**	**25.5**
		Mass (mg per cohort)	**2.7**			**36.0**			**80.9**			**105**			**87.4**			**312**

Data were calculated according to the scheme shown in Table 1.

Table 4 Mean masses of N (mg) gained by, lost from, and contained in 1st- and 2nd-order root cohorts of two white clover cultivars of different ages.

Cultivar	Order	Plant age (weeks)	Root cohort number														
			3			6			9			12			15			
			Mass	Gain	Loss	Mass	Gain	Loss	Mass	Gain	Loss	Mass	Gain	Loss	Mass	Gain	Loss	
Alice	**1**	3	1.7	0.0	0.0													**Loss (mg per 3 wk)**
		6	1.7	0.0	0.0	3.3	4.1	0.0										**0.0**
		9	1.7	0.1	0.0	7.4	0.1	0.0	7.6	2.1	0.1							**0.0**
		12	1.8	0.0	0.0	7.5	0.0	0.0	9.6	0.0	0.1	4.6	0.2	0.1				**0.1**
		15	1.8	0.0	0.1	7.5	0.0	0.4	9.5	0.0	0.3	4.7	0.0	0.4	1.4	0.5	0.0	**0.2**
		18	1.7			7.1			9.2			4.3			1.9			**1.2**
																		Total (mg)
		Loss (mg per cohort)			**0.1**			**0.4**			**0.5**			**0.5**			**0.0**	**1.5**
		Mass (mg per cohort)	**1.8**			**7.5**			**9.7**			**5**			**1.9**			**25.7**
	2	3	0.5	0.0	0.0													**Loss (mg per 3 wk)**
		6	0.5	0.0	0.1	4.1	0.5	0.1										**0.0**
		9	0.4	0.0	0.1	4.5	0.0	0.8	16.0	2.4	0.3							**0.2**
		12	0.3	0.0	0.2	3.7	0.0	1.1	18.1	0.5	1.1	19.3	0.4	0.4				**1.2**
		15	0.1	0.0	0.1	2.6	0.0	0.5	17.5	0.0	1.3	19.3	0.0	1.1	7.1	0.2	0.0	**2.8**
		18	0.0			2.1			16.2			18.2			7.3			**3.0**
																		Total (mg)
		Loss (mg per cohort)			**0.5**			**2.5**			**2.7**			**1.5**			**0.0**	**7.2**
		Mass (mg per cohort)	**0.5**			**4.6**			**18.9**			**19.7**			**7.3**			**51.0**
S184	**1**	3	2.1	0.6	0.0													**Loss (mg per 3 wk)**
		6	2.7	0.0	0.0	7.6	2.9	0.0										**0.0**
		9	2.7	0.0	0.0	10.5	0.6	0.0	5.2	3.3	0.0							**0.0**
		12	2.7	0.0	0.0	11.1	0.0	0.3	8.5	0.0	0.0	7.2	2.1	0.0				**0.0**
		15	2.7	0.0	0.0	10.8	0.0	0.2	8.5	0.0	0.1	9.3	0.2	0.0	2.8	1.6	0.0	**0.3**
		18	2.7			10.6			8.4			9.5			4.4			**0.3**
																		Total (mg)
		Loss (mg per cohort)			**0.0**			**0.5**			**0.1**			**0.0**			**0.0**	**0.6**
		Mass (mg per cohort)	**2.7**			**11.1**			**8.5**			**9.5**			**4.4**			**36.2**
	2	3	0.2	0.0	0.0													**Loss (mg per 3 wk)**
		6	0.2	0.0	0.0	2.2	0.2	0.0										**0.0**
		9	0.2	0.0	0.1	2.4	0.0	0.1	4.0	1.4	0.0							**0.0**
		12	0.1	0.0	0.0	2.3	0.0	0.4	5.4	0.1	0.2	6.6	0.5	0.1				**0.2**
		15	0.1	0.0	0.0	1.9	0.0	0.3	5.3	0.0	0.4	7.0	0.0	0.1	4.9	1.0	0.0	**0.7**
		18	0.1			1.6			4.9			6.9			5.9			**0.8**
																		Total (mg)
		Loss (mg per cohort)			**0.1**			**0.8**			**0.6**			**0.2**			**0.0**	**1.7**
		Mass (mg per cohort)	**0.2**			**2.4**			**5.5**			**7.1**			**5.9**			**21.1**

Data were calculated according to the scheme shown in Table 1.

Table 5 C:N ratios of material gained by, lost from, and contained in 1st- and 2nd-order root cohorts of two white clover cultivars of different ages.

Cultivar	Order	Plant age (weeks)	Root cohort number														Mean ± s.e. (mg)
			3			6			9			12			15			
			Mass	Gain	Loss	Mass	Gain	Loss	Mass	Gain	Loss	Mass	Gain	Loss	Mass	Gain	Loss	
Alice	**1**	3	17.3														
		6	17.8			17.9	17.6										
		9	17.8	7.0		17.7	27.0		17.6	16.9	17.0						
		12	17.2			17.7			17.5		26.0	17.6	22.0	22.0			
		15	17.2			17.7		19.3	17.5		16.3	17.7		18.8	17.9	18.2	
		18	18.2			17.6			17.6			17.7			17.9			
		Loss						**19.3**			**19.8**			**20.4**			**19.8 ± 0.32**
		Mass	**17.6**			**17.7**			**17.5**			**17.6**			**17.9**			**17.7 ± 0.07**
Alice	**2**	3	15.6														
		6	15.8			14.7	10.4										
		9	16.5			14.3			14.9	14.5	12.7						
		12	16.0			14.0			14.9		14.9	14.9	14.0	15.3			
		15	24.0			13.8		15.4	14.9		14.8	14.9		15.5	14.9	17.0	
		18				13.4			15.0			15.0			15.0			
		Loss						**15.4**			**14.1**			**15.4**			**15.0 ± 0.43**
		Mass	**17.6**			**14.0**			**14.9**			**14.9**			**15.0**			**15.3 ± 0.60**
S184	**1**	3	18.7														
		6	18.5			18.4	18.7										
		9	18.5			18.5			18.5	18.3							
		12	18.5			18.5			18.4			18.5	18.5				
		15	18.3			18.6		17.5	18.4		26.0	18.5			18.5	17.9	
		18	18.3			18.6			18.4			18.4			18.3			
		Loss						**17.5**			**26.0**						**21.8 ± 4.25**
		Mass	**18.5**			**18.5**			**18.4**			**18.4**			**18.4**			**18.5 ± 0.03**
S184	**2**	3	13.0														
		6	13.0			15.0	14.0										
		9	13.0			14.9			14.9	14.6							
		12	16.0			14.8			14.7			14.6	14.8				
		15	12.0			14.8		15.7	14.7		13.5	14.7			14.8	14.9	
		18	10.0			14.7			14.8			14.7			14.8			
		Loss						**15.7**			**13.5**						**14.6 ±1.08**
		Mass	**12.8**			**14.8**			**14.8**			**14.7**			**14.8**			**14.4 ±0.39**

No entries reflect zero or near-zero values in either Table 3 or 4, from which these C:N ratios were derived.