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N_2 fixation in three perennial *Trifolium* species in experimental grasslands of varied plant species richness and composition

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Abstract This study is the first to investigate quantitative effects of plant community composition and diversity on N_2 fixation in legumes. N_2 fixation in three perennial *Trifolium* species grown in field plots with varied number of neighbouring species was evaluated with the ^{15}N natural abundance method (two field sites, several growing seasons, no N addition) and the isotope dilution method (one site, one growing season, 5 g N m^{-2}). The proportion of plant N derived from N_2 fixation, pNdfa, was generally high, but the N addition decreased pNdfa, especially in species-poor communities. Also following N addition, the presence of grasses in species-rich communities increased pNdfa in *T. hybridum* and *T. repens* L., while legume abundance had the opposite effect. In *T. repens*, competition for light from grasses appeared to limit growth and thereby the amount of N_2 fixed at the plant level, expressed as mg N_2 fixed per sown seed. We conclude that the occurrence of diversity effects seems to be largely context dependent, with soil N availability being a major determinant, and that species composition and functional traits are more important than species richness regarding how neighbouring plant species influence N_2 fixation in legumes.

Keywords BIODEPTH · Biodiversity · Clover · Functional traits · ^{15}N natural abundance · ^{15}N isotope dilution

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Introduction

Biological N_2 fixation in symbioses between legumes and rhizobia provide valuable inputs of N that becomes available to neighbouring as well as succeeding plants (Høgh-Jensen and Schjørring 1997; Mulder et al. 2002; Spehn et al. 2002; Stopes et al. 1996; Temperton et al. 2007). Results from biodiversity manipulations in natural meadow

communities indicated that the transfer of fixed N from legumes to non-legumes increased with increasing plant species richness (Temperton et al. 2007). However, any effects of species richness and composition of the neighbouring vegetation on the N_2 fixation process in legumes have so far not been investigated quantitatively. Most previous studies of N_2 fixation in legume/grass mixtures have comprised only one or a few grass species together with one or a few legume species, and have typically covered the first 1–3 years after establishment in leys harvested several times during the growing season (Boller and Nösberger 1987; Farnham and George 1994; Heichel and Henjum 1991; Høgh-Jensen and Schjørring 1994). Little is thus known about how neighbouring species other than grasses and the species richness of neighbouring vegetation influence N_2 fixation in more extensively managed grasslands. Several findings indicate that the capture of limiting resources, aboveground as well as belowground, is intensified in species-rich plant communities, via competition and/or facilitative interactions (e.g. Fargione et al. 2007; Oelmann et al. 2007; Spehn et al. 2005). Positive relationships have been found between plant species richness and light interception (Jumpponen et al. 2005; Spehn et al. 2005), plant N uptake (Tilman et al. 1996), plant N yield (Mulder et al. 2002; Spehn et al. 2005; Temperton et al. 2007) and water use efficiency (Verheyen et al. 2008), while a negative relationship was found between species richness and soil NO_3^- leaching (Scherer-Lorenzen et al. 2003) or soil NO_3^- pool size (Palmborg et al. 2005).

An increased soil N uptake with increasing plant species richness should be expected to increase the proportion of N derived from N_2 fixation (pNdfa) in legumes. In addition, different species or different functional groups (e.g. legumes, grasses and non-leguminous forbs) may influence the N_2 fixation rates in neighbouring legumes differently depending on how strongly they compete for light and nutrients. Grasses, with their extensive and finely divided root system, take up soil N effectively and reduce soil N concentrations to lower levels than forbs (Craine et al. 2002; Fargione and Tilman 2006; Palmborg et al. 2005; Scherer-Lorenzen et al. 2003). The tall grass *Phalaris arundinacea* (L.) has been found to be a particularly strong competitor for inorganic soil N (Palmborg et al. 2005). The efficient uptake of soil N by grasses has been found to cause an increased legume dependence on N_2 fixation when grasses are present, both in species-poor agricultural fields and in more diverse semi-natural meadows (Brophy et al. 1987; Carlsson and Huss-Danell 2003; Hartwig 1998; Kahmen et al. 2006; Loiseau et al. 2001; Sanford et al. 1995; Xiao et al. 2004). In addition, Temperton et al. (2007) found strong species-specific effects on N transfer between legumes and neighbouring non-legumes, with grasses showing the largest benefit of fixed N.

The aim of this study was to examine the effects of plant diversity, both in terms of species richness and number of functional groups, and species composition on N_2 fixation in the three perennial *Trifolium* species *T. hybridum* L. (alsike clover), *T. pratense* L. (red clover) and *T. repens* L. (white clover). *Trifolium pratense* and *T. repens* are among the most widely used forage legumes in Europe, and *T. hybridum* is considered to be useful at sites where the soil is too wet, acid or infertile for *T. pratense* (Frame et al. 1998). The *Trifolium* species were grown in experimental grasslands at two sites, in Sweden and Germany. These fields were maintained for up to 7 years and harvested only once or twice each year. We used these field experiments to elucidate the consistency of the observed high pNdfa in perennial forage legumes (Carlsson and Huss-Danell 2003; Huss-Danell and Chaia 2005). Because there was a decrease in the abundance of each individual species with increasing species richness inherent in the experimental design, we calculated the amount of N_2 fixed expressed as N_2 fixation per sown seed in each *Trifolium* species. This measurement of N_2 fixation describes variation both in pNdfa and in biomass production. We thus expect N_2 fixation per sown seed to be influenced by aboveground competition from neighbouring plant species, as opposed to pNdfa which we believe is mainly controlled by soil N availability and thus belowground competition.

Our hypotheses were (1) that pNdfa in the studied *Trifolium* species increases in communities with greater plant species richness or functional diversity, with grasses having a particularly strong impact due to their efficient uptake of soil N; (2) that the standardized amount of N_2 fixed, N_2 fixation per sown seed, would be positively influenced by plant diversity due to a positive influence on pNdfa as well as a potential decrease in intra-specific competition among legumes with increasing diversity and (3) that

N_2 fixation per sown seed would be negatively influenced by presence of tall grasses as a result of aboveground competition (shading) and thus limited biomass production and lower amount of N_2 fixed at the plant level.

Materials and methods

Sites and experimental design

The experimental fields, established as parts of the European BIODEPTH project (Hector et al. 1999), were located in Umeå (63°49′N, 20°17′E, 12 m a.s.l.), northern Sweden, and in Bayreuth (49°55′N, 11°35′E, 355 m a.s.l.), south-eastern Germany.

The Swedish site is situated in the boreal zone. The short growing season has long days with good water availability while winters are long with frozen topsoil covered by snow. The 1961–1990 mean annual precipitation at this site is 591 mm and the mean annual temperature is 2.6°C. The soil is a fine silty sand with low clay content (Mulder et al. 2002). The uppermost 15 cm of the soil, collected at the time of sowing, contained 0.15 ± 0.02% N (N_{total}, mean ± SD, $n = 34$) with a $\delta^{15}N$ of 4.75 ± 0.74 (Mulder et al. 2002).

At the German site, located in the temperate zone, the 1951–1990 mean annual precipitation is 706 mm and the mean annual temperature is 7.8°C. The soil at the site varies between a loamy sand and a sandy clay and its N_{total} content is 0.08 ± 0.01% (mean ± SD, $n = 64$) (Scherer-Lorenzen et al. 2003).

Both experiments started in the spring 1996. Field plots were 2.2 by 5 m in Sweden and 2 by 2 m in Germany. They were randomly assigned to 1, 2, 4, 8 or 12 (16 instead of 12 in Germany) perennial plant species belonging to three functional groups: legumes, grasses and non-leguminous forbs. Each species was grown in monocultures as well as in mixtures of varied plant species composition and diversity. In the present N_2 fixation study we only used plots containing *Trifolium* species and plots containing the selected reference species. Tables 1 and 2 describes the composition of the plots used in this study. *Rhizobium leguminosarum* bv. *trifolii* was known to be present in the Swedish soil, and *Trifolium* seeds were therefore not inoculated. Although the top soil was initially steam-sterilized in situ at the German site, all legumes were nodulated shortly after sowing. Both experiments were replicated in two blocks. In addition, in Sweden some species combinations were established in triplicates, and the mixtures with the highest species richness were replicated six times in Sweden (Table 1). The total seed density was held constant at 2,000 seeds m^{-2} across all levels of species richness at both sites. To maintain the species composition for the entire study period, weeds were manually removed throughout the growing seasons. The Swedish experiment is situated on a typical coastal plain in northern Sweden, where winter freeze–thaw damage, causing plant death, is a common phenomenon. Therefore, since the intention was to maintain the initial species composition of each plot over several years, re-seeding was necessary when winter damage had occurred. The Swedish plots were re-sown in spring 1999 and spring 2000 (Palmborg et al. 2005). At both sites, plots were separated with 1.5-m wide borders sown with a non-clonal grass. For further details of the experimental design, see Mulder et al. (2002) for the Swedish site and Scherer-Lorenzen et al. (2003) for the German site.

Measurements and calculation

The Swedish site

The experimental plots were harvested once each year, in mid-August. All plant biomass above 5 cm in a central area of 0.2 by 0.5 m in each plot was cut by hand, sorted to species, dried at 60°C for 24 h and weighed. All remaining vegetation was then cut to 5 cm and clippings were removed. Data obtained from all plots grown with *Trifolium* spp. from the three growing seasons 1996, 1998 and 2000 were used for calculations of N_2 fixation with the ^{15}N natural abundance (NA) method (Amarger et al. 1979). The fourth legume included in the experiment, *Lotus corniculatus* L. (birdsfoot trefoil; Table 1), did not establish well until 2000. Due to its weak performance during a large part of the experiment, we do not include N_2 fixation data for *Lotus corniculatus* here. *Phleum pratense*, *Leucanthemum vulgare* and *Ranunculus acris* were used as non-N_2-fixing reference species for the estimations of N_2 fixation (Table 1). All samples of *Trifolium* spp. and reference species from the three harvests (1996, 1998 and 2000) were

Table 1 Species composition of the Swedish experimental plots used for the N_2 fixation measurements

Legumes	Grasses	Forbs	No. of spp.	% Legumes	% Grasses	% Forbs	Replicates
TH			1	100			2 (2)
TP			1	100			2 (2)
TR			1	100			2 (2)
	PP[a]		1		100		2
		LV[a]	1			100	2
		RaA[a]	1			100	2
TH, TR			2	100			2 (2)
TP	PP[a]		2	50	50		3 (3)
LC, TP	PA, PP[a]		4	50	50		3 (3)
TH, TR		LV[a], RaA[a]	4	50		50	2 (2)
TH, TR	FO	AM	4	50	25	25	2 (2)
TP	PP[a]	RaA[a], RuA	4	25	25	50	3
LC, TH, TP, TR	DG, FO	RuA, AM	8	50	25	25	2
LC, TP	PA, PP[a]	LV[a], RaA[a], RuA, AM	8	25	25	50	3 (2)
TH, TR	DG, PA, PP[a], FO	LV[a], RaA[a]	8	25	50	25	3 (2)
LC, TH, TP, TR	DG, PA, PP[a], FO	LV[a], RaA[a], RuA, AM	12	33	33	33	6 (4)

Species (spp.) were LC, *Lotus corniculatus* L.; TH, *Trifolium hybridum* L. cv. Stena; TP, *Trifolium pratense* L. cv. Betty; TR, *Trifolium repens* L. cv. Undrom; DG, *Dactylis glomerata* L.; PA, *Phalaris arundinacea* L.; PP, *Phleum pratense* L. cv. Jonatan; FO, *Festuca ovina* L.; LV, *Leucantemum vulgare* Lam.; RaA, *Ranunculus acris* L.; RuA *Rumex acetosa* L.; AM, *Achillea millefolium* L. Plots included in the ID experiment (2002) are indicated by the numbers in parenthesis in the last column

[a] Used as reference species

ground in a ball mill and analysed for ^{15}N abundance and N concentration. Analyses were performed using an online CN analyzer (Europa Scientific ANCA-NT) coupled to an isotope ratio mass spectrometer (Europa Scientific Europa 20-20) at the IRMS laboratory, Dept. of Forest Ecology and Management, SLU, Umeå (Ohlsson and Wallmark 1999). In 1996, *T. hybridum* could not be distinguished from *T. repens* and *Phalaris arundinacea* could not be distinguished from *Phleum pratense* with certainty, so measurements on these species taken in 1996 are excluded from the data presented. The ^{15}N abundance in legumes and reference species were used to calculate pNdfa according to the NA method (following the rationale of Amarger et al. 1979):

$$\text{pNdfa} = \left(\delta^{15}N_{\text{ref}} - \delta^{15}N_{\text{fix}}\right)/\left(\delta^{15}N_{\text{ref}} - B\right),$$

where $\delta^{15}N_{\text{ref}}$ and $\delta^{15}N_{\text{fix}}$ are the ^{15}N abundance in the reference species and the N_2-fixing species, respectively, expressed as parts per thousand deviation from the $^{15}N/^{14}N$ ratio in atmospheric N_2 (Hauck 1973). B is the $\delta^{15}N$ of the N_2-fixing species when grown with N_2 in air as the sole N source, included to account for ^{15}N isotope discrimination in the N_2-fixing plant (Amarger et al. 1979). The use of a correct B-value and reference species is crucial for the accuracy of the NA method (Carranca et al. 1999; Høgh-Jensen and Schjørring 1994). Measured B values for the studied *Trifolium* cultivars have been established in a controlled experiment, showing significant effects by *Rhizobium* genotype on the measured B values but a close similarity between the mean of B values for different *Rhizobium* genotypes and the lowest detected $\delta^{15}N$ in field-grown *Trifolium* species (Carlsson et al. 2006) The results of the same study also implied that the same B value should be used for all years in perennial systems, since there was only a very small change in B after a simulated winter in *T. pratense*. Some *Trifolium* samples from this field experiment had $\delta^{15}N$ values that were slightly lower than the measured B values. To avoid unrealistic values of pNdfa (>1), B was set to the lowest detected $\delta^{15}N$ (−1.9 in *T. hybridum*, −1.8 in *T. pratense* and −1.9 in *T. repens*), as proposed by Hansen and Vinther (2001) and Carlsson et al. (2006).

Several plots included more than one of the reference species (Table 1). Data from these plots were used to test for differences between pNdfa

Table 2 Species composition of the German experimental plots used for the N_2 fixation measurements

Legumes	Grasses	Forbs	No. of spp.	% Legumes	% Grasses	% Forbs	Replicates
TP			1	100			2
TR			1	100			2
	AE[a]		1		100		2
	DG[a]		1		100		2
		PL[a]	1			100	2
TP	FR		2	50	50		2
TR	DG[a]		2	50	50		2
TP	FR, LP	RaA	4	25	50	25	2
TR	AE[a], AP, FR		4	25	75		2
TP, TR	AE[a], DG[a], FR, HL, LP, PP		8	25	75		2
LA, TP	AE[a], AP, FP, FR	PL[a], RuA	8	25	50	25	2
LC, TP, TR, VC	AE[a], AO. AP, BH, FR, HL, LP, PP	CJ, KA, PL[a], PM	16	25	50	25	2
LA, LC, TP, VC	AE[a], AO, BH, CC, DG[a], HL, LP, PP	AM, CB, CP, LV	16	25	50	25	2
LA, LC, TR, VC	AO, AP, CC, DG[a], FP, FR, LP, PP	GP, LeA, LF, RaA	16	25	50	25	2

Species (spp.) were LA, *Lathyrus pratensis* L.; LC, *Lotus corniculatus* L.; TP, *Trifolium pratense* L.; TR, *Trifolium repens* L.; VC, *Vicia cracca* L.; AE, *Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl; AO, *Anthoxanthum odoratum* L.; AP, *Alopecurus pratense* L.; BH, *Bromus hordeaceus* L.; CC, *Cynosurus cristatus* L.; DG, *Dactylis glomerata* L.; FP, *Festuca pratensis* Huds.; FR, *Festuca rubra* L.; HL, *Holcus lanatus* L.; LP, *Lolium perenne* L.; PP, *Phleum pratense* L.; AM, *Achillea millefolium* L.; CP, *Campanula patula* L.; CB, *Crepis biennis* L.; CJ, *Centaurea jacea* L.; GP, *Geranium pratense* L.; KA, *Knautia arvensis* (L.) Coult.; LeA, *Leontodon autumnalis* L.; LF, *Lychnis flos-cuculi* L.; LV, *Leucantemum vulgare* Lam.; PL, *Plantago lanceolata* L.; PM, *Pimpinella major* (L.) Huds.; RaA, *Ranunculus acris* L.; RuA *Rumex acetosa* L

[a] Used as reference species

values obtained using different reference species. In all subsequent calculations and data analyses, the mean $\delta^{15}N_{ref}$ were used in plots where more than one reference species were present. Since the reference species is supposed to reflect the $\delta^{15}N$ of the soil N available to the studied legume, and soil $\delta^{15}N$ may vary spatially (Bremer and van Kessel 1990), we used reference species grown in the same plot as the sampled *Trifolium* species. However, some plots did not include a reference species (Table 1), and in some cases the reference species did not produce enough sample for further analyses. In such cases, the mean $\delta^{15}N_{ref}$ values from the three spatially closest plots containing at least one reference species were used.

Fertilizers were not applied to the fields during the period 1996–2001, but in 2002 a majority of the experimental plots were included in a ^{15}N isotope dilution (ID) experiment (Table 1). In early June 2002, a central area of 1 by 1 m in each included plot received $^{15}NH_4$ $^{15}NO_3$ (5 atom % ^{15}N excess) corresponding to 5 g N m^{-2}. This level of N addition corresponds to the recommended N fertilization to extensively managed grasslands (one harvest per year) in northern Sweden yielding about 400 g DM m^{-2} $year^{-1}$ and with legume proportions of at least 30% (Ericson 2005). Plant biomass from the ID plots was sampled in mid-August, prepared, and analysed as described above for the natural abundance samples. Using the same reference species as with the NA method, pNdfa was calculated according to the ID method (following the rationale of Fried and Middelboe 1977):

$$\text{pNdfa} = 1 - \left({}^{15}N_{fix} / {}^{15}N_{ref} \right),$$

where $^{15}N_{fix}$ is the atom % ^{15}N excess in the N_2-fixing species and $^{15}N_{ref}$ is the atom % ^{15}N excess in the reference species.

Amount of N_2 fixed per m^2 and year (Nfix) were then calculated:

$$\text{Nfix} = \text{harvested DM}\left(\text{g m}^{-2}\,\text{year}^{-1}\right) \times \text{N concentration}\left(\text{g Ng DM}^{-1}\right) \times \text{pNdfa}.$$

To account for the decreasing number of seeds sown per m^2 of each individual species with increasing

species richness, we calculated Nfix as the amount of N_2 fixed per sown seed (Nfix seed^{-1}). This was done by dividing the amount of N_2 fixed per m^2 and year with the amount of sown seeds per m^2 for each *Trifolium* species.

The German site

Plant biomass was sampled twice during each growing season (late June and mid-September) at 5 cm height in two areas of 0.2 by 0.5 m each within a permanent quadrate placed randomly within the centre 1.4 by 1.4 m of each plot. Data obtained from all plots including *T. pratense* and *T. repens* from the first harvest in 1997 (late June) were used for calculations of N_2 fixation. *Arrhenatherum elatius*, *Dactylis glomerata* and *Plantago lanceolata* were used as non-N_2-fixing reference species (Table 2). The experimental plots did not receive any fertilization during the period included in our study. The variables describing N_2 fixation (pNdfa, Nfix and Nfix seed^{-1}) were estimated using the NA method as described above for the Swedish site. *B* values for the two *Trifolium* species were set at the lowest detected δ^{15}N at the site: −0.9 in *T. pratense* and −1.6 in *T. repens*.

Statistical analyses

Changes in plant δ^{15}N over time were tested with linear regression, and the relationships between pNdfa values obtained with different reference species were tested with correlation analyses. The effects of number of species and functional groups on pNdfa and Nfix seed^{-1} were analysed with repeated measures ANOVA as part of a general linear model (GLM, type I sum of squares). The effects of time (*T*, only at the Swedish site), block (*B*), plant species richness (*S*), and number of plant functional groups (*FG*) were tested in a hierarchical order. All analyses were done with two orders of fitting: one which maximises the effect of species richness, and the other of the number of functional groups, to take into account that *S* and *FG* were not fully orthogonal (Schmid et al. 2002). *F*-values given in text and tables refer to those where the respective factor was fitted first. The significance level $P < 0.05$ was used in all these analyses, which were performed using SPSS 16.0.1 (SPSS Inc., Chicago, USA) and Minitab 14.1 (Minitab Inc., State College, PA, USA).

Effects of community composition on pNdfa and Nfix seed^{-1} in each *Trifolium* species were analysed with the multivariate approach projection to latent structures by means of partial least squares (PLS). PLS is a regression extension of principal component analysis (PCA), and is useful when the aim is to connect information in two blocks of variables, *X* and *Y*, to each other (Eriksson et al. 1999). By PLS modelling, relationships within complex datasets can be illustrated in PLS loading plots, which show how the *X* and *Y* variables are distributed along one or several model components. The number of components that a model consists of depends on the predictive ability, which is tested for each component by cross-validation (excluding observations and comparing predicted and actual response values, *Y*, for excluded observations). PLS has the advantages, as a multivariate method, that it can handle co-varying variables and optimises the fraction of explained variation in *Y* (Rougoor et al. 2000). Using the software package Simca P 10.0 (Umetrics AB, Umeå, Sweden), one PLS model was constructed for each of the *Y*-variables pNdfa and Nfix seed^{-1} for each *Trifolium* species at each site. The PLS analyses were done separately for results obtained with the NA and ID methods at the Swedish site. The biomass of individual species, expressed as the proportion of total community biomass, and sown proportion of each functional group were used as *X* variables in the PLS models. All proportion data (including pNdfa) were arcsin-transformed prior to the analyses, and Nfix seed^{-1} data were $\log_{10}$-transformed, to attain an approximately normal distribution of the data. The dataset was divided into seven subsets, and each subset was excluded once during the cross-validation. To include a component in the PLS model, the significance limit 0.05 was used. The multivariate data analyses resulted in a large number of PLS models; we only present models that represent at least 50% explained variation in the variables describing N_2 fixation.

Results

Unless otherwise stated, the results refer to data obtained with the NA method (no N addition).

N_2 fixation expressed as the proportion of N derived from N_2 fixation (pNdfa)

At the Swedish site there was an overall linear decline in δ^{15}N values over time during the period without N addition, 1996–2000, both in legumes and in reference species (Fig. 1). However, the decline in δ^{15}N over time in *Phleum pratense* and *Ranunculus acris* was significant only when these species were growing with legumes, while the decline in *Leucanthemum vulgare* was significant both with and without legumes (linear regression analyses, $P < 0.05$). In 2000, δ^{15}N in *Phleum pratense* and *Ranunculus acris* were significantly lower in communities containing legumes as compared to in monocultures (*t*-test). The decline in δ^{15}N over time in the *Trifolium* species was significant only when they were grown in mixture with plants of other functional groups (Fig. 1). Including data from both with and without N addition, estimates of pNdfa obtained using each of the three reference species separately generally correlated very well with each other ($P < 0.001$, $r > 0.9$, except for the correlation between pNdfa values obtained with *P. pratense* and *R. acris* as reference species: $P < 0.001$, $r = 0.64$). Using *P. pratense* as reference species resulted in higher pNdfa values (range 0.3–0.9) than with either of the two forbs, and the pNdfa values obtained with *L. vulgare* (range 0.2–0.9), were higher than values

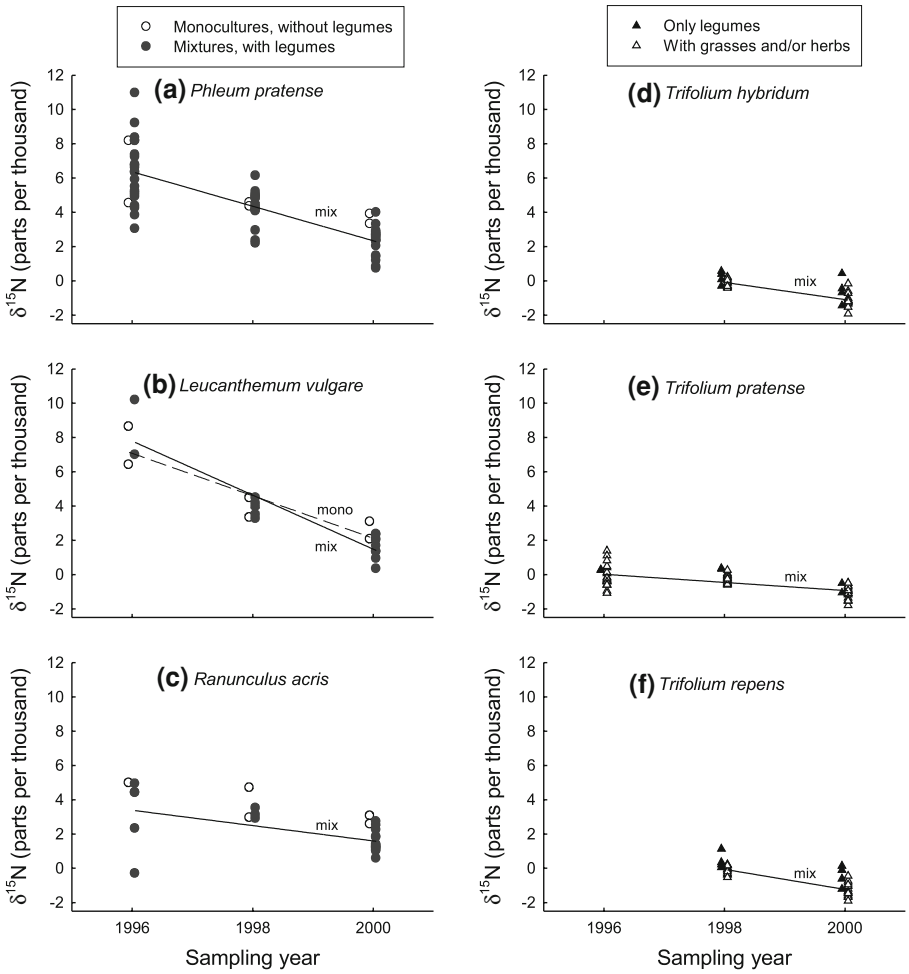

Fig. 1 δ^{15}N in shoots (>5 cm aboveground) of reference species (**a**–**c**) and *Trifolium* species (**d**–**f**) in the Swedish experiment during the unfertilized period. Each point represents one experimental plot. Regression lines indicate significant changes over time analysed by linear regression ($P < 0.05$)

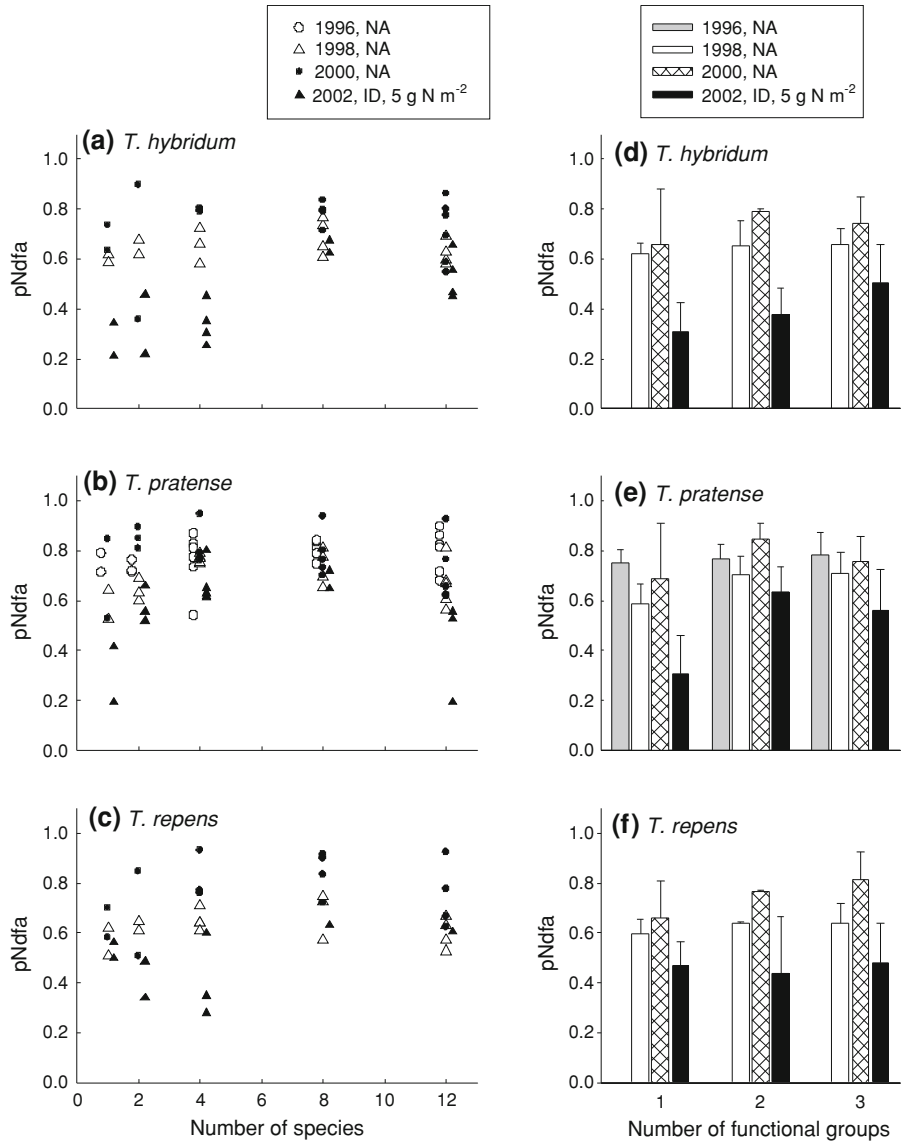

Fig. 2 The proportion of N derived from N_2 fixation (pNdfa) in the Swedish experiment in relation to the number of sown plant species (**a–c**) and number of functional groups (**d–f**, mean ± SD). Data are based on shoot (>5 cm aboveground) analyses. Each point represents one experimental plot. *NA* natural abundance method, *ID* isotope dilution method

obtained with *Ranunculus acris* as reference species (range 0.1–0.9, paired *t*-tests, $P < 0.05$ for all three comparisons).

During the period without N addition, all three *Trifolium* species acquired a large proportion of their N from N_2 fixation across all levels of species richness (Fig. 2). The mean pNdfa value at the Swedish site across the 3 years 1996, 1998 and 2000, and all three species was 0.7. Analyses of data from experimental plots where two or three *Trifolium* species were grown together showed that pNdfa was very similar in all three species, but pNdfa in *T. hybridum* was slightly lower than in *T. repens*, both with and without N addition (paired *t*-test, data not shown). There was no relationship between number of species or number of functional groups and pNdfa in any of the three *Trifolium* species (Table 3). There were significant differences in pNdfa between years in *T. pratense* and *T. repens*, with the highest values in 1996 (only *T. pratense*) and

Table 3 Repeated measures ANOVA table of *F*-values for effects of plant diversity on pNdfa and Nfix seed^{-1} estimated with the NA method at the Swedish site (no N addition)

Source of variation	*Trifolium hybridum*			*Trifolium pratense*			*Trifolium repens*		
	DF	pNdfa *F*	Nfix seed^{-1} *F*	DF	pNdfa *F*	Nfix seed^{-1} *F*	DF	pNdfa *F*	Nfix seed^{-1} *F*
Between subject									
Block (*B*)	1	1.066	2.454	1	1.115	*3.646*	1	1.233	0.094
Species richness (*S*)	4	1.269	1.524	4	1.526	1.228	4	1.627	0.053
Functional groups (*FG*)	2	1.008	1.365	2	1.478	1.485	2	1.658	0.006
Within subject									
Time (*T*)	1	2.648	**8.990**	2	**15.568**	**8.159**	1	**10.053**	0.174
$T \times B$	1	2.009	**8.965**	2	2.183	**4.989**	1	0.589	0.068
$T \times S$	1	0.313	2.834	8	**3.501**	1.516	1	0.224	1.104
$T \times FG$	2	0.396	2.765	4	1.910	0.714	2	0.402	0.479

DF degrees of freedom

F-values for species richness and for the number of functional groups represent those if fitted prior to the other factor, respectively. Significant effects at $P < 0.05$ are in bold, those at $P < 0.1$ are in italics

Table 4 ANOVA table of *F*-values for effects of plant diversity on pNdfa and Nfix seed^{-1} estimated with the ID method at the Swedish site in 2002, with the addition of 5 g N m^{-2}

Source of variation	*Trifolium hybridum*			*Trifolium pratense*		*Trifolium repens*	
	DF	pNdfa *F*	Nfix seed^{-1} *F*	pNdfa *F*	Nfix seed^{-1} *F*	pNdfa *F*	Nfix seed^{-1} *F*
Block (*B*)	1	0.018	1.287	*3.428*	3.262	1.550	*8.204*
Species richness (*S*)	4	**5.244**	1.894	**4.794**	0.511	0.779	2.969
Functional groups (*FG*)	2	**4.867**	1.352	**7.290**	2.372	0.039	4.386

DF degrees of freedom

F-values for species richness and for the number of functional groups represent those if fitted prior to the other factor, respectively. Significant effects at $P < 0.05$ are in bold, those at $P < 0.1$ are in italics

2000, and lowest values in 1998 (Fig. 2a–c, Table 3). There was no significant difference in pNdfa between years in *T. hybridum* (Table 3). The time by species richness interaction was significant for *T. pratense*, i.e. the slope of the species richness—pNdfa relationship changed over time. For the ID experiment (2002), when the plots received 5 g N m^{-2}, pNdfa was significantly lower, averaging 0.5 across all three *Trifolium* species. Following N addition, pNdfa in *T. hybridum* and *T. pratense* was significantly affected by both species richness and number of functional groups (Table 4), with a linear increase in *T. hybridum*, and an unimodal relationship with highest pNdfa values with eight species and two functional groups in *T. pratense* (Fig. 2a, b, d and e).

All PLS analysis of the data obtained with the NA method (no N addition) at the Swedish site showed no or very low levels of explained variation in pNdfa, and we therefore do not present any of these PLS models. On the other hand, the PLS models for pNdfa based on data from the ID experiment (with 5 g N added per m^2) showed that the proportion of the grasses *Dactylis glomerata*, *Phalaris arundinacea* and *Phleum pratense* in the harvested biomass were positively correlated with pNdfa in *T. hybridum* and *T. repens* (Fig. 3: DG, PA and PP all have positive values in the loading plot). In *T. hybridum*, pNdfa estimated with the ID method was positively correlated with sown grass proportion and negatively correlated with sown legume proportion (Fig. 3a).

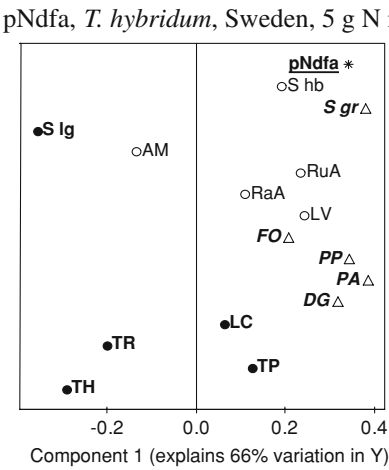

Fig. 3 PLS loadings for *X* and *Y* (pNdfa; *star*) variables in the Swedish isotope dilution (ID) experiment. *X* variables: biomass of individual species as a proportion of total harvested biomass (see Table 1 for abbreviations; legumes are given in *bold* with *dots*, grasses in *bold italic* with *open triangles*, and forbs with *circles*), and sown proportion of the three functional groups legumes, grasses and non-leguminous forbs (S-lg, S-gr and S-hb). Since the models only had one significant component, the variables are distributed along the *Y*-axis in the order they were inserted in the design matrix. *X* variables that are positively correlated with *Y* have positive values in the PLS component, and *X* variables that are negatively correlated with *Y* have negative values

Biomass proportion of *T. hybridum* as well as the non-leguminous forb *Achillea millefolium* were negatively correlated with pNdfa in both *T. hybridum* and *T. repens* after the N addition, while the correlations between pNdfa and sown grass and legume proportions were less obvious in *T. repens* (Fig. 3).

At the German site the $\delta^{15}N$ ranged from −1.6 to 1.1 in *Trifolium* shoots, and from 1.0 to 7.7 in shoots of the reference plants. Estimates of pNdfa obtained with the three reference species separately correlated well with each other ($P < 0.01$, $r > 0.9$), and there was no significant difference between pNdfa values obtained with different reference species (paired *t*-test). Both *T. pratense* and *T. repens* always acquired more than 60% of their N from N_2 fixation, and pNdfa was slightly higher in *T. pratense* (on average 0.9) than in *T. repens* (on average 0.8, paired *t*-test). There was no relationship between species or functional richness and pNdfa in either of the two *Trifolium* species (Fig. 4, Table 5).

Amount of N_2 fixed

In all *Trifolium* species, the amount of N_2 fixed per m^2 and year measured in plant parts >5 cm aboveground was highest in plots with low species richness and high legume abundance. At the Swedish site, Nfix in *T. hybridum*, ranged from <0.1 to 8 g N m^{-2} in 1998 and 2000, and from 0.1 to 3 g N m^{-2} in 2002 (ID method). In *T. pratense*, Nfix ranged from <0.1 to 4 g N m^{-2} in 1996 and 2000, from 0.1 to 10 g N m^{-2} in 1998 and from 0.1 to 9 g N m^{-2} in 2002 (ID). In *T. repens*, Nfix ranged from <0.1 to 6 g N m^{-2} in 1998 and 2002 (ID) and from <0.1 to 9 g N m^{-2} in 2000.

In all three *Trifolium* species at the Swedish site, the amount of N_2 fixed per sown seed (Nfix $seed^{-1}$) showed no response to increasing number of neither species nor functional groups, neither with the NA method nor with the ID method (Fig. 5, Tables 3 and 4).

At the German site the amount of N_2 fixed per m^2 and year was often more than twice the amount at the Swedish site, especially in *T. pratense* (range 3.5–26 g N m^{-2} $year^{-1}$ in *T. pratense*, 0.3–11 g N m^{-2} $year^{-1}$ in *T. repens*). For *T. pratense*, N_2 fixation per sown seed increased with both species and functional group richness (Fig. 6a), although the differences were only marginally significant with $P < 0.1$ due to a rather high variability within the 16-species level (Table 5). Based on the ID data at the Swedish site, the PLS analysis showed that N_2 fixation per sown seed in *T. repens* receiving N correlated positively with its own contribution to total community biomass and negatively with abundance of grasses (Fig. 7a). Also biomass proportion of *T. hybridum* and *T. pratense* correlated negatively with Nfix $seed^{-1}$

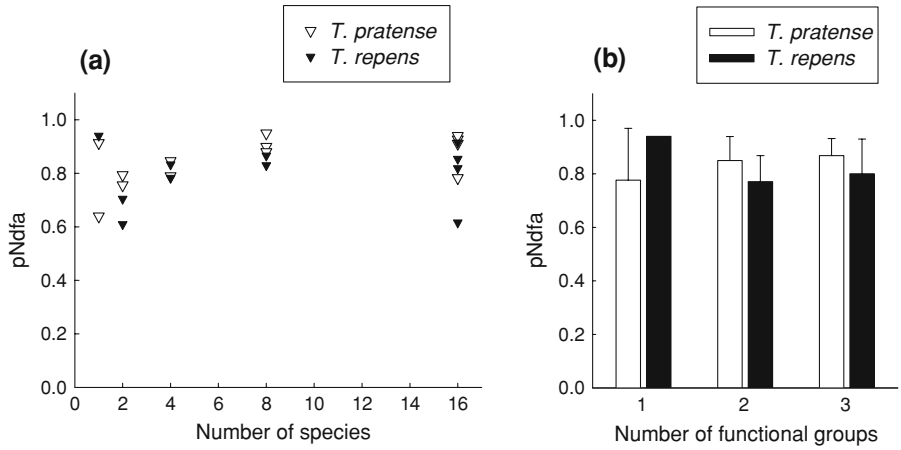

Fig. 4 The proportion of N derived from N_2 fixation (pNdfa) in the German experiment in relation to the number of sown plant species (**a**) and number of functional groups (**b**, mean ± SD). Data are based on shoot (>5 cm aboveground) analyses. Each point represents one experimental plot

Table 5 ANOVA table of *F*-values for effects of plant diversity on pNdfa and Nfix seed^{-1} estimated with the NA method at the German site (no N addition)

Source of variation	*Trifolium pratense*			*Trifolium repens*		
	DF	pNdfa *F*	Nfix seed^{-1} *F*	DF	pNdfa *F*	Nfix seed^{-1} *F*
Block (*B*)	1	0.169	2.534	1	1.522	0.862
Species richness (*S*)	4	1.877	*3.365*	3	1.140	0.225
Functional groups (*FG*)	2	1.917	*4.086*	1	0.376	0.262

DF degrees of freedom

F-values for species richness and for the number of functional groups represent those if fitted prior to the other factor, respectively. Significant effects at $P < 0.1$ are in italics

in *T. repens* after N addition (Fig. 7a). For the German site, the PLS analyses showed that biomass proportion of *T. pratense* correlated negatively with Nfix seed^{-1} in both *T. pratense* and *T. repens* (Fig. 7b, c). In *T. pratense*, Nfix seed^{-1} was positively correlated with grass abundance and negatively correlated with sown proportion of legumes, while the opposite was found in *T. repens* (Fig. 7b, c).

Discussion

The present study is the first effort to quantify effects of plant species richness and community composition on N_2 fixation in legumes. Without N addition, the studied *Trifolium* species consistently acquired the main part of their N from N_2 fixation, on average 70% at the Swedish site and 80–90% at the German site. These values are similar to previous studies in legume/grass leys receiving various amount of fertilizers and subject to several harvests per year (Carlsson and Huss-Danell 2003; Huss-Danell et al. 2007). The consistently high pNdfa in perennial *Trifolium* species highlights their value as N contributors, in diverse as well as in species-poor grasslands, harvested once or several times per season.

Effects of plant diversity on N_2 fixation

We found only partial support for our first hypothesis that pNdfa increases with increasing species richness. Even though species richness had no significant effect on pNdfa during the period without N addition, pNdfa in *T. pratense* responded differently to species richness in different years at the Swedish site, with a slight increase in pNdfa with increasing species richness in 1998 (Fig. 2b). Apart from this interaction (Table 3), plant diversity influenced pNdfa only after

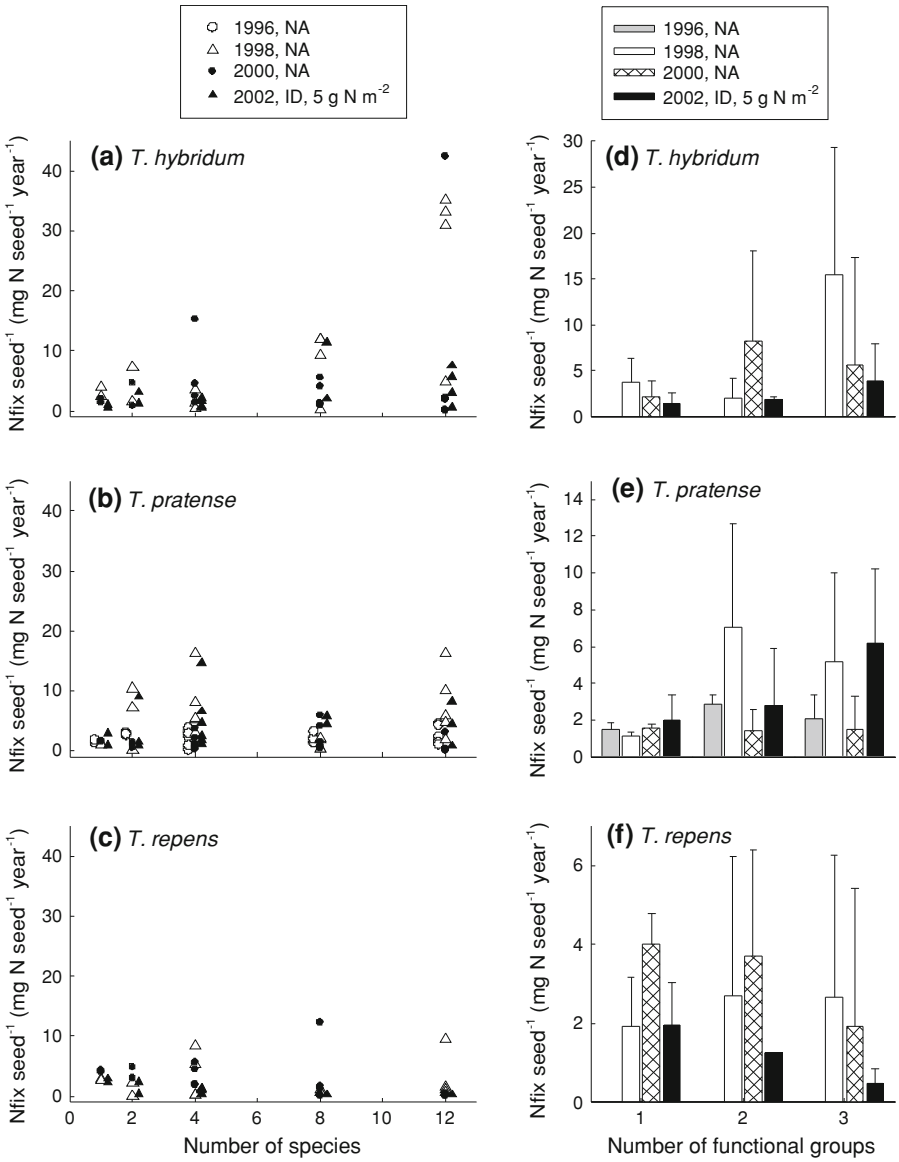

Fig. 5 The amount of N_2 fixed per sown seed (Nfix seed^{-1}) in the Swedish experiment in relation to the number of sown plant species (**a**–**c**) and number of functional groups (**d**–**f**, mean ± SD). Data are based on shoot (>5 cm aboveground) analyses. Each point represents one experimental plot. *NA* natural abundance method, *ID* isotope dilution method

the addition of 5 g N m^{-2} year^{-1} (Table 4). The N addition associated with the ID experiment resulted in higher plant biomass yields in communities without legumes, but not in communities with legumes (Palmborg et al. 2004), which indicates that communities with legumes did not suffer from N starvation despite the lack of N fertilization between 1996 and 2001. Therefore, the relatively small dose of added N created a temporary surplus of plant-available soil N so that the legumes could reduce their reliance on N_2 fixation, especially in species-poor communities without presence of grasses. In contrast, high diversity of coexisting non-leguminous species obviously lead to high competition for soil N, so that the *Trifolium* species had to increase their reliance upon symbiotically fixed N almost to similar levels as under conditions without N addition. Thus, diversity effects on N_2 fixation are obviously context dependent, with soil N availability being a major factor influencing pNdfa.

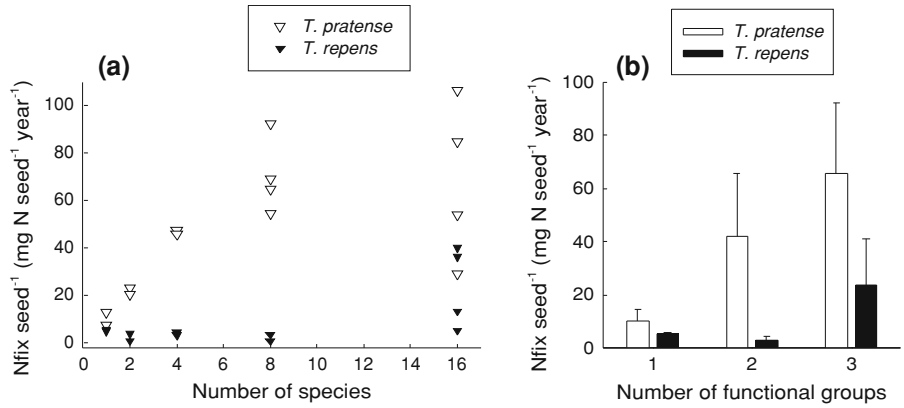

Fig. 6 The amount of N_2 fixed per sown seed (Nfix seed^{-1}) in the German experiment in relation to the number of sown plant species (**a**) and number of functional groups (**b**, mean ± SD). Data are based on shoot (>5 cm aboveground) analyses. Each point represents one experimental plot

Partially confirming our second hypothesis, the standardized measurement of the amount of N_2 fixed per sown seed (Nfix seed^{-1}) correlated positively with species richness in *T. pratense* at the German site (Fig. 7a, Table 5). Since pNdfa was unaffected by plant diversity in situations without N addition, this positive diversity effect on Nfix seed^{-1} obviously was related to the decreasing abundance of legumes along the diversity gradient: reduced intra-specific competition in species-rich communities may have allowed a higher proportion of sown *T. pratense* plants to reach high N_2 fixation per plant as compared to in species-poor communities. It is not clear why the positive diversity effect on Nfix seed^{-1} in *T. pratense* was not consistent between the two sites. Genotypic differences and climate probably contributed to this variation, since it has been shown at our sites that plant genotypes perform very differently across a wide geographical range (Joshi et al. 2001).

Effects of community composition on N_2 fixation

Our approach to include the biomass proportions of individual species in the multivariate analyses has highlighted the importance of community composition and functional traits in plant species interactions. In general, grass and legume abundance, but not abundance of non-leguminous forbs, often correlated with the analysed N_2 fixation parameters. The PLS analysis showed that biomass proportions of the grasses were positively correlated with pNdfa in *T. hybridum* and *T. repens* receiving N (Fig. 3a, b), most likely because grasses, with their large root biomass (Huss-Danell et al. 2007), efficiently reduced the soil mineral nitrogen levels at our study site (Palmborg et al. 2005). This corroborates previous studies showing that N uptake by grasses increase legumes reliance on N_2 fixation (Brophy et al. 1987; Carlsson and Huss-Danell 2003; Hartwig 1998; Kahmen et al. 2006; Loiseau et al. 2001; Sanford et al. 1995; Xiao et al. 2004). Clearly, our results support the importance of complementarity for plant utilization of limiting resources (Fargione et al. 2007; Oelmann et al. 2007; Temperton et al 2007). In particular, facilitative interactions between nitrogen fixers (legumes) and plants with efficient N uptake (grasses) may improve the input and retention of N, and thereby the soil fertility of the ecosystem (Dybzinski et al. 2008).

In line with our third hypothesis, sown grass proportion correlated negatively with Nfix seed^{-1} in *T. repens*, both at the Swedish site in the ID experiment (with N addition) and at the German site without N addition (Figs. 6, 7c). In the studied plant communities, analyses of the proportion of transmitted light within the photosynthetically active range and the $\delta^{13}C$ in *Trifolium* shoots have implied that *Trifolium* species tended to be shaded in species-rich communities (Jumpponen et al. 2005; Spehn et al. 2005). Thus, consistent with our third hypothesis, competition for light by tall grasses may have reduced legume growth and N_2 fixation, and this effect may have been particularly strong in *T. repens* with its creeping growth pattern. On the other hand,

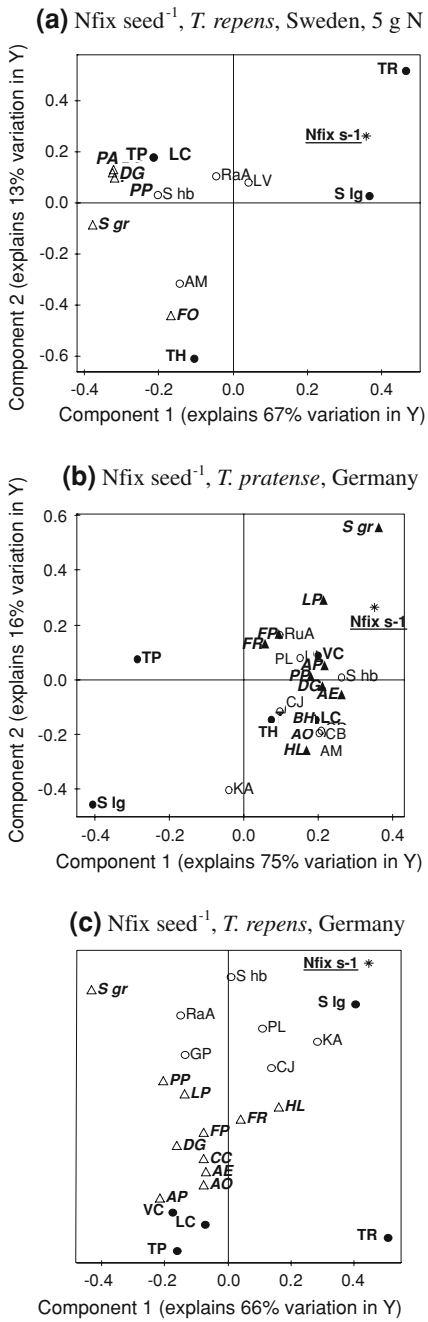

Fig. 7 PLS loadings for *X* and *Y* (Nfix seed^{-1} = Nfix s^{-1}; *star*) variables in the Swedish ID experiment (**a**) and the German experiment (**b**, **c**). For further details, see legend of Fig. 3

effects of community composition on Nfix seed^{-1} differed between *T. pratense* and *T. repens*. Grass abundance correlated positively with Nfix seed^{-1} in *T. pratense* at the German site (Fig. 7b). This finding supports the mechanism that increasing plant diversity and/or grass abundance—and thereby decreasing legume abundance—may lead to reduced intra-specific competition between *T. pratense* plants and thus higher N_2 fixation per sown seed. These findings thus imply that grasses may on one hand increase N_2 fixation in neighbouring legumes via reduced soil N availability and intra-specific competition, and on the other hand reduce legume growth and N_2 fixation due to shading, which would be particularly important for low-growing legumes like *T. repens*.

Sown legume proportion or biomass proportion of individual *Trifolium* species sometimes correlated negatively with N_2 fixation (Figs. 3, 6, 7b). According to the N feedback mechanism proposed by Hartwig (1998), the low C/N ratio of legume litter that releases plant-available N through mineralization may both down-regulate N_2 fixation in legumes (reduce pNdfa) and make co-existing non-legumes more competitive. In addition, our results show that abundance of one *Trifolium* species may decrease pNdfa and/or Nfix seed^{-1} in co-occurring *Trifolium* species, which implies that this N feedback mechanism may be extended to legume–legume interactions, thereby complementing the model by Hartwig (1998).

The negative correlations between biomass proportion of the forb *Achillea millefolium* and pNdfa in *T. hybridum* and *T. repens* in the ID experiment at the Swedish site (Fig. 3) were likely caused by the lack of strong competitors for soil N (tall grasses) in these communities. Mixtures of *A. millefolium*, *T. hybridum*, *T. repens* and the low-growing grass *Festuca ovina* resulted in large biomass proportions of the legumes and *A. millefolium*. Since this forb probably is a weaker competitor for soil N than tall grasses, the legumes were allowed to rely on soil N to a higher degree in these communities, and thereby reduce their pNdfa. We therefore suggest that the abundance of legumes and grasses had a strong influence on N_2 fixation in legumes, especially after N addition, while non-leguminous forbs had little or no effect.

Other implications of the study

Shoot δ^{15}N decreased over time both in the *Trifolium* species and the reference species (Fig. 1). An overall decrease in the ^{15}N-enrichment of plant-available soil N over time could be caused by decreased ^{15}N

discrimination via nitrification and N losses (Högberg 1997), since the yearly removal of shoot biomass and lack of N fertilization probably reduced the amount of N available for these processes (Mulder et al. 2002). In addition, legume-containing communities may benefit from inputs of fixed N, which has a relatively low $\delta^{15}N$ signature, via the degradation of legume litter. Fornara et al. (2009) found that fine roots of legumes and non-leguminous forbs decomposed and released N faster than grass fine roots, and suggested that the presence of legumes may enhance N cycling in diverse plant communities. In our studied plant communities, legume presence had a positive effect on soil inorganic N contents (Palmborg et al. 2005), supporting the idea that N fixed by legumes provided the soil with inputs of N with low $\delta^{15}N$. Indeed, our finding that $\delta^{15}N$ in *Phleum pratense* and *Ranunculus acris* declined more rapidly when these species grew together with legumes than when grown without legumes (Fig. 1a, c), provide evidence for such N transfer from legumes to non-legumes. The possibility that all of the plant species in the studied communities increasingly rely on leguminous N_2 fixation over time was proposed by Mulder et al. (2002)—a hypothesis supported by the additional data presented here.

Estimates of pNdfa differed slightly depending on which reference species that was used at the Swedish site. Different species have been found to take up soil N at different rates depending on soil depth and N pool (Jumpponen et al. 2002; Näsholm et al. 2000). Since it is nearly impossible to know the "true" $\delta^{15}N$ of the soil N available to the legume, and equally difficult to identify one optimal reference plant, it has been suggested that the best choice is to use several reference species (Jacot et al. 2000), as we have done in the present work. Using the mean $\delta^{15}N$ of several reference plants reduces the risk of comparing the N_2-fixing plant with a plant that has very different soil N uptake characteristics. Another important question is whether N transfer from legumes to reference plants undermines the accuracy of N_2 fixation measurements obtained with the NA and ID methods (Brophy et al. 1987). In 2000, $\delta^{15}N$ was lower in two of our reference species when they were growing together with legumes compared to in monoculture, indicating that these species had taken up N transferred from legumes. While direct unidirectional N transfer from a legume to a co-cultivated reference plant may confound pNdfa estimates, using a monocropped reference plant will impose more severe errors since it fails to accurately mimic the ^{15}N of the soil N available to the legume. Detailed studies in clover–grass mixtures have provided evidence that N transfer is a bi-directional process, i.e. it does not only occur from legumes to non-legumes but also in the opposite direction (Gylfadóttir et al. 2007; Rasmussen et al. 2007). In addition, these studies showed that the amount of soil N deposited by *Trifolium* plants (rhizodeposition, degradation of litter, etc.) was almost as large as the transfer of N from legumes to grass. We are thus convinced that any soil N derived from N_2 fixation via rhizodeposition or degradation of legume litter will be equally available to the legume as to the reference plant, stressing the need to use reference plants grown in the same plots as the legume.

Conclusions

This study is the first detailed and quantitative investigation of the effects of plant diversity and species composition on N_2 fixation in legumes. Without N addition, the studied genotypes of the three *Trifolium* species relied primarily on N_2 fixation for their N supply, regardless of community composition and species richness. Nevertheless, especially under conditions of slightly increased soil nitrogen availability, trait differences among co-occurring species influenced N_2 fixation rates through changes in resource availability. Grasses may on the one hand increase pNdfa in neighbouring legumes via competition for soil N, and on the other hand reduce growth and N_2 fixation in low-growing legumes like *T. repens* via strong competition for light. Increasing the diversity of neighbouring plants—and thus trait diversity—can obviously modulate these interactions between legumes and non-legumes, leading to higher N_2 fixation under certain conditions by reducing the amount of plant-available soil N and reducing the intensity of intra-specific competition among legumes.

The implications of our study are thus (1) that the N_2-fixation in perennial *Trifolium* species can be relied upon in a broad range of conditions, in species-rich communities with few harvests per season as studied here, as well as in species-poor communities

with several harvests per season, (2) that the role of plant diversity, both in terms of species richness and of number of a priori defined functional groups for the N_2 fixation process seems to be context dependent and (3) that community composition and functional traits of coexisting species do exert a strong control on N_2 fixation. We conclude that biomass proportions of legumes and grasses need to be well balanced for an optimal use of N_2 fixation in grasslands.

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