

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

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

### 14 **Abstract**

15 **Background and aims:** In mixed stands of forage legumes and non-legumes, substantial amounts of legume-derived  
16 Nitrogen (N) are relocated to neighboring non-legumes. However, there is sparse information about how deep rooted  
17 non-legume forage herbs (forbs) influence N transfer in multi-species grasslands.

18 **Methodology:** Six three-species grassland mixtures each containing perennial ryegrass (*Lolium perenne* L.) and red  
19 clover (*Trifolium pratense* L.) in different proportions were grown together with one of three forb species: chicory  
20 (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.) or caraway (*Carum carvi* L.). Red clover leaves were  
21 labeled with <sup>15</sup>N-urea to determine the N transfer from red clover to companion ryegrass and forbs.

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

25 **Conclusions:** Grass relied to a great extent on clover N, whereas forbs relied on soil N. Soil <sup>15</sup>N-enrichment indicated  
26 that N transfer occurred in the upper soil layers and that a dependence on clover-derived N did not necessarily give  
27 grass a growth advantage.

28 **Key words:** grassland mixture; red clover; perennial ryegrass; non-leguminous forb; <sup>15</sup>N-leaf labeling

### 29 **Introduction**

30 In agriculture, forage legumes have been shown to provide a high-quality forage (Luscher et al, 2014), promote the  
31 buildup of soil N fertility and increase plant productivity (Carlsson and Huss-Danell 2003). In grassland mixtures of  
32 forage legumes and non-legumes, the legumes improve N supply and thereby the growth of companion non-legume  
33 species through biological N<sub>2</sub>-fixation and N rhizodeposition (Dahlin and Stenberg 2010; Høgh-Jensen and Schjoerring  
34 2001). Rhizodeposition occurs via the decomposition of dead plant parts including nodules and roots cells and plant  
35 root exudation of soluble N compounds (Fustec et al. 2010). In addition, direct facilitative N transfer has been shown to  
36 occur from N-rich legume plants to companion non-legume plants (Høgh-Jensen and Schjoerring 2000) and vice-versa  
37 (Gylfadottir et al. 2007; Carlsson and Huss-Danell 2014). It occurs either through the exudation of low-molecular-  
38 weight soluble organic (Paynel et al. 2001; Rasmussen et al. 2013) or inorganic (Paynel et al. 2008) N compounds from

39 living root cells and subsequently uptake by companion species through direct root contact or interconnecting roots  
40 between arbuscular-mycorrhizal fungi and the plant species (Haystead et al. 1988). In addition, interspecific root  
41 interaction between legume and non-legume enhances nodulation and N<sub>2</sub>-fixation of legume due to the stimulatory  
42 effect of root exudates from non-legume (Li et al. 2016)

43 Studies have shown that significant amounts of N of up to 15 -50% of legume N can be introduced into the soil N pool  
44 through transfer of legume-derived N to neighboring non-legume species (Dahlin and Stenberg 2010; Gylfadóttir et al.  
45 2007; Rasmussen et al. 2007; Rasmussen et al. 2013). Under field conditions, the transfer is affected by climatic or  
46 seasonal variation in plant growth and soil conditions (Rasmussen et al. 2013) and internal factors such as plant root  
47 system development and distribution in the soil (Pirhofer-Walzl et al. 2012), traits controlling root turnover and residue  
48 quality such as carbon to nitrogen ratio and lignin content (Louarn et al. 2015), the ratio between legume and non-  
49 legume biomass, C allocation within the non-legume and turnover rates of the plant roots in the soil (Rasmussen et al.  
50 2007), and the ratio of legume to non-legume N (Dahlin and Stenberg 2010). The N transfer is also influenced by  
51 management factors such as selection of legume and non-legume plant species and genotypes, age of the sward and  
52 harvesting time of plant materials (Carlsson and Huss-Danell 2014; Chalk et al. 2014). In addition, the plant species  
53 diversity of the grassland mixture has been found to affect the direction and magnitude of N transfer due to differences  
54 in the ability of non-legumes to acquire soil- and legume-derived N (Høgh-Jensen 2006; Pirhofer-Walzl et al. 2012), in  
55 the growth strength of the legume in the mixture (Rasmussen et al. 2013) and in the rate of legume N<sub>2</sub>-fixation (Paynel  
56 et al. 2008).

57 Temporary grasslands in mixed rotations are usually dominated by two plant functional groups: grasses and forage  
58 legumes. To date, studies conducted on grasslands have mostly focused on simple mixtures of forage legumes and  
59 grasses, but more recent studies have involved a multi-species mixture in temporary grasslands with a greater range of  
60 plant species, including non-leguminous dicot forage herbs (forbs). Chicory, ribwort plantain and caraway are three  
61 such promising forage herbs because of their high competitive ability (Eriksen et al. 2011; Sjøgaard et al. 2013) and  
62 tolerance of adverse weather conditions (Younie 2012). They have the potential to increase plant diversity and herbage  
63 yield (Sanderson et al. 2005; Sjøgaard et al. 2011) and also herbage quality in terms of its mineral nutrition (Pirhofer-  
64 Walzl et al. 2011), effect on animal health (Younie 2012) and palatability (Sjøgaard et al. 2008). In addition, their  
65 physiological and morphological characteristics include deep and diverse root systems (Eriksen et al. 2012; Li and  
66 Kemp 2005; Sjøgaard et al. 2013; Stewart 1996). Studies have suggested that deep-rooted species are efficient at

67 utilizing N from deeper soil layers (Pirhofer-Walzl et al. 2013; Thorup-Kristensen 2006), and that the plant diversity of  
68 deep and diverse root system confers variability to the pattern of N acquisition from soil and N transferred from N-rich  
69 companion legume plants (Lesuffleur et al. 2007; Pirhofer-Walzl et al. 2013; Pirhofer-Walzl et al. 2012). However,  
70 these deep-rooted forbs are still very rarely included in grassland mixtures in Europe and the dynamics of N processes  
71 in multi-species grasslands including forbs are very rarely investigated.

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

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

## 82 **Materials and methods**

### 83 **Experimental site and design**

84 The field experiment was conducted at Foulumgaard experimental station, Aarhus University in central Jutland,  
85 Denmark (09° 34'E and 56°29'N). The experimental field formed part of a dairy crop rotation with a history of grass-  
86 arable cropping and managed according to EU organic standards since 1987. The soil is a loamy sand characterized as a  
87 typical Hapludult, containing 7.7% clay, 1.6% carbon (Eriksen et al. 2015) and 0.12-0.16% total N. The monthly  
88 precipitation and mean monthly temperatures during the experimental period between April and October were 35– 117  
89 mm, and 8– 19°C, respectively. June, July and August were the warmest months, while May, August and October were  
90 the wettest (Figure 1).

91 Six grassland mixtures were established in spring 2013, each including one of the three forb species of chicory  
92 (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.) or caraway (*Carum carvi* L.) in addition to the  
93 traditional grassland mixture of perennial ryegrass (*Lolium perenne* L.) and red clover (*Trifolium pratense* L.). The

94 species were sown based on their seeding rate in a pure stand: 15, 4 and 12 kg ha<sup>-1</sup> for perennial ryegrass, red clover and  
95 forbs, respectively. The mixtures were either dominated by clover (mixtures 1-3) or grass (mixtures 4-6) at 80 percent  
96 of their pure stand seed rates (Table 1). The experimental plots were harvested twice during the year of establishment,  
97 and the experiment was conducted in the following growing season.

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

### 102 **Leaf-labeling**

103 Leaf-labeling with <sup>15</sup>N-enriched urea was used to determine N transfer (Ledgard et al. 1985). Five fully-developed  
104 medium sized clover leaves in each cylinder were gently inserted into 2-ml Eppendorf tubes containing 1 ml urea  
105 solution (0.5% w/v and 98 atom % <sup>15</sup>N). The vials were sealed with sticky-tack sealing materials (taking care not to  
106 damage leaf petioles or other parts of the plant) to avoid the loss of <sup>15</sup>N (e.g. evaporation) and intrusion of rainwater.  
107 After three to four days, the leaves were detached at the petioles and the vials were removed along with the entire leaf.  
108 At the end of labeling we observed that many tubes contained the same or even increased amounts of <sup>15</sup>N solution than  
109 applied. During labeling and removal of tubes, care was taken to prevent contaminating soil and plants with the labeling  
110 solution. Two labeling sessions were conducted during each of four growth periods (May, June, July to mid-August,  
111 mid-August to early October). The number of clover plants in the cylinder varied between one and five. One leaf from  
112 each plant was labeled when five plants were present in the cylinder, but two to five leaves from the same plants were  
113 labeled when numbers of plants were less than five in the cylinder. Leaves from the same plant but from another part  
114 were selected at the second labeling. Hence, clover plants were labeled eight times during the growing season,  
115 amounting to 40 leaves in total and resulting in 40 ml urea labeling solution in each cylinder. This supplied a total of  
116 1.36 g N m<sup>-2</sup>.

### 117 **Plant sampling and analysis**

118 Each growth period was terminated approximately two weeks after the second labeling by harvesting all above-ground  
119 plant material. The sampling was done manually by hand cutting with scissors to a stubble height of 5 cm. The first  
120 sample was taken on 27 May following labeling on 1 and 12 May; the second on 30 June following labeling on 23 and

121 27 June; the third on 15 August, with labeling on 11 and 28 July; and the final sampling on 3 October following  
122 labeling on 12 and 23 September. The interval between two labeling events and between the second labeling and  
123 sampling was shorter for the second sampling event than for the others. Unlabeled plant samples were collected from  
124 each plot, at a distance of at least 50 cm from the cylinder, for analyzing background  $^{15}\text{N}$ -abundance. The plant samples  
125 were sorted into individual species, dried at  $80^{\circ}\text{C}$  for 24 hours, and dry matter (DM) weight determined. Samples were  
126 then coarsely milled, subsampled and ball-milled into a fine powder, and packed into tin capsules for analyses of total N  
127 and atom %  $^{15}\text{N}$ .

128 Root and soil samples were taken at termination of the experiment after the last plant sampling in October, by collecting  
129 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  
130 sample was dried at  $105^{\circ}\text{C}$  for 24 hours and DM amount was recorded. The remaining soil in each cylinder was passed  
131 through a 1-mm sieve, liberated from plant material by removing visible roots and other debris with the help of  
132 tweezers and dried at  $80^{\circ}\text{C}$  for 24 hours. Immediately after the soil sampling, the cylinders were manually excavated  
133 along with the intact soil and plant materials and weighted. All plants were removed with intact root systems up to the  
134 depth of cylinder and the bulk soil was homogenized. Representative samples of 1 kg fresh soil were obtained from  
135 each cylinder using a Rifle-splitter. All root fractions were recovered manually by washing roots on a  $500\text{-}\mu\text{m}$  sieve.  
136 The roots with intact plants were sorted into different species, and those that could not be identified were collected and  
137 bagged separately. The samples were dried at  $80^{\circ}\text{C}$  for 24 hours and weighed. The background soil and root atom%  $^{15}\text{N}$   
138 was estimated in the samples collected in the same plots but at a minimum 50 cm from the cylinder. The root and soil  
139 samples were separately ground to a fine powder and packed into tin capsules. All the above-ground plant, root and soil  
140 samples were analyzed for total N and atom%  $^{15}\text{N}$  at UC Davis Stable Isotope Facility, University of California, USA  
141 on an ANCA-SL Elemental Analyzer coupled to a 20-20 Mass Spectrometer using the Dumas dry-combustion method.

## 142 **Calculations**

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

149 receiving plants at harvest using the following equation in Ledgard et al. (1985) and modified in Pirhofer-Walzl et al.  
150 (2012):

$$151 \quad \% \text{ Ndfd} = \frac{N_{\text{receiver (1-2)}} \times \text{Excess atom\% } ^{15}\text{N}_{\text{receiver (1-2)}}}{(N_{\text{receiver (1-2)}} \times \text{Excess atom\% } ^{15}\text{N}_{\text{receiver (1-2)}}) + (N_{\text{donor}} \times \text{Excess atom\% } ^{15}\text{N}_{\text{donor}})} \times 100$$

152

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

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

$$166 \quad \% \text{ Ndfr} = \frac{\text{Excess atom \% } ^{15}\text{N}_{\text{soil}}}{\text{Excess atom \% } ^{15}\text{N}_{\text{root}}} \times 100$$

167

168 where %Ndfr denotes the percentage of N in the soil derived from roots, and the excess atom %  $^{15}\text{N}$  of soil and roots is  
169 calculated as the atom %  $^{15}\text{N}$  in roots and soil in the cylinder minus the atom %  $^{15}\text{N}$  measured in soil and respective  
170 plant roots adjacent to the cylinder. The clover roots were not separated from all fractions of the recovered roots in the  
171 cylinder. Hence, the excess atom %  $^{15}\text{N}$  of clover roots analyzed in separated fractions was used for the calculation. The  
172 average atom %  $^{15}\text{N}$  in clover roots and soil measured in six plots was used as background, at 0.3665 for clover and  
173 0.3689 for soil. The amount of N deposited was quantified as the product of %Ndfr and soil N content. The soil atom %  
174  $^{15}\text{N}$  was measured in two different soil profiles, 0-10 cm and 10-30 cm. Therefore, the below-ground measurements  
175 including soil N content and amount of rhizodeposits were made up to the depth of cylinder assuming one third of total

176 soil from the upper and two thirds from the lower soil profile. Then the weighted average, based on total rhizodeposits  
177 and N content in both soil profiles, was calculated to express %Ndf in each cylinder. The root density was calculated as  
178 the ratio of total root DM to the total soil dry weight in each cylinder and expressed as g root kg<sup>-1</sup> dry soil.

## 179 **Statistical analyses**

180 Data were analyzed using the open source statistical program R (R Core Team 2014) (version 3.1.0).. When normally  
181 distributed data were required, log transformation was used to minimize heteroscedasticity. An one way analysis of  
182 variance was used to calculate the effect of seed mixture on each dependent variable (biomass yield, total N  
183 accumulation, proportion and amount of N transferred from clover to non-legumes and soil), The effect of seed mixture  
184 and soil depth on soil excess atom% <sup>15</sup>N were tested using two way analysis of variance. The effect of cutting time on  
185 DM yield, N accumulation, excess atom% <sup>15</sup>N, %Ndfd and amount of N transferred was analyzed using a linear mixed  
186 model, where seed mixture (fixed effect) and cutting time (repeated fixed effect) were independent variables and  
187 cylinders random effect. Pairwise comparisons were made between least square means using the adjusted Tukey  
188 method. The confidence level used was 0.95, and the probability of rejection of hypothesis was set at P<0.05.  
189 Correlations were tested using Pearson's correlation analysis.

## 190 **Results**

### 191 **Above-ground DM yield, botanical composition and N accumulation**

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

194 The DM yield of mixtures was mainly dominated by red clover even when grass was the main component in the seed  
195 mixture. On an annual basis, the DM yield of the clover component ranged from 630 to 1460 g m<sup>-2</sup> and constituted on  
196 average 60% (range 42–74%) of the total DM production. The clover produced the highest DM yields (P<0.001) in the  
197 first and third cuts and highest in clover-dominated mixtures, especially when grown with plantain (Figure 2a). The  
198 proportion of ryegrass ranged from 3 to 46% in different mixtures and cuts. The yield of the grass component was  
199 highest in the first cut (P<0.001) and decreased in succeeding cuts. The grass proportion was particularly suppressed  
200 when grown in mixtures containing chicory and plantain. The grass DM yield was generally highest with caraway, but  
201 it was unaffected by the seeding densities of grass and clover (Figure 2a). The proportions of forbs in the mixtures



202 varied between 2 and 61% of total DM production, depending on cut and species. The yield of the specific species was  
203 not affected by the seeding density of grass and clover. Chicory and plantain constituted larger proportions of the total  
204 biomass production than grass, which was more pronounced in grass dominated seed mixture. However, the caraway  
205 always had a significantly lower DM yield ( $P < 0.001$ ) than chicory and plantain (Figure 2a). There was a negative  
206 correlation between total seasonal DM yield of forbs and grass ( $P < 0.001$ ,  $R^2 = 0.45$ ).

207 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<sup>-2</sup>,  
208 respectively. The pattern of N yield and proportions in the mixture closely resembled the pattern of DM production. The  
209 clover in different mixtures constituted on average 72% of the total seasonal N accumulation, ranging from 76 to 83%  
210 in clover and from 57 to 75% in grass-dominated seed mixtures (Figure 2b).

### 211 **Above-ground <sup>15</sup>N-enrichment**

212 The atom % <sup>15</sup>N in both clover and companion non-legume species was substantially above the natural abundance,  
213 ranging from 0.37 to 2.5 depending on plant species and cutting times, which revealed that the labeling was successful  
214 and that some of the clover N was transferred to the companion non-legume species.

215 The excess atom % <sup>15</sup>N measured in all species was comparatively low in the first cut, but generally increased when  
216 DM yield was low. A correlation analysis of weighted atom % <sup>15</sup>N from all four cuts with the respective total seasonal  
217 DM ( $P < 0.001$ ,  $R^2 = 0.46$ ) and N yield ( $P < 0.001$ ,  $R^2 = 0.49$ ) showed a negative relation in clover.

### 218 **Clover <sup>15</sup>N-enrichment and N transfer**

219 The average excess atom % <sup>15</sup>N from four replications in red clover varied between 0.9332 and 0.0686 depending on  
220 cut and mixture, but without any interaction between mixture and cut (Figure 3a). It increased from first to second cut,  
221 with a significant ( $P < 0.001$ ) difference in grass-dominated seed mixtures, but decreased at the third and fourth cuts. As  
222 the applied <sup>15</sup>N was distributed among fewer clover plants in the cylinder, the clover was more enriched in the grass-  
223 dominated seed mixtures than the clover-dominated mixtures. However, the <sup>15</sup>N-enrichment was not affected by the  
224 species of forb used in the seed mixtures.

225 The proportion of clover N transferred (%Ndfd) to companion non-legume species varied between 7 and 33%,  
226 depending on time of cut and mixture composition (Figure 3b), but without interactions. The amount of clover N  
227 transferred to grass and forbs at different cutting times ranged between 0.34 and 1.98 g N m<sup>-2</sup>, with no interactions

228 between mixture and time of cut (Figure 3c). Unlike %Ndfd, the size of the N transfer was found to be closely  
229 influenced by N accumulation in clover. However, neither the proportion nor the amount of clover N transferred was  
230 significantly affected by seed mixture.

### 231 **<sup>15</sup>N-enrichment and N transfer in non-legumes**

232 Grass had a greater ability to absorb N from clover compared to forbs (Figure 4). The average excess atom % <sup>15</sup>N from  
233 four replications in grass varied between 1.0398 and 0.0357 at the different cuts and mixtures, whereas in forbs it  
234 ranged between 0.07 and 0.01 and always significantly lower ( $P < 0.001$ ) than in grass (data not shown). The excess  
235 atom % <sup>15</sup>N in grass was generally highest in grass-dominated mixtures, and lower when grown with caraway. The  
236 excess atom% <sup>15</sup>N in forbs was not affected by either species or the seeding densities of the grass and clover measured  
237 in all four cuts.

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

### 245 **Seasonal %Ndfd and N transfer**

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

250 Of the total amount of N transferred from clover to non-legumes, grass received a very high proportion, ranging from  
251 63 to 94%. This transfer was unaffected by grass and clover seeding densities and the identity of the forb species  
252 present in the mixture. The total seasonal N transfer estimated in forbs ranged between 0.24 and 1.33 g N m<sup>-2</sup>,  
253 equivalent to 0.6 to 4% of clover N. The proportions of clover N transferred to chicory and plantain was significantly  
254 higher ( $P < 0.001$ ) than to caraway (Figure 5a).

## 255 **Below-ground DM and N yield**

256 Total root DM and N accumulation varied between 252 and 352 g DM m<sup>-2</sup>, and 4.5 and 7.6 g N m<sup>-2</sup>, respectively, with  
257 the tendency towards higher yields in clover dominated seed mixtures. However, there was no significant effect of seed  
258 mixture for both DM and N yields. There was a positive correlation between total root DM and total seasonal shoot DM  
259 yield (P<0.001 and R<sup>2</sup>= 0.52), and total root N accumulation and total seasonal shoot N accumulation (P<0.001 and R<sup>2</sup>=  
260 0.66). Similarly, the estimated total seasonal N transfer in shoots was positively correlated with root DM yield (P<0.01,  
261 R<sup>2</sup>=0.29), root N accumulation (P<0.001, R<sup>2</sup>=0.4) and root density (P<0.01, R<sup>2</sup>=0.34).

## 262 **<sup>15</sup>N-enrichment in clover root, %Ndf and amount of rhizodeposition**

263 The excess <sup>15</sup>N atom % measured in clover roots ranged from 0.0474 to 0.1674 (Figure 6a) and was strongly correlated  
264 to the excess atom % <sup>15</sup>N in clover shoots (P<0.001 and R<sup>2</sup>= 0.7). Similar to shoots <sup>15</sup>N, the excess atom% <sup>15</sup>N in roots  
265 was also negatively correlated (P<0.001, R<sup>2</sup>= 0.48) to clover shoot N yield; and similar to shoots, forbs roots were less  
266 enriched than grass roots.

267 The excess atom % <sup>15</sup>N in soil was higher (P<0.05) in the top 10 cm soil compared to the 10-30 cm layer, but there was  
268 no significant difference between the mixtures (Figure 6b). The proportion and amount of soil N deposited from clover  
269 roots varied widely between the mixtures, ranging from 0.56 to 4.3% of soil N, equivalent to 3 to 24 g N m<sup>-2</sup> (Figure  
270 7c). Generally, more than 80% of the rhizodeposition occurred in the upper soil horizon between 0-10 cm. There was a  
271 correlation between the rhizodeposition and total root DM (P<0.05, R<sup>2</sup>= 0.22), root N accumulation (P<0.01, R<sup>2</sup>= 0.30),  
272 and root density (P<0.05, R<sup>2</sup>= 0.16). A correlation was also found between total seasonal N transfer measured in shoots  
273 and N deposited to the soil (P<0.05 and R<sup>2</sup>=0.24).

## 274 **Discussion**

### 275 **Critical reflection on methodology**

276 In our study, nearly all the N accumulated in grass grown in a clover-dominated seed mixture with chicory and plantain  
277 was found to be transferred from clover. In some cases, it even exceeded the total amount of N accumulated in grass.  
278 This is of course not possible and clearly highlights an overestimation of N transfer (Chalk et al. 2014) Chalk and Smith  
279 1997. In other studies on N transfer (e.g. Rasmussen et al. (2007), (2013) and Gylfadottir et al (2007) the proportions of  
280 grass in the mixture were close to 50% or more. Since our system was strongly clover-dominated and the

281 overestimation mainly occurred when the grass proportions were very low, , it shows that the leaf-feeding <sup>15</sup>N-based  
282 method for estimating the proportion of non-legume N derived from transfer should be used with caution in swards  
283 heavily dominated by N-rich, N<sub>2</sub>-fixing species. In addition, the method used to assess the N rhizodeposition often does  
284 not meet the assumptions and question the accuracy of measurement. It confronts multiple bias caused by unrecovered  
285 root parts in soil, spatial and temporal variations in root <sup>15</sup>N enrichment and different <sup>15</sup>N enrichment in roots and  
286 rhizodeposits leading to overestimation of rhizodeposited N (Rasmussen et al. 2011).

### 287 **Growth dynamics of clover, grass and forbs**

288 The swards were dominated by red clover irrespective of seeding densities of red clover and perennial ryegrass. Hence,  
289 red clover defined the DM and N yield of the swards, with a strong tendency for greater DM and N yield in mixtures  
290 with high red clover seeding densities. These observations reflect the strong ability of red clover to compete for  
291 available light and soil resources (Rasmussen et al. 2012) and the competitive advantage of the N<sub>2</sub>-fixing legume under  
292 unfertilized conditions (Carlsson and Huss-Danell 2003)

293 The growth dynamics among the non-legumes showed poor growth of perennial ryegrass as compared to chicory and  
294 plantain, even when it was the main component in the seeded mixtures. The significant negative correlation between  
295 forb and grass DM yield showed that chicory and plantain utilized the available resources more successfully than grass.  
296 One explanation could be the competition for light due to different plant architecture of grass and forbs (Søegaard et al.  
297 2013) that both the chicory and plantain grows up above the grass canopy and overtopped the grass for light  
298 interception due to their tall plant and wide rosette leaves . This explanation is supported by the fact that grass grew  
299 much better in mixtures with caraway. It was in line with Søegaard et al. (2013), who found caraway to grow less  
300 during first and second year of ley establishment.. Caraway is known to initially invest in its root system and increase  
301 its competitiveness with sward age (Hakala et al. 2009; Pirhofer-Walzl et al. 2012; Søegaard et al. 2013). Another  
302 explanation for the observed differences in grass-forb competitiveness could be the individual species' ability to acquire  
303 N from companion red clover or from the soil. The greater N yield of chicory and plantain indicates that these two  
304 species may have been more efficient to acquire available N resources than grass. The <sup>15</sup>N-leaf-labeling of red clover  
305 made it possible to study the uptake of red clover derived N in neighboring non-legume species and hence indirectly  
306 their ability to use soil N.

### 307 **Grass and forbs acquisition of clover-derived N**

308 Perennial ryegrass showed a strong ability to absorb red-clover-derived N as compared to the three forb species. The  
309 ability of grass to receive N from clover has been demonstrated in previous studies with mixtures of grass and chicory  
310 grown together with white clover, lucerne or birdsfoot trefoil (Høgh-Jensen et al. 2006), in four-species mixtures of two  
311 grasses, red clover and chicory or lucerne (Frankow-Lindberg and Dahlin 2013) and in multi-species mixtures of  
312 different grasses, forbs and forage legumes (Pirhofer-Walzl et al. 2012). The ability of the grasses to compete for clover  
313 N can be explained by a combination of the localization of red clover N deposits in the upper part of the soil profile and  
314 the presence of dense grass roots in this layer, where our data on soil <sup>15</sup>N-excess showed that red clover predominantly  
315 deposited N in the uppermost soil layer (0-10 cm). Similar observations on the process of clover N deposition and  
316 subsequent grass acquisition of N in the upper soil layer have been reported by (Frankow-Lindberg and Dahlin 2013).  
317 Frankow-Lindberg and Dahlin (2013) and Pirhofer-Walzl (2012) and colleague further suggested grass has a large  
318 fibrous root system covering a large surface area and soil volume especially in the upper soil layers. This may have  
319 facilitated the close intermingling with neighboring clover roots and favored absorption of the clover-derived N (Xiao et  
320 al.2004), whereas the forbs may be more flexible to explore the N in lower soil profile and less competitive in the upper  
321 soil layer due to their deep and thick tap or fibrous root system limiting to make close root intermix with clover. In  
322 addition, as suggested by Rasmussen et al. (2013), the horizontal root distribution may play a more important role than  
323 rooting depth for interspecies N transfer. This suggests that root architecture is one of the most important factors  
324 influencing the competition between grass and forbs for clover-derived N.

325 The excess atom % <sup>15</sup>N in the three forbs demonstrated similar abilities to absorb the N transferred from clover, with the  
326 amount of N received from clover closely related to the total N yield of each of the forbs. Hence, the forbs must have  
327 relied on soil N to a much greater extent than clover-derived N, and vice versa for grass. Frankow-Lindberg and Dahlin  
328 (2013) also observed that forbs rely more on soil N and compete less with the grasses for clover-derived N in upper soil  
329 horizon. In the present study, one explanation could be a double N-sparring effect, where the N<sub>2</sub>-fixing clover allowed  
330 companion non-legume species better access to soil N in the upper soil layer, and the grass uptake of clover-derived N  
331 allowed the forbs to acquire soil N especially from deeper soil layers; hence we could have niche complementarity  
332 between grass and forbs in terms of N source used, rather than spatial niche complementary with depth (Hoekstra et al.  
333 2015). Another explanation, however, could be that the forbs, especially chicory and plantain, in the present experiment  
334 established rapidly and competed more successfully for soil N in all soil layers due to their deep and larger root system  
335 (Collins and McCoy 1997; Li and Kemp 2005; Stewart 1996; Thorup-Kristensen 2006) forcing grass to seek the clover-  
336 derived N. In contrast to (Daudin and Sierra 2008), despite a strong ability to receive N from clover the poor growth of

337 the grass in our study indicated a higher energy investment in the take-up of clover-derived N. Thus, our results  
338 demonstrated that a strong reliance on N transfer from companion N-rich species does not always give the receiving  
339 species an advantage in terms of plant growth and productivity.

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

342 There are two ways of looking at the clover N transfer to companion species: the proportions (%Ndfd) and the amounts  
343 transferred. We observed a general trend towards a higher %Ndfd with higher proportions of non-legume DM and N  
344 yield in the mixture, which was in line with our first hypothesis. This indicates that the better ability of non-legumes for  
345 clover-derived N, allowing clover less chance to re-assimilate deposited N and suggested that the plant growth of non-  
346 legumes in the mixture play an important role for legume N transfer to non-legumes. The observed tendency could have  
347 been even stronger if the forbs, especially chicory and plantain, had better ability to absorb clover-derived N. With  
348 regard to the amount of N transferred from clover, this was defined mainly by the DM and N accumulated in clover  
349 shoots, which was also reflected in higher soil <sup>15</sup>N-enrichment and root N accumulation under high clover seeding  
350 densities. As discussed in Rasmussen et al. (2013) and (2007), this highlights the importance of the growth of the  
351 donating legumes in the mixture for understanding the N transfer source and sink dynamics. Thus, our study suggests  
352 that the extent of N transfer is influenced both by the ability of non-legumes to acquire clover-derived N and by the  
353 ability of both legume and non-legume plants for DM production and N accumulation.

354 We observed temporal dynamics in the N transfer, with low levels in the first cut, highest levels at second cut, and an  
355 increase in N transfer to forbs at the third and fourth cuts. Despite higher DM and N yields in both clover and non-  
356 legumes, we observed the lowest proportions and amounts of N transferred at the first cut. This could be because of  
357 clover investing N in above-ground growth or it could be a methodological issue, where <sup>15</sup>N from the first labeling  
358 event did only represent short-term routes of N transfer, whereas at later cuts the <sup>15</sup>N from the early labeling event  
359 would be available for more long-term deposition routes, e.g. fine root turnover (Haystead and Marriott 1979) or leaf  
360 die off (Dahlin and Stenberg 2010). At the second cut, the high N transfer could be due to low clover proportions  
361 relative to grass, especially for the high seeding densities of grass. N transfer to forbs increased in the last two cuts,  
362 especially the third cut, indicating an increased ability of forbs to acquire clover-derived N later in the growing season.  
363 The reasons for this may be twofold: that their root development and function were better established later in the season

364 and that chicory and plantain peaked in DM production at the third cut and caraway at the fourth cut, which combined  
365 with a low proportion of grass late in the season allowed the forbs greater access to clover-derived N.

366 We found that root DM and N at the end of the growing season correlated well with total shoot DM and N yield, with  
367 total seasonal N transfer measured in shoots, and with N deposited to soil. There was also a strong correlation between  
368 the excess atom%  $^{15}\text{N}$  in clover shoots and in roots. These were in line with our second hypothesis. These tendencies  
369 towards higher root DM and N in clover-dominated mixtures show that above-ground DM yield is an indicator of root  
370 biomass. The correlation between  $^{15}\text{N}$  in clover shoot and root and between  $^{15}\text{N}$  in root and total N transfer underlined  
371 that the internal allocation of N in clover was a key factor controlling N transfer. This was in line with the findings of  
372 Rasmussen et al. (2007). Furthermore, the internal  $^{15}\text{N}$  allocation in clover was linked to the soil excess atom%  $^{15}\text{N}$  and  
373 total N deposition. The amount of rhizodeposition estimated in the present study was higher than in the Gylfadottir et al.  
374 (2007) study and lower than in the study by Høgh-Jensen and Schjoerring (2001). It could have been influenced by the  
375 different plant species or composition of seed mixture (Høgh-Jensen and Schjoerring 2001). One explanation for the  
376 higher deposition could be that our system had a higher proportion of clover DM than in the study by Gylfadottir and  
377 colleagues. However, as pointed out by (Rasmussen 2011), total N deposition may be overestimated due to uneven  
378 temporal and spatial distribution of  $^{15}\text{N}$  in sampled roots, or due to unrecovered roots in the soil samples (Gardener et al.  
379 2012) as soil  $^{15}\text{N}$  enrichment and rhizodeposition was observed higher in clover dominated mixtures and in upper soil  
380 layer between 0 and 10 cm.

## 381 **Conclusions**

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

385 Forbs differed in their biomass production and N accumulation, chicory and plantain had stronger growth than caraway,  
386 but showed no differences in their ability to absorb clover-derived N. Hence, our study demonstrated that forbs in  
387 grassland did not rely on N transferred from red clover and that this did not limit their growth, revealing that the forbs  
388 mainly acquired N from the soil pool. Despite the strong ability of grass to assimilate N transferred from red clover, its  
389 low biomass production when in mixture with chicory and plantain demonstrated that a high reliance on N transfer does  
390 not necessarily give the species an advantage for growth and resource utilization including N acquisition from the soil.

391 The dynamics of N transfer were positively influenced by root biomass and N accumulation in red clover, which  
392 confirms the importance of below-ground productivity in plant-soil systems. To increase our understanding of grassland  
393 N transfer dynamics future studies need to include both legume and non-legume behavior in relation to e.g. N  
394 deposition and root growth.

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#### 484 **Figure legends**

485 **Fig. 1** Monthly precipitation and mean monthly air temperature during the experimental period from April to October in  
486 2014 measured at a climate station near the experimental field.

487 **Fig. 2** Above-ground dry matter (a) and Nitrogen (b) yield of red clover, perennial ryegrass and non-legume forb  
488 mixtures grown in PVC cylinders in the field. Values are mean ( $\pm$  SE, n = 4) measured at four cuts during the 2014  
489 growing season. Different letters indicate statistically significant differences at the 0.05 level and the bars with no letter  
490 for the total yields indicates no significant difference. CH: chicory, PL: ribwort plantain, CA: caraway. RC: clover-  
491 dominated seed mixture, GR: grass-dominated seed mixture

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

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

500 **Fig. 5** Total seasonal estimation of percentage (a) and amount (b) of red clover N transferred to perennial ryegrass and  
501 non-legume forb species: chicory, ribwort plantain and caraway grown in PVC cylinders in the field. The %Ndfd is

502 weighted mean from four cuts ( $\pm$  SE, n= 4) and amount is the sum of N transfer measured in four cut ( $\pm$  SE, n= 4)  
503 during the 2014 growing season. Different letters indicate statistically significant differences between the non-legume  
504 forbs at the 0.05 level and the bars with no letter indicates no significant difference. CH: chicory, PL: ribwort plantain,  
505 CA: caraway. RC: clover-dominated seed mixture, GR: grass-dominated seed mixture.

506 **Fig. 6** Excess atom%  $^{15}\text{N}$  in red clover roots (a), and percentage (b) and amount (c) of soil N deposited from red clover  
507 root grown in PVC cylinder in the field. Values are mean ( $\pm$  SE, n = 4) measured at the end of the 2014 growing season.  
508 Different letters indicate statistically significant differences at the 0.05 level and no letter indicates no significant  
509 difference. CH: chicory, PL: ribwort plantain, CA: caraway. RC: clover-dominated seed mixture, GR: grass-dominated  
510 seed mixture.

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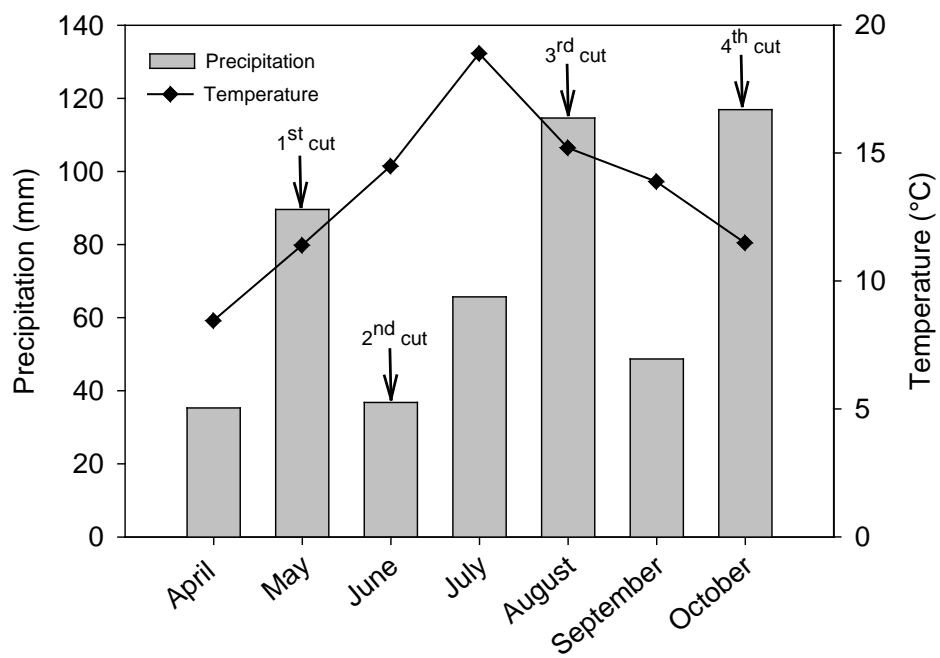
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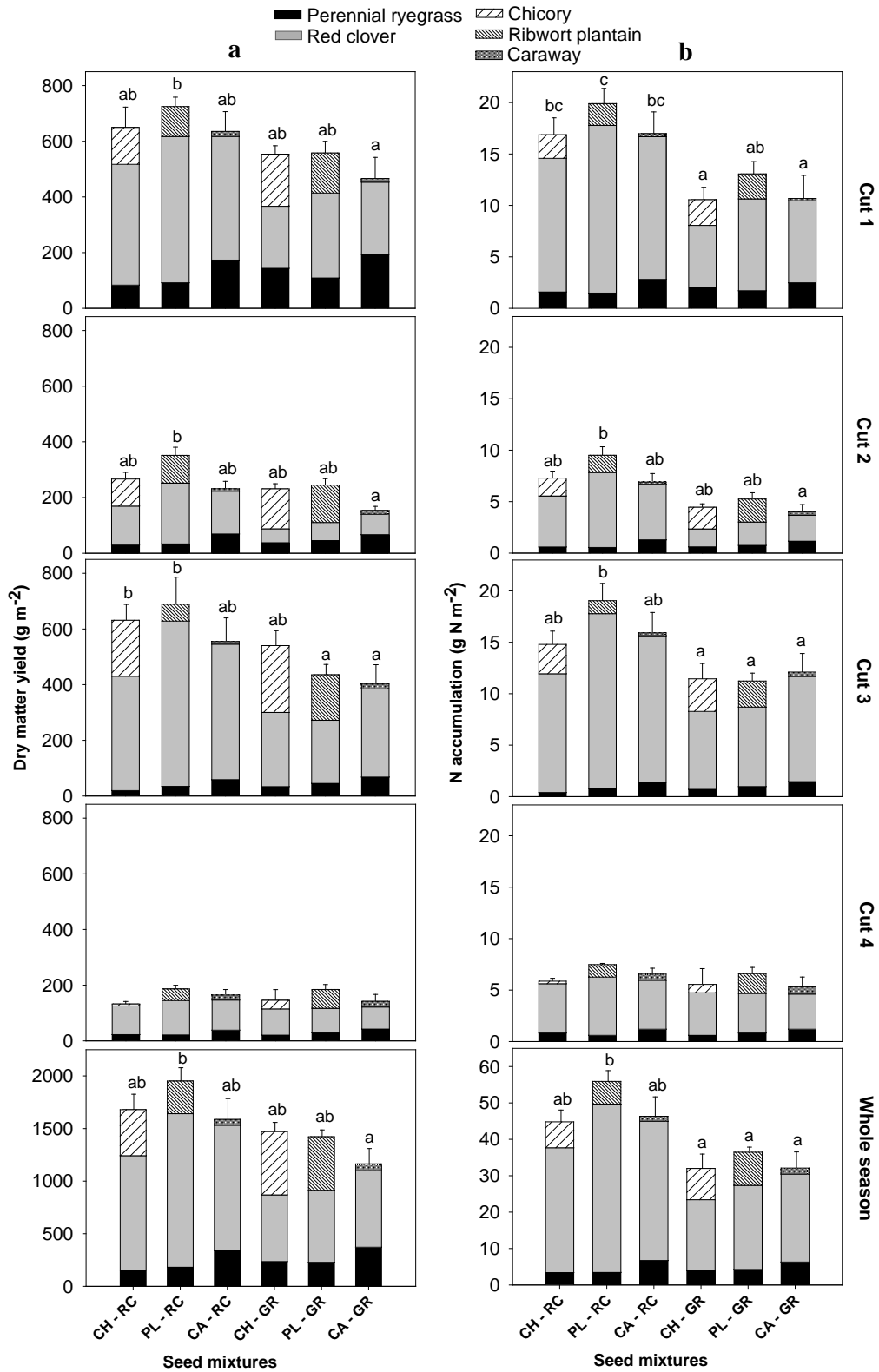
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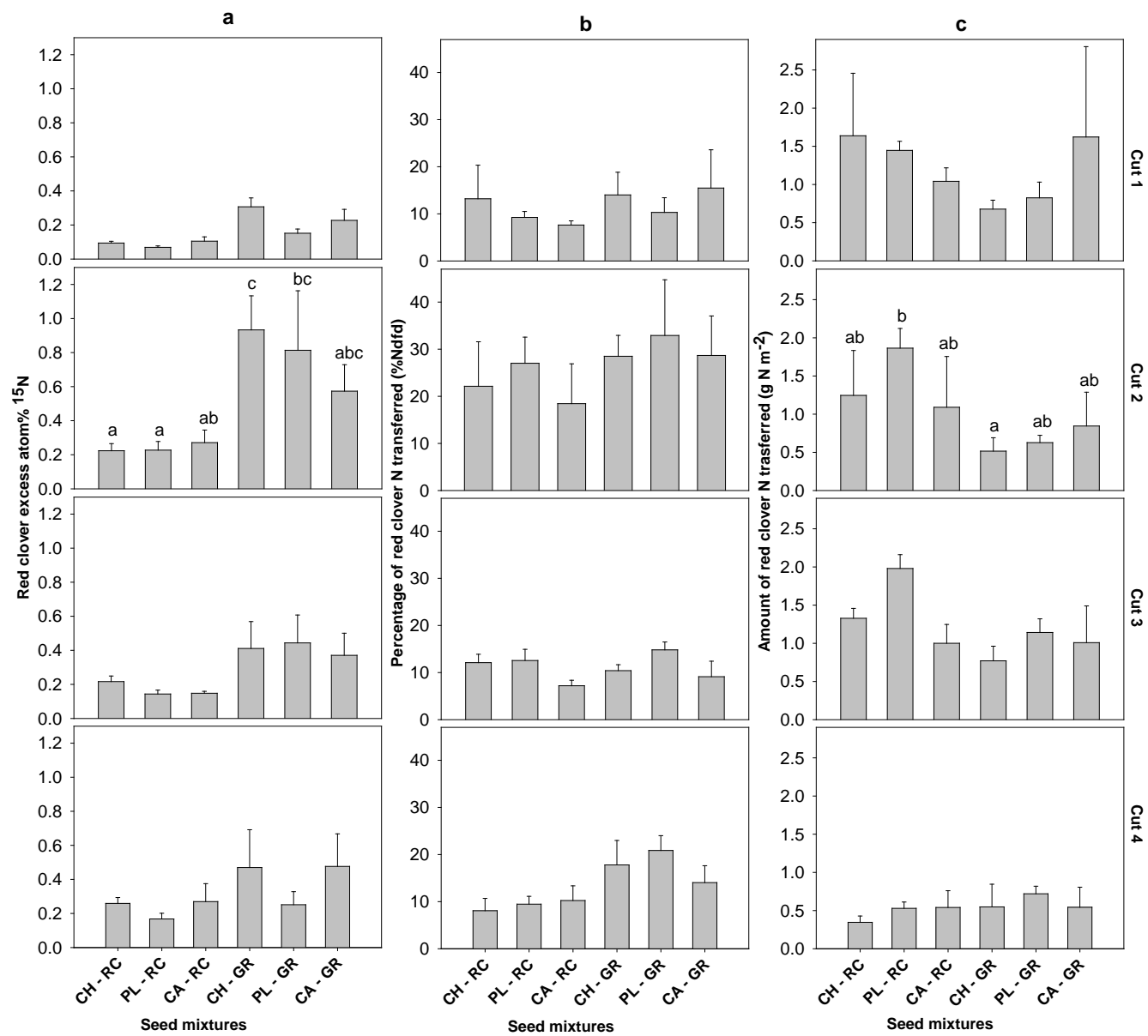
Fig. 1



**Fig. 2**



**Fig. 3**



**Fig. 4**

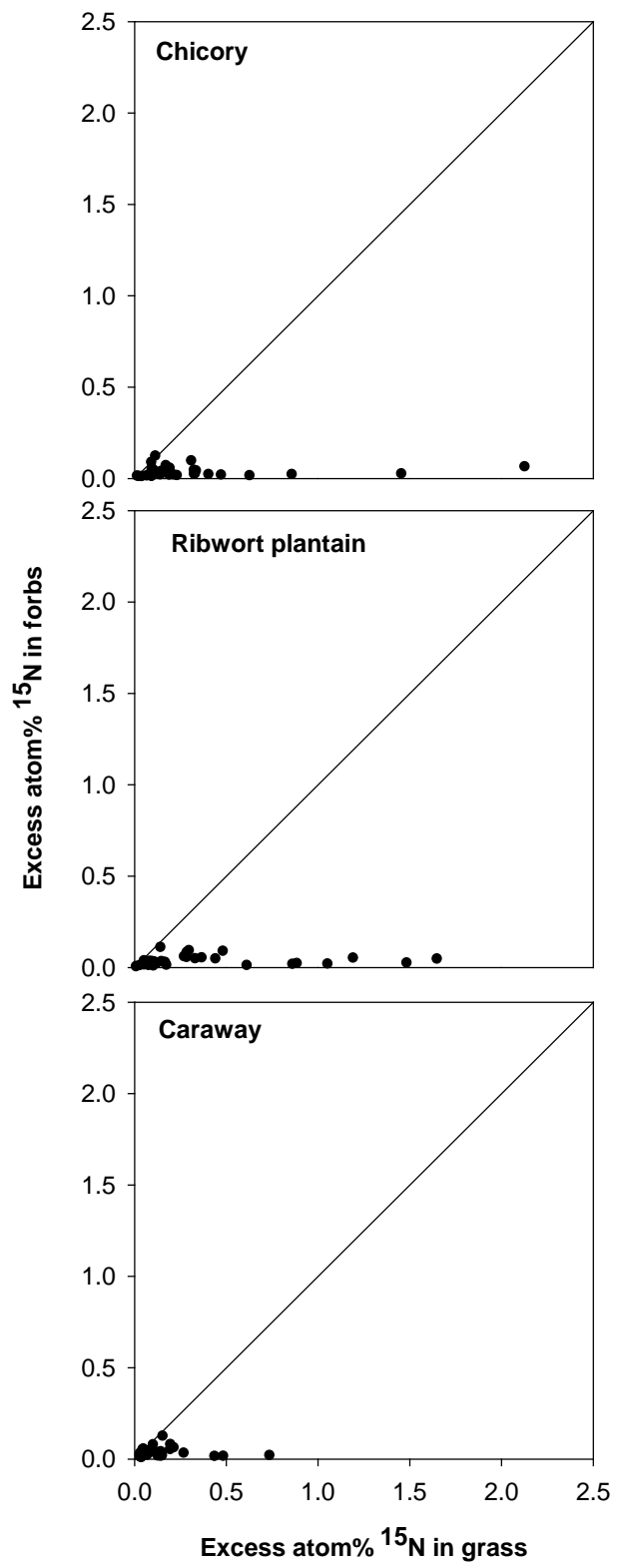
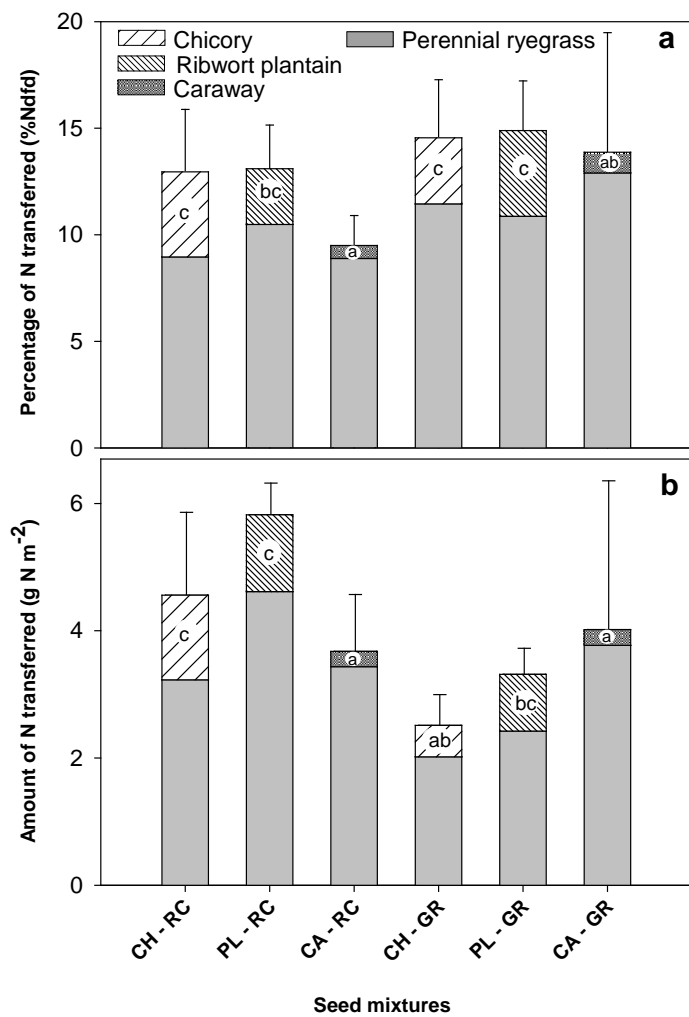






Fig. 5



**Fig. 6**

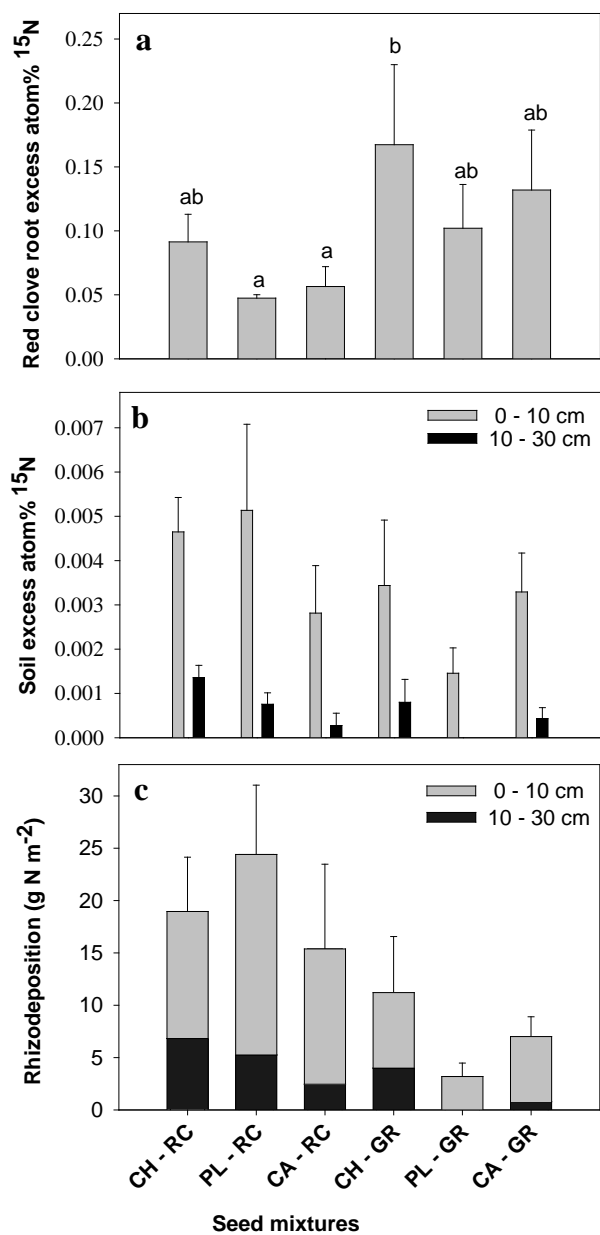


Table 1: Composition of seed mixtures (percentage is based on each species seeding rate in pure stand)

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