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RESEARCH ARTICLE

Optimising grass-legume mixtures based on growth strategies for high N-yield and low N loss in fertilised grasslands

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Abstract
Aims Managed grasslands are important agro-ecosystems, consisting of grass monocultures with high nitrogen (N) fertiliser inputs. This management results in low N use efficiency and high N losses to the environment. Growing mixtures of plant species with diverse N acquisition strategies can reduce N losses and maintain high grassland productivity, yet determining the best mixture remains a challenge. The aim of this study was to investigate how grass-legume mixtures with contrasting growth strategies affect plant productivity, N use efficiency, N uptake, and soil mineral N, and how these effects depend on the N-fertilisation level.

Methods Two complementing field experiments were established: the first determined how monocultures and mixtures (two and four grass-legume mixtures) with contrasting growth strategies (fast- *vs*. slow-growing) affect productivity and N-cycling. The second determined the effect of fertilisation level on productivity and N-cycling in monocultures and two-species mixtures.

Results We found that productivity and N uptake of the four-species mixture was as high as the most productive monoculture and two-species mixtures. This was associated with an increase in legume N fixation and high N use efficiency of the plant community. Fast-growing grass and legume combination increased productivity and reduced soil mineral N, thus reducing the risk of N loss at both N-fertilisation levels, while combining a fast-growing grass with a

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slow-growing legume promoted high legume N fixation under low N-fertilisation.

Conclusions This study shows that productivity and N-cycling decreases via complementarity effects when growing mixtures of fast- and slow-growing grasses and a fast-growing legume at moderate level of N-fertilisation.

Keywords Grass-legume mixtures · Growth strategies · Managed grassland · Nitrogen cycling · Nitrogen use efficiency · Plant productivity

Introduction

Managed grasslands are globally important ecosystems, essential for food production, biodiversity maintenance, and soil carbon storage (Bengtsson et al. 2019). However, intensification by growing monocultures of high-yielding grass species together with large inputs of inorganic nitrogen (N) fertiliser causes negative environmental effects due to substantial N losses via nitrate leaching and nitrous oxide emissions (Ledgard et al. 2009; Peeters 2009). A promising way to improve sustainability is to increase N use efficiency via increasing plant community diversity (Abalos et al. 2021). However, the relative importance of plant species richness, functional group richness, or functional trait diversity on grassland primary productivity and N use efficiency is not fully understood. Disentangling these facets of plant community composition diversity is needed to attain the goals of modern agriculture: maintaining high productivity, producing quality fodder, and reducing reliance on N fertilisers.

Increasing plant species richness promotes productivity in semi-natural grasslands due to complementarity effects, i.e. when the productivity of mixtures is on average higher than expected based on the average productivity of the monocultures (Cardinale et al. 2007; Schaub et al. 2020). The positive diversity-productivity relationship has been shown in fertilised grasslands (De Deyn et al. 2009; Lüscher et al. 2014; Suter et al. 2021). However, the increase in plant productivity may depend on plant functional group composition rather than species richness per se (De Deyn et al. 2009; Abalos et al. 2014). Legumes play an important role in grassland N-cycling by increasing soil N via biological N fixation and increasing N uptake of neighbouring plants (Pirhofer-Walzl et al. 2012). Thus, at a given soil fertility level, grass-legume mixtures are generally more productive than grass monocultures (Nyfeler et al. 2011). In addition, differences in root architecture between grasses and legumes could promote plant species complementarity, increase nutrient acquisition, and thereby plant community productivity (Abalos et al. 2014). Thus, designing grass-legume mixtures with specific traits that can enhance complementarity effects may provide a key to enhance resource utilisation (Nyfeler et al. 2011; Mason et al. 2020) and primary productivity (Finn et al. 2013; Suter et al. 2015), and minimise N loss to the environment.

Functional trait diversity can also affect primary productivity and N use efficiency in grasslands. Plant traits can be used to characterise plant growth strategies, i.e. the trade-off between prioritising growth *vs* defence (Wright et al. 2004). Species with a fast-growing strategy typically have high specific leaf area (SLA) and leaf N content, and low leaf dry matter content (LDMC) and leaf $\delta^{13}C$ and show a high growth rate but do not persist long over time. In contrast, species with a slow-growing strategy typically have low leaf N content and SLA, a high LDMC and leaf $\delta^{13}C$, and have a slow growth rate, but persist longer (Wright et al. 2004; Oram et al. 2021). Slow-growing species might have higher water use efficiency being more resistant to drought compared to fast-growing species (Walde et al. 2021). Studies have shown that combining slow-growing legumes with grasses enhances plant productivity (Finn et al. 2013). However, the extent of enhanced productivity in legume-grass mixtures can depend on the plant growth strategies of the species in both functional groups. The traits of grasses and legumes characterise their growth strategies and therefore can be used to predict their interactions in species mixtures (Mason et al. 2016).

As part of the functional trait diversity, the increase in productivity in specific plant-species combinations could also attributed to intraspecific trait plasticity. When grown in mixtures, compared to monocultures, previous research has shown intraspecific variation in N uptake (Nyfeler et al. 2011), leaf N content (Thein et al. 2008), SLA (Roscher et al. 2015), and traits associated with light acquisition and N nutrition in forbs mixtures (Lipowsky et al. 2015). Trait plasticity in grass species can often be seen in response to increased soil N availability due to biological N fixation by legumes in grass-legume mixtures, leading to lower leaf C:N ratio (Chen et al. 2005) and higher SLA (Al Haj Khaled et al. 2005) in grass species. In contrast, legumes tend to maintain their leaf C:N ratio when grown in mixtures with grasses, because they regulate the level of N fixation (Nyfeler et al. 2011).

Community-level plant traits (via plant species composition and trait plasticity) has been shown to influence soil N-cycling and N losses. For example, shifts in plant traits due to plant species composition and plant species interactions can also influence ecosystem processes, such as soil N-cycling, with potential consequences for the environment through N losses. For instance, fast-growing grasses may reduce soil mineral N levels by increasing plant N uptake, thereby reducing nitrous oxide emissions (Abalos et al. 2018) and N leaching (de Vries And Bardgett 2016). Conversely, slow-growing plant species with low plant N uptake can result in higher risk of soil mineral N loss in the short term (Abalos et al. 2018). When combining fast- and slow-growing grasses, soil mineral N can be lower compared to grass monocultures (van Eekeren et al. 2010), and decrease nitrous oxide emissions compared to legume monocultures, whilst maintaining high forage quality (Abalos et al. 2021).

The optimum plant community composition to increase productivity and N use efficiency and reduce N losses likely depends on the level of N fertilisation. Grass-legume mixtures may promote a better use of N resources compared to grass or legume monocultures, thereby reducing the need for N fertiliser to obtain high forage yield (Suter et al. 2015; Fuchs et al. 2018), and consequently reducing N losses (Fuchs et al. 2018; Suter et al. 2021). Growth strategy may determine how beneficial grass-legume mixtures are to N use efficiency and productivity because of species-specific responses of N fixation to soil fertility (Vitousek et al. 2002; Pirhofer-Walzl et al. 2012). Despite this, only a few studies have considered more than one legume species in mixtures with grasses (Nyfeler et al. 2009; Finn et al. 2013; Suter et al. 2015), and the interaction between N fertilisation and legume growth strategies or trait plasticity is not well known.

In this study, we investigated how mixtures of grass and legume species with contrasting growth strategies affect plant productivity, plant N uptake, and soil mineral N availability at two levels of N fertilisation. We tested the following hypotheses: H1. Increasing the diversity in plant strategies in species mixtures will relate positively with higher complementarity, N use efficiency and productivity; H2. Plant communities with fast-growing species (more acquisitive resource acquisition strategy) will have higher N use efficiency and maintain productivity under lower N fertilisation inputs (lower N soil levels). We tested our hypotheses in two complementing field experiments: 1) to determine the effect of plant growth strategy we grew two grass and two legume species with different growth strategies (fast- *vs.* slow-growing species) in mixtures of two and four-species with one level of N-fertilisation (100 kg N ha^{-1}), and 2) to determine the effect of fertiliser level in interaction with different plant growth strategies we grew two legumes and the fast-growing grass species in monocultures and two-species mixtures each at two levels of N-fertilisation (50 and 100 kg N ha^{-1}).

Materials and methods

Study site

The experimental site was located at Nergena, Wageningen, the Netherlands (51° 59′ 43.3"N, 5° 39′ 17.6"E, 9 m a.s.l.). The site is under maritime temperate climatic conditions, with mean annual temperature of 9.4 °C and mean annual precipitation of 780 mm (Fig. S1). The soil is a typic endoaquoll (Soil Survey Staff 2014) with 84% sand, 10% silt and 6% clay. Initial analyses of the properties of the upper 15 cm of the soil profile were: total N content 1.5 g kg^{-1}, total organic C content 21 g kg^{-1}, C:N ratio 14, plant available P 7.2 mg kg $^{-1}$, pH$_{\mathrm{CaCl2}}$ 5.6, and bulk density 1.25 g cm^{-3}.

Experimental design

The field experiment was established in September 2019 and consisted of two complementing studies each following a full-factorial, randomised block design. In Experiment 1, we tested the effects of plant community composition (11 plant communities with species richness ranging from 1 to 4) on productivity and N uptake at one level of fertilisation (100 kg N ha^{-1}). In Experiment 2, we tested the potential interactive effects of plant community composition (five plant community compositions) and N fertilisation level (50 and 100 kg N ha^{-1}) (Table 1). Both experiments comprised five replicates per treatment, randomly allocated across five blocks. Each block consisted of 16 plots of 9 m^2 each, totalling 80 plots (Fig. S2). Seeds of two grasses (*Lolium perenne* cv Barhoney and *Festuca arundinacea* cv Bardoux) and two legumes (*Trifolium pratense* cv Lemmon and *Lotus corniculatus* cv Lotar) were sown in monocultures, all two-species combinations, and a four-species mixture. Seeds were sourced from Barenbrug Holland B.V. (the Netherlands). The seeding density (recommended by the seed company) was 1,500 viable seeds m^{-2}, divided equally among the species of each mixture (i.e. 750 seeds m^{-2} for each species in the two-species mixtures, and 375 seeds m^{-2} for each species in the four-species mixture). The species are common in European grasslands and have contrasting growth strategies and traits (Oram et al. 2021). The two slow-growing species were *L. corniculatus* and *F. arundinacea,* while the fast-growing species were *L. perenne* and *T. pratense* (specific leaf area was higher in *L. perenne* compared to *F. arundinacea* (SLA = 54 *vs* 49 cm^2 g^{-1}, respectively), and higher in *T. pratense* compared to *L. corniculatus* (SLA = 32 *vs* 27 cm^2 g^{-1}, respectively). The two legumes differ in their capacity to fix N via biological N fixation (*L. corniculatus* Ndfa = 32%, *T. pratense* Ndfa = 58% (Table S4 from Oram et al. 2021). After sowing, the field experiment was weeded by hand to remove the non-target plant species as needed (primarily in March–April 2020) to maintain the original plant community composition. The plots were harvested three times during the growing season by cutting the vegetation on May 11, 2020 (T0), July 6, 2020 (T1), and August 10, 2020 (T2). We applied N fertiliser at two timepoints namely shortly after the harvests in May and July. We added calcium ammonium nitrate at a rate of 25 kg N ha^{-1} in the low fertilisation treatment (totalling 50 kg N ha^{-1}) or 50 kg N ha^{-1} in the high fertilisation treatment (totalling 100 kg N ha^{-1}). In May 2020 the plots were also fertilised with potassium sulphate at a rate of 31.5 kg K ha^{-1} in the low fertilisation treatment and 63 kg K ha^{-1} in the high fertilisation treatment.

Plant productivity and leaf traits

To quantify plant productivity of each species in the different plant communities, aboveground biomass was harvested at T0, T1, and T2 by clipping the vegetation 2 cm above ground level, sorting per plant species, drying at 70 °C, and weighing. To quantify the C and N content in the above-ground biomass and the level of N derived from N fixation from harvest at T2, dried samples were ground (ball-milled) and analysed for leaf C and N content, and for natural abundance of $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20–20 IRMS (Sercon Ltd., Cheshire, UK). Species-specific plant N uptake was calculated for the T2 sampling by multiplying the N concentration (%) by the above-ground biomass of each plant species.

Table 1 Overview of the treatment factors of the two complementing experiments. Experiment 1: plant community composition effects, and Experiment 2: plant community composition and fertilisation level effects on plant and soil response variables. All treatments were replicated five times. Plant species: grasses *Lolium perenne* (Lp) and *Festuca arundinacea* (Fa); legumes *Lotus corniculatus* (Lc) and *Trifolium pratense* (Tp). Schematic design can be found in Figure S2

Treatment factor	Experiment 1	Experiment 2
Plant community composition	11 different communities	5 different communities
- monocultures	4: *Lolium perenne*, *Festuca arundinacea*, *Trifolium pratense*, *Lotus corniculatus*	3: *Lolium perenne*, *Trifolium pratense*, *Lotus corniculatus*
- two-species mixtures	6: *Festuca arundinacea* + *Lolium perenne*, *Lolium perenne* + *Trifolium pratense*, *Lolium perenne* + *Lotus corniculatus*, *Festuca arundinacea* + *Trifolium pratense*, *Festuca arundinacea* + *Lotus corniculatus*, *Trifolium pratense* + *Lotus corniculatus*	2: *Lolium perenne* + *Trifolium pratense*, *Lolium perenne* + *Lotus corniculatus*
- four-species mixture	1: *Festuca arundinacea* + *Lolium perenne* + *Trifolium pratense* + *Lotus corniculatus*	–
Fertilisation level (kg N ha^{-1})	100	50 and 100

Specific leaf area (SLA, $cm^2\ g^{-1}$) was determined at T2 by sampling the youngest, fully expanded leaf from seven plants per plant species per plot, according to Pérez-Harguindeguy et al. (2013). Leaves were saturated by placing in moist paper towels in plastic containers, storing at 4 °C overnight, blotting dry and weighed. Saturated leaves were then scanned (Epson Perfection V700/750), area was determined with ImageJ (https://imagej.nih.gov/ij/https://imagej.nih.gov/ij/), and dried leaves (70 °C for 48 h) were weighed.

Soil mineral N analysis

We quantified soil mineral N at T0, T1, and T2 by taking four soil cores (Ø = 1.5 cm, depth = 25 cm) from each plot directly after each plant biomass harvest and before N fertilisation. Soil mineral N (NH_4^+ and NO_3^-) availability was determined by extracting 40 °C oven-dried soil with 0.01 M $CaCl_2$ (Houba et al. 2000) in a 1:10 ratio (soil weight: extractant volume, dry weight basis) and analysed by colorimetry (Brann en LuebbeTrAAcs 800 Autoanalyzer, Skalar Analytical B.V. Breda). Soil gravimetric moisture content was determined after drying fresh soil at 105 °C for 24 h.

Data analysis

The N use efficiency (NUE) was estimated as the total biomass produced per unit plant N (An et al. 2005; Egan et al. 2019):

$$\mathrm{NUE} = \frac{biomass(i)}{biomass(i) * totalN(i)} \tag{1}$$

where biomass refers to above-ground biomass, and total N refers to leaf N concentration for each sample.

The percent of N derived from biological N fixation (Ndfa) was calculated using the $\delta^{15}N$ method (Boddey et al. 2000) from T2 only:

$$\%N_{dfa} = \frac{(\delta^{15}N_{ref-}\delta^{15}N_{legume})}{(\delta^{15}N_{ref-}B)} \times 100 \tag{2}$$

where $\delta^{15}N_{ref}$ is the mean $\delta^{15}N$ value of the monoculture grasses, $\delta^{15}N_{legume}$ is the $\delta^{15}N$ value of the legumes in our experiment, and B is the $\delta^{15}N$ value of legumes inoculated with rhizobia and grown in N-free quartz sand, values from (Oram et al. 2021). The %Ndfa for mixtures containing both legume species were calculated as the average between the two legumes.

Functional trait diversity was calculated as 'functional dispersion' according to Laliberté and Legendre (2010) with the R function *fd_fdis* from the package *fundiversity* (Grenié And Gruson 2022). We included the following traits in the functional dispersion calculation: SLA, LDMC, leaf N concentration, leaf C concentration, leaf $\delta^{15}N$ and leaf $\delta^{13}C$. Community resource acquisition strategy was calculated by incorporating the community weighted mean (CWM) of SLA, LDMC, and leaf N into a principal component analysis using the *rda* function from the R package vegan (Oksanen et al. 2017). All traits were sourced from a greenhouse experiment using the same plant species grown in monoculture (Oram et al. 2021).

Plant resource acquisition strategy was determined using principal component analysis of the CWM of SLA, LDMC, and leaf N measured in the field, using the function *rda* from the R package *vegan* (Oksanen et al. 2017). CWM traits were scaled. Scores were extracted and PC1 was used as a measure of plant community resource acquisition strategy.

The relative mixture effect on plant species trait changes (i.e. trait shifts due to growing in a species mixture *vs.* in monoculture) was based on the trait values quantified on plant samples collected in the field experiments and calculated following Jung et al. (2010) for leaf N concentration, leaf C concentration, leaf C:N ratio, SLA, leaf $\delta^{13}C$ (a constant was added to negative values to make them positive) and Ndfa as shown below:

$$Relative\ mixture\ effect\ species\ (i) = \frac{mixture\ trait\ value\ species(i) - monoculture\ trait\ value\ species(i)}{monoculture\ trait\ value\ species(i)} \tag{3}$$

Diversity effects were calculated based on Loreau and Hector (2001). A positive net diversity effect occurs if species productivity in a mixture is on average higher than expected based on the average of the monoculture productivity of the component species. The net diversity effect results from complementarity and selection effects. A positive selection effect occurs when a species with high monoculture yields dominates a mixture. A positive complementarity effect occurs when species are generally more productive than expected in mixtures.

The complementarity was calculated as the difference between the net effect and the selection, and the selection effect was assessed by determining the covariance between the species monoculture productivity and their relative trait change in above-ground biomass from monoculture to mixture. The net effect refers to net biodiversity effect on the productivity of the plant species mixture. The net effect is the difference in productivity between observed productivity of plant species mixtures and the expected productivity based on the productivity of the monocultures taking into account the proportional seeding densities. The expected mixture productivity (PE) was calculated based on Loreau and Hector (2001) as shown in *Eq.* (4):

$$PE = \sum \left(RP_{E,i} X M_i \right) \tag{4}$$

where PE is the expected productivity of a mixture, based on the productivity of the monocultures of the component species; $RP_{E,i}$ is the expected relative contribution of species i to productivity in the mixture (the expected contribution of each species was assumed to be proportional to the proportion of seed sown for each species in the species mixture, i.e. 1:2 in the two-species mixtures and 1:4 in the four-species mixtures); Mi is the productivity of species i in monoculture.

Statistical analyses

Experiment 1

Linear mixed effects (LME) models (*nlme* package, Pinheiro et al. (2017) were used to test the effect of plant species richness (monoculture, two, and four-species mixtures) or plant community composition on above-ground biomass (separately for T0, T1, T2, and the cumulative biomass of the three harvests combined), plant N uptake, overyielding, N use efficiency, Ndfa, soil NH_4^+-N and NO_3^--N concentrations (mean of two sampling times), and diversity effects (complementarity, selection and net effect). Fixed effects were species richness or plant community composition, and the random effect was block. Two out of ten above-ground biomass samples of *L. corniculatus* at T2 (*L. corniculatus* in the four-species mixture and in the mixture with *L. perenne*) were too small to be analysed for C, N, $\delta^{13}C$ and $\delta^{15}N$, thus these were not included in the analyses of plant N uptake, Ndfa and N use efficiency of these plots.

LME models were also used to test the effect of plant functional group (grass or legume) and growth strategy (fast- and slow-growing species) and time-point (T0, T1, and T2) on above-ground biomass in Experiment 1. Fixed effects were functional group or plant strategy, time-point and their interaction, and random effect was block/plot.

LM models were used to test the relative importance functional traits and growth strategy. Fixed effects were functional traits and growth strategy.

Experiment 2

We used LME models to test the interactive effect of plant community composition (five plant communities) and fertiliser level (low or high) on overyielding, soil mineral N (NH_4^+-N + NO_3^--N, T2), N use efficiency, and Ndfa. Fixed effects were plant community composition and fertiliser level, and random effect was block.

Experiments 1 & 2

All data were checked for normality and equal variances using residual plots and log-transformed where necessary before analysis (i.e. above-ground biomass, plant N uptake, N use efficiency). We used the weight function *varIdent* from R package *nlme* (Pinheiro et al. 2017) to account for unequal residual variances following (Zuur et al. 2011). This was necessary to improve model fit for the following response variables: above-ground biomass, plant N uptake, Ndfa, soil NH_4^+-N and diversity effects (net, complementarity, and selection effects). The significance of the fixed effects was determined by comparing models with and without the factor of interest using a likelihood ratio test. We determined pairwise comparisons with Tukey post hoc using *emmeans* (Lenth 2020). All statistical analysis was carried out in the R version 4.0.2 (R Core Team 2020).

Results

Functional dispersion and plant species richness explaining diversity effects

We found that increasing species richness from one to four species significantly increased above-ground biomass, plant N uptake, N use efficiency, and Ndfa (Fig. 1 abcd, Table S2), and decreased soil NO_3^--N (Fig. 1 f, Table S2). Furthermore, increasing species richness from two to four species significantly increased complementarity effects (Fig. S3, Table S2).

Above-ground biomass and complementarity effects significantly increased with increasing functional trait dispersion (Fig. 2 ac) but were not related with plant community resource acquisition strategy (Fig. 3 ab). Functional trait dispersion of traits or strategies was not related with N use efficiency (Fig. 2 ef), nor did plant community resource acquisition strategy influence N use efficiency (Fig. 3 c).

Functional group was more important in explaining above-ground biomass, plant N uptake, N use efficiency, Ndfa and soil mineral N compared to growth strategy (Table 2). There was no difference in the explanatory power of functional traits or strategies on complementarity effects (Table 2).

Specific plant community composition and its effect on trait plasticity

The cumulative plant productivity across all harvests was highest in the mixture *F. arundinacea* + *T. pratense* (1249 g m^{-2}), exceeding that of most other plant communities (1134 g m^{-2}), although it was not significantly higher than that of the other treatments in which *T. pratense* was present (Fig. 4a). Plant N uptake was higher in both legumes, and in combinations with the fast-growing legume *T. pratense* (Fig. 4b). Nitrogen use efficiency was higher in four-species mixture, followed by mixtures containing the slow-growing grass *F. arundinacea* (*F. arundinacea* + *L. perenne* and *F. arundinacea* + *L. corniculatus*) (Fig. 4c). Plant community composition did not affect soil NH_4^+-N levels (Fig. 4d). However, levels of NO_3^--N in the soil differed significantly and legume monocultures and combinations containing *T. pratense* had higher soil NO_3^--N (Fig. 4e). Combinations of *T. pratense* with either fast- or slow-growing grass species, and the four-species mixture, resulted in overyielding ($P < 0.05$, Table S2c). The complementarity effect was highest in combinations with *T. pratense* ($P < 0.05$, Table S2).

Mixtures including *L. corniculatus* and either the fast- or slow-growing grass, or the mixture with both grasses had the lowest soil mineral N and also the lowest plant N uptake (Fig. 5). The mixture of *T. pratense* and *L. corniculatus* had the highest plant N uptake and the highest soil mineral N (Fig. 5). In contrast, combining *T. pratense* with either *F. arundinacea* or *L. perenne* had high plant N uptake, and relatively low soil mineral N (Fig. 5).

Plant species differed in their trait values and for several species there was also an intraspecific shift in trait values for several plant traits between individual plants growing in mixture compared to when grown in monoculture (Fig. 6). The slow-growing grass *F. arundinacea* had a higher leaf N content, SLA, and lower leaf C:N ratio when growing with *T. pratense* compared to *F. arundinacea* grown in monoculture ($P < 0.05$, Fig. 6). *F. arundinacea* also had lower leaf C:N ratio, leaf $\delta^{13}C$, and higher SLA when growing in the four-species mixture compared to *F. arundinacea* grown in monoculture ($P < 0.05$, Fig. 6). The slow-growing legume *L. corniculatus* increased Ndfa when growing with grasses and in the four-species mixture and decreased it when growing with *T. pratense*. The fast-growing legume *T. pratense* had higher Ndfa in grass mixtures compared to monocultures ($P < 0.05$, Fig. 6).

Interactions between plant community composition and fertiliser level

Both above-ground biomass and plant N uptake were higher when 100 kg N ha^{-1} was applied compared to 50 kg N ha^{-1}, regardless of plant community composition ($P < 0.05$, FERT, Table 3).

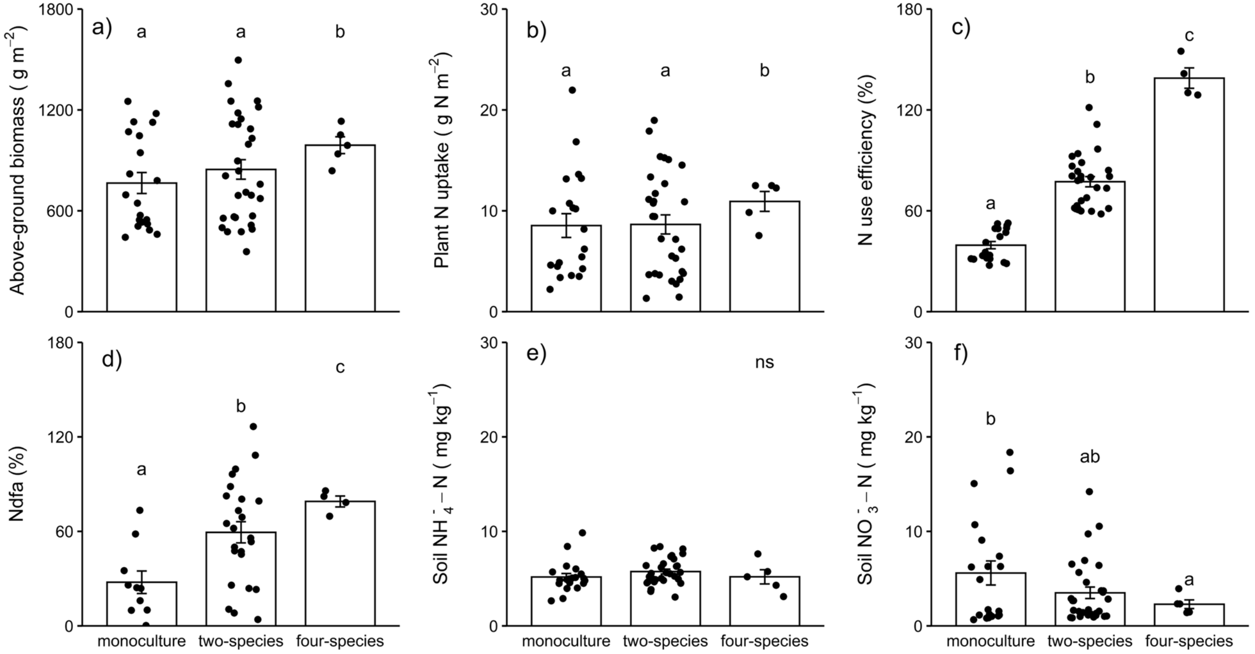


Fig. 1 Plant monoculture and species mixture (two and four-species) effects on a) above-ground biomass (cumulative for the three harvests), b) plant N uptake (final harvest), c) N use efficiency (final harvest), d) N fixation, expressed as N derived from the atmosphere (Ndfa, %), e) soil NH_4^+-N and f) soil $NO_3.^-$-N (mean of two soil samplings). Bars are mean ± SE ($n = 20$ for monoculture, $n = 30$ for two-species and n = 5 for four-species mixtures, plant N uptake, NUE, Ndfa $n = 4$) from Experiment 1. Dots indicate values of individual plots. Different letters above the bars indicate significant differences between species richness levels ($P < 0.05$) based on a Tukey posthoc test (ns: non-significant). Statistical analyses can be found in Fig. S1

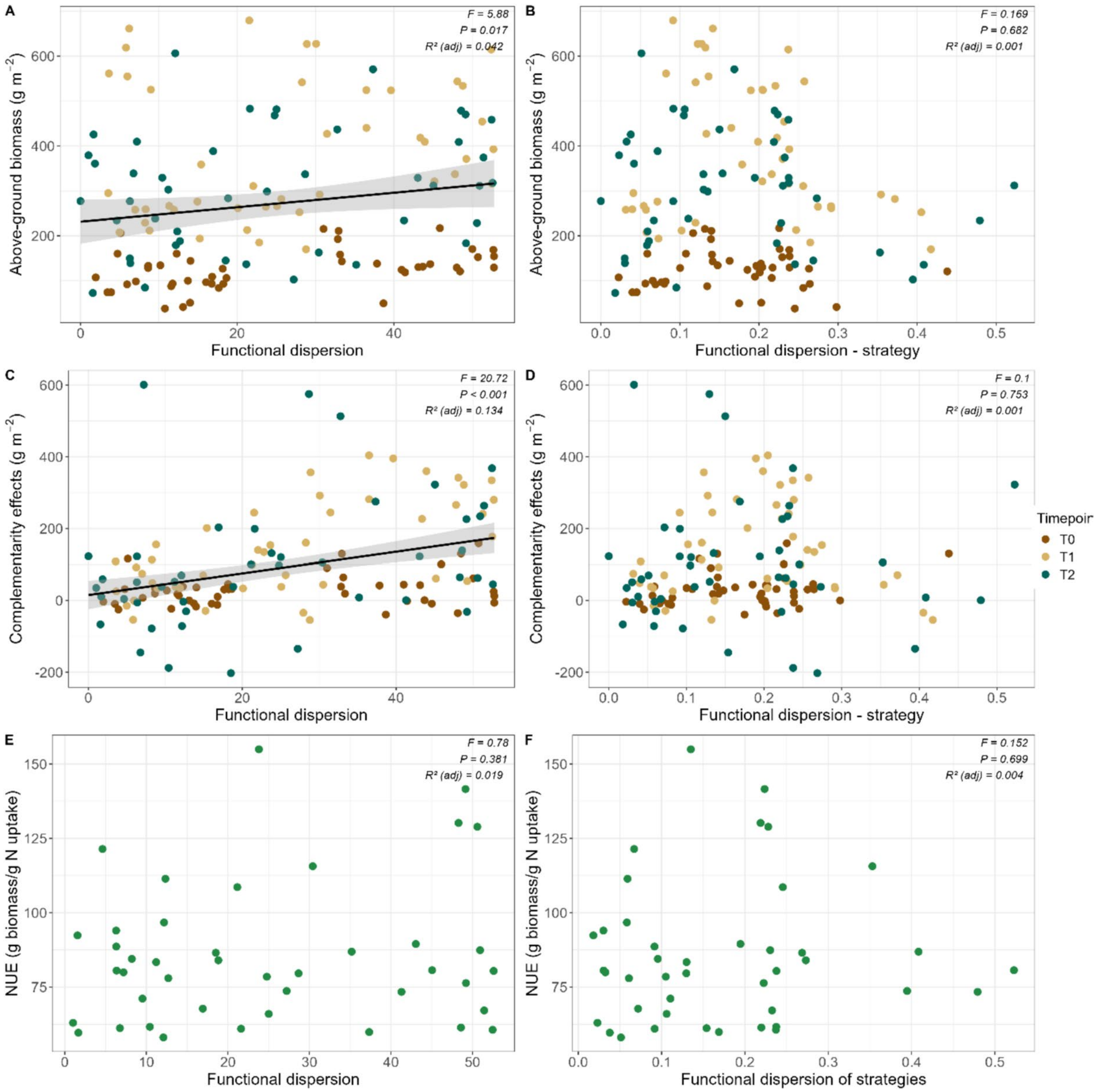


Fig. 2 Relationship between above-ground biomass, complementarity effects (Loreau & Hector 2001), nitrogen use efficiency (NUE) on functional dispersion (Laliberté & Legendre 2010). Effects were tested with linear mixed effects models with block/plot as a random structure to account for variation across the field site (block) and pseudo replication (plot). Interactions with fertilisation (low/high) and timepoint (T0, T1, T2) were tested and were never significant. Thus, the remaining model contained only one explanatory variable. F- and P-values, and model R^2 (explanatory power of the model) are shown on each panel. Traits considered in functional dispersion were specific leaf area (SLA), leaf dry matter content (LDMC), leaf C and N concentrations (% C and N in above-ground biomass), leaf natural abundance δ^{13}C and δ^{15}N. Functional dispersion of plant community strategy was calculated by determining the mean 'strategy' of each species (mean value of PC1, Figure S4). Traits were taken from a greenhouse experiment (Oram et al. 2021) and weighed by the relative abundance of above-ground biomass of each plant species in the field experiment

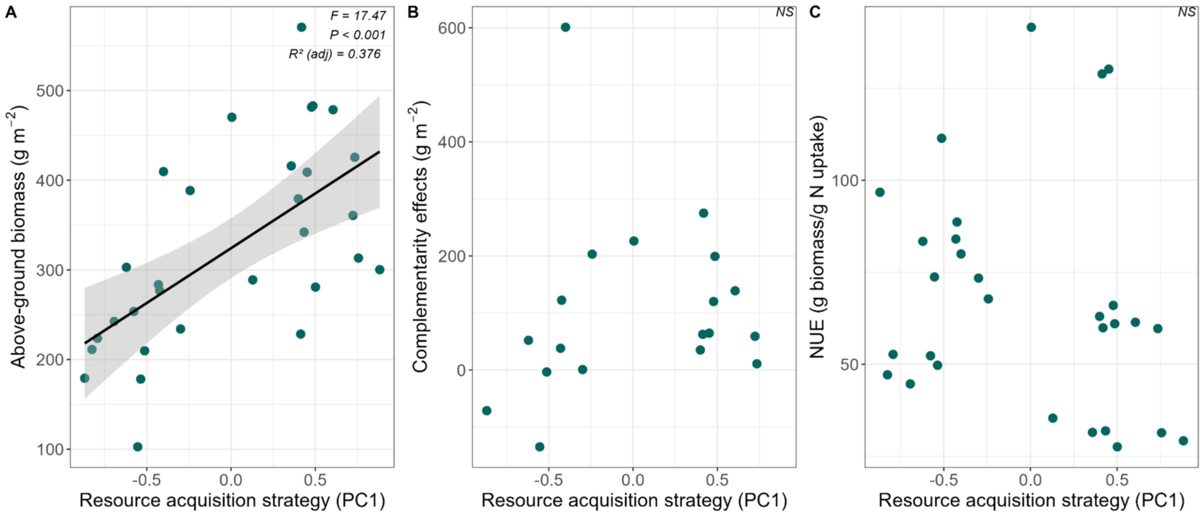


Fig. 3 Relationships between resource acquisition strategy (PC1) and (A) above-ground biomass, (B) complementarity effects, and (C) nitrogen use efficiency (NUE) at T2. A high PC1 value indicates a more acquisitive plant community (see Fig. S5). Ns: non-significant. F- and *P*-values, and model R^2 (explanatory power of the model) are shown on the panel

Table 2 Effect of functional groups *versus* growth strategy on above-ground biomass, plant N uptake, N use efficiency, Ndfa (%), complementarity effects and soil mineral N concentration from T2-end of experiment. Linear models were used to test the relative importance functional traits and growth strategy. Fixed effects were functional traits and growth strategy (significant effects are in bold, $P < 0.05$)

Factor		Above-ground biomass	Plant N uptake	N use efficiency	Ndfa	Complementarity effects	Soil mineral N
Functional group	F	7.27	9.22	18.6	15.4	4.1	30.8
	P	**0.0001**	**0.00001**	**< 0.0001**	**< 0.0001**	**0.03**	**< 0.0001**
Growth strategy	F	4.43	5.72	2.1	0.8	7.0	4.3
	P	**0.008**	**0.002**	0.11	0.51	**0.004**	**0.02**
	R^2	0.39	0.46	0.60	0.53	0.40	0.71

Monocultures of *T. pratense* and *L. corniculatus* had the highest above-ground biomass in both fertiliser treatments ($P < 0.05$, Table 3). The mixture of *L. perenne* and *T. pratense* was as productive as the monoculture *T. pratense*, and twice as productive as *L. perenne* in monoculture ($P < 0.05$, Table 3). There was an interactive effect between fertilisation rate and plant community composition on overyielding of plant N uptake. The two-species mixture *L. perenne* and *T. pratense* showed N overyielding, at the high level of fertilisation (100 kg N ha^{-1}) but not at low fertilisation level ($P = 0.04$, FERT * PLANT COMP, Fig. S6, Table 3). Soil mineral N (NH_4^+-N + NO_3^--N) concentrations were highest in the legume monocultures, irrespective of fertilisation level ($P < 0.05$, PLANT COMP, Table 3).

The N use efficiency of the plant communities was dependent on the interaction between plant species composition and fertilisation rate ($P < 0.05$, FERT * PLANT COMP, Table 3). Both mixtures with *L. perenne* (combined with either *T. pratense* or *L. corniculatus*) and *L. perenne* monoculture decreased N use efficiency with an increase in N fertiliser level (100 kg N ha^{-1}), while both legumes were not affected (Fig. S6).

There was a significant interactive effect between plant community composition and fertiliser rate on the level of N fixation as quantified by Ndfa (*P = 0.05*, FERT * PLANT COMP, Table 3). In the

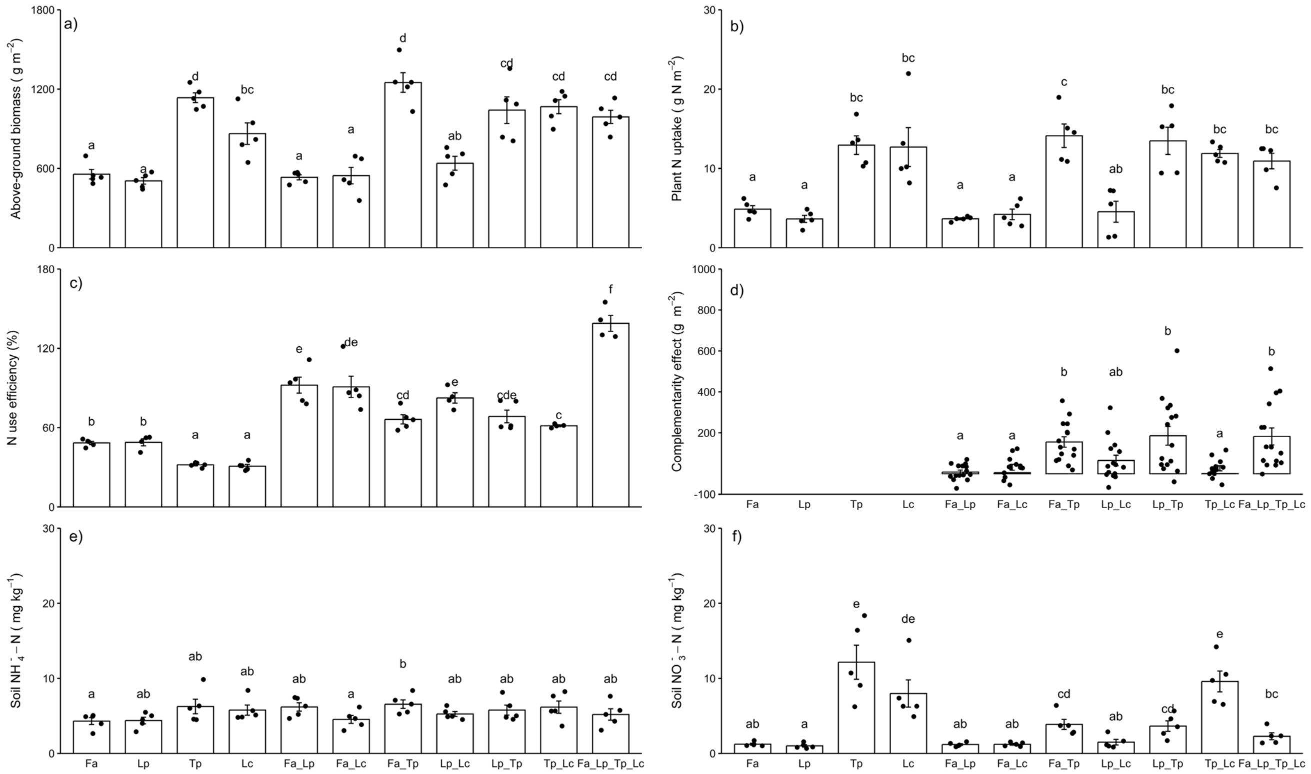
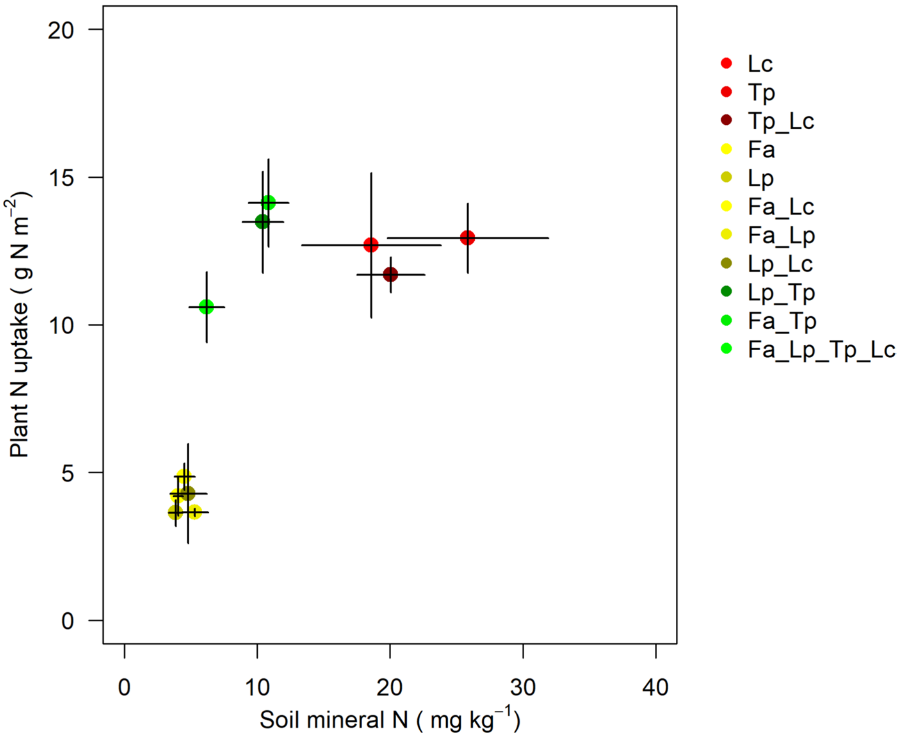


Fig. 4 Plant species composition effect on a) above-ground biomass (sum of the three harvests), b) plant N uptake, c) N use efficiency, d) complementarity effect, e) soil NH_4^+-N and f) soil $NO_3.^-$-N. Dots indicate values of individual plots. A linear mixed effect model was used to test the treatment effect. *Lolium perenne* (Lp); *Festuca arundinacea* (Fa); *Lotus corniculatus* (Lc); *Trifolium pratense* (Tp). Different letters indicate significant differences between the plant communities within each panel ($P < 0.05$) based on a Tukey posthoc test. Statistical analyses can be found in Fig. S1

Fig. 5 Relationship between plant N uptake (g N m^{-2}) and soil mineral N (mg kg.$^{-1}$) for each plant community composition at the final harvest-T2 of Experiment 1. *Lolium perenne* (Lp), *Festuca arundinacea* (Fa), *Lotus corniculatus* (Lc), *Trifolium pratense* (Tp). It shows clearly three clusters while dots represent mean of five replicates, bars represent the variation in standard error (SE)



grass-legume mixture, *L. perenne* and *T. pratense*, the legume maintained its level of N fixation irrespective of the level of N fertilisation, whereas *L. corniculatus* (in the *L. corniculatus* and *L. perenne* mixture) showed higher levels of N fixation at lower level of N fertilisation (69% under 50 kg N ha^{-1} and 98% under 100 kg N ha^{-1}; Fig. S6, Table 3).

Discussion

The aim of this study was to investigate how mixtures of plant species from different functional groups (grasses or legumes) with contrasting growth strategies (fast- or slow-growing species) affect plant productivity, N uptake, and soil mineral N, and how these effects depend on N fertilisation level in a managed grassland system. We found that increasing species richness from one to four species increased above-ground biomass and plant N uptake. The growth strategies of the legumes were of prime importance for these diversity effects; overyielding was only achieved when combining the fast-growing legume (higher Ndfa) with either slow- or fast-growing grass species, whereas the slow growing legume (*L. corniculatus*) biomass decreased in the mixtures at high N fertilisation (100 kg N ha^{-1}). At low level of N fertilisation, the slow-growing legume had very high N fixation when growing in combination with the fast-growing grass compared to growing alone. This suggests that slow-growing legumes like *L. corniculatus* could contribute to reducing the reliance on N fertiliser in managed grasslands, provided they get a chance to establish well with sufficient time and optimal conditions to develop a strong root system.

Higher diversity in plant strategies in plant species mixtures increased diversity effects (Experiment 1)

We found that functional groups were more important in explaining above-ground biomass, plant N uptake, N use efficiency, Ndfa and soil mineral N compared to growth strategy (Table 2). Plant communities with a diverse range of functional trait values exhibit improved N response efficiency. This enhancement is primarily due to increased N uptake efficiency, attributed to the presence of functionally diverse species that optimise resource use. Plant communities with specific functional traits can regulate N-cycling

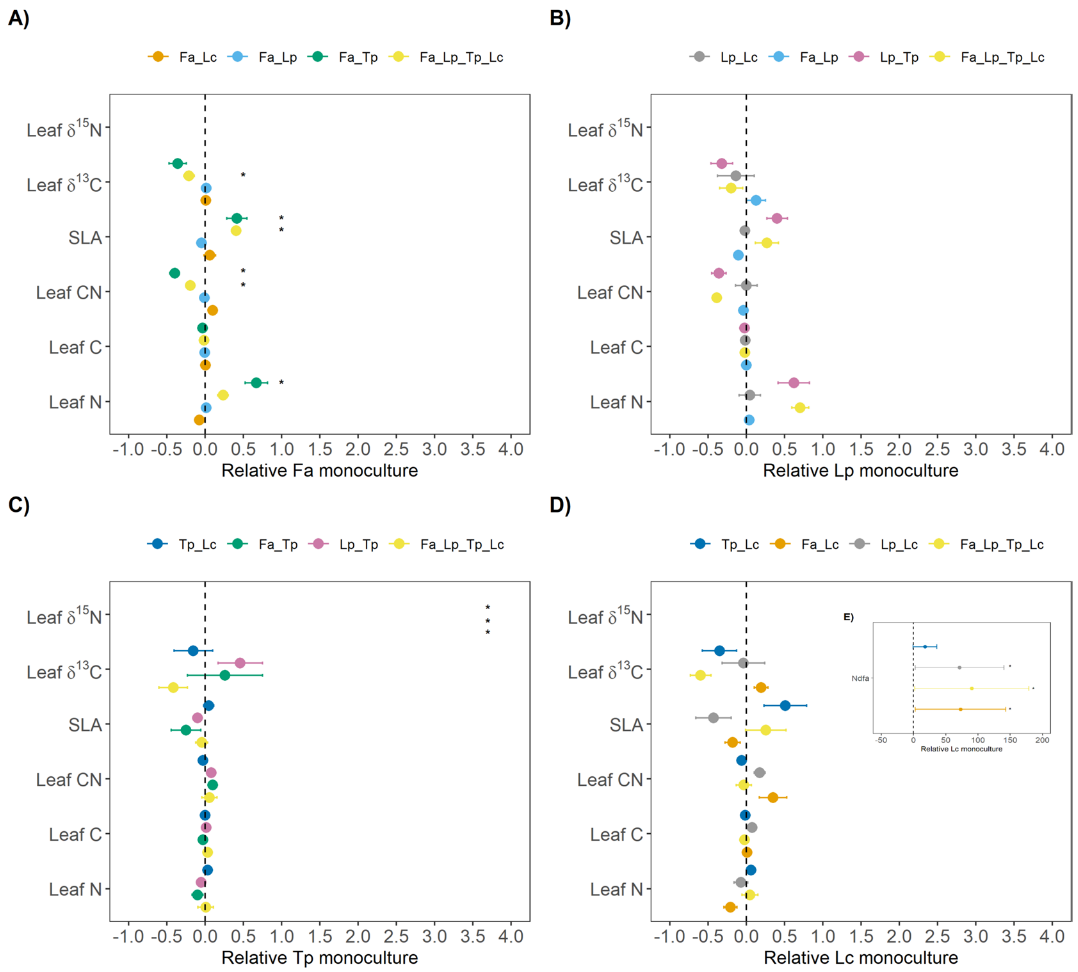


Fig. 6 The effect of growing in a species mixture on plant traits relative to the plant traits of the monoculture for A) *Festuca arundinacea* (Fa), B) *Lolium perenne* (Lp), C) *Trifolium pratense* (Tp), D) *Lotus corniculatus* (Lc) and E) *Lotus corniculatus* (Lc) for Ndfa only, from Experiment 1. Asterisks indicate a significant difference in the trait of that plant species between its monoculture and when grown in the plant species mixture. The reference trait value of the monoculture is indicated by the dashed line at x = 0. Values above 0 indicate that the trait value of the plant species is higher in the mixture than in the monoculture, and below 0 indicate that the trait value is lower

in intensively managed grasslands. For instance, incorporating legumes alongside grasses with high root length density can enhance plant N uptake and reduce soil mineral N levels, leading to more efficient N-cycling (Abalos et al. 2021). Although we did not measure root traits in our experiment, our grasses may exhibit contrasting root traits; for example, root length density and specific root length can be higher for *L. perenne* compared to *F. arundinacea*, indicating a higher ability to access resources (Abalos et al. 2021; Velasco-Sánchez et al. 2024).

The four-species mixture had higher above-ground biomass and plant N uptake than the average of the monocultures. Over the entire experiment, mixtures had significantly higher productivity compared to the average of the four species monocultures (overyielding), and the combination between *T. pratense* and *F. arundinacea* had higher productivity compared to the most productive species' monoculture (*T. pratense*), i.e. transgressive overyielding. These findings are consistent with other studies in fertilised grasslands that show overyielding effects (Kirwan et al.

Table 3 Effects of fertilisation level (FERT) and plant community composition (PLANT COMP) on cumulative above-ground biomass (sum of three harvests), plant N uptake (last harvest), overyielding, soil mineral N (last soil sampling, T2), N use efficiency, and N derived from atmosphere (Ndfa), in Experiment 2. Data are mean ± SE ($n = 5$). *Lolium perenne* (Lp), *Festuca arundinacea* (Fa), *Lotus corniculatus* (Lc), *Trifolium pratense* (Tp). Significance tests using likelihood ratio test (LRT) comparing models with or without parameter of interest where degree of freedom shows the difference in degrees of freedom between the models. Significant effects ($P < 0.05$) are shown in bold. Letters indicate per response variable significant differences between the plant communities ($P < 0.05$) based on Tukey posthoc test. Interactions between fertilisation level (FERT) and plant community composition (PLANT COMP) on overyielding, nitrogen use efficiency and Ndfa are shown in Fig. S6

	Cumulative above-ground biomass	Plant N uptake	Overyielding net effect		Soil mineral N (NH_4^+-N + NO_3^--N)	N use efficiency	Ndfa
	g m^{-2}	g N m^{-2}	g m^{-2}	g N m^{-2}	mg kg^{-1}	%	%
FERT							
50	661.4 ± 48	6.3 ± 0.7	15.7 ± 29	−0.3 ± 0.9	8.7 ± 1.1	60.4 ± 5.3	53.4 ± 8.0
100	825.6 ± 58	9.4 ± 1.1	51.3 ± 44	1.2 ± 1.9	12.7 ± 2.3	51.3 ± 4.5	49.4 ± 7.4
PLANT COMP							
Lp	453.6 ± 23 ^{a}	2.8 ± 0.4 ^{a}	-	-	4.0 ± 0.4 ^{a}	57.1 ± 2.9	-
Tp	1063.8 ± 47 ^{c}	11.8 ± 0.7 ^{b}	-	-	20.3 ± 3.6 ^{c}	32.3 ± 0.4	34.3 ± 6.2
Lc	749.9 ± 59 ^{b}	10.3 ± 1.5 ^{b}	-	-	15.1 ± 2.9 ^{bc}	31.6 ± 1.2	16.0 ± 3.5
LpLc	530.6 ± 49 ^{a}	3.7 ± 0.7 ^{a}	−40.4 ± 19 ^{a}	−2.9 ± 0.7	4.3 ± 0.7 ^{a}	88.5 ± 4.9	87.8 ± 8.3
LpTp	919.6 ± 67 ^{bc}	10.7 ± 1.3 ^{b}	107.5 ± 35 ^{b}	3.4 ± 1.2	9.9 ± 0.9 ^{b}	74.2 ± 3.5	71.2 ± 4.4
FERT	LRT = 18, **$P < 0.0001$**	LRT = 15, **$P = 0.0001$**	LRT = 1, $P = 0.35$	LRT = 0.1, $P = 0.7$	LRT = 4, **$P = 0.03$**	LRT = 7, **$P = 0.008$**	LRT = 0.5, $P = 0.4$
PLANT COMP	LRT = 74, **$P < 0.0001$**	LRT = 48, **$P < 0.0001$**	LRT = 10, **$P = 0.0008$**	LRT = 12, **$P = 0.0004$**	LRT = 61, **$P < 0.0001$**	LRT = 118, **$P < 0.0001$**	LRT = 57, **$P < 0.0001$**
FERT * PLANT COMP	LRT = 3, $P = 0.54$	LRT = 6, $P = 0.18$	LRT = 1, $P = 0.2$	LRT = 4, **$P = 0.04$**	LRT = 4, $P = 0.3$	LRT = 11.5, **$P = 0.02$***	LRT = 8, **$P = 0.05$***

2007; Nyfeler et al. 2009; Suter et al. 2015; Abalos et al. 2021). We suggest that the potential mechanism explaining this increase in productivity could be the increase in N use efficiency, and in complementarity use of soil N by the grasses and N obtained through N fixation by the legumes. The positive diversity effects were due to positive complementarity effects (rather than the chance of including a highly productive species, i.e. selection effects), agreeing with previous studies in semi-natural and fertilised grassland (Barry et al. 2019; Mason et al. 2020). The increase in biological N fixation can lead to a reduction in leaf C:N ratio of the species mixtures, indicating reduced competition for soil mineral N with a facilitative role for legumes (Temperton et al. 2007). In our fertilised grassland experiment, we found a decrease in the leaf C:N ratio of the grasses and thereby increased forage (high N content) quality when growing in mixtures with the fast-growing legume, but this was not the case when grown with the slow-growing legume. As the legume grows slowly it does not compete much directly with the grasses, but the grasses compete a lot amongst each other for a limited pool of N. When there is more N fixed there is more total N in the soil system and relatively more N from the grasses so somewhat less intraspecific competition in the grass enabling lower leaf C:N. Although there was only one four-species mixtures in our experiment at one fertilisation level (100 kg N ha^{-1}), and caution is needed when drawing general conclusions, our results align with other studies showing increases in N use efficiency and biological N fixation with increasing plant species richness (Cummins et al. 2021; Grange et al. 2021).

Legume growth strategy was a key driver of above-ground biomass and soil mineral N in our experiment. Combinations with *T. pratense*, the fast-growing legume, were associated with overyielding and could be explained by plant trait plasticity (Mason et al. 2020). Biological N fixation by *T. pratense* increased when it was growing with grasses. This grass-legume combination resulted in reduced soil mineral N due to high soil N uptake by the grasses, which stimulated the fast-growing legume to fix more N from the atmosphere (i.e. increase Ndfa, Fig. 3). The overyielding observed in the specific combinations between *T. pratense* and *F. arundinacea*, and in the four-species mixture, could be influenced by intraspecific trait shifts. In these plant species combinations, *F. arundinacea* plants had lower leaf C:N ratio and higher SLA compared to its monoculture plants (Fig. 6), that could suggest lower N limitation and larger light interception for *F. arundinacea* in this plant community. This was also observed in other studies under N rich conditions: a decrease in leaf C:N in two dominant grass species (Chen et al. 2005) and an increase in SLA (Al Haj Khaled et al. 2005). Trait plasticity has been linked to overyielding in other studies (Thein et al. 2008; Roscher et al. 2018; Yang et al. 2022), although (Mason et al. 2020) found only a weak relationship between intraspecific trait plasticity and overyielding relative to resource use efficiency (water, N, and light).

In our experiment, although *L. corniculatus,* the slow-growing legume*,* had high productivity in monocultures, it was suppressed when grown in mixtures. This contrasts with the high productivity observed for this species in species mixtures in other experiments (De Deyn et al. 2009). Frequently, the mixtures performance can be closely related to the performance of individual species i.e. their establishment rate, and their different shoot and root traits (Egan et al. 2019). However, the slow establishment rate of *L. corniculatus* in our experiment may have allowed other species in the mixture (i.e. fast-growing species, *T. pratense* and *L. perenne*) to out-compete it for light and nutrients. In addition, seeds were sown in our field experiment to represent agricultural practice, whereas in De Deyn et al. (2009) the plant communities were not established by seeding but by planting seedlings, with an equal number of individuals per plant species per area, and plants were not fertilised during the experiment. These reasons could have contributed to the poor (but possibly more realistic) competitivity of *L. corniculatus* in species mixtures in our fertilised grassland experiment.

Overall, taking into consideration plant N uptake and N loss to the environment, we found that legume monocultures and their combination (*T. pratense* + *L. corniculatus*) had the highest plant productivity, but they also had the highest soil mineral N levels (Fig. 5). This is not a surprise as many studies have shown that legume species can increase mineral N due to the release of fixed N from their roots via decomposition, and their inefficiency in acquiring soil mineral N alone (Niklaus et al. 2006; Barneze et al. 2020). Yet, we found that mixing the legumes with either grass species was an effective way to reduce soil mineral N strongly compared to legume monocultures, regardless of the grass growing strategy. This is an important finding, because decreasing soil mineral N availability is paramount for sustainable production systems, as this N pool is highly susceptible to be lost from the agroecosystem in the form of nitrate leaching or as nitrous oxide. Previous studies have shown reductions of soil mineral N with increasing plant biomass production (Abalos et al. 2014, 2018). Although it is difficult to generalise and to transfer the results from our study to other fast- *vs* slow-growing grasses and N fixers because we only had two grasses and two legumes, the selected species are very common in managed grasslands and therefore these findings are relevant for on-farm applications in temperate grasslands.

Fertiliser level determines the performance of legumes in grass-legume mixtures (Experiment 2)

At low fertilisation level (50 kg N ha^{-1}) we did not observe an increase in plant productivity (or plant N uptake) in the plant community with a more acquisitive resource acquisition strategy (*L. perenne* + *T. pratense*), contradicting our second hypothesis. However, at the higher fertilisation level, the *L. perenne* + *T. pratense* combination (together with the legumes) showed the highest plant community productivity (above-ground biomass) and N uptake, yet with high soil mineral N level at potential risk of getting lost. There was an interaction effect between N use efficiency and N fertilisation level, at low fertilisation level, N use efficiency was higher in the mixtures (*L. perenne* + *T. pratense* and *L. perenne* + *L. corniculatus*) compared to the monocultures, partly agreeing with our second hypothesis.

Overall, these results confirm the challenges of simultaneously achieving high plant biomass production quantity and quality and low soil mineral N levels, to reduce risks of N losses, in grasslands. Recommendations for optimum grass-legume mixtures will therefore depend on the specific priority for a given site: either a farmer-driven focus on production, or a policy-driven minimisation of N losses. Nevertheless grass-legume mixtures are preferable over grass monocultures as the N fixation by the legumes especially in grass-legume combinations can (partly) replace the use of mineral N fertiliser and therefore the greenhouse gas emissions associated with fertiliser production.

We observed higher overyielding in the mixture with the slow-growing legume (*L. perenne* + *L. corniculatus*) at low fertilisation level relative to high fertilisation level (Fig. S6). Although the grass-legume mixture with *T. pratense* had 1.6 times higher productivity compared to the mixture with *L. corniculatus*, the slow-growing legume was able to increase its N supply from atmospheric N by 11% in the treatment with 50 kg N ha^{-1} compared to 100 kg N ha^{-1} (Ndfa = 98%; Table 3, Fig. S6). This increase in biological nitrogen fixation by *L. corniculatus* grown with *L. perenne* suggests potential for reducing N fertilisation without compromising plant community N uptake or plant productivity. However, a key knowledge gap remains in understanding the conditions (e.g. seeding rates, fertiliser application timing) that support better establishment and development of species with lower competitive ability in the short-term but with high potential to contribute to plant productivity and N provisioning on the longer-term.

While this study offers important findings into plant species interactions over a growing season, its short duration may not fully reflect the longer-term dynamics of perennial grassland systems. This is particularly relevant for slow-growing legumes like *L. corniculatus*, whose competitive potential may only become evident over multiple years. Given that perennial plant species often exhibit delayed responses to interspecific interactions and environmental variability, caution is needed when interpreting short-term results. These temporal limitations highlight the importance of longer-term studies to better understand the sustainability and resilience of diverse, low-input grassland systems.

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Data availability The datasets generated during the current study are available from the corresponding author on request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose. The authors declare no conflict of interest.

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