Nitrogen Turnover and Assimilation during Regrowth in *Trifolium subterraneum* L. and *Bromus mollis* L.

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ABSTRACT

Subterranean clover (*Trifolium subterraneum* L. cv Woogenellup) and soft chess grass (*Bromus mollis* L. cv Blando) were grown in monocultures with $^{14}$NH$_4$Cl added to the soil to study nitrogen movement during regrowth following shoot removal. Four clipping treatments were imposed. Essentially all available $^{14}$N was assimilated from the soil prior to the first shoot harvest. Measurements of total reduced nitrogen and $^{15}$N contained within that nitrogen fraction in roots, crowns, and shoots at each harvest showed large, significant ($P < 0.001$) declines in excess $^{14}$N of crowns and roots in both species between the first and fourth harvests. There was no significant decline in total reduced nitrogen in the same organs over that period. Similar responses were evident in plants defoliated three times. The simplest interpretation of these data is that reduced nitrogen compounds turn over in plant roots and shoots during regrowth. Calculations for grass and clover plants clipped four times during the growing season indicated that 100 to 143% of the nitrogen present in crowns and roots turned over between the first and fourth shoot harvest in both species, assuming nitrogen in those organs was replaced with nitrogen containing the lowest available concentration of $^{14}$N. If other potential sources of nitrogen were used for the calculations, it was necessary to postulate that larger amounts of total nitrogen flowed through the crown and root to produce the measured dilution of $^{15}$N compounds. These data provide the first quantitative estimates of the amount of internal nitrogen used by plants, in addition to soil nitrogen or $N_b$, to regenerate shoots after defoliation.

It is well established that organic reserves contribute to regrowth of grazed or defoliated plants (19). The classic discussion of this concept (11) emphasized the importance of carbohydrates as a subclass of organic reserves but also showed that total nitrogen and nitrogen concentration in alfalfa roots were affected by clipping frequency. Other workers have recorded that root nitrogen concentration fluctuated after forage harvests in alfalfa (12) and grasses (15, 21, 22).

Classical techniques using changes in total dry weight and nitrogen of roots, crowns, and shoots during regrowth generally support the concept that organic reserves are mobilized to the new foliage (20), but such results are complicated under normal conditions by new carbon and nitrogen assimilation. Direct evidence for mobilization of $^{14}$C from root reserves to new shoots has been provided by several groups (19). Similar data are not available for nitrogen compounds. Indirect evidence that nitrogen from root proteins may be available for translocation from regenerating roots was reported by Davidson and Milthorpe (6, 7). Those workers concluded from changes in root carbohydrate and total CO$_2$ respired during regrowth that *Dactylis glomerata* L., probably degraded proteins as well as carbohydrates to produce the large quantities of CO$_2$ respired.

During shoot regrowth following defoliation, nitrogen is potentially available from several sources: soil nitrogen, symbiotic N$_2$ fixation in legumes, and nitrogen reserves. In some legumes, normal soil nitrogen probably is supplemented with organic nitrogen from roots and nodules that degenerate after clipping (4). In other legumes, root nodules remain intact after defoliation and symbiotic N$_2$ fixation continues at depressed levels until new foliage develops (5, 9, 23). Although it has been suggested that organic nitrogen reserves are degraded during regrowth (6, 7), data available in the literature do not allow one to estimate the amount of root or crown nitrogen that is used during shoot regrowth. As a first step toward providing the latter information, an experiment with isotopically labeled nitrogen was conducted in defoliated clover and grass plants.

MATERIALS AND METHODS

Growth Conditions. Subterranean clover (*Trifolium subterraneum* L. cv Woogenellup) and soft chess grass (*Bromus mollis* L. cv Blando) were grown as monocultures or 50:50 mixtures at 2000 seeds m$^{-2}$ in 7.6-L pots (22.5-cm diameter) containing 10 kg of air-dry Laughlin loam, which was excavated adjacent to a field site on which related studies have been conducted (17). Pots were supplemented with 1.3 g Na$_2$SO$_4$/pot, 943 mg KH$_2$PO$_4$/pot, 211 mg NH$_4$Cl/pot containing 3.00 atom % excess $^{15}$N, and 1.7 mg Na$_2$MoO$_4$/pot. Plants were maintained at the University of California Hopland Field Station in a greenhouse under natural illumination with temperatures that fluctuated between 13 and 32°C. The pots were watered as needed to maintain soil moisture near field capacity, as determined by weight. Clover seeds coated with a commercial peat *Rhizobium* inoculum and grass seeds were sown November 6, 1979, at a time when the same plants were germinating in the natural rangeland environment outside the greenhouse. Plants were clipped 2 cm above the soil on four dates: (1) December 20, 1979; (2) January 17, 1980; (3) February 21, 1980; and (4) March 18, 1980. Clipping treatments consisted of shoot removal on clipping dates 1 through 4, 2 through 4, 3 and 4, or 4 only. Clipping treatments and mixtures or monocultures were arranged in a randomized complete block design with five replications.

Each time a pot was clipped, a comparable pot was harvested by washing away the soil and separating plants into root, crown, and shoot fractions. Crown samples also included stem and leaf bases in the grass and stolon bases in the clover. Clover root samples included root nodules. All samples were dried to a constant weight at 70°C.

Compositional Analyses. Dry weight data were collected separately for various species and organs within each pot of all five replicates. Samples were ground in a Wiley mill to pass through a 40 mesh screen, and total reduced nitrogen was determined in three replicates by Kjeldahl analysis (3). Distillates from the Kjeldahl procedure were acidified and evaporated to dryness for...
storage. On the day mass analyses were conducted, samples were redissolved in distilled H₂O and oxidized to N₂ by alkaline hypobromite treatment in vacuo (3). The N₂ was analyzed for ¹⁵N concentration with a VG Micromass 602E isotope ratio mass spectrometer. All ¹⁵N concentrations were referred to a laboratory standard which was corrected to the normal value for atmospheric N₂ of 0.3663 atom % ¹⁵N.

Calculations. Kjeldahl nitrogen and ¹⁵N content data were subjected to a one-way analysis of variance for each plant organ within each monoculture to test for effects of clipping treatments.

Nitrogen incorporated between clipping dates was calculated by summing root, crown, and shoot total reduced N or ¹⁵N excess for clipping date n and subtracting total reduced N or ¹⁵N excess present in the root and crown tissue of comparable plants at the n-1 clipping date.

Minimum net flows of soil nitrogen through reduced N fractions of grass root tissue during three regrowth cycles were calculated in the following manner

\[
N_{R_n} = N_{R_{n-1}} - Y_R + X_R
\]

(1)

\[
¹⁵N_{R_n} = ¹⁵N_{R_{n-1}} - a Y_R + b X_R
\]

(2)

where \(N_{R_n}\) = total reduced N content of root system measured at time \(t\); \(¹⁵N_{R_n}\) = excess ¹⁵N content of root system measured at time \(t\); \(X_R\) = net soil N transferred to root between t1 and t4; \(Y_R\) = root N transferred to shoot between t1 and t4; \(t\) = clipping date 1; \(t_4\) = clipping date 4; and \(a\) = mean excess ¹⁵N concentration in the organ between t1 and t4; and \(b\) = mean ¹⁵N concentration of soil N incorporated between t1 and t4.

\[
a = (\frac{1}{2}) \left( [¹⁵N]_{R_1} + [¹⁵N]_{R_4} \right)
\]

(3)

\[
b = \frac{\text{total plant } ¹⁵N_n - \text{total plant } ¹⁵N_1}{\text{total plant } N_1 - \text{total plant } N_4}
\]

(4)

When shoot N and ¹⁵N removed in clippings were identified as \(N_s\) and \(¹⁵N_s\) respectively, and crown N and ¹⁵N were indicated as \(N_c\) and \(¹⁵N_c\), then

\[
b = \left( [¹⁵N]_{R_1} - [¹⁵N]_{R_4} \right) / \left( [N]_{R_1} - [N]_{R_4} + [¹⁵N]_{R_1} - [¹⁵N]_{R_4} \right)
\]

Net N transfers for grass crowns (\(Y_c\) and \(X_c\)) were calculated by substituting \([¹⁵N]_{C_1}\) and \([¹⁵N]_{C_4}\) into Equation 3 and using the measured values for \(N_{C_1}\), \(N_{C_4}\), \(¹⁵N_{C_1}\), and \(¹⁵N_{C_4}\) in Equations 1 and 2. The value of \(b\) from Equation 4 was identical for both root and crown calculations.

Two assumptions underlie these calculations. First, it was assumed that a simple linear dilution model fairly reflected the value of \(a\) or \(b\) in Equation 3. Second, an absence of significant isotopic fractionation was assumed, and therefore the net transfer of soil ¹⁵N to the root or crown was calculated as a proportion of the net transfer of total soil N (\(X_R\) or \(X_c\)) by using the factors \(b X_R\) or \(b X_c\).

In the case of clover root and crown calculations, it was assumed that any N that moved out of those organs was replaced by N₂-derived N (\(Z_R\) or \(Z_c\)), because \(N_2\) had a lower concentration of ¹⁵N than soil N. In fact, by definition, there was no excess ¹⁵N in the atmospheric N₂. Thus, for clover roots, the relationships comparable to Equations 1 and 2 were

\[
N_{R_n} = N_{R_{n-1}} - Y_R + Z_R
\]

(5)

\[
¹⁵N_{R_n} = ¹⁵N_{R_{n-1}} - a Y_R
\]

(6)

Similar relationships were used to calculate net N transfers for clover crowns (\(Y_c\) and \(Z_c\)) by substituting \([¹⁵N]_{C_1}\) and \([¹⁵N]_{C_4}\) into equation 3 and using measured values for \(N_{C_1}\), \(N_{C_4}\), \(¹⁵N_{C_1}\), and \(¹⁵N_{C_4}\) to replace corresponding root values in Equations 5 and 6. The contribution of soil N to the clover shoot N removed on clipping dates 2 through 4 was calculated by assuming that (soil ¹⁵N taken up)₂₋₄/(total soil N taken up)₁₋₄ was the same for both grass and clover monocultures over that period. Total \(N_2\) fixed (\(N_2 N\)) by clover on clipping dates 2 through 4 was calculated as, \(N_2 N_{1-4} = (N_{R_1} + N_{S_1} + N_{C_1}) + (N_{R_4} - N_{R_1}) + (N_{C_1} - N_{C_4}) - (\text{soil N taken up})_{1-4}.

RESULTS

Primary Data. The warmer temperatures experienced by the experimental plants inside the greenhouse relative to the same species growing outside under natural conditions accelerated growth and development. The flowering stages of the experimental plants at the final harvest on March 18, 1980, were comparable to those normally observed in field plots during the first week of April.

Nearly all of the available excess ¹⁵N was incorporated into grass and clover monocultures between planting and the first clipping date (Fig. 1). The incremental gains in ¹⁵N measured at subsequent clipping dates were not significantly different from zero incorporation (\(P \leq 0.05\)). There was, however, significant (\(P \leq 0.05\)) assimilation of soil N by grass monocultures after clipping date 1. The difference between N incorporation by grass and clover pots between clipping dates 2 and 3 and 4 showed that considerable symbiotic N₂ fixation occurred. Of the total 1660 µg excess ¹⁵N supplied to each pot, 103 to 372 µg excess or 6 to 22% of the applied ¹⁵N was recovered in plant material.

Increasing the number of harvests decreased the N assimilated by clover monocultures (\(P \leq 0.005\)) but promoted total N incorporation by grass monocultures (\(P \leq 0.005\)) (Table I). Shoot and total plant dry weight declined in both species with increasing clipping frequency (data not shown).
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Table I. Total Reduced Nitrogen and $^{15}$N Enrichment of Organs in Plants Subjected to Various Numbers of Shoot Harvests

Each value represents the mean of three pots planted with 80 seeds of either Trifolium subterraneum L. or Bromus mollis L. The amount of total reduced N was determined by Kjeldahl analyses. Excess $^{15}$N in the same samples was measured relative to the predicted $^{15}$N concentration if all N had been derived from atmospheric N$_2$ (0.3663 atom % $^{15}$N).

<table>
<thead>
<tr>
<th>Plant Organ</th>
<th>Clipping Date</th>
<th>Clipping Date</th>
<th>Clipping Date</th>
<th>Clipping Date</th>
<th>LSD (0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Crown</td>
<td>2987</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The total reduced N contained in crowns and roots at each clipping date varied with plant species, clipping treatment, and clipping date (Table I). There was, however, no significant decline (P \( \leq 0.05 \)) in total reduced N of crowns or roots between the first and last clipping date in either species for those plants clipped two, three, or four times.

The excess $^{15}$N measured in crowns and roots at each clipping date also varied with plant species, clipping treatment, and clipping date (Table I). In contrast to total reduced N, however, very large and significant (P \( \leq 0.001 \)) declines in excess $^{15}$N of crops and roots were measured between the first and fourth clipping date in both species for those plants clipped four times. Similar declines were evident in crowns and roots of plants clipped three times. No significant block effect was detected for excess $^{15}$N or total reduced N in any clipping treatment of either species.

Large declines in excess $^{15}$N content of crops and roots with no significant decreases in total reduced N also were measured between the first and fourth harvest in grass and clover mixtures and in replicated mixtures and monocultures grown without supplemental sulfur (data not shown).

**DISCUSSION**

These experimental results provide the first direct evidence for the turnover of reduced nitrogen reserves in crowns and roots during vegetative regrowth following defoliation. The movement of $^{15}$N from crowns and roots to new shoots and the replenishment of those N pools with soil N or N$_2$-derived N were measured directly (Table I and Fig. 1). The calculated net minimum flows that were required to produce the measured dilution of $^{15}$N in the crown and root showed that very large turnovers of nitrogenous compounds were necessary (Fig. 2). In fact, using the conservative calculation of diluting crown and root N with the source of N containing the lowest concentration of $^{15}$N produced values indicating that 100 to 143% of the N present in the crowns and roots turned over between the first and fourth harvests. If one makes the reasonable assumption that some soil N assimilated by the clover over that period passed through the root and crown pools of nitrogenous compounds, then the net flows calculated would be somewhat larger because a small amount of excess $^{15}$N was taken up from the soil in that period. If one postulates that some nitrogenous compounds known to be transported from the shoot to the root in many plants (16) were incorporated into the crown and root, then the calculations are mathematically more complex, but the fraction of N turned over in those organs would be larger. In all cases, only net flows of N can be considered because of possible reassimilation of N lost from degrading roots.

No direct comparison between the calculated flow of reduced N in this study (Fig. 2) and those reported by other workers is
NITROGEN TURNOVER IN CLOVER AND GRASS

![Diagram of nitrogen turnover in clover and grass]

FIG. 2. Minimum net flows of soil nitrogen or atmospheric N₂ through crown and root tissue during three regrowth cycles of annual grass and clover plants. Values shown in mg total reduced nitrogen were calculated from three replicate pots planted with 80 seeds of Trifolium subterraneum L. or Bromus mollis L. Nitrogen content of roots and crowns are the measured values reported in Table 1, when the first of four shoot harvests was made. Shoot nitrogen removed in harvests two through four also was measured. Nitrogen flows from N₂ and soil nitrogen were calculated to account for the measured dilution of ¹⁵N in the crown and root between the first and fourth harvests reported in Table 1. The measured total nitrogen content of plant organs at the fourth harvest, reported in Table 1, can be calculated by summing inputs, outputs, and original nitrogen values. All flows were calculated by assuming that crown or root nitrogen was diluted with nitrogen containing the lowest available concentration of ¹⁵N. Flows directly to the shoot from the soil or root nodules represent nitrogenous compounds moving in the xylem stream. Arrows going from the soil or root nodules to roots or crowns indicate nitrogen that entered into metabolic pools.

Possible. Ashley et al. (1) concluded that pools of reduced ¹⁵N compounds in wheat seedling roots reached a stable equilibrium several hours after transfer to ¹⁴N-nitrate solutions. If one assumes that the 100 to 145% turnover in reduced N of the crowns and roots of the present study occurred uniformly over the 89-d period between the first and fourth clipping, then the 1 to 2% N turnover each day probably would not have been detected by Ashley et al. (1). The 1 to 2% N turnover rate estimated by such a speculative calculation is somewhat less than the 6 to 9% of the total N reported to turn over daily in fully developed rice seedling leaves (18). Data from maize suggest that about 1% of the total N may turn over each day in stem and leaf fractions during grain filling, but no significant turnover was evident in roots during the 7-week experimental period (10). Unfortunately, the type of N movement data available from xylem and phloem samples in legumes (16) does not permit calculations of N turnover rates in metabolic pools of crowns or roots.

A critical assumption in calculations used in the present study is that the depletion of ¹⁵N in crowns and roots did not result from isotopic fractionation. This assumption is justified for two reasons. First, measurements of isotopic discrimination between ¹⁴N and ¹⁵N show that such phenomena are too small to have a significant effect on the calculated values in Figure 2. Second, most measurements of biological isotope effects show that metabolic processes discriminate against ¹⁵N atoms (β > 1.0) rather than favor its turnover as shown in Table 1. Delwiche and Stenly (8) measured β values of 1.0039, 1.0260, 1.0173, and 1.0011 to 1.0148 for N₂ fixation, nitrification, denitrification, and NH₄⁺ assimilation, respectively. Although no direct determinations of isotopic discrimination have been reported for ammonification, the process most relevant for this study, the combined effects of ammonification and nitrification in a small watershed favored ¹⁴N slightly (2). However, an isotopic effect favoring ¹⁵N (β = 0.99902) has been reported for symbiotic N₂ fixation by Trifolium pratense L. (14), and the possibility of other such phenomena cannot be disregarded. Any results of isotopic discrimination phenomena would be orders of magnitude smaller than the disappearances of ¹⁵N measured in roots and crowns of plants in the present study.

Another assumption underlying the calculation of data in Figure 2 is that the value of α, the mean excess ¹⁵N concentration in each organ, declined in a linear manner between the first and fourth harvest. This simplification was reasonably valid for the roots and crowns analyzed for Figure 2. Analysis of the ¹⁵N concentration in those organs at the second and third harvest suggested that the linear model overestimated the value of α by approximately 13%. If the true value of α were 13% less than that used for calculations in this study, then the actual amount of N transferred to produce the observed dilution of ¹⁵N would have been proportionately larger. This fact emphasizes once more the minimal nature of the calculated flows reported in Figure 2.

Table 1 shows that nitrogenous pools turn over in the crowns and roots of plants subjected to several clippings, but the causative factors associated with that phenomenon are not clear. Nitrogen may be available for translocation because those root proteins were respired to provide energy in the absence of current photosynthesis (6, 7). Alternatively, protein degradation may represent a mechanism for providing N required to synthesize new leaves at a time of decreased N₂ fixation and/or soil N uptake. Another possibility is that N molecules always are moving among metabolic pools in different plant organs (18). The classic demonstration of isotope movement and dilution in plants was made with ³²P in intact maize (13). The data in Table 1 suggest that for intact plants in the present study there was a similar turnover of ¹³N in crowns of both species and in clover roots but not in grass roots. A comprehensive comparison of nitrogen turnover in clipped versus intact plants would be best accomplished in hydroponic studies that allow a rapid, definitive change from ¹⁴N to ¹³N. The advantage of the current experiment over such artificial manipulations is that our data establish that nitrogenous pools turn over in crowns and roots of clipped plants under normal edaphic conditions.

LITERATURE CITED


