Does nitrogen transfer between plants confound ^{15}N-based quantifications of N_2 fixation?

Georg Carlsson · Kerstin Huss-Danell

Abstract

Background and aims Transfer of fixed N from legumes to non-legume reference plants may alter the ^{15}N signature of the reference plant as compared to the soil N available to the legume. This study investigates how N transfer influences the result of ^{15}N-based N_2 fixation measurements.

Methods We labelled either legumes or non-legumes with ^{15}N and performed detailed analyses of ^{15}N enrichment in mixed plant communities in the field. The results were used in a conceptual model comparing how different N transfer scenarios influenced the ^{15}N signatures of legumes and reference plants, and how the resulting N_2 fixation estimate was influenced by using reference plants in pure stand or in mixture with the legume.

Results Based on isotopic signatures, N transfer was detected in all directions: from legume to legume, from legume to non-legume, from non-legume to legume, from non-legume to non-legume. In the scenario of multidirectional N transfer, N_2 fixation was overestimated by using a reference plant in pure stand.

Conclusions Fixed N transferred to neighbouring reference plants modifies the ^{15}N signature of the soil N available both to the reference plant and the N_2-fixing legume. This provides strong support for using reference plants growing in mixture with the legumes for reliable quantifications of N_2 fixation.

Abbreviations

$\delta^{15}N$	Part per thousands deviation from the $^{15}N/^{14}N$ ratio of atmospheric N_2
ID	Isotope dilution
NA	Natural abundance
pNdfa	Proportion of N derived from atmosphere (*i.e.* from N_2 fixation)

G. Carlsson (✉) · K. Huss-Danell
Department of Agricultural Research for Northern Sweden,
Swedish University of Agricultural Sciences,
901 83 Umeå, Sweden
e-mail: georg.carlsson@slu.se

G. Carlsson
Department of Biosystems and Technology, Swedish
University of Agricultural Sciences,
PO Box 103, 230 53 Alnarp, Sweden

Introduction

Biological nitrogen (N_2) fixation in the symbiotic association between legumes and rhizobia is a valuable N source to both natural and managed ecosystems, and a crucial factor for increased sustainability of agricultural production with minimal or no application of industrial N fertilizers (Carlsson and Huss-Danell 2003; Jensen et al. 2012). Fixed N is made available to neighbouring and succeeding plants via

degradation of litter (both below and above ground), animal excreta and via N transfer in soil mediated by mycorrhiza and rhizodeposition (*e.g.* Ledgard and Steele 1992; Johansen and Jensen 1996; Høgh-Jensen and Schjoerring 1997, 2000; Mårtensson et al. 1998; Wivstad 1999; Hauggaard-Nielsen and Jensen 2005).

Below-ground N transfer has been studied in particular in the direction from N_2-fixing legumes to neighbouring non-legumes, and has been observed in several studies using the indirect ^{15}N isotope dilution (ID) method (*e.g.* Brophy et al. 1987; McNeill and Wood 1990; Ledgard 1991; Høgh-Jensen and Schjoerring 1997, 2000; Dahlin and Stenberg 2010) and the direct ^{15}N leaf-feeding method (*e.g.* Ledgard et al. 1985; Russell and Fillery 1996; McNeill et al. 1997; Høgh-Jensen and Schjoerring 2000, 2001; Dahlin and Stenberg 2010; Isaac et al. 2012). Analyses of the ^{15}N natural abundance (NA) in plant tissues also showed that the $\delta^{15}N$ values in grass and barley plants grown within the rooting zones of N_2-fixing legumes was more similar to the $\delta^{15}N$ of the legumes than when the non-legumes were grown at distance from the legumes, indicating a likely transfer of fixed N to these non-legumes (Pate et al. 1994).

Transfer of fixed N from legumes to non-legumes has been suggested to cause an underestimation of N_2 fixation when the NA and ID methods are used (Broadbent et al. 1982; Ledgard et al. 1985; Brophy et al. 1987; McNeill and Wood 1990). The proposed mechanism for such an underestimation is that if the non-legume reference plant derives a part of its N via transfer of fixed N from the legume it does not reflect the ^{15}N in soil N being available to the legume. There may thus be a trade-off between using reference plants grown close to or in mixture with the legume, with the risk of confounding effects of N transfer, and using reference plants grown in pure stand with the risk that spatial variations in soil ^{15}N between the legume plot and the reference plot affect the estimate (Bremer and Van Kessel 1990; Nesheim and Øyen 1994; Jacot et al. 2000; Holdensen et al. 2007).

Ledgard and Steele (1992) suggested that N transfer may confound ^{15}N-based quantifications of N_2 fixation if it occurs directly from the legume to the non-legume. However, studies using direct leaf-feeding of ^{15}N (Ledgard et al. 1985) have shown that N transfer also occurs in the direction from non-legume to legume, albeit at a lower rate than from legume to non-legume (Johansen and Jensen 1996; Høgh-Jensen and

Schjoerring 2000; Gylfadóttir et al. 2007; Rasmussen et al. 2007; Pirhofer-Walzl et al. 2012). In addition, recent findings have shown that N transfer also occurs between different legume species grown in mixture (Pirhofer-Walzl et al. 2012). Multidirectional N transfer might be mediated by mycorrhizal hyphae connecting several plants, or via rhizodeposition and mineralization of legume litter which contribute to a soil N pool that is equally available both to the legume and the non-legume reference plant. The importance of N transfer via a common soil N pool is exemplified by findings that rates of rhizodeposition may be about as high or higher than the measured rates of N transfer from legumes to non-legumes (Høgh-Jensen and Schjoerring 2001; Gylfadóttir et al. 2007; Rasmussen et al. 2007).

In light of the growing number of studies pointing to the multidirectional nature of N transfer, there is a need for a conceptual investigation of the consequences that different N transfer scenarios in combinations with different use of reference plants will have on ^{15}N-based quantifications of N_2 fixation. The aim of this study was to analyze the occurrence of N transfer between plants of the same species as well as between species in field experiments with pea-oat and clover-grass mixtures. Further, we have performed calculations of N_2 fixation based on empirical data and theoretical examples, showing the effect of using reference plants in mixture with the legume or in pure stand under different scenarios of N transfer: no transfer; unidirectional transfer from legume to reference plant; and multidirectional transfer, including from legume to legume. We use this analysis for a thorough discussion about how the strategy in choosing reference plant may influence the accuracy of ^{15}N-based measurements of N_2 fixation.

Materials and methods

Field sites and experimental design

Experiments were performed in agricultural fields at two sites in Sweden, Röbäcksdalen in Umeå (63°49′N, 20°17′E, 13 m above sea level) and Ås in Östersund (63°15′N, 14°34′E, 392 m above sea level), during 2 years (2007 and 2008). The site in Umeå is situated on a coastal plain where the soil is a clayey silt loam with a soil pH around 6. The site Ås is an inland locality

situated on a south-west facing slope with a gravely loam soil with a pH around 6.4. At each site, twelve 1.5×1.5 m plots were sown with a mixture of pea (legume; *Pisum sativum* L. cv. Capella) and oat (cereal; *Avena sativa* L. cv. Cilla) in late May–early June 2007. At the same time, 48 1.5×1.5 m plots were sown with a mixture of red clover (legume; *Trifolium pratense* L. cv. Betty) and timothy (grass; *Phleum pratense* L. cv. Jonatan) at both sites. Twenty-four new pea/oat plots were established in early June 2008, while the red clover/timothy plots started in 2007 were also used in 2008. The entire experiment thus comprised 36 pea/oat plots and 48 red clover/timothy plots per site. The pea/oat intercrop consisted of a commercial mixture of 80 % (w/w of seeds) pea (cv. Capella in 2007 and cv. Clara in 2008) and 20 % oat. The red clover /timothy mixture contained 27 % (w/w of seeds) red clover and 73 % timothy. Seeds were obtained from the Swedish company Lantmännen SW seeds. Seeds were mixed (pea together with oat, red clover together with timothy) before sowing the plots using a 1.5 m wide tractor-driven machine which distributed the seeds evenly in rows at 12.5 cm row spacing.

Soil from the top 10 cm soil layer was sampled by a 15 mm diameter soil corer at the end of each experiment, *i.e.* in September 2007 and in August 2008 in pea/oat plots and in August 2008 in red clover/timothy plots. The soil samples were stored frozen (−20 °C) until used for descriptive analyses of total N and total C concentrations. After freeze-drying and homogenization, a composite sample from each site and each experiment (pea/oat and red clover/timothy, respectively) was analyzed using an Elemental analyzer (Flash EA 2000, Thermo Fisher Scientific) at the Department of Forest Ecology and Management, Swedish University of Agricultural Sciences, Umeå. At the site in Umeå, the composite sample of the top 10 cm soil from the pea/oat experiments contained 0.17 % N and 2.4 % C, and the corresponding soil sample from the red clover/timothy experiment contained 0.25 % N and 3.3 % C. At the site in Ås, the composite sample of the top 10 cm soil from the pea/oat experiment contained 0.34 % N and 4.0 % C, and the corresponding soil sample from the red clover/timothy experiment contained 0.39 % N and 4.5 % C.

^{15}N labelling

Each species was sown in outdoor sand-filled plots adjacent to the field plots. The sand plots were constructed of four wooden frames measuring 1×1 m and 50 cm height. The bottom of each frame was covered with a water-permeable barrier against weed growth before the frames were filled with a 40 cm layer of sand and the four species were sown at 2 cm (red clover, timothy) and 5 cm (pea, oat) depths in separate rows. A complete nutrient solution containing ammonium nitrate ($^{15}NH_4$$^{15}NO_3$, 10 atom% ^{15}N) was added to the sand plots to obtain plants with high and uniform ^{15}N content. In 2007, pea and oat plants received a total of 5 g N m^{-2} divided into 2.5 g N m^{-2} in late May, shortly after sowing, and 2.5 g N m^{-2} in late June to early July. In 2008, 6.5 g N m^{-2} in total were added to pea and oat plants, divided into three additions from late May to early July. Clover and timothy plants received 7.3 g N m^{-2} divided into three additions in 2007 (late May to early August) and 4 g N m^{-2} in 2008 (2.5 g N m^{-2} in mid-May and 1.5 g N m^{-2} in early June). The resulting level of ^{15}N enrichment in the ^{15}N-fertilized plants ranged from 0.4 % to 6 % and varied depending on species (lower in pea and clover than in oat and timothy) and time of transplantation.

Transplantation and sampling

Each single pea/oat field plot was randomly assigned to one of three treatment periods (one period in 2007 and two periods in 2008; Table 1) and one of three transplantation treatments. Similarly, each red clover/timothy field plot was randomly assigned to one of four treatment periods (Table 1) and one of three transplantation treatments. The treatments consisted of a reciprocal transplantation design: in each treatment period (Table 1) four replicate plots received ^{15}N-labelled legume plants, four other plots received ^{15}N-labelled non-legume plants, and four plots served as control plots not receiving any ^{15}N-labelled plant. At the starting date of each treatment period ^{15}N-labelled plants were gently dug up from the sand culture with a hand shovel and root systems rinsed in tap water to remove adhering sand. The plants were then carefully planted in the centre of the assigned field plots. In control plots, a legume or non-legume plant was gently dug up from the centre of the plot, shaken free from adhering soil and planted back in its original position, to mimic the treatment to soil and plants in plots receiving ^{15}N-labelled plants. Plastic labels of different colours were used to mark the transplanted

Table 1 Dates and time for potential transfer of each treatment period in the different experiments at the two sites. Each treatment period comprised 12 field experimental plots randomly assigned to receiving a ^{15}N-labelled legume (4 plots), a ^{15}N-labelled non-legume (4 plots), or no ^{15}N-labelled plants (control; 4 plots)

Site	Treatment period	Transplantation	Final sampling	Time for potential transfer (days)
Pea/oat experiment				
Umeå	1	4 July 2007	22 Aug 2007	49
	2	15 June 2008	18 July 2008	33
	3	1 July 2008	15 Aug 2008	45
Ås	1	2 July 2007	30 Aug 2007	59
	2	17 June 2008	22 July 2008	35
	3	2 July 2008	11 Aug 2008	40
Red clover/timothy experiment				
Umeå	1	5 July 2007	12 September 2007	69
	2	22 Aug 2007	30 June 2008	312
	3	5 May 2008	30 June 2008	56
	4	15 June 2008	30 June 2008	15
Ås	1	2 July 2007	28 August 2007	56
	2	29 Aug 2007	2 July 2008	307
	3	7 May 2008	2 July 2008	56
	4	17 June 2008	2 July 2008	15

plants in order to find them at the sampling occasions.

At 2 to 3 weeks intervals following transplantation, shoots of individual legume and non-legume plants were sampled at 10 and 20 cm distances from the centre of each field plot (*i.e.* from transplanted and control plants). The same plots were thus used for the analyses of the ^{15}N signature (expressed in δ^{15}N units) in plants at 10 and 20 cm distance from the transplanted and control plants. In the pea/oat crop the same plots were used for analysis of δ^{15}N-values in pea and in oat, and in the clover/timothy crop the same plots were used for analysis of δ^{15}N-values in clover and in timothy. Different plots were used for different treatment periods (Table 1). On the last sampling occasion of each treatment period, plants were dug up with their root systems intact. Roots were easily identified since they were still attached to the shoots. This allowed detection of ^{15}N enrichment in neighbouring plants, both aboveground and belowground, and ^{15}N dynamics in the labelled and transplanted plants to be analysed.

Analyses

All plant samples were dried at 60 °C for 24 h and then finely ground. Analyses of ^{15}N enrichment, expressed as δ^{15}N units, were performed with a PDZ Europa ANCA-GSL elemental analyzer interfaced to a Sercon 20–20 IRMS with SysCon electronics (Sercon Ltd., Cheshire, UK), at the Laboratory of Applied Physical Chemistry, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium. Differences in plant δ^{15}N and proportion of N derived from atmosphere (pNdfa) were analysed statistically with the Wilcoxon rank sum test using the function *wilcox.test* in the R open source software, version 2.12.1 (R Development Core Team 2010). Significant ($p<0.05$) ^{15}N enrichment in plants sampled next to a ^{15}N-labelled plant as compared to control plots was taken as evidence for the occurrence of N transfer.

Conceptual model of N transfer-mediated changes in plant δ^{15}N

Literature data on rates of N transfer, together with the results from our field experiments, were used to establish a conceptual model for schematic illustrations of how plant ^{15}N signatures of legumes and reference plants would change between a scenario where no N transfer occurs and a scenario with multidirectional N transfer (both legumes and reference plants have access to N transferred from neighbouring legumes). The settings used for calculating the changes in plant δ^{15}N

were: a mixed crop (legumes and reference plants growing together) where the legumes obtained 75 or 25 % of their N from N_2 fixation, respectively. The first step was to set the starting-point $\delta^{15}N$ value of plant-available soil N to 4, and, assuming no discrimination against ^{15}N during uptake of soil N, the non-legume $\delta^{15}N$ was initially also set to 4. In the second step, N transfer from plant roots to a shared pool of plant-available soil N was introduced by adding N with the same $\delta^{15}N$ as the source plants. Third, the $\delta^{15}N$ of N in the shared pool of plant-available soil N was recalculated based on the assumption that legume-derived N comprises 20 % of this shared N pool and that the remaining 80 % originate from soil and transfer from non-legumes (both having the same initial $\delta^{15}N$). The proportion of legume-derived N in the shared pool of plant-available soil N was set to 20 % based on the frequent observations in the scientific literature that non-legumes may obtain 20 % or more of their N via transfer from legumes (Ledgard 1991; Høgh-Jensen and Schjoerring 2000; Gylfadóttir et al. 2007; Rasmussen et al. 2007; Dahlin and Stenberg 2010). In the fourth and last step, non-legume $\delta^{15}N$ was set to the same value as the shared pool of plant-available soil N after N transfer. Legume $\delta^{15}N$ was recalculated according to 75 or 25 % of its N being derived from N_2 fixation (according to the model settings described above) and the remaining N having the same $\delta^{15}N$ as the shared pool of plant-available soil N. The four steps describing the change in plant $\delta^{15}N$ caused by N transfer were illustrated in a schematic figure showing the $\delta^{15}N$ values in the setting with pNdfa=0.75 and pNdfa=0.25, respectively (Fig. 4).

Effects of N transfer and use of reference plant on pNdfa

Calculations of pNdfa according to the NA method were performed based on theoretical data from the conceptual model or empirical data from a field experiment (Carlsson et al. 2009). The equation established by Amarger et al. (1979) was used to calculate pNdfa as follows: pNdfa=$(\delta^{15}N_{ref} - \delta^{15}N_{fix})/(\delta^{15}N_{ref} - B)$, where $\delta^{15}N_{ref}$ and $\delta^{15}N_{fix}$ are the ^{15}N abundance in the reference species and the N_2-fixing legume, respectively, and B is the $\delta^{15}N$ in the legume when obtaining 100 % of its N from N_2 fixation. In the theoretical example, a situation with no N transfer was compared with two N transfer scenarios: unidirectional transfer from legume

to non-legume and multidirectional transfer via a shared pool of plant-available soil N. The calculations also compared the use of reference plants grown in pure stand ($\delta^{15}N_{ref}$ set to the value at step 1 in the conceptual model, i.e. no N transfer) with reference plants grown in mixture with a legume ($\delta^{15}N_{ref}$ changing by N transfer and set to the value at step 4 in the conceptual model). In the unidirectional N transfer scenario, $\delta^{15}N_{fix}$ was set to the value in step 1 in the conceptual model while $\delta^{15}N_{ref}$ was set to the value in step 4. In the multidirectional N transfer scenario, both $\delta^{15}N_{ref}$ and $\delta^{15}N_{fix}$ were set to the corresponding values in step 4 in the conceptual model. The B value was set to 0 in all these situations and scenarios.

The empirical data set was obtained in a field experiment manipulating plant species richness and composition (Carlsson et al. 2009) and was used to calculate pNdfa in red clover during 3 years by using timothy grown in mixture with red clover or timothy grown in pure stand as reference plants. All parameters in the equation were set to measured values in samples from the field experiment. This comparison between using reference plants grown in mixture with the legume or in pure stand was not part of the original publication (Carlsson et al. 2009).

The variations in pNdfa caused by the different use of reference plants under the multidirectional N transfer scenario were compared with other sources of variation in pNdfa estimated with the NA method. Effects of spatial variability in $\delta^{15}N_{ref}$ caused by sampling reference plants at different distance from the legume were calculated by applying the reported range of 3.9 $\delta^{15}N$ units (‰) between the highest and lowest $\delta^{15}N$ values among barley plants sampled along a 4 m row in a field experiment with pea and barley (Holdensen et al. 2007). The range within which the pNdfa would vary as a consequence of this variability was obtained by calculating pNdfa using $\delta^{15}N_{ref}$ values 2‰ above and below the initially set $\delta^{15}N_{ref}$ value, respectively. For an estimation of the effects of variability in the B value, we calculated pNdfa by applying the lowest and highest B values measured in red clover by Carlsson et al. (2006).

Results

The analyses from the first treatment period in the red clover/timothy plots showed no significant effect on

δ^{15}N in plants next to ^{15}N-labelled plants as compared to control plants (not shown). Similarly, a majority of the analyses in pea and oat after potential transfer periods shorter than 30 days showed no significant ^{15}N enrichment in plants sampled next to ^{15}N-labelled pea or oat plants compared to control plants (not shown).

After transfer periods longer than 30 days, δ^{15}N-values were considerably, often significantly, higher in pea plants sampled at 10 cm from ^{15}N-labelled oats or ^{15}N-labelled peas, than in pea plants sampled 10 cm from non-labelled (controls) plants (Fig. 1a). This was observed in shoots at all three sampling times, representing different years and different times from transplantation to sampling (Fig. 1a, Table 1). In general, a similar pattern was found at both sites and in pea shoots sampled at 20 cm from ^{15}N-labelled pea and oat plants, although the δ^{15}N was lower and not as often significantly different from control plots as was the case at 10 cm (Fig. 1b). The δ^{15}N values in pea roots and stubble showed the same general pattern as in shoots (Fig. 1), and was often significantly higher at 10 cm from ^{15}N-labelled pea and oat plants than at 10 cm from control plants (Fig. 1c). The control pea samples from the earliest sampling date, Umeå 18 July 2008, had a consistently higher δ^{15}N than control samples taken later in the growing season (Fig. 1). It is likely that N_2 fixation was not yet highly active early in the season at this high latitude, and that a relatively higher reliance on seed and soil N would lead to a slightly higher δ^{15}N in control plants on this sampling date.

Oat plants were treated (Table 1) and sampled on the same dates as pea on the two sites (Fig. 2). As can be expected from a non-N_2-fixing plant that relies entirely on soil N, the δ^{15}N-values in oat were often higher (Fig. 2) than in N_2-fixing pea (Fig. 1). Similar to the situation in pea, oat plants had increased shoot δ^{15}N-values when sampled 10 cm from ^{15}N-labelled oat or pea as compared to 10 cm from unlabelled (control) plants (Fig. 2a). At all sampling times in Umeå and in mid-August in Ås the largest ^{15}N enrichment relative to control plants was found in oat sampled at 10 cm from ^{15}N-labelled pea plants (Fig. 2a). Only small differences between shoots sampled 20 cm from labelled plants and shoots sampled 20 cm from control plants were noticed, especially in 2008 (Fig. 2b). The δ^{15}N in oat roots and stubble showed similar patterns as in oat shoots, although there were
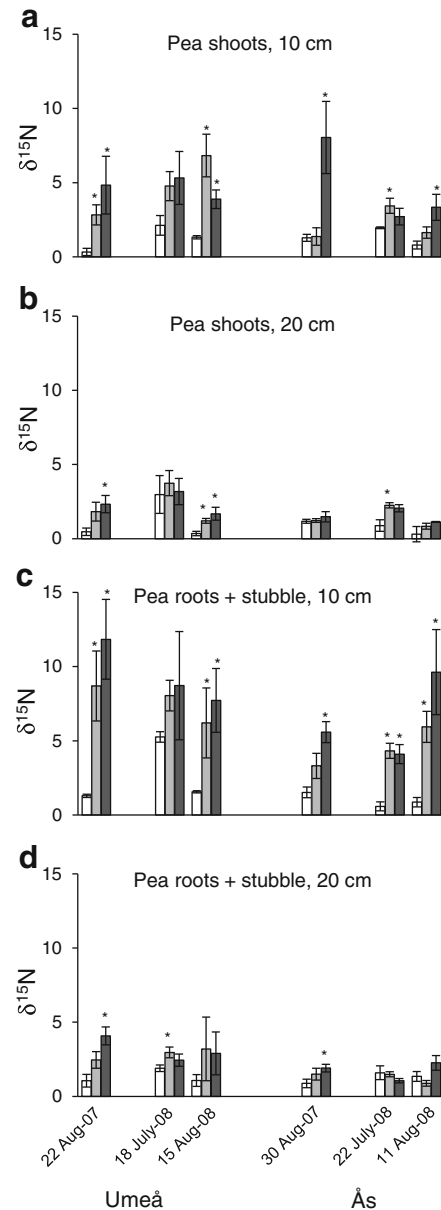

Fig. 1 δ^{15}N-values in shoots (**a**, **b**) and roots and stubble (**c**, **d**) of pea plants sampled at 10 (**a**, **c**) and 20 (**b**, **d**) cm distance from transplanted plants. *Empty bars* represent control plots (no ^{15}N-labelling), *light-grey bars* represent plots with ^{15}N-labelled oat plants and dark-*grey bars* represent plots with ^{15}N-labelled pea plants. *Bars* are mean values ± SE, $n=4$ (except for shoots at 10 cm in Umeå where $n=3$ for control on 22 August 2007 and roots and stubble at 10 cm in Umeå where $n=3$ for control on 22 August 2007 and for 15N-labelled pea on 18 July 2008). An * indicates significantly higher δ^{15}N-value than the control treatment on the same date (Wilcoxon test, $p<0.05$)

larger variations and less clear patterns in the root data (Fig. 2c and d).

Irrespective of site and time since transplantation, red clover shoots sampled 10 and 20 cm from ^{15}N-labelled timothy or ^{15}N-labelled red clover had always increased δ^{15}N-values compared to red clover shoots sampled 10 and 20 cm from non-labelled plants (Fig. 3a and b). There was, unfortunately, a low density of red clover plants in the centre of some experimental plots at the site in Umeå, which led to few replicates and lack of statistically significant differences at 10 cm from labelled and control plants in Umeå (Fig. 3a). Timothy shoots sampled 10 and 20 cm from labelled timothy or red clover had a tendency towards higher δ^{15}N-values than timothy sampled at 10 and 20 cm from control plants at 6 weeks after transplantation, and these differences were clearly significant when the transplantation was done 10 months ago, in the preceding season (Fig. 3c and d).

Step-by-step calculations of changes in plant-available soil δ^{15}N caused by N transfer and subsequent changes in plant δ^{15}N were based on two situations, one where the legume derived 75 % of its N from N_2 fixation (Fig. 4a–d) and one where the legume reliance on N_2 fixation was only 25 % (Fig. 4e–h). These theoretical calculations showed that in a situation with high legume reliance on N_2 fixation, non-legume δ^{15}N decreased from 4 to 3.4 and legume δ^{15}N decreased from 1 to 0.85 as a result of N transfer when the two plants had access to the same pool of plant-available soil N (Fig. 4a–d). In the situation with low legume reliance on N_2 fixation, non-legume δ^{15}N decreased from 4 to 3.8 and legume δ^{15}N decreased from 3 to 2.85 as a result of N transfer to the shared pool of available soil N (Fig. 4e–h).

Calculations of pNdfa based on the different N transfer scenarios and uses of reference plant showed that using a reference plant grown in mixture with the legume when unidirectional N transfer occurs would underestimate pNdfa. In contrast, in the scenario of multidirectional N transfer the use of a reference plant grown in mixture with the legume would give the correct result while using a reference plant grown in pure stand would overestimate pNdfa (Table 2). The difference between the lowest and highest pNdfa was the same in situations of high and low legume reliance on N_2 fixation, which means that the relative variations were higher in the situation of low legume reliance on N_2 fixation (Table 2). The empirical data indicated that pNdfa increased when using a reference
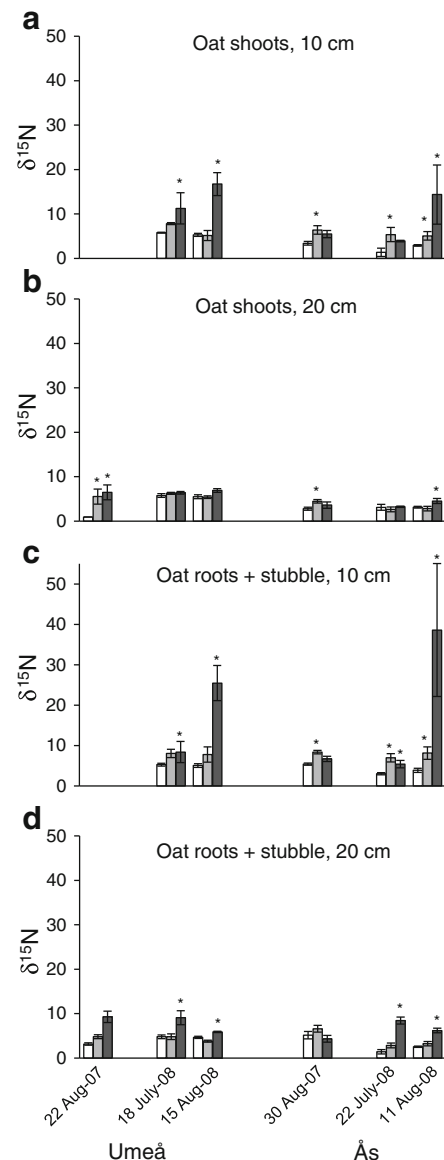

Fig. 2 δ^{15}N-values in shoots (**a**, **b**) and roots and stubble (**c**, **d**) of oat plants sampled at 10 (**a**, **c**) and 20 (**b**, **d**) cm distance from transplanted plants. *Empty bars* represent control plots (no ^{15}N-labelling), *light-grey bars* represent plots with ^{15}N-labelled oat plants and *dark-grey bars* represent plots with ^{15}N-labelled pea plants. Data from Umeå on 22 August 2007 are lacking in **a** and **c** because no oat plants were found at 10 cm from transplanted plants. *Bars* are mean values ± SE, $n=4$ (except for roots and stubble at 10 cm in Ås on 30 August 2007 where $n=3$ for ^{15}N-labelled pea, and for shoots and roots and stubble at 20 cm in Umeå in 22 August 2007 where $n=3$ for ^{15}N-labelled oat and ^{15}N-labelled pea). An * indicates significantly higher δ^{15}N-value than the control treatment on the same date (Wilcoxon test, $p<0.05$)

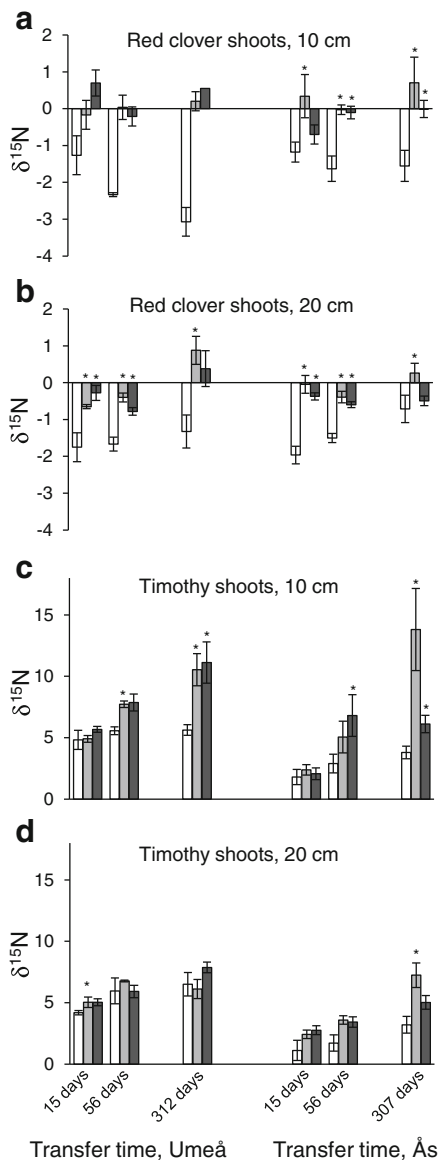plant in pure stand versus using a reference plant grown together with the legume, which is the same

Fig. 3 δ^{15}N-values in shoots of red clover (**a**, **b**) and timothy (**c**, **d**) sampled at 10 (**a**, **c**) and 20 (**b**, **d**) cm distance from transplanted plants. *Empty bars* represent control plots (no ^{15}N-labelling), *light-grey bars* represent plots with ^{15}N-labelled timothy plants and *dark-grey bars* represent plots with ^{15}N-labelled red clover plants. *Bars* are mean values ± SE, *n*=4 but with several exceptions (for red clover in Umeå at 10 cm: *n*=2 for control at 2 and 6 weeks, *n*=3 for ^{15}N-labelled timothy at 2 weeks, *n*=2 for ^{15}N-labelled timothy at 10 months and *n*=1 for ^{15}N-labelled clover at 10 months; for red clover in Ås at 10 cm: *n*=3 for control at 6 weeks; for red clover in Umeå at 20 cm: *n*=3 for control at 2 weeks and for ^{15}N-labelled clover at 10 months; for red clover in Ås at 20 cm: *n*=3 for control at 10 months). An * indicates significantly higher δ^{15}N-value than the control treatment on the same date (Wilcoxon test, *p*<0.05)

pattern as in the theoretical scenarios (Table 2). However, the differences in pNdfa caused by the different use of reference plants in the empirical data set were not statistically significant. The variations in calculated pNdfa values caused by spatial variability in reference plant δ^{15}N or different B values were larger than variations caused by using a reference plant in mixture with the legume versus a reference plant in pure stand (Table 3).

Discussion

Our experimental design allowed studies of N transfer in four different directions: from legume to legume, from legume to non-legume, from non-legume to legume and from non-legume to non-legume. In the red clover/timothy experimental plots, no evidence for N transfer was detected during the first year, probably because these perennial species did not establish extended root systems quickly enough for N transfer to be significant already during the year of establishment. On the other hand, after more extended periods of potential N transfer (longer than 30 days in pea/oat and during the second in red clover/timothy) we found consistently higher δ^{15}N-values in sampled plants growing 10 cm from a ^{15}N-labelled legume or non-legume as compared to the same distance from a non-labelled plant. This demonstrates that nitrogen in all studied species becomes available to all studied neighbouring plants. Our data also indicate that N transfer is not restricted to a distance of 10 cm but occurs to some extent also between plants growing 20 cm apart from each other. Further, we used both annual (pea, oat) and perennial plants (red clover, timothy) and replicated our experiments in 2 years at two sites, a condition that strengthens the generalization of our findings.

Most other studies of N transfer in field experiments have used the direct leaf-feeding technique and kept the plants enclosed in cylinders in the soil. We were interested in how far ^{15}N could spread from a labelled plant and therefore we used plants raised in sand fertilized with ^{15}N to obtain high and uniform levels of ^{15}N enrichment in the labelled plants. We cannot exclude that the transplantation caused a disturbance to the plants, and that breakdown of material from transplanted plants was different from that in undisturbed plants. However, if the transplantation had caused a serious disturbance to the plant
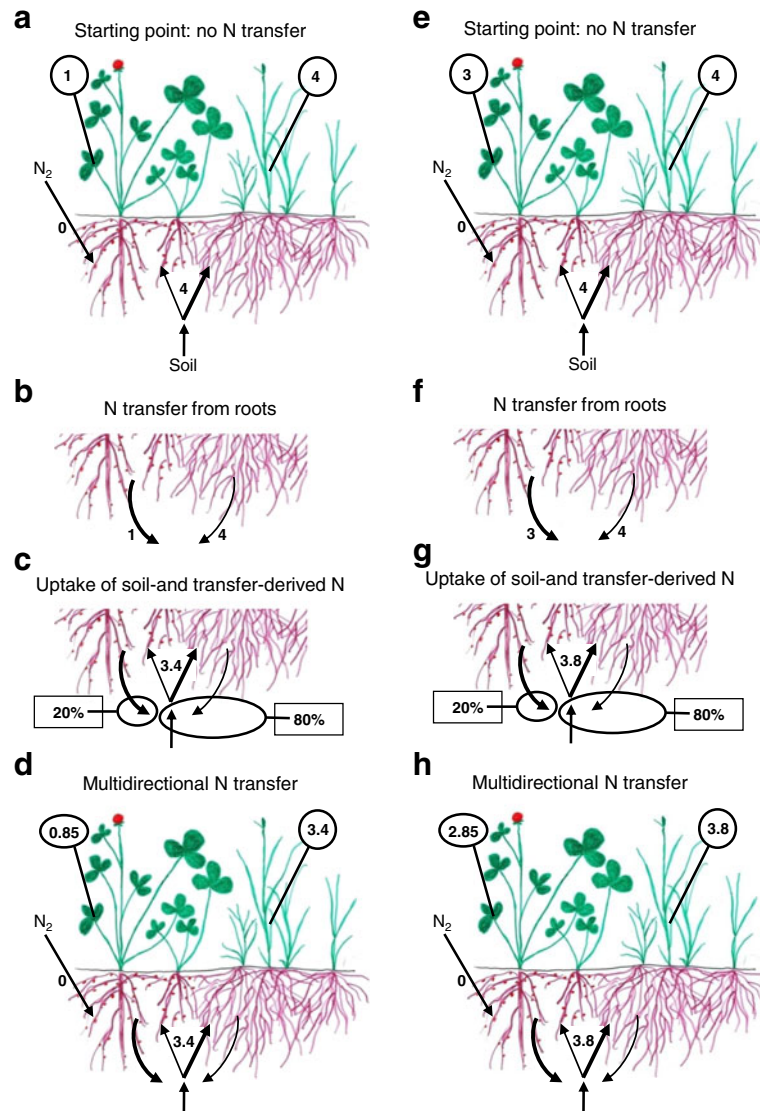
Fig. 4 Conceptual model of expected changes in $\delta^{15}N$ in legumes and reference plants grown together in a situation of multidirectional N transfer (legume-derived N becomes available to both legumes and reference plants) where the legume obtains 75 % (**a**–**d**) or 25 % (**e**–**h**) of its N from N_2 fixation. Numbers in circles indicate plant $\delta^{15}N$ values without N transfer (**a**, **e**) and after multidirectional N transfer (**d**, **h**). Numbers next to arrows indicate $\delta^{15}N$ values of plant-available soil N (**a**, **e**), N derived from N_2 fixation (**a**, **d**, **e**, **h**), N transferred from plants to the pool of plant-available soil N (**b**, **f**) and plant-available soil N after transfer (**c**, **d**, **g**, **h**). Different thickness of arrows illustrates the generally higher soil N uptake by non-legumes and higher contribution to N transfer from legumes. The model assumes that N transferred from any plant becomes equally available for all plants sharing the same rooting zone, and

that legume-derived N accounts for 20 % of the plant-available soil N after N transfer. The multidirectional N transfer will thus lead to a change in the $\delta^{15}N$ of plant-available soil N from 4 to 3.4 in the high-pNdfa scenario (**c**; 80 %×4+20 %×1=3.4) and from 4 to 3.8 in the low-pNdfa scenario (**g**; 80 %×4 + 20 %×3=3.8). Consequently, legume $\delta^{15}N$ changes from 1 to 0.85 in the high-pNdfa scenario (**d**; 0.75×0+0.25×3.4=0.85) and from 3 to 2.85 in the low-pNdfa scenario (**h**; 0.25×0+0.75×3.8=2.85). Reference plant $\delta^{15}N$ changes from 4 to 3.4 in the high-pNdfa scenario (**d**; 100 %×3.4) and from 4 to 3.8 in the low-pNdfa scenario (**h**; 100 %×3.8). The model assumes no ^{15}N discrimination in uptake and transfer. N transferred from the non-legume (**b**, **f**) is therefore supposed to have the same $\delta^{15}N$ as plant-available soil N and would not impose any changes in the $\delta^{15}N$ of plant-available soil N

Table 2 Effects of different N-transfer scenarios and use of reference plant (in mixture with the N_2-fixing legume or in pure stand) on pNdfa calculated according to the ^{15}N natural abundance method

Scenario of N transfer	$\delta^{15}N$ of soil N available to legume	pNdfa calculated using reference plant	
		in mixture	in pure stand
Theoretical data from conceptual model, high pNdfa (Fig. 4a–d)			
No transfer	4.0	0.75	0.75
Direct, unidirectional legume to ref	4.0	0.71	0.75
Multidirectional	3.4	0.75	0.79
Theoretical data from conceptual model, low pNdfa (Fig. 4e–h)			
No transfer	4.0	0.25	0.25
Direct, unidirectional legume to ref	4.0	0.21	0.25
Multidirectional	3.8	0.25	0.29
Empirical data from field experiment with red clover and timothy (Carlsson et al. 2009). pNdfa in red clover was calculated using timothy grown in mixture with red clover or timothy in pure stand as reference plant (mean ± SE, n=4). Mean values within a same row followed by the same letter are not significantly different (p<0.05) according to Wilcoxon test.			
Not determined[a], first year	Not determined	0.81±0.01 a	0.82±0.01 a
Not determined, third year	Not determined	0.80±0.03 a	0.85±0.02 a
Not determined, fifth year	Not determined	0.73±0.06 a	0.84±0.03 a

[a] The field experiment (Carlsson et al. 2009) included measurements of N concentration and $\delta^{15}N$ in aboveground tissues, while analyses of uni- or multidirectional N transfer was not included

we would expect a rapid (*i.e.* quicker than 30 days) enrichment of ^{15}N in neighbouring plants, a situation which we rarely observed. Furthermore, transplanted plants did in general not differ in visual observations of plant growth as compared to non-transplanted plants, which indicates that a disturbance was not severe. N-transfer is not only a matter of breakdown of plant material but can also be mediated by leakage of N, rhizodeposition or mycorrhiza.

Even though we did not quantify the amounts of N transferred in our experiments, our results support previous findings that N is transferred not only from

legumes to neighbouring non-legume plants but also in the opposite direction, from non-legumes to legumes (Høgh-Jensen and Schjoerring 2000; Gylfadóttir et al. 2007; Rasmussen et al. 2007) and that N derived from a legume plant is available both to neighbouring non-legumes and to neighbouring legumes (Pirhofer-Walzl et al. 2012). As a consequence, N transferred from a legume plant should be considered equally available for re-uptake by the legume plant itself as for neighbouring legume and non-legume plants.

According to our conceptual model, multidirectional N transfer changes both legume and non-legume

Table 3 Ranges of estimated pNdfa values caused by different sources of variation in the calculation of pNdfa according to the ^{15}N natural abundance method

Source of variation	Range in pNdfa estimates	Reference
High reliance on N_2 fixation (legume $\delta^{15}N$=0.85, reference plant $\delta^{15}N$=3.4, B=0)		
Reference plant in mixture versus in pure stand	0.75–0.79	This study
Spatial variations in reference plant $\delta^{15}N$ (± 2)	0.40–0.84	Holdensen et al. 2007
Range in B values (−2.6 to 0)	0.43–0.75	Carlsson et al. 2006
Low reliance on N_2 fixation (legume $\delta^{15}N$=2.85, reference plant $\delta^{15}N$=3.8, B=0)		
Reference plant in mixture versus in pure stand	0.25–0.29	This study
Spatial variations in reference plant $\delta^{15}N$ (± 2)	0–0.50	Holdensen et al. 2007
Range in B values (−2.6 to 0)	0.15–0.25	Carlsson et al. 2006

δ^{15}N as compared to when no transfer occurs (Fig. 4). Our theoretical calculations were based on the assumption that legumes and non-legumes obtain identical proportions of their soil-derived N from transfer. This means that a legume that obtains 75 % of its N from N_2 fixation would obtain 5 % of its total N via transfer from neighbouring legumes (25 % from the shared pool of plant-available soil N, which in turn contains 20 % N transferred from legumes). This level corresponds well with measured rates of N transfer from non-legumes to legumes (Høgh-Jensen and Schjoerring 2000; Høgh-Jensen et al. 2006; Gylfadóttir et al. 2007; Rasmussen et al. 2007), while Pirhofer-Walzl et al. (2012) detected lower rates of N transfer from legumes to legumes: ranging from nearly 0 up to around 3 %. On the other hand, the rates of N transfer from legumes to non-legumes were markedly lower in the study by Pirhofer-Walzl et al. (2012) than was found by Høgh-Jensen and Schjoerring (2000), Gylfadóttir et al. (2007), Rasmussen et al. (2007) and Dahlin and Stenberg (2010). This difference in the extent of N transfer between studies might be due to differences in stand age, soil conditions, species identities and management. Irrespective of the actual rates of N transfer, we are confident in our assumption that in mixed herbaceous plant communities legumes acquire similar proportions of their soil-derived N via transfer from neighbouring legume plants as do non-legumes.

When we calculate pNdfa with different N transfer scenarios based on our conceptual model we find that using reference plants grown in mixture with the studied legume would underestimate N_2 fixation if N transfer exclusively occurs in the direction from legumes to non-legumes (Table 2). This would support the recommendation to use reference plants grown in pure stand (Broadbent et al. 1982; Ledgard et al. 1985; Brophy et al. 1987; McNeill and Wood 1990). However, both our present results and previously published analyses of multidirectional N transfer and rhizodeposition (Høgh-Jensen and Schjoerring 2000; Gylfadóttir et al. 2007; Rasmussen et al. 2007; Pirhofer-Walzl et al. 2012) strongly imply that N transfer is multidirectional. These findings support the assumption that any N transferred from a legume for uptake by a co-cultivated non-legume will be equally available to the legume crop itself. When we base our pNdfa calculations on the multidirectional N transfer scenario (Fig. 4d, h), we find that using reference plants in mixture with the legume results in the "correct" result (pNdfa=0.75 and 0.25 in the

situations of high and low reliance on N_2 fixation, respectively; Table 2). Results showing that N transfer rates were reduced if mycorrhizal networks were prevented (Johansen and Jensen 1996) imply that indirect N transfer would be of relatively low importance, lower than direct transfer such as via mycorrhiza. Nevertheless, we are not aware of any evidence claiming that mycorrhizal networks would only facilitate unidirectional transfer; the multidirectional N transfer observed in our study and by others may also be mediated by mycorrhiza. Consequently, our empirical and conceptual analyses provide convincing evidence that the reference plants should be grown as close as possible to the legume (ideally at the same distance as between individual legume plants) for correct and reliable ^{15}N-based estimations of N_2 fixation. Placing the reference plant within the legume stand is also recommended in order to avoid effects of spatial variability in plant ^{15}N composition that may be considerable if the reference plant is grown far from the studied legume (Holdensen et al. 2007).

Contrasting opinions do exist among N_2 fixation scientists as whether reference plants should be grown in pure stand or in mixture with the N_2-fixing plants. One argument for using reference plants grown in pure stand is that the N transferred to non-legumes should be accounted for as being a part of the N_2 fixation estimate, and that this N is overlooked when using reference plants grown in mixture with the studied legume. We agree that a decrease in legume δ^{15}N caused by transfer from a neighbouring legume plant would be due mainly to re-uptake of originally fixed N, and as such the transferred N represents a part of the amount of N_2 fixed. Indeed, we find an increasing discrepancy with time between using reference plants (timothy) in mixture with the legume (red clover) or in pure stand when analysing empirical data from the field experiment described in Carlsson et al. (2009), although the differences were not statistically significant (Table 2). Plant δ^{15}N decreased globally with time, both in legumes and in non-legumes, but the decrease was more pronounced in plant communities containing legumes (Carlsson et al. 2009). Thus, with time, the N content in soil under legumes likely increased via N inputs from N_2 fixation. But, the proportion of N derived from N_2 fixation, pNdfa, does not increase if the N_2-fixing plant takes up soil N originally fixed by its neighbours. In the example based on theoretical data in Table 2, we show that the δ^{15}N in both legume and reference plants decrease as a result of

uptake of N originally fixed by neighbouring legumes. In this situation of multidirectional N transfer, using a reference plant grown in pure stand would overestimate pNdfa (Table 2). In other words, the reliance on N_2 fixation must not be confounded by supply of N_2 fixed by a neighbour.

The correct way to account for fixed N that is transferred to neighbouring plants, and thereby "hidden" from the quantification of N_2 fixation, is to quantify N transfer separately (Ledgard and Steele 1992). Estimations of the quantity of N transferred from a N_2-fixing legume to a neighbouring non-legume can be obtained by the direct ^{15}N leaf-feeding method (Ledgard et al. 1985) or indirectly with the ID method (Brophy et al. 1987). Applying the ID method for quantifications of N transfer requires that plants of the same reference species are grown both in mixture with the legume and in pure stand. The pure stand reference plants should be grown close to the mixture plots in order to minimize effects of different soil conditions and environment. Preferably, pure stand reference plants should be grown adjacent to the mixture, with attention to make sure that the pure stand plants are well outside the rooting zone of the legume (Pate et al. 1994). Differences in ^{15}N enrichment in reference plants grown in mixture with the legume and reference plants grown in pure stand can then be used to calculate the transfer of fixed N. In a similar way, differences in $\delta^{15}N$ between reference plants grown in mixture with the legume and in pure stand, detected with the NA method, might indicate that N transfer occurs (Temperton et al. 2007). However, since several other mechanisms also cause changes in plant $\delta^{15}N$ in legume/non-legume mixtures as compared to pure stands of non-legumes (*e.g.* Högberg 1997), and since the NA method is based on very small differences in ^{15}N abundance, one must be very careful in how to interpret changes in non-legume $\delta^{15}N$. We therefore recommend using the ID method rather than the NA method for quantifying N transfer.

The major assumption of ^{15}N-based quantifications of N_2 fixation is that the legume and the reference plant utilize soil N of the same isotopic composition. Since the ^{15}N abundance of plant-available soil N may vary temporally during a growing season and spatially with soil depth, errors may also occur if N uptake patterns differ between the legume and the reference plant. Observed variations in the ^{15}N composition among different reference species grown in the same substrate (Pate et al. 1994) imply that root depth or N uptake patterns may differ between different non-legume species. Due to such difficulties to identify one reference species which optimally matches the N uptake characteristics of the studied legume, it has been suggested that the best choice would be to use several reference species (Jacot et al. 2000; Carlsson et al. 2009). The recommendation to use reference plants grown in mixture with the N_2-fixing legume is of course problematic if the aim is to measure N_2 fixation in a legume growing in pure stand. For such studies, we recommend using weeds or to sow/plant non-legume reference plants within the legume pure stand plots. The non-legumes should be placed in confined parts of the legume plots and not broadcasted within the legume stand, since non-legume presence is likely to change the legume reliance on N_2 fixation. The essential is that reference plants are able to reflect the soil- and site-specific properties in order to provide an integrated measurement of the ^{15}N composition of plant-available soil N.

Lastly, we cannot exclude that unidirectional N transfer of fixed N from legumes to non-legumes may occur in some ecosystems where root systems of different species are stratified in different soil depths and an important N transfer route is via aboveground litter, like in forestry or agroforestry systems. We are nevertheless confident in our recommendation, based on multidirectional N transfer in herbaceous cropping systems, that reference plants should be grown in mixture with the legume, a situation which is valid for a vast majority of N_2 fixation assessments in agricultural systems. One must also be aware of other sources of uncertainty when quantifying N_2 fixation with the ID and NA methods (*e.g.* Ledgard and Steele 1992; Carlsson and Huss-Danell 2003; Carlsson et al. 2009). Indeed, the differences in pNdfa caused by using reference plants grown in mixture with the legumes or in pure stand in our examples might fall within the margin of methodological uncertainty, as exemplified by applying documented ranges of spatial variability in reference plant $\delta^{15}N$ (Holdensen et al. 2007) or variability in measured B values (Carlsson et al. 2006) in the theoretical calculations of pNdfa (Table 3). However, referring to other, potentially larger sources of errors does not justify an erroneous use of reference plants in the calculation of pNdfa. One must also be aware that the range of variations in pNdfa caused by different uses of reference plants

(Tables 2 and 3) are calculated from specific theoretical examples without replication or hypothesis testing. The results presented in Tables 2 and 3 provide a conceptual illustration of how the calculated pNdfa would change in response to different sources of variation, but should not be used for general conclusions in a wider context.

Conclusions

We present data from a field experiment and analyses of literature data which both provide evidence for multidirectional N transfer in mixed legume/non-legume communities. A conceptual model was used to describe and analyze changes in legume and reference plant ^{15}N resulting from multidirectional N transfer. The results showed that using reference plants grown in pure stand introduces an error when calculating pNdfa with ^{15}N-based methods. Based on our analyses and previous findings that reference plant ^{15}N may be subject to large spatial variability, we recommend that reference plants should be grown in mixture with the studied legume for reliable ^{15}N-based quantifications of N$_2$ fixation in herbaceous cropping systems. Ideally, reference plants should be located at the same distance to the sampled legume as the closest neighbouring legume plant, i.e. the reference plant should be located in the same environment as the legume. To measure N$_2$ fixation in a legume growing in pure stand, we recommend using weeds or to sow/plant non-legume reference plants in confined parts of the legume pure stand plots. Transfer of fixed N to neighbouring plants is not a part of the N$_2$ fixation process. If the aim is to assess the total N benefits from legumes in an intercropping system, N transfer should therefore be quantified separately and added to the amount of N$_2$ fixed.

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