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# The influence of below-ground herbivory and defoliation of a legume on nitrogen transfer to neighbouring plants

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**Summary**

**1.** Both foliar and root herbivory can alter the exudation of carbon from plant roots, which in turn can affect nitrogen availability in the soil. However, few studies have investigated the effects of herbivory on N fluxes from roots, which can directly increase N availability in the soil and uptake by neighbouring plants. Moreover, the combined effects of foliar and root herbivory on N fluxes remains unexplored.

**2.** We subjected the legume white clover (*Trifolium repens* L.) to defoliation (through clipping) and root herbivory (by an obligate root-feeding nematode, *Heterodera trifolii* Goggart) to examine how these stresses individually, and simultaneously, affected the transfer of *T. repens*-derived N to neighbouring perennial ryegrass (*Lolium perenne* L.) plants using $^{15}$N stable-isotope techniques. We also examined the effects of defoliation and root herbivory on the size of the soil microbial community and the growth response of *L. perenne*.

**3.** Neither defoliation nor root herbivory negatively affected *T. repens* biomass. On the contrary, defoliation increased root biomass (34%) and total shoot production by *T. repens* (100%). Furthermore, defoliation resulted in a fivefold increase in *T. repens*-derived $^{15}$N recovered in *L. perenne* roots, and increased the size of the soil microbial biomass (77%). In contrast, root herbivory by *H. trifolii* slightly reduced $^{15}$N transfer from *T. repens* to *L. perenne* when *T. repens* root $^{15}$N concentration was included as a covariate, and root herbivory did not affect microbial biomass. Growth of *L. perenne* was not affected by any of the treatments.

**4.** Our findings demonstrate that defoliation of a common grassland legume can substantially increase the transfer of its N to neighbouring plants by directly affecting below-ground N fluxes. These finding require further examination under field conditions but, given the prevalence of N-limitation of plant productivity in terrestrial ecosystems, increased transfer of N from legumes to non-N-fixing species could alter competitive interactions, with implications for plant community structure.

*Key-words*: legume, nitrogen dynamics, nitrogen limitation, plant-parasitic nematodes, rhizodeposition

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## Introduction

Ecologists have long recognized that both above- and below-ground herbivory can have major consequences for primary producers, altering plant community structure and influencing rates of vegetation succession (McNaughton *et al*. 1989; Brown & Gange 1990; Brown & Gange 1992; Van der Putten, Van Dijk & Peters 1993; Bardgett 2005). However, over the past decade there has been a growing appreciation of the importance of indirect effects of above- and below-ground herbivory on plant communities, via their influence on soil biological properties and processes (Bardgett, Wardle & Yeates 1998; Bardgett & Wardle 2003; Wardle *et al*. 2004; Bardgett *et al*. 2005). In particular, it has become apparent that plant physiological responses to both foliar and root herbivory that alter rates of root exudation can influence the activity of

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rhizosphere microbes, which rely on exudates as their primary nutrient source, thereby altering rates of nutrient mineralization (Yeates *et al*. 1998, 1999; Hamilton & Frank 2001). Such responses of plants to herbivory therefore have the potential to alter nutrient availability and acquisition by plants, thereby influencing local-scale plant–plant interactions.

To date, most studies investigating the relationship between herbivory and plant inputs below ground have focused on carbon rhizodeposition. For instance, at the individual plant level, defoliation of a range of plants has been shown to increase the exudation of isotopically labelled C from roots into the soil (Holland, Chang & Crossley 1996; Hamilton & Frank 2001; Paterson *et al*. 2003, 2005; Murray *et al*. 2004; but cf. Todorovic *et al*. 1999; Mikola & Kytöviita 2002; Dilkes, Jones & Farrar 2004), which can stimulate microbial activity in the root zone (Mawdsley & Bardgett 1997; Guitian & Bardgett 2000) and increase soil nitrogen availability and its uptake by defoliated plants (Hamilton & Frank 2001; Ayres *et al*. 2004). Similarly, below-ground herbivory of clover has been shown to enhance transfer of C from plant roots to the rhizosphere, resulting in a stimulation of soil microbial biomass (Yeates *et al*. 1998, 1999; Bardgett, Denton & Cook 1999; Denton *et al*. 1999). These studies point to an indirect and short-term pathway whereby both above- and below-ground herbivory can enhance soil N availability to plants, via increased C rhizodeposition, leading to a stimulation of microbial populations and enhanced rates of N mineralization. However, few studies have investigated the direct effect of herbivory on the rhizodeposition of N from plants, which may also enhance N availability in the soil. For instance, below-ground herbivory of clover by root-feeding nematodes has been shown to increase the exudation of N into the soil, resulting in increased transfer of clover-derived N to a neighbouring grass species, stimulating its growth (Bardgett *et al*. 1999; Dromph *et al*. 2006). The effect of above-ground herbivory, and combined effects of root and foliar herbivory, on short-term N rhizodeposition and below-ground N transfer have not been investigated.

In this study, we investigated the effects of both defoliation through clipping and root herbivory on the transfer of N from a legume (*Trifolium repens*) to a neighbouring grass (*Lolium perenne*). We also measured the growth response of each plant species to defoliation and root herbivory of *T. repens*, and effects on the soil microbial biomass. *Trifolium repens* is the most abundant legume in European and New Zealand grasslands, and often co-occurs with grass species, including *L. perenne* (Whitehead 1995). Moreover, *T. repens* is frequently subject to both defoliation (by livestock and other herbivores, as well as cutting) and root herbivory (Cook *et al*. 1992; Whitehead 1995). In addition, up to 80% of grass N may be derived from clover (Broadbent, Nakashima & Chang 1982; Boller & Nösberger 1987; Ledgard 1991). In this study, we experimentally controlled root herbivory by inoculating microcosms with *Heterodera trifolii*, a widespread obligate root-feeding nematode that parasitizes *T. repens* throughout the UK and New Zealand, and is associated with reduced *T. repens* biomass (Skipp & Christensen 1983; Cook & York 1985; Cook *et al*. 1992). We tested the hypothesis that both above-ground clipping and below-ground herbivory would increase the transfer of *T. repens*-derived N to neighbouring *L. perenne* plants, thereby benefiting its growth. We predicted that the transfer of *T. repens*-derived N to neighbouring plants would be greatest when defoliation and root herbivory operate together. These hypotheses were tested in model *T. repens–L. perenne* systems, using *T. repens* plants whose foliage was labelled with $^{15}N$ to enable measurement of N transfer.

## Materials and methods

A brown earth soil was collected from an unfertilized hay meadow at Colt Park, Ingleborough National Nature Reserve, north-west England (54°12′ N, 2°21′ W). Colt Park meadow is an *L. perenne–Cynosurus cristatus* grassland on a soil of moderate–high residual fertility (Smith *et al*. 2003). The soil was passed through a 6-mm sieve and then defaunated by three freezing (–20 °C) and heating (50 °C) cycles. Several other studies have employed a similar method to defaunate soil (Laakso & Setälä 1999a, 1999b; Liiri *et al*. 2002). Although this defaunation procedure results in the death of some microbes and may alter their community composition, a soil microbial community remains (Laakso & Setälä 1999a). Defaunated soil was added to 40 0·75-l pots (10 cm diameter) into which two *T. repens* cuttings and two *L. perenne* tillers were planted. The plants were placed in a growth chamber maintained at 15 °C with 16/8 h light/dark cycles.

The treatments (± *H. trifolii*, ± clipping and ± labelling with $^{15}N$) were applied in a fully factorial randomized block design with five replicate blocks. Treatments were not replicated within each block. After 90 days, half the pots were inoculated with *H. trifolii* (an obligate root-feeding nematode of *T. repens* that does not attack *L. perenne*) by adding a suspension of ≈1000 infective juveniles to the soil. The *H. trifolii* inoculum was prepared from full cysts extracted from pots of sandy soil planted with *T. repens*. Clean cysts were placed on sieves standing in shallow trays filled with water, and infective juveniles emerging from eggs within these cysts were collected daily and stored at 2 °C. Suspensions of juveniles were bulked and concentrated by settling and decanting at 2 °C, and aliquots taken from the suspension were pipetted into soil around the *T. repens* plants. The clipping treatment of *T. repens* (removal of ≈50% of leaves) was applied on two occasions (days 174 and 186); *L. perenne* plants were not clipped. Removal of 50% leaf area was considered appropriate as it is similar to defoliation of clover-dominated pasture (Orr *et al*. 2004). The short time between

clipping treatment and labelling, compared with the *H. trifolii* treatment, was due to the ephemeral (days) nature of defoliation on nutrient fluxes from plant roots to soil (Paterson & Sim 1999, 2000). In contrast, *H. trifolii* needed time to colonize the roots of *T. repens* after inoculation. Half the pots were labelled with $^{15}N$ for 7 days starting on day 188; the remaining unlabelled pots were used to determine the natural abundance of $^{15}N$ stable isotopes and were not included in the statistical analysis of any measure. Labelling with $^{15}N$ consisted of immersing three trifoliate leaves of each *T. repens* plant into a 10 at % $^{15}N$-labelled 300-mM $KNO_3$ solution for 7 days immediately before the end of the experiment (Bardgett *et al*. 1999).

The pots were destructively harvested 195 days after establishment, immediately after labelling with $^{15}N$. Roots from each species were carefully separated from the soil. Nodules were clearly visible on clover roots, indicating that they had successfully formed an association with *Rhizobium*. Shoot and root biomass for each species was determined by drying and weighing plant material (70 °C). A subsample of shoot and root material was ground for stable isotope analysis, which was conducted on a Carlo Erba elemental analyser (CE Elantech, Lakewood, USA) coupled to a isotope ratio mass spectrometer (Denis Leigh Technologies, Manchester, UK). Separate clover and grass-root subsamples, taken from block 1 pots only, were analysed for root infestation by *H. trifolii*: only a single measurement of clover and grass-root infestation was made for each treatment. Roots were cleared, mixed with distilled water (25 ml) and acid fuchsin stain solution (1 ml), and boiled for 30 s (Byrd, Kirkpatrick & Barker 1983), then stained nematodes within the roots were counted using a stereomicroscope. In addition, *H. trifolii* cysts were extracted from 100 g (FW) soil from each microcosm by elutriation for 90 s at 4·5 l $min^{-1}$, collected from the floated debris by hand under a stereomicroscope, and counted. Soil microbial biomass C and N were determined using the fumigation–extraction procedure (Brookes *et al*. 1985; Vance, Brookes & Jenkinson 1987).

The concentration of $^{15}N$ in plant tissues was calculated using the following equation:

$$^{15}N_{conc} = N_{conc} \times (^{15}N_{at\%}/100) \qquad \text{eqn 1}$$

where $^{15}N_{conc}$ is the concentration of $^{15}N$ in the plant tissue, $N_{conc}$ is the concentration of N in the plant tissue, and $^{15}N_{at\%}$ is the percentage of N atoms in the plant tissue that are $^{15}N$ rather than $^{14}N$. The natural abundance of $^{15}N$ in unlabelled plants was averaged across the five replicates of each treatment combination, and this value was subtracted from the $^{15}N$ concentration of each of the labelled plants subjected to that treatment, to determine excess $^{15}N$ concentrations.

Data were analysed using generalized linear models (Nelder & Wedderburn 1972) with the GENMOD procedure in SAS ver. 8·0 (SAS Institute 1990). Defoliation, *H. trifolii* and block were independent variables in each analysis, and *T. repens* root excess $^{15}N$ concentration was used as a covariate for analysis of grass excess $^{15}N$ data, as $^{15}N$ transfer to *L. perenne* was expected to relate to the concentration of $^{15}N$ in *T. repens* roots (Bardgett *et al*. 1999; Dromph *et al*. 2006). *Heterodera trifolii* inoculation was not included as an independent variable for the analysis of *H. trifolii* cyst abundance in soil, as only a single cyst was found in the uninoculated pots. The number of *H. trifolii* cysts in the soil, *T. repens* and *L. perenne* shoot and root biomass, and microbial biomass C and N were log-transformed to meet assumptions of normality and homogeneity of variance. Statistical significance was taken as $P < 0{\cdot}05$.

## Results

Inoculation of *T. repens* with the root-feeding nematode was successful, resulting in an average of 917 cysts per kg soil in the *H. trifolii* treatments; only a single cyst was found in one sample of the uninoculated soil, confirming that the initial defaunation procedure had been successful. Defoliation did not significantly affect cyst abundance in the inoculated soil; mean ± SE number of cysts per kg dry soil were 587 ± 237 in the unclipped treatment and 1248 ± 415 in the clipped treatment ($F_{1,4} = 0{\cdot}1$, $P > 0{\cdot}1$). Consistent with the soil data, no *H. trifolii* were observed in *T. repens* roots from uninoculated pots, whereas 1318 *H. trifolii* per g root were observed in roots of undefoliated *T. repens*, and 411 *H. trifolii* per g root were observed in roots of defoliated *T. repens*. This level of infection is equivalent to 66 nematodes per plant in the undefoliated treatment and 21 nematodes per plant in the defoliated treatment; around 25 nematodes per plant is typical of lightly infested grass–clover pastures in the UK (Cook *et al*. 1992). No *H. trifolii* were observed in *L. perenne* roots from any of the treatments.

Neither defoliation nor infection with *H. trifolii* affected *T. repens* shoot biomass at the end of the experiment (Fig. 1a). However, defoliation doubled *T. repens* total shoot biomass production, when calculated as the biomass of accumulated clippings taken from the two defoliation events and shoot mass at the end of the experiment ($F_{1,4} = 43{\cdot}9$, $P = 0{\cdot}003$; Fig. 1b), indicating that *T. repens* compensated for the tissue lost as a result of defoliation. Defoliation also increased *T. repens* root biomass by 34% at the end of the experiment ($F_{1,4} = 5{\cdot}1$, $P = 0{\cdot}024$; Fig. 1c). Labelling of *T. repens* with $^{15}N$ was successful: clover-root excess $^{15}N$ values were 3·9 μg per g root when averaged across all treatments (Fig. 1d). The allocation of $^{15}N$ to roots of *T. repens* was unaffected by any of the treatments.

Defoliation of *T. repens* significantly increased the size of the soil microbial biomass (microbial biomass C) by 77% ($F_{1,4} = 15{\cdot}4$, $P < 0{\cdot}001$), but had no effect on the amount of N contained within microbes (microbial biomass N) (Fig. 2). Inoculation with *H. trifolii*, and

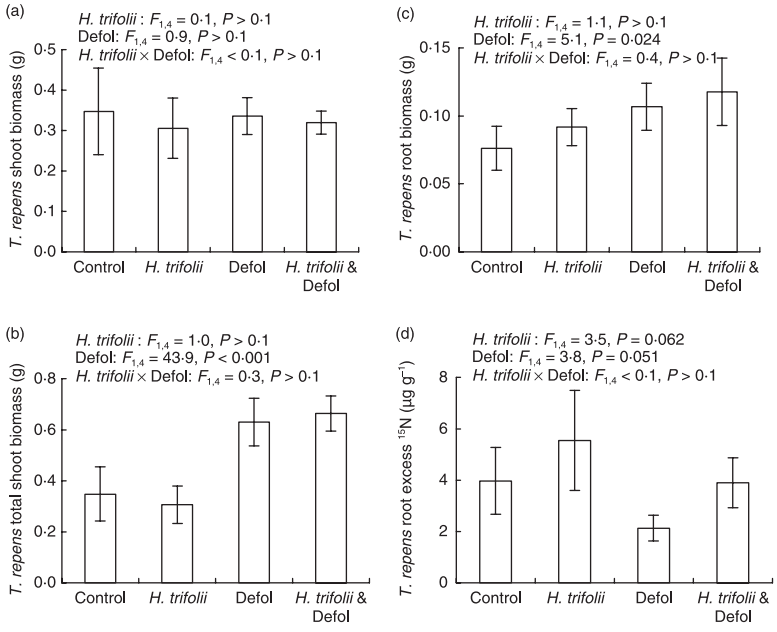


**Fig. 1.** Effect of the root herbivore *Heterodera trifolii* and defoliation on *Trifolium repens* (a) shoot biomass; (b) total shoot biomass production (shoot biomass plus clippings); (c) root biomass; and (d) root excess $^{15}$N at end of experiment. Results from a generalized linear model are also shown (main effects and interaction). Values are means (± SE) of five replicates. Defol, defoliation.

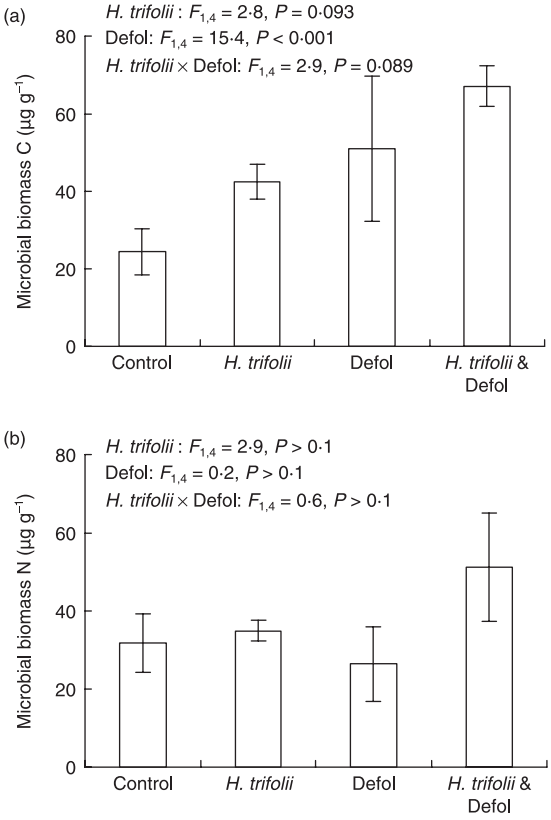


**Fig. 2.** Effect of the root herbivore *Heterodera trifolii* and defoliation on microbial biomass (a) carbon; (b) nitrogen. Results from a generalized linear model are also shown (main effects and interaction). Values are means (± SE) of five replicates. Defol, defoliation.

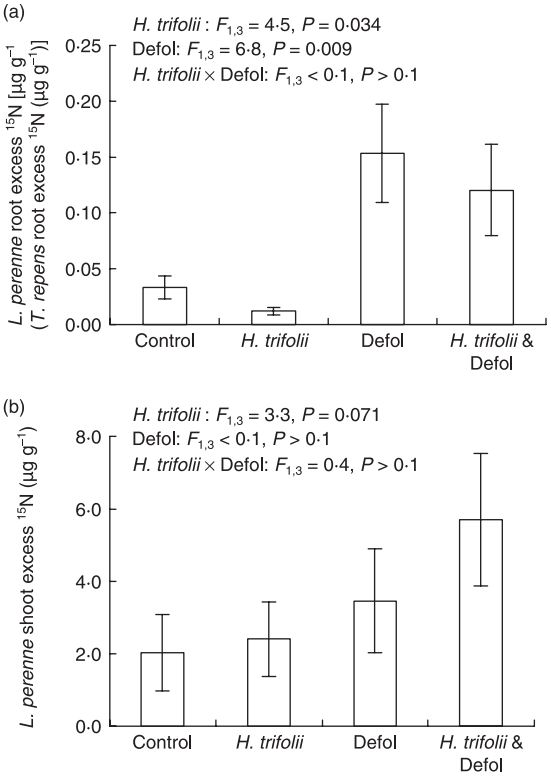


**Fig. 3.** Effect of the root herbivore *Heterodera trifolii* and defoliation on *Trifolium repens*-derived excess $^{15}$N concentration in *L. perenne* (a) roots per unit excess $^{15}$N in *T. repens* roots, and (b) shoots. Results from a generalized linear model are also shown (main effects and interaction). Values are means (± SE) of five replicates. Defol, defoliation. Denominator df = 3 in these analyses because *T. repens* root excess $^{15}$N was included as a covariate.

the interaction between *H. trifolii* and defoliation, did not affect microbial biomass C or N.

Both inoculation of *T. repens* with *H. trifolii* and defoliation significantly affected the recovery of *T. repens*-derived $^{15}$N in grass roots when *T. repens* root $^{15}$N concentration was included as a covariate (*H. trifolii*, $F_{1,3} = 4{\cdot}5$, $P = 0{\cdot}034$; defoliation, $F_{1,3} = 6{\cdot}8$, $P = 0{\cdot}009$; Fig. 3a). Defoliation of *T. repens* resulted in a fivefold increase of $^{15}$N recovery in *L. perenne* roots, while inoculation with *H. trifolii* reduced $^{15}$N transfer from *T. repens* to *L. perenne* by 13%. The concentration of *T. repens*-derived $^{15}$N in grass shoots was not affected by the treatments (Fig. 3b), and neither treatment altered *L. perenne* root or shoot biomass at the end of the experiment (Fig. 4).

## Discussion

The aim of our study was to test the hypothesis that both defoliation and root herbivory of clover (*T. repens*) stimulates soil microbial biomass and the flux of *T. repens*-derived N to neighbouring plants. This was achieved by measuring the effect of defoliation and root herbivory on the soil microbial biomass and the transfer of *T. repens*-derived $^{15}$N to the neighbouring

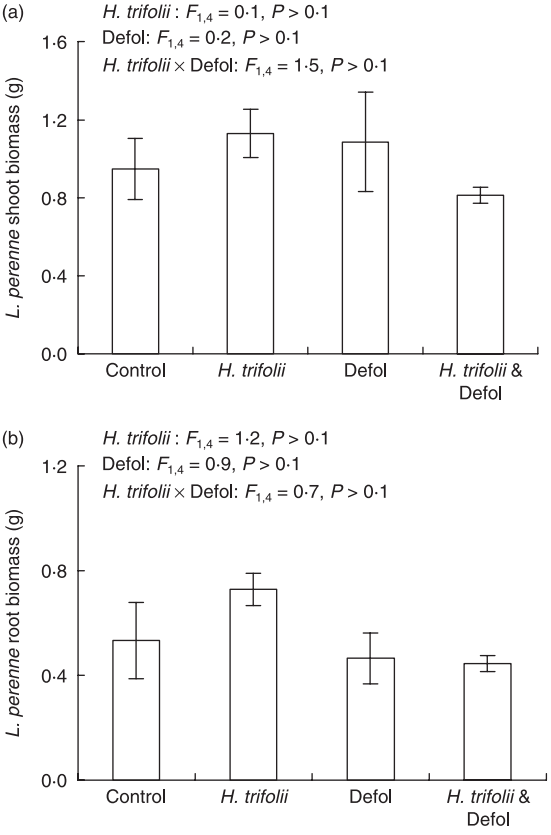


**Fig. 4.** Effect of the root herbivore *Heterodera trifolii* and defoliation on *Lolium perenne* (a) shoot, and (b) root biomass. Results from a generalized linear model are also shown (main effects and interaction). Values are means (± SE) of five replicates. Defol, defoliation.

grass *L. perenne*. Defoliation resulted in a fivefold increase in concentrations of $^{15}$N in *L. perenne* roots, indicating increased transfer of N from *T. repens*. The concentration of $^{15}$N in *L. perenne* shoots was also greater in the defoliated treatment; however, this increase was not significant, possibly because the shoots are an extra 'step' removed from the source of $^{15}$N. Although we cannot be certain of the mechanism behind the increase in N transfer from *T. repens* to *L. perenne*, the short period between $^{15}$N labelling of *T. repens* and $^{15}$N sampling of *L. perenne* suggests a direct physiological mechanism, such as increased exudation of N to the soil and/or transfer of N by mycorrhizae. Nitrogen transfer from legumes to non-legumes via mycorrhizae has been demonstrated in other studies, for example, from *Trifolium alexandrinum* to *Zea mays* (Frey & Schüepp 1992). Regardless of the mechanism, this is the first time, as far as we are aware, that defoliation has been shown to increase N transfer from one plant species to a neighbouring plant species ('direct pathway', Fig. 5). Defoliation has been shown to stimulate soil N availability through effects on the physiology of trees (Ayres *et al*. 2004) and a grazing-tolerant grass (Hamilton & Frank 2001). However, this was thought to be the result of increased C inputs enhancing the mineralization of soil organic N via a stimulation of the soil microbial community ('indirect pathway', Fig. 5; Hamilton & Frank 2001), rather than increased N inputs into the soil by the defoliated plants. The positive effect of defoliation on microbial biomass that we observed indicates that the indirect pathway may also have operated in this study, although we did not measure N mineralization in the soil. The results presented here, in combination with evidence from the literature, suggest that defoliation can increase N availability via both a direct and indirect pathway (Fig. 5). First, defoliated plants can increase N inputs below-ground, resulting in enhanced transfer to neighbouring plants (direct pathway). Second, defoliated plants often increase soil C inputs, stimulating the activity of the soil microbial community that mediates N-mineralization rates (indirect pathway) (Hamilton & Frank 2001). Further studies are required to determine if defoliation of other legumes, and non-leguminous species, influences N transfer to neighbouring plants.

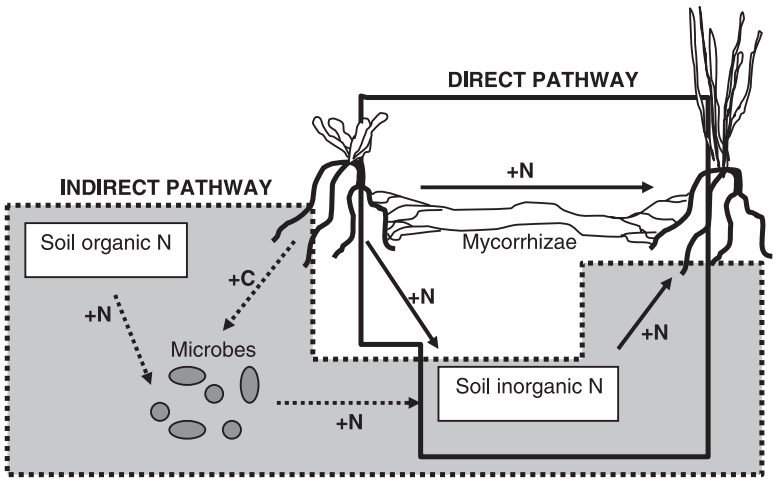


**Fig. 5.** Conceptual diagram showing how defoliation can increase soil nitrogen availability via direct (grey dotted box) and indirect (solid lined box) pathways.

Inoculation of *T. repens* with *H. trifolii* also altered N transfer from *T. repens* to *L. perenne*, resulting in a 13% reduction in N transfer when *T. repens* root excess $^{15}$N concentration was used as a covariate. This is surprising given that two other studies, using a similar approach, have shown increased $^{15}$N transfer from *T. repens* to *L. perenne* in response to root feeding by nematodes. Bardgett *et al*. (1999) observed that *H. trifolii* increased $^{15}$N flux from *T. repens* to *L. perenne*; and Dromph *et al*. (2006) observed a positive relationship between the density of two root-feeding nematodes (*H. trifolii* and *Pratylenchus* sp.) in *T. repens* roots, and $^{15}$N transfer to, and growth of, *L. perenne*. The study by Bardgett *et al*. (1999) had a long period between labelling and sampling (84 days), whereas we sampled immediately after labelling with $^{15}$N. Thus our contrasting findings may indicate that increased transfer of *T. repens*-derived N as a result of root herbivory may become apparent only after several weeks or months. Dromph *et al*. (2006) observed increased *T. repens*-derived

N transfer associated with greater densities of plant-parasitic nematodes; however, two nematode species were present in this study (*H. trifolii* and *Pratylenchus* sp.), which may have caused the different results.

We found that the growth of *T. repens*, measured as shoot and root biomass at the end of the experiment, was unaffected by root herbivory by an obligate root-feeding nematode. In contrast, defoliation of *T. repens* significantly increased both total accumulated shoot biomass (100%), indicating compensatory growth, and root biomass (34%), relative to undefoliated plants. Denton *et al*. (1999) found that low-level infestation of *T. repens* with *H. trifolii* did not affect shoot or root biomass, but high-level infestation reduced shoot biomass (Denton *et al*. 1999). In contrast, Bardgett *et al*. (1999) observed greater clover root biomass in the presence of *H. trifolii* 84 days after inoculation. It has long been known that defoliation can promote compensatory growth in a range of plants (McNaughton 1985; Hamilton & Frank 2001; Ayres *et al*. 2004) including *T. repens* (del Val & Crawley 2004). Our findings indicate that moderate rates of defoliation can promote root and shoot production by *T. repens*, and simultaneously increase N transfer to *L. perenne*. Given that N-limitation is common in terrestrial ecosystems (Vitousek & Howarth 1991), increased N supply has the potential to influence plant production in grassland ecosystems. Several studies have observed positive effects of herbivory on plant production in natural ecosystems, which have been attributed to a variety of mechanisms, such as selective feeding altering plant community structure, or stimulation of soil nutrient availability due to the addition of labile organic matter in animal waste (McNaughton *et al*. 1997a, 1997b; Augustine & McNaughton 1998; Frank & Groffman 1998); although negative effects of herbivory on plant productivity are also common (Pastor *et al*. 1993; Bardgett & Wardle 2003). Our finding may represent a previously unrecognized mechanism for the stimulation of primary productivity, which requires further testing under field conditions.

The growth of *L. perenne*, measured as shoot and root biomass, was not affected by any of the experimental treatments. This is surprising given the increase in N transfer to *L. perenne* as a result of defoliation and the N-responsive nature of this species (Daepp, Nösberger & Lüscher 2001; Wagner *et al*. 2001). However, the lack of response may be due to the relatively short period between the first defoliation event and the end of the experiment (3 weeks), and/or the inherently high availability of N in the soil due to its moderate–high fertility and a pretreatment of sieving and defaunation (several freeze–thaw cycles). Both these disturbances are likely to have resulted in a flush of N to the soil which, in combination with the N fixed by rhizobia on the roots of *T. repens*, may have meant that *L. perenne* was not N-limited. Contrary to our findings, Bardgett *et al*. (1999) and Dromph *et al*. (2006) observed increased *L. perenne* growth when neighbouring *T. repens* was subjected to root herbivory. In these experiments, increased growth of *L. perenne* coincided with increased transfer of *T. repens*-derived N to *L. perenne* (Bardgett *et al*. 1999; Dromph *et al*. 2006). Both Bardgett *et al*. (1999) and Dromph *et al*. (2006) used soil of low N status, which might have resulted in N-limitation of *L. perenne*, and potentially explains why *L. perenne* biomass increased in response to enhanced N transfer from *T. repens* in their studies, but not in ours.

As far as we are aware, this study is the first to investigate the effects of both defoliation and below-ground herbivory on nutrient fluxes to neighbouring plants. The results show that both types of herbivory may influence N transfer between plant species. However, they do not support our hypothesis that defoliation and below-ground herbivory interact positively to increase N transfer from *T. repens* to *L. perenne*. Consistent with our findings, studies of plant responses to above- and below-ground herbivory often report few interactive effects (Müller-Schärer & Brown 1995; Maron 1998; Rudgers & Hoeksema 2003), despite evidence that above- and below-ground herbivores of the same plant can influence each other (Moran & Whitham 1990; Masters & Brown 1992; Masters, Brown & Gange 1993). The results presented here warrant further investigation under field conditions and with real above-ground herbivores. However, given the prevalence of N-fixing plants in grassland and early successional communities (Stevens & Walker 1970; Whitehead 1995), foliar herbivory could have important implications for plant community structure and succession, as increased N transfer from N-fixers to neighbouring non-N-fixing species might alter their competitive interactions.

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