NRC Research Press

ARTICLE

Interactions between cultivars of legume species (*Trifolium pratense* L., *Medicago sativa* L.) and grasses (*Phleum pratense* L., *Lolium perenne* L.) under different nitrogen levels

M.S. McElroy, Y.A. Papadopoulos, K.E. Glover, Z. Dong, S.A.E. Fillmore, and M.O. Johnston

Abstract: The transfer of nitrogen (N) from legumes to grasses is an important process in low-input forage production systems, and may be improved by selecting compatible species and cultivars. This study sought to examine what effect species and cultivar have on plant growth and N accumulation in temperate grass-legume mixtures under a range of nitrogen fertility levels. A pot study using two cultivars each of alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), perennial ryegrass (*Lolium perenne* L.), and timothy (*Phleum pratense* L.) in all grass-legume combinations was devised. Compatibility indices, based on plant performance grown in combination versus alone, were used to quantify the net impact legumes and grasses had on each other. The presence of legumes had an overall negative effect on the growth of grasses (87% compared with growing alone), but did improve tissue N content by weight and total accumulated N. Improvements in total N were highest in a single timothy cultivar (Champ; 169%), but highest net total N was achieved in a ryegrass cultivar (Bastion; 1.92 mg N). Results indicate that grass N accumulation in legume-grass mixtures may be influenced more by grass N demand than legume supply, which suggests that competition between grasses and legumes may be a major determinant of N transfer efficiency.

Key words: nitrogen transfer, legume-grass interaction, forages, red clover, alfalfa, perennial ryegrass, timothy.

Résumé : Le transfert d'azote (N) des légumineuses aux graminées est un processus important dans les régimes de culture fourragère à faibles intrants et on pourrait l'intensifier en sélectionnant des espèces et des cultivars compatibles. Cette étude devait préciser dans quelle mesure l'espèce et le cultivar agissent sur la croissance de la plante et l'accumulation de N dans les mélanges combinant graminées et légumineuses pour climats tempérés, compte tenu d'un degré de fertilité variable pour l'azote. Les auteurs ont réalisé une expérience en pot sur deux cultivars, dans chaque cas, de luzerne (*Medicago sativa* L.), de trèfle rouge (*Trifolium pratense* L.), de raygrass vivace (*Lolium perenne* L.) et de fléole (*Phleum pratense* L.) en combinaisons graminées-légumineuses. Ils se sont servis d'indices de compatibilité s'appuyant sur la performance des plantes cultivées ensemble par rapport à leur performance lorsqu'elles sont cultivées seules, afin de quantifier l'impact net des légumineuses sur les graminées, et vice-versa. Les légumineuses ont un effet généralement négatif sur la croissance des graminées (87 % par rapport à la croissance observée sans compagnon), mais elles rehaussent la teneur en N dans les tissus selon le poids ainsi que la quantité totale de N accumulée. La hausse du N total la plus élevée a été observée chez un unique cultivar de fléole (Champ; 169 %), mais la plus forte hausse nette du N total a été obtenue avec un cultivar de raygrass (Bastion; 1,92 mg de N). Ces résultats indiquent que l'accumulation de N dans les mélanges de graminées et de vivaces pourrait être plus influencée par la demande de N de la graminée que par l'apport de N des légumineuses, ce qui laisse croire que la compétition entre graminées et légumineuses pourrait être un paramètre majeur pour mesurer l'efficacité avec laquelle il y a transfert de N. [Traduit par la Rédaction]

Mots-clés : transfert d'azote, interactions légumineuses-graminées, cultures fourragères, trèfle rouge, luzerne, raygrass vivace, fléole.

Received 24 April 2016. Accepted 2 August 2016.

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Can. J. Plant Sci. **97:** 1–12 (2017) dx.doi.org/10.1139/cjps-2016-0130

Introduction

Pasture legumes and grasses provide an interesting example of the complex relationship between plants inhabiting a common space. At an individual level, the legume-grass relationship is often characterized as competitive (Haynes 1980), with local soil N availability identified as a major determinant of competitive outcome (Schwinning and Parsons 1996; Thornley 2001). From an agronomic viewpoint, the relationship between the two plant types is often framed as a mutualistic association; pasture swards can maintain productivity without the application of nitrogen (N) fertilizer because of the ability of legumes to fix atmospheric nitrogen, the growth-limiting nutrient in most terrestrial systems (LeBauer and Treseder 2008). The presence of legumes can also increase the yields of non-legumes, either through decreased competition for soil N (“N-sparing effect”; Vallis et al. 1967; Herridge et al. 1995; Chalk 1998; Peoples et al. 2012) or through a process known as “N transfer” (Paynel et al. 2008; Fustec et al. 2010; Thilakarathna et al. 2012), in which legume-fixed N is taken up by non-legumes. Over the long term (i.e., over months and years), legume-derived N is made available to grasses via the turnover of legume root debris, whose protein is mineralized by soil microbes into low-molecular weight (LMW) N compounds available for uptake by grasses (Fustec et al. 2010). However, “direct” N transfer, via the excretion of LMW N from legumes roots, has been identified as an important flow of N in cool grasslands in the early season; Gylfadóttir et al. (2007) and Rasmussen et al. (2013) both demonstrated significant N transfer in northern European grasslands that surpassed the predicted mineralization rate of N in the soil, suggesting direct uptake of legume-exuded N by grasses was responsible for the difference.

Direct transfer of N depends on root exudation, a process universal across all plants in which compounds are passively or actively excreted into the surrounding soil (Walker et al. 2003). While most LMW exudates are hydrocarbons, amino acids and ammonium are common. Generally, nodulated legumes tend to release exudates more rich in LMW N than non-N-fixing plants (Rovira 1956; Paynel et al. 2001; Phillips et al. 2006; Lesuffleur et al. 2007), which can be subsequently taken up by grasses. Legume species (Brophy et al. 1987; Ofosu-Budu et al. 1992) and cultivars (Thilakarathna et al. 2013) can vary in their N exudation rate, making some individuals potentially better “N-donors” in the pathway. On the other side, it has been demonstrated that temperate perennial grasses can differ in their role as “N-receivers”: species vary in their nitrogen use efficiency (NUE; Vazquez de Aldana and Berendse 1997) and their ability to take up specific forms of N (Wiegelt et al. 2005). Nitrogen use efficiency and N-form preference has also been observed within species (Clárk 1983; Jarvis and MacDuff 1989; Kuo et al. 1999; Brégard et al. 2001). Theoretically, matching N-donors with high exudation rates of LMW N with receivers capable of rapid, more efficient direct N transfer could be achieved.

Unlike the decomposition pathway of N transfer, however, direct N transfer requires the close proximity of N-donors and receivers, which presents another important factor: competition. Models exploring the legume-grass dynamic in pasture systems (Schwinning and Parsons 1996; Thornley 2001) predict that competition in grass-legume systems will depend on soil mineral N availability: when available N is low, the relative growth rate of legumes is higher, allowing them to outcompete grasses for light and other resources, and vice-versa when soil N is abundant. However, the magnitude of competition for grass-legume swards can be diminished through the identification of compatible genotypes. Turkington and Harper (1979) demonstrated that selected white clover (*Trifolium repens*) genotypes behaved less “aggressively” towards the species of grass they were found growing adjacent to in an established pasture, suggesting micro-scale genotype sorting over time, resulting in less competitive grass-legume pairs. Further studies in similar systems (Aarssen and Turkington 1985; Barthram 1997; Adams and Vellend 2011) confirmed that “compatible” genotypes of legumes and grass were more productive than mismatched neighbours. Compatible growth, therefore, could be another important factor in N transfer efficiency.

To test for variation in grass N accumulation in different grass-legume mixtures, a pot experiment was devised to examine the interactions between cultivars of two species legumes (alfalfa, *Medicago sativa* L.; red clover, *Trifolium pratense* L.) and perennial forage grasses (perennial ryegrass, *Lolium perenne* L.; timothy, *Phleum pratense* L.). The effect of pairings on grass and legume dry matter yield and N accumulation was measured and compared with each other and to relative performance when grown alone, giving an inferred measure of net N-transfer as well as an evaluation of compatibility. We predicted that the N content and total N of grasses would be improved by the presence of legumes as a result of the additional N provided by legume exudates and that this effect would be influenced by the species/cultivar identity of the N-donor (legume), the N-receiver (grass), or a combination of both.

Materials and Methods

Growing conditions and experimental design

To capture some of the diversity of plants found in temperate pastures, two diverse cultivars of two distinct forage species were selected for the experiment. For red clover (RC), A.C. Christie, a diploid variety (Martin et al. 1999) and Tempus, a tetraploid, (Oseva Uni., Czech Republic, released 1988; http://www.osevauni.cz/pdf/Oseva-Uni-vlastni-odrudy.pdf) were selected. For alfalfa (Af), Apica, a tap-rooted tetraploid (Michaud and Richard 1983) and CRS1001, a rhizomatous tetraploid, (Y.A. Papadopoulos, AAFC, unpublished data) were selected. For perennial ryegrass (PR), Bastion, an early season tetraploid (http://www.sroseed.com/resources/pdfs/bastion.pdf) and Feeder, a mid-season diploid (http://www.uwex.edu/ces/forage/pubs/vargrassinfo.htm) were selected. For timothy (Tm) Champ, a mid-season variety (Childers et al. 1978) and Richmond, a late season variety (http://extension.psu.edu/plants/crops/forages/species/timothy) were selected. Seeds of all cultivars were surface sterilized for 2 min with 2% hypochlorite solution, then rinsed in distilled water three times. Seeds were germinated on moist filter paper in Petri plates covered with aluminium foil at 20 °C for 2 d.

Once germinated, seedlings were transferred to standard 10.2 cm-diameter pots (approx. 700 mL volume) filled with acid-washed sand according to the design described below. Each plant was placed approximately 2 cm from the edge of the pot; each pot contained two plants on opposite sides, or one plant on one side in "No Legume"/"No Grass" treatments. Performance data of the single plants grown alone was not included in the statistical analysis, but was used to generate the compatibility indices as described below.

To reduce the impacts of competition, the experiment was deliberately made sparse, with only a single plant of each type (N-donor and N-receiver) in each pot. The planting design consisted of replicated latinized arrays, with legumes (Af-Apica, Af-CRS1001, RC-Christie, RC-Tempus, and "No Legume") as columns and grasses (PR-Bastion, PR-Feeder, Tm-Champ, Tm-Richmond, "No Grass") as rows. This five-by-five pot arrangement included all pairwise combinations of legumes and grasses, as well as each legume and grass alone, and one empty pot for symmetry. Each array was replicated four times each within a growth chamber to accommodate one of four nutrient treatments (main plots), containing each combination of legume and grass (sub plots), and repeated in four separate growth chambers, which were used as blocks.

Growth chambers were maintained at a photoperiod of 16 h of daylight at 425 μmol m^{-2} s^{-1} at 21 ± 2 °C and 8 h of dark (16 D: 8 N) at 16 ± 2 °C. Pots containing clover or alfalfa were inoculated with 2 mL of *Rhizobium leguminosarum* biovar *trifolii* (ATCC 14480) or *Sinorhizobium meliloti* (Rm 1021), respectively, with a cell density of 10^8 cells mL^{-1} ($OD_{600} = 0.1$). Pots were watered daily for 1 wk, and then thinned to ensure that only one individual plant of each type was present in each pot. After the first week, plants received a daily fertilization of 40 mL of 10% "Hoagland's No. 2 Basal Salt Mixture without Nitrogen" (http://www.caissonlabs.com/product.php?id=313) adjusted to pH 5.8, and amended with $(NH_4)_2SO_4$ to give an addition of 0.6 mg N wk^{-1} pot^{-1}.

Nitrogen treatments

After 4 wk, arrays were assigned one of four N treatments; "Baseline", "No N", "Half N", and "Full N". The first treatment array ("Baseline") was harvested immediately to measure growth during the establishment phase. The remaining arrays were allowed to grow for another 4 wk fertilized at the same rate with the same No-N Hoagland, adjusted to give three different N fertility levels: 0.6 mg N wk^{-1} pot^{-1}, the same as in the establishment phase ("Full N"); 0.3 mg N wk^{-1} pot^{-1}, half of what was supplied during the establishment phase ("Half N"); and 0 mg N wk^{-1} pot^{-1} ("No N").

Harvesting and data collection

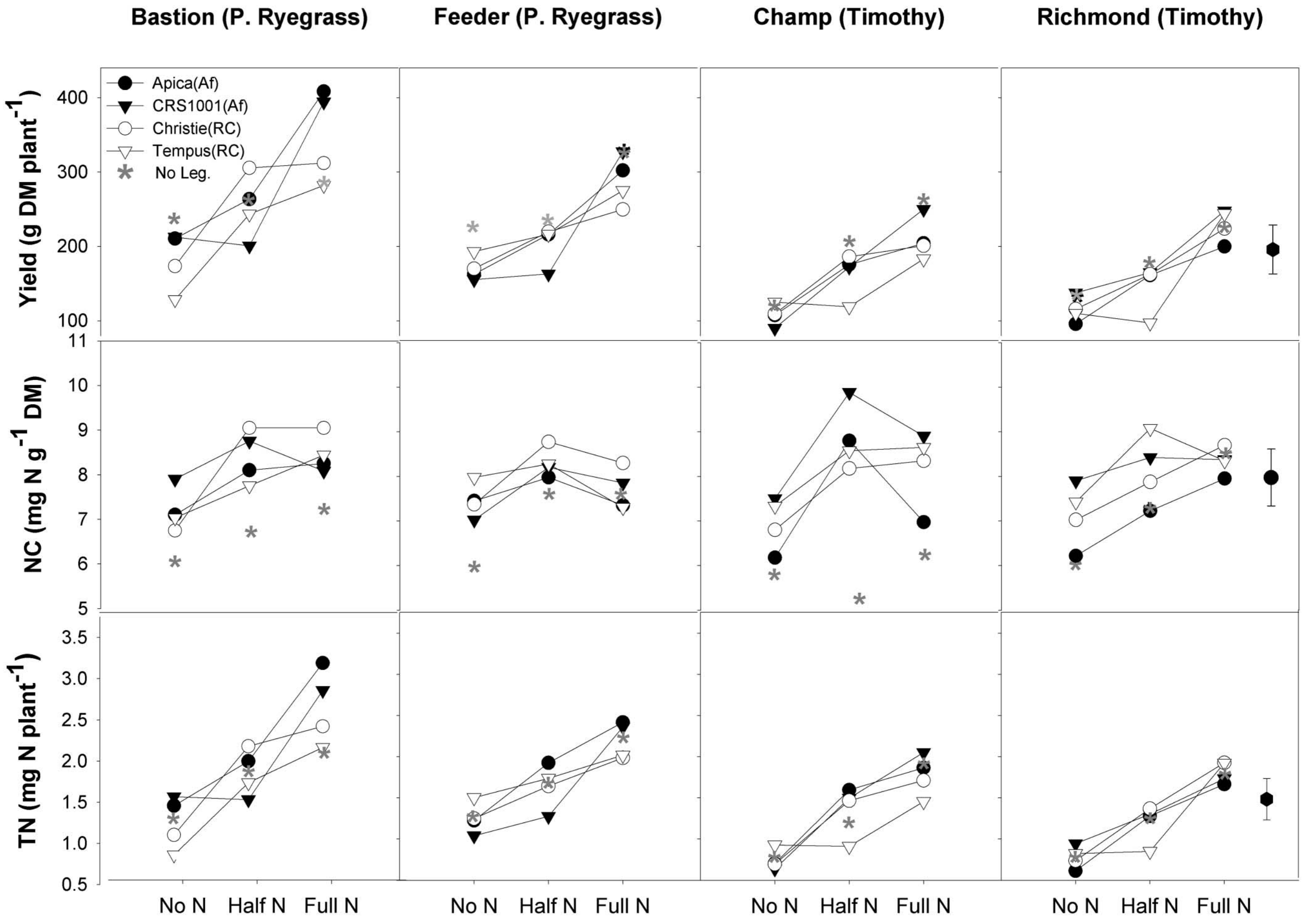
Pots were destructively harvested at four ("Baseline") or 8 wk ("No N", "Half N", "Full N"). Plant tissue was dried in a hot air oven at 65 °C for 3 d and weighed to measure dry matter (DM) yield. Legumes roots were scored for nodules number. After measuring total plant yield (mg DM), tissue was ground to pass through a 1 mm sieve in a micro Wiley mill, standard model 3 (Arthur H Thomas Co., Philadelphia, PA, USA). Total nitrogen (TN; mg N $plant^{-1}$) in plant tissue was determined using the combustion method on a LECO protein/N determinator FP-528 according to the Dumas method (Williams et al. 1998) using the entire plant tissue (mg N $plant^{-1}$). Total N was divided by total DM yield to calculate tissue N content (mg N g^{-1} DM).

Statistical analysis

The "Baseline" treatment was not included in the final analysis, but was measured to determine growth and N accumulation during the initial 4 wk establishment period. Analysis of variance (ANOVA) of variables in that treatment found no significant differences between blocks or companion plant treatments, so a mean value of total DM yield (mg DM $plant^{-1}$) and total N (TN; mg N $plant^{-1}$) of each grass and legume cultivar was determined and subtracted from each corresponding variable from the 8 wk plants to obtain a standardized change in total DM yield and N during the final 4-wk treatment period. Thus, grass and legume yield and total N yield represent a change from week 4 to week 8, during the treatment period. Nitrogen content and nodule number represent the true measured values of the plants harvested.

As the specific objective of the study was to compare N-donor/N-receiver DM yield and N accumulation in combination, plants grown alone ("No Legume" and "No Grass" treatments) were not used in the analysis, but were used to generate compatibility indices (CI) for the remaining plants. The index was based on the "relative yield" index of de Wit (1960), expanded to include measurements other than herbage biomass and assume values above and below "1". For each value of DM yield, N content, and total N, a CI was generated by dividing it by the corresponding value of the same cultivar grown alone in the same array (i.e., $CI = x_{combination}/x_{alone}$). A score of <1 indicates a negative effect on growth in combination compared with growing alone in similar

Fig. 1. Grass total dry matter (DM) yield, nitrogen content (NC), and total plant nitrogen (TN) by grass cultivar, legume companion cultivar, and nitrogen fertilization treatment. Hexagonal points in the far right column indicate the grand mean for each attribute; error bars represent the SEM for the three-way interaction of grass cultivar × legume cultivar × nitrogen.



conditions, while a score of >1 indicates a positive effect. Treatment means were assessed via one-sample *t*-test to determine if they differed significantly from 1. For total N of grasses, a significant positive CI value would indicate apparent net N transfer, as the amount of non-fixed N available to the grasses remained constant between the “combination” and “alone” treatments, and thus is assumed to be legume-derived.

“Inferred N transfer” from grass to legume was measured as the difference between N accumulation in grasses paired with a legume versus their N accumulation alone under the same N treatment. While the most direct measure of N transfer is done by N_{15} isotope studies (Høgh-Jensen 2006), our planting design were not of equal density, removing the problem of “N-sparing” (Chalk 1998; Chalk et al. 2014). Given that grasses had access to the same amount of N when alone and in combination, differences in N accumulation were taken as a conservative measure of the net amount of N supplied to them by legumes.

Data were analyzed in GenStat (VSN International 2011), as a split-plot multifactor ANOVA with Latinized subplots, using N fertilization treatments (“No N”, “Half N”, “Full N”) as the main effect, and legume cultivar (‘Af-Apica’, ‘Af-CRS1001’, ‘RC-Christie’, ‘RC-Tempus’) and grass cultivars (‘PR-Bastion’, ‘PR-Feeder’, ‘Tm-Champ’, ‘Tm-Richmond’) as the subplot factor. Orthogonal contrasts were used to determine differences within main effects. *P* values below 0.05 were considered significant.

Results

Grass cultivar response

The addition of N had an overall positive effect on the growth of grasses (Fig. 1), with a significant increase in accumulated yield over N treatments (Table 1). Mean grass yield CI values remained similar over N treatment levels indicating no significant change in mean relative performance (Table 1). Nitrogen fertilization also affected plant N status with positive responses in N content and total N, respectively, with somewhat similar patterns of increasing values with increasing N fertilization. Compatibility indices for N content and total N were not significantly impacted by N treatments, and

Table 1. Grass mean total yield, nitrogen content, and total nitrogen under different N fertility treatments and in different legume and grass cultivar combinations.

		DM yield[a]		N content		Total N	
Treatments		mg DM	CI[b]	mg N g^{-1}	CI[b]	mg N	CI[b]
Nitrogen	No N	144	0.89	7.2	1.15	1.01	1.11
	1/2 N	192	0.85	8.4	**1.48**	1.52	1.37
	Full N	269	0.92	8.2	1.24	2.11	1.12
	SEM	17.9	0.133	0.33	0.136	0.157	0.294
Grass cultivar	Bastion (PR)	261	0.92	7.5	1.15	1.92	1.17
	Feeder (PR)	221	0.84	8.2	1.17	1.70	0.99
	Champ (Tm)	160	0.93	8.0	**1.71**	1.29	**1.69**
	Rich. (Tm)	163	0.84	8.0	1.12	1.26	0.94
	SEM	9.8	0.084	0.21	0.172	0.072	0.267
Legume cultivar	Apica (Af)	209	0.91	8.0	**1.34**	1.67	1.23
	CRS1001 (Af)	210	0.89	7.9	1.27	1.57	1.20
	AC Chris. (RC)	202	0.91	8.0	1.28	1.54	1.18
	Tempus (RC)	185	0.84	7.9	1.27	1.41	1.19
	SEM	16.5	0.105	0.36	0.178	0.14	0.281
	Grand mean	201	0.89	7.9	1.29	1.54	1.20
***F*-probabilities**[c]							
Nitrogen treatment		**0.01**	0.93	0.08	0.28	**0.01**	0.77
Legume cultivar		0.28	0.68	0.81	0.29	0.31	0.96
Af vs. RC		0.13	0.64	0.88	0.28	0.15	0.68
Nitrogen × legume		0.19	0.39	0.33	0.60	0.56	0.19
Grass cultivar		**<0.005**	0.84	0.09	0.07	**<0.005**	0.20
Tm vs. PR		**<0.005**	0.91	0.43	0.15	**<0.005**	0.39
Nitrogen × grass		0.47	0.11	0.89	0.29	0.50	0.51
Legume × grass		**0.04**	0.24	0.41	0.45	0.05	**<0.005**
Nitrogen × legume × grass		**<0.005**	**0.03**	0.86	0.40	**<0.005**	**<0.005**

[a] Combined root and shoot DM.

[b] CI, compatibility index ($V_{\text{combination}}/V_{\text{alone}}$). A score of <1 indicates a negative effect on growth in combination compared with growing alone in similar conditions, while a score of >1 indicates a positive effect. CI mean values in bold indicate a significant difference ($P < 0.05$) from 1.0.

[c] *F*-probabilities significant at the $P < 0.05$ level are given in bold.

only one mean value (N content at "Half N" level) was significantly greater than 1.

Grass species and cultivars varied in yield and total N with contrasts revealing a large difference between species. Ryegrass cultivars had significantly greater yield than timothy cultivars (241 and 162 mg DW, respectively), with corresponding greater total N (Table 1). Grass N concentration was nearly significant for the grass cultivar term ($P = 0.09$), which was due to a single cultivar (Bastion) showing a lower overall mean N concentration (Table 1). Grass N content CI value was nearly significant ($P = 0.065$), with one grass cultivar (Champ) showing much higher values over the rest, both of which were significantly different than 1.0. In contrast to grass cultivars, the legume companion cultivar on its own had no significant effect on grass yield, N content or total N (Table 1).

Interaction terms demonstrated a complex relationship between treatments with many attributes being affected significantly by the combination of grass and companion legume cultivars in an N-dependent context. Grass DM yield and total N accumulation, and their respective CI values, were significantly affected by the three-way grass-legume-nitrogen interaction (Table 1). Patterns of DM yield and total N accumulation over N levels tended to be similar across grass/species interactions, with ryegrass cultivars showing roughly similar patterns of rapid growth and N accumulation at high N-fertility when paired with alfalfa cultivars compared with clover, while timothy cultivars show more reduced growth in the presence of Tempus (RC) than any other legume, only at the "Half N" treatment level (Figs. 1, 3). This is reflected in grass yield and TN CI trends; particularly in the legume species-specific interactions of Champ at lower N fertility levels (Fig. 4).

Legume cultivar response

In contrast to grass cultivars, the addition of N had a mainly negative effect on the DM yield and total N accumulation of legumes at the highest level, while CI values dropped to a point significantly below 1 in "Half N" and

Table 2. Legume mean total yield, nitrogen content, and total nitrogen under different N-fertility treatments and in different legume-grass cultivar combinations.

		DM yield[a]		N content (mg N g^{-1})		Total N		
Treatments		mg DM	CI[b]	DM	CI[b]	mg N	CI[b]	Sp. Nod. (nod. g^{-1} root DM)
Nitrogen	No N	347	1.01	19.7	1.03	7.23	1.07	305
	1/2 N	346	0.97	19.4	1.00	6.82	1.07	236
	Full N	247	**0.64**	18.5	1.01	4.63	**0.65**	275
	SEM	23.2	0.136	0.43	0.049	0.597	0.179	38.9
Legume cultivar	Apica (Af)	279	1.12	19.7	0.96	5.31	1.16	250
	CRS1001 (Af)	294	0.88	19.6	1.05	6.08	0.98	236
	AC Chris. (RC)	280	0.77	18.8	1.03	5.45	0.87	409
	Tempus (RC)	402	0.72	18.9	1.01	8.08	0.73	192
	SEM	27.2	0.157	0.04	0.049	0.585	0.174	34.8
Grass cultivar	Bastion (PR)	301	**0.84**	19.5	1.02	5.91	0.91	273
	Feeder (PR)	301	**0.83**	19.5	**1.04**	6.07	0.92	265
	Champ (Tm)	330	0.93	19.4	1.02	6.74	0.96	286
	Rich. (Tm)	323	0.89	18.3	0.98	6.19	0.95	264
	SEM	23.2	0.063	0.49	0.023	0.46	0.074	20.9
	Grand mean	313	0.87	19.2	1.01	6.25	0.93	272
***F*-probabilities**[c]								
Nitrogen treatment		**0.01**	0.93	0.08	0.28	**0.01**	0.77	0.50
Legume cultivar		0.28	0.68	0.81	0.29	0.31	0.96	**<0.005**
Af vs. RC		0.13	0.64	0.88	0.28	0.15	0.68	0.11
Nitrogen × legume		0.19	0.39	0.33	0.60	0.56	0.19	0.85
Grass cultivar		**<0.005**	0.84	0.09	0.07	**<0.005**	0.2	0.87
Tm vs. PR		**<0.005**	0.91	0.43	0.15	**<0.005**	0.39	0.78
Nitrogen × grass		0.47	0.11	0.89	0.29	0.50	0.51	0.84
Legume × grass		**0.04**	0.24	0.41	0.45	0.05	**<0.005**	**0.04**
Nitrogen × legume × grass		**<0.005**	**0.03**	0.86	0.40	**<0.005**	**<0.005**	**<0.005**

[a] Combined root and shoot DM.

[b] CI, compatibility index ($V_{\mathrm{combination}}/V_{\mathrm{alone}}$). A score of <1 indicates a negative effect on growth in combination compared with growing alone in similar conditions, while a score of >1 indicates a positive effect. CI mean values in bold indicate a significant difference ($P < 0.05$) from 1.0.

[c] *F*-probabilities significant at the $P < 0.05$ are given in bold.

"Full N" treatments (0.64 and 0.65, respectively; Table 2). Legume N content, on the other hand, remained similar across N levels, while CI values showed that the N content was not significantly different to plants grown alone.

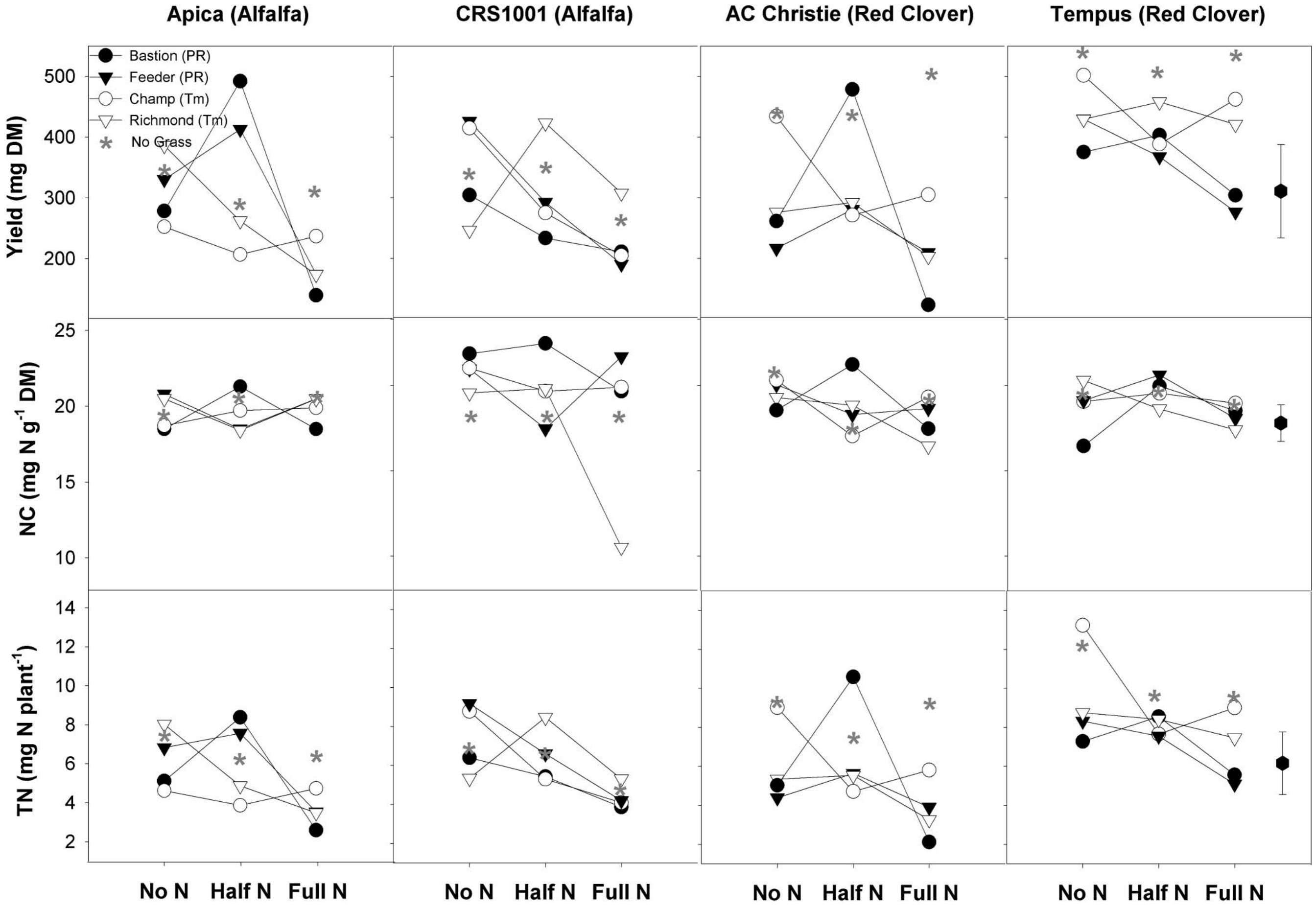
Legume cultivars were similar in DM yield and total N yield, with the exception of the red clover cultivar Tempus, which had higher arithmetic DM yield and total N (Table 2, Fig. 2), though the differences were not significant ($P = 0.28$ and 0.30, respectively; Table 2). Mean specific nodulation rate was highest in one clover cultivar (AC Christie; 409 nodules g^{-1} root DM) and lowest in the other (Tempus; 198 nodules g^{-1} root DM). Grass companion cultivar had a significant effect on the growth of legumes, which appeared to be species-specific: ryegrass cultivars decreased the DM yield of legumes more than timothy cultivars (301 vs. 327 mg DM), while CI scores for legumes coupled with ryegrass were significantly lower than plants grown alone (mean CI = 0.84). This pattern was also present in the total N (Table 1).

As with grasses, legumes cultivars showed distinct patterns depending on the grass companions and the N provided. Legume total N seemed principally a response to the nitrogen × legume × grass interaction, with one cultivar in particular (Bastion) associated with large levels of N accumulation at "Half N" levels in some cultivars (Apica, Christie), while not others (Fig. 2). Grass cultivar had no significant effect on nodulation (Table 2).

Legume-grass combinations

To explore the relative growth and nitrogen accumulation of individual grass-legume combinations, grass total yield was plotted against two other important variables: legume total yield and grass nitrogen content in both real values (Fig. 3) and relative to their performance alone (CI values; Fig. 4). Because the influence of

Fig. 2. Legume total dry matter (DM) yield, N content (NC), and total plant nitrogen (TN) by legume cultivar, grass companion cultivar, and nitrogen fertilization treatment. Hexagonal points in the far right column indicate the grand mean for each attribute; error bars represent the SEM for the three-way interaction of grass × legume × nitrogen treatment.



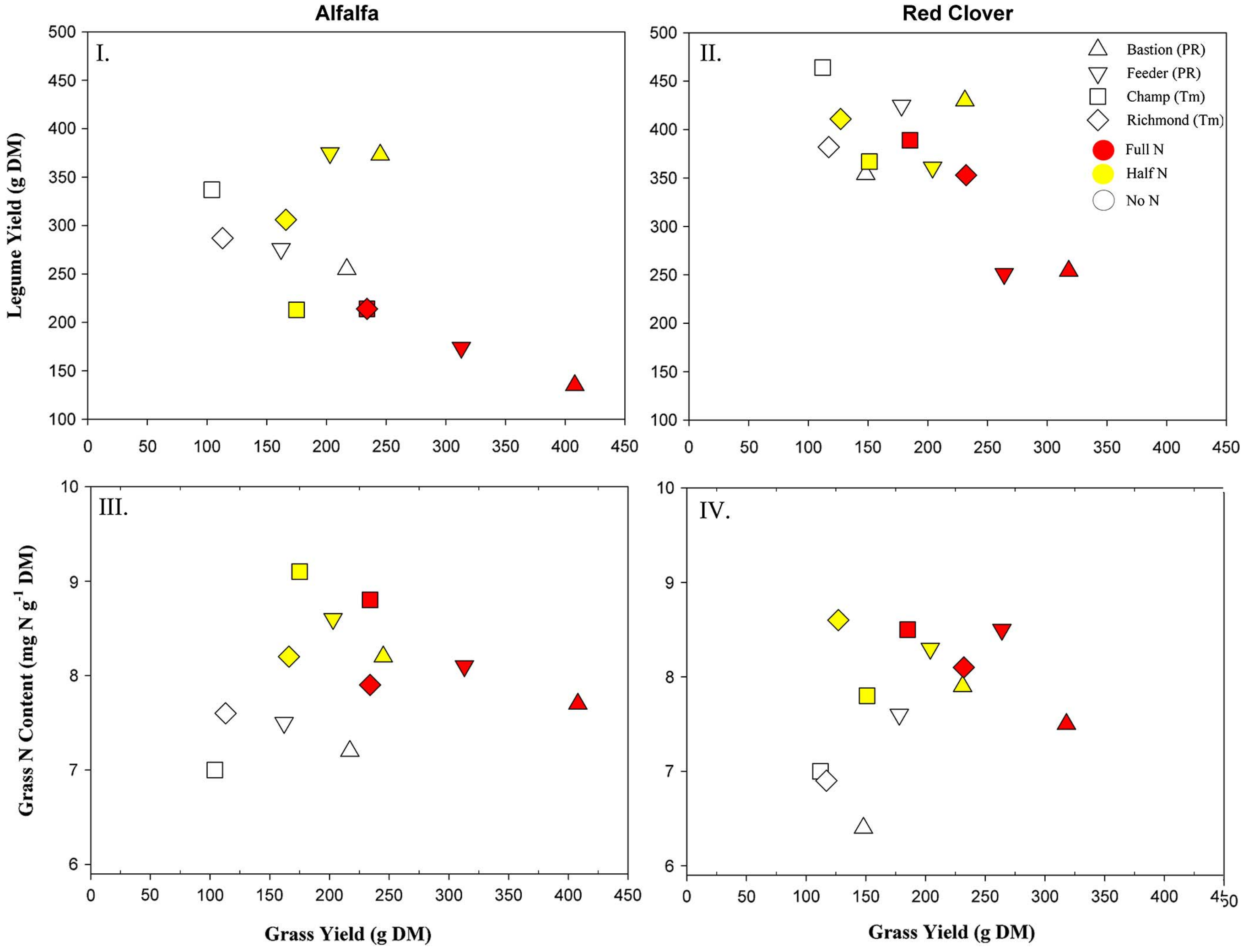
individual legume cultivars was minimal, the mean values of each grass cultivar by legume species by nitrogen treatment were used. In addition, an isocline was added to each graph with the equation "$x \times y = 1$". For the graphs showing the relationship between grass and legume yield CI values (Figs. 4I, 4II), this line represents the point at which total relative growth is neutral compared with growth alone, with any relative gain in one plant corresponding to a similar decline in the other plant. For the graphs showing the relationship between grass, yield, and N content CI values (Figs. 4III, 4IV), this line represents the point at which grass total N is the same as when grown alone, where changes in grass N content change inversely proportional to grass yield.

A generally negative relationship between the yield of grasses and legumes was observed, demonstrating the effect of the larger red clover plants on grass growth (Figs. 3I, 3II). As the results above previously suggested, higher N treatments favoured the growth of grasses at the expense of legumes. The relationship between the yield of grasses and their N content (Figs. 3III, 3IV) shows a positive influence of N additions from the "No N" to "Low N" treatments, but little change from the medium to highest N treatment ("High N"), even as yield increased.

In terms of relative performance given by the CI values, both grasses and legumes generally yielded less than when grown alone (i.e., CI < 1; Fig. 4I), with most treatment groups falling well below the total relative yield isoclines. Alfalfa cultivars at a "Half N" treatment had CI values higher than 1 (with the exception of those paired with Champ) and, given their position relative to the isoclines, increased their yield at the same relative rate as grasses decreased theirs. Combinations of red clover and Bastion (Fig. 3II) showed very similar growth in both plants at the "Half N" level, while clover-Champ combinations at the "No N" treatment showed large gains in relative yield (Gr Yield CI = 1.34), with legume yield largely unchanged (Leg Yield CI = 0.96).

The relationship between relative grass yield and N content (Figs. 4III, 4IV) remained generally close to the total N isoclines, indicating a likely trade-off between grass yield and N content. Champ combinations at the "Half N" level, however, saw large gains in relative N content (CI = 2.60 and 2.32 for alfalfa and clover combinations, respectively) that were larger in magnitude

Fig. 3. Relationship between grass and legume yield (mg DM; I, II) and grass nitrogen content (mg N g^{-1} DM; III and IV) of two cultivars of perennial ryegrass (PR) and timothy (Tm) paired with alfalfa (left) and red clover (right) under three nitrogen treatments.



than their loss in yield, resulting in improved total N. Champ at the "No N" treatment showed an increase in relative yield (CI = 1.37) and N content (CI = 1.23) when paired with red clover, but not alfalfa.

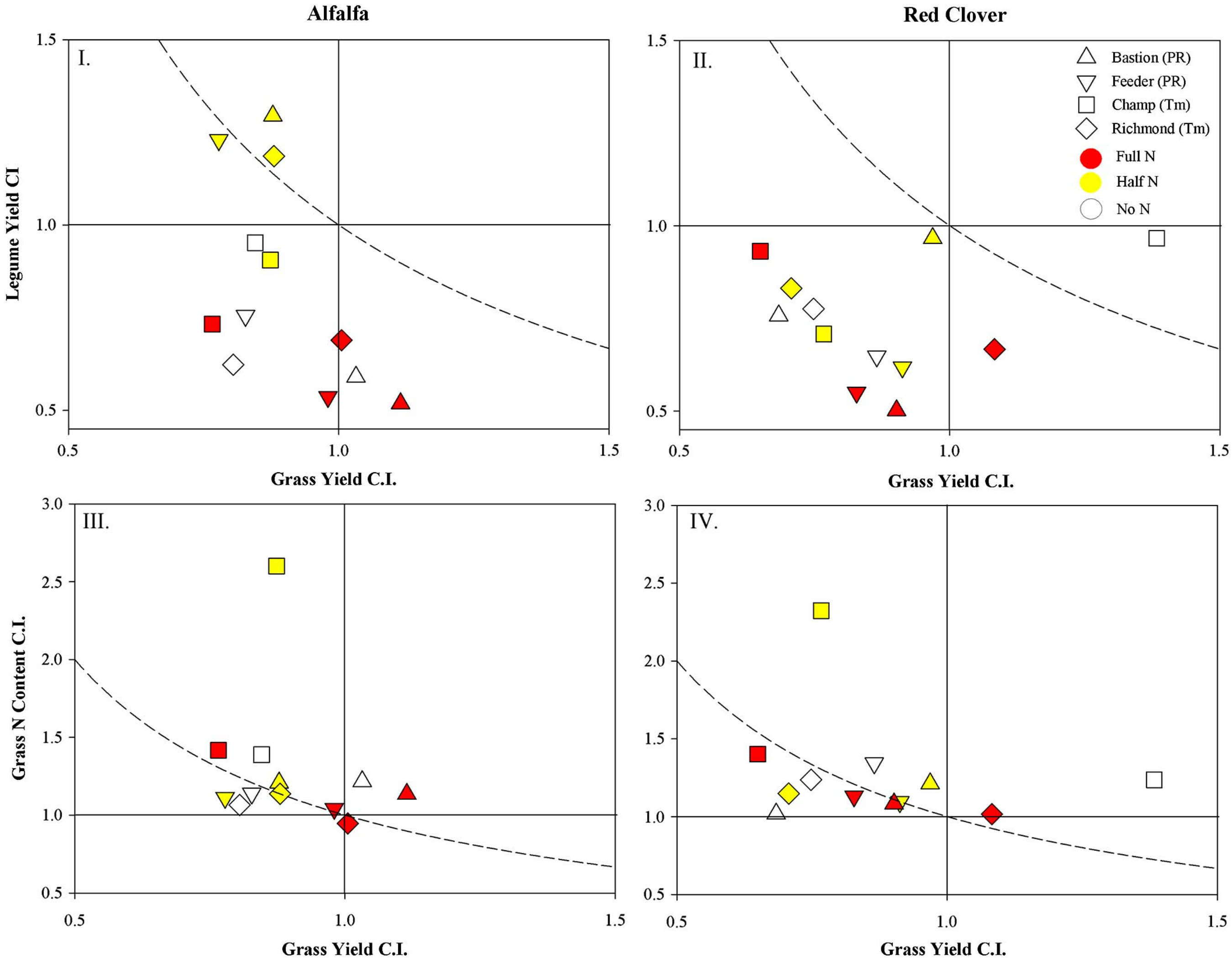
Discussion

The aim of this study was to gauge the relative benefits of legumes to grasses with respect to N accumulation and growth, and how this relationship may change with increasing N-additions. By keeping plant density low relative to available substrate, and providing ample non-N resources (water, light, nutrients), we predicted that the grasses would benefit from the presence of legumes with increased N content and total N relative to growth alone, especially at low N additions, and that this effect would be more pronounced in more compatible cultivar pairs.

As predicted, the N status of grasses was improved with the addition of legumes, both in terms of N content and total N; legume N content stayed consistent throughout, meaning total N was a direct product of biomass accumulation. The addition of N had a consistent positive effect on the growth of grasses, as evidenced by growing yields over N treatments even as grass yield CI values remain similar. Legumes, on the other hand, showed a sharp reduction in yield at the highest level of N, suggesting that decreased growth of legumes was a response to faster growing grasses than the addition of N itself. Contrary to our hypothesis, grasses benefitted most from the presence of legumes in the intermediate N level ("Half N"), although the variation in responses from individual cultivars meant that this was not a significant difference in the main effect.

In terms of DM yield, grasses and legumes respectively produced 89% and 87% when grown in combination than alone. This was somewhat surprising, given that the non-N resources generally cited in grass-legume competitions (light, P; Haynes 1980; Marriott and Zuazua 1996)

Fig. 4. Relationship between relative grass yield (grass yield compatibility index; C.I.), relative legume yield (Legume Yield C.I.; I and II) and relative grass nitrogen content (grass N C.I.; III and IV) of two cultivars of perennial ryegrass (P.R.) and timothy (Tm) paired with alfalfa (left) and red clover (right) under three nitrogen treatments. Dashed lines are isoclines of relative grass total nitrogen (TN CI = 1.0) and total dry matter (TDM CI = 1.0).



were given in ample supply given the density. As for N itself, it was definitely a limiting nutrient for grasses, but our results indicate that grass N content and total N were improved by the presence of legumes, even when yield was not, making competition for N seem unlikely in this scenario. Root space, a common problem in pot experiments, may be another possibility; the total plant biomass for all pots was less than the 1.0 g L^{-1} substrate cited by Poorter et al. (2012) as ideal conditions for pot-grown plants, but in the absence of competition for any specific resource, this seems to be a likely reason for the diminished growth of the plants grown with a partner.

The relationship between grass and legume relative yield over N treatments (Figs. 4I, 4II) show that the effect of suppressed growth was not dramatically affected by the rates of N applied to the pots, nor was there a trend of "trade-off" between grass and legume yield (indicated by movement along the isoclines, instead of away from it). While the uneven density design of this experiment used could not test for true transgressive overyielding (Trenbath 1974), the positions of most of the treatments beneath the grass/legume yield isocline suggest that the gains in yield that some plants make in combination would be unlikely to match the yield of the individual plants if the density were the same.

The observed results for grass cultivars are in agreement with higher plant density pot (25 plants) studies conducted by Butler and Lad (1985) and Beschow et al. (2000), which found that ryegrass grown in combination with legumes (*M. littoralis* and *M. sativa*, respectively) both had reduced growth and improved N nutrition. McNeil and Wood (1990), in an eight-plant per pot red clover-ryegrass study, found a similar reduction in biomass but increased tissue N content when compared with pure grass pots of similar density. In contrasts to these results, Ta and Faris (1987) found that grasses and legumes in eight-plant combination pots produced more biomass per plant in combination than in monoculture over a range of environmental conditions. Similar pot experiments performed with perennial ryegrass and white clover in sand, also in eight-plant designs (Paynel et al. 2001), reported high rates of N transfer (3%–23%) from legume to grass at fertilization rates similar to our study, though dry matter production over the experiment was not given.

Ultimately, we hoped to find compatible cultivars of grasses and legumes that would maximize N accumulation in grasses. We felt that the best measure of this would be change in total N in combination versus alone (as opposed to N content, which may be sensitive to differences in plant size as a result of growing conditions). ANOVA did reveal differences in grass total N CI, which point to two distinct interactions.

First of all, the timothy cultivar ‘Champ’ showed large improvements in total N with all legumes it was paired with, when compared with plants grown alone. This is almost entirely due to its large gains in N content when grown in conjunction with legumes, and largely when N was supplied at a moderate (“Half N” treatment) rate (Fig. 3). While the large CI values may seem impressive, the actual values of N content and total N for Champ grown with a legume are not dissimilar from those of Richmond, the other timothy cultivar used. This suggests that while Champ benefits from the presence of legumes, its poor performance alone is the major factor in its inflated CI score. On the other hand, alfalfa plants grown with Champ also showed a tendency towards lower relative growth compared with other grass cultivars (Fig. 4I), which may have affected the N available in the soil as well. Field studies using this cultivar have shown that even under N-limiting conditions, Champ did not differ much in N content than a diverse group of other timothy cultivars (Brégard et al. 2001). Nitrogen-limiting conditions in field soil, however, may be less severe than those used in this experiment, and our range of grass N content values more closely matched the range of other short-term growth chamber studies using other forage grass species (5–10 mg N g^{-1} DM; Paynel et al. 2008) than what was recorded from that field trial (~22 mg N g^{-1} DM).

Second of all, the perennial ryegrass cultivar ‘Bastion’ showed more moderate improvements in total N when paired with alfalfa cultivars, particularly ‘Apica’ (Figs. 2, 3). Unlike Champ, however, these gains came largely from difference in DM yield, as Bastion was able to maintain relatively high levels of growth while maintaining similar improvements to N content (Figs. 3III, 3IV). This may be due to the lower “aggressiveness” of alfalfa, as compared with red clover, towards grasses reflected in the relative dry matter yield CI (Figs. 3I, 3II). The difference between the two cultivars is also demonstrated by how they react to N additions: Champ benefits most from legumes at the “Half N” treatments, where N was more limiting, while Bastion is able to increase its total relative yield in the “High N” treatment without losing any N content (Figs. 3, 4). These results are in agreement with several N_{15} field studies that have directly measured an increase in the amount of N transferred from legumes to grasses under higher soil N conditions. Høgh-Jensen and Schjoerring (1997) and Elgersma et al. (2000) both showed an increase in N-transfer from clover to associated grasses after N applications in the field, while Nyfeler et al. (2011) showed that grasses benefited most from N transfer from legumes when N fertilization was at moderate levels (150 kg N ha^{-1}) and legumes and grasses were in equal proportions. Pirhofer-Walzl et al. (2012), in a multispecies N transfer study, noted specific differences between the species used here, specifically that alfalfa doubled its transfer of N to grasses after fertilization, while red clover increased their transfer by less than half. In a coupled pot-hydroponic study, Paynel et al. (2008) demonstrated that ryegrass took up more fixed N from clover plants as N fertilization increased, even as the rate of fixation in clover decreased, and was associated with high grass biomass accumulation. These studies suggest that access to soil N stimulates further uptake of N by increasing grass biomass and root length, allowing for greater access to legume-derived N. The rate of growth of grasses therefore becomes an important factor in accumulation, and, as our study shows, is affected by the choice of both grass and legume species and (or) cultivar. While grass identity can affect the rate of growth in a given time, legume identity may also limit it depending on how competitive it is.

In terms of legume response to grasses, one of the more interesting results noted was the positive effect of grass presence on the yield (and ultimately total N) of alfalfa cultivars in “No N” and “Half N” treatments (Table 4, Fig. 3), particularly Apica. While grasses have been shown in increase nodulation rate (Craig et al. 1981) and N-fixation rate (Nyfeler et al. 2011), specific nodulation did not differ much between plants grown with grasses and those without (data not shown), so this is unlikely to be a reason. It is possible that under certain circumstances, plants may sense a potential competitor (Schenk et al. 1999) and increase their growth rate to compensate, but there is no evidence within the literature to confirm suspicions.

From a practical standpoint, these results indicate that there is the potential for selection of superior forage species and cultivar combinations that will allow for more efficient growth and plant N accumulation in forage swards in early growth stages. As the ‘Champ’ results indicate, grasses more sensitive to low soil N levels will likely benefit the most from intercropping with legumes by taking advantage of the extra N provided by legumes compared with growth alone. This may add an additional dimension to consider when choosing species and cultivars for forage production systems selecting for cultivar pairs, and outlines the importance of evaluating performance of forages under a range of soil N levels.

The superior growth of 'Bastion' with alfalfa, on the other hand, highlights another interesting point in the grass-legume relationship: the role of competitiveness. While the probable factor responsible for the reduced growth of plants in this experiment (substrate size) is unlikely to occur in the field, other environmental factors likely will keep the relationship competitive. Results of the perennial ryegrass pots show that, in some cases, potential benefits of increased access to N can be limited by grass size, which means that excess legume N may be lost through leaching, an environmental problem already associated with swards with high legume content (Loiseau et al. 2001; Scherer-Lorenzen et al. 2003).

Conclusion

In this pot study of grass-legume combinations over a range of N fertility levels, perennial ryegrass and timothy cultivars accumulated less DM, when paired with alfalfa and red clover cultivars than when grown alone, but showed higher tissue N content at moderate N levels. One particular cultivar of timothy ('Champ') had large improvements in total N when paired with alfalfa cultivars, but the relative increase was mostly due to its poor performance when grown alone. One cultivar of perennial ryegrass ('Bastion') managed to benefit the most in terms of total N alfalfa cultivars, but generally only when added mineral N was sufficient to promote a higher rate of growth, increasing its capacity for N-uptake and accumulation. Results indicate that certain legume/grass species and cultivars might be more suited to intercropping, but that the benefits of legume for grass N status may be limited as much by grass capacity as legume N supply.

Acknowledgements

We acknowledge the support of the staff of Agriculture and Agri-Food Canada (AAFC), in particular, Matthew Crouse, Mike Pulsifer, Donald Ells, May Ward, and David Baldwin. This work was supported through a Beef Cluster grant by Agriculture and Agri-Food Canada and Beef Cattle Research Council, a division of the Canadian Cattlemen's Association.

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