***Highlights**

Research highlights,

> The N_2-fixation of four forage legumes was quantified in terms of biomass yield and residual N effect > Red clover fixed more than 300 kg N ha^{-1} $year^{-1}$ in the above ground biomass > Red clover gave the highest residual N effect, and bird's foot trefoil the lowest > Lucerne had twice the N_2-fixation than white clover, yet their residual N effects were similar.

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N_2-fixation and residual N effect of four legume species and four companion grass species.

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Abstract

Inclusion of forage legumes in low-input forage mixtures improves herbage production and soil fertility through addition of nitrogen (N) from N_2-fixation. The impact of different grass-legume mixtures on the N contribution of the forage mixture has rarely been investigated under comparable soil and climatic conditions. We conducted a field experiment on a sandy soil at two nitrogen levels with seven two-species forage mixtures: alfalfa, bird's-foot trefoil, red clover, or white clover in mixture with perennial ryegrass, and white clover in mixture with meadow fescue, timothy, or hybrid ryegrass. We found high N_2-fixation of more than 300 kg N ha^{-1} from both red clover and alfalfa even when the two mixtures received 300 kg total-N ha^{-1} in cattle slurry. The addition of cattle slurry N fertilizer lowered N_2-fixation for white clover and red clover as expected, but for bird's-foot trefoil and alfalfa no changes in the proportion of N derived from N_2-fixation was observed. We conclude that the competition for available soil N from perennial ryegrass in mixture was an important factor for the proportion of N in alfalfa, white clover, and bird's-foot trefoil obtained from N_2-fixation. White clover had a high proportion of N derived from atmosphere for all companion grasses despite significant differences in white clover proportion. Although the perennial ryegrass-alfalfa mixture in the grass phase yielded more than twice the N from N_2-fixation compared to white clover in the perennial ryegrass mixture, this did not in the following year lead to higher residual N effects of alfalfa. Both in terms of N yield in the grass phase and N yield in the subsequent spring barley red clover contributed most to the improvement of soil N fertility.

Keywords: N_2-fixation, N transfer, residual N effect, companion species, forage legume

1. Introduction

The world population is predicted to increase to nine billion people by 2050, and by then the world food production needs to double to meet the demand (Godfray et al., 2010). At the same time we face the challenges of reducing the climatic and environmental impact of human production systems (Canfield et al., 2010); thus, a sustainable intensification of the agricultural production is needed (The Royal Society, 2009). Achieving higher yields whilst neither being able to increase the size of the farmed area nor the use of mineral fertilizers is one of the greatest challenges for agricultural scientists. A suggested method to achieve this goal is to increase the inclusion of legumes in crop rotations (Canfield et al., 2010; [Anon], 2010) in order to replace fertilizer-N by biologically fixed N – with N being a major limiting factor for plant growth

(Fustec et al., 2010). Today, N_2-fixation contributes at least 16% of the global N supply (Liu et al., 2010), with grain and forage legumes being the main contributors of biologically fixed N (Herridge et al., 2008).

In production systems based on temporary grasslands the inclusion of forage legumes has long been recognized as a means of reducing N fertilizers. In a crop rotation system N_2-fixation of forage legumes adds N in both the grass phase and in the subsequent arable crop phase. In the grass phase the presence of forage legumes in the harvested biomass increases the N yield and feed quality of the sward. In addition, the forage legumes increase available N for the companion non-legumes either via N sparing (Kumar and Goh, 2000b) or through direct and indirect N transfer (Høgh-Jensen, 2006; Rasmussen et al., 2007). For subsequent crops forage legumes contribute N both directly from forage legume residues and indirectly through mineralization of soil N pools build up during the grass phase (Eriksen et al., 2008; Vertés et al., 2007).

Alfalfa, red clover and white clover are the main forage legumes used worldwide today, whereas bird's-foot trefoil is mainly important under temperate conditions (Fustec et al., 2010). In previous studies, the N input from forage legume N_2-fixation in harvested biomass has been estimated to be up to 350, 373, 545, and 223 kg N ha^{-1} $year^{-1}$ for alfalfa, red clover, white clover, and bird's-foot trefoil, respectively (Carlsson and Huss-Danell, 2003; Ledgard and Giller, 1995). In general, forage legumes grown in a mixture with grass receive most (> 80%) of their N supply via N_2-fixation (Heichel and Henjum, 1991; Kumar and Goh, 2000a), which implies that the amount of N from N_2-fixation depends on the forage legume dry matter production (Unkovich et al., 2010). The dry matter production is highly site-specific, i.e. due to differences in the length of the growth season, or in climatic or environmental conditions (Carlsson and Huss-Danell, 2003). In addition, the management of the grass-legume mixture affects N_2-fixation, e.g. N fertilization reduces the proportion of N that the forage legume derives from N_2-fixation (Cherney and Duxbury, 1994; Mallarino and Wedin, 1990; Nesheim et al., 1990) or competition from the companion grass affects the growth of the forage legume (Carlsson et al., 2009; Nesheim and Boller, 1991; Woledge et al., 1992).

The residual N effect of forage legumes on the N yield of subsequent crops is well studied in terms of the total N fertilizer effect and the direct N contribution from crop residues. Generally, the largest amount of N becomes available the first year after the grass phase (Eriksen, 2001; Nevens and Reheul, 2002; Ta and Faris, 1990), with studies showing that up to 25% of N in incorporated crop residues can be found in the subsequent crop (e.g. Kumar and Goh, 2000b; Muller and Sundman, 1988). The decomposition of crop residues and the build-up of the soil N pool depends on quality measures such as the C/N-ratio, lignin-, polyphenol-, and cellulose-content (Fillery, 2001; Wichern et al., 2008). Thus, the N availability for subsequent crops depends both on the amount of N added in crop residues and N input to the soil during the grass phase and the chemical characteristics of residues and the build-up of soil organic N. Since 70-80% of the total N released after ploughing-in of a the grass sward originates from the build-up of the soil N pool (Vertés et al., 2007), for improved management of this N pool it is essential that the soil N build-up during the grass phase can be estimated well. Studies have shown that the N yields in the grass phase are a good indicator of soil organic N build-up and the subsequent residual N effect (Alvarez et al., 1998; Høgh-Jensen and Schjoerring, 1997b).

In this paper we give the results of a field experiment with different two-species mixtures of temporary grasslands consisting of perennial ryegrass with one out of four different forage legumes respectively, and white clover with one out of four different grass species. With the objective of improving the nitrogen use efficiency of agricultural systems through an enhanced use of forage legume N_2-fixation we tested the hypotheses, that:

(i) addition of N fertilizer reduces the N_2-fixation of forage legumes both in relation to proportion and amount of N fixed,

(ii) increased growth of the companion grass species when fertilized increases the proportion of N that white clover derives from N_2-fixation and at the same time reduces the growth of white clover,

(iii) the residual N effect observed in the subsequent crop following cultivation reflects the N yield of the two-species mixtures and the estimated N_2-fixation of the forage legume obtained during the grass phase.

2. Materials and Methods

2.1. Soil and site history

The experimental area is located at Foulumgaard Experimental Station in Jutland, Denmark (9°34'E, 56°29'N). The soil is a loamy sand classified as a Typic Hapludult with the Ap-horizon (0-25 cm) containing 7.7% clay (<2 μm), 11% silt (2-20 μm), 47% fine sand (20-200 μm), 32% coarse sand (200-2000 μm), and 1.6% carbon. The mean annual temperature and precipitation at the site are 7.3°C and 627 mm, respectively (1961-1990). Temperature, rainfall and irrigation in 2008 and 2009 are shown in Figure 1. The site had been under cereal cropping for five years before the experiment was established.

2.2. Establishment, treatments and measurements in the forage mixtures

In spring 2006 seven forage mixtures and a perennial ryegrass pure stand were undersown in spring barley in a randomized block design with four replications. The seven forage mixtures were: perennial ryegrass (*Lolium perenne*, cv. Mikado) / red clover (*Trifolium pratense*, cv. Rajah) (PR/RC), perennial ryegrass / alfalfa (*Medicago sativa*, cv. Pondus) (PR/A), perennial ryegrass / bird's-foot trefoil (*Lotus corniculatus*, cv. Lotanova) (PR/BT), perennial ryegrass / white clover (*Trifolium repens*, cv. Milo) (PR/WC), meadow fescue (*Festuca pratensis*, cv. Laura) / white clover (MF/WC), timothy (*Phleum pratense*, cv. Tundra) / white clover (T/WC), and hybrid ryegrass (*Lolium hybridum*, cv. Solid) / white clover (HR/WC); and in addition, perennial ryegrass in pure stand (PRpure). The seeding rates are given in Table 1. The spring barley cover crop was harvested in August 2006. The forage mixtures were cut once in the autumn in 2006, and four times in 2007. In 2007 all plots were fertilized with 300 kg total-N ha^{-1} in cattle slurry divided into four applications during the growth season.

In spring 2008 each plot (7.5 × 8m) was divided into a main plot (6 × 8m) and a subplot (1.5 × 8m) (Figure 2) with the subplot receiving no additional fertilization and the main plot receiving a fertilization of 290 kg total-N ha^{-1}, 54 kg P ha^{-1}, and 263 kg K ha^{-1} in cattle slurry given as 93, 80, 59, and 58 kg total-N ha^{-1} in spring and after the first, second and third cut, respectively, using trail hose application. In the cattle slurry 61% of the total-N was in the form of NH_4. The forage mixtures were harvested four times during the season: late May, early July, middle of August, and early October. At each harvest, herbage dry matter (DM) yield and botanical composition, after hand separation, were determined per plot.

Legume N_2-fixation was determined by the ^{15}N-isotopic dilution method (McNeill et al., 1994). Briefly, ^{15}N-$(NH_4)_2SO_4$ (98 atom%) was irrigated at a rate of 0.9 kg ^{15}N ha^{-1} to 1-m squares in both the main plot and the subplot at the beginning of April. Measurements of total N and ^{15}N-enrichment of grasses and legumes from the areas irrigated with ^{15}N-$(NH_4)_2SO_4$ was determined for grass and legume shoot material at each harvest. Plant samples were dried and ball-milled before being packed into tin capsules and analysed by mass spectrometry (Stable Isotope Facility Lab, UC Davis, CA). Total N and ^{15}N-enrichment in the samples were analysed using continuous flow isotope rate mass spectrometry after combustion to N_2 gas at 1000°C in an on-line elemental analyzer (PDZ Europe, Northwick, England).

2.3. Spring barley test crop

In late March 2009 all forage mixtures were ploughed and planted to spring barley one week later. All plots received a basic dressing of 500 kg ha^{-1} 0-4-21 N-P-K. In two of the four former forage mixture blocks the spring barley received no additional fertilizer (0N), while the other two forage mixture blocks received a dressing of 300 kg ha^{-1} 24-7 N-S, corresponding to 70 kg N ha^{-1} (70 N; Figure 2). The plots were not irrigated as drought stress did not appear (Figure 1). The barley was harvested at maturity in August in a subplot of 1.5 x 7 m in the middle of the plot, i.e. the plots that in the grass phase in 2008 had received 300 kg total-N ha^{-1} in cattle slurry, The DM yields of grain and straw were determined and total N in dry matter was determined on a LECO CNS-100 elemental analyzer.

2.4. Calculations and statistics

The percentage of N derived from N_2-fixation (%Ndfa) was calculated using the equation of McNeill et al. (1994):

$$\%Ndfa = \left(1 - \frac{legume\ atom\%\ excess}{grass\ atom\%\ excess}\right) \times 100$$

where legume and companion grass atom% excess are calculated by subtracting the ^{15}N-atom% enrichment of legume or companion grass in unlabelled plots from the ^{15}N-atom% enrichment of legume or companion grass in the ^{15}N-dilution plots.

For forage dry matter and N yields, N derived from the atmosphere (Ndfa) and legume proportion of the sward, and for barley yields, analysis of variance was carried out using the General Linear Model (GLM) procedure of SAS (SAS institute Inc., 1999) to estimate differences between treatments.

3. Results

3.1. Dry matter yields and botanical composition of forage mixtures

The total dry matter production as well as the botanical composition differed significantly among the four perennial ryegrass / legume mixtures, both unfertilized and when fertilized with 300 kg total-N ha^{-1} (Table 2). The red clover (PR/RC) mixture had the highest dry matter yields followed by alfalfa (PR/A), white clover (PR/WC), and bird's-foot trefoil (PR/BT). The same sequence was found for the legume proportion of the swards (Table 2, Figure 3). The differences in total dry matter yields among the four perennial ryegrass / legume mixtures were caused by differences in total dry matter yields of both the legumes and perennial ryegrass in the mixtures;

red clover in particular was high-yielding, almost outcompeting perennial ryegrass at the last harvests, and when fertilized red clover increased growth in contrast to the other three legumes.

The total dry matter production and the botanical composition among the four grass / white clover mixtures were only significantly different when fertilized (Table 2). Meadow fescue (MF/WC) had the highest total dry matter yield, followed by hybrid ryegrass (HR/WC), perennial ryegrass (PR/WC) and timothy (T/WC). The perennial ryegrass (PR/WC) mixture had a significantly higher proportion of white clover in the sward than the other three grass / white clover mixtures, both with and without fertilization (Table 2). There were no significant differences between the proportion of white clover in mixture with meadow fescue (MF/WC), timothy (T/WC), and hybrid ryegrass (HR/WC) when not fertilized, but with fertilization the proportion of white clover was significantly higher when in mixture with timothy than with meadow fescue (Table 2, Figure 3). Fertilization caused for both perennial ryegrass (PR/WC) and timothy (T/WC) an increase in grass dry matter yields without a simultaneous decrease in white clover dry matter yields, whereas for both meadow fescue (MF/WC) and hybrid ryegrass (HR/WC) the increases in grass dry matter yields were accompanied by a decrease in white clover dry matter yield (Figure 4).

The perennial ryegrass in pure stand (PRpure) had significantly lower dry matter yields than all grass / legume mixtures both with and without fertilization (Table 2). In addition, perennial ryegrass in pure stand fertilized with 300 kg total-N ha^{-1} had significantly lower total dry matter yields than the unfertilized grass / legumes mixtures. The growth of perennial ryegrass in pure stand was in the last part of the growing season in 2008 to some degree affected by the presence of white clover not sown in the plot.

3.2. N_2-fixation and N yields in forage mixtures

The total N yield and the amount of N in the harvested herbage originating from legume N_2-fixation followed total dry matter yields for the four perennial ryegrass / legumes mixtures (Table 2). Red clover (PR/RC) and alfalfa (PR/A) fixed well above 300 kg N ha^{-1}, both with and without fertilization. Fertilization with 300 kg total-N ha^{-1} increased total N yields significantly for the white clover (PR/WC) and red clover (PR/RC) swards. However, the amount of N derived from N_2-fixation was not significantly affected by fertilization in white clover (PR/WC), red clover (PR/RC), or alfalfa (PR/A). Only for bird's-foot trefoil did fertilization significantly lower the amount of N derived from N_2-fixation, i.e. from 121 to 81 kg N ha^{-1}. Averaged over the growing season the percentage of N in legume coming from N_2-fixation was above 90% for alfalfa (PR/A), bird's-foot trefoil (PR/BT), and white clover (PR/WC), both with and without fertilization (Table 3), and was fairly constant over the four harvests. Addition of 300 kg total-N ha^{-1} did not lower the percentage of N from N_2-fixation for alfalfa (PR/A), and bird's-foot trefoil (PR/BT), but did so for white clover (PR/WC) and red clover (PR/RC). Red clover (PR/RC) obtained 89% of its N from N_2-fixation without fertilization and 73% under fertilized conditions. Furthermore, the percentage of N from N_2-fixation in red clover (PR/RC) was lower for the third and fourth harvest compared to the first and second harvest, both with and without fertilization (Table 3).

Without fertilization, total N yield of the four grass / white clover mixtures was significantly different (Table 2) with meadow fescue (MF/WC) and hybrid ryegrass (HR/WC) having the greatest yields and perennial ryegrass (PR/WC) the lowest. When 300 kg total-N ha^{-1} was added, the N yields for perennial ryegrass (PR/WC) and timothy (T/WC) increased by 83 kg N ha^{-1} and 68 kg N ha^{-1}, respectively, somewhat higher than the N yield increases of 43 kg N ha^{-1} and 10 kg N ha^{-1} for meadow fescue (MF/WC) and hybrid ryegrass (HR/WC), respectively. Differences in the amount of N from N_2-fixation reflected the differences in white clover dry matter yields among the four grass / white clover mixtures (Table 2). Fertilization did not suppress white clover dry matter yields and the amount of N derived from N_2-fixation only slightly when grown in mixture with timothy (T/WC) and perennial ryegrass (PR/WC), in contrast to the decrease of 43 and 45 kg N ha^{-1} in N_2-fixation in meadow fescue (MF/WC) and hybrid ryegrass (HR/WC). For white clover, the proportion of N derived from N_2-fixation was not significantly different among the four companion grasses without fertilization (Table 3). When fertilized the proportion of N derived from N_2-fixation significantly decreased for white clover in mixture with perennial ryegrass (PR/WC) and timothy (T/WC). Simultaneously, there was a significant increase in soil N uptake by white clover in mixture with timothy (T/WC) (Table 4).

The total N yield in perennial ryegrass in pure stand (PRpure) was significantly lower than the seven grass / legume mixtures both under fertilized and unfertilized conditions (Table 2). Fertilizing the perennial ryegrass in pure stand with 300 kg total-N ha^{-1} in slurry increased the total N yield from 126 kg N ha^{-1} to 229 kg N ha^{-1}, corresponding to a N use efficiency of 56% of the applied ammonium-N in the slurry.

3.3. Spring barley dry matter and N yields

The N yields in unfertilized spring barley following the four perennial ryegrass / legume pre-crops showed a tendency for higher grain N yield after red clover (PR/RC) than after alfalfa (PR/A) and white clover (PR/WC), all being significantly higher than the grain yield following the bird's-foot trefoil mixture (PR/BT) (Table 5). There was no significant difference in straw N yields for the unfertilized barley. When barley was fertilized with 70 kg N ha^{-1} the grain N yield was highest after white clover (PR/WC) followed by red clover (PR/RC), but the difference was not significant. The grain N yield with fertilization was significantly lower after alfalfa (PR/A) than after white clover, and significantly lower yet again after bird's-foot trefoil (PR/BT) (Table 5). The straw N yield for the fertilized barley was significantly higher after white clover (PR/WC), red clover (PR/RC), and alfalfa (PR/A) than after bird's-foot trefoil (PR/BT).

The grain N yields in unfertilized barley following the four grass / white clover mixtures were highest after perennial ryegrass (PR/WC), followed by meadow fescue (MF/WC) and hybrid ryegrass (HR/WC), being higher than timothy (T/WC) (Table 5). The N yields in straw did not follow the same order, as barley after meadow fescue (MF/WC) had a higher straw N yield than after the other three grass / white clover mixtures, although this was only significant for timothy (T/WC). When barley was fertilized with 70 kg N ha^{-1}, perennial ryegrass (PR/WC) and hybrid ryegrass (HR/WC) gave significantly higher grain N yields than timothy (T/WC).

The barley N yields following cultivation of the perennial ryegrass in pure stand (PRpure) was lower than the seven grass / legume mixtures, except for bird's-foot trefoil (PR/BT) and timothy (T/WC), the latter only when spring barley was fertilized (Table 5).

Using perennial ryegrass in pure stand (PRpure) as reference the residual N effect of the grass / legume mixtures on spring barley N yields was in the range 6 – 21 kg N ha^{-1} for unfertilized barley and 5 – 28 kg N ha^{-1} for fertilized barley. Fertilization of spring barley with 70 kg N ha^{-1} increased total N yields by 44 – 58 kg N ha^{-1}, giving a fertilizer use efficiency of 62 – 82%. Spring barley after perennial ryegrass / white clover (PR/WC) and hybrid ryegrass / white clover (HR/WC) responded significantly better to fertilization than when grown after the other five grassland mixtures. Using perennial ryegrass in pure stand (PRpure) as reference, hybrid ryegrass / white clover (HR/WC) and perennial ryegrass / white clover (PR/WC) resulted in an additional N yield of the fertilized spring barley of 11 and 14 kg N ha^{-1}, respectively.

4. Discussion

The impact of different forage legumes on N yield in the grass phase of temporary grasslands and on soil N fertility which is reflected by N yields in crops in the subsequent arable phase is an important research issue in order to improve the N use efficiency of agricultural systems. In the present study we compared the grassland N yields and N_2-fixations of four forage legumes and their residual N effects, and furthermore the influence of the companion grass on the growth and N_2-fixation of white clover, and N yield in the following spring barley. We estimated the N_2-fixation based on above ground dry matter yields, knowing that this underestimates the total N_2-fixation (Carlsson and Huss-Danell, 2003). The N from N_2-fixation in below-ground plant material is, however, represented in the determinations of residual N effect of the two-species forage mixtures.

4.1. Forage legume N_2-fixation and the effect of companion grass

The N yields and N_2-fixation of all four forage legumes were within the range of what has previously been reported, with red clover and alfalfa being in the high end of previous studies (Carlsson and Huss-Danell, 2003; Ledgard and Giller, 1995).

Based on the existing literature our first hypothesis was that the addition of fertilizer N in slurry would lower both the proportion and the amount of N from N_2-fixation for all four forage legumes. Our data did only support the hypothesis on the amount of N from N_2-fixation for bird's-foot trefoil and on the proportion of N from N_2-fixation for red clover (PR/RC) and white clover (PR/WC). Surprisingly, neither alfalfa (PR/A) nor bird's-foot trefoil (PR/BT) had decreasing proportions of N derived from the atmosphere when fertilized, which contradicts with previous studies, that for all four forage legumes have shown reductions in the proportion of N derived from the atmosphere when fertilized with N (e.g. Cherney and Duxbury, 1994; Høgh-Jensen and Schjoerring, 1997a; Mallarino and Wedin, 1990; Nesheim et al., 1990). In order to elucidate the underlying mechanisms for the lacking effect of fertilization on alfalfa and bird's-foot trefoil we need to look at the dynamics between legume and grass in the four mixtures with and without fertilization, since the determination of N_2-fixation with the ^{15}N-dilution method is a de facto comparison of the plants' competition for soil N. The high proportion of N derived from N_2-fixation for all four legumes without fertilization points to a strong competition for available soil N from the companion perennial ryegrass in low soil N conditions. Due to red clover and alfalfa having more above-ground upright growth it is clear that they have competitive advantages towards the companion grass compared to white clover and bird's-foot trefoil. This is reflected in the higher legume proportions in red clover and alfalfa mixtures. The proportion of red clover in the sward was unaffected by fertilization, whereas alfalfa when fertilized had a lower proportion at all four harvests caused by an increased growth of the companion grass, which, however, did not negatively affect alfalfa growth. A negative effect of increased grass growth was clearly present in the bird's-foot trefoil mixture, where grass strongly suppressed the growth of bird's-foot trefoil, especially from the second harvest onwards. Thus, bird's-foot trefoil was outcompeted for soil N by the companion grass, implying that bird's-foot trefoil had to rely on N_2-fixation to acquire N. The opposite was the case for red clover where fertilization increased its soil N uptake from 47 to 115 kg N ha^{-1} (Table 4) in strong competition with the companion grass; reducing red clovers need for N_2-fixation. In the present study, alfalfa did not compete strongly for soil N, which is very surprising, as alfalfa in other studies has been found to be a strong competitor for soil N (Haby et al., 2006; Tomm et al., 1995).

The impact of grass-legume competition on legume N_2-fixation was studied in detail for white clover in mixtures with four different companion grass species. Our second hypothesis was that increased growth of the companion grass species would increase the proportion of N in white clover derived from N_2-fixation and at the same time reduce the growth of white clover. The present data could not fully support such a relation between companion grass growth and white clover N_2-fixation. Both with and without fertilization there were significant differences in the growth of the four grasses. Without fertilization perennial ryegrass (PR/WC) had a N yield in grass of 29-41 kg N ha^{-1} lower than the other three grasses, without having any effect on the N_2-fixation of white clover, neither did the growth of white clover differ as compared to meadow fescue (MF/WC) and hybrid ryegrass (HR/WC). When fertilizer was added all four companion grass increased their growth with perennial ryegrass and meadow fescue having the highest N yield increases. But only for meadow fescue (MF/WC) and hybrid ryegrass (HR/WC) decreased white clover growth; a decreased white clover growth that was not accompanied by changes in the proportion of N derived from N_2-fixation. For perennial ryegrass (PR/WC) and timothy (T/WC) fertilizer addition significantly reduced the proportion of N in white clover derived from N_2-fixation, due to increased white clover uptake of soil N. Thus, the present study showed that high yielding grasses did not stimulate white clover to change its reliance from soil N to N from N_2-fixation. Only in mixture with two out of four companion grass species was the N_2-fixation in white clover lowered by fertilizer addition.

The competition between grass and legumes can be further elucidated by comparing the present results with two-species mixtures to the results from an experiment done in a neighboring field where the four forage legumes were grown in a multi-species mixture (Pirhofer-Walzl et al., *in press*). The N_2-fixation was also measured in 2008 with the same method as in the present study, as well as the effect of slurry application was studied; although the multi-species mixtures only received a fertilization of 200 kg total-N ha^{-1} in cattle slurry. In the present study only red clover of the four legumes showed a markedly decreased proportion of N derived from N_2-fixation when fertilizer was added, i.e. from 90% to 74%. When multiple companion species were present red clover showed a similar reduction, i.e. from 90% to 75%, but furthermore there was a clear effect of fertilizer application for the other three forage legumes; with the proportion of N derived from N_2-fixation decreasing from 89% to 78%, 95% to 89%, and 87% to 55% for white clover, alfalfa, and bird's-foot trefoil, respectively. The high levels of N derived from N_2-fixation for all legumes in the present study point to perennial ryegrass being a strong competitor for soil N, but the comparison of the four companion grasses did not show perennial ryegrass as a particularly strong competitor for soil N among the grasses. However, the observed decrease in the proportion of N derived from N_2-fixation for alfalfa and bird's-foot trefoil when in the multi-species mixture strongly indicates that the lower number and thus larger distance between strong competitors for soil N in the multi-species mixtures makes more soil N available for uptake by alfalfa, white clover, and bird's-foot trefoil. Thus, the present findings of high N_2-fixation by alfalfa and bird's-foot trefoil when fertilized was most likely caused by strong companion grass competition.

4.2. Forage legume residual N effect

A build-up of the soil N pool during the grass phase of grassland mixtures including forage legumes compared to pure grass mixtures (Christensen et al., 2009; Høgh-Jensen and Schjoerring, 1997b) is a key reason for including forage legumes in a grassland sward. Based on previous studies (Alvarez et al., 1998; Høgh-Jensen and Schjoerring, 1997b) our hypothesis was that the build-up of soil organic N as indicated by the residual N effect measured in the subsequent crop would be related to the N yields in the grass phase. From this we would expect the residual N effect to decrease in the order red clover (PR/RC) > alfalfa (PR/A) >> white clover (PR/WC) > bird's-foot trefoil. However, the actual residual N effects measured in unfertilized spring barley did not follow the expected order. Red clover (PR/RC) gave the highest residual N effect and bird's-foot trefoil (PR/BT) the lowest, whereas white clover (PR/WC) and alfalfa (PR/A) had similar residual N effects. This similar residual N effect was unexpected as total N yields and N yields from N_2-fixation for alfalfa were more than twice as high as those for white clover.

The N availability for the subsequent spring barley relates not only to the amount of N added by the forage legume to the soil N pool during the grass phase or in incorporated residues, but also to N losses during the off season, the build-up of the soil C pool during the grass phase or the C content of the residues, and to synchrony between soil N mineralization and spring barley N demand (Kumar and Goh, 2000b; Vertés et al., 2007). In the present study alfalfa (PR/A) not only had higher N yields, but also higher dry matter yields during the grass phase than white clover (PR/WC), which indicates both higher N and C additions to the soil pools (Alvarez et al., 1998). Although not determined in this study, a higher C/N-ratio of alfalfa deposits and residues than those of white clover, as shown by Bolger et al. (2003) for alfalfa and subterraneum clover, would result in a higher C limitation of soil organic N and residue decomposition after alfalfa than after white clover (Hauggaard-Nielsen et al., 1998); thus, more N would be mineralized and available for plant uptake after white clover (Alvarez et al., 1998). This was supported by the residual N effects found for the fertilized spring barley, where more plant-available N was present after white clover (PR/WC) than after alfalfa (PR/A); this also explains why N yields were higher for fertilized spring barley following white clover (PR/WC) than following red clover (PR/RC). Furthermore, since the N contents of both grain and straw were similar following white clover (PR/WC) and alfalfa (PR/A), there seemed to be no difference in the synchrony of N mineralization (Eriksen et al., 2006), which was for white clover in the four grass mixtures. Keeping in mind, that alfalfa was unaffected by fertilizer addition, these results of the residual N effects also show that white clover (PR/WC) had a higher soil N input (build-up + residues) relative to above-ground N yield than alfalfa (PR/A). White clover has previously been reported to have high relative soil N inputs (Høgh-Jensen and Schjoerring, 2001; Sturite et al., 2006), but the present findings that white clover (PR/WC) residual N effects match those of alfalfa (PR/A), underlines our knowledge gaps of processes behind a build-up of the soil N pool under forage mixture and, in particular, the N deposition and root longevity of the forage legumes.

The residual N effect of white clover in mixture with the four grasses was different both for the unfertilized and fertilized spring barley. There were, however, no clear relations between the grass phase production measures and the residual N effect. Timothy (T/WC) gave the lowest residual N effect, and when spring barley was fertilized, perennial ryegrass (PR/WC) as a pre-crop led to the highest spring barley N yields. But a thorough discussion would need knowledge of e.g. C input to the soil in the grass phase and N leaching during the winter.

4.3. Evaluation of legume N impact

Substitution of chemically fixed N with biological N fixation will enhance the sustainability of the agricultural production, in terms of N fertility. The N_2-fixation values found for the four forage legumes in this study show that the choice of forage legume strongly affects the N input to an agricultural production system. Only a few studies compare more than two forage legumes under similar management, environmental and climatic conditions (Askegaard and Eriksen, 2007; Heichel and Henjum, 1991; Mallarino and Wedin, 1990; Mallarino et al., 1990a and b; Ross et al., 2009; Ta and Faris, 1987). In relation to the N yield from forage legume N_2-fixation we conclude that alfalfa and red clover are superior compared to white clover and bird's-foot trefoil (see summary in Table 6; Heichel and Henjum, 1991; Mallarino et al., 1990a; Ta and Faris, 1987). To evaluate the cropping system the residual N effect on the subsequent crop should also be included. The residual N effect in the first year following the grass phase is estimated to represent 10-25% of the N added in residues and to the soil N pool during the grass phase (Kumar and Goh, 2000b; Ta and Faris, 1990; Wichern et al., 2008). Using these proportions in the present study the net residual N effect of including forage legumes in the grassland (Figure 5) corresponds to a N addition in mineral fertilizer of 40-100 kg N ha^{-1} for bird's-foot trefoil, 88-220 kg N ha^{-1} for white clover and alfalfa, and 140-350 kg N ha^{-1} for red clover. Hence, red clover in relation to both N yields in the grass phase and the subsequent barley contributed the most to soil N fertility.

5. Conclusions

We found high N yields from N_2-fixation in red clover and alfalfa. Unexpectedly, N fertilization did not lower the proportions of N derived from the N_2-fixation for alfalfa and bird's-foot trefoil. The impact of companion grass species on white clover N_2-fixation indicated that above-ground competition for light was not the main process controlling forage legume N_2-fixation behavior. Instead we conclude that the companion grasses significantly affect the available soil N, as shown for bird's-foot trefoil which was outcompeted by grass and reduced in growth, and for alfalfa relying on N_2-fixation to obtain N; although still having large biomass productions and N yields. Unexpectedly, the large differences in total N yields and N yields from N_2-fixation among the forage legume mixtures in the grass phase were not reflected in the residual N effects measured in the subsequent barley crop. In particular, white clover gave higher residual N effects than expected from its N yields in the grass phase. We conclude that red clover under the present settings was the best choice in terms of N fertility impact on the agricultural production system.

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Figure captions

Figure 1. Daily temperature, monthly rainfall and irrigation in 2008 and 2009.

Figure 2. Schematic representation of treatments in the forage mixture: 2007 and 2008, and in the spring barley: 2009.

Figure 3. Temporal development in legume proportion of harvested dry matter the four legume-perennial ryegrass mixtures, (○) white clover (PR/WC), (▽) red clover (PR/RC), (△) bird's-foot trefoil (PR/BT), and (□) alfalfa (PR/A), without slurry (A) and with application of in total 300 kg N ha^{-1} as cattle slurry (B); and the legume proportion of harvested sward for the four white clover-grass mixtures, (○) perennial ryegrass (PR/WC), (▼) meadow fescue (MF/WC), (▲) timothy (T/WC), and (■) hybrid ryegrass (HR/WC), without slurry (C) and with application of in total 300 kg N ha^{-1} as cattle slurry (D).

Figure 4. Dry matter yields of grass (black) and white clover (grey) in the four grass-white clover mixtures without fertilization, and the change in dry matter yield of grass and white clover when fertilized with 300 kg total-N ha^{-1} in cattle slurry.

Figure 5. Estimated net residual N effect in barley of red clover (PR/RC), alfalfa (PR/A), white clover (PR/WC), and bird's-foot trefoil (PR/BT). Estimation based on total barley N yields fertilized with 0 and 70 kg N ha^{-1} with pure stand perennial ryegrass (PRpure) as reference pre-crop.

Figure

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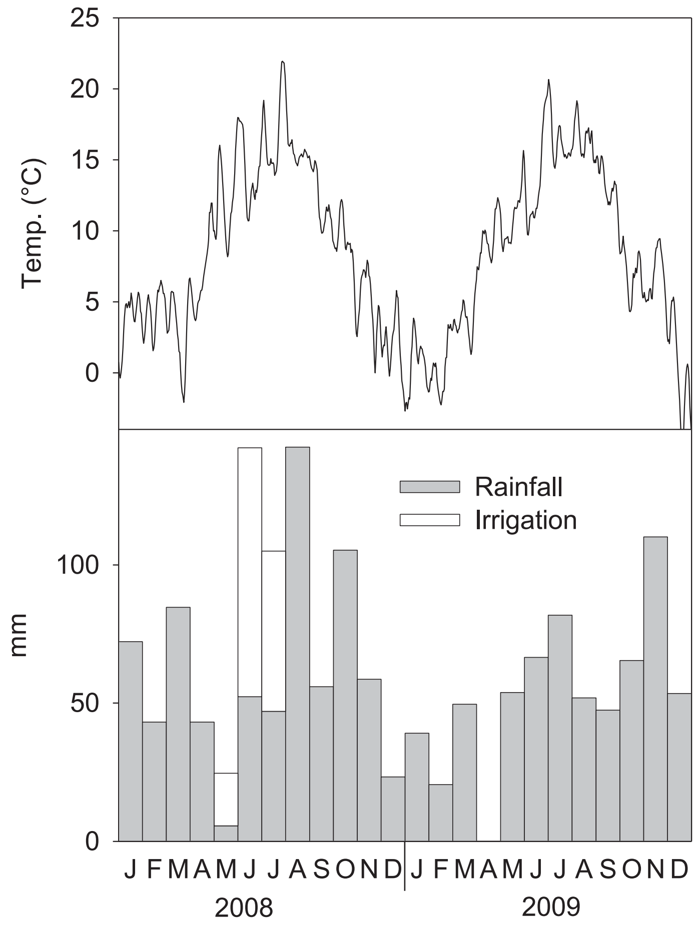

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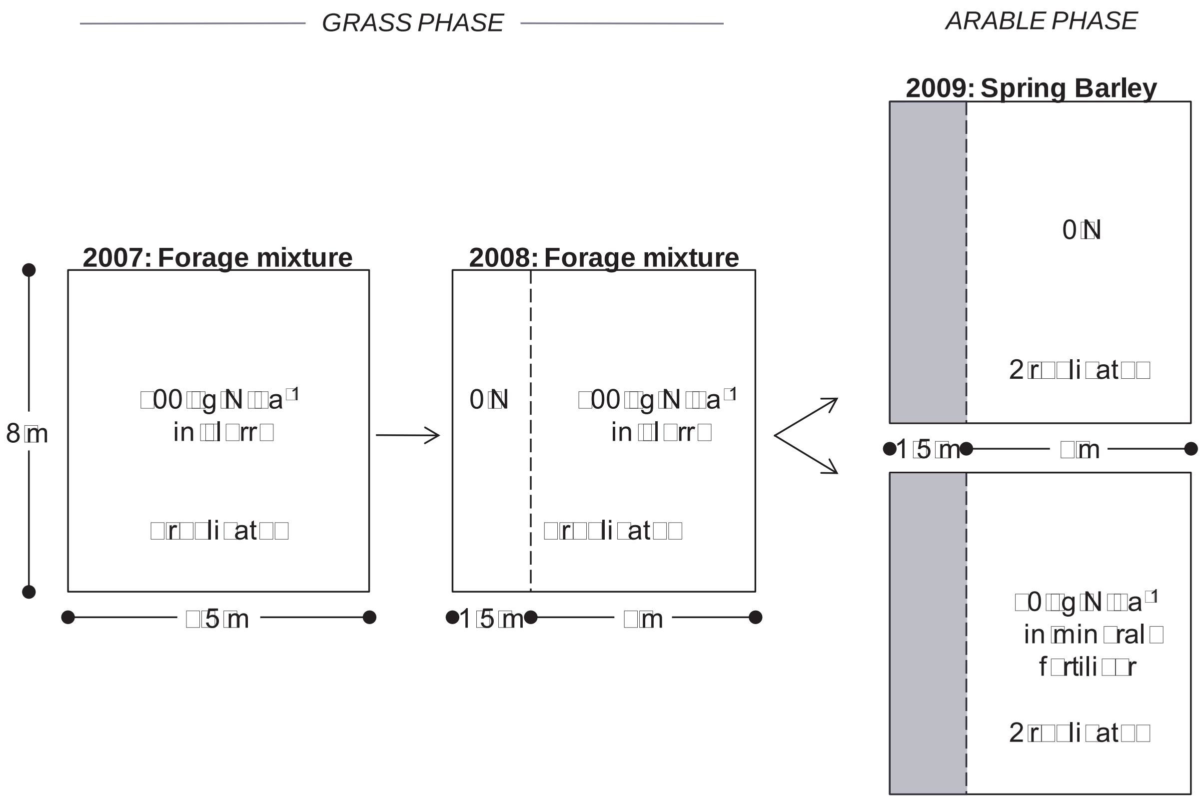

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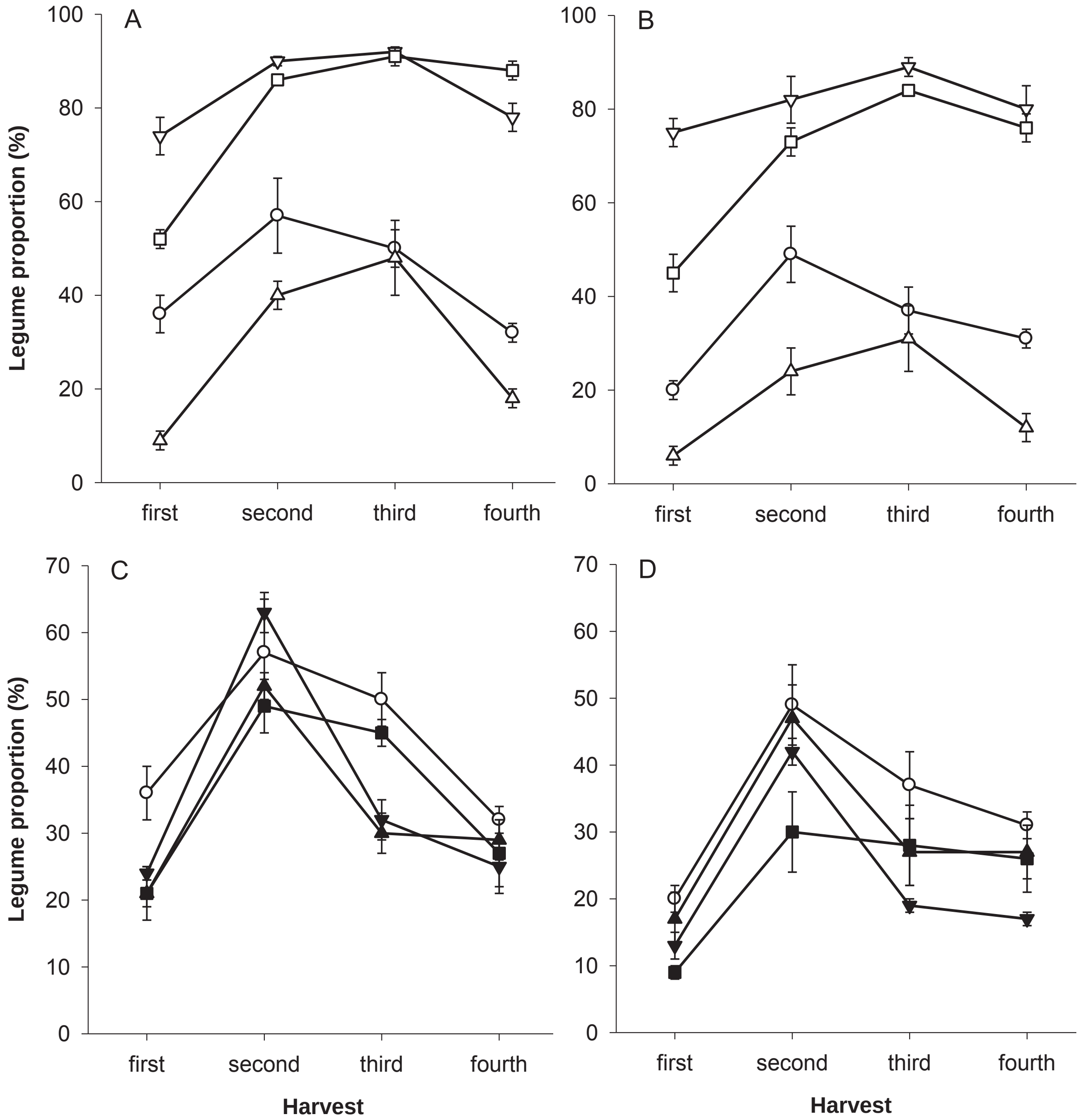

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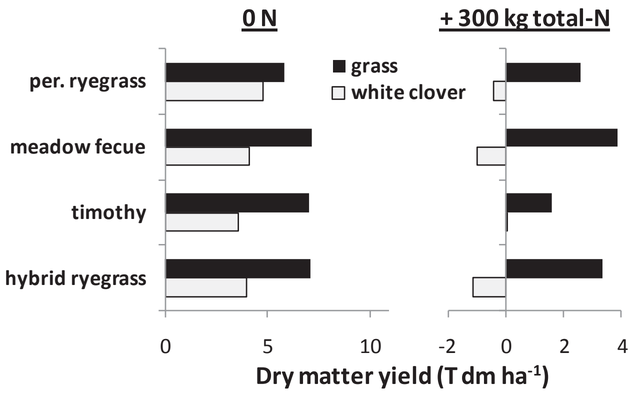

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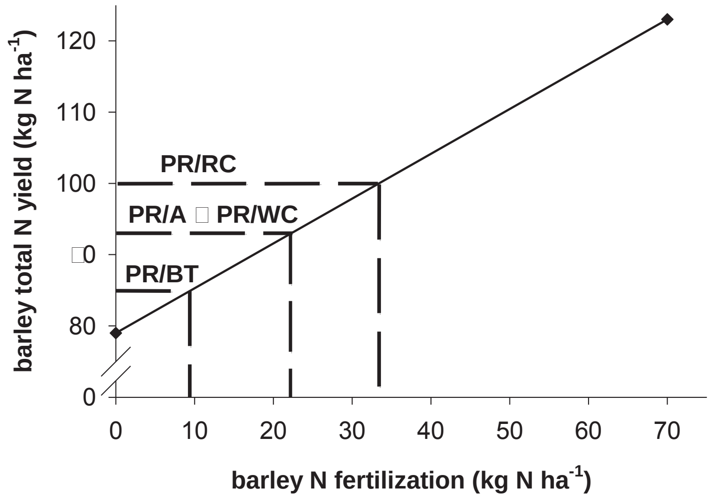

Table

Table 1. Seed amounts sown at establishment of the seven grass-legume mixtures at establishment in 2006.

Grass specie	Legume specie	Abbreviation	Seed amount (kg seed ha^{-1}) Grass	Legume
Perennial ryegrass	White clover	PR/WC	20	5
Perennial ryegrass	Red clover	PR/RC	20	5
Perennial ryegrass	Alfalfa	PR/A	15	20
Perennial ryegrass	Bird's-foot trefoil	PR/BT	20	12
Meadow fescue	White clover	MF/WC	25	5
Timothy	White clover	T/WC	15	5
Hybrid ryegrass	White clover	HR/WC	30	5
Perennial ryegrass		PRpure	25	

Note: There was a lower seed amount of perennial ryegrass in mixture with alfalfa (PR/A) in order to help a good establishment of alfalfa.

Table

Table 2. Total dry matter (DM) and grass, legume, and total N yields, N yields derived from atmospheric N_2-fixation, and the botanical compositions shown as the legume proportion of the dry matter in the harvest biomass in 2008 for 0 N and 300 kg total-N ha^{-1} cattle slurry fertilization.

	DM yield		N yield								Botanical	
	Total		Total		Grass		Legume		N_2-fixation		composition	
	t ha^{-1}		kg N ha^{-1}		kg N ha^{-1}		kg N ha^{-1}		kg N ha^{-1}		Legume %	
	0 N	300 N	0 N	300 N	0 N	300 N	0 N	300 N	0 N	300 N	0 N	300 N
Legume species												
Per. ryegrass / White clover	10.6^{e}	12.8^{c}	278^{e}	361^{d}	112^{c}	202^{a}	166^{c}	159^{cd}	157^{b}	144^{b}	44^{c}	34^{d}
Per. ryegrass / Red clover	15.2^{b}	16.6^{a}	460^{b}	524^{a}	56^{e}	86^{d}	404^{a}	438^{a}	357^{a}	324^{a}	84^{a}	81^{a}
Per. ryegrass / Alfalfa	13.5^{c}	14.8^{b}	406^{c}	431^{bc}	47^{e}	93^{cd}	359^{b}	338^{b}	343^{a}	320^{a}	79^{a}	70^{b}
Per. ryegrass / Bird's-foot trefoil	9.5^{f}	11.5^{d}	275^{e}	294^{e}	151^{b}	211^{a}	124^{d}	83^{e}	121^{b}	81^{c}	29^{d}	18^{e}
Grass species												
Per. ryegrass / White clover	10.6^{d}	12.8^{bc}	278^{c}	361^{a}	112^{d}	202^{b}	166^{a}	159^{a}	157^{ab}	144^{ab}	44^{a}	34^{b}
Meadow fescue / White clover	11.3^{d}	14.1^{a}	324^{a}	367^{a}	153^{c}	240^{a}	171^{a}	126^{a}	156^{ab}	113^{b}	36^{b}	23^{d}
Timothy / White clover	10.6^{d}	12.2^{c}	284^{bc}	352^{a}	141^{c}	205^{b}	143^{a}	147^{a}	135^{ab}	129^{ab}	33^{b}	29^{bc}
Hybrid ryegrass / White clover	11.1^{d}	13.3^{b}	325^{ab}	335^{a}	152^{c}	205^{b}	173^{a}	130^{a}	159^{a}	114^{ab}	35^{b}	23^{cd}
Per. ryegrass pure stand	6.1	9.3	126	229								

Values with the same letter within each variable and species group are not significantly different ($P>0.05$).

Table 3. Forage legume N_2-fixation proportion (%Ndfa) at the four harvests and on weighted average in 2008. S.E. based on four replicates.

Harvest	1st	2nd	3rd	4th	Average[2]
			0 N		
Per. ryegrass / White clover	95.5 ±0.9	97.0 ±0.3	93.0 ±1.2	92.5 ±1.4	94.8 ±0.2[ab]
Per. ryegrass / Red clover	93.6 ±1.4	97.1 ±0.5	86.4 ±2.1	82.7 ±1.2	88.6 ±2.9[c]
Per. ryegrass / Alfalfa	97.1 ±0.5	99.3 ±0.0	95.5 ±0.7	91.5 ±1.5	95.6 ±0.6[ab]
Per. ryegrass / Bird's-foot trefoil	96.8 ±0.8	99.8 ±0.1	96.4 ±1.2	94.6 ±0.8	97.6 ±0.6[a]
			300 kg total-N ha^{-1}		
Per. ryegrass / White clover	91.5 ±1.4	94.4 ±1.1	85.5 ±1.8	89.5 ±2.1	90.6 ±0.3[bc]
Per. ryegrass / Red clover	84.7 ±3.0	88.7 ±2.2	55.9 ±7.0	57.4 ±7.0[1]	73.4 ±3.7[d]
Per. ryegrass / Alfalfa	96.4 ±0.3	99.8 ±0.2	95.0 ±1.3	87.7 ±3.5	94.7 ±1.1[ab]
Per. ryegrass / Bird's-foot trefoil	91.2 ±1.7	99.8 ±0.3	98.2 ±0.7	98.5 ±0.3	98.2 ±0.5[a]

Harvest	1st	2nd	3rd	4th	Average
			0 N		
Per. ryegrass / White clover	95.5 ±0.9	97.0 ±0.3	93.0 ±1.2	92.5 ±1.4	94.8 ±0.2[a]
Meadow fescue / White clover	87.5 ±6.0	96.3 ±0.2	87.0 ±4.9	90.7 ±1.7	91.6 ±2.2[ab]
Timothy / White clover	95.2 ±1.4	97.0 ±0.6	91.3 ±1.5	91.6 ±1.2	94.4 ±0.6[a]
Hybrid ryegrass / White clover	95.6 ±1.1	95.8 ±0.8	86.2 ±1.6	87.7 ±2.5	92.1 ±0.8[ab]
			300 kg total-N ha^{-1}		
Per. ryegrass / White clover	91.5 ±1.4	94.4 ±1.1	85.5 ±1.8	89.5 ±2.1	90.6 ±0.3[b]
Meadow fescue / White clover	89.0 ±2.6	92.0 ±1.2	87.6 ±3.5	89.3 ±2.5	89.9 ±1.6[bc]
Timothy / White clover	90.4 ±2.2	91.1 ±1.7	78.2 ±4.5	82.9 ±2.4	86.7 ±2.3[c]
Hybrid ryegrass / White clover	89.8 ±0.8	90.2 ±1.6	86.8 ±4.9	89.6 ±1.9	88.7 ±2.5[bc]

[1]No reference ryegrass could be harvested as red clover was strongly dominating; instead the reference ryegrass from 3rd harvest was used as reference.
[2]Values with the same letter within each species group are not significantly different ($P>0.05$).

Table

Table 4. Legume soil N uptake in the grass-phase for 0 N and 300 kg N ha^{-1} cattle slurry fertilization.

	Legume soil N uptake	
	0 N	300 N
	Legume species	
Per. ryegrass / White clover	9^{c}	15^{c}
Per. ryegrass / Red clover	47^{b}	115^{a}
Per. ryegrass / Alfalfa	16^{c}	18^{c}
Per. ryegrass / Bird's-foot trefoil	3^{c}	2^{c}
	Grass species	
Per. ryegrass / White clover	9^{b}	15^{ab}
Meadow fescue / White clover	15^{ab}	13^{ab}
Timothy / White clover	8^{b}	19^{a}
Hybrid ryegrass / White clover	14^{ab}	15^{ab}

Values with the same letter within each species group are not significantly different ($P>0.05$).

Table 5. Barley N yields (kg N ha^{-1}) in 2009 following the eight forage mixtures. Barley N yields from forage mixture plots added 300 kg total-N ha^{-1} slurry in 2008.

Barley N fertilization level	0 N			70 kg N ha^{-1}		
Harvest	grain	straw	total	grain	straw	total
Per. ryegrass / White clover	81^{ab}	12^{ab}	93^{ab}	127^{a}	24^{a}	151^{a}
Per. ryegrass / Red clover	87^{a}	13^{ab}	100^{a}	123^{abc}	22^{ab}	146^{a}
Per. ryegrass / Alfalfa	81^{ab}	12^{ab}	93^{ab}	118^{bc}	22^{ab}	140^{ab}
Per. ryegrass / Bird's-foot trefoil	74^{cd}	11^{b}	85^{cd}	110^{de}	18^{c}	128^{c}
Per. ryegrass / White clover	81^{ab}	12^{ab}	93^{ab}	127^{a}	24^{a}	151^{a}
Meadow fescue / White clover	79^{bc}	15^{a}	94^{ab}	118^{cd}	22^{ab}	141^{ab}
Timothy / White clover	76^{bcd}	11^{b}	87^{bc}	111^{de}	20^{bc}	131^{bc}
Hybrid ryegrass / White clover	79^{bc}	11^{ab}	91^{bc}	126^{ab}	20^{bc}	146^{a}
Per. ryegrass pure stand	69^{d}	10^{b}	79^{d}	105^{e}	18^{c}	123^{c}

Values with the same letter (a-e) within each column are not significantly different (P>0.05).

Table 6. Summary of relative performance of the four forage legumes tested in the present study in regard to: legume proportion of the sward, amount (kg N ha^{-1}) derived from N_2-fixation, the effect of slurry application on proportion of N derived from fixation (%Ndfa), and the residual N effect in the first year after plough-in of the forage mixture.

Specie	Proportion	N_2-fixation	Slurry effect	Residual N effect
White clover	Medium	Medium	Low	Medium
Red clover	High	High	High	High
Alfalfa	High	High	No	Medium
Bird's-foot trefoil	Low	Low	No	Low