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Nitrogen dynamics in temporary multi-species grasslands

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Preface

This dissertation, as part fulfilment of the requirements of the Doctor of Philosophy (PhD) degree, is submitted to the Faculty of Science and Technology, Aarhus University, Denmark. The main objective of the study was to investigate how inclusion of the non-leguminous forb species chicory, ribwort plantain or caraway in the traditional grass-clover mixture would influence red clover N_2 fixation and contribute to soil N fertility, and how species diversity in swards containing only forage legumes (red clover, white clover or lucerne) would influence biomass production and N_2 fixation. The study was supported financially by the Green Development and Demonstration Programme (GUDP project MultiPlant) as part of the Organic RDD-2 programme, coordinated by the International Centre for Research in Organic Food Systems (ICROFS) and the Graduate School of Science and Technology (GSST). The study was carried out under the supervision of Prof. Jørgen Eriksen, Dr. Jim Rasmussen, Dr. Karen Søegaard from the Department of Agroecology at Aarhus University and Dr. Georg Carlsson from the Department of Biosystems and Technology at the Swedish University of Agricultural Sciences, Sweden.

This dissertation combines the outcomes from four field and semi-field experiments carried out at the Department of Agroeoclogy, Aarhus University, Foulum over a period of three years. The details of individual experiments have been presented in four research articles and are attached as appendices to this dissertation. The published paper is printed with publishers' permission. The papers are referred to by Roman numerals in the entire thesis.

LIST OF PAPERS

Paper I: Dhamala, N. R., Rasmussen, J., Carlsson, G., Søegaard, K., Eriksen, J. Increasing plant species diversity with inclusion of non-leguminous forbs in grass-clover mixtures does not affect red clover N2-fixation and total N acquisition. *Plant and Soil, Submitted.*

Paper II: Dhamala, N. R., Rasmussen, J., Carlsson, G., Søegaard, K., Eriksen, J. (2017). N transfer in three species grass-clover mixtures with chicory, ribwort plantain or caraway. *Plant and Soil*, 413 (1): 217–230.

Paper III: Dhamala, N. R., Rasmussen, J., Cong, W., Carlsson, G., Søegaard, K., Eriksen, J. Inclusion of non-leguminous forbs in grass-clover mixtures does not affect soil N fertility for the subsequent cereal crop. *Soil Use and Management, Submitted.*

Paper IV: Dhamala, N. R., Eriksen, J., Carlsson, G., Søegaard, K., Rasmussen, J. Highly productive forage legume stands show no positive biodiversity effect on yield and N_2-fixation. *Plant and Soil, Accepted.* doi: 10.1007/s11104-017-3249-2

Summary

Plant diversity is often low in high-yielding temporary grasslands in Europe, with grass-clover being the traditional dominant mixture. Non-leguminous dicot forage herbs have potential as complementary species to traditional grassland species. Chicory (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.) and caraway (*Carum carvi* L.) are the three non-leguminous dicot forage herbs (hereafter called forbs) that have attracted most attention. Another form of grassland production of increasing interest is the practice of growing species mixtures containing only forage legumes for low-input production of biomass with high protein content.

In this study, grassland nitrogen (N) dynamics were studied in a sward that included the forbs chicory, plantain and caraway in a traditional mixture of perennial ryegrass (*Lolium perenne* L.) and red clover (*Trifolium pratense* L.), with fertilizer application as an additional factor, and in a sward containing species mixtures of only forage legumes red clover, white clover (*Trifolium repens* L.) and lucerne (*Medicago sativa* L.). The objectives of the study were to determine how inclusion of forbs in ryegrass-red clover mixtures would influence sward production and red clover biological N_2 fixation (BNF), red clover N transfer to the companion non-legumes and residual soil N fertility, and to investigate how species mixtures of only forage legumes would influence sward production and BNF. The BNF and N transfer studies were performed in situ using the ^{15}N isotope dilution and ^{15}N leaf-labelling method, respectively. The residual N effect was determined in terms of soil initial inorganic N and mineralizable organic N contents, and biomass production and N uptake in the subsequent spring barley test crop in a pot experiment.

Low-input swards containing forbs in ryegrass-red clover or only forage legumes were highly productive with seasonal herbage dry-matter (DM) yields of up to 17 t ha^{-1}. Neither the inclusion of forbs in the ryegrass-red clover, nor the cultivation of only forage legumes in species mixtures demonstrated any species-diversity effect on percentage of legume N derived from BNF (%Ndfa). The three forbs displayed different competitive strengths when included in the ryegrass-red clover, where chicory and plantain were more competitive than caraway. Plantain and caraway appeared more compatible with ryegrass-red clover, where the sward DM yield and BNF of ryegrass-red clover-plantain or caraway were comparable to ryegrass-red clover. A high seeding proportion of chicory did, however reduce the biomass proportion of red clover in the mixture, thus suppressing the seasonal amount of BNF by up to 60% and decreasing sward DM production in unfertilized plots by 20%. Fertilization did not affect %Ndfa in the mixtures, indicating a low level of soil N or non-legume competition for plant-available soil N, but tended to decrease the amount of BNF by up to 35%, suppressing red clover growth to the benefit of grass. Red clover transferred up to 15% of its N to the companion ryegrass and forbs. Forbs included in the ryegrass-red clover relied much less on N transferred from red clover – 0.6 to 4% of the red clover N – than the ryegrass, and showed no effect on red clover N transferred to co-existing non-legume species or on N rhizodeposition in the soil. In addition, including forbs in the ryegrass-red clover did not change the residual N fertility in the subsequent spring barley crop. In the sward containing only forage legumes, red clover appeared to be the strongest species. Mixtures containing red clover showed a similar potential of sward production and input of N from BNF to red clover pure stand. The herbage DM yield, N accumulation and amount of BNF increased by 25 to 50%, 11 to 34%, and 7- 36%, respectively, in the mixtures containing red clover compared to white clover or lucerne in pure stands, or a white clover-lucerne mixture.

The study concludes that when used in right proportions, the non-leguminous forbs chicory, ribwort plantain or caraway can be included in the traditional perennial ryegrass-red clover mixture with no negative effects on herbage production, red clover BNF, red clover N transfer to non-legumes or short-term residual soil N fertility. However, to achieve a balance between legume and non-legume species in the sward, it is important that the mixture does not include a high seeding proportion of chicory. Similarly, the forage legumes red clover, white clover and lucerne can be grown in mixtures without compromising herbage production or input of N from BNF compared to the pure stand of red clover, as long as red clover is included in the mixture. Thus, the study has provided new insights into the design and implementation of multi-species temporary grasslands for increasing biodiversity and improving N self-sufficiency and productivity of low-input grass-arable cropping systems.

Sammendrag

Plantediversiteten er ofte lav i højtproduktive græsmarker i omdrift i Europa, hvor kløvergræs traditionelt er den dominerende blanding. Ikke-bælgplante tokimbladede urter har potentiale til at komplementere de traditionelle græsmarksarter. Cikorie (*Cichorium intybus* L.), lancetbladet vejbred (*Plantago lanceolata* L.) og kommen (*Carum carvi* L.) er tre ikke-bælgplante tokimbladede urter, som har tiltrukket opmærksomhed. En anden type græsmarksproduktion, som får øget opmærksomhed er dyrkning af blandinger, som udelukkende består af græsmarksbælgplanter til lav-input produktion af biomasse med højt proteinindhold.

I dette studie blev undersøgt kvælstof (N) dynamik i græsmarker med cikorie, vejbred og kommen iblandet en traditionel blanding af almindelig rajgræs (*Lolium perenne* L.) og rødkløver (*Trifolium pratense* L.) med gødningstilførsel som en yderligere faktor og i blandinger, som kun består af græsmarksbælgplanterne rødkløver, hvidkløver (*Trifolium repens* L.) og lucerne (*Medicago sativa* L.). Undersøgelsens formål var at bestemme hvordan iblanding af urterne i rødkløver-rajgræsblandinger påvirkede produktion, rødkløvers biologiske N_2 fiksering (BNF) og N-overførsel til ikke-bælgplanter samt kvælstofeftervirkning, og at undersøge hvordan blandinger med kun græsmarksbælgplanter påvirkede produktion og BNF. BNF og N-overførselsstudierne blev udført in situ vha. henholdsvis ^{15}N-isotopfortynding og ^{15}N-bladmærkning. N-eftervirkningen blev bestemt i form af uorganisk jord-N, mineralisérbart organisk N, biomasseproduktion og N-optag i den efterfølgende vårbyg testafgrøde i et potteforsøg.

Lav-input rødkløver-rajgræsmarker med urter iblandet eller marker kun med bælgplanter var meget produktive med årsudbytter op til 17 t ha^{-1}. Hverken iblanding af urter eller dyrkning af græsmarker med kun bælgplanter viste nogen positiv effekt af øget diversitet på andelen af bælgplante-N, som kom fra BNF (%Ndfa). De tre urter havde forskellig konkurrenceevne iblandet rødkløver-rajgræs, hvor cikorie og vejbred var mere konkurrencedygtige end kommen. Vejbred og kommen var mere kompatible med rødkløver-rajgræs og udbytter og BNF var sammenlignelige med rødkløver-rajgræs. En stor udsædsmængde af cikorie reducerede imidlertid biomasseproduktionen af rødkløver i blandingen og reducerede dermed den årlige BNF med op til 60 % og reducerede tørstofproduktionen i ugødede parceller med 20 %. Gødning påvirkede ikke %Ndfa i blandingerne, hvilket indikerer et lavt niveau af jord-N eller ikke-bælgplante konkurrence om plantetilgængeligt jord-N, men med tendens til at reducere mængden af BNF med op til 35 % ved at undertrykke rødkløvers vækst på bekostning af græs. Rødkløver overførte op til 15 % af dets N til rajgræs og urter i blandingen. Urter i rødkløver-rajgræs var langt mindre afhængige af N overført fra rødkløver – 0.6 til 4 % af rødkløver-N – end rajgræs og havde ikke nogen effekt på rødkløver-N overført til ikke-bælgplanter eller på N rhizodeposition i jorden. Inklusionen af urter

i rødkløver-rajgræs påvirkede ikke N-eftervirkningen i den efterfølgende vårbyg. I marker med kun græsmarksbælgplanter var rødkløver stærkest. Blandinger med rødkløver havde samme udbyttepotentiale og input af N fra BNF som rødkløver i renbestand. Tørstofudbytte, N-optagelse og mængde af BNF blev forøget henholdsvis fra 25 til 50 %, 11 til 34 %, og 7- 36 % i blandinger med rødkløver i forhold til hvidkløver eller lucerne i renbestand eller hvidkløver-lucerne-blandingen.

Undersøgelsen viste, at tilsat i de rigtige proportioner kan urterne cikorie, vejbred og kommen inkluderes i en traditionel rødkløver-rajgræsblanding uden negative effekter på produktionen, rødkløver BNF, rødkløver-N overførsel til ikke-bælgplanter eller korttids N-eftervirkning. For at opnå en balance mellem bælgplanter og ikke-bælgplanter i marken er det vigtigt at blandingerne ikke indeholder en stor andel af cikorie. Tilsvarende kan græsmarksbælgplanterne rødkløver, hvidkløver og luverne dyrkes i blandinger uden at kompromittere produktion eller N fra BNF sammenlignet med rødkløver i renbestand så længe rødkløver er inkluderet i blandingen. Denne undersøgelse har givet ny indsigt i, hvordan mangearts-græsmarker i omdrift kan designes og implementeres for at øge biodiversiteten, selvforsyningen og produktiviteten i lav-input sædskifter med græs.

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Abbreviations

BNF, Biological nitrogen fixation
C/N ratio, Carbon to Nitrogen ratio
C, Carbon
DM, Dry matter
EU, European Union
GHG, Greenhouse gas
ID, ^{15}N Isotope dilution
N, Nitrogen
^{15}N, Rare stable isotope of N
%Ndfa, Percentage of N derived from atmospheric N_2 fixation
%Ndfr, Percentage of N derived from root
NUE, Nitrogen use efficiency
RYT, Relative yield total
SOM, Soil organic matter
t, Tonne (1000 kg)

1. General introduction

1.1 Grassland agriculture

Grassland production system is the largest and most common use of land in the world. It is broadly accepted as one of the important pillars of sustainable agriculture and characterized by multiple values and functions (Panunzi 2008; Stypinski 2011; O'Mara 2012). Grasslands, including permanent meadows and pastures, occupy 37% of the global terrestrial area and 69% of the agricultural area (O'Mara 2012). Grasslands cover 33 and 40% of the agricultural area in the EU and Western Europe, respectively, where semi-natural grassland forms an integral part of livestock production systems (Stypinski 2011; O'Mara 2012; Bedoin 2013). Temporary grasslands integrated in arable cropping systems is an important land-use practice in Europe. In the EU-28, grasslands in arable rotations cover over 10% of the arable land (Eurostat, 2010). In Denmark, grass and green fodder production is second only to cereal production, where temporary grasslands included in arable rotations occupy about 10% of the total agricultural land (Pedersen 2015).

Perennial forage legumes are principal components of temporary grasslands (leys) for feed production, due to their underpinning role of providing protein-rich forage, improving soil fertility and increasing plant productivity (Frame 2005; Lüscher et al. 2014). Recent studies have focused on the ability of species-rich leys including perennial forage legumes and non-legumes to provide ecosystem services such as resource-efficient biomass production (Carlsson et al. 2017), increasing sward productivity (Nyfeler et al. 2009; Nyfeler et al. 2011) and persistency (Jing et al. 2017), improving nutritive value (Sanderson 2010), enhancing biodiversity (Søegaard et al. 2011), carbon (C) and nitrogen (N) sequestration in the soil (De Deyn et al. 2009), proportions of biological N_2 fixation (BNF) (Carlsson and Huss-Danell 2003), reducing nitrate leaching (Scherer-Lorenzen et al. 2003; Palmborg et al. 2005) and greenhouse gas (GHG) emissions (Carlsson et al. 2017) and improving resilience to environmental stress (Sanderson et al. 2005). However, most of the high-yielding temporary grasslands in many parts of Europe are dominated by just a few clover and grass species and their binary mixtures mainly due to the positive effect on quantity and quality forage and N self-sufficiency of agricultural systems (Peyraud et al. 2009). Recent studies have shown that non-leguminous dicotyledonous forage herbs might complement traditional grassland species under varied management and agro-climatic conditions (Sanderson et al. 2005; Labreveux et al. 2006; Skinner 2008; Søegaard et al. 2008; 2011).

1.2 Forb-based multi-species grassland

With a growing interest in increasing plant diversity in grasslands, several non-leguminous dicotyledonous forage herbs have begun to be included in multi-species swards. Chicory (*Cichorium intybus* L.), ribwort plantain (plantain*; Plantago lanceolata* L.) and caraway (*Carum carvi* L.) are the three non-leguminous dicot forage herbs (hereafter called forbs) that have received special attention. They have shown good competitive strength (Mortensen et al. 2012; Søegaard et al. 2013) and an ability to establish well in the swards (Søegaard et al. 2011; Mortensen et al. 2012) and grow under adverse weather conditions (Skinner 2008; Younie 2012). When included in the swards, the forbs increase forage yield (Sanderson et al. 2005; Skinner 2008; Søegaard et al. 2011) and belowground biomass (Eriksen et al. 2012), improve animal performance in terms of milk production, live-weight gains, meat quality and animal health (Stewart 1996; Li and Kemp 2005; Younie 2012; Somasiri et al. 2015), feed palatability (Søegaard et al. 2008) and mineral nutrition (Pirhofer-Walzl et al. 2011).

Key features of chicory, plantain and caraway

Chicory is a deep-rooted, warm-season perennial herb belonging to the *Asteraceae* family. It forms a rosette plant with broad prostrate leaves, has a deep and thick taproot system (Fig. 1) that facilitates water and nutrient uptake from deep soil layers and is a good companion species for shallow-rooted species in its complementary utilization of above- and below-ground resources (Li and Kemp 2005; Thorup-Kristensen 2006; Pirhofer-Walzl et al. 2013). This property makes it tolerant to drought and helps to reduce deep drainage and nitrate leaching, which simultaneously leads to mitigation of soil acidity and dry-land salinity (Li and Kemp 2005; Thorup-Kristensen 2006). Chicory establishes fast and grows rapidly and vigorously, which makes it highly productive (Hume et al. 1995; Collins and McCoy 1997; Li and Kemp 2005). It performs well in combination with forage legumes and grass species (Hume et al. 1995; Li and Kemp 2005). Furthermore, Li and Kemp (2005) reported that animal performance in chicory-based pastures is similar to legume-based and superior to grass-based pastures due to the greater quantity and quality of forages. In addition, chicory has been shown to improve the leaf water relation and growth of companion forage legume species in the mixture (Labreveux et al. 2004).

Plantain is a perennial herb belonging to the *Plantaginaceae* family. It has upright rosette growth with a deep and dense adventitious root system (Fig. 1). It is widely distributed in temperate regions and has adapted well in a wide range of soil conditions including low-fertility soils, and has the ability to utilize water and nutrients from deep soil layers (Stewart 1996). These features makes it tolerant to adverse weather conditions, especially drought, and productive in a range of agro-climatic regions (Stewart 1996). Plantain is easy and rapid to establish and the forage produced is palatable. It has demonstrated a competitive advantage over grasses in low-fertility soils and increased root biomass when grown in mixture (Stewart 1996).

Caraway is a perennial herb of the *Apiaceae* family, with feathery and finely divided leaves (Plant world Seed, 2015) (Fig. 1). It has a tuberous root system (Kiviniemi 2009), with a large root biomass (Eriksen et al. 2012; Søegaard et al. 2013) and a high root to shoot ratio where more than half of the total biomass is allocated to belowground plant parts (Hakala et al. 2009).

Chicory Ribwort plantain Caraway

Fig. 1 Plant architecture of the forbs species chicory, ribwort plantain, and caraway. Photos: NR Dhamala

The three forbs differ in several attributes such as competitiveness, persistency, response to different management factors and nutritive value. Chicory has demonstrated a higher winter survival (Skinner and Gustine 2002), persistency and productivity than plantain (Labreveux et al. 2004; Sanderson et al. 2003a). Chicory and plantain are more competitive than caraway, especially during the initial years of establishment (Søegaard et al. 2008; Mortensen et al. 2012; Søegaard et al. 2013), whereas caraway grows better with increasing sward age compared to chicory and plantain (Søegaard et al. 2011). They differ in their response to the applied management practices such as cutting, grazing and fertilization. Plantain and caraway grow better under cutting, whereas chicory performs better under grazing. Chicory and caraway are more responsive to N fertilization than plantain (Søegaard et al. 2008; 2011). Caraway or plantain developed a greater root biomass than chicory when added to a grass-clover mixture (in a parallel study by Cong et al. *unpublished*). Further, they have different nutritive values such as mineral nutrient concentrations, fiber contents and digestibility of organic matter (Sanderson et al. 2003b; Labreveux et al. 2006; Søegaard et al. 2008; 2011).

The three forbs, therefore, differ among each other and from grass in a range of key traits such as above- and below-ground plant architecture, nutrient uptake pattern, forage quality and nutritive value, and ability to grow in warmer and water-stressed conditions. These differences in the traits are likely to make them promising candidates as complementary species to traditional, species-poor mixtures of grass and clover in order to improve important production factors such as sward productivity, persistency, nutrient use efficiency and nutritional quality (Hume et al. 1995; Pirhofer-Walzl et al. 2011; Pirhofer-Walzl et al. 2013; Jing et al. 2017). However, they are not yet widely grown in grasslands in Europe, and used to a very low extent in grasslands in Denmark. Only a small number of studies have measured N dynamics in swards including non-legume forbs (e.g. Høgh-Jensen et al. 2006; Pirhofer-Walzl et al. 2012; Frankow-Lindberg and Dahlin 2013; Pirhofer-Walzl et al. 2013). Hence, investigating N processes in temporary grass-clover swards that include the three forbs is expected to generate new knowledge on grassland production, agricultural N cycling and efficient N nutrition management in grass-arable cropping systems. **This led to the objective of the first part of this study of how the forb species chicory, plantain or caraway included in the traditional perennial ryegrass (ryegrass; *Lolium perenne* L.) and red clover (*Trifolium pratense* L.) mixture (hereafter called ryegrass-red clover) would affect sward production, red clover BNF, red clover N transfer to companion non-legumes and residual soil N fertility to the subsequent crop in the crop rotation.**

1.3 Multi-species mixture of forage legumes

Forage legumes are a common and key component of grassland farming. Red clover, white clover (*Trifolium repens* L.) and lucerne (*Medicago sativa* L.) are three forage legume crops most widely used on a global level (Phelan et al. 2015) and are adapted to a diverse range of soil and environmental conditions (Frame, 2005). They dominate the temperate grasslands by virtue of their potential as a protein-rich feed for livestock (Frame 2005) and N input from BNF for plant production (Carlsson and Huss-Danell 2003; Rasmussen et al. 2012).

The EU is largely dependent on imports of protein feed such as soybean meal for livestock production, and the demand for homegrown protein sources as an alternative to imported soybean meal is growing. For an increased domestic production of plant protein for monogastrics, new research projects in collaboration with Danish feed industry has started to investigate the extraction of protein from forage legume biomass. Since red clover, white clover and lucerne differ in their growth habits and extractable protein concentrations, this research also includes analyses of the potential protein production in mixtures of forage legumes without grasses (Solati et al. 2017).

Key features of red clover, white clover and lucerne

Red clover forms an upright and erect growth habit with solitary crowns (Black et al. 2009; Younie 2012) and has a deep taproot system. Its crown and taproots store N and carbohydrates which is remobilized for shoot regrowth (Black et al, 2009). It assimilates N from the deep soil layer and has a high forage yield potential (Frame, 2005). Red clover has been shown to be more competitive than white clover and lucerne in the grass-legume mixture (Black et al. 2009; Elgersma and Søegaard 2016). It forms larger shoots but a less dense shoot biomass than white clover (Black et al., 2009) and has a larger leaf area, total root length with more branch roots and nodulation, but lower root-to-shoot ratio than lucerne (McElroy 2015). It intercepts more light and greater distribution of leaf area in the intermediate layer of the canopy (Black et al., 2009). Transfer of N from legumes to neighboring plants in species mixtures has been found to be intermediate in red clover, *i.e.* lower than in white clover but higher than in lucerne, while red clover was more efficient at assimilating N transferred from companion species than white clover and lucerne (Pirhofer-Walzl et al. 2012).

White clover has a stoloniferous (creeping) growth habit (Younie, 2012) and an ability to produce leaves faster from the stem (Black et al., 2009). This property makes it more resistant to frequent cutting than red clover (Black et al., 2009) and lucerne, and offers good persistence (Younie, 2012). The adventitious shallow root systems of white clover favours N uptake mainly from the top soil layer (Frame, 2005). The horizontal leaves of the white clover intercept more light at the top of the canopy (Black et al. 2009). It has a greater ability for transfer of fixed N to the companion species than red clover and lucerne (Høgh-Jensen and Schjoerring 2000; Pirhofer-Walzl et al. 2012). In addition, white clover has a larger proportion of fine roots with a low C/N ratio than lucerne, creating conditions more conducive to rapid residue decomposition and release of N from mineralization (Louarn et al. 2015).

Lucerne forms an upright growth habit with a deep taproot system (Younie 2012) and an ability to explore N from the deep soil layer (Kelner et al. 1997). Lucerne is longer lived than red clover and grows much deeper on suitable soil such as well-drained (Frame 2005) and calcareous soils (Younie 2012). Its root system confers better water use efficiency and resistance to drought compared to red clover and white clover (Frame 2005), favours the storage and remobilization of N and carbohydrates for shoot regrowth (Barber 1996; Frame 2005) and competes strongly for the recycled N via decomposition of plant material (Tomm et al. 1995). It competes better for the soil mineral N than red clover (Frankow-Lindberg and Dahlin 2013). It can tolerate high levels of soil N for BNF (Kelner et al. 1997). Lucerne, however, has demonstrated relatively low rates of N transfer to companion species as well as low assimilation of N transferred from other companion legume species (Pirhofer-Walzl et al. 2012; Frankow-Lindberg and Dahlin 2013; Louarn et al. 2015).

The three forage legumes differ in their above-and below-ground plant architecture, canopy characteristics, rooting depth, growth habit and competitive ability in a mixture, N uptake pattern, tissue composition, N storage in roots and remobilization, competitiveness for the recycled N, transfer of fixed N, re-uptake or assimilation of N transferred from companion species. These differences between the species are expected to complement each other by combining niche optimization and efficient utilization of above- and below-ground resources including available soil N, thereby enhancing sward production and reliance upon BNF when grown in a mixture. **This led to the objective of the second part of the study, of how sward production and BNF would be affected when forage legume species red clover, white clover or lucerne are grown in mixtures without non-legumes.**

1.4 Biological N_2 fixation

Biological N_2 fixation is a microbiological process, where atmospheric molecular di-nitrogen (N_2) gas is converted to plant-usable form. It is carried out by specific N-fixing bacteria, either free-living in soil or water or associated with the leguminous plants. In legume plants, the *Rhizobia* species of bacteria living in symbiotic association with their root nodules perform this process (Carlsson 2005; Haygarth et al. 2013), where plants supply carbohydrate to fuel the bacterial growth in return for N (Phillips 1980; Schulze 2004). The symbiosis between legumes and *Rhizobium* constitutes a major source of N from BNF in most cropping systems with a potential to complement or substitute N fertilizer (Garg 2007; Fustec et al. 2010). Input of N from legume BNF in agro-ecosystems is an important contributor to N nutrition in agricultural soil, providing environmental and resource benefits (Jensen et al. 2012; Reckling et al. 2016).

Biological N_2 fixation, however, is dynamic in nature and there is considerable spatial and temporal variation in legume contribution to soil N fertility through BNF (Carlsson and Huss-Danell 2003; Hauggaard-Nielsen et al. 2010). It is influenced by a range of internal and external factors including selection of plant species, genotype and their interaction with surrounding environment (Ledgard and Steele 1992; Vinther and Jensen 2000; Carlsson and Huss-Danell 2003). Biological N_2 fixation is affected by variation in legume dry matter (DM) production (Kumar and Goh 2000; Carlsson and Huss-Danell 2003), and applied management strategies such as cutting (Dahlin and Mårtensson 2008; Dahlin and Stenberg 2010a), grazing (Unkovich and Pate 2000), fertilization (Paynel et al. 2008) and the diversity of legume and non-legume plant species (Carlsson and Huss-Danell 2003; Hauggaard-Nielsen et al. 2009).

It is well established that the percentage of legume N derived from the atmosphere (%Ndfa) is primarily regulated by soil N availability. A high content of plant-available mineral N in the soil generally negatively affects nodulation and the BNF (Jørgensen et al. 1999; Unkovich and Pate 2000) and vice versa with low soil N levels (Ledgard and Steele 1992; Høgh-Jensen and Schjoerring 1994) due to a smaller investment of energy to take up soil N as compared to N derived from BNF (Phillips 1980).

In diversified leys, an interaction between forage legume and non-legume species influences the available N status and thus legume dependency on BNF, where legumes have shown fertilizing functions and the non-legumes N-retention functions (Palmborg et al. 2005; Fargione et al. 2007). Nitrogen uptake from the soil is intensified in diversified systems due to non-legume competition for plant-available soil N, forcing legumes to rely more on BNF to fulfil their N requirements (Ledgard and Steele 1992; Carlsson and Huss-Danell 2003; Rasmussen et al. 2012).

In the present study, different above- and below-ground plant architectures, growth habits and competitive abilities of the three forbs are expected to enhance complementary utilization of available above- and below-ground resources with ryegrass-red clover. The deep and dense root systems of forbs are expected to offer greater depth penetration and flexibility to take up N from varied soil depths and large soil volume (Thorup-Kristensen 2006; Pirhofer-Walzl et al. 2013) leading to more competition for plant-available soil N compared to the red clover grown in a mixture with ryegrass with a shallow adventitious root system. **This led to the hypothesis that the inclusion of forbs in the ryegrass-red clover will increase sward production and red clover %Ndfa due to functional complementarity between the species, and fertilization will decrease %Ndfa, increasing red clover access to available soil N.**

Likewise, differences in the above- and below-ground plant architecture, growth habit and N uptake pattern of the red clover, white clover and lucerne are expected to enhance complementary utilization of above-and below-ground resources including efficient uptake of N from the soil profiles, leading to a greater depletion of the plant-available soil N than when they are grown in pure stands. **This led to the hypothesis that forage legume species red clover, white clover or lucerne grown in a mixture will increase sward production and %Ndfa compared to pure stands due to functional complementarity between the species.**

1.5 Nitrogen transfer

Nitrogen transfer is the process of deposition and assimilation or direct movement of N compounds from one plant to another. It is an important biological pathway involved in N cycling via both above- and below-ground routes. Aboveground N transfer is accomplished through grazing animals via urine and dung (Ledgard and Steele 1992), leaf die-off (Dahlin and Stenberg 2010b), and decomposition and mineralization of green manure shoot and senesced residues (Peoples et al. 2015). The belowground N transfer occurs indirectly through N rhizodeposition-release of N compounds in the rhizosphere. Major pathways of N rhizodeposition include turnover of legume roots and nodules and legume root exudation of soluble N compounds in the rhizosphere (Fustec et al. 2010). The N rhizodeposition constitutes a large pool of soil N of up to 70% of total plant N (Fustec et al. 2010) and above 80% of the below ground plant N (Høgh-Jensen and Schjoerring 2001; Wichern et al. 2008; Fustec et al. 2010), with a significant effect on N nutrition of co-existing non-legume species (Høgh-Jensen and Schjoerring 2000; 2001) and the following crop in mixed farming systems (Mayer et al. 2003).

Recent studies have evidenced the direct short-term pathway of inter-plant N transfer that occurs either through the exudation of low-molecular-weight soluble organic (Paynel et al. 2001; Rasmussen et al. 2013) or inorganic (Paynel et al. 2008) N compounds from living root cells and subsequent uptake by companion plants. These compounds include amino acids, hormones and enzymes, but major N compounds released are mostly in the form of ammonium, amino acids and ureides in the rhizosphere (Fustec et al. 2010). The N transferred is taken up by the companion species through direct root contact, a common mycorrhizal network of arbuscular mycorrhizal (AM) fungi interconnecting roots between the plant species (Haystead et al. 1988; Moyer-Henry et al. 2006) or via mass flow of N compounds to the vicinity of the roots with the soil solution (Jalonen 2012). N transfer is multi-directional (Carlsson and Huss-Danell 2014), but it is larger from legume to non-legume (Rasmussen et al. 2007). In mixed stands of forage legumes and non-legumes, up to 50% (Gylfadóttir et al. 2007; Dahlin and Stenberg 2010b; Rasmussen et al. 2013) and 40% (Rasmussen et al. 2007) of legume N was transferred to companion non-legumes. In addition, legume-derived N has been found to contribute up to 50% (Gylfadóttir et al. 2007) and 80% of non-legume N (Moyer-Henry et al. 2006). Hence, estimation of N transfer helps to understand the legume contribution to long-term soil N pool formation as well as early growth and establishment of the grassland species.

Nitrogen rhizodepostion and transfer varies due to several factors. Nitrogen released in the rhizosphere is influenced by plant community structure in terms of species composition in the mixture (Høgh-Jensen and Schjoerring 2001), plant N content (Wichern et al. 2008; Fustec et al. 2010), root biomass (Phillips et al. 2006) and nutritional status in the soil (Wichern et al. 2008). Inter-plant N transfer between legume and non-legume varies due to the influence of root architecture and distribution in the soil (Pirhofer-Walzl et al. 2012; Rasmussen et al. 2013), biomass production (Rasmussen et al. 2007) and N accumulation (Dahlin and Stenberg 2010b) in legume and non-legume species in the mixture, C allocation within the non-legume species and turnover rates of the plant roots in the soil (Rasmussen et al. 2007). In addition, N transfer is influenced by choice of legume and non-legume species, and the plant diversity and composition of the species in the mixtures (Høgh-Jensen 2006). Plant diversity affects N transfer because of changes to species competition, biomass production and N accumulation, root growth and architecture of the different species included in the mixture, ability of non-legumes to take up soil- and legume-derived N (Høgh-Jensen 2006; Pirhofer-Walzl et al. 2012; Rasmussen et al. 2013), and rate of legume BNF (Paynel et al. 2008) which affects the amount and composition of N release.

In this study, the addition of three forbs - with contrasting growth, competitive strength, root architecture and N uptake patterns - to the ryegrass-red clover mixture is expected to influence sward production, botanical composition, legume N rhizodeposition and transfer to companion non-legumes, as well as non-legume N assimilation from soil pools and transferred from companion legume plant. The dynamics of N rhizodeposition and transfer might differ with different abundances of legume and non-legume species in the sward due to changes in the competition for soil- and legume-derived N. **This led to the hypothesis that the inclusion of forbs in a ryegrass-red clover sward will affect red clover N transfer to companion non-legume species, depending on choice of the forbs, due to their varied growth, competitive ability and N uptake pattern, and higher proportions of non-legumes in the sward increases the competition for red clover derived N.**

1.6 Residual N fertility of grassland pre-crop

Grassland provides a valuable means of improving soil N fertility and plant productivity for the following crop in rotation. During the grassland phase, the forage legumes increase the N pools in agricultural soil through BNF and transfer of fixed N to the soil via above- and below-ground routes. The legume-fixed N retained in plant residues and rhizodeposits or immobilized in soil organic matter, microbial biomass and microbial residues is mineralized and contribute to the build-up of a reserve N pool for the subsequent crops in the rotation when the grassland is cultivated (Eriksen 2001; Vertès et al. 2007; Lemaire et al. 2015). Hence, precise estimation and efficient use of residual N have implications for appropriate N budgeting and the N economy of cropping systems (Hansen et al. 2005; Rasmussen et al. 2012).

The residual N available to the succeeding crop depends on the microbial decomposition and remineralization of the organic N pool formed during the pre-crop phase, which is influenced by the amount and quality of residues and rhizodeposits, with more N available with larger populations and activity of soil microorganisms (Høgh-Jensen and Schjoerring 2001; Kumar and Goh 2002; Wichern et al. 2008). The phenomenon of accumulation of a soil organic N pool, N release and N balance in the cropping system is influenced by the range of applied management practices including the species composition of the grassland pre-crop (Kumar and Goh 2002; Hansen et al. 2005; Eriksen et al. 2008). In addition, the residual N fertility is affected by legume productivity, BNF and N yield in the grassland phase (Peoples et al. 2009; Vrignon-Brenas et al. 2016), and residue quality such as the C/N ratio of the residues (Nykänen et al. 2008; Louarn et al. 2015). Residues with a low C/N ratio increases the N required for microbial growth, resulting in net N mineralization (Kumar and Goh 2002).

Belowground tissue holds a large proportion of the accumulated N in the plant and provides a substantial residual fertilization effect to the following crop in the rotation (Jørgensen and Ledgard 1997; Huss-Danell et al. 2007). Hector et al. (2000) and Cong et al. (2015) found higher rates of root decomposition in the soil previously under a diverse plant community mixture compared to soil from species-poor vegetation due to changes in abiotic and biotic attributes of decomposition microenvironment. Hence, the inclusion of forbs in ryegrass-red clover mixtures may be beneficial for the next crop both via improved residue quality (diversity of different tissues) and thanks to their large and deep root systems that add more belowground biomass (Eriksen et al. 2012) and improve soil structure (Younie 2012). In addition, the three forbs are expected to influence the proportion of legumes due to their different competitive ability in the mixture. **This led to the hypothesis that the inclusion of forbs in the ryegrass-red clover sward will increase the residual N effect and belowground residues, and the effect would differ depending on the forbs species present in the mixture due to their different residue quantities and qualities and influence on the red clover biomass proportions.**

1.7 Aim, objective and hypothesis

This study aimed to improve the understanding of plant productivity and efficient N use in low-input grass-arable cropping systems for their more sustainable management.

The overall objective of the study was to investigate how non-legume forbs when included in a grass-clover sward and a sward containing only forage legumes would affect plant production and grassland N dynamics.

The objectives of the study were to determine:

1. how the inclusion of forb species chicory, plantain or caraway in the conventional ryegrass-red clover mixture would affect
   a) sward production and red clover BNF (Paper I),
   b) red clover N transfer to companion non-legume species (Paper II), and

  c) the residual soil N fertility of a grassland pre-crop for the succeeding cereal crop in the rotation (Paper III), and

2. how sward production and BNF would be affected when forage legume species red clover, white clover or lucerne are grown in mixtures without non-legumes (Paper IV).

The following main hypotheses were tested:

1. The inclusion of non-legume forbs chicory, plantain or caraway in ryegrass-red clover mixtures will
   a) increase sward production and red clover %Ndfa due to functional complementarity between the species, and fertilization will reduce %Ndfa, increasing red clover access to available soil N,
   b) affect red clover N transfer to companion non-legume species, depending on the choice of forbs, due to their varied growth, competitive ability and N uptake pattern, and higher proportions of non-legumes in the sward increases the competition for red clover derived N,
   c) increase the residual N effect, as will belowground residues, and the effect will differ depending on the forbs present in the mixture due to their different residue quantities and qualities and influence on red clover biomass proportions.
2. Forage legume species red clover, white clover or lucerne grown in a mixture will increase sward production and %Ndfa compared to pure stands due to functional complementarity between the species.

2. Description of overall methodology

2.1 Overview of the experiments

The experiments were conducted at Foulumgaard Experimental Station, Aarhus University, in Central Jutland, Denmark. The first three studies (Papers I-III) were conducted in the ley established in 2013 with forb species chicory (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.) and caraway (*Carum carvi* L.) mixed with the traditional grassland species perennial ryegrass (*Lolium perenne* L.) and red clover (*Trifolium pratense* L.) (hereafter called forb-based sward). The swards comprised pure stands of each species and a broad range of species combinations of two to five species with different seeding proportions of individual species in a replacement design with three replicate blocks.

In the first study (Paper I), herbage yield, N yield and red clover BNF were evaluated at two fertilization levels - zero and equivalent to 216 kg total N ha^{-1} yr^{-1} in cattle slurry. In the second study (Paper II), red clover N transfer to companion ryegrass and forbs was evaluated among the species combinations with two different seeding proportions of ryegrass and red clover including one of the forb species: chicory, plantain or caraway. In the third study (Paper III), changes in soil N fertility and plant productivity caused by various species combinations of a grassland pre-crop including forbs were evaluated.

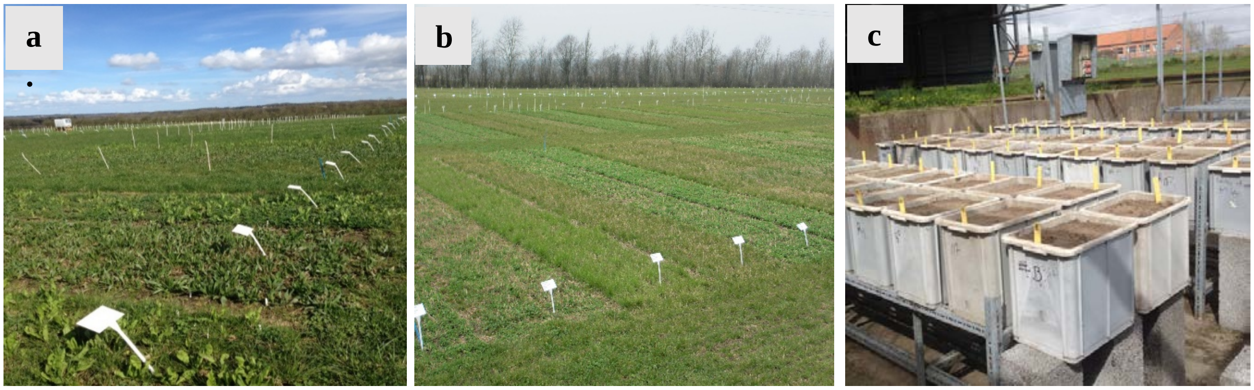

Fig. 2 Experimental layout of N2 fixation and N transfer study in sward containing (a) perennial ryegrass, red clover and non-legume forb chicory, ribwort plantain or caraway, (b) N2 fixation in sward containing only forage legumes red clover, white clover and lucerne in the field, and (c) semi-field measurement of residual N fertility of grassland pre-crop containing grass, clover and forbs in the spring barley test crop. Photos: NR Dhamala

The BNF (Paper I) and N transfer (Paper II) studies were performed during one growing season, comprising four cuts from May to October in the first production year (2014) of the sward. The residual soil N fertility effect (Paper III) was determined at the start of the second production year, from May to August in 2015, following the measurement of BNF.

The fourth study (Paper IV) was conducted in the swards established in 2014 including the three forage legume species red clover (*Trifolium pratense* L.), white clover (*Trifolium repens* L.) and lucerne (*Medicago sativa* L.) sown in pure stands and in two- and three-species combinations based on the seeding rate of each species in a pure stand in a replacement design with four replicates (hereafter called forage legume sward). The BNF study was performed at the time of four cuts during the growing season between May and October in 2015.

2.2 Methods

2.2.1 Measurement of BNF

The BNF was determined using the ^{15}N isotope dilution (ID) method as applied by Rasmussen et al. (2012) by labelling soil with ^{15}N-enriched N fertilizer in the early growing season followed by harvesting and sorting of the above ground biomass (Fig. 3).

Fig. 3 Soil labelling with ^{15}N enriched ammonium sulphate (a), plant sampling in the field (b), and sorting of plant samples (c). Photos: NR Dhamala and D Croft

The samples were then dried, weighted and analyzed for total N concentration and atom fraction ^{15}N. This method provides a yield-independent and time-integrated measure of BNF (Unkovich and Pate 2000). In this method, %Ndfa is estimated by assessing the small difference in ^{15}N enrichment between atmospheric N_2 and ^{15}N-enriched soil N. The underlying assumption is that the N_2-fixing and non-fixing species take up soil N with identical ^{15}N enrichment, and that the BNF process dilutes the ^{15}N enrichment of legume N in proportion to the amount of N_2 fixed from the atmosphere (Unkovich et al. 2008). The ^{15}N enrichment of non-N_2-fixing (reference) plants grown together with fixing plants reflects the ^{15}N enrichment of legume-N derived from soil (Carlsson and Huss-Danell 2003). Hence, the precision of this method requires that the legume and non-legume species follow the identical N uptake pattern and take up soil N with the same ^{15}N enrichment (Jørgensen et al. 1999; Unkovich and Pate 2000).

2.2.2 Measurement of N transfer and N rhizodeposition

The N transfer study was carried out using the leaf feeding method with ^{15}N-enriched urea (Ledgard et al. 1985). Poly Vinyl Chloride (PVC) cylinders were installed, enclosing plants of all the species in the mixture to a confined area where N transfer could be measured (Fig. 4a). The red clover leaves in each cylinder were labelled with ^{15}N enriched urea solution contained in Eppendorf tubes (Fig. 4b). Aboveground plant biomass was harvested, sorted into individual species, dried, weighted and analyzed for total N concentration and atom fraction ^{15}N.

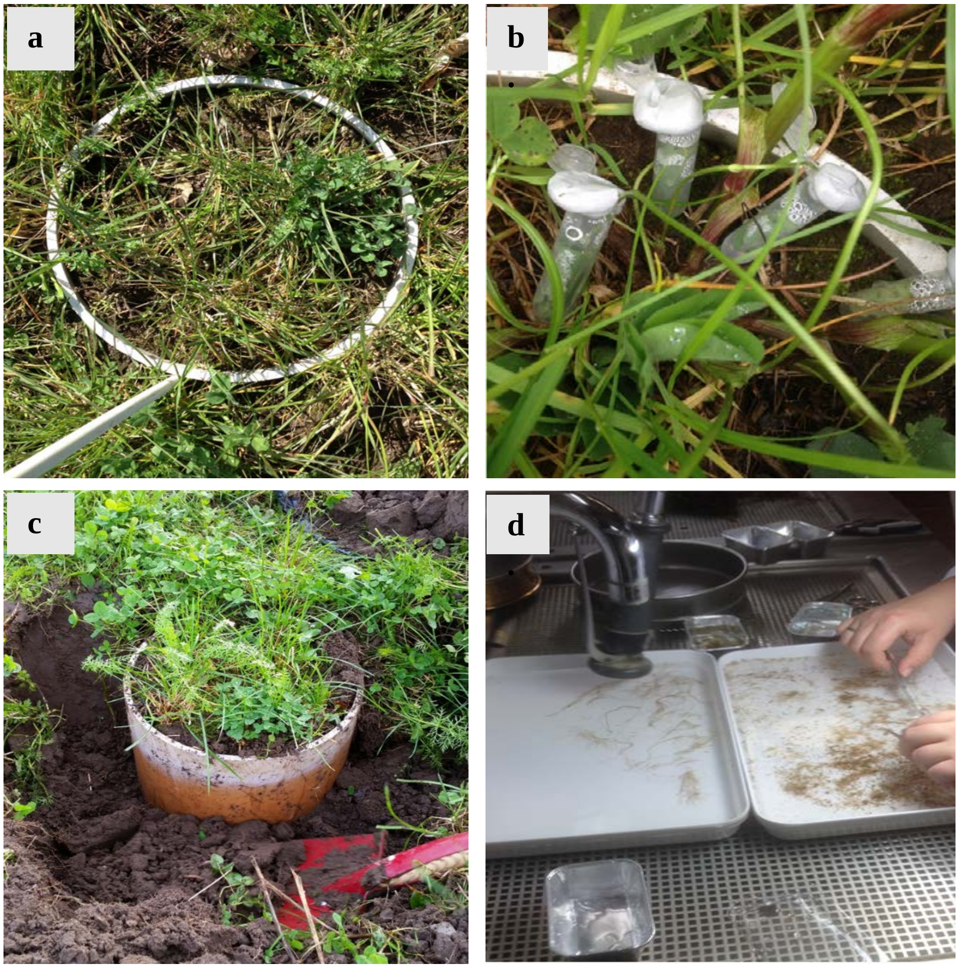

Fig. 4 PVC cylinder installed to confine area of red clover N transfer (a), red clover leaf labelling with ^{15}N-enriched urea (b), and cylinder excavation (c), and recovery of roots (d). Photos: NR Dhamala

This is a yield-dependent and direct method of measuring inter-species N transfer in situ, where the N transfer is quantified from the fate of ^{15}N in the receiving and donating species. The principal assumption is that the ^{15}N absorbed by donor leaves labels all the N compounds within the plant, and that are subjected to transfer to companion receiver species (Ledgard et al. 1985). Hence, the N transfer is quantified as the proportion of total ^{15}N-labelled N in the harvested biomass present in receiver plants. The quantification assumes that ^{15}N in donor plants at the beginning of the growth period is equal to the sum of ^{15}N detected in the labelled donor and unlabelled receiver plants at the end of growth period, where the loss of ^{15}N to the soil-plant system is assumed to be negligible. Applying this method, the plant root system is not disturbed and estimation of N rhizodeposition can also be made (Wichern et al. 2008).

The N rhizodepsition was estimated at the end of the growing season by taking root and soil samples followed by excavation of the cylinders and recovery of the roots using root washing method (Fig. 4c and 4d). The soil and root samples were then dried and analyzed for total N concentration and atom% ^{15}N. The percentage of total N in the soil derived from roots (%Ndfr) was calculated based on ^{15}N-enrichment in legume roots and soil using the equation proposed by (Janzen and Bruinsma 1989), assuming that rhizodeposits and roots have identical ^{15}N-enrichment at harvest, that root ^{15}N-enrichment is stable over the growing season and that the ^{15}N tracer is homogenously distributed in the root system (Mayer et al. 2003). N rhizodeposition from red clover, in this study, was estimated as root-deposited N in the soil free from visible roots and debris.

2.2.3 Measurement of residual N fertility

The residual N effect is studied either by assessing N released from residue decomposition, N uptake in following crop or ^{15}N-labelling of pre-crop residues followed by assessing the fate of the ^{15}N in the soil and plants originating from labelled residues (Crews and Peoples 2005). Hansen et al. (2005) and Rasmussen et al. (2012) suggested that the residual N effect is best evaluated based on the yield and N use of the subsequent crop in the rotation. Plant N uptake is influenced both by the inorganic N content in the soil (Eriksen and Jensen 2001) and mineralization of organic N pools (Vertès et al. 2007), more information about the residual soil N fertility can be obtained by measuring soil inorganic N content and N mineralization in addition to the yield and N accumulation of the following crop (Hansen et al. 2005). In the present study, changes in soil N fertility and plant productivity caused by various mixtures of a preceding grassland crop was measured in terms of biomass production and N uptake in a spring barley test crop in a pot experiment, combined with measurements of potentially mineralizable soil N in an anaerobic incubation experiment as applied by Hansen et al. (2005). Briefly, soil with plant residues was manually collected in each plot from the plough layer (20 cm) (Fig. 5a). Plant residues were separated from the soil, chopped with a chopping machine (Fig 5b) and mixed back into the respective samples. Representative sub-samples were taken and analyzed for initial inorganic N concentration and potentially mineralizable N. Then the pot experiment was established using the homogenized soil from each plot and sown with spring barley (Fig. 5c). The spring barley crop was harvested at maturity (Fig. 5d), dried, herbage dry-matter (DM) weights were recorded, and both grain and straw samples were analyzed for total N concentration.

Fig. 5 Collection of the soil from grassland plots (a), chopping and mixing of plant residues with the soil (b), and spring barley test crop at vegetative growth (c) and at maturity (d). Photos: NR Dhamala and J Eriksen

Further details of the materials and methods for each experiment are provided in the respective papers in the appendix of this dissertation.

3. Results and discussion

3.1 Sward production and botanical composition

Swards containing forbs or only forage legumes showed none of the expected species-diversity effects on sward production

The forbs included in the ryegrass-red clover increased herbage and N yield compared to the non-legumes in pure stands, but did not show yield advantages compared to red clover in pure stand and ryegrass-red clover mixture (Table 1). Total seasonal DM yield tended to increase in fertilized plots, but that was not statistically significant (Table 2; Paper I). In the forage legume sward, herbage yield increased in the mixtures containing red clover compared to white clover or lucerne in pure stands or white clover paired with lucerne (Fig. 2; Paper IV). However, none of the mixtures performed better than red clover in the pure stand. Hence, neither the inclusion of forbs nor the mixture of forage legume species in swards without non-legumes showed any evidence to support the hypothesized yield advantages from increased species diversity.

Table 1 Relative changes in total seasonal herbage dry-matter (DM) yield, Nitrogen(N) accumulation, red clover DM yield and N accumulation, and amount of red clover N_2 fixation compared to the ryegrass-red clover mixture in the forb-based sward, measured in shoots at two levels of fertilization, 0N and 216 kg total N ha^{-1} yr^{-1} in cattle slurry. Numbers are given in percentage of the perennial ryegrass-red clover mixture, which are set to 100 %.

Seed mixtures		**Total**				**Red clover**					
		DM yield		**N yield**		**DM yield**		**N yield**		**N_2 fixation**	
		0N	**216N**	**0N**	**216N**	**0N**	**216N**	**0N**	**216N**	**0N**	**216N**
Two species	GR+RC	100	100	100	100	100	100	100	100	100	100
Three species	GR+RC+60CH	78	106	61	88	43	66	40	60	41	62
	GR+RC+20CH	96	106	79	90	61	59	58	59	60	57
	GR+RC+60PL	99	110	84	104	64	73	61	81	64	84
	GR+RC+20PL	100	115	89	105	74	90	72	87	74	88
	GR+RC+60CA	92	101	83	88	75	80	72	74	76	78
	GR+RC+20CA	94	107	91	102	81	98	81	94	82	95
Five species	GR+RC+20CCP	87	114	72	101	48	77	48	73	49	73
	GR+RC+60CCP	85	104	69	83	45	54	44	49	45	51
	GR+RC+80CCP	73	106	52	83	27	52	26	49	27	51

GR: Perennial ryegrass, RC: Red clover, CH: Chicory, PL: Ribwort plantain, CA: Caraway, CCP: Chicory-Caraway-Plantain

Table 2 Relative changes in total seasonal herbage dry-matter (DM) yield, N yield and amount of N_2 fixation compared to red clover in the sward containing only forage legumes, measured in shoots. Numbers are given in percentage of the pure stand of red clover, which are set to 100 %.

Seed mixtures		**DM yield**	**N yield**	**N_2 fixation**
Pure stand	RC	100	100	100
	WC	65	74	73
	LU	67	76	80
Two species	RC+WC	97	97	98
	WC+LU	68	76	75
	RC+LU	87	85	85
Three species	80RC+WC+LU	91	91	92
	RC+80WC+LU	87	91	92
	RC+WC+80LU	95	99	99
	RC+ WC+LU	98	96	97

RC: Red clover, WC: White clover, LU: Lucerne and 80: percentage of total seeds in the mixture

Vigorous growth of red clover defined the sward production

Red clover was often the most dominant species in the mixtures, in both types of swards. In the forb-based sward, red clover contributed 30 to 80% and 30 to 60% to total seasonal DM yield without and with fertilization, respectively, and 65 to 95% to total seasonal DM in the forage legume sward. This is evidence of its strong ability, under the conditions of the present study, to exploit above- and below-ground resources. In the unfertilized forb-based sward (Paper I), this could be explained by N_2 fixing species having a competitive advantage over non-fixing species by being able to sustain N nutrition (Carlsson and Huss-Danell 2003; Rasmussen et al. 2012). The red clover proportions were suppressed in the three-species ryegrass-red clover-chicory and five-species mixtures with three forbs (Fig. 2; Paper 1). In unfertilized plots, a decrease in red clover proportions in these mixtures tended to reduce total seasonal herbage DM yield by 4 to 27% and N yield by 20 to 48% compared to ryegrass-red clover (Table 1). In the forage legume sward, mixed stands containing red clover gave herbage DM and N yields, respectively, that were 25 to 50% and 11 to 34% higher than a white clover pure stand, lucerne pure stand and white clover-lucerne mixture (Table 2). Thus, herbage production in both swards may be influenced by the system design or a selection effect where the most competitive species dominates the biomass proportion and defines the productivity of the sward (Loreau and Hector 2001) and where complementarity of the species is probably masked by the strong species (Fargione et al. 2007).

The total seasonal herbage DM yield increased by 1-15% in the three- and five-species mixtures in the fertilized forb-based sward (Table 1), and the relative yield total (RYT) was highest (1.2) in the three-species mixture with an 80% seeding proportion of lucerne in the forage legume sward (Supplementary table 1; Paper IV). In both swards, biomass yield of the species in these mixtures were more even than in other species combinations (Fig.2; Papers I and IV). As suggested by Kirwan et al. (2007) and Roscher et al. (2008), it seems likely that complementary resource utilization may require an evenness in growth and resource partitioning among the species in the mixture and a balance between the dominant and non-dominant species (Jing et al. 2017). An alternative explanation could be that competitiveness of the non-dominant species, *e.g.* white clover, lucerne and caraway, may increase over time and therefore not observed in the early phase of the swards studied here. Thus, complementarity effects due to better establishment and resource utilization of weak species could potentially become evident in later stages of the sward (Cardinale et al. 2007; Fargione et al. 2007), which calls for future studies over multiple growing seasons.

Grass and forbs varied in their competitive ability and resource utilization

Chicory and plantain were more competitive regardless of their seeding proportions and fertilization rate than ryegrass and caraway (Fig. 2, Paper I). This reflects the competitive advantage of chicory and plantain to utilize above- and below-ground resources. Their tall plant architecture may have intercepted more of the light (Søegaard et al. 2013), and their deep and dense root systems (Stewart 1996; Li and Kemp 2005) provided the flexibility to take up nutrients from deeper soil layers (Thorup-Kristensen 2006; Pirhofer-Walzl et al. 2013). This explanation is confirmed by the better growth of the ryegrass grown in mixtures with caraway (Fig. 2; Paper I). Poor growth of caraway corroborated the findings of Hakala et al. (2009) and Pirhofer-Walzl et al. (2012) who suggested that caraway is initially less competitive because of its energy investment in establishing a large root system.

Table 3 Seasonal mean percentage of total shoot dry matter yield of species estimated in five-species mixtures containing perennial ryegrass, red clover, chicory, ribwort plantain and caraway under 0N and 216 kg total N ha^{-1} year^{-1} (216N) in cattle slurry.

Fertilization level	Perennial ryegrass	Red clover	Chicory	Ribwort plantain	Caraway
0N	10	40	24	24	2
216N	20	35	27	16	2

Ryegrass showed a greater response to fertilization than forbs, with an increase in N uptake from 50 to 225% (Tables 2 and 5; Paper I) and an increase in the mean proportion of total seasonal herbage DM yield in five-species mixtures from 10 to 20% (Table 3). Fertilization increased the N uptake of chicory by up to 102% and of caraway by up to 150%. In line with Søegaard et al. (2011), plantain appeared to be less responsive to fertilization, with an increase in N uptake by up to 54% (Tables 2 and 5; Paper I). Fertilization decreased N uptake of plantain by 36 and 21% in the five-species mixtures with the lowest and highest seeding proportion of forbs, respectively, and with a decrease in mean proportion of total seasonal herbage DM yield in five-species mixtures from 24 to 16% (Table 3). This indicated a better ability of plantain to perform at low N fertility levels and a better ability of chicory and caraway to exploit applied N fertilizer. The more moderate response of forbs than ryegrass to fertilization may be related to their ability to take up nutrients from the deeper soil layer.

Different competitive strengths of the forbs affected red clover growth

Red clover growth was suppressed when grown with chicory, despite a nearly similar biomass proportions of chicory and plantain (Fig. 2; Paper I). In the three-species ryegrass-red clover-chicory mixture, red clover herbage DM yield decreased by up to 57 % and 41% without and with fertilization, respectively, compared to ryegrass-red clover (Table 1). The corresponding decrease in the three-species ryegrass-red clover-plantain or caraway was up to 36% without fertilization and 27% with fertilization. In addition, in fertilized plots, seasonal DM proportions of the chicory correlated negatively with DM proportions of the clover (R^2= 0.33). Such relation was not observed with plantain. This indicates that reduced red clover growth in five-species mixtures, despite similar seeding proportion (20 and 40%) of red clover to three-species mixtures, was likely caused by the competition from chicory for resource utilization. It is likely that the taller growth and broad leaves of the chicory overtopped and competed more strongly for canopy light than red clover, whereas the upright leaves of plantain may have offered a more compatible leaf morphology with red clover for light interception (Søegaard et al. 2013). Likewise, in the forage legume sward, the stronger growth of red clover may be favoured by its taller growth and large root system, resulting in dominance over white clover for the canopy light and soil resources. This was supported by the fact that the white clover proportion increased at later cuts at the expense of red clover. The poor performance of lucerne, despite its large root system and strong growth habit, could be due to environmental and management factors such as soil (e.g. soil pH and availability of nutrients other than N) and temperature not being optimal for lucerne as opposed to for red clover which can thrive under a broad range of environmental conditions (Frame 2005). Another reason is that lucerne might be less tolerant than red clover to the high cutting frequency applied in the present study (Frame 2005). Thus, the results on yield and botanical composition indicate that the plant architecture likely influenced above- and below-ground resource utilization and thus growth and competitiveness of the species in the swards.

Swards containing forbs or only forage legumes were highly productive

In the forb-based sward, mixtures including forbs resulted in seasonal herbage DM yields from 12 to 17 t ha^{-1} and N yields from 250 to 400 kg ha^{-1}, which were comparable to ryegrass-red clover mixture. Corresponding values ranged from 11 to 16 t ha^{-1} and 450 to 590 kg ha^{-1} for the forage legume sward, where mixtures containing red clover produced herbage DM and N yields as high as in the highest-yielding pure stand (red clover in the present study). The herbage DM yields from both swards were higher or comparable to the reported grassland productions in the various studies conducted at the same location (e.g. Pirhofer-Walzl et al. 2012; Rasmussen et al. 2012; Elgersma and Søegaard 2016) or other locations in Europe (e.g. Oberson et al. 2013; Pirhofer-Walzl et al. 2013; Anglade et al. 2015). This demonstrated that swards containing forbs or only forage legumes were highly productive in the present low-input production system. Thus, including forbs in ryegrass-red clover mixture or growing forage legumes in a mixture does not negatively affect the total herbage production and N accumulation, provided that the ryegrass-red clover-forb seed mixture does not include a high proportion of chicory and that the forage legume sward contains at least a small proportion of red clover in the seed mixture.

3.2 Percentage and amount of N derived from BNF

In the present study, on a seasonal basis, red clover grown in a mixture with ryegrass or ryegrass and forbs derived over 90% of its N from BNF even when fertilized with 216 kg total N ha^{-1} in cattle slurry (Paper I). In the forage legume sward, over 80% of the seasonal N accumulated was derived from BNF regardless of species composition (Paper IV). In agreement with the several previous studies (e.g. Carlsson and Huss-Danell 2003; Rasmussen et al. 2012; Anglade et al. 2015), the three forage legume species demonstrated a high BNF potential in temperate temporary grassland.

Sward containing forbs or only forage legumes showed no species-diversity effect on %Ndfa

Inclusion of forbs in ryegrass-red clover mixture in forb-based sward and species mixtures of forage legumes in forage legume sward showed none of the expected positive species-diversity effect on %Ndfa. Red clover %Ndfa increased significantly more in ryegrass-red clover than in the pure stand. However, the inclusion of forbs in the reygrass-red clover did not change red clover %Ndfa, regardless of species composition and seeding proportions of the red clover (Tables 3 and 4; Paper

I). Similarly, mixed stands of forage legumes did not influence %Ndfa as compared to the pure stands (Tables 3 and 4; Paper IV), regardless of the species composition and seeding proportions of the species. Therefore, both the hypotheses that the inclusion of forbs in the ryegrass-red clover mixture (Paper I) or that mixed stands containing only forage legumes (Paper IV) would increase %Ndfa were not confirmed.

In forb-based sward (Paper I), a comparison of %Ndfa between a red clover pure stand and ryegrass-red clover corroborate the findings of previous authors that non-legume competition for plant-available soil N stimulates legume dependence on BNF (e.g. Ledgard and Steele 1992; Carlsson and Huss-Danell 2003; Oberson et al. 2013). Most of the previous studies tended to include different grass species as companion non-legumes that are known to compete strongly for the plant-available soil N (e.g. Carlsson et al. 2009; Nyfeler et al. 2011; Rasmussen et al. 2012) and for the N transferred from companion legumes (e.g. Høgh-Jensen et al. 2006; Pirhofer-Walzl et al. 2012). Hence, no effect on %Ndfa, despite large variations in the red clover proportions (Fig. 6) and regardless of the number of forb species included, indicates that ryegrass was likely the main factor regulating %Ndfa in the forb-based stands. This may be due to the different abilities of the ryegrass and forbs to compete with the legumes for plant-available soil N and their N rhizodeposits (root exudates).

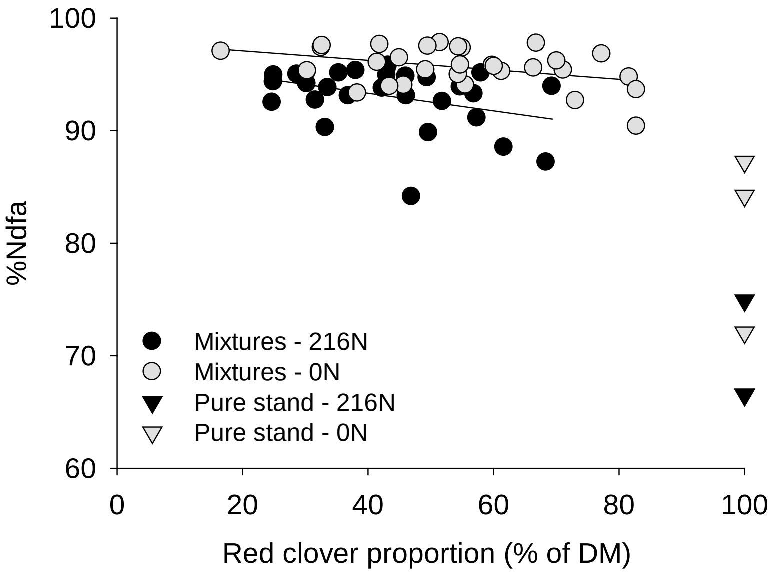

Fig. 6 Relationship between seasonal red clover proportions of total herbage dry-matter (DM) yield and percentage of N derived from the atmosphere (%Ndfa) measured in shoots at two levels of fertilization, 0N and 216 kg total N ha^{-1} in cattle slurry, and at four cuts during the 2014 growing season.

Legume re-assimilation of their N rhizodeposits likely influences %Ndfa

The results from the present study led to a hypothetical explanation that non-legume competition with legume plants for uptake of legume root exudation of N compounds may be a regulatory mechanism for %Ndfa. Legume N rhizodeposits may be re-uptaken by the legume roots or absorbed by the companion non-legume plants (Høgh-Jensen and Schjoerring 2001; Jensen 1996). Studies have demonstrated a competitive advantage of grass over forbs for uptake of legume N rhizodeposits (e.g. Paper II; Høgh-Jensen et al. 2006; Pirhofer-Walzl et al. 2012; Frankow-Lindberg and Dahlin 2013). In addition, in the present study, the red clover N transferred to the companion ryegrass component and N rhizodeposition in the soil estimated at the end of the growing season did not differ depending on which forb species was present in the mixture (Figs. 5 and 6; Paper II). The forbs likely explored additional soil N pools by virtue of their large deep root systems, and the grass mainly competed for and reduced red clover re-uptake of its rhizodeposits, resulting in a stronger reliance on BNF. Similarly, the lack of an expected mixture effect on %Ndfa in the forage legume sward (Paper IV) could be due to the absence of a non-legume competitor for their N rhizodeposits. This was supported by the fact that the %Ndfa in lucerne tended to increase in the pure stand, where weed abundance was significantly higher than in other mixtures. Thus, different functionalities of ryegrass and forbs (Paper I), and forage legumes (Paper IV) showed no evidence of change in legume competition for plant-available soil N. The growth and performance differences between the ryegrass and forb, and forage legumes grown in a mixture may have been mediated by their ability to compete for resources other than N such as light and water with no regulatory effect of species combination on %Ndfa.

N fertilization did not affect %Ndfa in non-legume mixtures

The large majority of previous authors have reported that the rate of legume BNF varies in response to soil N level where a high level of plant-available soil N has a negative feedback on %Ndfa (e.g. Carlsson and Huss-Danell 2003; Paynel et al. 2008; Peoples et al. 2013). In the present study, besides a large range of red clover proportion (Fig. 6), no effect of fertilization was observed in the mixtures containing ryegrass or ryegrass and forbs (Table 4; Paper 1) where the %Ndfa remained above 80% throughout the experimental period. The consistently high %Ndfa and competitive advantage of red clover over non-legumes was likely due to a low level of plant-available soil N. This was supported also by the fact that red clover %Ndfa was up to 75% in the pure stand even when fertilized with 216 kg total N ha^{-1} cattle slurry (Table 4; Paper I). Previous studies have shown that forage legumes often derive less than 60% of their N from BNF when grown in pure stands and fertilized with N (e.g. Nyfeler et al. 2011; Peoples et al. 2013; Carlsson and Huss-Danell 2014). Another explanation could be an N-sparing effect whereby the red clover may have relied mostly on BNF and the non-legume competed better for the plant-available soil N (Peoples et al. 2013). This was supported by significant negative fertilization effect on red clover %Ndfa in the pure stand. In addition, fertilization did not significantly change N uptake of the red clover and forbs in the mixtures, but ryegrass N uptake increased significantly (Table 5; Paper I). This indicated that ryegrass competed for the increased level of plant-available N to support its growth without increasing red clover access to soil N. Hence, as observed in the previous studies (e.g. Palmborg et al. 2005; Carlsson et al. 2009; Rasmussen et al. 2012), grass competition for the available soil N may have a regulatory effect on red clover %Ndfa. Thus, the study showed that inclusion of forbs in the ryegrass-red clover mixtures and species mixtures of forage legumes does not affect legume dependence on BNF, and highlights the importance to include grasses in multi-species grasslands for efficient use of both BNF and soil N.

Competitiveness of the species in the sward affected quantity of BNF

The amount of BNF varied in both swards (Paper I and IV) due to the change in botanical composition caused by the different competitive abilities of the species in the mixtures. The amount of legume BNF varies due to variations in the %Ndfa and legume productivity (Carlsson and Huss-Danell 2003). Since the variations in %Ndfa across the species composition in most cases were very small (Table 4; Paper I and IV), as documented in many previous studies (e.g. Carlsson and Huss-Danell 2003; Hauggaard-Nielsen et al. 2009; Anglade et al. 2015), a strong positive correlation was observed between legume N accumulation and the amount of BNF (R^2= 0.97 in both swards and at both levels of fertilization in forb-based sward).

In the forb-based sward (Paper I), the amount of BNF decreased in response to the high competitive ability of chicory or greater seeding proportions (60% or above) of the forbs and due to a negative effect on clover biomass proportions. In the fertilized plots, a negative correlation was observed between seasonal herbage DM yield of chicory and the amount of red clover BNF (R^2= 0.22), but a weak positive correlation was found with the DM yield of plantain (R^2= 0.1). In the ryegrass-red clover-chicory and five-species mixtures, the amount of red clover BNF fell by 40 to 73% and 27 to 50% without and with fertilization, respectively, compared to ryegrass-red clover on its own (Table 1), whilst the three-species mixtures containing plantain or caraway in most cases fixed comparable amounts of N_2 to that of ryegrass-red clover. Fertilization decreased the red clover seasonal N accumulation and amount of BNF by up to 30 and 35%, respectively, restricting red clover biomass proportion and N accumulation, being highest in the three-species ryegrass-red clover-chicory with 20% seeding proportion of chicory followed by ryegrass-red clover. Regarding herbage DM yield, N accumulation and botanical composition, the study suggested that the amount of red clover BNF was influenced by the factors that affect red clover growth. This suggests that the choice of forb species and their appropriate seeding proportions - depending on the competitive ability - are important when considering integration of forbs in multi-species swards. Thus, among the three forbs, plantain appeared to be an important component to achieve a balance between legume and non-legume abundance and retaining a similar level of sward production and input of N from BNF to ryegrass-red clover mixture without losing its biomass proportion.

In the forage legume sward (Paper IV), mixtures containing the strongest species, red clover, fixed as much BNF as red clover in the pure stand (Table 3; Paper IV). Compared to the pure stands of white clover, lucerne and their two-species mixture, the seasonal BNF increased in the two- and three-species mixtures containing red clover by 16 to 36%, 7 to 25% and 14 to 32%, respectively, which in most cases was statistically significant, except for lucerne in the pure stand (Table 3; Paper IV). This showed that the amount of BNF is not compromised in the mixture containing red clover and could be improved if (at least a small amount of) red clover is included in the mixture compared to the pure stand of white clover and lucerne and their two-species mixture.

Seasonal BNF in the forb-based sward in the three-species mixtures containing plantain or caraway was above 200 kg ha^{-1} even when fertilized with 216 kg N ha^{-1} in cattle slurry and in the forage legume sward it was above 300 and as much as 500 kg ha^{-1}. These amounts were higher or within the range of previously estimated BNF levels in European grasslands (Carlsson and Huss-Danell 2003; Lüscher et al. 2014; Anglade et al. 2015). Thus, the study showed that swards containing forbs or only forage legumes have potential to acquire a large input of N from BNF, and that the BNF is not compromised when including forbs in the ryegrass-red clover in controlled proportions or growing forage legumes in a mixture with red clover.

3.3 Red clover N transfer and N rhizodeposition

Forbs relied less on red clover-derived N and did not influence red clover N transfer to the companion non-legume species and N rhizodeposition in the soil

Red clover transferred up to 15% of its N to neighbouring ryegrass and forb species over a growing season in which the ryegrass absorbed 63 to 94% of the total amount transferred. In accordance with previous studies (Høgh-Jensen et al. 2006; Pirhofer-Walzl et al. 2012; Frankow-Lindberg and Dahlin 2013), ryegrass was a stronger receiver of red clover deposits than the forbs. The three forbs demonstrated a similar ability to absorb the N transferred from red clover, ranging from 0.6 to 4% of red clover N, regardless of seeding proportions of ryegrass and red clover and did not affect the amount of N transferred to the grass component. The amount of N transferred to chicory and plantain was higher than to caraway, which was associated with their better growth and N accumulation. Hence, the results from this study (Paper II) failed to support the hypothesis that the choice of forb species and different seeding proportions of ryegrass and red clover would influence red clover N transfer to companion non-legume species.

The functional difference between the ryegrass and forbs in their abilities to absorb N transferred from the red clover was likely associated with legume root exudation of the N compounds taking place mainly in the uppermost soil layer, where the grasses have a large fibrous root network and develop a close inter-connection with the legume roots (Pirhofer-Walzl et al. 2012; Frankow-Lindberg and Dahlin 2013;). This was supported by larger proportions, 65 to 100%, of red clover N rhizodeposition in the upper 0-10 cm soil layer and transfer of red clover N predominantly to the ryegrass component (Figs. 5 and 6; Paper II). On the other hand, such facilitative interaction might have been less in forbs due to their deep and thick tap or adventitious root system. It appears that forbs may have relied substantially on the N from soil pools due to their ability to assimilate N from the deeper soil layer (Thorup-Kristensen 2006; Pirhofer-Walzl et al. 2013). This was supported by the fact that the strong growth of chicory and plantain was not generally affected either by variations in amount of red clover BNF or by fertilization, whereas ryegrass biomass proportions and N uptake was greatly increased with fertilization (Fig. 2 and Table 5; Paper I). Despite the functional differences between the ryegrass and forbs in the ability to assimilate N transferred from the red clover, ryegrass did not show a growth advantage compared to chicory and plantain. It seems that the chicory and plantain either competed strongly with the ryegrass for uptake of soil N in all soil layers or that the ryegrass growth was limited by the utilization of above- and below-ground resources other than N.

The dynamics of N transfer tended to vary with biomass production and N accumulation in red clover and non-legumes. The percentage of red clover N transfer tended to increase with an increase in non-legume N accumulation, that the quantity of N transferred and rhizodeposition tended to be higher with large red clover herbage yield and N accumulation (Figs. 5 and 6; Paper II). In addition, total seasonal shoot DM and N yield correlated well with the root DM and N yield estimated at the end of the growing season. A positive correlation was also found between seasonal N transfer measured in shoots and the amount of N rhizodeposition. Hence, this study suggests that legume N transfer to companion non-legume species is likely a combined effect of localization of donor N rhizodeposits, root architecture of the receiving species, and the competition between donor and receiver plants for above- and below-ground biomass production and N accumulation. Thus, the inclusion of forbs in the ryegrass-red clover does not influence red clover N transfer to companion non-legumes. It is, therefore, important that grass is included in the multi-species swards to enhance assimilation of legume-deposited N and tight internal N cycle of the grassland agricultural systems.

The forbs assimilated more of the N transferred from red clover by the third and fourth cuts. It is not fully clear if this was caused by the better establishment of the forbs or reduced growth of the ryegrass. Frankow-Lindberg and Dahlin (2013) found that most of the legume-derived N was transferred to grass in first harvest year and that the N transfer to chicory increased in the second harvest year. Likewise, %Ndfa tended to increase at the fourth cut in the two-and three-species mixtures containing forbs compared to ryegrass-red clover, especially in unfertilized plots (Fig. 4; Paper I). These could therefore be indications that the dynamics of BNF and N transfer change over time. These aspects need to be further investigated by analyzing dynamics in BNF and N transfer in forb-based swards over multiple growing seasons.

3.4 Residual soil N fertility of grassland pre-crop containing forbs

Including forbs in the ryegrass-red clover mixture did not influence residual soil N fertility

Biomass yield and N uptake in the subsequent spring barley crop increased by 40 to 70% and 70 to 104%, respectively, when grown in the grassland-based soil than in unfertilized reference soil with a history of cereal cropping. However, all mixtures, regardless of species composition, showed a stable initial potentially available soil N level, biomass yield and N uptake of a spring barley test crop (Figs. 1 and 2; Paper III). Therefore, the hypothesis that the inclusion of forbs in ryegrass-red clover would increase residual N effect to the subsequent crop in rotation was not confirmed (Paper III).

Previous studies have suggested that legume biomass production, BNF, N accumulation (e.g. Høgh-Jensen and Schjoerring 1997; Vrignon-Brenas et al. 2016), and residue quality such as C/N ratio from the grassland pre-crop phase (Nykänen et al. 2008) are good early indicators of N released to the succeeding crop in rotation. Palmborg et al. (2005) found a positive association between legume biomass and soil inorganic N content in the soil. Many studies have reported a higher residual N effect in the presence of forage legumes in the swards than plant communities without legumes (e.g. Høgh-Jensen and Schjoerring 1997; Kumar and Goh 2000; Kumar et al. 2001). Interestingly, no correlation was observed between measured parameters during the grassland phase (such as total biomass production, N yield, red clover biomass proportion, input of N from BNF, N balance) and the N fertility for the subsequent cereal crop, with a similar N pre-crop value of red clover in the pure stand and the ryegrass-red clover mixture.

Eriksen et al. (2015) suggested that N availability to the subsequent crop via mineralization of plant residues is a slow-release process and could take some years to have the visible effect. Kumar and Goh (2002) observed that mineralization of residues is influenced by its C/N ratio as soil microbes need more N to decompose C-rich materials. They suggested that decomposition and mineralization of the residues would be faster for the legume residues due to a lower C/N ratio. It is likely that N could initially have been immobilized during the decomposition of the large belowground biomass of the forbs. Additionally, as suggested by (Crews and Peoples 2005), all potentially available N released from the pre-crop may not have been available to the succeeding crop due to the possible loss of N from the system via leaching and denitrification, this loss being larger from the clover-dominated plots (Kušlienė et al. 2015). Since the spring barley biomass and N uptake when grown in grassland soil were higher than in the reference soil, the N fertility of the grassland system may have been caused by a positive pre-crop effect of perennial grassland crops compared to cereal crops, regardless of the species composition of the grassland crop. The N fertility of the succeeding crop may increase in the following years. This calls for future studies covering multiple growing seasons to improve understanding and to generate a more robust conclusion. Thus, the study showed that the inclusion of any of the three forbs in the ryegrass-red clover mixture does not affect the short-term residual N effect of the grassland pre-crop.

3.5 Methodological reflections

N2 fixation measurement

Uneven spatiotemporal distribution of ^{15}N in the soil profile is an important source of uncertainty in the %Ndfa estimates obtained using ^{15}N ID method (Unkovich et al. 2008; Burchill et al. 2014). Jørgensen et al. (1999) highlighted similar N uptake pattern of legume and non-legume species is more important than the temporal variation in the soil ^{15}N enrichment for reliable estimation of %Ndfa. In the present study, to minimize the risk of large bias caused by spatiotemporal variations in soil ^{15}N enrichment and contrasting N acquisition patterns between legumes and non-legume reference plants, as suggested in previous studies (e.g. Jacot et al. (2000), Unkovich et al. (2008) and Carlsson and Huss-Danell (2014)), the average atom% ^{15}N value of all non-legumes grown in the same plot as red clover were used as the reference value to estimate %Ndfa in the forb-based sward. Average atom% 15N excess of all non-legumes grown in mixtures constituting at least 40% seeding proportions of red clover was used as reference value to estimate %Ndfa in pure stand of red clover. Similarly, an approach of using the average ^{15}N of several reference species (pooled samples of all weeds present in the sampled plots, representing both grasses and dicotyledon species) were applied to estimate %Ndfa in forage legume sward. It is possible that the amount of BNF could have been overestimated by the fact that sampling for biomass and BNF measurements were made in small ^{15}N dilution plots. However, the biomass yield measured in the dilution plots in Paper I was close to the yield measured in the whole plots (Dhamala et al. 2015).

Nitrogen transfer measurement

It is not always possible to meet all assumptions behind the ^{15}N leaf labelling method for quantifying N transfer, which might be associated with multiple methodological biases (Chalk et al. 2014). This study showed a clear tendency for overestimation of the proportions of non-legume N derived from transfer of red clover N, especially in ryegrass. The uncertainty in the amount of N transfer in ryegrass was more pronounced when biomass proportions of ryegrass was very low and the biomass proportions of clover was high. The study suggests that caution should be taken to apply the method in the swards heavily dominated by legume plants. Methodological bias may also be caused by temporal non-uniformity in the ^{15}N enrichment of legume due to continuous growth and dilation of ^{15}N by N_2 fixation. This bias may be minimized with more frequent labeling of legume and shortening the gap between the termination of labelling and plant sampling.

Residual N fertility measurement

The applied methods to determine the residual N fertility provide measures of potentially available soil inorganic N and mineralizable organic N accumulated in soil organic matter (SOM) during the grassland pre-crop phase, and mineralized N taken up by the subsequent crop. These provide the means of evaluating agronomic utility of grassland pre-crop and N nutrition management of grass-arable cropping systems (Crews and Peoples 2005). However, it is not clear from the applied methodology what factor are most important in controlling the residual N effect of the different treatments. Although many studies have highlighted that C/N ratio and microbial decomposition of the residues are instrumental in determining the residual N fertility, such measurements were not part of this study. Furthermore, nitrate leaching and denitrification losses of N were not measured during the grassland phase, and these flows would also be valuable to include for a complete determination of the N balance of the grassland pre-crop.

4. Summary of main results

This study showed that:

- The inclusion of non-leguminous forbs chicory, ribwort plantain or caraway in the ryegrass-red clover mixture did not affect red clover %Ndfa. Different competitive abilities of the forbs affected the sward production and quantity of BNF through their impact on red clover abundance in the mixtures. A comparable herbage and N yield and input of N from BNF to ryegrass-red clover was achieved when including plantain or caraway. However, a high seeding proportion of chicory reduced the seasonal amount of BNF by up to 60% and decreased sward herbage DM yield in unfertilized plots by ca. 20%, decreasing biomass proportions of red clover.
- Plots receiving ca. 220 kg total N ha^{-1} in cattle slurry did not down-regulate %Ndfa, indicating a low soil N level or non-legume competition for plant-available soil N. Fertilization lowered amount of BNF by up to 35%, decreasing red clover growth.
- Red clover transferred up to 15% of its N to the companion non-legume species. When including the forbs in a ryegrass-red clover mixture, they relied much less on red clover-derived N, 0.6 to 4% of red clover N, than the ryegrass and did not influence the amount of red clover N transferred to the non-legumes. The forbs showed similar abilities to absorb the N transferred from red clover and the choice of forb and the seeding proportions of the ryegrass and red clover did not influence the percentage of red clover N transferred to ryegrass.
- Species mixtures of forage legumes behaved like pure stands with no evidence of a species-diversity effect on %Ndfa. Mixtures containing red clover reached a comparable amount of herbage DM and BNF to the red clover pure stand, the strongest species under the conditions of the present study. In the mixtures containing red clover, herbage DM yield, N accumulation and BNF increased by 25 to 50%, 11 to 34%, and 7- 36%, respectively, as compared to pure stands of white clover and lucerne and their two-species mixture.
- Biomass yield and N uptake in the subsequent spring barley crop increased by up to 70% and 104%, respectively, when grown in the grassland-based soil compared to unfertilized reference soil with a history of cereal cropping. However, the very large span in red clover biomass proportions and thus input of N from BNF in the grasslands with different forbs did not influence residual N fertility in the subsequent cereal crop in the crop rotation.

5. Conclusions

The present study concludes that if used in the right proportions, the non-leguminous forbs chicory, ribwort plantain or caraway may be included in the traditional perennial ryegrass-red clover mixture for purposes such as enhancing acquisition of water and nutrients from deeper soil layers, mineral nutrition, forage quality and biodiversity without negative effects on herbage yield, BNF, N transfer from legume to the non-legumes and short-term residual soil N effect. Since the strong competitive ability of chicory reduced the red clover proportion and amount of BNF, a high seeding proportions of the chicory should be avoided for a balance between legume and non-legume proportions in the sward. Similarly, red clover, white clover and lucerne can be grown in mixtures with no negative effect on herbage production, N accumulation and input of N from BNF as compared to the strongest species in pure stand (red clover), provided that the mixtures contains at least a small proportion of red clover. Additionally, it appears that grass is an important component in multispecies swards because of its complementarity with legumes and forbs.

6. Perspectives of the study

- This study investigated plant production and N dynamics in the swards including a range of multi-species mixtures integrating plant species of the same or different functional groups. This provides an important management tool for the farmers for designing and implementing new multi-species temporary grasslands to optimize plant diversity and efficient use of N resources in grassland-arable cropping systems.
- Large variations in red clover proportions and fertilization did not significantly influence sward productivity, %Ndfa, total N yield or the N fertility of the subsequent crop. Hence, the inclusion of a large seeding proportion of a strong forage legume such as red clover can be avoided in forb-based high-yielding grasslands in order to efficiently utilize the legume-fixed N and minimizing risk of excessive N loss from the agricultural soil.
- The study revealed that a forb-based sward and a sward containing only forage legumes can be integrated into a low-input arable crop rotation and provide synchrony between the supply of N from legume BNF and crop uptake for plant production. This provides an important tool for farmers for crop management and N fertilization to enhance self-sufficiency in N resources and improve the N economy by using an alternative to N fertilizer, thus lowering the cost of animal feed production.
- Both swards under this study were highly productive. Hence, forbs can be grown in ryegrass-red clover and forage legume only in mixed stands, and integrated in arable cropping systems to produce larger and more stable yields of green biomass for bioenergy or nutrient-rich ruminant fodder without applying N fertilizer. This may be an important tool towards protein self-sufficiency in Europe.
- Low-input high-yielding forb-based sward and sward containing only forage legumes provide an important insight towards using marginal land for biomass production.

Future studies

- It is not fully clear from this study whether the forbs were outcompeted by the ryegrass for the red clover-derived N and in regulating %Ndfa and whether they would have different abilities when grown in the absence of ryegrass. Hence, both BNF and N transfer studies could be performed in mixtures containing forage legumes and forbs only to enhance the understanding of the role of forbs in the dynamics of N transfer and BNF.
- Future studies should focus on including a range of species mixtures such as other forbs in the forb-based sward and forage legumes in the sward containing only forage legumes, with different growth patterns and root architectures for a more illustrative and clearer picture of the underlying mechanisms of the studied N processes.

- The study could be performed under a range of management conditions such as cutting, grazing, cutting vs. grazing, N fertilization and under conditions such as high or low water stress and temperature to enhance the understandings of potentials of new mixtures to perform in changing management, resource availability and environmental conditions.
- Dynamics of N transfer may have been affected by the form of N released from red clover and the different N uptake patterns of ryegrass and forbs including access to mineral N in the soil. Hence, further work to elucidate the form of N released from red clover and N uptake patterns of ryegrass and forb species could help to better understand the dynamics of N transfer between forage legume and non-legume and extend our understanding of whether forbs rely on N uptake from deep soil layers or compete in all soil layers.
- All the measurements in the present study were made in aboveground plant parts. Hence, a future study could be carried out that incorporates belowground biomass to extend our understanding and make stronger recommendation, especially for the residual N effect.
- Assessment of N transformation processes such as mineralization, immobilization, nitrification and denitrification and a simultaneous study of leaching of N and nitrous oxide (N_2O) emission could be done in different mixtures to extend our understanding of how new swards containing forbs in grass-clover or only forage legumes help to improve NUE, thereby reducing N leaching and GHG emissions from the agricultural systems.
- Since chicory and plantain appear to establish and grow fast, future studies could examine the capacities of forb-based catch and cover crop swards, including chicory or plantain, to retain residual N and recycle it in the following crop.
- Biogas potential or changes in nutritional quality of the green biomass in the sward caused by different species combinations could be investigated to potentially utilize the harvested biomass from these low-input high-yielding swards.
- A future study could concentrate on the effect of inclusion of different forbs on residue quality such as C/N ratio and microbial biomass to enhance the understanding of forb's role in influencing residual N and SOM.

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8. Supporting papers

8.1 Paper I

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Increasing plant species diversity with inclusion of non-leguminous forbs in grass-clover mixtures does not affect red clover N_2-fixation and total N acquisition

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Abstract

Background: Legume N_2-fixation is stimulated by a diverse plant community, but studies of legume N_2-fixation in temporary grasslands including deep-rooted non-leguminous forage herb (forb) species are rare.

Methodology: We investigated N_2-fixation and nitrogen (N) yield in a range of grassland mixtures consisting three forb species – chicory (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.) and caraway (*Carum carvi* L.) – mixed into a traditional grassland mixture of perennial ryegrass (*Lolium perenne* L.) and red clover (*Trifolium pratense* L.) at two fertilisation levels.

Results: The percentage of red clover N derived from the atmosphere (%Ndfa) was higher in mixtures than in pure stand, but did not increase with inclusion of forbs. Red clover in all the mixtures derived over 90% of its N from fixation even when fertilised with 216 kg total N ha^{-1}. Forbs affected the amount of N_2-fixation by affecting the clover content in the harvested biomass.

Conclusions: We conclude that forbs can be included in temporary grassland mixtures to increase N use efficiency and herbage production without affecting legume N_2-fixation. The lacking %Ndfa decline with fertilisation showed that non-legumes either competed hard for soil N or that other mechanisms for regulation of %Ndfa were at play.

Keywords: plant diversity; temporary grassland; chicory; ribwort plantain; caraway; percentage of N_2-fixation (%Ndfa)

Introduction

The inclusion of forage legumes in grassland production systems has been shown to improve forage quality (Lüscher et al. 2014), enhance soil N fertility (Fustec et al. 2010) and increase plant productivity (Nyfeler et al. 2011). Soil N fertility is enhanced through the process of biological N_2-fixation (BNF), N rhizodeposition (Høgh-Jensen and Schjoerring 2001; Rasmussen et al. 2007) and turnover of above- and below-ground plant residues (Dahlin and Stenberg 2010b; Rasmussen et al. 2008; Rasmussen et al. 2012). The N fertility effect of forage legumes also includes their green manuring and catch crop capacities in temporary grassland included in rotation, where the soil N pool built up under them is mineralised upon termination of the swards, rendering it available to the subsequent crop in the rotation (Eriksen et al. 2008).

Globally, forage legumes are the second largest source of BNF after grain legumes (Herridge et al. 2008). However, there are considerable spatial and temporal variations in their abilities of BNF (Anglade et al. 2015; Lüscher et al. 2014). The extent of legume BNF is influenced by several internal and external factors such as legume plant and rhizobium genotype and their interaction with the surrounding environment, including management practices such as cutting, grazing, fertilisation and species composition (Carlsson and Huss-Danell 2003).

Numerous studies have shown that plant diversification by growing forage legumes in mixtures with non-legumes, notably forage grasses, has a direct effect on the extent to which legumes rely on BNF for their N acquisition (*e.g.* Carlsson and Huss-Danell 2003; Hauggaard-Nielsen et al. 2009; Høgh-Jensen and Schjoerring 1997). The inclusion of a non-legume increases the competition for available soil N and increases legume dependence on BNF compared to legumes grown in pure stands (Carlsson and Huss-Danell 2003; Hauggaard-Nielsen et al. 2009; Høgh-Jensen and Schjoerring 1997). Therefore, many studies have focused on legume and non-legume diversity in grasslands to improve BNF and soil N fertility. However, the majority of studies on N dynamics in grasslands tend to be confined to binary mixtures of grass and clover. Studies on BNF in multi-species grasslands including deep-rooted non-leguminous forage herbs (forbs) are scarce (exceptions: Frankow-Lindberg and Dahlin 2013; Pirhofer-Walzl et al. 2012).

Chicory (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.), hereafter plantain, and caraway (*Carum carvi* L.) are three forbs that may be adopted in grassland mixtures. They have potential as important components of grasslands due to their high competitive abilities in mixtures with different forage legume and non-legume species (Søegaard et al. 2013). They increase herbage production (Søegaard et al. 2008, 2011), forage quality (Høgh-Jensen et al. 2006; Søegaard et al. 2008), mineral nutrition (Pirhofer-Walzl et al. 2011) and can tolerate adverse weather conditions (Younie 2012). They have deep and diverse root systems (Li and Kemp 2005; Stewart 1996) and can take up N from deeper soil layers (Pirhofer-Walzl et al. 2013; Thorup-Kristensen 2006). Thus, the synergistic effects of including forbs with different above- and below-ground traits in grass-clover mixtures are expected to increase soil N acquisition and the competition for available soil N with accompanying legume species, thereby affecting the dependence of the legume species on BNF. However, forbs are not widely included in grassland mixtures in Europe, and little is known about how different forbs in grass-clover mixtures influence legume BNF. Here we conducted an experiment with the objectives of determining how the inclusion of non-leguminous forb species in grassland mixtures of red clover (*Trifolium pratense* L.) and perennial ryegrass (*Lolium perenne* L.), hereafter grass and clover, respectively, would affect sward composition (clover, grass, forbs) in terms of dry matter (DM) production and N accumulation as well as the percentage (%Ndfa) and amounts of clover BNF when exposed to two levels of fertilisation. The following hypotheses were tested: 1) percentage of clover N derived from the BNF (%Ndfa) can be increased by increasing plant species diversity via the inclusion of companion non-legume forbs, and 2) cattle slurry application reduces the clover dependency on BNF.

Materials and methods

Experimental site

The field experiment was carried out at Foulumgaard Experimental Station, Aarhus University, in Central Jutland, Denmark (56° 29°N and 09° 34°E). The experimental field was part of an organic dairy crop rotation with a cropping history of both grassland and arable crops since 1987. The soil is a loamy sand characterised as Typic Hapludult with 7.7% clay and 1.6% carbon (Eriksen et al. 2015) and 0.14 % total N. The mean monthly temperatures during the experimental period (April-October, 2014) were between 8 and 19 °C, with July the warmest months. The monthly precipitation varied between 35 and 117 mm, with May, August and October being relatively damp (Fig. 1).

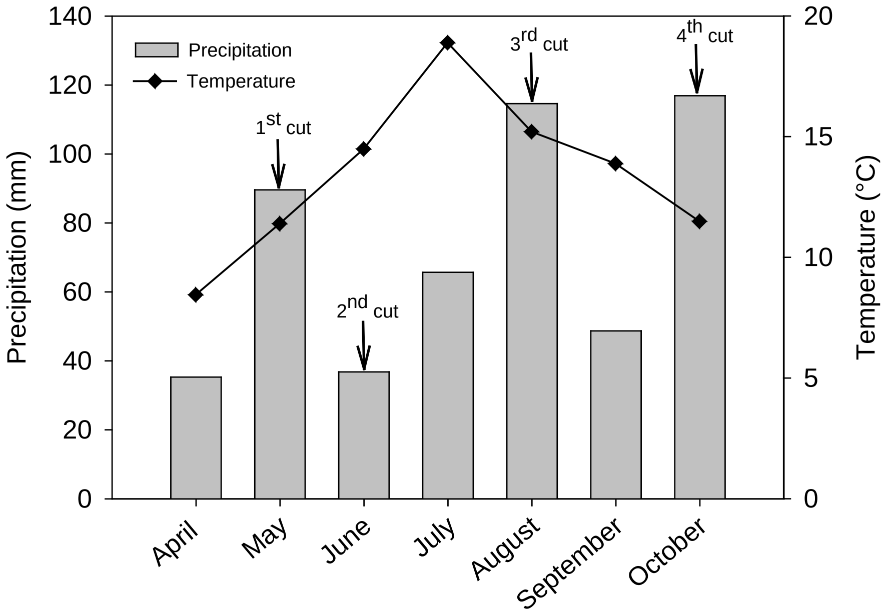

Fig. 1 Mean monthly air temperature and monthly precipitation during the experimental period from April to October 2014 measured at a climatic station near the experimental field.

Experimental design and establishment of experimental plots

Sixteen seed mixtures composed of different combinations of perennial ryegrass (*Lolium perenne* L., cultivar Stefani), red clover (*Trifolium pratense* L., cultivar Rajah) and three non-leguminous forbs: chicory (*Cichorium intybus* L., cultivar Spadona), ribwort plantain (*Plantago lanceolata* L., wild type) and caraway (*Carum carvi* L., cultivar Volhouden) were established in spring 2013 (Table 1).

Table 1 Composition of the seed in the mixtures (percentage and amount are based on the seeding rate of each species in a pure stand)

Seed mixtures		**Percentage of seed in the mixture (%)**					**Seeding rate (kg ha^{-1})**				
		GR	**RC**	**CH**	**PL**	**CA**	**GR**	**RC**	**CH**	**PL**	**CA**
Pure stand	Perennial ryegrass (GR)	100					15				
	Red clover (RC)		100					4			
	Chicory (CH)			100					12		
	Ribwort plantain (PL)				100					12	
	Caraway (CA)					100					12
Two species	50GR+50RC	50	50				7.5	2			
Three species	33CH+33PL+33CA			33	33	33			4	4	4
	20GR+20RC+60CH	20	20	60			3	0.8	7.2		
	40GR+40RC+20CH	40	40	20			6	1.6	2.4		
	20GR+20RC+60PL	20	20		60		3	0.8		7.2	
	40GR+40RC+20PL	40	40		20		6	1.6		2.4	
	20GR+20RC+60CA	20	20			60	3	0.8			7.2
	40GR+40RC+20CA	40	40			20	6	1.6			2.4
Five species	40GR+40RC+7CH+7PL+7CA	40	40	7	7	7	6	1.6	0.84	0.84	0.84
	20GR+20RC+20CH+20PL+2+20CA	20	20	20	20	20	3	0.8	2.4	2.4	2.4
	10GR+10RC+33CH+33PL+33CA	10	10	27	27	27	1.5	0.4	3.24	3.24	3.24

The species were sown in a replacement design based on the proportion of each species' seeding rate in a pure stand of 15, 4 and 12 kg ha^{-1} for grass, clover and forbs, respectively, in 1.5 x 8 m plots in three replicates. Each mixture was treated with two levels of N fertiliser, 0 and 216 kg total N ha^{-1}, in the form of cattle slurry applied in four split doses – 91 kg at the start of the growing season in early April, and the rest after the first, second and third cuts in nearly equal amounts (39, 44 and 42 kg ha^{-1}, respectively). The plots were irrigated after the first and second cuts. The quantitative analysis of clover BNF over a growing season was carried out in 2014 using the ^{15}N isotope dilution method (e.g. Rasmussen et al., 2012). For this purpose, a subplot measuring 1×1 m was demarcated in each experimental plot and the soil was labelled with ammonium sulphate 0.1 g N m^{-2} (atom% ^{15}N = 98) in early April 2014 to artificially enrich the soil ^{15}N above natural abundance.

Plant sampling and analysis

The shoot biomass was sampled by harvesting by hand to 5 cm stubble height in one 0.25 m^2 subplot per experimental plot four times during the growing season on 27 May, 30 June, 18 August and 3 October. At each cut, unlabelled plant samples were collected adjacent to the experimental plots (but at least 5 m from the ^{15}N-labeled subplots). The biomass samples were sorted into individual species, dried at 80 °C for 24 hours and weighed. The dried samples were milled to a fine powder, packed in small tin capsules and analysed for total N and atom% ^{15}N at the UC Davis Stable Isotope Facility, University of California, USA, on an ANCA-SL Elemental Analyzer coupled to a 20-20 Mass Spectrometer using the Dumas dry-combustion method. The total N yield was quantified based on N concentrations and shoot DM yields of each species in the subplot.

Calculations

BNF was quantified based on excess atom% ^{15}N in legume and non-legume species, here grass and forbs grown in the same plot as clover were used as non-legume reference plants, *i.e.* to estimate how much excess atom% ^{15}N clover was derived from soil. The percentage of clover N derived from the atmosphere (%Ndfa) was calculated using the following equation (McNeill et al., 1994):

$$\%\mathrm{Ndfa} = (1 - (\text{excess atom\% }^{15}\text{N legume} / \text{excess atom\% }^{15}\text{N reference})) \times 100$$

where excess atom% ^{15}N was calculated by subtracting the atom% ^{15}N of the legume and companion non-legume species in unlabelled plots (background atom% ^{15}N) from the atom% ^{15}N of these species in ^{15}N-labelled plots. The background atom% ^{15}N values measured in both clover and non-legume species were affected by neither cutting time nor plant species, so the average values measured in clover of 0.3664 atom% and in grass and forb species of 0.3678 atom% were used as background. In mixtures containing more than one non-legume species, the average value for excess atom% ^{15}N of all non-legumes species was used as reference value. Since the reference plants should reflect the ^{15}N signature of the soil N available for uptake by the legume (Carlsson and Huss-Danell 2014), the average excess atom% ^{15}N value of all non-legume species grown in mixtures containing at least 40% clover (according to seeding rate) was used as the reference value to estimate %Ndfa in pure stands of clover. The amount of BNF was calculated by multiplying %Ndfa with clover N accumulation in shoots for each cut separately. The average %Ndfa for the whole growing season was estimated by dividing the total amount of BNF over the growing season by the total amount of clover shoot N accumulated.

Data analysis

The data were analysed in the open-source statistical program R (R Core Team, 2016) (Version 3.1.0). Seasonal clover N yield and N uptake, and seasonal total DM and N yield data were log-transformed before analysis to obtain a normal distribution of residuals. A two-way analysis of variance was used to determine the effect of the two fixed factors (sown species composition and slurry application) on each of the dependent variables (DM yield, N yield, %Ndfa and amounts of BNF). The effect of cutting time on DM yield, N yield, %Ndfa and amount of BNF for each slurry level was analysed using the linear mixed model, where sown species composition (fixed effect) and cutting time (repeated fixed effect) were independent variables with the blocks as a random variable and the plots were nested in the blocks. The model was tested using ANOVA. The pairwise comparisons were made by *lsmean* using the adjusted Tukey method. The probability of hypothesis rejection was tested at the 0.95 confidence level ($P<0.05$).

Results

The weather conditions measured at the experimental site during the growing season (May to early October; Fig. 1) showed that the temperature was similar to the 30-year average, while the mean monthly precipitation was about 17% higher than the 30-year average at the same experimental station.

Dry matter production and botanical composition

Clover had significantly higher DM yields than the other pure stands in plots without slurry application, and significantly higher than grass and caraway in plots with slurry application (Table 2). The three- and five-species mixtures did not achieve significantly higher DM yields than the two-species grass-clover mixture, either with or without slurry application. The total DM production measured over the growing season was generally highest at the first and third cuts

($P<0.001$) and lowest at the fourth cut and the difference was most pronounced without slurry application.

Table 2 Total seasonal total shoot dry matter (DM) and N yields measured under two levels of slurry application, 0N and 216 kg total N ha^{-1} year^{-1}. Values are means (± SE; n = 3), with different letters within each column indicating a statistically significant ($p<0.05$) difference between species compositions and '*' indicating a significant ($P<0.05$) effect of slurry application within each variable.

Seed mixtures			**DM yield (t ha^{-1})**		**N yield (kg ha^{-1})**	
			0N	**216N**	**0N**	**216N**
Pure stand	Perennial ryegrass	(GR)	4.5±0.1^{a}	7.9±0.8^{a}*	76±5^{a}	127±12^{a}*
	Red clover	(RC)	15.4±0.7^{e}	14.8±0.9^{bc}	479±7^{e}	491±68^{d}
	Chicory	(CH)	7.1±1.6^{ab}	10.8±0.9^{ab}*	106±26^{ab}	180±23^{a}*
	Ribwort plantain	(PL)	8.3±0.9^{bc}	12.1±1.0^{bc}*	124±15^{ab}	190±16^{ab}*
	Caraway	(CA)	4.7±0.3^{a}	7.4±0.4^{a}*	93±6^{ab}	134±8^{a}*
Two species	50GR+50RC		15.8±0.9^{e}	14.5±0.3^{bc}	468±16^{e}	397±28^{d}
Three species	33CH+33PL+33CA		8.9±0.9^{bcd}	12.8±0.7^{bc}*	135±10^{b}	208±15^{abc}*
	20GR+20RC+60CH		12.4±1.4^{cde}	15.3±1.0^{bc}	283±34^{cd}	349±36^{cd}
	40GR+40RC+20CH		15.2±0.5^{e}	15.4±0.6^{bc}	372±29^{cde}	359±28^{d}
	20GR+20RC+60PL		15.6±1.4^{e}	15.9±1.0^{bc}	392±52^{cde}	411±28^{d}
	40GR+40RC+20PL		15.8±1.0^{e}	16.7±0.1^{c}	416±26de	418±33^{d}
	20GR+20RC+60CA		14.5±1.7^{e}	14.4±1.8^{bc}	389±53^{cde}	349±52^{cd}
	40GR+40RC+20CA		14.8±0.6^{e}	15.5±0.9^{bc}	426±9de	403±22^{d}
Five species	40GR+40RC+7CH+7PL+7CA		13.7±0.1^{e}	16.5±1.2^{c}	337±1^{cde}	402±39^{d}
	20GR+20RC+20CH+20PL+20CA		13.4±1.5^{de}	15.0±0.1^{bc}	324±54^{cde}	331±14^{cd}
	10GR+10RC+27CH+27PL+27CA		11.6±0.9^{cde}	15.3±0.4^{bc}*	243±29^{c}	331±26^{bcd}*

On an annual basis, slurry application significantly increased total DM yield by 43 to 75% in pure stands of non-legumes and the three species mixture of forbs ($P<0.001$). In the grass-clover-forb mixtures, the changes in yield ranged between -8 and 32%, with the two highest values in the five-species mixture containing 27% of each forb ($P<0.001$) and the three-species mixture with 60% seeding density of chicory (Table 2).

The proportion of clover in harvested biomass was often higher than the seeded proportion. Over the growing season, the clover produced the largest DM yield at the first and third cuts. The clover had the highest proportions of total DM yield in the two-species mixture (Fig. 2).

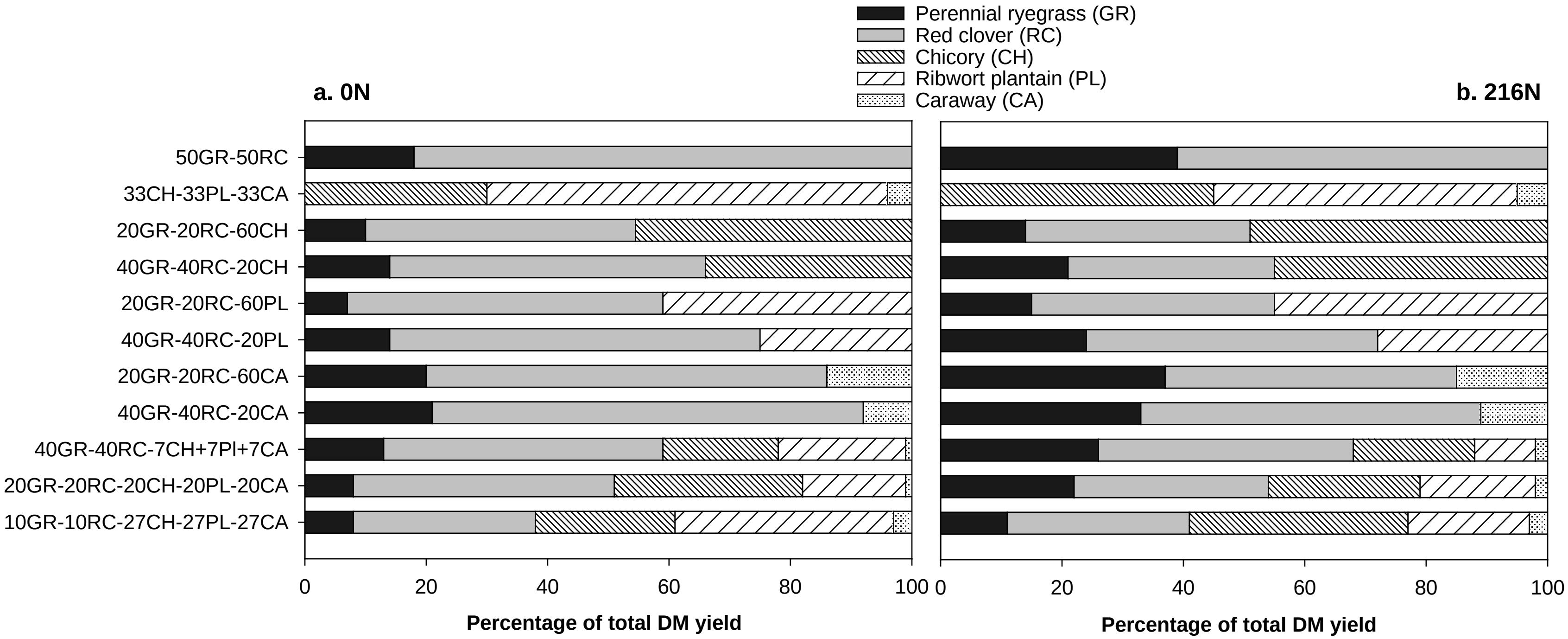

Fig. 2 Botanical composition of shoot herbage dry matter yield of different species mixtures grown without (0N) and with (216 kg total N ha^{-1}) slurry application (n=3) sampled four times over the growing season in 2014.

In the three-species mixtures, the clover generally had the highest proportion of total DM yield in the grass-clover-caraway combination followed by grass-clover-plantain mixtures. The clover proportions often decreased in the grass-clover-chicory and five-species mixtures. Slurry application decreased the clover proportion, especially in the two-species (grass-clover) and grass-clover-chicory mixtures (Fig. 2).

Grass generally had higher DM yields in a pure stand followed by the two-species mixture. In the grass-clover-forb mixtures, the grass yielded more in the three species grass-clover-caraway mixture (Fig. 2). Slurry application increased grass growth ($P<0.001$) in the majority of the mixtures with the proportions of grass generally increasing at the expense of clover (Fig. 2).

Similar to grass and clover, the forb species also had the highest DM yields in pure stands. Chicory, in general, produced the highest yield in the mixture followed by plantain, whereas caraway generally produced relatively little DM (Fig. 2). Slurry application had a similar effect on chicory and plantain in three species mixture. In the five-species mixtures, it benefitted chicory DM yield at the expense of plantain.

N yield

Total seasonal N accumulation in different mixtures varied from 76 to 479 kg N ha^{-1} without and 126 to 491 kg N ha^{-1} with slurry application (Table 2). As the clover dominated the mixtures, the differences in total N yields of the mixtures were mainly dependent on the clover content. On an annual basis, clover in pure stands produced the highest N yields followed by the two-species mixture without slurry and the three-species grass-clover-plantain or caraway mixtures with a high seeding density of clover with slurry application. The total N yields did not differ significantly between the mixtures containing clover with slurry application. Without slurry application, the N yield in the grass-clover-chicory and a five-species mixture with the highest seeding density of forbs was significantly lower ($P<0.001$) than in the two-species mixture. Slurry application increased ($P<0.001$) the seasonal total N yield for pure stands of non-legumes and the three-species mixture of forbs by 44 to 70%, but always much less than the 216 kg N applied. The changes in N yield of grass-clover-forb mixtures ranged between -5 and 36%, with a greater effect in the five species mixture with a high seeding density of forbs.

Percentage and amount of red clover N derived from BNF

The atom% ^{15}N measured in all five species at all four sampling occasions was sufficiently above the background and there was a clear distinction between clover and non-legumes to calculate the

%Ndfa. The excess atom% ^{15}N was highest at the first cut and decreased for succeeding cuts. In mixtures, the grass was often more enriched compared to forbs (data not shown).

The percentage of N in clover derived from BNF significantly increased ($P<0.001$) in mixtures compared to the pure stands of clover (Table 3). The seasonal %Ndfa in clover grown in mixtures was consistently above 90%, and there was no significant difference between mixture compositions with and without slurry application (Table 3).

Table 3 Total seasonal red clover N yields, and percentage (%Ndfa) and amount of red clover N derived from the atmosphere measured in shoots under two levels of slurry application, 0N and 216 kg total N ha^{-1} year^{-1}. Values are means (± SE; n = 3), with different letters within each column indicating statistically significant ($p<0.05$) differences between species compositions and '*' indicating a significant ($P<0.05$) effect of slurry application within each variable.

Seed mixtures		**Red clover N yield (kg ha^{-1})**		**%Ndfa**		**N$_2$-fixation (kg ha^{-1})**	
		0N	**216N**	**0N**	**216N**	**0N**	**216N**
Pure stand	Red clover	479±7^{d}	491±68^{b}	81.2±4.6^{a}	69.3±2.8^{a}*	389±21^{e}	339±42^{b}
Two species	50GR+50RC	412±14^{cd}	283±40^{ab}*	92.9±1.3^{b}	91.4±2.1^{b}	383±18de	258±36^{ab}*
Three species	20GR+20RC+60CH	164±33^{ab}	170±44^{a}	96.8±0.7^{b}	94.0±0.8^{b}	159±33^{ab}	159±39^{a}
	40GR+40RC+20CH	240±34^{bcd}	167±35^{a}	95.6±1.0^{b}	89.8±2.8^{b}*	229±32^{abcd}	148±26^{a}
	20GR+20RC+60PL	253±49^{bcd}	230±24^{ab}	96.9±0.6^{b}	94.4±0.7^{b}	245±46^{abcde}	217±22^{ab}
	40GR+40RC+20PL	297±33^{bcd}	247±48^{ab}	95.1±0.5^{b}	92.3±1.9^{b}	283±32^{bcde}	226±41^{ab}
	20GR+20RC+60CA	298±57^{bcd}	210±47^{a}	97.3±0.3^{b}	95.5±0.2^{b}	290±55^{bcde}	200±45^{ab}
	40GR+40RC+20CA	333±10^{bcd}	265±13^{ab}	94.7±1.0^{b}	92.7±0.8^{b}	315±7^{cde}	245±10^{ab}
Five species	40GR+40RC+7CH+7PL+7CA	197±13^{abc}	207±33^{a}	94.9±0.9^{b}	91.3±1.2^{b}	187±13^{abc}	189±28^{ab}
	20GR+20RC+20CH+20PL+20CA	183±53^{ab}	139±23^{a}	95.0±0.6^{b}	94.7±0.2^{b}	174±51^{abc}	132±22^{a}
	10GR+10RC+27CH+27PL+27CA	108±32^{a}	138±28^{a}	96.9±0.4^{b}	95.0±0.1^{b}	105±30^{a}	131±27^{a}

GR: Perennial ryegrass, RC: Red clover, CH: Chicory, PL: Ribwort plantain, CA: Caraway

Table 4 Percentage of red clover N derived from the atmosphere (%Ndfa) measured in shoots at two levels of slurry application, 0N and 216 kg total N ha^{-1} year^{-1}. Values are means (± SE; n = 3), with different letters within each column indicating statistically significant ($p<0.05$) differences between species compositions and '*' indicating a significant ($p<0.05$) effect of slurry application within each cut.

Seed mixtures		**%Ndfa**							
		Cut 1		**Cut 2**		**Cut 3**		**Cut 4**	
		0N	**216N**	**0N**	**216N**	**0N**	**216N**	**0N**	**216N**
Pure stand	Red clover	86.0±3.0^{a}	70.6±5.0^{a}*	83.2±4.0^{a}	64.3±2.3^{a}*	80.6±5.6^{a}	75.0±3.0^{a}*	58.1±7.0^{a}	53.7±4.7^{a}
Two species	50GR+50RC	97.5±0.1^{b}	96.7±0.4^{c}	96.3±0.1^{b}	95.5±1.3^{c}	92.3±1.6^{b}	92.9±2.2^{b}	76.6±5.0^{b}	75.2±4.9^{b}
Three species	20GR+20RC+60CH	97.1±0.4^{b}	93.9±0.4^{bc}*	96.7±0.8^{b}	93.7±0.5^{bc}	97.9±0.5^{b}	95.8±0.9^{b}	90.4±3.4^{cd}	83.7±2.4^{bc}
	40GR+40RC+20CH	96.3±1.0^{b}	92.3±1.4^{b}*	95.4±1.1^{b}	90.4±0.7^{b}*	96.7±0.7^{b}	88.9±6.7^{b}*	89.0±1.9^{cd}	80.5±3.5^{bc}*
	20GR+20RC+60PL	97.5±0.6^{b}	96.3±0.3^{bc}	96.9±0.7^{b}	95.6±0.6^{c}	97.6±0.6^{b}	95.7±1.0^{b}	92.4±1.0^{cd}	84.7±3.1^{bc}
	40GR+40RC+20PL	97.4±0.4^{b}	96.0±0.9^{bc}	96.3±0.4^{b}	94.6±0.7^{c}	94.8±1.0^{b}	93.4±2.2^{b}	83.3±2.0^{bc}	79.3±5.0^{bc}
	20GR+20RC+60CA	97.6±0.3^{b}	96.1±0.4^{bc}	97.1±0.1^{b}	93.8±0.5^{bc}	97.7±0.4^{b}	96.7±0.3^{b}	94.6±0.4^{d}	87.4±1.6^{c}
	40GR+40RC+20CA	97.1±0.5^{b}	96.0±0.5^{bc}	94.9±1.2^{b}	93.8±0.6^{bc}	94.4±1.2^{b}	92.6±0.8^{b}	85.4±3.8^{bcd}	79.7±1.5^{bc}
Five species	40GR+40RC+7CH+7PL+7CA	96.5±0.6^{b}	93.7±0.5^{bc}	94.8±1.7^{b}	91.7±1.7^{bc}	95.7±0.9^{b}	93.4±0.9^{b}	88.5±0.9^{cd}	77.2±2.7^{bc}*
	20GR+20RC+20CH+20PL+20CA	95.5±0.2^{b}	94.6±0.6^{bc}	95.1±0.7^{b}	95.5±0.2^{c}	95.5±1.0^{b}	96.6±0.4^{b}	90.6±2.0^{cd}	80.5±2.8^{bc}*
	10GR+10RC+27CH+27PL+27CA	96.8±0.6^{b}	95.1±0.7^{bc}	97.0±0.6^{b}	95.4±0.7^{c}	97.9±0.2^{b}	96.6±0.5^{b}	92.6±0.4^{cd}	89.1±1.2^{c}

GR: Perennial ryegrass, RC: Red clover, CH: Chicory, PL: Ribwort plantain, CA: Caraway

The percentages of clover BNF were consistently high during the first three cuts and lower at the fourth cut ($P<0.001$) (Table 4). The species composition of the mixture in the unfertilised treatment had no significant effect on clover %Ndfa in the first three cuts, whereas at the fourth cut %Ndfa in the unfertilised grass-clover-forb mixtures was mostly significantly higher than in the grass-clover mixture. Clover %Ndfa showed nearly the same pattern in the treatments with slurry application, with little effect of mixture composition during the first three cuts. In the fourth cut, %Ndfa was higher in the grass-clover-caraway mixture with 20% clover and the five-species mixture with 10% clover than in the grass-clover mixture (Table 4).

Slurry applications of 216 kg total N ha^{-1} generally did not significantly lower %Ndfa in the mixtures, although there were individual exceptions (Table 4). The effect on the specific mixture varied depending on cutting time and composition of seed mixtures with a significant interactive effect ($P<0.001$) for both levels of slurry application. On a seasonal basis, the slurry application significantly ($P<0.001$) decreased %Ndfa in the pure stand of clover and in the grass-clover-chicory mixture with a high seeding density of clover (Table 3).

The amount of BNF differed depending on seed mixture and cut, with significant interactive effects, especially with slurry application ($P<0.05$). The amount of BNF was closely related to the pattern of clover DM and N accumulation. There was a strong linear association between the amount of N_2 fixed and both total N yield ($R^2= 0.81$ with slurry and $R^2= 0.94$ without slurry) and clover N yield ($R^2= 0.97$ both with and without slurry). The total amount of seasonal BNF in different mixtures ranged between 105 and 390 kg N ha^{-1} without and from 130 to 340 kg N ha^{-1} with slurry application (Table 3). The amount of BNF was highest in the pure stand of clover followed by the two-species grass-clover mixture. The clover in the three-species mixtures with plantain or caraway fixed in excess of 200 kg N ha^{-1} yr^{-1} at both levels of slurry application, which in most cases was not significantly different from clover in the pure stand and the two-species clover-grass mixture. The amount of BNF was suppressed in the grass-clover-chicory and five-species mixtures, especially with high a seeding density of forbs, compared to other species compositions (Table 3), which was mainly caused by a decrease in the proportion of clover in the harvested biomass (Fig. 2).

Slurry application generally lowered the amount of BNF in the majority of the mixtures, which was most pronounced at the first and second cuts (data not shown). The amount of BNF declined mainly due to a decrease in the clover content in the harvested biomass in treatments with slurry application (Fig. 2). On an annual basis, slurry application lowered the seasonal BNF by up to 35%, with a larger effect in the two- and three-species mixtures than in the five-species mixtures. In three-species mixtures there was a tendency for the effect of slurry application to be larger with a high seeding density of clover. The effect of slurry application was, however, significant only in the two-species mixture ($P<0.001$).

N uptake in red clover and non-legumes

The uptake of soil N in clover was highest in the pure stand followed by the two-species mixture of clover and grass (Table 5). The range of difference due to slurry application was small across all the treatments and was not statistically significant for clover, and all the forb species in the mixtures.

When included in unfertilised grass-clover-forb mixtures with a 20% or lower proportion in the seed mixture, clover took up significantly less soil N than in the two-species mixture with grass (Table 5). While all non-legume pure stands significantly increased their N uptake in response to slurry application (Table 2), only grass (*i.e.* none of the forbs) significantly increased its N uptake with slurry application when grown in grass-clover-forb mixtures. Chicory and plantain in grass-clover-forb mixtures almost always took up more N than perennial ryegrass – the only exception was the fertilised five-species mixtures with 40% of grass (Table 5).

Table 5 Seasonal N uptake from soil pools in red clover, perennial ryegrass, chicory, ribwort plantain and caraway measured under two levels of slurry application, 0N and 216 Kg total N ha^{-1} year^{-1}. Values are means (± SE; n = 3), with different letters within each column indicating statistically significant ($p<0.05$) differences between species compositions and '*' indicating a significant ($P<0.05$) effect of slurry application within each variable.

Seed mixtures		**N uptake (kg ha^{-1})**									
		Red clover		**Perennial ryegrass**		**Chicory**		**Ribwort plantain**		**Caraway**	
		0N	**216N**	**0N**	**216N**	**0N**	**216N**	**0N**	**216N**	**0N**	**216N**
Pure stand	Red clover	90 ± 22^{e}	152 ± 30^{c}								
Two species	50GR+50RC	29 ± 4^{d}	25 ± 8^{b}	56 ± 3^{bc}	$114\pm13^{e}*$						
Three species	33CH+33PL+33CA					45 ± 25^{a}	98 ± 27^{ab}	83 ± 15^{ab}	98 ± 2^{bc}	8 ± 1^{ab}	12 ± 2^{a}
	20GR+20RC+60CH	5 ± 1^{ab}	11 ± 4^{ab}	22 ± 6^{ab}	40 ± 11^{ab}	98 ± 9^{a}	138 ± 21^{ab}				
	40GR+40RC+20CH	11 ± 3^{abcd}	19 ± 9^{ab}	39 ± 2^{abc}	$63\pm14^{abcd}*$	93 ± 12^{a}	130 ± 9^{ab}				
	20GR+20RC+60PL	8 ± 3^{abc}	13 ± 3^{ab}	21 ± 2^{ab}	$46\pm9^{abc}*$			119 ± 6^{b}	135 ± 7^{c}		
	40GR+40RC+20PL	14 ± 1^{bcd}	20 ± 8^{ab}	45 ± 5^{abc}	$75\pm17^{bcd}*$			73 ± 10^{ab}	97 ± 4^{bc}		
	20GR+20RC+60CA	8 ± 2^{abc}	10 ± 3^{ab}	43 ± 5^{abc}	$88\pm3^{de}*$					48 ± 3^{c}	51 ± 6^{b}
	40GR+40RC+20CA	18 ± 4^{cd}	19 ± 3^{ab}	63 ± 10^{c}	$93\pm5^{de}*$					30 ± 11^{bc}	$45\pm10^{b}*$
Five species	40GR+40RC+7CH+7PL+7CA	10 ± 1^{abcd}	19 ± 5^{ab}	34 ± 5^{abc}	80 ± 11^{cde}	46 ± 23^{a}	68 ± 20^{a}	56 ± 22^{a}	36 ± 4^{a}	4 ± 2^{a}	10 ± 6^{a}
	20GR+20RC+20CH+20PL+20CA	9 ± 2^{abc}	7 ± 1^{a}	18 ± 5^{a}	$59\pm5^{abcd}*$	77 ± 1^{a}	74 ± 20^{a}	42 ± 1^{a}	53 ± 4^{ab}	5 ± 1^{a}	7 ± 1^{a}
	10GR+10RC+27CH+27PL+27CA	4 ± 1^{a}	7 ± 1^{a}	14 ± 5^{a}	32 ± 6^{a}	48 ± 9^{a}	97 ± 13^{ab}	66 ± 8^{a}	52 ± 2^{ab}	7 ± 2^{ab}	12 ± 2^{a}

GR: Perennial ryegrass, RC: Red clover, CH: Chicory, PL: Ribwort plantain, CA: Caraway

Discussion

Our focus was on investigating how new non-legume species with different functional traits influence legume growth, competition for available soil N and BNF when included in the conventional grass-clover mixture.

Red clover growth dynamics and sward competition

We found a yield advantage in mixtures containing clover compared to non-legume pure stands and the three-species mixture of forbs, but in the presence of clover the total DM and N yield were generally not affected by plant species diversity, seeding density of clover or slurry treatment. The total DM and N yield of the mixtures were comparable to similar two-species mixtures of grass and clover (Rasmussen et al. 2012), but higher than in a multi-species forage legume, grass and non-legume forb mixture (Pirhofer-Walzl et al. 2012) previously measured at the same location. Hence, the present study indicates that a potential yield advantage expected from increasing the number of species may be overshadowed by the high productivity of a competitive forage legume – in this case clover.

Clover generally dominated in the mixtures regardless of species composition and seeding densities of clover and non-legumes, and thus defined the DM and N yield of the mixtures, especially without slurry application. This reflects the strong competitive ability of clover for above- and below-ground resources (Rasmussen et al. 2012) and the competitive advantage from BNF under zero fertilisation (Carlsson and Huss-Danell 2003). The clover proportions of total DM and N yield were suppressed in grass-clover-chicory and five-species mixtures, but clover showed a competitive advantage when grown with plantain and caraway in three-species mixtures. Our results showed that chicory competed strongly with the other crops in herbage production, which is in line with the observations of Goh and Bruce (2005) and may be explained by the plant functional traits. The tall rosette plant and broad prostrate leaves of chicory would have shaded neighbouring plants (Søegaard et al. 2013), and its deep-growing roots may compete successfully for soil and water resources (Pirhofer-Walzl et al. 2013; Thorup-Kristensen 2006). Despite better competitiveness in the mixture, the plantain may have favoured the growth of clover by its more upright leaves letting in more light (Søegaard et al. 2013). We observed increased growth of caraway later in the growing season, which could be related to its initial energy investment for establishing a large root system (Hakala et al. 2008; Søegaard et al. 2013).

Comparing grass and clover, the main finding was that addition of slurry increased the proportion of grass at the expense of clover in the sward. Thus, although clover generally dominated the swards, its growth varied with non-legume seeding proportions and slurry application. The clover proportion varied from 30 to 80% of the sward biomass, which may be expected to affect the availability of soil N and therefore, in theory, also the proportion of N that clover derived from BNF (%Ndfa).

Proportion of red clover N derived from BNF (%Ndfa)

The proportion of N in clover derived from BNF (%Ndfa) was stimulated in all the mixtures compared to the pure stand of clover, but it was remarkably consistent across the two-, three- and five-species mixtures, both with and without slurry application. Hence, we could not confirm our first hypothesis that clover reliance on BNF would increase with increasing species diversity of companion non-legumes. Previous studies have reported that the %Ndfa is primarily influenced by legume production, soil N availability and competition for the available soil N among co-existing non-legumes (e.g. Carlsson and Huss-Danell 2003; Høgh-Jensen and Schjoerring 1997; Ledgard and Steele 1992; Nyfeler et al. 2011). Carlsson et al. (2009), Nyfeler et al. (2011) and Oberson et al. (2013) have shown a stimulatory effect of higher proportions of N sinks (non-legumes) on %Ndfa. Carlsson et al. (2009) further suggested that in diversified leys species composition and functional traits (*e.g.* efficient soil N uptake by competitive grasses) are more important than species richness *per se* for an effect of companion non-legumes on legume BNF. In contrast to those studies, we found no relation between species diversity and composition on %Ndfa in clover for the two-, three- and five-species mixtures. Furthermore, we found no effect of slurry application on %Ndfa in the majority of the mixtures, even in the mixtures dominated by clover (*i.e.*, a significant effect of slurry application on %Ndfa was only detected in the clover pure stand). This fails to support our second hypothesis and contrast with the previous findings that demonstrated negative effects of elevated soil N levels (N fertilisation) on %Ndfa in red clover (e.g. Pirhofer-Walzl et al. 2012; Rasmussen et al. 2012) and white clover (e.g. Høgh-Jensen and Schjoerring 1997), also when grown in mixtures with non-legumes. Carlsson et al. (2009) found that increasing soil N levels affect %Ndfa, depending on companion non-legume species' competition for available soil N. They found %Ndfa to decrease with increases in soil N level in species-poor communities and to increase in species-rich communities containing grass. However, we found no such response to slurry application when comparing the proportion of different functional groups in the different mixtures included in the present study.

The lack of an effect of slurry on %Ndfa when clover was growing in a mixture with grasses and forbs could be due to very low soil N levels. If the soil N status was initially very low, then application of fertiliser N would benefit the non-legumes of the sward, which was indeed the case in our experiment. However, in most mixtures it was only the proportion of grass in harvested DM which increased with slurry application, whereas the proportion of forbs in most cases did not increase. Also, the increased soil N uptake in response to slurry application was significant only for grass in the mixtures, not for forbs. These observations point to the likely explanation that soil N availability was limiting only for grass and that the forbs could acquire sufficient amounts of N – potentially by uptake from deeper soil layers – for their growth even without slurry application. This is in line with a parallel study on N transfer from red clover to the grass and forbs, showing that forbs to a greater extent relied on soil N compared to grass, which also relies on clover-derived N (Dhamala et al. 2017). The observed increases in the proportion of harvested DM and N uptake by grass in slurry-fertilised mixtures support the hypothesis that grass efficiently competes for available soil N, thereby stimulating high %Ndfa in companion legumes even if the mixture is fertilised with N (*e.g.* Carlsson and Huss-Danell 2003; Carlsson et al. 2009; Palmborg et al. 2005). Furthermore, since the percentage of clover in the sward varied greatly, it seems unlikely that the clover was not able to compete for soil N. It implies that an explanation other than a regulatory effect of available soil N is needed.

In addition to the complementarity between soil N uptake and BNF, we suggest that the grass is also a strong competitor for N derived from clover via rhizodeposition and N transfer (Dhamala et al. 2017). Consequently, when growing in mixtures, clover would not be able to re-assimilate its deposited N (root exudates), which could have provoked the clover to rely on its own BNF. Hence, grass competition with clover for clover exudations of N could explain the observed pattern of high %Ndfa in all treatments. In contrast, since the forbs were shown to rely less on red clover-derived N than grass (Dhamala et al. 2017), it is logical that the presence of forbs in the three- and five-species mixtures did not have a significant regulatory effect on %Ndfa.

We observed a seasonal variation in %Ndfa, where %Ndfa decreased at the fourth cut, especially in clover pure stands and the two-species mixtures of grass and clover. The reduction in the %Ndfa could be related to a low carbohydrate supply to the root nodules and thus lower nitrogenase activity, due to the reduction in photosynthetic activity with the fall in light intensity and temperature at the fourth cut in October (Gralle and Heichel 1982; Roughley and Dart 1970). Furthermore, it could be that the reduction in non-legume growth, especially of grass, likely increased clover access to N from the soil. A lower N demand of grass later in the growing season was also found by Nyfeler et al. (2009; 2011), but contrast with the study of Frankow-Lindberg and Dahlin (2013) who found a greater reliance of grasses on legume-derived N towards the latter part of the growing season. In summary, the %Ndfa of clover was mostly at a high level in all mixtures even with slurry application, which implies that the studied non-legumes in general competed efficiently for soil N, thereby stimulating a high %Ndfa by clover.

The amount of red clover N derived from BNF

Since we observed a consistently high %Ndfa across two-, three- and five-species mixtures, the amount of BNF in mixtures generally followed the same trend as the DM and N yield of the clover. This confirms that legume DM production is the main factor controlling the amount of N derived from BNF, as shown in several previous studies (*e.g.*, Anglade et al. 2015; Carlsson and Huss-Danell 2003; Dahlin and Stenberg 2010a; Unkovich et al. 2010). The clover in grass, clover and plantain or caraway mixtures fixed in excess of 200 kg N ha^{-1}, which was comparable to clover in a pure stand and the two-species grass-clover mixture. The only treatment that affected the amount of BNF was for the mixture with a high chicory content and with slurry application, which reduced the proportion of clover in the sward.

The amount of shoot N derived from clover BNF (105– 400 kg N ha^{-1} yr^{-1}) in the present experiment was lower than the highest amount recorded (545 kg N ha^{-1}yr^{-1}) in red clover in Europe (Anglade et al. 2015). However, it was within the previously reported range in legumes of European grasslands (100-380 kg N ha^{-1}yr^{-1}) (Lüscher et al. 2014) and comparable to the reported amounts of BNF in red clover in northern European grasslands (373 kg N ha^{-1}yr^{-1}, Carlsson and Huss-Danell 2003; 324 with and 357 kg N ha^{-1} yr^{-1} without slurry, Rasmussen at al. 2012). Thus, the present organic temporary grassland system obtained large amounts of N from clover BNF and forb species and seeding density in the mixture were the main influences on the amount of BNF via their effect on the share of legumes in the harvested biomass.

Conclusions

Our study did not show any strong effect of plant species diversity on red clover dependency on biological N$_2$-fixation when grown in two-, three- and five-species mixtures with grass and non-legume forbs. The presence of a negative effect of slurry on the proportion of red clover N derived from biological N$_2$-fixation when in a pure stand but not in most of the mixtures – even with the high share of red clover – indicates that either the non-legumes competed well for soil N or soil N availability was not the main regulating mechanism for %Ndfa. The red clover content in the harvested biomass defined, as previously reported, the amount of N originating from biological N$_2$-fixation, with some variation due to the seeding densities of the species and application of slurry.

We conclude that forbs can be included in a temporary grassland mixtures without negative effects on biological N_2-fixation and N yield, provided that the mixtures do not include very high seeding densities of chicory. The study suggests that forb species and seeding density are important factors in the design and implementation of multi-functional multi-species grasslands that help to combine N input from legume biological N_2-fixation and efficient N use to produce herbage and quality forage.

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8.2 Paper II

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N transfer in three-species grass-clover mixture with chicory, ribwort plantain or caraway

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REGULAR ARTICLE

N transfer in three-species grass-clover mixtures with chicory, ribwort plantain or caraway

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Abstract

Background and aims There is substantial evidence that legume-derived Nitrogen (N) is transferred to neighboring non-legumes in grassland mixtures. However, there is sparse information about how deep rooted non-legume forage herbs (forbs) influence N transfer in multi-species grasslands.

Methodology Red clover (*Trifolium pretense* L.) was grown together with perennial ryegrass (*Lolium perenne* L.) and one of three forb species: chicory (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.) or caraway (*Carum carvi* L.) in a field experiment. During the first year after the establishment, red clover leaves were labeled with ^{15}N-urea to determine the N transfer from red clover to companion ryegrass and forbs.

Results On an annual basis, up to 15 % of red clover N was transferred to the companion ryegrass and forbs, but predominantly to the grass. The forb species did not differ in their ability to take up clover N, but biomass production and soil N acquisition was higher in chicory and plantain than in caraway.

Conclusions Grass relied to a great extent on clover N, whereas forbs relied on soil N. Soil ^{15}N-enrichment indicated that N transfer occurred in the upper soil layers and that a dependence on clover-derived N did not necessarily give grass a growth advantage.

Keywords Grassland mixture · Red clover · Perennial ryegrass · Non-leguminous forb · ^{15}N-leaf labeling

Introduction

In agriculture, forage legumes have been shown to provide a high-quality forage (Lüscher et al. 2014), promote the buildup of soil N fertility and increase plant productivity (Carlsson and Huss-Danell 2003). In grassland mixtures of forage legumes and non-legumes, the legumes improve N supply and thereby the growth of companion non-legume species through biological N_2-fixation and N rhizodeposition (Dahlin and Stenberg 2010; Høgh-Jensen and Schjoerring 2001). Rhizodeposition occurs via the decomposition of dead plant parts including nodules and roots cells and plant root exudation of soluble N compounds (Fustec et al. 2010). In addition, direct facilitative N transfer has been shown to occur from N-rich legume plants to companion non-legume plants (Høgh-Jensen and Schjoerring 2000) and vice-versa (Gylfadóttir et al. 2007; Carlsson and Huss-Danell 2014). It occurs either through the exudation of low-molecular-weight soluble organic (Paynel et al. 2001; Rasmussen et al. 2013) or inorganic (Paynel et al. 2008) N compounds from living root cells

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and subsequently uptake by companion species through direct root contact or interconnecting roots between arbuscular-mycorrhizal fungi and the plant species (Haystead et al. 1988).

Studies have shown that significant amounts of N of up to 15–50 % of legume N can be transferred to neighboring non-legume species (Dahlin and Stenberg 2010; Gylfadóttir et al. 2007; Rasmussen et al. 2007; Rasmussen et al. 2013). Under field conditions, the transfer is affected by climatic or seasonal variation in plant growth and soil conditions (Rasmussen et al. 2013) and internal factors such as plant root type and distribution in the soil (Pirhofer-Walzl et al. 2012), traits controlling root turnover and residue quality such as carbon to nitrogen ratio and lignin content (Louarn et al. 2015), the ratio between legume and non-legume biomass, C allocation within the non-legume and turnover rates of the plant roots in the soil (Rasmussen et al. 2007), and the ratio of legume to non-legume N (Dahlin and Stenberg 2010). The N transfer is also influenced by management factors such as selection of legume and non-legume plant species and genotypes, age of the sward and harvesting time of plant materials (Carlsson and Huss-Danell 2014; Chalk et al. 2014). In addition, the plant species diversity of the grassland mixture has been found to affect the direction and magnitude of N transfer due to differences in the ability of non-legumes to acquire soil- and legume-derived N (Høgh-Jensen 2006; Pirhofer-Walzl et al. 2012) as well as variations in the rates of legume growth and N_2-fixation (Paynel et al. 2008; Rasmussen et al. 2013).

Temporary grasslands in mixed rotations are usually dominated by two plant functional groups: grasses and forage legumes. To date, studies conducted on grasslands have mostly focused on simple mixtures of forage legumes and grasses, but more recent studies have involved a multi-species mixture in temporary grasslands with a greater range of plant species, including non-leguminous dicot forage herbs (forbs). Chicory, ribwort plantain and caraway are three such promising forage herbs because of their high competitive ability (Eriksen et al. 2011; Søegaard et al. 2013) and tolerance of adverse weather conditions (Younie 2012). They have the potential to increase plant diversity and herbage yield (Sanderson et al. 2005; Søegaard et al. 2011) and also herbage quality in terms of its mineral nutrition (Pirhofer-Walzl et al. 2011), effect on animal health (Younie 2012) and palatability (Søegaard et al. 2008). In addition, their physiological and morphological characteristics include diverse root systems e. g. deep-rooted plant species (Eriksen et al. 2012; Li and Kemp 2005; Søegaard et al. 2013; Stewart 1996). Studies have suggested that deep-rooted species are efficient at utilizing N from deeper soil layers (Pirhofer-Walzl et al. 2013; Thorup-Kristensen 2006), and that the plant diversity of diverse root system confers variability to the pattern of N acquisition from soil and N transferred from N-rich companion legume plants (Lesuffleur et al. 2007; Pirhofer-Walzl et al. 2013; Pirhofer-Walzl et al. 2012). However, these deep-rooted forbs are still very rarely included in grassland mixtures in Europe and the dynamics of N processes in multi-species grasslands including forbs are very rarely investigated.

A field experiment was therefore conducted to investigate how the inclusion of a non-legume forb species (chicory, ribwort plantain or caraway) in a mixture with perennial ryegrass and red clover would influence the dynamics of interspecies N transfer. The main objectives of the study were to determine how the choice of forb species and the seeding proportions of grass and clover in the seed mixture would affect: 1) The shoot biomass and N accumulation of ryegrass, red clover and forbs, 2) the N transfer from red clover to grass and forbs, and 3) the total root biomass and root N accumulation. The following main hypotheses were tested:

1. The proportion of clover N transfer increases with increasing above-ground biomass production and N accumulation in companion grass and forb species, and
2. The total seasonal N transfer to above-ground plant parts and to the soil increases with an increase in total root biomass and N accumulation in legume and non-legume species in the mixture.

Materials and methods

Experimental site and design

The field experiment was conducted at Foulumgaard Experimental Station, Aarhus University in central Jutland, Denmark (09° 34° E and 56° 29° N). The experimental field formed part of a dairy crop rotation with a history of grass-arable cropping and managed according to EU organic standards since 1987. The soil is a loamy sand characterized as a typical Hapludult, containing

7.7 % clay, 1.6 % carbon (Eriksen et al. 2015) and 0.12–0.16 % total N. The monthly precipitation and mean monthly temperatures during the experimental period between April and October were 35–117 mm, and 8–19 °C, respectively. June, July and August were the warmest months, while May, August and October were the wettest (Fig. 1).

Six grassland mixtures were established in spring 2013, each including one of the three forb species of chicory (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.) or caraway (*Carum carvi* L.) in addition to the traditional grassland mixture of perennial ryegrass (*Lolium perenne* L.) and red clover (*Trifolium pratense* L.). The species were sown based on their seeding rate in a pure stand: 15, 4 and 12 kg ha^{-1} for perennial ryegrass, red clover and forbs, respectively. The mixtures were either dominated by clover (mixtures 1–3) or grass (mixtures 4–6) at 80 % of their pure stand seeding rates (Table 1). The experimental plots were harvested twice during the year of establishment, and the experiment was conducted in the following growing season.

In early April 2014, polyvinyl chloride (PVC) cylinders of 30 cm internal diameter and 30 cm height were inserted 28 cm into the soil enclosing all three species in the mixture to confine the area of N transfer. Four replicate cylinders were randomly installed within an area of each seed mixture measuring approximately 1.5 × 8 m and at least 50 cm apart to avoid cross-contamination.

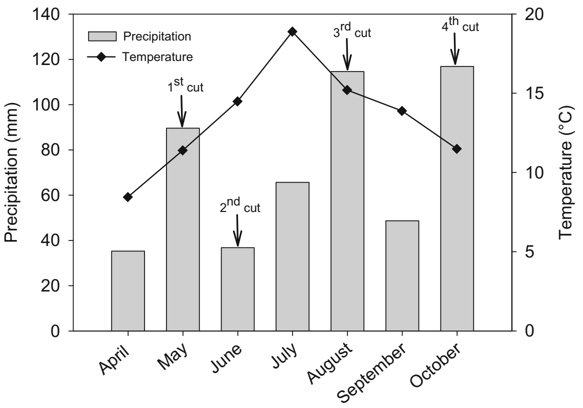


Fig. 1 Monthly precipitation and mean monthly air temperature during the experimental period from April to October in 2014 measured at a climate station near the experimental field

Leaf-labeling

Leaf-labeling with ^{15}N-enriched urea was used to determine N transfer (Ledgard et al. 1985). Five fully-developed medium sized clover leaves in each cylinder were gently inserted into 2-ml Eppendorf tubes containing 1 ml urea solution (0.5 % *w/v* and 98 atom %^{15}N). The tubes were sealed with sticky-tack sealing materials (taking care not to damage leaf petioles or other parts of the plant) to avoid the loss of ^{15}N (e.g. evaporation) and intrusion of rainwater. After 3–4 days, the leaves were detached at the petioles and the tubes were removed along with the entire leaf. During labeling and removal of tubes, care was taken to prevent contaminating soil and plants with the labeling solution. Two labeling sessions were conducted during each of four growth periods (May, June, July to mid-August, mid-August to early October). The number of clover plants in the cylinder varied between one and five. One leaf from each plant was labeled when five plants were present in the cylinder, and two to five leaves from the same plants were labeled when numbers of plants were less than five in the cylinder. Different leaves from the same plant were selected at the second labeling. Hence, clover plants were labeled eight times during the growing season, amounting to 40 leaves in total and resulting in 40 ml urea labeling solution in each cylinder. This supplied a total of 1.36 g N m^{-2}.

Plant sampling and analysis

Each growth period was terminated approximately 2 weeks after the second labeling by harvesting all above-ground plant material. The sampling was done manually by hand cutting with scissors to a stubble height of 5 cm. The first sample was taken on 27 May following labeling on 1 and 12 May; the second on 30 June following labeling on 23 and 27 June; the third on 15 August, with labeling on 11 and 28 July; and the final sampling on 3 October following labeling on 12 and 23 September. The interval between two labeling events and between the second labeling and sampling was shorter for the second sampling event than for the others. Unlabeled plant samples were collected from each plot, at a distance of at least 50 cm from the cylinder, for analyzing background ^{15}N-abundance. The plant samples were sorted into individual species, dried at 80 °C for 24 h, and dry matter (DM) weight determined. Samples were then coarsely milled,

Table 1 Composition of seed mixtures (percentage is based on each species seeding rate in pure stand)

Seed mixtures			Percentage of seed in the mixture (%)					Seeding rate (Kg ha^{-1})				
			GR	RC	CH	PL	CA	GR	RC	CH	PL	CA
1	Clover dominated	CH-RC	10	80	10			1.5	3.2	1.2		
2		PL-RC	10	80		10		1.5	3.2		1.2	
3		CA-RC	10	80			10	1.5	3.2			1.2
4	Grass dominated	CH-GR	80	10	10			12	0.4	1.2		
5		PL-GR	80	10		10		12	0.4		1.2	
6		CA-GR	80	10			10	12	0.4			1.2

GR Perennial ryegrass, *RC* Red clover, *CH* Chicory, *PL* Ribwort plantain, *CA* Caraway

subsampled and ball-milled into a fine powder, and packed into tin capsules for analyses of total N and atom % ^{15}N.

Root and soil samples were taken at termination of the experiment after the last plant sampling in October, by collecting three soil cores (2 cm diameter) from each cylinder at 0–10 cm and 10–30 cm depth, respectively, and 5 g soil from each cylinder and depth was dried at 105 °C for 24 h and DM amount was recorded. The remaining soil in each cylinder was passed through a 1-mm sieve, liberated from plant material by removing visible roots and other debris with the help of tweezers and dried at 80 °C for 24 h for analyzing total N concentration and atom% ^{15}N. Immediately after the soil sampling, the cylinders were manually excavated along with the intact soil and plant materials and weighted. All plants were removed with intact root systems up to the depth of cylinder and the bulk soil was homogenized. Representative samples of 1 kg fresh soil were obtained from each cylinder using a Rifle-splitter and roots were recovered manually by washing roots on a 500-μm sieve. The roots with intact plants were sorted into different species, and those that could not be identified were collected and bagged separately. The samples were dried at 80 °C for 24 h and weighed. The background soil and root atom% ^{15}N was estimated in the samples collected in the same plots but at a minimum 50 cm from the cylinder. The root and soil samples were separately ground to a fine powder and packed into tin capsules. All the above-ground plant, root and soil samples were analyzed for total N and atom% ^{15}N at UC Davis Stable Isotope Facility, University of California, USA on an ANCA-SL Elemental Analyzer coupled to a 20-20 Mass Spectrometer using the Dumas dry-combustion method.

Calculations

The ^{15}N was applied to clover plants as multiple pulses. The N transfer from ^{15}N-labeled clover to unlabeled grass and forbs shoots was estimated based on differences in atom % ^{15}N excess and N content in above-ground plant tissues of clover and grass or forb species. The principle assumption was that the loss of ^{15}N in the soil and plant system would be minimal, and the ^{15}N assimilated by the legume would label all the N compounds subjected to transfer to companion non-legume species (Ledgard et al. 1985). The proportion of N transferred was estimated based on the assumption that clover N at the beginning of the growth period is equal to the ^{15}N measured in clover plus the ^{15}N transferred to receiving plants at harvest using the following equation in Ledgard et al. (1985) and modified in Pirhofer-Walzl et al. (2012):

$$\%\mathrm{Ndfd} = \frac{\mathrm{N}_{\mathrm{receiver}(1\text{-}2)} \times \mathrm{Excess\ \ atom}\%^{15}\mathrm{N}_{\mathrm{receiver}(1-2)}}{\left(\mathrm{N}_{\mathrm{receiver}(1-2)} \times \mathrm{Excess\ \ atom}\%^{15}\mathrm{N}_{\mathrm{receiver}(1-2)} + \left(\mathrm{N}_{\mathrm{donor}} \times \mathrm{Excess\ \ atom}\%^{15}\mathrm{N}_{\mathrm{donor}}\right)\right.} \times 100$$

where %Ndfd denotes the percentage of red clover N transferred to grass and forb plants, $\mathrm{N}_{\mathrm{receiver}}$ and $\mathrm{N}_{\mathrm{donor}}$ are amounts of N accumulated in the above-ground tissue of unlabeled grass or forb species (receivers) and ^{15}N-labeled clover (donor), respectively. Receiver (1–2) denotes two non-legume species grass and forbs. Excess atom % $^{15}N_{receiver}$ and $^{15}N_{donor}$ are the atom % ^{15}N of grass or forbs and clover grown inside the cylinder minus the atom % ^{15}N in samples of the corresponding plant species grown adjacent to the cylinder. The background atom % ^{15}N measured in clover was not affected ($P > 0.05$) by cutting time, and that measured in non-legume species was also unaffected ($P > 0.05$) by both cutting time and plant species. Therefore, the average values from the four cutting times were used as background atom % ^{15}N, which were 0.3664 and 0.3678 for clover and all non-legumes, respectively. Then the amount of legume N transfer was calculated by multiplying %Ndfd with the N accumulated in clover. Similarly, the weighted mean of %Ndfd and N accumulation in clover over four cuts were used to express the total amount of N transferred over the season.

The N deposited to the soil was estimated based on excess atom % ^{15}N in clover roots and soil using the following equation proposed by (Janzen and Bruinsma 1989), assuming that the sampled clover roots and deposited N have the same excess atom% ^{15}N and the ^{15}N tracer is uniformly distributed throughout the root system (Mayer et al. 2003):

$$\%\mathrm{Ndfr} = \frac{\text{Excess atom } \%^{15}\mathrm{N_{soil}}}{\text{Excess atom } \%^{15}\mathrm{N_{root}}} \times 100$$

where %Ndfr denotes the percentage of N in the soil derived from roots, and the excess atom % ^{15}N of soil and roots is calculated as the atom % ^{15}N in roots and soil in the cylinder minus the atom % ^{15}N measured in soil and respective plant roots adjacent to the cylinder. The clover roots were not separated from all recovered roots in the cylinder. Hence, the excess atom % ^{15}N of clover roots analyzed in separated fractions was used for the calculation. The average atom % ^{15}N in clover roots and soil measured in six plots was used as background, at 0.3665 for clover and 0.3689 for soil. The amount of N deposited was quantified as the product of %Ndfr and soil N content. The soil atom % ^{15}N was measured in two different soil profiles, 0–10 cm and 10–30 cm. Therefore, the below-ground measurements including soil N content and amount of rhizodeposits were made up to the depth of cylinder assuming one third of total soil from the upper and two thirds from the lower soil profile. Then the weighted average, based on total rhizodeposits and N content in both soil profiles, was calculated to express %Ndfr in each cylinder. The root density was calculated as the ratio of total root DM to the total soil dry weight in each cylinder and expressed as g root kg^{-1} dry soil.

Statistical analyses

Data were analyzed using the open source statistical program R (R Core Team 2014) (version 3.1.0). The normal distribution of residuals was tested and, when required, improved by log transformation of data. One-way analysis of variance was used to statistically test the effect of seed mixture on each dependent variable (biomass yield, N accumulation, excess atom% ^{15}N, and proportion and amount of N transferred from clover to non-legumes and soil). The effect of seed mixture and soil depth on soil excess atom% ^{15}N were tested using two-way analysis of variance. The effect of cutting time on DM yield, N accumulation, excess atom% ^{15}N, %Ndfd and amount of N transferred was analyzed using a linear mixed model, where seed mixture (fixed effect) and cutting time (repeated fixed effect) were independent variables and cylinders as random effect. Pairwise comparisons were made between least square means using the adjusted Tukey method. The confidence level used was 0.95, and the probability of rejection of hypothesis was set at P 0.05. Correlations were tested using Pearson's correlation analysis.

Results

Above-ground DM yield, botanical composition and N accumulation

The above-ground DM yield was significantly higher in the first and third cuts compared to the second and fourth cuts ($P < 0.001$), and was highest in the clover-dominated seed mixtures with chicory or plantain (Fig. 2a).

The DM yield of mixtures was mainly dominated by red clover even when grass was the main component in the seed mixture (Fig. 2a). The clover produced the highest DM yields ($P < 0.001$) in the first and third cuts, while the grass DM yield was highest in the first cut ($P < 0.001$) and decreased in succeeding cuts. The grass proportion was particularly suppressed when grown in mixtures containing chicory and plantain (Fig. 2a). The

DM yield between forb species was not significantly affected by the seeding proportions of grass and clover. Chicory and plantain constituted larger proportions of the total biomass production than grass, which was more pronounced in grass dominated seed mixture. However, the caraway always had a significantly lower DM yield ($P < 0.001$) than chicory and plantain (Fig. 2a). There was a negative correlation between total seasonal DM yield of forbs and grass ($P < 0.001$, $R^2 = 0.45$).

On an annual basis red clover, ryegrass and forbs yielded between 19 and 46, 3.4 and 6.7, and 1.3 and 9 g N m^{-2}, respectively. The pattern of N yield and proportions in the mixture closely resembled the pattern of DM production (Fig. 2b). The clover in different mixtures constituted on average 72 % of the total seasonal N accumulation, ranging from 76 to 83 % in clover-dominated and from 57 to 75 % in grass-dominated seed mixtures (Fig. 2b).

Above-ground ^{15}N-enrichment

The atom % ^{15}N in both clover and companion non-legume species was substantially above the natural abundance, ranging from 0.37 to 2.5 depending on plant species and cutting times, which revealed that the labeling was successful and that some of the clover N was transferred to the companion non-legume species. The excess atom % ^{15}N measured in all species was comparatively low in the first cut, ranging from 0.0031 to 0.4611, but generally increased in later cuts when DM yield was low.

Clover ^{15}N-enrichment and N transfer per cut

The average excess atom % ^{15}N in red clover varied between 0.0686 and 0.9332 depending on cut and mixture (Fig. 3a). The clover was more enriched in the grass-dominated seed mixtures than the clover-dominated mixtures, but clover ^{15}N-enrichment was not affected by the species of forb used in the seed mixtures. A correlation analysis of weighted atom % ^{15}N from all four cuts with the respective total seasonal DM ($P < 0.001$, $R^2 = 0.46$) and N yield ($P < 0.001$, $R^2 = 0.49$) showed a negative relation in clover.

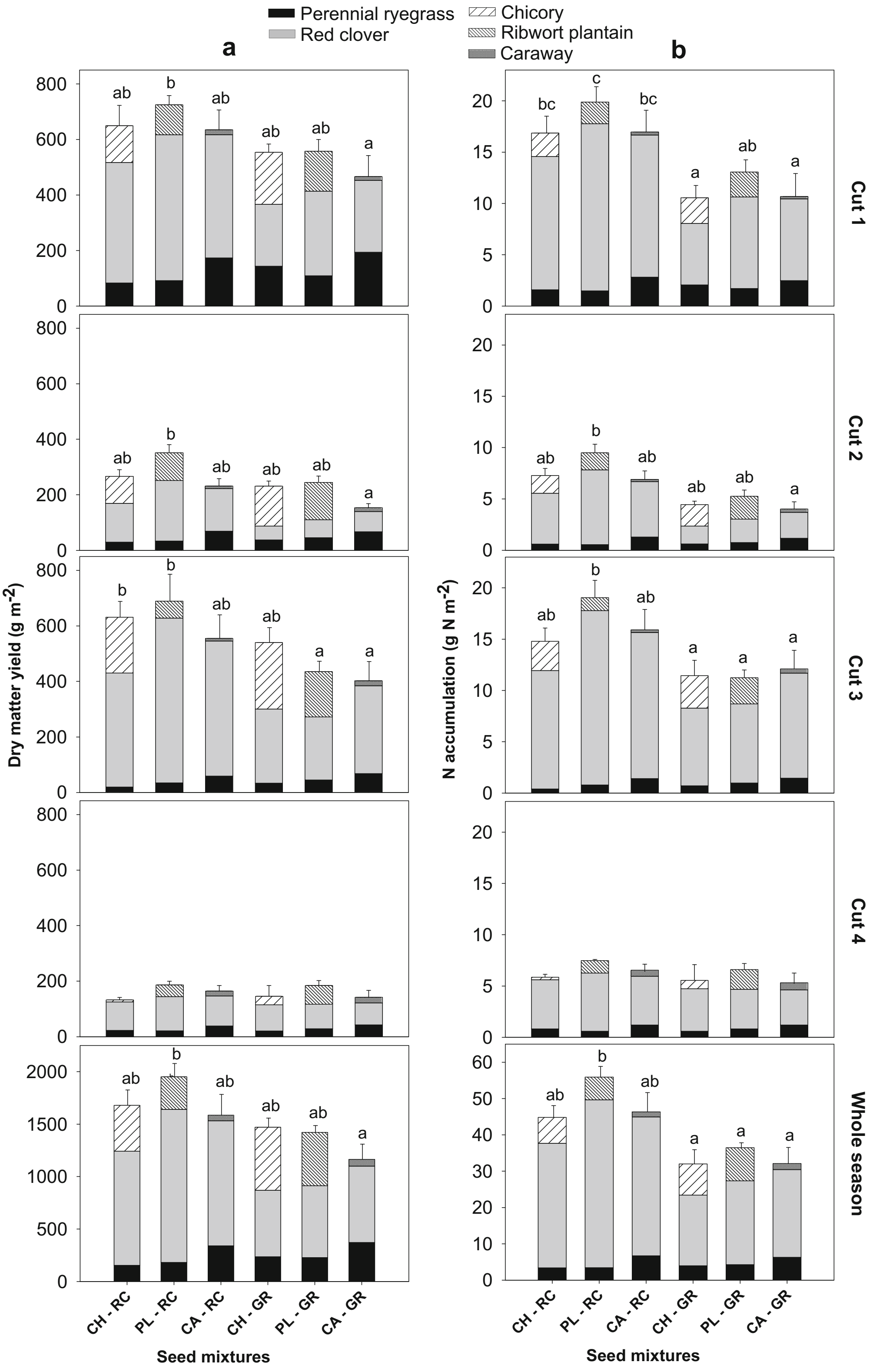
The proportion of clover N transferred (%Ndfd) to companion non-legume species varied between 7 and 33 %, depending on time of cut and mixture (Fig. 3b), but without interactions. The amount of clover N transferred to grass and forbs at different cutting times ranged between 0.34 and 1.98 g N m^{-2}, with no interactions between mixture and time of cut (Fig. 3c). Unlike %Ndfd, the amount of the N transfer was found to be closely influenced by N accumulation in clover. However, neither the proportion nor the amount of clover N transferred was significantly affected by seed mixture.

^{15}N-enrichment and N transfer in grass and forbs

Grass had a greater ability to absorb N from clover compared to forbs (Fig. 4), since the excess atom % ^{15}N in forbs was consistently lower ($P < 0.001$) than in grass (data not shown). The average excess atom % ^{15}N in grass varied between 0.0357 and 1.0398 at the different cuts and mixtures, whereas in forbs it ranged between 0.01 and 0.07. The excess atom % ^{15}N in grass was generally highest in grass-dominated mixtures, and lower when grown with caraway. The excess atom% ^{15}N in forbs was not affected by either species or the seeding proportions of the grass and clover measured in all four cuts.

The proportion of clover N (%Ndfd) transferred to grass was highest at the second cut ($P < 0.001$), but there was no effect of seed mixture at any of the cuts. The amount of N transferred was generally not affected by either time of cut or by mixture (data not shown). The N transferred to forbs varied depending on mixture and cut, with significant interaction ($P < 0.001$). The N transferred was comparatively low at the first and second cuts, and increased significantly at the third and fourth cuts in chicory and at the third cut in plantain ($P < 0.001$). Chicory and plantain took up significantly more of the N transferred from clover than caraway ($P < 0.001$) but the N transferred from clover to the forbs was not affected by the different seeding proportions of grass and clover.

Fig. 2 Above-ground dry matter (**a**) and Nitrogen (**b**) yield of red clover, perennial ryegrass and non-legume forb mixtures grown in PVC cylinders in the field. Values are mean (± SE, $n = 4$) measured at four cuts during the 2014 growing season. Different letters indicate statistically significant differences at the 0.05 level. Absence of letter above the bars means that there was no significant difference between the treatments. CH: chicory, PL: ribwort plantain, CA: caraway. RC: clover-dominated seed mixture, GR: grass-dominated seed mixture ▶


Perennial ryegrass
Red clover
Chicory
Ribwort plantain
Caraway
a
b
Dry matter yield (g m^{-2})
N accumulation (g N m^{-2})
Cut 1
Cut 2
Cut 3
Cut 4
Whole season
0
200
400
600
800
0
500
1000
1500
2000
0
5
10
15
20
0
10
20
30
40
50
60
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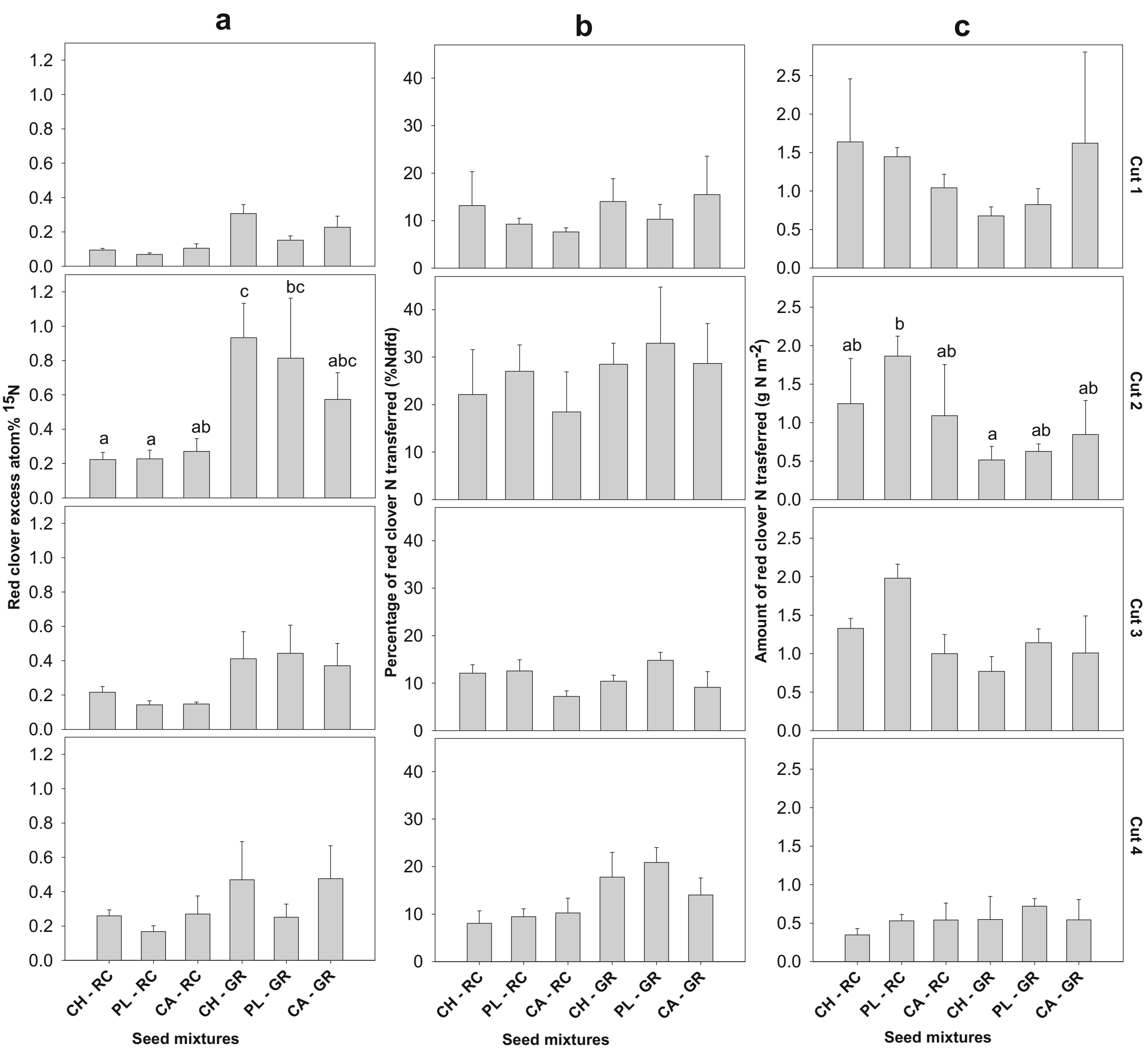

Fig. 3 Excess atom fractions ^{15}N in red clover (**a**), and percentage (**b**) and amount of red clover N transferred (**c**) to perennial ryegrass and non-legume forb species: chicory, ribwort plantain and caraway grown in PVC cylinders in the field. Values are mean (± SE, $n = 4$) measured at four cuts during the 2014 growing season. Different letters indicate statistically significant differences at the 0.05 level. Absence of letter above the bars means that there was no significant difference between the treatments. CH: chicory, PL: ribwort plantain, CA: caraway. RC: clover-dominated seed mixture, GR: grass-dominated seed

Seasonal %Ndfd and N transfer

On an annual basis, 9.5 to 15 % of clover N, equivalent to 2.5 to 5.8 g N m^{-2}, was found to be transferred to grass and forbs (Fig. 5a and b). The %Ndfd generally increased with increasing non-legume N accumulation, while the amount of N transferred correlated positively with clover N accumulation ($P < 0.05$, $R^2 = 0.35$). However, neither the proportion nor the amount of total clover N transferred was affected by the seed mixture (Fig. 5a and b).

Of the total amount of N transferred from clover to non-legumes, grass received a very high proportion, ranging from 63 to 94 %. This transfer was unaffected by grass and clover seeding proportions and the identity of the forb species present in the mixture. The total seasonal N transfer estimated in forbs ranged from 0.24 to 1.33 g N m^{-2}, equivalent to 0.6 to 4 % of clover

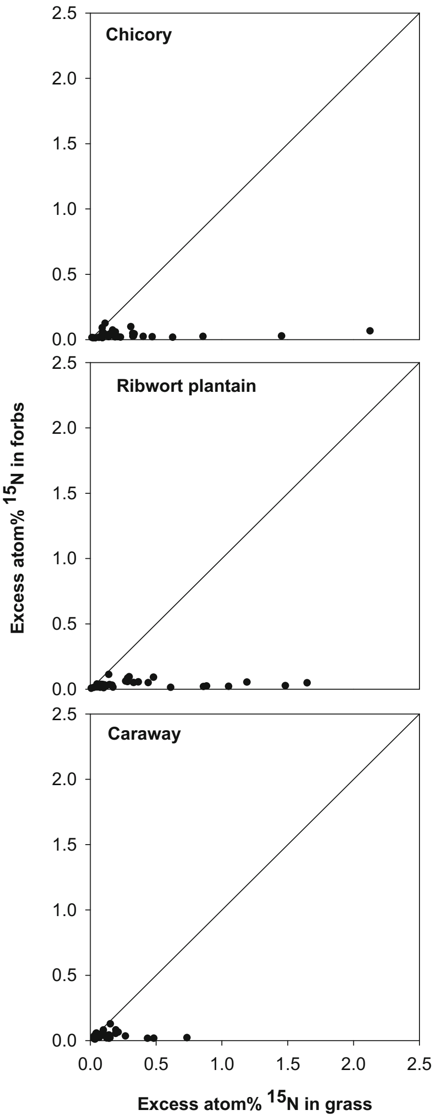

Fig. 4 Excess atom% ^{15}N in above ground plant parts of perennial ryegrass and non-legume forb species: chicory, ribwort plantain and caraway grown in PVC cylinder in the field. Values are measured at four cutting times during a growing season in 2014

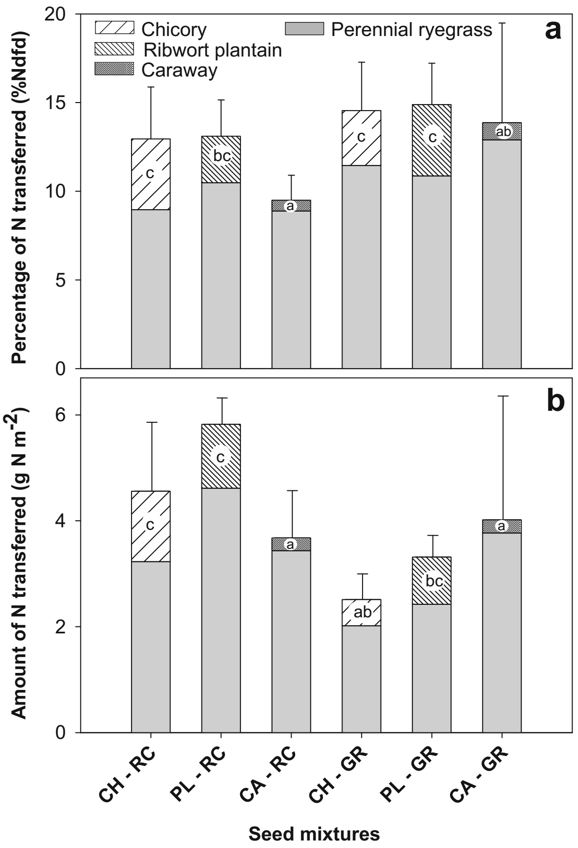

Fig. 5 Total seasonal estimation of percentage (**a**) and amount (**b**) of red clover N transferred to perennial ryegrass and non-legume forb species: chicory, ribwort plantain and caraway grown in PVC cylinders in the field. The %Ndfd is weighted mean from four cuts (± SE, $n = 4$) and amount is the sum of N transfer measured in four cut (± SE, $n = 4$) during the 2014 growing season. Different letters indicate statistically significant differences between the non-legume forbs at the 0.05 level Absence of letter above the bars means that there was no significant difference between the treatments. CH: chicory, PL: ribwort plantain, CA: caraway. RC: clover-dominated seed mixture, GR: grass-dominated seed mixture

N. The proportions of clover N transferred to chicory and plantain was significantly higher ($P < 0.001$) than to caraway (Fig. 5a).

Below-ground DM and N yield

Total root DM and N accumulation varied between 252 and 352 g DM m^{-2}, and 4.5 and 7.6 g N m^{-2}, respectively, with the tendency towards higher yields in clover dominated seed mixtures. However, there was no significant effect of seed mixture for neither DM nor N yields. There was a positive correlation between total root DM and total seasonal shoot DM yield ($P < 0.001$ and $R^2 = 0.52$), and total root N accumulation and total seasonal shoot N accumulation ($P < 0.001$ and $R^2 = 0.66$). Similarly, the estimated total seasonal N transfer in shoots was positively correlated with root DM yield ($P < 0.01$, $R^2 = 0.29$), root N accumulation ($P < 0.001$, $R^2 = 0.4$) and root density ($P < 0.01$, $R^2 = 0.34$).

^{15}N-enrichment in clover roots, %Ndfr and amount of rhizodeposition

The excess atom % ^{15}N measured in clover roots ranged from 0.0474 to 0.1674 (Fig. 6a) and was strongly correlated to the excess atom % ^{15}N in clover shoots ($P < 0.001$ and $R^2 = 0.7$). Similar to shoots ^{15}N, the excess atom% ^{15}N in roots was also negatively correlated ($P < 0.001$, $R^2 = 0.48$) to clover shoot N yield; and similar to shoots, forbs roots were less enriched than grass roots.

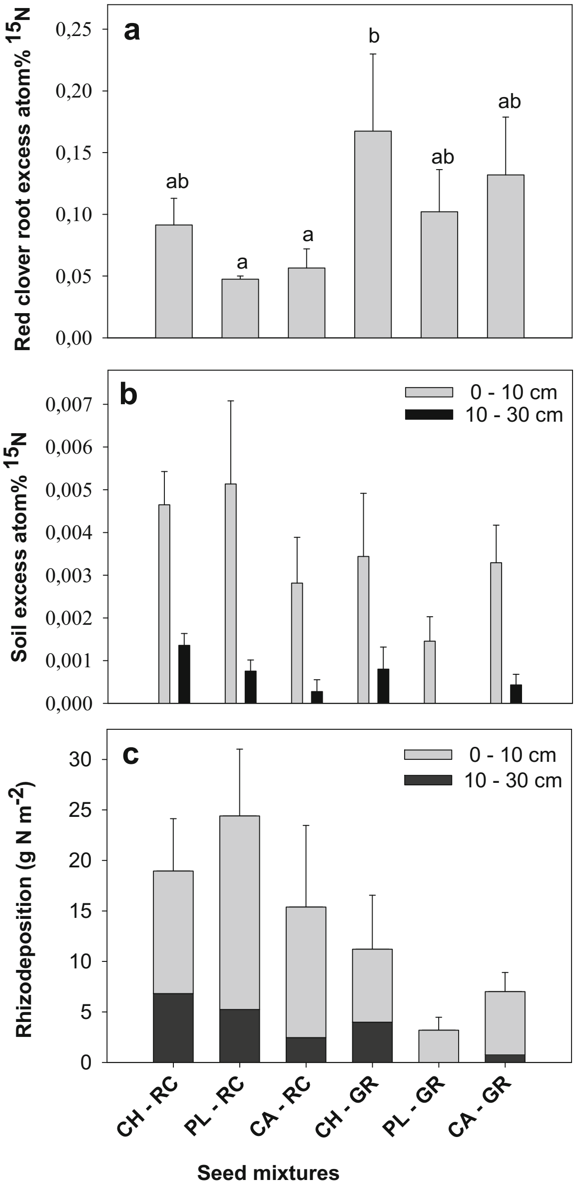

Fig. 6 Excess atom% ^{15}N in red clover roots (**a**), and percentage (**b**) and amount (**c**) of soil N deposited from red clover root grown in PVC cylinder in the field. Values are mean (± SE, $n = 4$) measured at the end of the 2014 growing season. Different letters indicate statistically significant differences at the 0.05 level. Absence of letter above the bars means that there was no significant difference between the treatments. CH: chicory, PL: ribwort plantain, CA: caraway. RC: clover-dominated seed mixture, GR: grass-dominated seed mixture

The excess atom % ^{15}N in soil was higher ($P < 0.05$) in the top 10 cm soil compared to the 10–30 cm layer, and in general more than 80 % of the rhizodeposition occurred in upper top 10 cm soil layer, but there was no significant difference between the mixtures (Fig. 6b). The proportion and amount of soil N deposited from clover roots varied widely between the mixtures, ranging from 0.56 to 4.3 % of soil N, equivalent to 3 to 24 g N m^{-2} (Fig. 6c). There was a correlation between the rhizodepsition and total root DM (P < 0.05, $R^2 =$ 0.22), root N accumulation ($P < 0.01$, $R^2 = 0.30$), and root density ($P < 0.05$, $R^2 = 0.16$). A correlation was also found between total seasonal N transfer measured in shoots and N deposited to the soil ($P < 0.05$ and $R^2 =$ 0.24).

Discussion

Critical reflection on methodology

In our study, nearly all the N accumulated in grass grown in a clover-dominated seed mixture with chicory and plantain was found to be transferred from clover. In some cases, it even exceeded the total amount of N accumulated in grass. This is of course not possible and clearly highlights an overestimation of N transfer (Chalk et al. 2014; Chalk and Smith 1997). In other studies on N transfer (e.g. Rasmussen et al. 2007, 2013 and Gylfadóttir et al. 2007) the proportions of grass in the mixture were close to 50 % or more. Since our system was strongly clover-dominated and the overestimation mainly occurred when the grass proportions were very low, it shows that the leaf-labeling ^{15}N-based method for estimating the proportion of non-legume N derived from transfer should be used with caution in swards heavily dominated by N-rich, N_2-fixing species. In addition, the method used to assess the N rhizodeposition does not meet the assumptions (Mayer et al. 2003) and question the accuracy of measurement. It confronts multiple bias caused by unrecovered root parts in soil, spatial and temporal variations in root ^{15}N enrichment and different ^{15}N enrichment in roots and rhizodeposits leading to overestimation of rhizodeposited N (Rasmussen 2011). Nevertheless, even though the possible methodological bias may have overestimated the quantities of N rhizodeposition and transfer, our results provide novel insights about the relative use of clover-derived N and soil N among ryegrass and the three studied forbs in an unfertilized organic system.

Growth dynamics of clover, grass and forbs

The swards were dominated by red clover irrespective of seeding proportions of red clover and perennial ryegrass. Hence, red clover defined the DM and N yield of the swards, with a strong tendency for greater DM and N yield in mixtures with high red clover seeding proportions. These observations reflect the strong ability of red clover to compete for available light and soil resources (Rasmussen et al. 2012) and the competitive advantage of the N_2-fixing legume over non-legume forage plant under unfertilized conditions (Carlsson and Huss-Danell 2003).

The growth dynamics among the non-legumes showed poor growth of perennial ryegrass as compared to chicory and plantain, even when it was the main component in the seeded mixtures. The significant negative correlation between forb and grass DM yield showed that chicory and plantain utilized the available resources more successfully than grass. One explanation could be the competition for light due to different plant architecture of grass and forbs (Søegaard et al. 2013) that both the chicory and plantain grow taller than ryegrass and may have a competitive advantage over grass for light interception. This explanation is supported by the fact that grass grew much better in mixtures with caraway, with a less dense canopy than chicory and plantain. It was in line with Søegaard et al. (2013), who found caraway to grow less during first and second year of ley establishment. Caraway is known to initially invest in its root system and increase its competitiveness with sward age (Hakala et al. 2009; Pirhofer-Walzl et al. 2012; Søegaard et al. 2013). Another explanation for the observed differences in grass-forb growth could be the individual species' ability to acquire N from companion red clover or from the soil. The greater N yield of chicory and plantain indicates that these two species may have been more efficient to acquire available N resources than grass. The ^{15}N-leaf-labeling of red clover made it possible to compare the uptake of red clover derived N in neighboring non-legume species and hence indirectly their ability to use soil N.

Grass and forbs acquisition of clover-derived N

Perennial ryegrass showed a strong ability to absorb red-clover-derived N as compared to the three forb species. The ability of grass to receive N from clover has been demonstrated in previous studies with mixtures of grass and chicory grown together with white clover, lucerne or birdsfoot trefoil (Høgh-Jensen et al. 2006), in four-species mixtures of two grasses, red clover and chicory or lucerne (Frankow-Lindberg and Dahlin 2013) and in multi-species mixtures of different grasses, forbs and forage legumes (Pirhofer-Walzl et al. 2012). The ability of the grasses to compete for clover N can be explained by a combination of the localization of red clover N deposits in the upper part of the soil profile and the previously documented presence of dense grass roots in this layer (Frankow-Lindberg and Dahlin 2013; Pirhofer-Walzl et al. 2012), where our data on soil ^{15}N-excess showed that red clover predominantly deposited N in the uppermost soil layer (0–10 cm). Similar observations on the process of clover N deposition and subsequent grass acquisition of N in the upper soil layer have been reported by Frankow-Lindberg and Dahlin (2013). The fibrous root system of perennial ryegrass may have facilitated the close intermingling with neighboring clover roots and favored absorption of the clover-derived N, as observed in faba bean-wheat intercropping by Xiao et al. (2004). In contrast, the forbs may be more adapted than grasses to acquire N in the lower soil profile due to their deep roots (Eriksen et al. 2012; Li and Kemp 2005; Søegaard et al. 2013; Stewart 1996). The root structure of forbs limits the intermingling with red clover roots and thereby reduces the forbs' capacity to acquire clover-derived N in the upper soil layer. In addition, as suggested by Rasmussen et al. (2013), the horizontal root distribution may play a more important role than rooting depth for interspecies N transfer. This suggests that root architecture is one of the most important factors influencing the competition between grass and forbs for clover-derived N.

The excess atom % ^{15}N in the three forbs demonstrated similar abilities to absorb the N transferred from clover, with the amount of N received from clover closely related to the total N yield of each of the forbs. Hence, forbs must have relied on soil N to a much greater extent than clover-derived N, and vice versa for grass. Frankow-Lindberg and Dahlin (2013) also observed that forbs rely more on soil N and compete less with the grasses for clover-derived N in upper soil horizon. In the present study, one explanation could be a double N-sparring effect, where the N_2-fixing clover allowed companion non-legume species greater access to soil N, and the grass was capable to take up clover-derived N in the upper soil layer. Hence we could have niche complementarity between grass and forbs in terms of N source used than instead of that spatial niche complementary with depth (Hoekstra et al. 2015). Another explanation, however, could be that the forbs, especially chicory and plantain, in the present experiment established rapidly and competed more successfully for soil N in all soil layers forcing grass to seek the clover-derived N. In contrast to Daudin and Sierra (2008), despite a strong ability to receive N from clover, the poor growth of the grass in our study indicated either a higher energy investment in the take-up of clover-derived N or that the availability of clover-derived N was not enough to support the grass growth under the conditions of the study. Thus, our results demonstrated that a strong reliance on N transfer from companion N-rich species does not always give the receiving species an advantage in terms of plant growth and productivity.

Red clover contributions to the N nutrition of companion species; correlations with above- and below-ground parameters

There are two ways of looking at the clover N transfer to companion species: the proportions (%Ndfd) and the amounts transferred. We observed a general trend towards a higher %Ndfd with higher proportions of non-legume DM and N yield in the mixture, which was in line with our first hypothesis. This indicates that the studied non-legumes, especially grass have a higher capacity to take up clover-derived N compared to clover itself, thus allowing clover less chance to re-assimilate deposited N, and suggested that the growth of non-legumes in the mixture play an important role for legume N transfer. The observed tendency could have been even stronger if the forbs, especially chicory and plantain, had a higher ability to absorb clover-derived N. The amount of N transferred from clover was mainly determined by the DM and N accumulated in clover shoots, which was also reflected in higher soil ^{15}N-enrichment and root N accumulation under high clover seeding proportions. As discussed in Rasmussen et al. (2013) and (2007), this highlights the importance of the growth of the donating legumes in the mixture for understanding the N transfer source and sink dynamics. Thus, our study suggests that the extent of N transfer is influenced both by the ability of non-legumes to acquire clover-derived N and by the ability of both legume and non-legume plants for DM production and N accumulation.

We observed temporal dynamics in the N transfer, with low levels in the first cut, highest levels at second cut, and an increase in N transfer to forbs at the third and fourth cuts. Despite higher DM and N yields in both clover and non-legumes, we observed the lowest proportions and amounts of N transferred at the first cut. This could be because of clover investing N in above-ground growth or it could be a methodological issue, where ^{15}N from the first labeling event did only represent short-term routes of N transfer, whereas at later cuts the ^{15}N from the early labeling event would be available for more long-term deposition routes, e.g. fine root turnover (Haystead and Marriott 1979) or leaf die off (Dahlin and Stenberg 2010). At the second cut, the high N transfer could be due to low clover proportions relative to grass, especially for the high seeding proportions of grass. N transfer to forbs increased in the last two cuts, especially the third cut, indicating an increased ability of forbs to acquire clover-derived N later in the growing season. The reasons for this may be twofold: that their roots were better established and functioned later in the season and that chicory and plantain peaked in DM production at the third cut and caraway at the fourth cut, which, combined with a low proportion of grass late in the season allowed forbs greater access to clover-derived N than at first and second cut.

We found that root DM and N at the end of the growing season correlated well with total shoot DM and N yield, with total seasonal N transfer measured in shoots, and with N deposited to soil, which support our second hypothesis. These tendencies towards higher root DM and N in clover-dominated mixtures show that above-ground DM yield is an indicator of root biomass. The correlation between ^{15}N in clover shoot and root and between ^{15}N in root and total N transfer underlined that the internal allocation of N in clover was a key factor controlling N transfer, in line with the findings of Rasmussen et al. (2007). Furthermore, the internal ^{15}N allocation in clover was linked to the soil excess atom% ^{15}N and total N deposition.

The amount of rhizodeposition estimated in the present study was higher than found by Gylfadóttir et al. (2007) but lower than in the study by Høgh-Jensen and Schjoerring (2001). In addition to the potential methodological bias when estimating rhizodeposition, as discussed above, the results could also have been influenced by differences in plant species and their relative proportions in the mixture, as soil ^{15}N enrichment and rhizodeposition was observed to be higher in clover-dominated mixtures and our system had a higher proportion of clover DM than in the study by Gylfadottir and colleagues. As pointed out by Rasmussen (2011), total N deposition may also be overestimated due to uneven temporal and spatial distribution of ^{15}N in sampled roots, or due to unrecovered roots in the soil samples (Gardener et al. 2012) as soil ^{15}N enrichment and rhizodeposition was observed higher in clover dominated mixtures and in upper soil layear between 0 and 10 cm.

Conclusions

Our study confirmed the results of previous studies on the ability of grass to absorb N transferred from neighboring legume plants, and provided new knowledge on the N competition between perennial ryegrass and three deep-rooted non-legume forb species: chicory, ribwort plantain and caraway.

Forbs differed in their biomass production and N accumulation, chicory and plantain had stronger growth than caraway, but showed no differences in their ability to absorb clover-derived N. Hence, our study demonstrated that forbs in grassland did not rely on N transferred from red clover and that this did not limit their growth, revealing that the forbs mainly acquired N from the soil pool. Despite the strong ability of grass to absorb N transferred from red clover, its low biomass production when in mixture with chicory and plantain demonstrated that a high reliance on N transfer does not necessarily give the species an advantage for growth and resource utilization including N acquisition from the soil. The dynamics of N transfer were positively influenced by root biomass and N accumulation in red clover, which confirms the importance of below-ground productivity in plant-soil systems. Hence, our study enables the disentangling of temporal and spatial dynamics of N transfer from forage legume to non-legumes and improve the knowledge on how to design and manage the multi-species temporary grassland including grass-clover-forb mixture. To increase our understanding of grassland N transfer dynamics future studies need to include both legume and non-legume behavior in relation to e.g. N deposition and root growth.

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8.3 Paper III

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Inclusion of non-leguminous forbs in grass-clover mixtures does not affect soil N fertility for the subsequent cereal crop

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Running title: Grassland residual N effect

Abstract

Increasing food production with minimum use of N fertilizer and environmental impact is a challenge. Temporary legume-based grasslands in crop rotations improve soil N fertility and substitute N fertilizer in both the grassland and arable crop phase. Here, we determined residual soil N fertility of grassland pre-crops containing clover, grass and non-legume forage herbs (forbs) and covering a large range in N_2-fixation inputs. Two measures of potentially available N, soil inorganic N and mineralizable organic N, were determined along with spring barley N uptake in a pot experiment using soil collected from the temporary grasslands. We estimated the fertilizer replacement value of the grassland mixtures by comparing barley N uptake to a neighboring soil with a history of cereal cropping. The residual soil N fertility was surprisingly similar across the pre-crops of red clover pure stand, grass-clover, and grass-clover-forb mixtures. However, biomass production and N uptake in spring barley was significantly higher following the grassland mixtures than reference soil with previous cereal cropping, corresponding to a fertiliser replacement value of about 100 kg N ha^{-1}. In conclusion, variation in N input from N_2-fixation and replacement of the grass with strong forb species in the preceding grass phase did not affect the residual soil N fertility in the subsequent cereal crop. Forbs that enhance mineral nutrition, forage quality and biodiversity can therefore be included in the grassland mixtures without negative effects on short- term soil N fertility.

Key words: grassland; residual effect, chicory, ribwort plantain, caraway

Introduction

Nitrogen (N) is one of the important yield determining elements of plant nutrition. Increasing human population and demand of food production without expanding arable land has been increasing the global demand of N resources in agriculture, which is mainly supplemented by industrial N fertilizer (Erisman et al., 2011). However, the efficiency of applied N resources, both mineral and organic, in agricultural production is often low (Baligar et al., 2001; Paustian et al., 2016) and carries the risk of several environmental problems (Fowler et al., 2013). Therefore, efficient use of N resources is essential to enhance sustainable N use in arable crop production system with minimum negative environmental impacts.

Legume based grasslands are widely considered an important mean of improving soil N fertility and plant productivity. In grassland-arable crop rotations, build-up of soil N pool and crop residues in the grassland phase provides an important input of N to the arable phase (Rasmussen et al., 2012; Bedoin, 2013, Christensen et al., 2009). During the grassland phase, forage legumes increases the soil N pool through the process of biological N_2-fixation (Rasmussen et al., 2012; Dhamala et al. *submitted*) and rhizodeposition (Høgh-Jensen & Schjoerring, 2001; Rasmussen et al., 2007). The part of legume derived N left in plant residues or immobilized in soil organic matter provides an additional soil N pool to the subsequent crops when decomposed (Vertès et al., 2007; Eriksen et al., 2008; Yang et al., 2010). Multiple studies have suggested that the residual N effect is highly influenced by cropping history and applied management practices such as cutting, grazing, ploughing and fertilization together with age and species composition of the grassland pre-crop (Høgh-Jensen & Schjoerring, 1997; Hansen et al., 2005; Eriksen et al., 2008; Bedoin, 2013). The soil N buildup depends on grassland production, legume content, N_2-fixation and total N incorporated in the soil during grassland phase (Høgh-Jensen & Schjoerring, 1997; Peoples et al., 2009; Vrignon-Brenas et al., 2016). Variations in the build-up and decomposition of the soil N pool in grasslands are also caused by differences in residue quality, depending on species composition (Wichern et al., 2008; Louarn et al., 2015).

Residual soil N fertility effects of grassland pre-crops have been widely studied with respect to forage legumes (Kumar & Goh, 2000; Kumar et al., 2001; Askegaard & Eriksen, 2008), grasses (Høgh-Jensen & Schjoerring, 1997; Kumar et al., 2001; Askegaard & Eriksen, 2008) and mixtures of forage legume and grasses (Høgh-Jensen & Schjoerring, 1997; Eriksen, 2001; Eriksen et al., 2008; Nykänen et al., 2008; Rasmussen et al., 2012). However, the residual soil N fertility effects of non-legume forage herbs (forbs) has not been studied. Chicory (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.) and caraway (*Carum carvi* L.) are three promising forb species for inclusion in grassland mixtures. The forbs are adopted in grasslands for plant diversity and herbage production (Søegaard et al., 2011; Dhamala et al., 2015), forage quality (Søegaard et al., 2008), mineral nutrition (Pirhofer-Walzl et al., 2011), and N utilization from deeper soil layers (Thorup-Kristensen, 2006; Pirhofer-Walzl et al., 2013). The different plant architecture of the forbs with deep and diverse root systems (Stewart, 1996; Li & Kemp, 2005) could affect the buildup of N under the grass sward and the quality of the residues left upon grassland termination. In this study, we investigated the residual N effect of grassland mixtures containing different forbs at varying proportions along with clover and grass. The following hypotheses were tested:

- The main driver for residual N effect is the content of red clover in the pre-crop grassland, and therefore mixtures with higher proportions of red clover will have a greater N input via N_2-fixation and build-up of soil N as compared to mixtures with lower proportions of red clover, and
- The inclusion of forbs in the pre-crop grassland will affect the quality of plant residues left in the soil upon termination of the grassland and therefore, the presence of different plant species will affect the release of N to a subsequent cereal crop.

Materials and methods

Experimental site and description of the grassland pre-crop

The soil for the experiment came from plots established in the organic dairy crop rotation long-term field experiment at Foulumgaard Experimental Station, Aarhus University, Central Jutland, Denmark (9°34′ E and 56° 29′ N). The soil is loamy sand characterized as a typic Hapludult containing 7.7% clay and 1.6% carbon (Eriksen et al., 2015). The pre-crop grassland mixtures, established in spring 2013, were composed of red clover (*Trifolium pratense* L., variety Rajah), perennial ryegrass (*Lolium perenne* L., variety Stefani), chicory (*Cichorium intybus* L., variety Spadona), ribwort plantain (*Plantago lanceolata* L., wild type) and caraway (*Carum carvi* L., variety Volhouden) in different seeding mixtures ranging from pure stands to five species (Table 1). Aboveground biomasses were harvested twice during the growing season (May to October) in 2013 and four times in 2014. In spring 2015, soil was sampled in selected grassland mixtures, including red clover pure stand and mixtures of varying species richness and composition (Table 1). Nearly 50 kg soil with plant residues was collected from the plough layer (20 cm) in each of the previous ^{15}N dilution plots. Plant residues were separated from the soil, cut into 0.5 cm pieces using a chopping machine and then mixed back into the soil from the respective samples.

In order to estimate the fertilizer replacement value of the dairy grassland based crop rotation a reference soil was taken from a neighboring field with a history of cereal production for at least the five years (since 2010) prior to sampling for the experiment. The cereal production soil had received N fertilizer ranging from 84 to 179 kg N ha^{-1} yr^{-1} in the preceding years in NPK fertilizer. About 750 kg reference soil was collected and prepared for the experiment as described above.

Characterization of available soil N pools

Three representative sub-samples were taken from each grassland soil as well as the reference soil. The sub-samples were used to determine the initial inorganic N concentration, water content and potentially mineralizable N.

Soil initial inorganic N

Initial inorganic N concentration in fresh soil was measured in a 100 g sub-sample, which was extracted in 200 ml of 1 M KCl and shaken for 1 hr at 30 rpm before filtration through GC-50 filter paper. The filtrates were analyzed for ammonium and nitrate concentration in the soil on a Technicon Auto-Analyzer III (Bran+Luebbe, Norderstedt, Germany). The dry matter content of the soil was determined by drying 20 g fresh soil at 105 °C for 24 hours and recording the dry weight.

Table 1 Species composition (proportion of the seed mixture), and seasonal red clover and total N yield, red clover percentage of total dry matter production, , total N input from fixation and N balance during the grassland pre-crop phase in 2014. Values are means (± SE; n = 3), with different letters within each column indicating a statistically significant ($p<0.05$) difference between the species compositions. The percentage and amount in the seed mixtures are based on the seeding rate of each species in a pure stand.

		2013					2014				
Seed mixtures		**Percentage of seed in the mixture sown in 2013**					**N yield (Kg N ha^{-1})**		**Red clover percent of total DM production**	**Total N fixation input**[1]	**N balance (kg N ha^{-1})**[2]
		GR	**RC**	**CH**	**CA**	**PL**	**Red clover**	**Total**			
Pure stand	Red clover		100				480±7^{c}	480±7	100±0^{c}	617±18^{b}	137±19^{c}
Two species	GR+RC	50	50				412±14^{bc}	468±16	82±1^{bc}	594±27^{b}	126±11^{c}
Three species	GR+RC+CH	20	20	60			164±33^{a}	283±34	45±6^{a}	246±50^{a}	-37±16^{ab}
	GR+RC+CA	20	20		60		298±57^{abc}	389±53	66±7^{ab}	450±85^{ab}	60±32^{bc}
	GR+RC+PL	20	20			60	253±49^{ab}	392±52	52±5^{a}	380±72^{ab}	-13±21^{ab}
Five species	GR+RC+60CCP	20	20	20	20	20	183±53^{a}	324±54	43±7^{a}	270±79^{a}	-54±26^{a}

GR: Perennial ryegrass, RC: Red clover, CH: Chicory, PL: Ribwort plantain, CA: Caraway, CCP: Chicory-Caraway-ribwort plantain.

[1] Total N input is the sum of N fixed in red clover shoot, roots and stubble including fixed N transferred to companion non-legume species and N immobilized in soil organic N pool calculated using an empirical module purposed by Høgh-Jensen et al. (2004).

[2] The N balance is the difference between total N input and removal by harvested aboveground biomass.

Potentially mineralizable N in anaerobic incubation

Potentially mineralizable N was estimated by an anaerobic incubation experiment (Waring & Bremner, 1964), modified by (Keeney, 1982). Seventy-five g of fresh soil sample was mixed with demineralized water in the ratio of 1:2.5 in a plastic bottle. The bottles were tightly closed and incubated at constant temperature (40 °C) for two weeks. Thereafter, ammonium was extracted with 4M KCL in a soil: KCl- ratio of 1:2.5 followed by shaking for 1 hr. and filtration through GC-50 filter paper. The filtrates were analyzed for the concentration of ammonium on a Technicon Auto-Analyzer III (Bran+Luebbe, Norderstedt, Germany). The potentially mineralizable N was estimated as the difference between the initial ammonium concentration and the concentration after two weeks of incubation.

Establishment of pot experiment with spring barley

A pot experiment was set up in rectangular pots (0.1 m^2 surface area, 31 L volume) with a 12 mm hole in the bottom and a PVC grid covered by a glass fiber mat placed on the bottom ensuring free drainage. Each pot was filled with 36 kg homogenized soil, from one of the grassland plots or reference soil, and randomly placed on metal frames installed in an outdoor experimental facility at Foulum, Aarhus University. Additionally, in order to produce an N response curve fifteen reference soil pots were prepared, placed randomly together with the pots with grassland soil and supplied with five different levels of N fertilisation: 0, 1, 2, 3, and 4 g N per pot, equivalent to 0, 100, 200, 300 and 400 kg total N ha^{-1}, in the form of $Ca(NO_3)_2$. The N fertilization was added 3.5 weeks after sowing the spring barley.

Spring barley *(Hordeum vulgare* L., variety evergreen*)* was sown in the last week of April (27 April) with 32 spring barley seeds per pot, at 5 cm soil depth. Upon germination, the spring barley was thinned to 26-28 seedlings in each pot. The pots were supplied with demineralized water to its water holding capacity in regular intervals. The drainage water was collected in individual reservoirs placed underneath each pot and recycled during the next watering. The pots received a basic fertilization of 0.25 g Mg, 0.025 g Mn and 0.05 g Cu, equivalent to 25, 2.5 and 0.5 kg ha^{-1} three weeks after sowing, followed by 0.5 g P, 1.75 g K, 0.2 g S and 0.001g Mo, equivalent to 50, 175, 20 and 0.1 kg ha^{-1}, at four weeks after sowing the spring barley, to ensure sufficient availability of all nutrients except N.

The pots were manually weeded every 3-5 days. Spring barley, especially in N fertilized reference pots, showed symptoms of fungal infection around the third week of June and therefore all pots were treated with the fungicides Proline EC 250 (0.2 lit ha^{-1}) and Comet Pro (0.15 lit ha^{-1}) on 2 July. The mean monthly air temperature during the pot experimental period from April to August 2015 were between 7 and 17 °C, with June, July and August being the warmest months.

Harvest and measurements of the spring barley

The spring barley was harvested at maturity in the last week of August (28 August 2015). The plants were dried at 60 °C for 48 hours and total DM yields were measured at constant weight. The grains were threshed and weighted. The grain and straw were separately milled to a fine powder, packed into tin capsules and analyzed for total N concentration and atom fraction ^{15}N at UC Davis Stable Isotope facility in University of California, USA on an ANCA-SL Elemental Analyzer couple to 20-20 Mass Spectrometer (Sercon Ltd., Cheshire, UK) using the Dumas dry-combustion method.

Calculations

The N balance in the grassland phase 2014 was estimated as the difference between N input and N output in the leys. The N input equaled N from red clover N_2-fixation, and the N output equaled the harvested N yield in forage legume and non-legume shoot biomass. The input of N from biological N_2-fixation was calculated using an empirical model (Høgh-Jensen et al., 2004) including the N fixed in legume shoots, roots and stubbles and additionally fixed N transferred to companion non-legume species and immobilized in the soil organic N pool.

The plant N uptake in above ground biomass was calculated as the product of above ground dry matter yield and N concentration. The N fertilizer replacement value of the grassland soils was calculated as the amount of N fertilizer required in reference soil to obtain the same spring barley N yield as spring barley grown in the grassland soil.

Data analysis

The results were analyzed in the open source statistical program R (Version 3.1.1) (R Core Team, 2016). The effect of species composition of grassland pre-crop on each of the dependent variables: soil initial inorganic N, potentially mineralizable N, biomass production and N uptake in spring barley, and N input and balance during grassland phase was tested using one-way analysis of variance (ANOVA). The comparisons between the seed mixtures were made with *least square means* using the adjusted Tukey method. The probability of rejection of hypothesis was tested at the confidence level 0.95 ($P<0.05$). Pearson's correlation analysis was used to test the correlation between the different dependent variables. The data violating the assumption of normality and homogeneity of variance were log transformed before analysis to minimize heteroscedasticity.

Results

N_2-fixation and N balance during the grassland phase

The grassland mixtures varied widely for the red clover proportion, input of N from N_2-fixation, N uptake in shoots and N balance depending on species composition and clover proportions in the seed mixtures (Table 1). The N_2-fixation ranged from 160 to 390 kg N ha^{-1}, total N input from 246 to 617 kg N ha^{-1} and N balance from -54 to 137 kg N ha^{-1}.

Initial N availability and potentially mineralizable N

The initial inorganic N concentration of the grassland soils ranged from 3.2 to 5.7 mg N kg^{-1} dry soil, with the concentration of nitrate being greater than ammonium (Figure 1a).

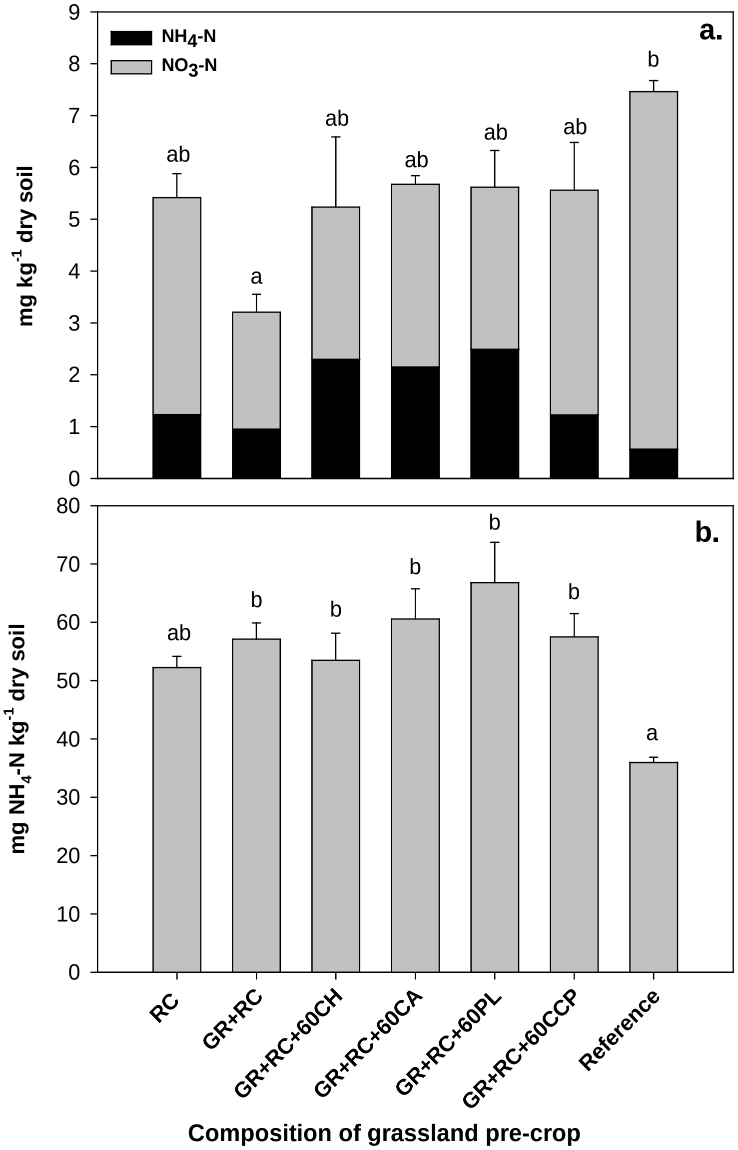

Fig. 1 Soil initial inorganic N concentration (a) and net N mineralized in anaerobic incubation (b) in the soil previously under different composition of grassland pre-crop measured at the beginning of the growing season at the third year of ley establishment in 2015. The values are means (±SE; n=3), with the bars following the different letters are significantly different at the level of 0.05. GR: perennial ryegrass, RC: red clover, CH: chicory, CA: Caraway, PL: ribwort plantain, CCP: Chicory-caraway-ribwort plantain

The mineral N concentration was generally lower in soils following the grassland mixtures than in the reference soil. However, no significant differences were seen in the inorganic N concentration across the six different grassland pre-crops.

The potentially mineralizable N was in general significantly higher in the soil following grasslands compared to the reference soil ($p<0.01$), with the N mineralized in the grassland soil ranging from 52 to 67 mg N kg^{-1} dry soil (Figure 1b). However, there was no significant differences in the potentially mineralizable N among the grassland pre-crops. A correlation analysis from the measurements in grassland soils showed no association between initial inorganic N concentration and N mineralized in anaerobic incubation ($R^2= 0.01$).

Spring barley dry matter and N yield

The N uptake in spring barley following the grassland mixtures ranged from 11 to 13 g N m^{-2}, which was predominately in grain (Figure 2b).

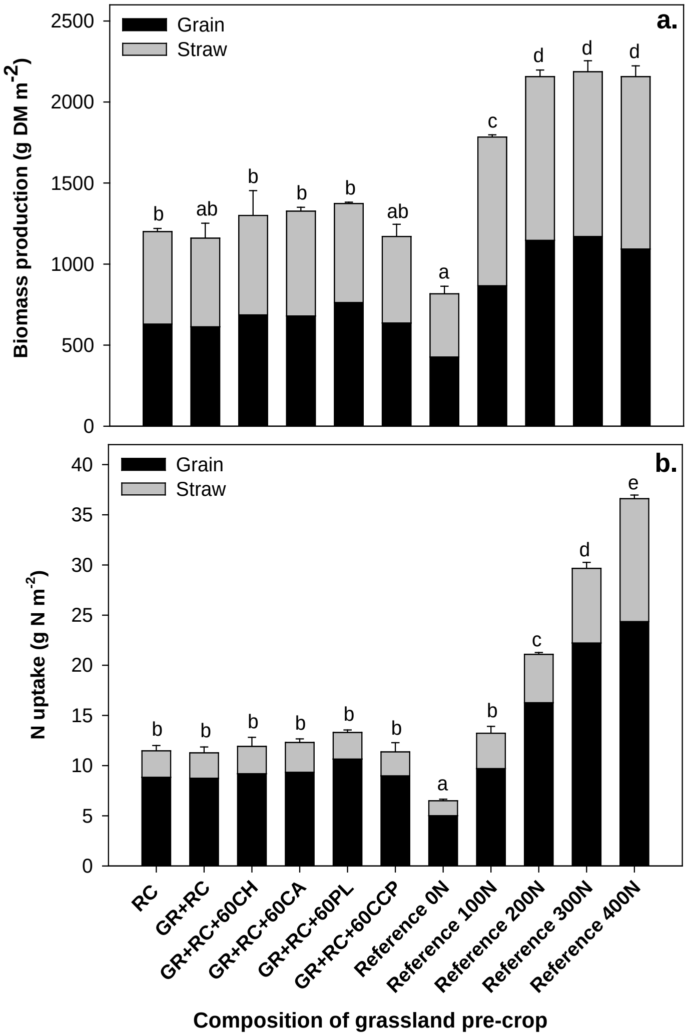

Fig. 2 Biomass mass production (a) and N uptake (b) in spring barely test crop grown in the soil previously under different composition of grassland pre-crop measured during the growing season at the third year of ley establishment in 2015 in pots in a semi-field experiment. Values are means (±SE; n=3), with the bars following the different letters are significantly different at the level of 0.05. GR: perennial ryegrass, RC: red clover, CH: chicory, CA: Caraway, PL: ribwort plantain and CCP: Mixture of Chicory, caraway and ribwort plantain. 0N, 100N, 200N, 300N, 400N indicates different levels of N fertilizer application, equivalent to 0, 100, 200, 300 and 400 kg total N ha^{-1}.

The total N uptake in spring barley following grassland pre-crops was significantly higher than N uptake in unfertilized reference soil ($p<0.001$) and similar to the reference soil fertilized with 100 kg total N ha^{-1}. However, N uptake was unaffected by the species composition of the grassland pre-crop. The increasing rate of N fertilization in the reference soil significantly increased N uptake of spring barley ($p<0.001$), with a significant linear association for both grain ($R^2=0.98$) and straw ($R^2=0.93$).

We observed no correlation between neither clover N_2-fixation or N yield, grassland total N yield nor the grassland N balance and the residual N effect as measured as initial inorganic N, N mineralized during anaerobic incubation, spring biomass production and N yield.

Discussion

In the present experiment, different species composition and the variation in red clover proportion, N_2-fixation, N yield, and total N input and N balance during the grassland phase did not affect the residual N effect for the subsequent spring barley crop. This is surprising since previous studies have shown that species composition of grassland pre-crop affects the residual soil N fertility of the succeeding crop the in rotation due to variations in input of N from N_2-fixation and total N incorporated in the soil (Høgh-Jensen & Schjoerring, 1997; Nykänen et al., 2008), and that pure legume pre-crops resulted in higher residual N fertility than legume-grass mixtures (Høgh-Jensen & Schjoerring, 1997; Kumar et al., 2001; Kumar & Goh, 2002; Askegaard & Eriksen, 2008). Further, Vertès et al. (2007) and Eriksen et al. (2008) suggested that residual N effect is influenced by the build-up of the C and N pools during the grassland phase, soil N losses, N mineralization from soil organic matter, together with the N demand of succeeding crop. Vrignon-Brenas et al. (2016) found that the biomass yield of preceding legumes correlate with the N gain in the following maize crop and suggests that the biomass of preceding legume is a good early indicator of N released to the subsequent crop in rotation. In the present experiment, red clover N yield, N_2-fixation and the N balance during the grassland phase differed significantly among the six forage mixtures (table 1). The estimates of red clover N yield from the pure stand of red clover and the two species grass-clover mixture was up to three times as high as from the mixtures containing forbs. These large differences would be expected to have larger effects on the measured parameters of residual soil N fertility than we observed.

The surprisingly similar residual N effect across the different grassland mixtures could be caused by a number of reasons. Firstly, the N balance for the pre-crop grassland phase is based on above-ground N yield data and a subsequent estimation of non-harvested N input using the empirical model of Høgh-Jensen *et al.* (2004). It may well be that there is not a fixed ratio between above-ground production and below-ground standing plant biomass and N build-up. In fact, a parallel study of N transfer and rhizodeposition in grasslands including forb containing mixtures showed no significant effect of grassland species composition on red clover rhizodeposition (Dhamala et al., 2017). Secondly, part of the N buildup during the grassland phase may have been lost via denitrification or leaching, with the leaching loss potentially being negatively correlated with the presence of non-legumes (Kušlienė et al., 2015) i.e. greater potential N leaching from the red clover pure stands and red clover dominated two-species mixtures as compared to the three- and five species mixtures. Thirdly, the input of C and N under the different mixtures may have differed in quality and quantity affecting the immobilization of N in soil, and then upon termination of the grassland also the release of N. A recent study shows that root biomass of grass-clover is increased by inclusion of caraway or plantain (Cong et al. *submitted*), and greater rates of root decomposition was found in soils previously cultivated by species-diverse mixtures (Hector et al. 2000; Cong et al., 2015) Finally, it could be that the active uptake period of the spring barley test crop was too short (mainly May and June) to detect any differences among the pre-crop mixtures. Eriksen et al. (2015) observed that the effect of different grassland managements (e.g. cut vs. grazing) was not reflected in the N uptake in the spring barley whole crop, but rater in the perennial ryegrass catch crop taking up N released after harvest of the spring barley. Therefore, the present study point to that the fertility effect of temporary grasslands should be seen at the cropping system level. This becomes clear when comparing the grassland mixtures with the reference soil from cereal based cropping system, where we observed that the long-term fertility buildup in the grassland based crop rotation corresponded to a fertilizer effect of about 100 kg N ha^{-1} in the spring barley test crop. The positive pre-crop effect is further highlighted by the fact that the grassland mixtures did not receive any N fertilizer, while the reference soil had been fertilized with up to 179kg N ha^{-1} yr^{-1} during the cereal phase.

Conclusions

In our study, variation in legume proportion and N input, and replacement of the grass component with forbs in preceding grassland phase did not change the residual soil N fertility in the subsequent spring barley test crop. However, we observed higher residual N effect following the cultivation of grasslands compared to unfertilized reference soil with a history of cereal production and N fertilization for at least for five years. We conclude the forbs can be included in the grassland mixtures for *e.g.* enhanced biodiversity and forage quality, without any negative effects on short-term soil N fertility for the succeeding crop. Future studies can be done to increase the understanding of the longer term residual N effect of grassland pre-crops including non-leguminous forbs.

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8.4 Paper IV

Status: Accepted

Journal: Plant and Soil

Highly productive forage legume stands show no positive biodiversity effect on yield and N_2-fixation

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Abstract

Background and aims: While N_2-fixation in diversified grasslands including forage legumes and non-legumes has been widely studied, N_2-fixation in swards containing only forage legumes remains unclear. In this study, we investigated N_2-fixation in pure stands and mixtures of three forage legumes.

Methodology: N_2-fixation, dry matter (DM) and nitrogen (N) yields were quantified in a field experiment for red clover (*Trifolium pratense* L.), white clover (*Trifolium repens* L.) and lucerne (*Medicago sativa* L.) pure stands and mixtures using the isotope dilution method.

Results: All three forage legume species derived most (around 85%) of their N from atmospheric N_2-fixation (%Ndfa). However, no positive effect of species diversity was found in any of the mixtures. Species composition of the forage legume mixtures affected the amount of N from N_2-fixation by affecting DM production and N accumulation of the species, where the seasonal amount of N_2-fixation ranged from 370 to 500 kg N ha^{-1}; which was highest in the presence of red clover.

Conclusions: We found that mixtures of the three forage legumes were highly productive, but did not show positive advantages compared to the red clover pure stands in terms of DM, N yield and %Ndfa.

Key words: grassland; forage legume mixture; percentage of N derived from atmosphere (%Ndfa); N yield

Introduction

The EU is nearly 30% self-sufficient in protein feed (Bouxin 2014), and there is an increasing demand for new homegrown sources of protein in the EU as alternatives to the import of soybean meal for livestock production. Nitrogen (N) is one of the most limiting crop nutrients, and to produce the required protein sources from crops requires large inputs of N. However, the efficiency of applied N resources is often low and such resources carry the risk of several environmental, economic and agronomic problems (Fowler et al. 2013). Hence, there is societal need to produce large amounts of plant protein without N fertilization or with more efficient use of available N resources. Compared to cropping systems containing only annual crops, grassland-based cropping systems that contain perennial legume-grass leys have shown more ecosystem benefits such as enhanced soil C and N sequestration, soil organic matter, soil fertility and biodiversity, reduced greenhouse gas emission and improved possibility to use marginal land for biomass prouduction (Tilman et al. 2006; Glover et al. 2010; Carlsson et al. 2017). In this context, forage legumes have the potential to produce N-rich plant biomass with no inputs of N fertilizer.

Forage legumes are widely used in agriculture as a valuable means of supplying protein-rich feed (Lüscher et al. 2014) and maintaining soil N fertility and plant productivity (Anglade et al. 2015). Forage legumes in cropping systems introduce atmospheric N_2 to the soil N pool through the process of biological N_2-fixation and improve the N supply to companion non-legume species (Fustec et al. 2010; Pirhofer-Walzl et al. 2012) and subsequent crops in the rotation (Eriksen et al. 2008; Rasmussen et al. 2012). However, multiple studies have reported wide spatial and temporal variations in legume N_2-fixation and contribution to soil N fertility (e.g. Carlsson and Huss-Danell 2003; Lüscher et al. 2014; Anglade et al. 2015). N_2-fixation is the result of internal and external factors such as legume species and genotype, their interaction with the environment, management practices such as cutting, grazing and fertilization, and plant species diversity and composition (Carlsson and Huss-Danell 2003; Carlsson et al. 2009; Rasmussen et al. 2012).

Numerous studies on N_2-fixation have shown that plant species diversity associating forage legumes with non-legumes in grasslands are some of the most efficient management factors that can be applied to increase legume reliance on N_2-fixation (e. g. Carlsson and Huss-Danell 2003; Carlsson et al. 2009; Nyfeler et al. 2011). The studies suggest that N_2-fixation is regulated by legume competition for available soil N, defined as the gap between N availability in the soil and the N demand of the plant species in the mixtures, where non-legume species compete for the available soil N forcing the legume species to acquire more N from biological N_2-fixation.

To date, the N dynamics in diversified grasslands with forage legume and forage grasses have been extensively studied. The dynamics of N_2-fixation in swards including only forage legume species remain poorly understood. In this new experiment, we investigated how each of the three forage legume species: red clover (RC; *Trifolium pratense* L.), white clover (WC; *Trifolium repens* L.) and lucerne (LU; *Medicago sativa* L.) in a sward influence the growth, N_2-fixation and N acquisition of the other legumes in the mixture.

These three forage legumes are widely cultivated and commercially important forage legumes across the globe (Phelan et al. 2015) and are potential biological N_2-fixers in temperate grasslands (Carlsson and Huss-Danell 2003; Rasmussen et al. 2012). They differ in several key traits that are likely to influence growth, biomass production and N dynamics when included in species mixtures. In contrast to the deep tap root systems and large erect shoots of RC and LU, WC has clonal or stoloniferous and shallow adventitious root systems and higher shoot biomass. WC also forms leaves from stem faster than RC and is more resistant to frequent cutting (Black et al. 2009). Regarding canopy structure (leaf position and angle) and light interception, WC has horizontal leaves, which favors more light interception at the top of the canopy, while RC has greater distribution of the leaf area and light interception in the intermediate layer of the canopy (Black et al. 2009). When included in the species mixtures, RC is more competitive to grass than LU and WC (Black et al. 2009; Elgersma and Søegaard 2016). The three species also differ in their patterns of N uptake from the soil, of build-up and utilization of the N reserve in roots and in their ability to compete for recycled N. LU has shown ability to assimilate N from deep soil layers (Kelner at al. 1997), WC with their shallow roots take up N from upper soil layers (Rasmussen et al. 2013; Younie 2012). RC and LU build-up and remobilize carbohydrates and N stored in their large tap root system for shoot growth (Black et al. 2009; Barber et al. 1996). Moreover, they differ in their ability to fix atmospheric N_2, to transfer and rhizodeposit fixed N, and to receive N transferred from companion species as well as re-uptake of rhizodeposited N. RC and LU have shown to fix higher amount of N_2 than WC (e.g. Rasmussen et al. 2012), whereas RC has been found to reach higher rate of transfer of fixed N to the companion species than RC and LU (Høgh-Jensen and Schjoerring 2000; Louarn et al. 2015; Pirhofer-Walzl et al. 2012). RC has demonstrated better ability to absorb N transferred from companion spcies than WC (Pirhofer-Walzl et al. 2012), while low rates of N transfer have been found in LU, both as donor (Frankow-Lindberg and Dahlin 2013; Louarn et al. 2015) and as receiver (Pirhofer-Walzl et al. 2012).

Varied above- and below-ground resource utilization and niche differentiation in space and time might occur between these species when grown in a mixture due to differences in plant architecture, and growth and N uptake patterns. In particular, the functional complementarity between forage legume species in a mixture might increase the utilization of soil N resources, making them stronger competitors for the available soil N in the rhizosphere, thereby increasing total plant production, N acquisition and the proportion of atmospheric N_2-fixation in the mixture compared to the species grown in pure stands. Hence, exploration of plant production and N_2-fixation in a mixture of forage legume species is expected to generate new knowledge towards achieving higher and more stable biomass and N yields. In addition, integration of sward containing forage legumes only in grassland is expected to increase the supply of protein without N fertilization. Thus, we conducted this study with the objectives of determining how the swards of forage legume species will affect: herbage yield, botanical composition, N yield and the percentage and amount of N derived from the atmosphere. The following hypotheses were tested: functional complementarity between the species with different above-and below-ground architecture increases 1) the herbage yield and N accumulation, and 2) the proportion of legume-N derived from the atmosphere in the forage legume mixtures compared to pure stands .

Materials and methods

Experimental site

This field experiment was conducted at Foulumgaard Experimental Station, Aarhus University, located in Central Jutland, Denmark (09° 34° E and 56° 29° N). The experimental field has grown cereals at least since 2010 prior to the establishment of the present experiment in 2014. The soil is loamy sand characterized as typic Hapludult comprising 7% clay, 10% silt, 81% sand, and 1.7% carbon in the upper soil layer (0- 20 cm) (Solati et al. 2017). Soil extractable P was 36 mg kg^{-1}, soil exchangeable K was 129 mg kg^{-1} and pH 5.9. The mean monthly air temperature during the experimental period between April and October 2015 ranged between 7 and 17 °C, with July and August the warmest months. The monthly precipitation ranged from 21 to 117 mm, where May, June, July and September were the wettest months (Fig. 1).

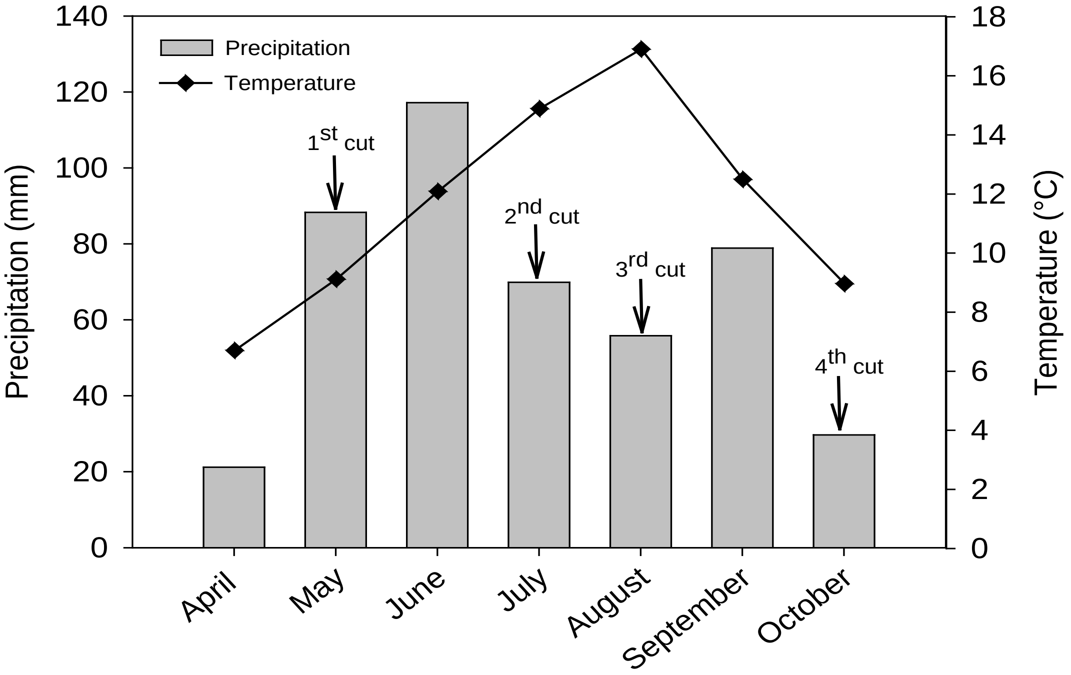


Fig. 1 Monthly precipitation and mean monthly air temperature during the experimental period from April to October in 2015 measured at a climate station near the experimental field.

Experimental design and establishment of experimental plots

Ten different species mixtures, including pure stands, two- and three-species mixtures, were established in spring 2014 including commonly used cultivars of three forage legume species: red clover (*Trifolium pratense* L. var. Suez), white clover (*Trifolium repens* L. var. Silvester) and lucerne (*Medicago sativa* L. var. Creno). The species were undersown in a spring barley cover crop in a replacement design, with different proportions of the species in the mixture based on their seeding rates in pure stands, in plots measuring 1.5 x 12 m and in four replicates. The seeding rates in pure stands were 10, 10 and 20 kg ha^{-1} for WC, RC and LU, respectively (Table 1).

Table 1 Composition of seed mixtures (percentage is based on each species seeding rate in pure stand)

Seed mixtures		**Percentage of seed in the mixture (%)**			**Seeding rate (Kg ha^{-1})**		
		RC	**WC**	**LU**	**RC**	**WC**	**LU**
Pure stands	RC	100			10		
	WC		100			10	
	LU			100			20
Two species	RC+WC	50	50		5	5	
	WC+LU		50	50		5	10
	RC+LU	50		50	5		10
Three species	80RC+WC+LU	80	10	10	8	1	2
	RC+ 80WC+LU	10	80	10	1	8	2
	RC+ WC+80LU	10	10	80	1	1	16
	RC+ WC +LU	33	33	33	3.3	3.3	6.6

RC: Red clover, WC: White clover, LU: Lucerne

The LU seeds were inoculated with rhizobium (Nitragin Gold) before sowing. The spring barley crop was harvested at maturity and N_2-fixation was determined in situ during the first production year over the growing season between April and October in 2015 using the ^{15}N isotope dilution method as applied by Rasmussen et al. (2012). In this method, N_2-fixation is measured by comparing the dilution of soil-derived ^{15}N by atmospheric N_2 in the N_2-fixating plan to the ^{15}N enrichment of plants that derived all their N from soil. The non-legume reference plants are thus assumed to reflect the ^{15}N enrichment of legume derived N from soil (Unkovich et al. 2008; Carlsson and Huss-Danell 2014). At the onset of the growing season, during the second week of April, a subplot (dilution plot) measuring 1×1 m was demarcated in each experimental plot and the soil was labeled with ammonium Sulphate 0.1 g N m^{-2} (^{15}N enriched to 98 atom%) to artificially enrich the soil with ^{15}N above the background natural ^{15}N abundance.

Plant sampling and analysis

The shoot biomass in each subplot was manually sampled four times during the growing season in a 0.25 m^2 area at a stubble height of 5 cm, following common agricultural practice in cut grassland. The first sampling was done on 22 May, the second on 1 July, the third on 17 August and the last on 5 October. The plant samples were manually sorted into individual species and weeds, air-dried at 80 °C for 24 hours and dry matter (DM) weight was recorded. The dried samples were milled, subsampled and ball-milled into a fine powder, packed into small tin capsules and analyzed for total N concentration and atom% ^{15}N at UC Davis Stable Isotope Facility, University of California, USA, on an ANCA-SL Elemental Analyzer coupled to a 20-20 Mass Spectrometer using the Dumas dry-combustion method. The N yield in each plot was determined as a product of shoot DM yield and N concentration in each species in the harvested biomass.

Calculations

The N_2-fixation was quantified based on excess atom% ^{15}N in legumes and non-legumes using the weed species (representing both grasses and dicotyledon weed species) growing together with the legume species as reference plants (Carlsson and Huss-Danell 2014), using pooled samples of different weed species to avoid potential bias caused by spatiotemporal unevenness in soil ^{15}N enrichment (Carlsson et al. 2009; Unkovich et al. 2008). The percentage of N derived from the atmosphere (%Ndfa) was calculated using the following equation (Chalk et al. 2016):

$$\%\text{Ndfa} = (1 - (\text{excess atom}\%^{15}\text{N legume} / \text{excess atom}\%^{15}\text{N reference})) \times 100$$

where, excess atom% ^{15}N was calculated by subtracting the background atom% ^{15}N (determined by analyzing ^{15}N in legumes and weed species grown in unlabeled field plots adjacent to the ^{15}N-labeled plots) from the atom% ^{15}N determined in the corresponding species in ^{15}N-labeled subplots. The average background atom% ^{15}N was 0.3676 in non-legumes and 0.3663 in legumes, and did not vary significantly across cutting occasion or legume species. Then the amount of N_2-fixation was expressed as a product of %Ndfa and N accumulation for the respective legume species. In the mixtures containing two or three legume species, the average %Ndfa for the whole mixture was estimated as the ratio of total amount of N_2-fixed to the total N accumulated in shoots, and the seasonal %Ndfa was estimated as the ratio of the total amount of N_2-fixed over the growing season to the total amount of shoot N accumulated. The soil N uptake was estimated subtracting the amount of N_2-fixation from the N accumulated in shoots.

The relative yield (RY) was calculated for each species as its DM yield in the mixture as a proportion of its DM yield in the pure stand, with relative yield total (RYT; the sum of RYs for all species included in the mixture) values higher than 1 indicating the presence of complementarity effects in the mixture (Hector 1998).

Data analysis

The data were analyzed using the open-source statistical program R (R Core Team 2016) (Version 3.1.1). A one-way analysis of variance (ANOVA) was used to determine the effect of sown species composition on each of the dependent variables: DM yield, N yield, atom% ^{15}N, %Ndfa, amounts of N_2-fixation, N uptake, RY and RYT, and the effect of two fixed factors (species composition and individual species) was tested using two-way ANOVA. The effect of time of cut on DM yield, N yield, %Ndfa and amount of N_2-fixation was tested using the linear mixed model. In the model, the composition of sown species (fixed effect) and time of cut (repeated fixed effect) were independent variables and block was a random factor, where plots were nested in the blocks. The model was then tested using ANOVA. For all dependent variables, the tests for significant differences between the seed mixtures were made using *least square means* in the adjusted Tukey method. The probability limit for rejection of the hypothesis was set at the confidence level 0.95 ($P<0.05$). The correlations between different dependent variables were tested using Pearson's correlation coefficient. The data violating the assumption of normal distribution were generally log-transformed before analysis to achieve a normal distribution of residuals.

Results

The weather at the experimental site measured during the experimental period from April to early October, 2015 (Fig. 1) showed similar temperatures to the 30-year average measured at the same experimental station, while the mean monthly precipitation was about 30% higher than the 30-year average.

Dry matter production and composition of the sward

The seasonal total DM yield of the mixtures ranged from 10.7 to 16 t ha^{-1}, with significant effect of species composition ($p<0.001$) (Fig. 2). RC produced the highest DM yield among the pure stand, all species mixtures containing RC produced higher than pure stands of WC and LU and the WC paired with LU (Fig. 2). However, there was no significant difference between the seed mixtures containing RC. Species composition and seeding density affected the DM production in WC and LU, with the higher DM yield in the pure stand followed by the two-species mixture and three-species mixture with their 80% seeding density. Weeds were most abundant in the pure stand of LU, which was significantly higher than in all other treatments and was strongly suppressed in the mixtures containing RC. The proportions of total DM yield differed markedly between the species across all mixtures. RC was the most productive of the three species, and the proportion of RC in the total harvested biomass was always much higher than its relative proportion in the seed mixture (Fig. 2). Hence, RC could be defined as the most competitive of the three legume species under the conditions of the present study. WC was always strongly suppressed by RC, but made up a higher proportion in the harvested biomass than its sown proportion in the two-species mixture with LU, and could thus be defined as less competitive than RC but more competitive than LU. LU was always strongly suppressed in all mixtures.

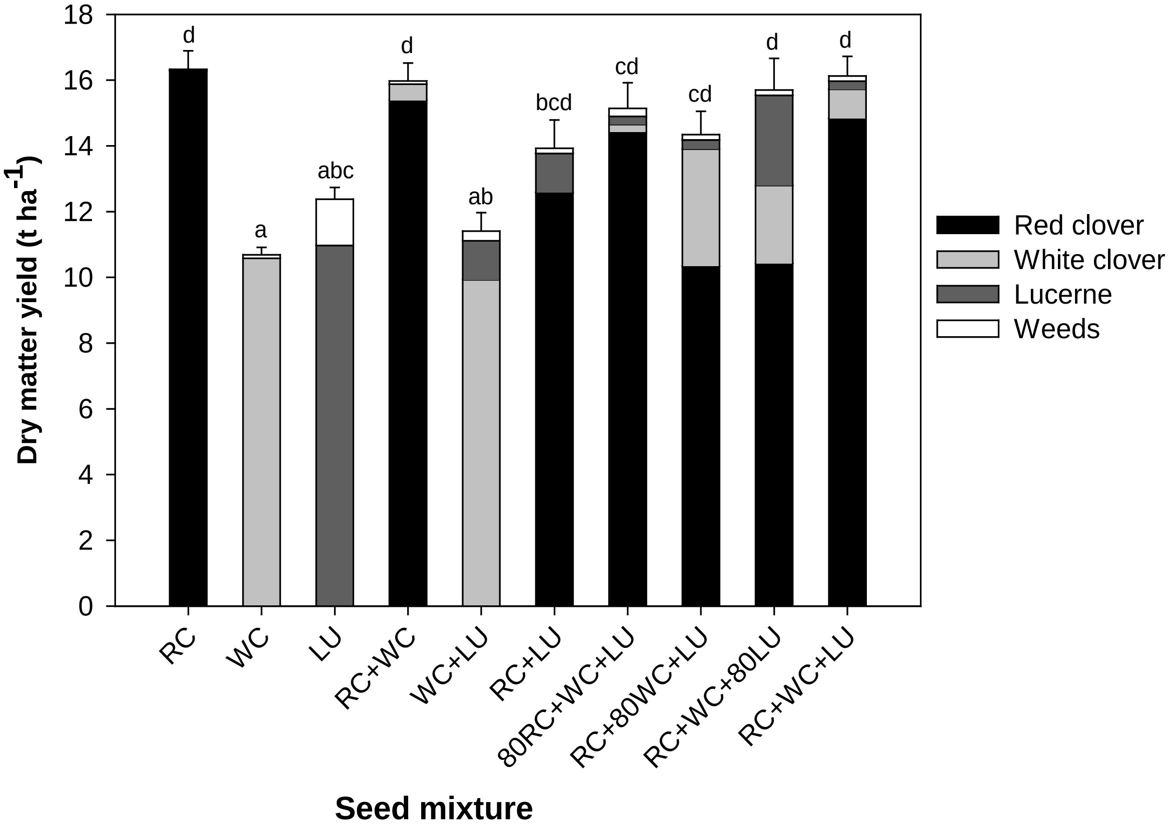


Fig. 2 Whole season shoot dry matter (DM) yield of red clover, white clover, lucerne and weeds grown in the field. Values are mean (±SE; n= 4) measured at four cutting times during the 2015 growing season. Different letters indicate statistically significant differences between species composition at the 0.05 level. RC: Red clover, WC: White clover, LU: Lucerne and 80: percentage of total seeds in the mixture.

The RYT values based on total seasonal DM yield were higher than 1 only in the WC+LU mixture (1.06) and the three-species mixture with 80% LU (1.2) (Supplementary Table 1). In two of the RC-dominated mixtures, RYT was lower than 1, *i.e.* 0.91 in RC+LU and 0.93 in the three-species mixture with 80% RC.

N accumulation

The average total seasonal N accumulation ranged from 440 to 595 kg ha^{-1}, with significant differences between seed mixtures ($p<0.001$). Since RC defined the DM yield of the mixtures, the total N accumulation of the mixtures mirrored the pattern of RC DM production, with considerable higher seasonal N accumulation in mixtures containing RC compared to pure stands of WC, LU and the WC+LU mixture, respectively (Table 3). WC accumulated from 10 to 440 kg N ha^{-1}, with the largest amount in the pure stand followed by WC+LU and RC+80WC+LU. Similarly, N accumulation in LU ranged from 10 to 450 kg ha^{-1} with the largest amount in the pure stand followed by RC+ WC+80LU and RC+LU.

Proportions and amounts of N_2-fixation

The values of atom% ^{15}N in forage legumes and reference species were substantially above the natural abundance, and differences between the ^{15}N enrichment of legumes and reference species were sufficient to estimate the %Ndfa. The ^{15}N enrichment was highest at the first cut and decreased from the second cut onwards. The excess atom% ^{15}N in the legumes ranged from 0.2087 to 3.5486 at the first cut and from 0.0718 to 0.9688, 0.0221 to 0.2687 and 0.0372 to 0.1679 at the second, third and fourth cuts, respectively (data not shown). The excess atom% ^{15}N in weed species did not show any significant difference between species composition treatments (Table 2), and the average weed excess atom% ^{15}N (used as reference plant values for the calculation of N_2-fixation at each cut) was 3.9784, 1.1790, 0.3386 and 0.2373 for the first, second, third and fourth cuts, respectively.

Table 2 Excess atom% ^{15}N in shoots of reference plants (pooled samples of weed species). Values are mean (±SE; n= 4) measured at four cuts during the 2015 growing season. No significant difference between the treatments was found at any of the cuts.

Seed mixtures		**Cut 1**	**Cut 2**	**Cut 3**	**Cut 4**
Pure stand	RC	4.484±1.250	1.302±0.280	0.333±0.043	0.278±0.024
	WC	3.458±0.986	1.215±0.101	0.219±0.044	0.206±0.011
	LU	2.830±0.920	0.835±0.039	0.253±0.006	0.214±0.008
Two species	RC+WC	5.632±0.568	1.399±0.100	0.428±0.065	0.283±0.034
	WC+LU	3.858±0.355	1.419±0.143	0.387±0.070	0.162±0.001
	RC+LU	3.645±1.107	1.265±0.250	0.328±0.035	0.261±0.029
Three species	80RC+WC+LU	4.034±0.199	1.274±0.127	0.374±0.032	0.253±0.028
	RC+80WC+LU	2.862±0.780	0.857±0.068	0.269±0.019	0.200±0.012
	RC+WC+80LU	3.742±0.383	1.031±0.078	0.351±0.060	0.246±0.024
	RC+ WC+LU	4.095±0.693	1.106±0.288	0.363±0.042	0.222±0.016

RC: Red clover, WC: White clover, LU: Lucerne and 80: percentage of total seeds in the mixture

Table 3 Whole season N accumulation in red clover, white clover and lucerne, percentage (%Ndfa) and amount of N_2-fixation in red clover, white clover, lucerne and in the whole mixture, and total N uptake in pure stands and the whole mixture measured in shoots. Values are mean (±SE; n= 4) measured at four cutting times during the 2015 growing season. Different letters within the same column indicate statistically significant differences between species compositions at the 0.05 level.

Seed mixtures		**N accumulation (kg N ha^{-1})**			**% Ndfa**				**N_2-fixation (kg N ha^{-1})**				**Total N uptake (kg N ha^{-1})**
		RC	**WC**	**LU**	**RC**	**WC**	**LU**	**Weighted**	**RC**	**WC**	**LU**	**Total**	
Pure stand	RC	595±18^{b}			85±0.3^{a}			85±0.3^{a}	506±15^{b}			506±15^{d}	89±3^{c}
	WC		440±8^{c}			84±0.9^{a}		84±0.9^{a}		370±7^{e}		370±7^{a}	71±4^{b}
	LU			450±10^{c}			90±0.5^{a}	90±0.5^{b}			405±10^{c}	405±10^{abc}	45±2^{a}
Two species	RC+ WC	558±22^{ab}	20±4^{a}		86±1.0^{a}	74±4.5^{a}		86±0.8^{ab}	480±20^{ab}	15±4^{ab}		495±17^{d}	82±5^{bc}
	WC+LU		410±9^{c}	45±18^{ab}		84±0.5^{a}	76±5.2^{a}	84±0.7^{a}		345±6^{e}	34±16^{ab}	380±15^{ab}	73±2^{b}
	RC+LU	453±49^{ab}		50±22^{ab}	86±1.3^{a}		77±8.5^{a}	86±1.4^{ab}	390±31^{ab}		40±20^{ab}	430±22^{abcd}	71±9^{b}
Three species	80RC+WC+LU	524±25^{ab}	10±3^{a}	10±7^{a}	86±0.8^{a}	77±7.5^{a}	81±4.0^{a}	86±0.9^{ab}	450±25^{ab}	8±3^{a}	8±6^{a}	466±22^{bcd}	75±2^{bc}
	RC+80WC+LU	376±48^{a}	155±34^{b}	12±7^{a}	87±0.6^{a}	84±1.3^{a}	75±9.0^{a}	86±0.9^{ab}	327±44^{a}	130±28de	9±6^{a}	466±28^{bcd}	76±6^{bc}
	RC+WC+80LU	374±81^{a}	100±37^{ab}	114±38^{b}	86±0.6^{a}	83±2.0^{a}	85±3.0^{a}	86±0.5^{ab}	322±71^{a}	83±31^{cd}	97±32^{b}	502±30^{d}	82±6^{bc}
	RC+ WC+LU	520±25^{ab}	37±8^{a}	12±8^{a}	87±0.4^{a}	87±1.0^{a}	60±13.0^{a}	86±1.0^{ab}	452±23^{ab}	32±7^{bc}	7±3^{a}	492±16^{cd}	78±8^{bc}

RC: Red clover, WC: White clover, LU: Lucerne and 80: percentage of total seeds in the mixture

On a seasonal basis (Table 3), all the three forage legume species in the pure stand derived above 80% of their N from atmospheric N_2-fixation (%Ndfa), which was similar for WC and RC. The %Ndfa in the pure stand of LU was significantly higher than in the pure stand of WC and RC and the mixture of WC+LU ($p<0.01$). In the two- and three-species mixtures, the %Ndfa in WC and RC was mostly above 80%, irrespective of composition of seed mixtures, but tended to decrease in LU (Table 3). However, the %Ndfa estimated in all three species was not affected by the composition of the seed mixture. Since RC dominated the mixtures, the weighted %Ndfa for the whole mixtures closely resembled the %Ndfa in RC, which was consistently above 80% with no significant difference between the two- and three-species mixtures.

The measured %Ndfa at each cut (Table 4) showed that RC and WC mostly derived above 80% of their N from fixation during the first three cuts. The %Ndfa in LU tended to be higher in the pure stand than in mixtures, and this difference between LU pure stands and LU in mixtures was more pronounced than the corresponding differences between pure stands and mixtures in RC and WC. However, the species composition, in general, did not affect the %Ndfa in either of the three species or the weighted average %Ndfa for the whole mixture.

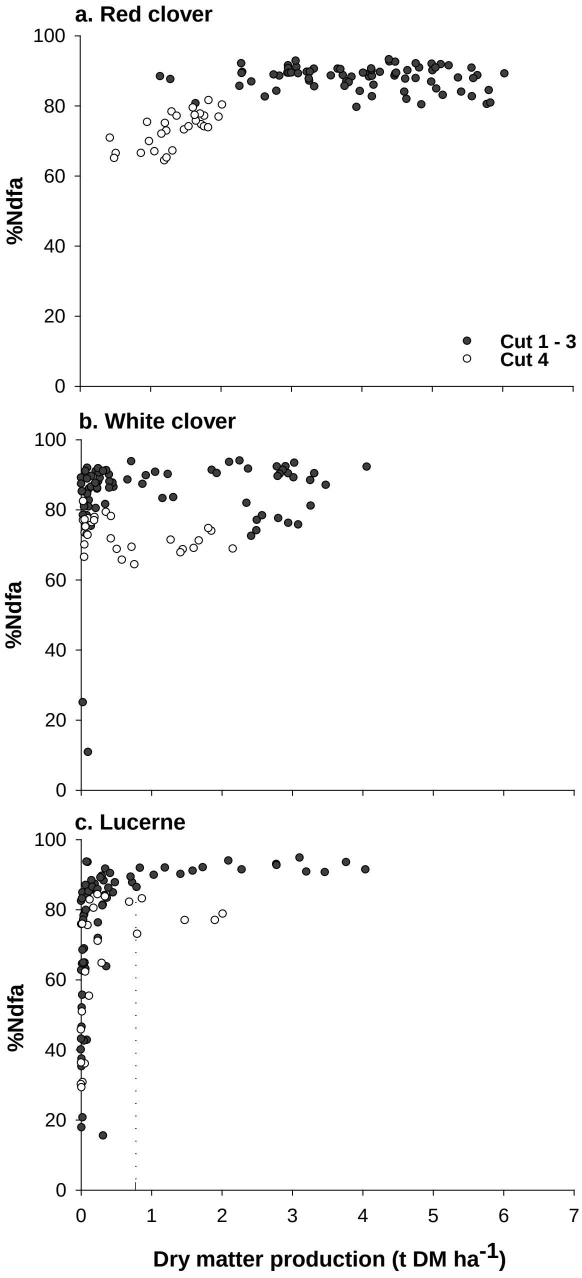
Table 4 Percentage of N derived from the atmosphere (%Ndfa) in shoots of red clover, white clover and lucerne. Values are mean (±SE; n= 4) measured at four cutting times during the 2015 growing season. Different letters within the same column indicate statistically significant differences between species compositions at the 0.05 level.

Seed mixtures		**Cut 1**			**Cut 2**			**Cut 3**			**Cut 4**		
		RC	**WC**	**LU**	**RC**	**WC**	**LU**	**RC**	**WC**	**LU**	**RC**	**WC**	**LU**
Pure stand	RC	88±0.5^{a}			88±0.5^{a}			83±0.9^{a}			75±0.6^{b}		
	WC		92±0.8^{b}			90±1.0^{a}			76±2.0^{a}			71±1.5^{ab}	
	LU			93±0.6^{a}			92±0.8^{a}			92±0.7^{a}			77±0.5^{a}
Two species	RC+WC	90±0.9^{a}	49±18.0^{a}		89±0.4^{a}	85±1.5^{a}		83±1.4^{a}	80±2.6^{ab}		77±1.0^{b}	73±1.3^{ab}	
	WC+LU		92±0.5^{b}	83±3.0^{a}		90±1.0^{a}	70±9.0^{a}		78±1.0^{a}	67±9.0^{a}		70±0.9^{a}	60±10.0 ^{a}
	RC+LU	90±0.8^{a}		84±7.0^{a}	90±0.8^{a}		79±5.5^{a}	84±1.8^{a}		73±11.0^{a}	71±3.9^{ab}		67±12.0^{a}
Three species	80RC+WC+LU	90±1.0^{a}	88±2.6^{b}	86±2.4^{a}	89±0.9^{a}	88±1.0^{a}	71±8.2^{a}	84±0.7^{a}	84±1.0^{ab}	62±12.0^{a}	75±2.8^{ab}	75±3.4^{ab}	57±19.0^{a}
	RC+80WC+LU	92±0.6^{a}	89±1.0^{b}	82±5.8^{a}	89±0.9^{a}	90±0.9^{a}	78±5.0^{a}	83±0.8^{a}	83±1.6^{ab}	55±15.0^{a}	69±2.7^{a}	72±2.5^{ab}	54±10.0 ^{a}
	RC+WC+80LU	90±1.0^{a}	88±1.0^{b}	88±3.2^{a}	89±0.4^{a}	89±1.7^{a}	82±5.8^{a}	84±2.4^{a}	82±2.2^{ab}	85±2.2^{a}	71±1.8^{ab}	71±3.8^{ab}	78±2.0^{a}
	RC+ WC+LU	91±0.8^{a}	89±1.2^{b}	58±19.0^{a}	90±0.3^{a}	90±0.9^{a}	49±15.0^{a}	85±0.3^{a}	87±0.9^{b}	55±13.0^{a}	73±3.0^{ab}	75±1.0^{b}	46±8.2^{a}

RC: Red clover, WC: White clover, LU: Lucerne and 80: percentage of total seeds in the mixture

The %Ndfa in RC and WC did not change with species composition, variation in DM production or botanical composition (Fig. 3). However, %Ndfa in LU appeared to be positively influenced by biomass yield at low yield levels, *i.e.* up to around 1 t DM ha^{-1}. At higher biomass yields, the %Ndfa tended to be more stable around or above 80% (Fig. 3).

Fig. 3 Relationship between the dry matter production and percentage of N derived from atmosphere (%Ndfa) in the three forage legume species measured at four cutting times during the 2015 growing season. The dashed vertical line in c. Lucerne represent threshold line for positive association between LU dry matter production and %Ndfa.



The amount of N_2-fixation varied between the mixtures and cuts, with a significant interaction effect ($p<0.001$). The N_2-fixation was generally higher in the pure RC stand and in the mixtures containing RC. The seasonal amount of N_2-fixation in the mixtures ranged from 370 to 500 kg N ha^{-1}, with significant differences between the seed mixtures ($p<0.001$) (Table 3). Since %Ndfa was not affected by the composition of the seed mixture, the amount of N_2 fixed in all three species was closely related to the N accumulation. The pure stand of RC, and the WC+RC and WC+RC+80LU mixtures fixed amounts of N that were significantly higher than in the pure stands of WC, LU and the WC+LU mixture. Similarly, all the three-species mixtures fixed significantly larger N amounts than the pure stand of WC.

When looking at each species, RC in the mixtures generally fixed as much N as in the pure stand, with the exception of the three-species mixture with the high seeding density of WC or LU. However, the seeding density influenced the N_2-fixation in WC and LU, affecting their proportion of total DM in the harvested biomass. On a seasonal basis, WC fixed more N when grown with LU in the two-species mixture, which was nearly similar to the WC in the pure stand. However, N_2-fixation in LU was suppressed in the mixtures, irrespective of seeding density, with significantly lower amounts of N_2-fixed compared to its pure stand (Table 3).

The seasonal soil N uptake was significantly lower in pure stand of LU ($p<0.01$), but did not differ across the two-and three-species mixtures (Table 3).

Discussion

Plant growth and sward competition

In the present study, the pure RC stand and mixtures containing RC showed a yield advantage compared to pure stands of WC and LU and the WC+LU mixture. However, there was no evidence of an effect of diversity leading to transgressive over-yielding (Palmborg et al. 2005), since none of the mixtures were more productive in terms of biomass yield, N accumulation or N_2-fixation than the highest-yielding pure stand (RC). One explanation for the lack of an over-yielding effect could be that we had no non-legume species in the mixture that would benefit from legume-fixed N (Dhamala et al. 2017; Nyfeler et al. 2011) and increase the complementarity of N use in mixtures with the fertilizing function of legumes and the uptake of this N by non-legumes (Palmborg et al. 2005). Since the WC and LU were outcompeted in mixtures with RC, another explanation for the lack of transgressive over-yielding could be a lack of evenness in the growth of the species in the mixture (Kirwan et al. 2007) or the lack of evenness in the resource partitioning among the species in the mixture (Roscher et al. 2008). In our study, the mixture with the most even biomass yield proportions of the three species, *i.e.* the three-species mixture with 80% LU, also had the highest RYT value (1.2). This observation supports previous findings that evenness of species proportions in mixtures enhances complementarity effects in resource use among the mixed species.

The total DM and N yields of the mixtures containing RC in the present experiment (i.e. without grass in the mixture) were comparable to previous studies of grass-legume mixtures at the same location (Elgersma and Søegaard 2016; Pirhofer-Walzl et al. 2012; Rasmussen et al. 2012) and higher or within the range of grassland production measured in various geographical regions in Europe (e. g. Anglade et al. 2015; Kirwan et al. 2007;Phelan et al. 2015). Thus, our study showed that when RC was present in the seed mixture, total DM and N yields were not compromised in the forage legume mixtures. Furthermore, the abundance of weeds was strongly suppressed in the pure stands of RC and WC as well as in the mixtures containing RC compared to the LU pure stand. This suggests that the mixtures containing competitive legumes attributed to lower weed invasion (Kirwan et al. 2007) and there was a high resource utilization efficiency of the sown species in the mixture (Sanderson et al. 2005).

We observed contrasting growth and competition between the three forage legume species in the mixture. RC consistently dominated in the mixtures irrespective of species composition and seeding densities of WC and LU; hence, RC defined the DM and N yields of the mixtures. This demonstrates the strong ability of RC to compete for above- and below-ground resources (Pirhofer-Walzl et al. 2012). We observed that RC was the most productive in the pure stand and always dominated in the mixture, with the RY value ranging from 0.63 to 0.94 and the RYT value below or near 1 in most of the mixtures, with the evidence that there was no complementarity between the species in the RC-dominated mixtures for the resource utilization (Hector 1998). Canopy characteristics of the species in the mixture was found to play an important role for light interception for photosynthesis and thereby growth in the study by Black et al. (2009), and an explanation for the restrained growth of the WC and LU in our study could be that they were shaded by the vigorous upright growth of RC (Frame 2005) and therefore outcompeted for light. The poor light interception could have lowered the leaf/stem ratio and the photosynthetic activity in LU and WC. Thompson and Harper (1988) showed that many growth attributes such as stolon branching, petiole and internode lengths and number of branched and rooted nodes in WC are affected by canopy light interception (quality of radiation transmitted) under the canopies of different grass species. We observed that the proportions of WC and LU increased with later cuts, while the RC generally showed the opposite trend with a significantly lower DM yield at the fourth cut. A second explanation could be environmental factors in that the mean monthly air temperature during the growing season (Fig. 1) remained lower than the optimum growth temperature required for the three species, the better performance of RC could be potentially due to its ability to grow in a wider range of temperatures than WC and LU (Frame 2005). A third explanation for the poor growth, especially of LU, could be the preference of LU for a less frequent cutting regime than the four cuts per year applied in this study (Frame 2005; Wolf and Smith

1964). Our result indicate that the dominant species, RC in the present experiment, was the better able of the three to exploit the available resources.

Proportions of N derived from the atmosphere (%Ndfa)

We observed that the three forage legume species relied mainly on N derived from atmospheric N_2-fixation, regardless of whether they were grown in pure stand or in two- or three-species mixtures. Hence, our second hypothesis that the proportion of legume-N derived from the atmosphere would increase in mixtures compared with pure stands was not confirmed. Numerous studies on N_2-fixation have demonstrated that the %Ndfa is positively influenced by plant diversity in grasslands that are made up of a mixture of forage legumes and non-legumes due to non-legume competition for available soil N (e.g. Carlsson and Huss-Danell 2003; Nyfeler et al. 2011; Rasmussen et al. 2012). Carlsson et al. (2009) and Palmborg et al. (2005) further suggested that functional traits of the species in the mixture play a more important role for efficient N uptake and stimulatory effect on %Ndfa. In this light, previous studies have shown that forage legumes in swards containing non-legumes, especially grasses, often derive up to 90% or more of their N from atmospheric N_2-fixation (Carlsson and Huss-Danell 2003; Rasmussen et al. 2012). The present study showed that the three studied forage legumes were equally good (or bad) competitors for soil N, *i.e.* that there was no difference between intra- and inter-specific competition for soil N, indicating that legume-legume mixtures behave like pure stands of legumes in terms of soil N acquisition. Hence, the documented trait differences between the three studied legumes (see references in introduction) in above- and below-ground growth, dynamics in acquisition and use of different N sources and competitive ability did not influence their N_2-fixation when grown together in mixture without non-legumes. Thus, in order to have a mixture effect on %Ndfa, the swards needs to contain non-legume species, which compete more efficiently for available soil N than the legumes.

The normally accepted regulation mechanism for %Ndfa is the availability of soil N, whereby a high N availability reduces %Ndfa and a low N availability increases %Ndfa. It is possible that the consistently high %Ndfa in our study was a consequence of generally low levels of plant-available soil N, since the field experiment was not fertilized, and that the legumes therefore relied to a large extent on N_2-fixation for their N acquisition also when grown in pure stands. The lack of the expected effect of mixture could also be explained, at least in part, by complementary rooting patterns among the three forage legume species reducing the direct competition for plant-available soil N. LU has a relatively higher capacity to absorb N from deep soil layers (Kelner et al. 1997), whereas WC has been shown to assimilate nutrients more easily from the upper soil layers with the help of stoloniferous (creeping) roots (Younie 2012; Rasmussen et al. 2013). The large root system of RC may have given it more flexibility to explore the N from both upper and lower soil profiles. Furthermore, RC and LU accumulate and use their large root system as an N reserve, and that their shoot regrowth is mainly supported by the supply of N from roots (Barber et al. 1996; Black et al. 2009). Thus, LU and RC, in the present experiment, could have built up more reserve N in their roots and therefore competed less with companion species for the available soil N. Given the fairly large variation in the proportions of the three legumes across the mixtures, the finding that none of the three legumes seemed to be affected by competition for available soil N indicates that the legumes competed mainly for other resources, such as light, water and nutrients other than N.

Previous studies have shown that all three forage legume species are net donors of N when in mixture with non-legume species. WC is a generous N-donor (Høgh-Jensen and Schjoerring 2000; Pirhofer-Walzl et al. 2012), but a poor receiver of the N transferred from companion species, while RC is an intermediate donor and a good receiver (Pirhofer-Walzl et al. 2012). LU has been shown to retain its plant N with a lower proportion being transferred (Frankow-Lindberg and Dahlin 2013; Louarn et al. 2015) and less fixed N being rhizodeposited (Louarn et al. 2015; Wichern et al. 2008), including a poorer ability to absorb the N transferred from companion species (Pirhofer-Walzl et al. 2012). Thus, we suggest an alternative regulation mechanism for %Ndfa that the re-uptake by the legume of its N exudates may be regulating %Ndfa . Our result indicates that the legumes, especially RC, could access their N exudates due to the absence of non-legume competition for the legume-derived N, as observed by the net transfer of N to companion species in mixtures with non-legumes (e.g. Rasmussen et al. 2013; Dhamala et al. 2017).

Interestingly, the %Ndfa of LU showed a dependency on DM yield, which was not found for RC and WC. The LU, in general, had a relatively lower %Ndfa than RC and WC in the mixtures, but this tended to be higher in the pure stand (Tables 2 and 3). Therefore, despite comparable levels of shoot yields of WC and LU, the %Ndfa tended to be lower in LU than WC. The differences in %Ndfa between the two species could partly be the result of differences in their pattern of N uptake. LU has been shown to compete more strongly for available soil N and act as both a soured clovere and sink for the recycled mineral N (Tomm et al. 1995). Therefore, LU in the present experiment when grown in mixtures must have extracted more N from the soil pool, resulting in less dependency on atmospheric N_2-fixation when the DM yield of LU was low. In contrast, with a higher biomass production in pure stand, soil N might have become limited and LU had to increase its reliance on N_2-fixation. Another possible explanation that the higher weed abundance in pure stand of LU may have led higher competition for the available N, thereby reducing its access to soil N and stimulating a higher %Ndfa in LU pure stand. Buildup and utilization of the N reserve in the roots may have been lower in WC because of a higher rate of transfer and rhizodepositon of fixed N (Pirhofer-Walzl et al. 2012; Louarn et al. 2015) and a fast turnover and N release from roots (Louarn et al. 2015). Hence, the dependency on N_2-fixation was in general higher for WC than for LU. Furthermore, the lower %Ndfa of LU at DM productions below 1 t DM ha^{-1} could also be related to a higher metabolic cost of N_2-fixation as the plants should be able to supply the necessary carbohydrates produced from photosynthesis for the N fixed from bacteria (Schulze 2004). This indicates that the growth of LU at low densities might have been limited by resoured cloveres other than soil N (*e.g.* light, water, other nutrients) caused by competition from RC and WC. In summary, our result suggests that the three forage legume species varied in their competitiveness, including their N acquisition strategies, but that these differences did not cause significant variations in %Ndfa when the studied legumes were grown together in different mixtures.

Variations in %Ndfa estimates obtained with the ^{15}N isotope dilution method might be confounded by spatiotemporal unevenness in soil ^{15}N enrichment after the addition of ^{15}N-labelled fertilizer, as documented by Burchill et al. (2014). Such unevenness would undermine the assumption that the excess atom% ^{15}N in the sampled reference plants represents the ^{15}N enrichment of soil N available to the legume, at least if the legume and the reference plant differ in their soil N uptake patterns. To avoid the risk of large bias caused by spatiotemporal variations in soil ^{15}N enrichment, we followed the approach to use the average excess atom% ^{15}N of several reference species (pooled samples of weeds, representing both grasses and dicotyledon species) as suggested by e.g. Carlsson et al. (2009), Jacot et al. (2000) and Unkovich et al. (2008). Furthermore, we avoid the risk of misinterpreting temporal variations in N_2-fixation as we analyze effects of the different species compositions at each individual cutting occasion or on the seasonal mean value instead of analyzing temporal variations between cutting occasions.

Amount of N_2-fixation

In the present study, given the similar levels of %Ndfa, the differences in N_2-fixation among the species were mainly driven by the differences in DM production and N accumulation, as observed in previous investigations and documented in reviews (e.g. Anglade et al. 2015; Carlsson and Huss-Danell 2003; Nyfeler et al. 2011).

The seasonal amount of N fixed in the whole mixture (370-500 kg ha^{-1}) in the present experiment was higher than a previously reported range of N_2-fixation (100–380 kg N ha^{-1} yr^{-1}) in European grasslands (Lüscher et al. 2014). The fixed amount of N was comparable to the highest amount of N_2-fixation recorded in RED CLOVER (545 kg ha^{-1}) and LU (443 kg ha^{-1}) in Europe (Anglade et al. 2015), and the reported highest amounts of N_2-fixation in WC (545 kg ha^{-1}), RC (373 kg ha^{-1}) and LU (350 kg ha^{-1}) in northern European grasslands (Carlsson and Huss-Danell 2003). Thus, the present organic temporary grassland demonstrated a high N input from N_2-fixation. Since we did not observe any transgressive over-yielding, none of the mixtures in the present study fixed more N than the highest performing pure stand (RC). However, the amounts of N_2-fixation in mixtures containing RC were comparable to the strongest species in the pure stands and mostly higher than the pure stands of WC and LU and the WC+LU mixture. Hence, the lower amount of N_2-fixed in the WC and LU pure stands and in the WC+LU mixture was compensated when RC was incorporated in the seed mixture, at least in a relatively small proportion. Thus, our study showed that forage legumes have the potential to deliver a high herbage production, N accumulation and N_2-fixation, and provide protein-rich biomass without the need for N fertilization. These perennial crops are therefore a strong tool in the challenge of increasing European protein self-sufficiency. In addition, mixtures of perennial legumes might be more stable in their biomass yields and amount of N_2-fixed when measured across more than one growing season- an aspect that calls for further researed cloverh since it was not included in the present study.

Conclusions

Our study showed that mixtures of forage legume species had high biomass productivity and N yield from N_2-fixation. The proportion of N derived from N_2-fixation, and soil N uptake in most cases, in the mixtures was similar to that of the respective pure stands; hence, we did not observe a mixture effect on N_2-fixation as known from mixtures of legumes and non-legumes. Red clover was highly competitive under the study conditions, and there was no indication of complementary resoured clovere use in red clover-dominated mixtures. We conclude that mixtures consisting of only forage legume species do not express strong complementarity or over-yielding. However, such mixtures can be grown without compromising herbage production, N accumulation and input of N from N_2-fixation, provided that the mixture contains the dominant species (red clover in the present study) at least as a small proportion in the seed mixture.

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Supplementary Table 1 Relative yield (RY) of red clover, white clover and lucerne, and relative yield total (RYT) calculated based on whole season shoot biomass. Values are mean (±SE; n= 4) measured at four cuts during the 2015 growing season. Different letters within the same column indicate statistically significant differences between species compositions at the 0.05 level.

Seed mixtures		**RY**			**RYT**
		Red clover	**White clover**	**Lucerne**	
Two species	RC+WC	0.94±0.020^{b}	0.05±0.009^{a}		0.99±0.022^{a}
	WC+LU		0.94±0.022^{d}	0.12±0.040^{ab}	1.06±0.016^{a}
	RC+LU	0.78±0.100^{ab}		0.14±0.038^{b}	0.91±0.090^{a}
Three species	80RC+WC+LU	0.89±0.043^{b}	0.02±0.006^{a}	0.03±0.020^{a}	0.93±0.030^{a}
	RC+80WC+LU	0.63±0.063^{a}	0.34±0.078^{c}	0.03±0.016^{a}	1.00±0.050^{a}
	RC+WC+80LU	0.64±0.145^{a}	0.23±0.093^{bc}	0.33±0.026^{c}	1.20±0.070^{a}
	RC+ WC+LU	0.91±0.038^{b}	0.08±0.015^{a}	0.03±0.020^{a}	1.02±0.054^{a}

RC: Red clover, WC: White clover, LU: Lucerne and 80: percentage of total seeds in the mixture