Transfer of Nitrogen from Legumes to Grasses in Perennial Forages

by

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Dedicated to Sam and Charlie,
two budding scientists who learn more about the world before lunch
than I do in a month...

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Abstract

Nitrogen (N)-fixing legumes are important in forage production not only for the high-quality herbage they provide, but also for their ability to provide a portion of their fixed N to surrounding non-fixing plants, such as grasses. This general process, known as 'N transfer', involves the net transfer of N from one plant (N-donor) to another (N-receiver). While much of this transfer is accomplished through the decomposition of root tissue, recent studies have suggested that a more direct route, via exudation of low-molecular weight N (LMW N) compounds by N-donors and subsequent uptake by N-receivers, may also be important.

To test what effect that plant species and cultivars have on the efficiency of direct N-transfer via exudates in temperate forage species, three key areas involved in its efficiency were examined: legume nodulation (Chapter 2), legume exudation (Chapter 3) and legume-grass compatibility (Chapter 4). Two genotypically different cultivars each of two common legume N-donors, alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*), as well as two N-receiver grasses, perennial ryegrass (*Lolium perenne*) and timothy (*Phleum pratense*) were chosen.

Legume nodulation rate varied mostly between species, with red clover nodulating much higher at lower N-fertility levels than alfalfa, but growth and N-accumulation were similar between the species, suggesting nodulation number might not be indicative of fixation rate. In testing legume N exudation, one specific clover cultivar released much more LMW N than other cultivars tested, which may have been related to its high nodule to dry matter (DM) ratio. Ultimately, however, exudation rate was a poor predictor of N transfer, as grasses grown with alfalfa tended to accumulate more N. Grass species and cultivar were also important, with more aggressively-growing grasses (ryegrass cultivars) accumulating more N.

Ultimately, it was found that two important legume traits for transfer (nodulation and exudation) varied both within and between species, but that the competitive aspects of the legume-grass relationship may limit the amount of N transferred via the exudation pathway. Further research into complimentary growth patterns in N-donor/-receiver pairs may help improve the efficiency in this complex relationship.

List of Abbreviations and Symbols Used

AAFC	Agriculture and Agri-Food Canada
Af	Alfalfa
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BNF	Biological Nitrogen Fixation
C	Carbon
CI	Compatibility Index
cv	Cultivar
DM	Dry Matter
DON	Dissolved Organic Nitrogen
Fe	Iron
GOGAT	Oxoglutarate Aminotransferase
GS	Glutamine Synthase
HPLC	High Performance Liquid Chromatagraphy
K	Potassium
Lin	Linear
N	Nitrogen
n	Number of replicates
NC	Nitrogen Content
NH_4^+	Ammonium
PC	Principal Component
PCA	Principal Component Analysis
PR	Perennial Rygrass

OD	Optical Density
P	Potassium
Quad	Quadratic
RC	Red Clover
T	Timothy
TN	Total Nitrogen

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Chapter 1. Introduction

As a growth-limiting nutrient in most terrestrial systems, nitrogen (N) is a critical factor in all agrosystems. Since the advent of the Haber–Bosch process (Smil 2004), the extensive use of synthetic N fertilizer (and crops responsive to them) has managed to double the number of people supported per hectar of land worldwide (Erisman et al. 2007). But while inorganic N fertilizers can assure higher yields, their production is energy intensive and increasingly expensive. In addition to this, research has show that their long-term use can result in environmental damage including degradation of soils by acidification (Bouman et al. 1995) depletion soil nitrogen (Mulvaney et al. 2009), eutrofication of water due to leaching (Di and Cameron 2002), and release of greenhouse gases (N_2O; Velthof et al. 2006). Given the high monetary and environmental costs, alternatives to synthetic N fertilizers are in demand.

One potential alternative to supply N to agrosystems is the use of legumes, which have the ability to derive a portion of their N requirements from atmosphere via N-fixation instead of the soil (Dixon and Kahn 2004). When grown in a polyculture system, such as multi-species forage production, this benefit can be further extended to non-legumes through a process known as 'N transfer' (Paynel et al. 2008), in which legumes supply a portion of their fixed N to surrounding plants below-ground, improving the surrounding plants' yield and quality (Pirhofer-Walzl et al. 2012). While this transfer can occur through multiple routes, recent research has suggested that at northern latitudes a direct pathway involving the excretion of nitrogenous compounds by legume roots (exudation), and subsequent capture by grasses (uptake), may constitute a major conduit by which

legume-fixed N is supplied to grasses (Gylfadóttir et al. 2007; Lessuffleur et al. 2008; Rasmussen et al. 2013). Given the species and population variation in both legume N exudation rate (Brophy and Heichel, 1989; Ta and Faris 1989; Ofosu-Budu et al. 1992; Thilakarathna 2013) and grass N uptake ability (Vazquez de Aldana and Berendse, 1997; Brégard et al. 2001; Weigelt et al. 2005), there exists a prime opportunity to improve upon the efficiency of this pathway and the productivity of low-input grass-legume stands.

1.1 Background

1.1.1 Biological Nitrogen Fixation

Biological N-fixation (BNF) generally refers to the reduction of atmospheric N into ammonia via the enzyme complex known as 'nitrogenase' (Halbeid and Ludden 2000). The diverse group of bacteria and archea that can perform this reaction are collectively known as 'diazotrophs', and of them, two main groups, the filamentous bacteria of the genus *Frankia* and two phyla of proteobacteria known collectively as *Rhizobia* (Fani et al. 2000), form symbiotic relationships with plants. While *Frankia* spp. can associate with a wide variety of plants, *Rhizobia* are restricted to certain members of the legume family (Fabaceae), with which they form very specific symbioses (Hirsch et al. 2001).

Through a complex exchange of molecular signals, similar to those used by mycorrhizae (Gherbi et al. 2008), rhizobia bacteria enter legume roots via 'infection threads' (Jones et al. 2007) and cause a rapid cell division in the cortex to form a structure known as a nodule (Sprent 2008). The structure of a nodule can vary greatly between legume species, but they share several key common elements, including an infection zone which houses

the symbiotic bacteria and a protective layer made up of an inner cortex and outer cortex (Serraj et al. 1999; Sprent 2008). Nodules are also noted for their production of a hemoglobin protein known as 'legheamoglobin' in the cells of the infection zone that regulate oxygen levels. Once inside the new nodule, Rhizobia change their structure to become bacteriods which, depending on their species, involves increasing their cell size, amplifying their genome and increasing their production of N-fixation-related proteins (Haag et al. 2013).

Nitrogen fixation is the conversion of di-nitrogen gas into ammonia, which is achieved in Rhizobia by the nitrogenase enzyme via the equation below (Dixon and Kahn 2004):

$$N_2 + 8\ e^- + 8\ H^+ + 16\ MgATP \rightarrow 2\ NH_3 + H_2 + 16\ MgADP + 16Pi$$

The ammonia produced is then assimilated into amino acids by the plant via the GS-GOGAT cycle (Lam et al. 1996) and then converted in a form suitable for transport in the xylem. In temperate legumes this is amides (typically apsaragine and glutamine) and in tropical legumes, ureides (allantoin and allantoic acids; Tegeder 2014). In return for the N, plants provide carbohydrates to the bacteroids that sustain their growth and powers fixation (Udvardi and Day 1997).

As a means of procuring N, fixation is much more energy-intensive than uptake from the soil and is only thought to be metabolically efficient under low-N conditions (Cannell and Thornley 2000). It is therefore crucial for legumes to regulate nodulation and N-fixation under conditions that do not favour it. Root exposure to mineral N, particularly nitrate, acts as a powerful negative feedback on several stages of fixation, including rhizobial infection (Coronado et al. 1995), nodule formation (Heidstra et al. 1997; Mohd-

Radzman et al. 2013) and fixation itself (Svenning et al. 1996; Fujikake et al. 2003). High levels of amino acids in the shoot can also downregulate fixation (Tegeder 2012) and nodulation (Sasaki et al. 2014).

1.1.2 Nitrogen Transfer

While legumes fix atmospheric N for their own use, non-fixing plants in their vicinity can also benefit from it through a process known as 'N-transfer' (Paynel et al. 2008). This process is the flow of N through the soil from one plant, known as the 'N-donor', to another, known as the 'N-receiver'. In legume-grass intercrops, the rate of transfer has been recorded from virtually none to 75–110 kg N ha^{-1} y^{-1} (Elgersma and Hassink 1997; Høgh-Jensen and Schjoerring 2000) and up to 80% of the grass N (Moyer-Henry et al. 2006; He et al. 2009).

Three main pathways are recognized as the major routes of N-transfer between plants (Fig. 1.1). First, the 'decomposition pathway', in which senesced root material from the N-donor is mineralized by soil microbes, releasing bound organic N into forms available for uptake by N-receivers (Wichern et al. 2008). This pathway is one of the major routes of N-transfer in the long-term, contributing up to 100 kg N ha^{-1} yr^{-1} in forage systems (Ledgard and Steele 1992). It is also, however, the slowest: it can take up to 20 days for legume tissue to decompose enough to release a significant amount of N (Bingham and Rees 2008), and this rate is dependent on environmental conditions, which can slow drastically at lower temperatures, soil moisture and nutrient availability (Goodman 1988). Second, the 'mycorrizhae pathway', in which N is transferred from an N-donor to an N-receiver linked by a common mycorrizhal network (Høgh-Jensen 2006). Thus far,

experimental evidence shows that transfer by this route in the field is relatively small, making up only a few percent of total N of the receiver (Rogers et al. 2001; Paynel et al. 2008), though reports of up to from 20-50% have been recorded in artificial systems (He 2003). Finally, there is the 'exudation' or 'direct pathway', which involves the exudation of soluble, low-molecular weight (LMW) N compounds from the N-donor which are then taken up by the N-receiver. Typically, these LMW N compounds consist of amino acids, ammonium, and occasionally nitrate and small peptides (Fustec et al. 2010).

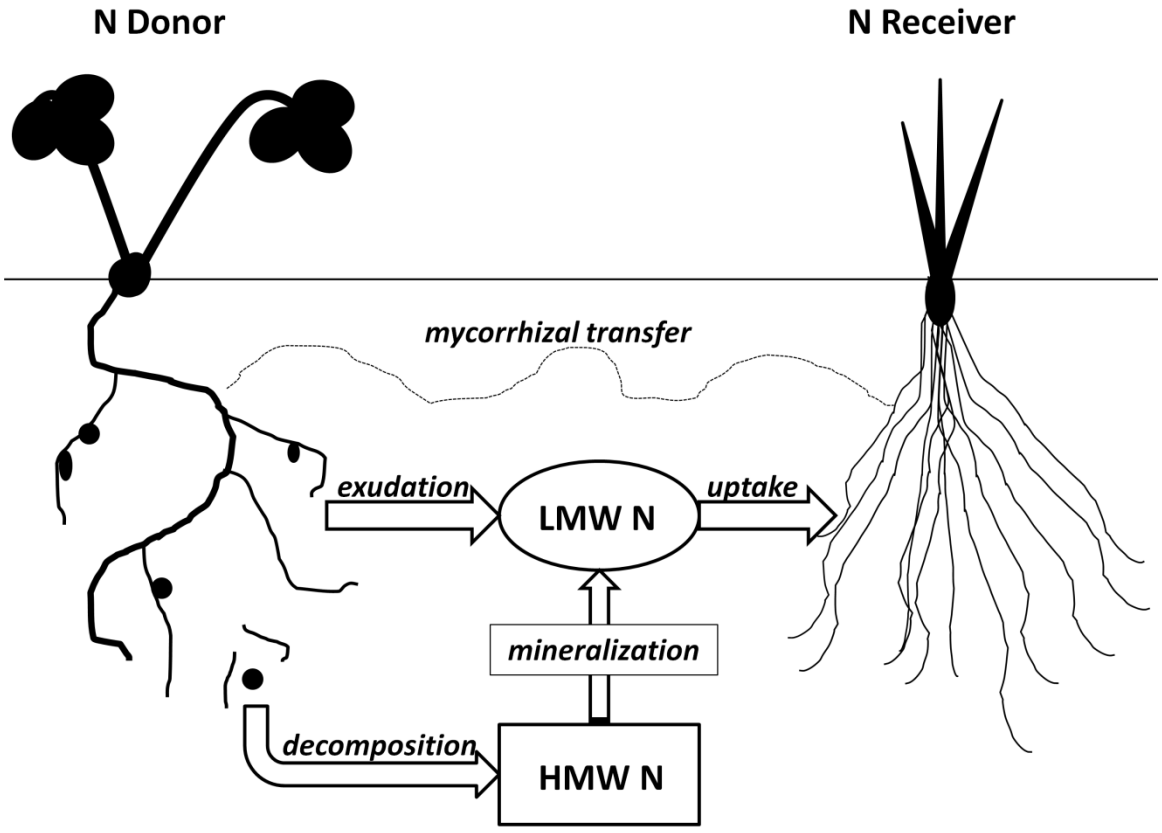

Figure 1.1 Three recognized routes of Nitrogen transfer between plants: decomposition of root material, exudation of nitrogenous substances from roots, and direct transfer via mycorrhizal connections. HMW N= high molecular weight nitrogen compounds; LMW= low molecular weight nitrogen compounds. (Adapted from Paynel et al. 2008.)

While difficult to measure directly in the field, experiments under controlled conditions have demonstrated that direct transfer can occur at levels meaningful to N-receiver plant growth (Tomm et al. 1994; Paynel et al., 2001; Paynel and Cliquet, 2003; Xiao et al. 2004).

1.1.3 Mechanisms of Exudation

Both the exudation and uptake of LMW N compounds depend on a set of linked processes moving them simultaneously in and out of the root: influx and efflux (Miller and Cramer, 2005). The ratio of influx to efflux of a compound at a point in time will determine if there is a net uptake (influx>efflux) or exudation (influx<efflux) (Phillips et al. 2004). Efflux of N compounds across the root membrane is largely the result of passive diffusion, facilitated by aquaporins and anion channels (Miller and Cramer 2004), though in cases where excess N is present in root cells, they may be actively effluxed (Kronzucker et al. 1999; Britto et al. 2001). Influx is primarily an active process, controlled by a variety of ATP-dependant membrane transport proteins (Williams and Miller 2001). Nitrate is taken up by two main transporter families: the Peptide Transporters (PTR or NRT1) and Nitrate-Nitrate Porters (NNP or NRT2), the former being primarily low-affinity transporters (Tsay et al., 2007) and the latter almost exclusively high-affinity transporters (Orsel et al. 2002), with both groups having inducible and constitutive members. Ammonium is transported by proteins of the Ammonium Transporter family (AMT), which occur in both low- and high-affinity forms (Ludewig et al. 2002) and are mostly inducible (von Wiren et al. 2000). Amino acids are taken up by a variety of transporters, which can generally be grouped into two groups:

The Amino Acid-Polyamine-Choline (APC) superfamily, and the Amino Acid/Auxin Permease family (AAAP).

The APC transporters in plants are not well studied, but are known to include the high-affinity basic amino acid transporter AtCAT1 (Frommer et al. 1995) while the AAAP family is thought to be responsible for the transport of neutral and acidic amino acids, however most of the transporters isolated from either group are not very specific, showing a low-affinity for a wide range of amino acids (Williams and Miller 2004). There have also been systems identified for the uptake of small (3-5 amino acid) peptides (Tegeder and Rentsch 2010), but less is known about how they contribute to overall N uptake.

Table 1.1 Quantification of LMW-N exudation across many studies by species. If multiple treatments or genotypes used, the range of averages is given.

Study	Species	N Exudation Rate	Culture System
Brophy and Heichel 1989	*Medicago sativa*	NH_4: 52.2-164.2 μg plant^{-1} d^{-1}	sterile sand microcosm
	Glycine max	NH_4: 818-2018 μg plant^{-1}d^{-1}	
Bowen 1969	*Pinus radiata*	TDN: 25-248 pM plant^{-1} (2 wks)	Hydroponic
Carvalhais et al. 2011	*Zea mays*	tAA:172-525 pM g^{-1} (FW) h^{-1}	Hydroponic
El-Baz, M. et al. 2004	*Cucumis sativus*	tAA: 292.11-568.63 μg g^{-1} root DW (4 h)	Hydroponic
Fan et al. 2001	*Hordeum vulgare*	tAA:4.57 μg g^{-1} root DW (3 wks)	Hydropnic
	Triticum	tAA: 0.47 μg g^{-1} root DW (3	

	aestivum	wks)	
	Oryza Sativa	tAA: 0.6 µg g^{-1} root DW (3 wks)	
Klein et al. 1988	*Agropyron cristatum*	tAA: 9.86-31.68 mg g^{-1} DW roots (90d)	sterile/non-sterile microcosms
	A. smithii	tAA:10.76-22.72 mg g^{-1} DW roots (90d)	
	Bouteloua gracilis	tAA: 26.25-43.42-mg g^{-1} DW roots (90d)	
Kraffczyk et al. 1984	*Z. mays*	tAA: 0.14-1.4 ng mg^{-1} root DW h^{-1}	axenic sand microcosm
Kremer et al. 2005	*G.max*	tAA: 1.1-2.5 mmol $plant^{-1}$ (16d)	
Ofosu-Budu et al. 1990	*G.max*	TDN: 74.3-305 µg N $plant^{-1}$ h^{-1} Ur: 6.7-26.5 µg N $plant^{-1}$ h^{-1} NH_4: 158.7-898.9 µg N $plant^{-1}$ d^{-1} tAA:44.5-85.3 µg N $plant^{-1}$ d^{-1}	Hydroponic
Ofosu-Budu et al. 1992	*G. max*	Ur: 36.8-37.8 µg N $plant^{-1}$ d^{-1} NH_4: 17.1-25.2 µg N $plant^{-1}$ d^{-1}	Hydroponic
	Sesbania cannabina	Ur: ND NH_4: 15.6-15.9 µg N $plant^{-1}$ d^{-1}	
	Astragahls sinicus	Ur: ND NH4: 18.7-23.5 µg N $plant^{-1}$ d^{-1}	
Paterson et al. 2003	*Festuca rubra*	NH4: 4.6 µg N $plant^{-1}$ d^{-1} tAA: 0.15 µg N $plant^{-1}$ d^{-1}	axenic sand microcosm
Paynel et al. 2001	*T.repens*	NH_4: 135 nM $plant^{-1}$ d^{-1} tAA: 2.0-25 nM $plant^{-1}$ d^{-1}	axenic sand microcosm

	Lolium perenne	NH_4: 131 nM plant^{-1} d^{-1} tAA:3.0-5.5 nM plant^{-1} d^{-1}	
Paynel and Cliquet 2003	*T. repens*	NH_4: 1.36-1.65 µg plant^{-1} d^{-1} tAA : 60-91 ng N·plant^{-1}d^{-1}	axenic sand microcosm
	L. perenne	NH_4: 0.41-2.19 µg plant^{-1} d^{-1} tAA : 77-84 ng N·plant^{-1} d^{-1}	
Paynel et al. 2008	*T. repens*	NH_4: 29 nM plant^{-1} d^{-1} tAA: 2.3 nM plant^{-1} d^{-1}	axenic sand microcosm
Rasouli-Sadaghiani et al. 2011	*H.vulgare*	tAA: 1.85-878 uµmol g^{-1} FW (28 d)	non-axenic pots
Ratnayake et al. 1978	*Sorghum vulgare*	tAA: 63-200 µg N g^{-1} plant DW (16-17h)	Hydroponic
	Citrus aurantium	tAA: 29-83 µg N g^{-1} plant DW (16-17h)	
Schroth et al. 1966	*Phaeolos vulgaris*	TDN:0.91-3.21 mg N g^{-1} DW d^{-1}	axenic sand microcosm
	Gossypium hirsutum	TDN: 1.13-7.2 mg N g^{-1}DW d^{-1}	
	Pisium sativum	TDN: 1.88-5.75 mg N g^{-1}DW d^{-1}	
Sundin et al. 1990	*Brassica Napus*	tAA: 1.1-2.1 µg g^{-1} DW (15 days)	axenic sand microcosm
Svenningsson et al. 1990	*B. Napus*	tAA: 553-982 µg g^{-1} DW (3 d)	axenic sand microcosm
Ta et al. 1986	*M. sativa*	15 µg N plant^{-1} day^{-1}	Hydroponic

Table 1.2 Review of the effects of selected treatments on LMW N exudation across multiple studies

Treatment	Effect on LMW N exudation	Species	Ref.	Notes
Temperature	+	*Solanum lycopersicum*	Rovira 1959	amino acid exudation increased with temperature
	+	*Trifolium subterraeum*		
	+	*Phalaris tuberosa*		
	+	*Phaseolus vulgaris*	Schroth et al. 1966	'Ninhydrin-positive' exudation increased with temperature
	+	*Gossypium hirsutum*		
	+	*Pisium sativum*		
	+	*Zea mays*	Vančura 1972	Amino acids exudation increased with temperature
	+	*Cucumis sativus*		
	+/-	*Glycine max*	Ofosubudu et al. 1992	Amino acid exudation increased with higher temperatures, ammonia and ureides stayed similar or decreased
	+/-	*Seshania cannabina*		
	+/-	*Astragalus sinicus*		
Light	+/-	*S. lycopersicum*	Rovira 1959	General decrease of amino acid exudation with light intensity; tomato exudation of serine and asparagine increased
	-	*T.subterraeum*		
	-	*P. tuberosa*		

	-	*Sorghum vulgare*	Furgeson and Menge 1982	decrease in amino acid exudation with increasing light intensity
	+	*Z. mays*	Melnitchouck et al. 2005	increase in amino acid exudation during light cycle
Carbon Dioxide	0 0 +	*Lolium multiflorum* *Medicago truncatula* *Z. mays*	Phillips et al. 2005	increase in amino acid efflux level in *Z. mays* with increased CO2
Defoliation	+	*G. max*	Ofosu-budsu et al. 1995	increase in total nitrogen exudation with defoliation
	+	*Festuca rubra*	Paterson et al. 2003	increase in ammonium exudation with defoliation
Water Stress	-	*Brassica napus*	Svenningson et al. 1990	decrease in amino acid exudation with water stress
Nitrogen	+	*Pinus radiata*	Bowen et al 1969	decrease in amino acid exudation with N deprivation
	+/-	*Trifolium repens*	Paynel et al. 2008	increase in ammonia exudation with nitrate

				fertilization, decrease in amino acids; no net change
	+	*Z. mays*	Carvalhais et al. 2011	decrease in amino acid exudation with N deprivation
Non-N Nutrients	+/-	*S. Lycopersicum* (Ca)	Rovira 1959	inconsistent effects of Ca additions
	+/-	*T.subterraeum* (Ca)		
	+/-	*P. tuberosa* (Ca)		
	-	*S. vulgare* (P)	Ratnayaki et al. 1978	decrease in amino acid exudation with P additions
	-	*S. vulgare* (P)	Schwab et al. 1981	decrease in amino acid exudation with P additions
	-	*Z. mays* (K)	Kraffczyk et al. 1984	decrease in amino acid exudation withK additions
	-	*Z. mays* (P, K Fe)	Carvalhais et al. 2011	increase in amino acid exudation with all nutrient deficiencies
Mycorrhizal infection	-	*S. vulgare*	Graham et al. 1981	decrease in amino acid exudation in infected plants
	-	*Pinus silvestrus*	Leyval and	increase of

+	*Fagus silvatica*	Berthelin 1993	total amino acid exudation in *F. Silvatica*, decrease in *P sylvestrus*.
0	*Z. mays*	Azaizeh et al. 1997	no change in amino acid exudation in infected plants

'+'= positive response to treatment, '-'=negative response to treatment; '0'=no effect of treatment, '+/-'= effects varied.

Influx and efflux rates can alter rapidly with environmental conditions, with plants changing from net uptake to net exudation of compounds in a relatively short time. Table 1.1 outlines some of the major factors that have been identified as altering the exudation rate of amino acids, ammonium or nitrate. Generally, exudation of LMW N, particularly amino acids, seem to increase when there is an interruption in photosynthesis during the light cycle (i.e. at lower light intensities or when defoliated) and during non-N macronutrient deficiencies. While the former is thought to be a result of decreased ATP levels in the root, leading to a decrease in active re-uptake levels (Ofosu-budu et al. 1995) it is not clear as to which portion of the influx-efflux system is affected in each case. In a study of the effects of microbial products on the amino acid exudation rate of corn (*Zea mays*), Phillips et al. (2004) discovered through the use of radioactive isotopes that one compound (2, 4-diacetylphloroglucinol) decreased the rate of influx while another (zearalenone) increased the rate of efflux, suggesting that both systems are potential control points for modulating exudates or uptake rates.

Plant genetics also play an important role in exudation and uptake. Interspecific differences in N exudation rates have been noted in a number of plant groups, including trees (Leyval and Berthelin 1993; Grayston et al. 1997), legumes (Brophy and Heichel, 1989; Ofosu-Budu et al. 1992), cereals (Vančura 1966) and other crop species (Rovira 1956; Vančura et al. 1972; Phillips et al. 2006; Lesuffleur et al. 2007). Table 1.2 outlines studies with estimates of LMW N exudation across many species. Generally, nodulated legumes tend to exude more N relative to their size than non-fixing plants (Rovira 1956; Paynel et al. 2001; Phillips et al. 2006; Lesuffleur et al. 2007). Within the former, tropical legumes tend to exude more N overall than temperate species, but usually in the form of amino acids and ureides instead of ammonia (Brophy and Heichel, 1989; Ofosu-Budu et al. 1992) which may be the result of the different N transport system from the nodules (see above). Part of the difference in exudation rates between species may be related to root morphology. Highly-branched root systems are thought to exude more LMW N compounds, both because of their higher surface to volume ratio and the number of root tips, which are thought to be the major site of exudation in most plants (Lesuffleur and Cliquet 2010).

Intraspecies differences in exudation have also been noted, though they are not as common. Mozafar et al. (1992) and El-baz (2004) each observed dissimilar amino acid content in the exudates of genotypes sensitive to iron deficiency in tomato and cucumber, respectively. Rasouli-Sadaghiani et al. (2012) observed similar differences in barley genotypes differing in Zn uptake efficiency while Li et al. (2009) noted similar differences in amino acid profile content between cultivars of cotton. Finally, Thilakarathna (2013) showed that different populations of red clover had different rates

of N exudation, and suggested that higher ploidy level (i.e. tetraploid versus diploid varieties) may result in higher exudation of N.

1.1.4 Direct N transfer in Grass-Legume Intercrops

The difficulty of separating the effects of each N transfer pathway in the field means that there are very few definitive studies on the impact of direct transfer of N via LMW exudates (Høgh-Jensen 2006). However, several studies have made a compelling case for its importance. Gylfadóttir et al. (2007), for example, reported high levels of N-transfer from white clover (*Trifolium repens*) to smooth meadow grass (*Poa pratensis*) (nearly 50% of the grasses' N-uptake) in a north European grassland over a period too brief to be accounted for by decomposition pathways. Rasmussen et al. (2013) found similar results using clover and perennial ryegrass (*Lolium perenne*), in a similar environment, while noting that plant competition between N-donors and receivers also played a critical role. Microcosm experiments mimicking field conditions have also provided evidence of significant levels of direct N transfer. In an isotope-tracer microcosm study, Lessuffleur et al. (2013) observed a significant difference of N accumulation in *L. perenne* grown with *T. repens* in 3 days while also noting an increase in clover-derived ammonia in the soil solution, confirming earlier reports by Paynel et al. (2001, 2008) using similar systems.

Given the evidence of the importance of N transfer via exudates in grass-legume intercrops, and the variation in LMW N exudation at both between and within legume species, there exists the potential to improve the efficiency of N transfer in the grass-legume relationship by selecting legumes that exude higher levels of LMW N, and

grasses that may be able to respond to this input. The effect of species and/or cultivar of both N-donor and N-receiver may be critical into improving the efficiency of forage production in low-input systems.

1.2 Research Hypothesis and Objectives

1.2.1 Research Hypothesis

Legumes and grasses species and cultivars differ in their ability to transfer N directly via exudates during growth.

1.2.2. Research Objectives

The main objective of this research is to investigate the variability among pairs of grasses and legumes for their efficiency in transferring N.

The specific objectives of this research were to:

1. Evaluate potential quality and quantity of N in the exudates of N-donor legume species and differences between cultivars within those species.
2. Characterize the transfer of N from N-donor legume species grown in combination with N-receiver species and differences between cultivars within those species.
3. Determine whether N-donor identity, N-receiver identity, or a combination of both is most critical for the efficient transfer of N between the two.

1.3 Research Approach

To be able to address the specific objectives set out for my research, I first selected two common pasture legumes (alfalfa, *Medigaco sativa*; red clover *Trifolium pratense*) and two common grasses (perennial ryegrass, *Lolium perenne*; timothy, *Phleum pratense*) as my N-donors and N-receivers, respectively. Two distinct cultivars were chosen from each to be able to gauge potential intraspecies variation. Details on the species and cultivars can be found in Table 1.3.

The investigation into N-transfer efficiency between these four potential N-donors and receivers was broken down into three main studies:

(1) **Characterization of Nodulation and Growth of Red Clover (*Trifolium pratense* L.) and Alfalfa (*Medicago sativa* L.) Cultivars Under Different Nitrogen Fertilization Levels (Chapter 2):** As the site of N-fixation, nodule number can directly impact the quantity of N a potential N donor could supply to receivers. In order to gauge the potential quantity of N that N-donors could provide, a growth chamber study was performed to determine the relative nodulation rate among legumes over a range of mineral N fertilization rates. This study also helped to provide information for subsequent experiments on how fertilization could restrict nodulation.

(2) **Characterization of Nitrogen Exudation from Two Cultivars of Red Clover (*Trifolium pratense* L.) and Alfalfa (*Medicago sativa* L.)(Chapter 3):** Besides quantity of N, it is important to characterize the quality of N present in donor exudates as receivers can vary in the capacity to uptake certain forms of N. This growth chamber study used microlysimeters to collect exudates from growing

legumes and test for levels of specific amino acids, ammonia and other forms of organic N, which would be available for uptake by receivers.

(3) **Interactions between Cultivars of Nitrogen-fixing Legumes Species (*Trifolium pratense* L., *Medicgo sativa* L.) and Grasses (*Phleum pratense* L., *Lolium perenne* L.) Under Different Nitrogen Levels (Chapter 4):** To test for compatibility and N-transfer efficiency between donors and receivers, all pairs of donors and receivers were grown in controlled conditions under several N fertilization levels. Effects on both donors and receivers were measured to determine the compatibility of the pairs.

In addition to this, a literature review on current knowledge on low molecular weight N exudation to investigate current theories on why this phenomenon occurs, and what the ecological function behind it might be, is included. This is given in chapter 5, and is titled: “Why Lose Nitrogen? Plant Exudation of Low Molecular Weight Nitrogen”

Table 1.3: Brief description of species and cultivars used as N-donors and N-receivers for this study.

N-Donors (legumes)	**Alfalfa (*Medicago sativa*)** is a perennial temperate legume, originating in central asia, cultivated for forage, particularly in hay/silage. Both diploid and tetraploid varieties exist. It is nodulated by the bacteriod *Sinorhizobium meliloti* on both the crown and the lateral roots	cv Apica: A tetraploid tap-rooted variety adapted for Eastern Canada (Michaud et al. 1983)
		cv CRS1001: A tetraploid rhizomotous variety adapted to Atlantic Canada for grazing (Papadopoulos, unpublished data)
	Red Clover (*Trifolium pratense*) is a perennial temperate legume originating in Europe, typically cultivated for pasture or as a plow-down crop. Both diploid and tetraploid varieties exist. It is nodulated by *Rhizobium leguminosarum* biovar *trifolii,* most commonly on lateral roots	cv AC Christie: early flowering diploid variety adapted for Atlantic Canada (Martin et al. 1999)
		cv Tempus: tetraploid variety known for persistence and high yields
N-Receivers (grasses)	**Perennial Ryegrass (*Lolium Perenne*)** is a perennial bunchgrass grown in temperate climates worldwide for forage production, but especially in Europe. Both diploid and tetraploid varieties exist.	cv Bastion: an early-maturing tetraploid variety developed in Holland for grazing tolerance and crown-rust resistance
		cv Feeder: a mid-maturing diploid variety
	Timothy (*Phleum pratense*) is a perennial bunchgrass native to Europe but wide naturalized in temperate areas across the northern hemisphere. A hexaploid, it is most often grown for hay	cv Champ: a mid-maturing hexaploid variety developed in Ontario, adapted for eastern Canada.
		cv Richmond: an early-maturing hexaploid variety

Chapter 2: Characterization of nodulation and growth of red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) cultivars under different nitrogen fertilization levels

2.1 Abstract

Nitrogen (N)-fixing legumes often suppress nodulation in response to elevated soil mineral N as a way of switching from an energetically-expensive method of acquiring N (N fixation) to a lower one (N uptake). While this may increase efficient use of resources within the plant itself, forage production in mixed-species grasslands can benefit from N-fixing legumes at higher levels of soil N so that they will not compete with non N-fixing plant for this resource. Past research has noted that different species and populations of legumes can vary in their nodulation response to available soil N, a potentially valuable trait for forage legume breeders. To investigate inter- and intra-species effects of N on nodulation, two diverse cultivars each of red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) were cultivated in a sand-culture growth chamber under four N fertilization treatments (0, 0.5, 1.0, 2.5 mg N $plant^{-1}$ wk^{-1}), with ammonia as the sole mineral N source. Increasing N negatively impacted nodulation, with plants in the 2.5 N mg N $plant^{-1}$ wk^{-1} treatment yielding on average only 39% of the total nodules produced when no N was supplied. A corresponding reduction in specific nodulation rate from 110.3 to 51.8 nodules g^{-1} root dry matter (DM) was observed over the same range. While red clover cultivars produced more nodules overall compared to alfalfa cultivars, they were also more sensitive to N additions, producing 74% fewer nodules at the 2.5 mg N $plant^{-1}$ wk^{-1} N level, compared to a 37% reduction in alfalfa nodules. Red clover morphology changed more drastically with increasing N, decreasing total size and leaf

area, in contrast to alfalfa cultivars, which stayed largely the same. All plants maintained similar levels of total N (13.37 mg N plant^{-1}) among treatments, suggesting that both species were able to maintain N accumulation rates, but in different ways. Results suggest that there may be important species differences in response to exposure to N which may be related to root morphology and nodule type.

2.2 Introduction

As a limiting nutrient of agro-systems worldwide, nitrogen (N) has been the focus of many attempts to improve the efficiency of agriculture while reducing inputs of expensive and often environmentally damaging fertilizers (Byrnes, 1990). One solution has been to harness the power of biological N fixation, a process resulting from the mutualistic association between legumes and symbiotic rhizobia bacteria, which have the ability to convert atmospheric N into forms available for plant use. In forage production, this process is particularly important, because N fixation of legumes can have positive effects on the growth of neighbouring non-fixing plants as well, in a process termed 'N transfer' (Paynel et al. 2008, Fustec et al. 2010, Thilakarathna et al. 2012a), which can help lower the cost of producing animal feed (Herridge et al. 2008; Nyfeler et al. 2011).

One of the major objectives of legume breeding is to select for populations with more efficient N fixation, which often involves increasing the size and number of nodules per plant (Herridge and Rose 2000). In forage legumes, studies have shown variation between lines for nodulation rate among white clover (Jones and Ardarson 1979; Abberton et al. 1998), alfalfa (Tan and Tan 1988, Hernández et al 1995; Charman et al 2008) vetch (Mytton et al. 1977), and red clover (Nutman and Reilley 1981;

Thilakarathna et al. 2012a). Nodulation is also, however, determined by environmental interactions, including soil salinity, pH, moisture, temperature, phosphorus (P) and N availability (Zahran 1999). The negative effect of available N on nodulation in legumes is well documented (Van Schreven 1959; Chambers et al. 1980; Caroll and Gresshoff 1983; Harper and Gibson 1984; Wielbo and Skorupska, 2008; Thilakarathna et al. 2012a) and is believed to be part of a regulatory system to keep plant N acquisition more efficient. Acquiring N by fixation has been estimated to cost as much as four times the photosynthate required to take up mineral N from the soil (Cannell and Thornley 2000) making the production of nodules when fixation is not required an unnecessary cost. Efficient use of resources means that a legume will have an optimum number of nodules in a given situation, which depends largely on its access to N (Oka-Kira and Kawaguchi 2006; Mortier et al. 2012) or its availability relative to other macronutrients, such as P (Hellsten and Huss-Danell 2000; Chmelíková et al. 2014).

Because of the importance of N availability to nodulation, the N-status of legumes plays a role in the regulation of nodulation development at multiple stages. It has been shown that alfalfa will suppress its production of rhizobia-attracting flavonoids in the presence of nitrate (Coronado et al. 1995), arresting the initiation of nodulation . After rhizobia have been attracted and nod genes induced, nodulation can still be blocked locally via the suppression of root hair deformation, which can occur through exposure to nitrate (Heidstra et al. 1997) or ammonia (Mohd-Radzman et al. 2013). Finally, studies of knock-out mutants in model legumes that produce ‘hypernodulating’ and ‘supernodulating’ phenotypes have identified three separate long-distance signalling systems of autoregulation of nodulation (AON), each linked to the presence of a specific

stimulus: light on the root surface (*astray* mutant; Nishimura et al. 2002), ethylene levels (*sickle* mutant; Penmetsa and Cook 1997) and N availability (*nitrogen-tolerant symbiosis* mutant; Duc and Messager 1989). While the precise mode of action of these regulation systems is not known, evidence points to a suppression of cortical cell division in the primordium (Reid et al. 2011). Nitrate is particularly inhibitive of nodulation and N fixation (Svenning et al. 1996; Fujikake et al. 2003), but high levels of ammonia can also adversely affect a legume's ability to produce its own source of N (Wang and Stacey, 1990; Gulden and Vessey, 1998). On the other hand, low concentrations of ammonia can stimulate the nodulation in legumes that produce the indeterminant nodules common to most temperate legumes (Guo et al. 1992; Waterer et al. 1992; Fei and Vessey 2003, 2009). It has been suggested that this is related to ammonium's effect on the balance of ethylene to auxins in the root tissues (Fei and Vessey 2009), an important determinant in nodule organogenesis (Ferguson and Mathesius 2003). Taken as a whole, this suggests that there are multiple potential areas of genetic diversity that could modulate legume genotype response to N exposure.

Because of its application in agricultural production, genetic variation in nodulation response to soil N has received much attention in crop species (Harper and Gibson, 1984). In the production of pulses, an over-abundance of nodules when soil N is available is often framed as a disadvantage, as nodules and seeds will compete for photosynthate during the pod-filling stage (Gresshoff et al. 1988; Rosendahl et al. 1989). In forage production, however, this could be a benefit, since the improved quality (i.e., higher protein content) of high-nodulating plants could offset reductions in shoot yield caused by resource investment in nodules (Novak 2010). Forage legumes that continue to

nodulate under a range of soil N levels may be more compatible in mixtures with non-fixers, by avoiding competition for soil mineral N (Schwinning and Parsons, 1996) and increasing the amount of N available for transfer (Fustec et al. 2010). Differential responses in terms of nodulation response to available N have been reported both between (Harper and Gibson, 1984; Guo et al. 1992) and within (Serraj et al. 1992; Cruz et al. 2011; Thilakarathna et al. 2012a, 2012b) legume species, making N-insensitive nodulation a potentially valuable trait for forage breeding. A parallel experiment of this study, which was conducted under the same conditions and treatments but focused exclusively on red clover cultivars (Thilakarathna 2013), found variation in nodulation rate among a range of cultivars, suggesting that populations of clover can have different responses to available N and, by extension, that some cultivars may be more suited to low- or high-N fertility soils.

This study examines the response in nodulation rate of two genotypically distinct cultivars each of two important temperate forage legumes, red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.), to a range of N fertilization treatments, and how this shift might affect plant morphology and N status. We chose to use ammonia (NH_4^+) instead of nitrate (NO_3^-) as an N source because the effects of nitrate on nodulation rate have already been well established (Harper and Gibson 1984). In addition, levels of ammonia in unfertilized grasslands may be higher than nitrate (Corre et al. 2002), particularly in the spring when the first legume nodules are being formed (Jackson et al. 1989; Lipson et al. 1999). Legumes that are able to nodulate in spring may be at an advantage later in the season when N deficiencies may limit growth. The ultimate objective is to measure variation in nodulation in response to available N between and

within legumes species to determine which might be an efficient nodulater over a range of N fertility levels.

2.3 Methods

2.3.1 Species, cultivars and growth conditions

Two cultivars of red clover ,AC Christie (Martin et al. 1999) and Tempus, (http://www.inspection.gc.ca/english/plaveg/variet/regvare.shtml) and two of alfalfa, , Apica (Michaud et al. 1983) and CRS 1001 (Papadopoulos, unpublished data) were chosen for the experiment. Cultivars of each species have been previously shown to be phenotypically distinct. Red clover cultivars different in ploidy level (AC Christie is diploid, Tempus tetraploid) and have different nodulation responses to nitrate applications (Thilakarathna 2013). The alfalfa cultivars are both tetraploid, but have different rooting morphologies, as pica is a tap-rooted variety, and CRS1001 a rhizomatous type.

Seeds were surface sterilized with 2% sodium hypochlorite for three minutes and washed with three changes of sterile distilled water. Seeds were pre-germinated on wet sterile filter paper under dark for two days before transferring to 125 mL plastic 'cone-tainers' (Stuewe and Sons Inc., Tangent, Oregon USA) filled with acid-washed sand. Cotton plugs were placed at the bottom of each cell to prevent loss of sand. Three pre-germinated seedlings were transferred into each cell followed by inoculation with 1 mL of rhizobia suspension (OD_{600} = 0.04; *Rhizobium leguminosarum* biovar *trifolii* ATCC 14480 and *Sinorhizobium meliloti* Rm 1021 for red clover and alfalfa, respectively). Plants were thinned seven days after seeds were transferred, leaving one plant per

conetainer and then re-inoculated with appropriate rhizobia to ensure the presence of rhizobia for nodulation. Plants were grown in a growth chamber with supplemental lighting maintained with a photoperiod of 16 hours of day light at 425 μmol m^{-2} s^{-1} and 8 hours of dark (16 D: 8 N) at 20 ± 2 ^{o}C.

2.3.2 Nitrogen treatments

Plants were assigned to one of four N treatments (0, 0.5, 1.0, 2.5 N mg $plant^{-1}wk^{-1}$) so that there were four reps of each clover and alfalfa cultivar in each treatment group. Treatments (cultivars × nitrogen level) were arranged in a resolvable row design, with N levels as rows and cultivars as columns within a single growth chamber. Plants were supplied with 5 mL of 'Hoagland's No. 2 Basal Salt Mixture without Nitrogen' (http://www.caissonlabs.com/catalog.php) weekly during the growth period, amended with the appropriate amount of N in the form ammonium sulfate ($(NH_4)_2SO_4$; Sigma Aldrich, Oakville), and adjusted to pH 5.8 before application. Deionised water was supplied according to plant demand for the duration of the experiment.

2.3.3 Harvesting and data collection

Plants were grown for eight weeks before harvesting. Roots were carefully washed using distilled water to remove sand. Active nodules were counted based on the pink colour of the nodules. The total leaf area was measured using Winfolia (Regents Instruments Inc., Quebec City) software system. Shoot and root dry matter (DM) weights were determined after drying the plant materials for three days at 65 ºC. Dried plant samples were ground using a micro Wiley mill, standard model 3 (Arthur H Thomas Co., Philadelphia, USA), to pass through a 1-mm sieve. Nitrogen was determined using the combustion method on

a LECO protein/ N determinator FP-528 according to the Dumas method (Williams et al. 1998) to determine 'N content' (mg N g^{-1} DM) for both root and shoots, and plant 'total N' (mg N $plant^{-1}$).

2.3.4 Statistical analysis

The experimentwas analyzed using ANOVA in a strip-plot design with the N levels as the main plot and cultivars as the sub-plots. Two variables (active nodule number and specific nodulation rate) were square-root transformed to improve normality. In addition to the ANOVA, a principle component analysis (PCA; Jolliffe 2003) was performed using all the plant yield, N and morphology variables recorded. Statistical analysis of data was carried out using GenStat® (VS.N International 2011).

2.4 Results

2.4.1 Species and cultivar differences

Red clover cultivars, on average, had a higher total number of active nodules (16.7 vs. 10.0 nodule $plant^{-1}$) and mean specific nodulation rate (93.1 vs. 40.3 nodules g^{-1} root DM) than alfalfa (Table 2.1). Nitrogen content was similar across both species for both roots (23.0 mg N g^{-1} DM) and shoots (27.4 mg N g^{-1}; Table 2.1). Plants accumulated a mean total N of 13.46 mg per plant, with no significant differences recorded between species (Table 2.1). The two legumes species accumulated a similar level of DM yield (530.8 mg DM, SEM=24.39; Table 2.1) but allocated it differently, with alfalfa showing a mean root-to-shoot ratio of 1.11 and clover 0.56 (Table 2.1). Leaf area was accordingly higher in clover cultivars (40.5cm^2) than alfalfa (26.1 cm^2; Table 2.1.)

Table 2.1: Mean yield, nitrogen content and nodulation by two cultivars of alfalfa (Af) and red clover (RC) over four nitrogen treatment levels (0, 0.5, 1.0, and 2.5 N mg plant^{-1} wk $^{-1}$).

Cultivar[+]	**Yield** (mg DM)	**Root: Shoot Ratio**	**Leaf Area** (cm^2)	**Shoot N** (mg N g^{-1} DM)	**Root N** (mg N g^{-1} DM)	**Total N** (mg N plant^{-1})	**Active Nodules**[*] (nod. plant^{-1})		**Spec. Nodulation**[*] (nod. g^{-1} root DM)	
							√	ut	√	ut
Apica (Af)	549.3	1.08	25.8	28.4	22.7	**14.2**	3.86	9.7	7.43	42.0
CRS1001 (Af)	478.4	1.14	26.3	27.3	22.3	**11.6**	3.83	10.3	7.95	38.6
Christie (RC)	549.1	0.58	41.5	26.3	22.4	13.4	5.44	18.9	12.24	95.9
Tempus (RC)	546.5	0.54	39.4	27.8	24.5	14.3	4.74	16.0	11.07	90.7
Alfalfa	513.9	**1.11**	**26.1**	27.9	22.5	12.9	**3.85**	9.98	**7.69**	40.3
Red Clover	547.8	**0.56**	**40.5**	27.1	23.5	13.9	**5.10**	17.5	**11.66**	93.3
Grand Mean	530.8	0.84	33.3	27.4	23.0	13.4	4.74	13.5	9.67	64.1
SEM (N=4)	25.39	0.069	2.42	0.66	0.69	0.39	0.261	-	0.790	-
***F*-probability**[+]										
Cultivar	0.478	**0.016**	**0.036**	0.209	0.137	**0.048**	0.053		**0.045**	
Af vs.RC[†]	0.651	**<0.005**	**0.010**	0.281	0.175	0.095	**0.017**		**0.012**	
Af[‡]	0.183	0.716	0.868	0.282	0.946	**0.021**	0.976		0.780	
RC[‡]	0.825	0.436	0.692	0.276	0.922	0.142	0.542		0.772	

*nodulation variables were square-root transformed for ANOVA analysis; both transformed (√) and untransformed (ut) values are given.

+*F*-probabilities in bold indicate significance at the $P<0.05$ level; Means in bold indicate a significant difference between two paired means, according to orthogonal contrasts (see below)

†Orthogonal contrast of alfalfa cultivars (Apica, CRS1001) versus red clover (Christie, Tempus)

‡Orthagonal contrasts between cultivars within a single species of alfalfa or red clover

Table 2.2: Mean legume yield, nitrogen content and nodulation by N fertility treatment and legume cultivar response.

N Treatment (mg N wk^{-1})	**Total Yield** (mg DM)	**Root: Shoot ratio**	**Leaf Area** (cm^2)	**Shoot N** (mg N g^{-1} DM)	**Root N** (mg N g^{-1} DM)	**Total N** (mg N $plant^{-1}$)	**Active Nodules*** (nod. $plant^{-1}$)		**Spec.Nod*** (nod. g^{-1} root DM)	
							√	ut	√	ut
0.0	579.6	0.85	41.5	24.5	22.0	13.5	5.04	25.4	10.50	110.3
0.5	526.6	0.77	34.1	27.9	25.0	13.8	5.00	24.9	11.18	124.4
1.0	530.7	0.94	32.0	26.7	19.8	12.5	4.69	22.0	9.81	96.2
2.5	486.4	0.77	25.4	30.7	25.2	13.7	3.14	9.84	7.20	51.8
Grand Mean	530.8	0.84	33.3	27.4	23.0	13.4	4.47	13.4	9.67	95.7
SEM (N=4)	35.17	0.082	3.25	0.173	0.290	1.15	0.147		0.391	
***F*-probabilities**+										
Nitrogen	0.301	0.310	**0.034**	0.141	0.522	0.812	**<0.005**		**<0.005**	
Nitrogen × Cultivar	0.555	0.145	**<0.005**	0.164	**0.028**	0.420	**0.023**		**0.028**	

*nodulation variables were square-root transformed for ANOVA analysis; both transformed (√) and untransformed (ut) values are given.
+*F*-probabilities significant at the $P<0.05$ level are presented in bold.
†Polynomial contrasts of linear (lin) and quadratic (quad) trends of alfalfa cultivars (Apica, CRS1001) versus red clover cultivars (Christie, Tempus)
‡ Polynomial contrasts of linear and quadratic trends between alfalfa cultivars (Apica, CRS1001) and red clover cultivars (Christie, Tempus, respectively)

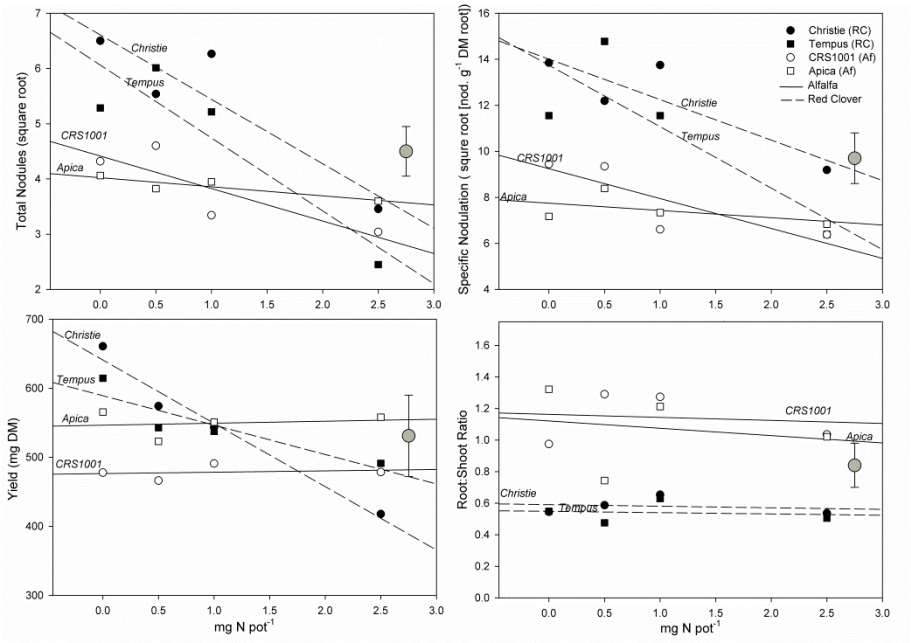

Figure 2.1: Cultivar responses to N fertility treatments. Response in total active nodules (nod. plant^{-1}; top left), specific nodulation rate (nod. g^{-1} root DM; top right) yield (mg DM; bottom left) and Root:Shoot ratio (bottom right) of red clover and alfalfa cultivars N fertility treatments. Both nodulation response variables are presented as transformed variables (square root) as used in ANOVA analysis. Linear regression lines are labelled for each cultivar. Large grey circles denote the grand mean and error bars the standard error (SEM).

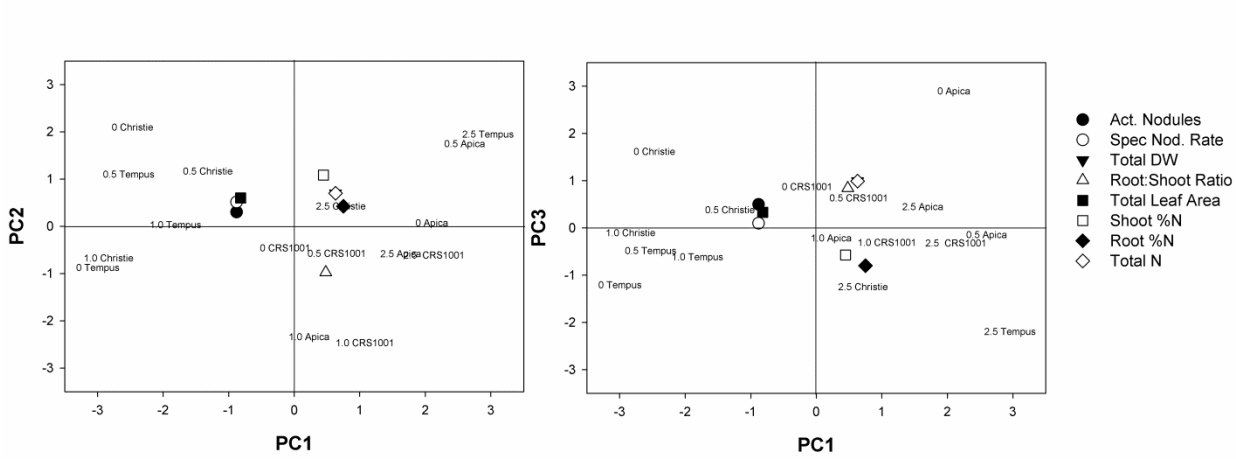

Figure 2.2: Principal Components Analysis (PCA) of legume cultivar attributes by N fertility treatments. Principal components 1, 2 and 3 account for 57%, 22% and 18% of the variance, respectively, for a total of 97%. For group scores, four cultivars are given, two of red clover (Christie, Tempus) and two of alfalfa (Apica, CRS1001), along with one of four nitrogen treatments (0, 0.5, 1.0, 2.5 mg N plant^{-1} wk $^{-1}$)

No significant differences were found between clover cultivars, though, similar to alfalfa, one cultivar (Tempus) had a higher, but not significantly different (P=0.142), mean of total N, which unlike clover, was likely due to higher N content (Table 2.1).

2.4.2 Species and cultivar response to N treatments

Nodulation decreased markedly with N additions, ranging from a mean of 25.4 nodules plant^{-1} at no added N to 9.8 at the highest N treatment level, a reduction of 61%. Likewise, specific nodulation decreased from 110.3 to 51.8 nodules g^{-1} root DM over the same range, for a reduction of 53%. Leaf area also decreased with higher N additions, dropping from 41.5 cm^2 to 25.4 cm^2 over the same range. In spite of this, no main trends were observed for N content, total N, DM yield or root:shoot ratio (Table 2.2).

Nitrogen × cultivar interaction contrasts revealed that the major difference between legumes were between, not within, species. Red clover showed a much steeper decline in total and specific nodulation, in comparison to alfalfa, which, while having fewer total nodules, retained a higher percentage of them as N fertility increased (Table 2.2, Fig. 2.1). Clover cultivars had a 74% reduction in mean total nodule number reduced from the lowest N level (0N) to the highest (2.5N) and their mean specific nodulation rate reduced by 61%, compared to alfalfa's losses of 37% and 38%, respectively. Root percent N and total leaf area had significant N × cultivar interaction. Clover DM yield also fell as N fertility increased (Fig 2.1) though the interaction term (N × Cultivar) was not significant (Table 2.2).

2.4.3 Principal component analysis

Three principal components had eigen values greater than 1 and were used for analysis. The first, second and third principal components accounted for 57%, 22% and 18% of the variance, respectively, for a total of 97% of the variance explained (Fig. 2.2). The first component discriminates between plants with large leaf areas and high nodulation (negative values) against those with a higher root N content, DM yield and total N. This largely separates the red clover (large leaves, high nodulation rate) from the alfalfa, though it is interesting to note that the red clover cultivars at high N treatments (2.5 N) cluster more with the alfalfa than clover at low N treatments. The second principal component discriminates between root:shoot ratio and other attributes, though that trait is also associated with positive values on PC1, putting it diametrically opposed to the nodulation/leaf attributes that define clover cultivars. This component shows that alfalfa cultivars at one particular N treatment (1.0 N) show a much higher root:shoot ratio than most treatment groups, especially compared to Tempus at the highest N treatment (2.5 N) Christie at the lowest N treatment (0 N), even if these two groups differ greatly on the first PC. Finally, PC3 shows an association between root and shoot N content, which is inversely related to plant size and total N. This component mainly discriminates N treatment groups within cultivars, showing the somewhat opposing patterns of growth and tissue N concentration. The two extremes of this component show Apica at 0 N treatment associated with high total N at low concentration within plant tissue, while Tempus at 2.5 N associating lower total N in relatively less biomass.

2.5 Discussion

Results agree with current knowledge of N fixation in temperate legumes, namely that high N availability will inhibit nodulation and reduce the rate of N fixation (Wang and Stacey 1990; Gulden and Vessey 1997). This response has typically been studied with nitrate, which has been long known as a potent repressor of nodulation (Streeter and Wong 1988), but our study confirms reports that other forms of N can also inhibit their formation in temperate forage legumes (Chambers et al. 1980; Wielbo and Skorupska 2008). Reduction in nodulation with increasing N is thought to be a general response mechanism to use to the least metabolically expensive N source: when mineral N is abundant, N fixation becomes less efficient because it requires the expenditure of more photosynthate than taking it up from the surrounding soil taking it up from the surrounding soil (Ledgard and Steele 1992; Høgh-Jensen et al. 2004). Results of this study, however, indicate that a less metabolically expensive source of N (in this case, ammonia) does not necessarily translate into higher biomass or N accumulation.

Overall, red clover cultivars in our experiment were found to produce more nodules, both on a total and per g root basis, a difference that has been observed in studies comparing these species before (Rice et al. 1976; Peters and Zbiba 1979; Sherer and Lange 1996; Staley and Belesky 2004). This may be related to the more branched root system of clover. This characteristic of alfalfa and its impact on nodulation has been noted before: as a tap-rooted legume with relatively fewer lateral roots, it is assumed that there are fewer sites for infection on alfalfa than on a highly branched root system such as clover (Chmelíková et al. 2015a; Chmelíková et al. 2015b). It has also been proposed that the regulatory mechanisms in root-branching and nodulation may have some common

elements: hypernodulating *Lotus japonicas* mutants also developed a 'bushy root' phenotype with much higher rates of lateral root formation, suggesting that regulation of root branching may also suppress nodulation (Kawaguchi et al. 2002). In our study, we had two alfalfa cultivars with distinct root phenotypes (rhizomatous and tap-rooted) but kept a similar nodulation rate, suggesting that root morphology alone may not explain difference between species. While not measured, alfalfa appeared to produce larger nodules. In a survey of European legumes, Chmelíková and Hejcman (2012) reported that alfalfa produced nodules typically above 4 mm in length, while red clover tended to produce nodules less than 2 mm, consistent with our observations. I also noted that alfalfa cultivars contained large 'fan-like' nodules in addition to the typically smaller 'spherical type' (Corby 1988) produced by clover, in agreement with previous studies (Chmelíková and Hejcman 2012). If compared we were to have compared the total mass of nodules per plant, we may have found similar reductions in alfalfa as N levels, as reduction in nodule size as a response to N has been noted before (Hussain et al. 1992; Yashima et al. 2003 Bollman et al. 2006).

In terms of legume nodulation response to N, it appears that the main difference occurred at the species level: both alfalfa cultivars, while producing fewer nodules altogether, produced a comparable amount over the range of N treatments in this study, while red clover greatly reduced its nodulation rate as N fertilization rate increased. Trends of nodulation reduction in clover match those observed by Thilakarathna (2013), who used a larger number of cultivars in a parallel study. As discussed above, this may be related to the different nodule morphologies of the two species. If we were to have compared the total mass of nodules per plant, we may have found similar reductions in alfalfa as N

levels increased, as reduction in nodule size in response to N has been noted before (Yashima et al. 2003; Bollman and Vessey 2006).

Results did show some potential differences in response to N between red clover cultivars, as evidenced by the different response in nodulation rate at the lower N treatment levels (Fig 2.1) and the near-significant polynomial linear contrasts between the cultivars in the ANOVA (P=0.063; Table 2.2). One cultivar of red clover, Tempus, had its peak total number of nodules and nodulation rate at the low-level N treatment (0.5 mg N plant^{-1}) while Christie had virtually the opposite response (see Fig. 2.1). Interestingly, an in vitro study of nodulation using these same two cultivars showed the opposite response, with Tempus showing a negative quadratic response over low levels of N fertility and Christie a positive one (Thilakarathna et al. 2012b). While plants were of similar age and were given similar levels of N, one crucial difference was the form of N used in the experiment (ammonium nitrate, versus ammonium used in our study), which can illicit different responses when it comes to nodulation suppression (Chambers 1980).

From the data we collected, it is not possible to determine which particular mechanism is responsible for the lower rates of nodulation (Nod factor down-regulating, root hair curling suppression, AON) associated with higher N fertilization levels (particularly in clover). It has been reported previously that ammonium-nitrate can suppress root-hair curling in red clover (Thilakarathna et al. 2012a), but this does not eliminate the possibility that other mechanisms may be involved as well. Alternately, it may be possible that nodulation rates were actually similar and only N-fixation rates were lowered. Only active nodules were counted in this study, largely because the small size of developing nodules on clover roots made them hard to identify without the pinkish colour

of leghemoglobin found in active nodules. It may be possible that clover plants also nodulated at constant levels over N treatments, but restricted the N-fixation process in response to the available N, resulting in fewer active nodules. It has been demonstrated that legumes can downregulate the synthesis of leghemoglobin when exposed to external N (Gallusci et al. 1991). Further experimentation measuring the expression of nodulation- and fixation-related genes would be required to demonstrate specifically which mechanism is being affected in clover, but not in alfalfa.

While cultivar, species and N level did have a large impact on nodulation profile, evidence for impacts on plant DM or N accumulation was weaker. There was an arithmetic trend towards smaller mean DM yield as N additions increased, but polynomial contrasts suggest that this was a trend restricted to red clover cultivars. Clover cultivars in high N treatments had higher root N content, as opposed to alfalfa, but total N remained similar between species, suggesting an N-concentration effect (i.e. same total N, less biomass). In fact, total N remained largely similar across all plants over all N levels, suggesting that no major interruption to N acquisition occurred over the different N treatments. This stands in contrast to field studies that have noted that an increase of N fertilization is associated with higher N content of shoots and roots in alfalfa (Trimble et al. 1987; Bélanger and Richard 2000), an effect we did not observe. Previous studies have reported that high nodulation and low soil nutrient levels are associated with more root biomass being produced (Voisin et al. 2007; Johnson and Biondini 2001; Belanger and Richard, 2000), but our results show that biomass allocation between above- and below-ground tissue remained the same over our N treatments (Fig 2.1).

Normally, it would be expected that additions of mineral N would increase biomass accumulation in legumes, particularly in the shoots, because less photosynthate would have to be spent on nodule formation and fixation (Brugge and Thornley 1989). Instead, a weak trend of decreasing biomass as N treatments increased was observed in this study, with no significant change in biomass allocation (root:shoot ratio). This may be explained by our choice of N compound: soil-derived ammonia is, theoretically, the least expensive form of mineral N to acquired, having none of the costs associated with the fixation of dinitrogen or reduction of nitrate before entering the nitrogen assimilation pathway(Miller and Cramer 2005). In practice, however, ammonia can be problematic as a sole source of N, because its toxic effect on cellular function means it must be immediately assimilated in the root (as opposed to nitrate, which can be stored in vacuoles) requiring constant stream of carbon for the amide production in the GO-GAT cycle (Ohyama and Kumazawa 1980). In addition, if the soil solution contains high levels of ammonia, it must be actively effluxed from roots to prevent membrane depolarization in a phenomenon known as 'futile nitrogen cycling' (Britto et al. 2001), a process which can further cause problems by acidifying the root zone (Britto and Kronzunker 2002). Studies comparing the effects of N form on nodulated peas noted that ammonia, when provided as the sole N source, increased root respiration much more than nitrate while also reducing nitrogenase activity (Mahon, 1977; Houwaard 1980). Given that our results show similar levels of both N and DM accumulation, we can assume that the photosynthate spent on nodulation and fixation in legumes at low N treatments was equal, or less than, the photosynthate required to protect against the negative effects of ammonia assimilation While somewhat counter-intuitive, this effectmay have been exacerbated by the growing conditions used: as a neutral medium, sand has no buffering capability or

cation exchange sites, meaning that H^+ ions effluxed during ammonium uptake could have turned the soil solution acidic much more quickly than in field soil.

While we observed species-specific responses to added N in the form of ammonium, both were able to compensate so that their accumulation of N was similar over all N-fertility levels. Alfalfa, with a high relative proportion of root tissue and a small number of nodules, is able to maintain a similar morphology over different N levels, while red clover has a more plastic strategy, lowering nodulation rate, and putting less resources towards shoot growth (lower leaf area) and more, presumably, towards root respiration. The PCA analysis reinforces this interpretation, showing that while alfalfa cultivars seem to group similarly, regardless of N level, clover cultivars at high N levels tend to score very differently than other groups, implying a much different morphology.

Results suggest that alfalfa and red clover may have contrasting strategies to cope with different N availability at early growth stages. The selected clover cultivars altered their morphology at higher N levels to reduce emphasis on photosynthate production associated with the high metabolic cost of N fixation (Vitousek and Field 1999) and focus resources instead on N uptake, which is dependent on root surface area (Garnett et al. 2009). The selected alfalfa cultivars, on the other hand, does not drastically alter its morphology or nodulation rate in response to N, suggesting that a high root:shoot ratio, coupled with fewer (but likely larger) nodules might serve as an efficient phenotype across a range of N availabilities. Within the conditions of the experiment, it seems as though both strategies are successful at coping with changes in N availability, as neither biomass nor N status changed significantly for any cultivar over the treatments. Previous studies have shown that plants from similar environments can have a range of responses

to changes in soil N as a result of different N uptake strategies (Johnson and Biondini 2001).

A parallel experiment of this study, which was conducted under the same conditions and treatments but focused exclusively on red clover cultivars (Thilakarathna 2013), found variation in nodulation rate among a range of cultivars, suggesting that certain genotypes of clover are more adapted to high- or low-N availability. While the range of response by alfalfa cultivars in this study was much smaller, results suggest that the potential exists to find high-nodulating cultivars that are less likely to be inhibited by higher levels soil mineral N and thus less likely to compete with grasses. Further research into a wider array of alfalfa cultivars could help identify cultivars that meet these criteria, and perhaps help improve the efficiency of grass-legume intercrops.

2.6 Conclusion

Two distinct cultivars each of red clover and alfalfa were evaluated for their response in nodulation to four levels of N fertilization. Higher levels of N reduced the total number of nodules and the specific nodulation rate of all plants, though more drastically in the higher-nodulating red clover cultivars. Compared to alfalfa, red clover cultivars also changed their morphology more with increasing N. All plants showed similar levels of N accumulation, suggesting the two species had different, yet equally successful, strategies for dealing with changes in N. Reductions in biomass accumulation in red clover at the highest N treatments suggest that ammonia could have a toxic effect at that level, related possibly to ammonia efflux or increasing soil acidity. Further experiments comparing

nodule development and N-fixation rate in these species may elucidate what specific mechanisms of control are used to modulate N-fixation in these two species.

Chapter 3: Characterization of nitrogen exudation from red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) cultivars

3.1 Abstract

The efficiency of direct nitrogen (N) transfer from N-fixing legumes to non-fixing grasses, an important process in mixed-species forage production, depends on the quantity and chemical profile of N in the exudates of the legumes. The exudation of different nitrogenous compounds of two cultivars each of red clover and alfalfa was evaluated to determine what differences in N exudation may occur between and within species of temperate legumes. Plants were grown in microlysimeters in non-sterile sand culture for six weeks before undergoing a 48-hour leachate collection period, the products of which were assessed for total dissolved N, ammonium and specific amino acid content. Total N collected in the leachates ranged from 14.64 to 32.57 μg N, but approximately 90% of this was in the form of high molecular weight organic N (HMW N; protein and peptides), and was thus unavailable for direct N-transfer to other plants. The remaining portion consisted mainly of ammonium (3.95 μg N) and only small amounts of amino acids (110 ng N). Compared to alfalfa, red clover leachates contained significantly higher amounts of ammonium and amino acids, with one amino acid in particular (tyrosine) driving the latter difference. Of the two cultivars of red clover AC Christie showed significantly higher amounts of ammonium than Tempus, both in total (6.30 vs. 2.60 μg N) and relative to size (14.76 vs. 4.43 μg N g^{-1} DM). This difference may be related to the former's smaller yield (449 vs. 595 mg DM) and higher specific nodulation rate (132 vs. 44 nodules g^{-1} root DM). Future research into the relationship between relative nodule

number and N-exudation rate could be valuable in identifying cultivars that are more suited to direct N-transfer to neighbouring plants in the field.

3.2 Introduction

Legumes play an important role in livestock production, not only through the production of high-quality forage, but also by supporting the growth of other forage species by providing them a source of soil N (Brophy et al. 1987). This phenomenon, known as nitrogen transfer (or 'N-transfer') can occur in one of three ways: by the decomposition of belowground legume tissues and subsequent uptake by neighbouring plants (Wichern et al. 2008; Fustec et al. 2010), by fungal hyphae between plants sharing a common mycorrhizal network (Haystead et al. 1988; Høgh-Jensen 2006) and via plant root exudates released by N-donors (legumes) and taken up by N-receivers (non-legumes) (Paynel et al. 2008; Fustec et al. 2010).While it is difficult to quantify the precise amount of N that is transferred by each pathway (Høgh-Jensen 2006), studies of N-transfer rates in the field have suggested that the majority of N transfer over the growing season occurs via the decomposition route, as estimates of root exudation are far too small to explain differences in N content observed in intercropped non-legumes (Russelle et al. 1994; Dubach and Russelle 1994). Still, the direct transfer of N via exudation may be critical for the early growth and establishment of forage stands (Gylfadóttir et al. 2007).

Major nitrogenous components of temperate legumes exudates are ammonium (NH_4^+) and amino acids, as well as small peptides and proteins (Paynel et al. 2001, 2008; Lesuffleur and Cliquet 2010).The N exuded from temperate legume roots finds its origin in several different processes. First, mineral or organic N acquired by plant roots can be

released back into the soil quickly after uptake, either unchanged or after conversion (i.e. NO_3^- released after being reduced to NH_4^+). Second, N can be derived from the productions of symbiotic N fixation which converts atmospheric N into ammonium, which in most temperate legumes is subsequently converted to amides (glutamine and asparagine) for transportion in the xylem (Atkins and Beevers 1990; Atkins and Smith 2002). Nodules can be major sources of ammonium exudation because, while transportation of amides from nodules is under plant control, fixation by bacteriods is not, leading to build-ups of ammonium within the nodule that is excreted from the plant (Schulze 2004; Udvardi and Poole 2013). Third, N that has been transported to the shoot and older sections of the root for conversion to amino acids is transported to the root tips via the phloem (Tegeder et al. 2014) where some of it is released as exudates (Jaegar et al. 1999).

The exudation of ammonium and amino acids has been shown to result from their release (efflux) across the cell membrane into the soil, either passively or actively (Miller and Cramer 2005; Badri and Vivanco 2009), while their uptake (influx) occurs by membrane-bound transporters (Näsholm et al. 2009). Nitrogen movement in or out of the root is thus determined by the difference between the rate of influx and efflux (I:E ratio, Phillips et al. 2010). Both influx and efflux rates can be modified by plant-controlled processes in response to environmental conditions and plant needs (Lesuffleur and Cliquet 2010; Carvalhais et al. 2011). In addition to controlling the quantity of N in exudates, plants can also alter the chemical profile via the selective release and/or re-uptake of particular forms of N. The difference in amino acid profiles of plant root exudates and xylem sap, for example (Phillips et al. 2004; Lesuffleur and Cliquet 2010), has demonstrated that

plants can be selective about what nitrogenous compounds are released via exudation. This may explain why certain amino acids are usually found in great abundance in legume exudates (e.g. serine, glycine) while other amino acids common in plant tissues (e.g. asparagines, glutamine) are not (Svenningsson et al. 1990; Paynel et al. 2001; Lesuffleur et al. 2007). The release of ammonium, another common component of the legume exudate N fraction (Fustec et al. 2010), is also regulated by the plant. Unlike amino acids, however, its release is thought to be related to its deleterious effects on cellular function. If the ammonium concentration in root cells becomes too high, either through external supply the reduction of nitrate or via N-fixation, plants will often actively efflux ammonium ions via low-affinity ammonium transport proteins (Howitt and Uvardi 2000). When external ammonium conditions are particularly acute, maintaining low cellular levels by efflux in the face of passive influx can be detrimental to plant growth, leading to a phenomenon known as 'futile ammonium cycling (Britto et al. 2001; Kronzucker et al. 2001).

Individual plants can modulate the release of N via exudation in response to environmental conditions such as nutrient deficiencies (Graham et al. 1981; Carvalhais et al. 2011) and increases in light intensity (Rovira, 1959; Ofosu-Budu et al. 1992) or to biological stimulus such as the presence of rhizosphere microbes (Leyval and Berthelin 1993; Phillips et al. 2004) or infection by mycorrhizae. There is also evidence of different N-exudation rates between species both in the quantity and form of N released. Differences in the rate of exudation of amino acids in particular have been noted between species of trees (Grayston et al. 1996), cereals (Fan et al. 2001), perennial grasses (Klein et al. 1988) and legume (Schroth et al. 1966, Brophy and Heichel 1989; Ofusu-budsu et

al, 1992). Comparisons of more distantly related species have suggested that legumes, in comparison to non-nodulating plants, release more N via exudates relative to their size (Paynel et al., 2001; Paynel and Cliquet 2003; Lesuffleur et al. 2007), though not always (Phillips et al. 2004). While there are few studies comparing temperate legume species, white clover (*Trifolium repens*) has been reported to exude more N than alfalfa (*Medicago sativa*; Lesuffleur et al. 2007)

Studies comparing differences in N exudation rate within species, however, are less common. In cucumber, differences in root exudates amino acid levels have been correlated with cultivars resistant to iron stress (El-baz et al. 2004) and susceptible to *Fusarium* wilt (Pan and Wu 2007). Efflux of nitrate from cultivars of rape (*Brassica napus*) has been shown to be associated with lines having low nitrogen accumulation efficiency (Huang et al. 2011). Thus far, however, very little work has been done determine if populations of forage legumes differ in exudates N composition. Thilikarathna (2013) reported that tetraploid cultivars of red clover (*T. pratense*) contained more inorganic N in their exudates than diploids, and that a single diploid cultivar ('A.C. Christie') exuded more dissolved organic N (DON) than all other cultivars tested.

In temperate forage legumes, high exudation rates of fixed N is a potentially valuable agronomic trait, as it would increase the amount of labile N available for uptake by associated non-fixing grasses. Quality of N could also be important, as grasses can vary in their efficiency to uptake particular forms of N. It has been reported, for example, that species adapted to high fertility conditions (e.g. *Lolium perenne*) have a greater affinity for inorganic nitrogen, while those from nutrient-poor environments (e.g. *Festuca ovina*)

favour direct uptake of organic N (Vazquez de Aldana and Berendse, 1997; Weigelt et al. 2005). In order to maximize potential direct N-transfer in legume-grass associations, it is therefore important to have an understanding of the relative N-exudation qualities of species and cultivars of legumes so that they could be matched properly with the uptake preferences of non-fixing grasses.

Here we examined the N exudate profile of two cultivars each of two common temperate forage species, red clover (*T. pratense* L.) and alfalfa (*M. sativa* L). The objective was to compare the N excreted by different pasture legume species and cultivars, and determine whether certain plant populations may be superior N-donors based on the quantity or composition of N-containing compounds in the leachates collected.

3.3 Materials and Methods

3.3.1 Growing Conditions

Two phenotypically distinct cultivars of each species were chosen for the experiment. For red clover, 'AC Christie' (Martin et al. 1999) and 'Tempus' (Oseva Uni, released 1988; http://www.osevauni.cz/vlastni-odrudy), a diploid and tetraploid variety, respectively, were chosen. For alfalfa, Apica (Michaud and Richard 1983) and CRS1001 (Papadopoulos, unpublished data) were selected, the former being a tap-rooted variety and the latter having a rhizomotous root growth habit. These varieties had previously been shown to have distinct nodulation profiles, with variation in nodulation rate between species (alfalfa<red clover; McElroy, Chapter 2) and within ('AC Christie'>'Tempus'; Thilakarathna et al. 2012a; Thilakarathna 2013).

Seeds were surface sterilized with 2% sodium hypochlorite for three minutes, washed with three changes of sterile distilled water and placed on moistened filter paper in a petri dish to germinate. After three days, seedlings were removed from the petri dish and transferred to micro-lysimeters.

Microlysimeters were based on a system designed by Paynel et al. (2001). The bottom tips of 50 mL polypropylene centrifuge tubes (B.D. Biosciences) were removed, leaving a hole approximately 0.5 cm in diameter, which was filled with a swab of sterile cotton. The tube was filled with acid-washed, sterilized fine-grain sand up to 45 mL. The sand was watered to capacity with half-strength 'Hoagland's No. 2 Basal Salt Mixture without Nitrogen' (http://www.caissonlabs.com/ catalog.php), and allowed to drain before adding the seedling to the surface. The 'field capacity' of the microlysimeters was determined to be approximately 30 mL of liquid. Plants were then inoculated with 1 mL of a cell suspension, adjusted to 108 cells ml^{-1} (OD_{600}=0.1) of *Rhizobium leguminosarum* biovar *trifolii* (ATCC 14480) and *Sinorhizobium meliloti* (1021), for red clover and alfalfa, respectively. The surface was then covered with vermiculite, and the microlysimeter suspended upright on a rack.

The microlysimeters were placed in a growth cabinet and plants were allowed to grow for four weeks in a growth cabinet with light provided at 125 μmol m^{-2} s^{-1} for 16 hours a day. Temperature was maintained at 21 °C, and 16 °C during the light and dark periods, respectively. Plants were watered with distilled water as required, and twice a week 10mL of half-strength Hoagland No-N (see above) nutrient solution was applied.

3.3.2 Collection of exudates and plant material

After six weeks of growth, four healthy-looking plants of each species/cultivar were selected for sampling. Before the collection period, two applications of 30 mL distilled water were slowly applied to remove the solution already in the microlysimeter. After the lysimeters were drained and the bottom drainage holes covered with paraffin wax, the microlysimeter was filled to field capacity with No-N Hoagland's solution and returned to the growth chamber at the beginning of the light cycle. After 12 hours, microlysimeters were removed from the growth chamber and the paraffin wax removed. Each microlysimeter was placed over a 50 mL Erlenmeyer flask and distilled water was applied until 30 mL of leachates were collected. The paraffin wax was re-applied and the nutrient solution was applied again, as above. This process was repeated three more times for a total of 120 mL collected over 48 hours. Samples were stored at 2 °C during the collection period, put through a 0.45 μm filter using a 50mL syringe, combined and then separated into two 50 mL batches and frozen at -20 °C. Once frozen, samples were lyophilized and re-suspended in 1 or10 mL of distilled water for one 50X for amino acid analysis, and one 5X concentrate sample for nitrate, ammonium and total dissolved nitrogen.

Once sampling was completed, plant material was destructively harvested. Active nodule counts were made by enumerating nodules with pink tissue visible. Plant material was separated into 'root' and 'shoot' components and dried at 65°C for three days to obtain a dry matter (DM) weight. Shoot and root were then recombined and ground to pass through a 1 mm sieve using a micro Wiley mill, standard model 3 (Arthur H Thomas Co., Philadelphia, USA). Total N in plant tissue was determined using a LECO protein/ N

determinator FP-528 according to the Dumas method (Williams et al. 1998) and divided by total DM to derive tissue N content (mg N g^{-1} DM).

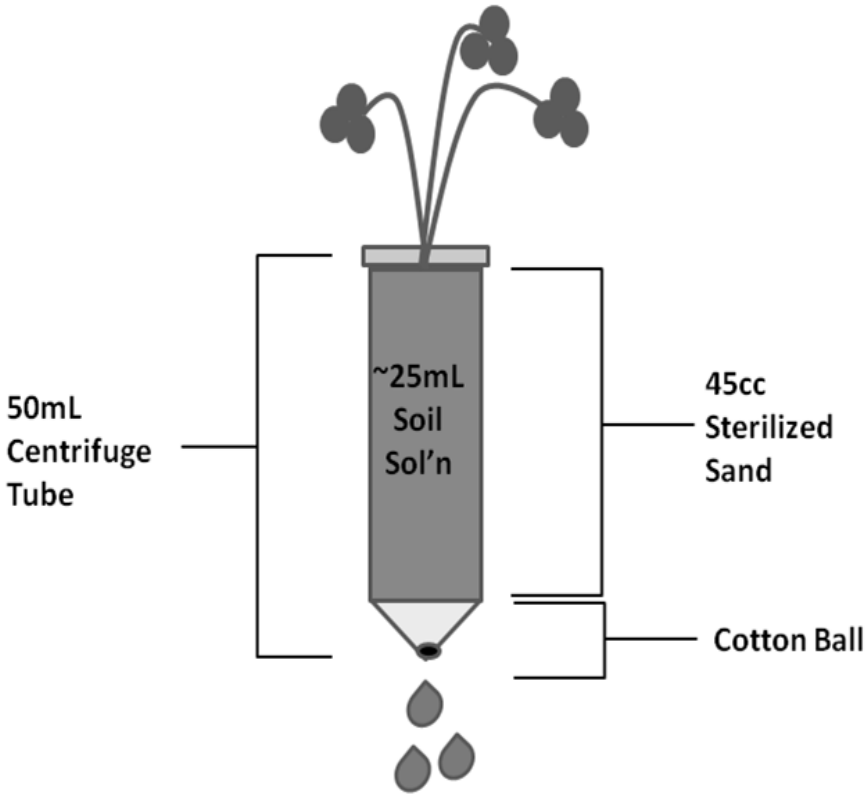

Figure 3.1: Mycrolysimeter design used for the collection of leachates from alfalfa and clover plants. Based on the design of Paynel et al. (2001)

3.3.3 Leachate analysis

Leachate samples were analyzed for nitrate and ammonium with ion analysis on a Lachat QuikChem 8500 (Lachat Instruments, Loveland, CO) using Lachat methods 10-107-06-1-X and 10-107-04-1-A (Lachat Instruments 2007; 2009). Standards and carrier were prepared from Hoagland's solution described above. Total dissolved N (TDN) was

determined similarly following potassium persulfate digestion at 121 ^{o}C and subsequent NO_3-N analysis with matrix-matched standards on the above described flow injection analyzer.

Amino acids were quantified by high performance liquid chromatography (HPLC) as *o*-phthaldialdehyde (OPA) derivatives, following the method of Umugat et al. (1982) with a slightly reduced run time (40 minutes). Over the course of the experiment, two C18 columns were used (Agilent Zorbax Eclipse XDB-C18 column; Phenomenex Gemini C18 110A), on an Agilent 1200 HPLC system, including a quaternary pump (product number G1311A), fluorescence detector (G1321A) diode array detector (G1315D) (Agilent Technologires Inc., Santa Clara CA, USA; Henderson et al., 2006) and a HTC PAL Autosampler (CTC Analytics, Zwingen, Switzerland). Empower 3 software (rev. 2.2.0; Waters Corp., Milford, Massachusetts, USA) was used to collect, integrate and analyze results. Standard curves using derivatized standards of 38 amino acids (Sigma, product no. A9906) were developed using a 5-point quantitation over the range 0.01-0.001 μmol ml^{-1}. Amino acid identification was confirmed through analysis of individual OPA-derivitized amino acid standards of interest (ammonium, α ketoglutaric acid, glycine, L-alanine, L-asparagine, L-aspartic acid, L-cystiene. L-isoline, L-proline,L-serine, L-threonine, L-valine, L-tryptophan, L-Tyrosine, urea). Where there was the possibility of peak overlap and amino acid designation uncertainty, the mixed standard was spiked with the potential individual amino acids for further confirmation. Derivatized Norvaline was used an internal standard in all leachate samples. See Appendix A for chromatograph examples.

The difference between TDN and the remaining N compounds (nitrate, ammonium, amino acids), assumed to be peptides and proteins, was termed 'organic N'.

3.3.4 Statistical analysis

All statistical analysis was performed in R (R Core team, 2013). The experiment was set up as a completely randomized design (CRD) analyzed using a one-way ANOVA with the four plant cultivars as treatment groups and four replicates per group. Comparison contrasts used to detect differences between ('Alfalfa vs. Red Clover cultivars') and within ('Apica vs. CRS1001', AC Christie vs. Tempus') species. *P*-values below 0.05 were considered significant.

3.4 Results

3.4.1 Leachate nitrogen content

The largest proportion of N found in the exudates of plants was organic N (26.46 μg N), followed by ammonium (3.98 μg N) and amino acids (110 ng N). No nitrate was detected (Table 3.1; Figure 3.1). Red clover showed higher levels of total N than alfalfa (36.16 vs. 23.61 μg N) though the difference was not statistically significant (Table 3.1). Red clover exudates also contained over three times as much amino acid N as alfalfa (166 vs. 55 ng N). These results were reflected in the specific N variables, where red clover leachates contained significantly more total N (78.28 vs. 37.84 μg N g^{-1} DM), ammonium (9.60 vs. 4.05 μg N g^{-1} DM), and amino acids (335 vs. 90 ng N g^{-1} DM) relative to their DM accumulation. In terms of differences within species, alfalfa cultivars had no significant differences between cultivars.

Table 3.1 Nitrogen content of leachate solution of six-week-old alfalfa (Af) and red clover (RC) cultivars collected over a 48 hour period.

	T.Nitrogen† **(µg N)**	**Ammonium (µg N)**	**Amino Acids (ng N)**	**D.O.N†** **(µg N)**
Apica (Af)	32.57	2.61	70	30.00
CRS1001 (Af)	14.64	2.45	40	12.39
AC Christie (RC)	45.10	**6.30**	178	38.80
Tempus (RC)	27.29	**2.60**	153	24.69
Alfalfa	23.61	2.53	**55**	21.20
Red Clover	36.16	4.45	**166**	31.75
Grand Mean	29.65	3.98	110	26.46
SEM (N=4)	7.556	0.905	29.1	6.897
F-Probabilities ‡				
Cultivar	0.074	**0.029**	**0.017**	0.104
Af vs. RC	0.109	0.056	**<0.005**	0.152
Within Af	0.102	0.904	0.480	0.097
Within RC	0.122	**0.014**	0.554	0.174
Specific Leachate Nitrogen+				
	T.Nitrogen† **(µg N g^{-1} DM)**	**Ammonium (µg N g^{-1} DM)**	**Amino Acids (ng N g^{-1} DM)**	**D.O.N. †** **(µg N g^{-1} DM)**
Apica (Af)	53.07	4.27	110	48.80
CRS1001 (Af)	22.60	3.83	70	18.77
AC Christie (RC)	**106.93**	**14.76**	403	92.17
Tempus (RC)	**49.63**	**4.43**	268	45.21
Alfalfa	**37.84**	**4.05**	**90**	33.79
Red Clover	**78.28**	**9.60**	**334**	68.69
Grand Mean	58.05	6.82	200	51.23
SEM (N=4)	18.071	2.357	52.4	16.357
F-Probabilities ‡				
Cultivar	**0.039**	**0.017**	**0.002**	0.051
Af vs. RC	**0.045**	**0.037**	**<0.005**	0.054
Within Af	0.256	0.898	0.578	0.219
Within RC	**0.044**	**0.009**	0.077	0.065

+ 'Total' (top) values represent total µg N in leachates, 'Specific' (bottom) values represent total µg N g^{-1} DM

‡ *F*-probabilities of ANOVA analysis of cultivar groups, plus comparison contrasts between ('Af vs. RC') and within ('within Af'; 'within RC'); significant values ($P<0.05$) are bolded. Means in bold indicate significant differences between pairs indicated by comparison contrasts.

†T. Nitrogen= 'Total Nitrogen'; D.O.N. = 'Dissolved Organic Nitrogen'

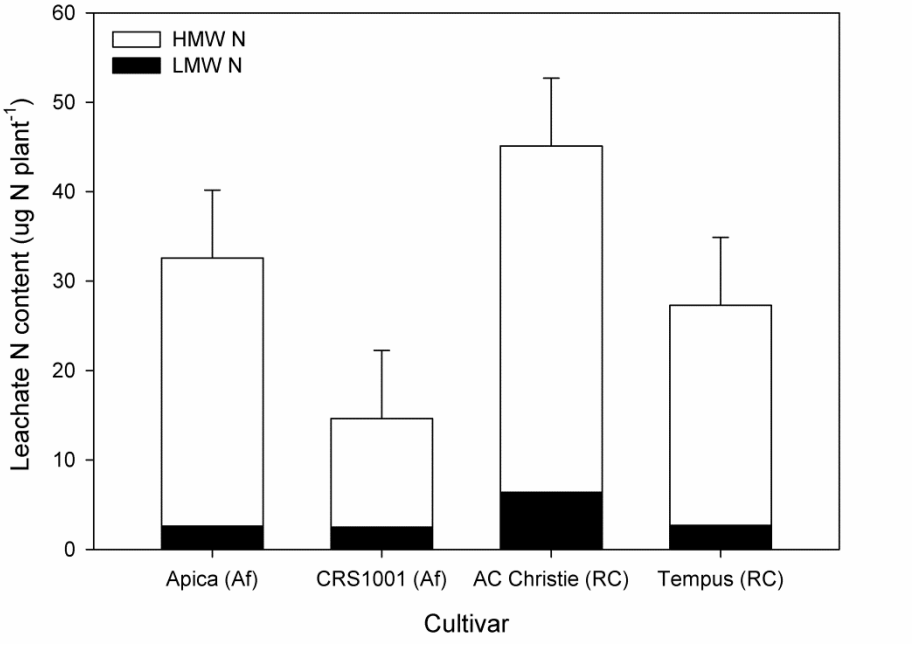

Figure 3.2: Nitrogen in leachates collected over 48h from two cultivars of alfalfa(Af) and red clover (RC) grown in microlysimeters, divided into low molecular weight N (NH_4^+ and Amino Acids) and high molecular weight N (protein, peptide) fractions.

Table 3.2: Percent amino acid composition of exudates of alfalfa and red clover cultivars

	Alfafa		**Red Clover**	
Amino Acid$^+$	**Apica**	**CRS1001**	**AC Christie**	**Tempus**
α-aminobutyric acid	3.4 (2.0)	-	2.0 (4.1)	21.2 (17.4)
Aspartic Acid	24.5 (10.3)	19.6 (11.1)	17.5 (8.7)	9.0 (4.4)
Cysteine	8.7 (5.6)	-	35.0 (33.4)	5.6 (3.8)
Glutamine	11.9(0.8)	13.9 (6.0)	5.4 (3.1)	6.1 (6.3)
Glycine	7.8(2.5)	9.6 (1.7)	0.8 (1.0)	2.2 (1.5)
Isoleucine	-	-	-	0.7 (0.8)
Leucine	2.7(1.9)	14.9 (5.3)	3.8 (4.6)	2.7 (4.2)
Serine	0.4 (0.4)	-	-	1.4 (1.0)
Tyrosine	5.3(3.5)	-	27.3 (33.4)	25.6 (18.6)
Valine	1.0 (1.0)	4.4 (2.3)	0.9 (1.0)	2.5 (1.9)
Other	34.3(7.5)	37.6 (7.3)	7.2 (2.6)	23.0 (4.9)

+ (N=4, SEM given in parentheses) 'Other' refers to total of peaks not identified from standard), '- '= none detected

Table 3.3: Dry matter yield, nitrogen content and nodulation rate of alfalfa (Af) and red clover (RC) cultivars grown in microlysimeters for 6 weeks.

	Yield (mg DM)	**Root:Shoot Ratio**	**Shoot (mg DM)**	**Root (mg DM)**	**N Content (mg N g^{-1} DM)**	**Total N (mg N $plant^{-1}$)**	**Total Nodules (Nod $plant^{-1}$)**	**†Spec. Nod (Nod g^{-1} root DM)**
Apica (Af)	622	1.04	316	307	**20.0**	12.4	26	86
CRS1001 (Af)	652	1.08	334	319	**19.3**	12.6	31	100
AC Christie (RC)	**449**	0.94	218	**231**	**19.4**	**8.7**	**31**	**132**
Tempus (RC)	**595**	0.76	254	**341**	**18.7**	**11.1**	**14**	**44**
Alfalfa	**637**	1.06	**325**	313	**19.7**	**12.5**	29	93
Red Clover	**522**	0.85	**236**	286	**19.1**	**9.9**	23	88
Grand Mean	579	0.95	280	299	19.4	11.2	25	91
SEM (N=4)	41.2	0.104	27.3	25.1	0.021	0.73	4.67	18.2
F-probabilities+								
Cultivar	**0.020**	0.160	**0.039**	0.089	**<0.005**	**0.009**	0.098	**0.029**
Af vs. RC	**0.016**	0.059	**0.007**	0.303	**<0.005**	**0.030**	0.254	0.780
Within Af	0.611	0.771	0.650	0.736	**0.016**	0.884	0.511	0.584
Within RC	**0.027**	0.210	0.376	**0.009**	**0.011**	**0.040**	**0.031**	**<0.005**

+*F*-probabilities of ANOVA analysis of cultivar groups, plus comparison contrasts between ('Af vs. RC') and within ('within Af'; 'within RC'); significant values ($P<0.05$) are bolded. Means in bold indicate significant differences between pairs indicated by comparison contrasts.

† 'Spec. Nodulation' = Specific Nodulation

Within red clover, AC Christie showed significantly higher levels of total ammonium, specific total N, and specific ammonium, with near-significant results for specific amino acids and organic N as well, compared to Tempus (Table 3.1).

Peaks of nine amino acids (α-aminobutyric acid, aspartic acid, cystein, glutamine, glycine, isoleucine, leucine, serine, tyrosine, and valine) were positively identified in the samples. Four unidentified peaks of probable amino acids, or similar nitrogenous compounds, were also noted, and were combined in the 'other' category.

Aspartic acid and glutamine appeared to be the most commonly found amino acids across the samples (Table 3.2) with others more common to a specific species (tyrosine in red clover; glycine in alfalfa), or specific cultivars (α-aminobutyric acid in Tempus; leucine in CRS1001).

3.4.2 Plant yield, N content and nodulation

Mean plant biomass yield was 579 g DM, with 19.4 mg N g^{-1} DM and 91 nodules g^{-1} root DM (Table 3.3). Significant species differences were detected between species, with alfalfa having higher yield (637 vs. 522 mg DM), shoot yield (325 vs. 236 mg DM), higher N content (19.7 vs. 19.1 mg N g^{-1}) and total N (12.5 vs. 9.9 mg N) than red clover (Table 3.3). There was little variation between alfalfa cultivars with respect to plant growth and N accumulation, with only N content differing between them (20.0 and mg N g^{-1} for Apica and CRS1001, respectively). There were more differences between red clover cultivars, with Tempus showing higher yield (595 vs. 449 mg DM), lower N content (19.4 vs. 18.7 mg N g^{-1} DM) and higher total N (8.7 vs. 11.1 mg N) than AC

Christie. In addition, AC Christie showed much higher levels of nodulation, both in terms of total nodules and specific nodulation rate than Tempus (Table 3.3)

3.5 Discussion

Nitrogenous compounds collected from the leachates of the forage legumes were primarily high molecular weight dissolved organic N (most likely proteins and peptides), followed by ammonium and small amounts of amino acids. Quantities of ammonium and amino acids matched the range expected from similar experiments done with nodulated forage legumes collected over a period of several days (Paynel et al. 2001; Ta et al. 1985).

With regard to total N measured in the leachates, one particular cultivar, AC Christie, ranked the highest both in total amount and relative to size (specific nitrogen exudation). It is also notable as the legume in the study with the lowest biomass accumulation and the one with the highest specific nodulation. These morphological traits may be important for two reasons. First, as an efficient nodulator relative to its biomass, its N fixation rate may be above its N demand. As an important site of N exudation, nodules may build up in small N compounds if not immediately transferred to the plant (Day et al. 2001). While the synthesis of amides for the transport of N from the nodules to the xylem may be repressed by the plant when soil N is available, the fixation of N by symbiotic bacteria is not under as strict control (Day et al. 2001; Schulze 2004), leading to situations where build-up of ammonium may occur within nodule tissue. In this case, a low N demand relative to nodules number could result more potential for release. Second, if N exudation is viewed as the net result of the influx and efflux of N compounds across the root cell membrane (Philips et al. 2004), then a smaller plant may be less likely to be able to re-

uptake those compounds in a relatively larger soil volume, leaving more in the soil solution. Within this study, a correlation analysis between between the specific nodulation rate and N exudation rate was not possible because the strong association of cultivar and nodulation rate, as well as the low number of plants measured per cultivar, meant that other genetic factors could not be ruled out. Further research into this rea with a larger sample size may provide more definitive evidence of the relationship between these traits.

It is important to note that the plant size and morphology recorded in this experiment differs from those observed in chapter 2 under similar nutrient levels. In that study, red clover cultivars were generally larger than alfalfa (RC=638, Af= 522 mg DM), root:shoot ratios were much more different (RC=1.15, Af=0.55), and there were large differences in specific nodulation (RC= 163, Af=70 nod g^{-1} root DM). In contrast, this experiment showed virtually no difference in root:shoot ratio, smaller differences in specific nodulation, and higher DM yields in alfalfa (Table 3.3). This is not altogether unexpected: plants in the previous experiment were allowed to grow for an additional 14 days and had a larger volume of substrate, which can have a significant impact on the growth distribution of plants (Poorter et al. 2011). If, however, exudation of ammonium and other nitrogenous compounds is related to the relationship between nodulation rate and the relative biomass of the legume as hypothesized above, then it may be difficult to extrapolate from these experiments how these specific cultivars will behave in the field. Temperate legumes in the field can be quite plastic in morphology, especially in response to the presence of neighbours (Turkington 1983; Marcuvitz and Turkington 2000; Thein et al. 2008).

The present results contrast somewhat with those of Thilakarathna (2013) who, using the same red clover cultivars in solution culture, found Tempus to excrete more N than AC Christie. The fact that the earlier experiment was carried out in growth pouches in a smaller volume of solution (25mL vs. 30mL) without a soil matrix lends some weight to the idea that re-uptake may play a role in the measured exudation rates of legumes, as a larger root system might prove to be more efficient re-uptaking N in a larger volume with potential obstructions. Field studies comparing the same two clover cultivars showed that in mixed swards of bluegrass (*Poa pratensis*) and clover, Tempus was associated with higher levels of nitrate in the top 15cm of soil (MacPherson, 2010;), where the majority of forage legume and grass roots are present, and a significantly higher transfer of N to surrounding grass compared to AC Christie (Thilakarathna et al 2012b). While this could be evidence that Tempus exudes more N that is retained in the rooting zone, it was not possible from those studies to determine if that N was derived from exudates or other N-rhizodeposits that were subsequently mineralized (Fustec et al. 2010) and thus part of the decomposition pathway.

While red clover cultivars did show higher levels of total N in their leachates than alfalfa, differences between species varied mostly in terms of N quality (i.e. the form of N present) rather than quantity. The main difference between the species appears to be the amount of amino acids present, which was higher in red clover in both net amount and relative to size (Table 3.1). This may be related to the high exudation rate of tyrosine from red clover plants, which made up a quarter of the mean amino acids found in the clover exudates, but was barely detected in alfalfa samples (Table 3.2). It should be noted, however, that amino acids made up only a small proportion of the total N found in

samples, two levels of magnitude lower than ammonium, the other main nitrogenous compound thought to be taken up directly by neighbouring plants (Kraiser et al. 2007). Therefore, its role as a potential means of N transfer may not be as important in these plants at this stage of life.

Results of amino acid content by percent also stand in contrast to other studies on similar species, which show much higher levels of glycine, and serine, and negligible amounts of aspartic acid, tyrosine and cysteine (Ta et al. 1985; Paynel et al. 2001; Paynel and Cliquet 2003; Lesuffleur et al. 2007). Studies on the ratio of exudation of amino acids show that in alfalfa and medic, tyrosine and aspartic acid may be some amino acids with the lowest levels of efflux relative to influx (Phillips et al. 2004). One crucial difference with the above-mentioned studies, however, was that they were performed with an external source of mineral N available, which can have impacts on the exudation rates of specific amino acids. Paynel et al.'s (2008) study on white clover exudation, for example, noted that certain amino acids, such as asparagine, showed a decrease in exudation rate as nitrate levels increased, while others, such as glutamate, increased. It is interesting to note that aspartic acid and glutamine, both found in substantial amounts in both species and cultivars, are important intermediates in the synthesis of asparagines, the main carrier of N from the nodule to the xylem (Prell and Poole 2006). This may suggest that the a large portion of the amino acids found in this study derive from the nodules, and not the root tips, which are thought to contain higher levels of glycine and serine, synthesized in the plastids (Tegeder 2014). Tyrosine, which was found in high levels in the clover but not alfalfa leachates, has been shown in *Trifolium* exudate studies previously (Paynel et al. 2008), and at levels similar to aspartate/glutamate, but not in alfalfa exudates (Ta et al.

1986). While most amino acids are synthesized across a range of plant tissues, the shikimate pathway, which is responsible for the production of all aromatic amino acids, is expressed mostly in flowers and roots (Weaver and Herrmann 1997). This suggests that the tyrosine is produced within the root instead of being exported directly from the shoot. Whether it is being excreted from the root for a specific function or being produced for internal functions could not be assessed.

While most studies use axenic conditions during their exudates collection (Jones and Darrah 1994; Phillips et al. 2004; Carvalhais et al. 2011), we chose not to keep our microlysimeters sterile to better match conditions in related experiments. It is possible, therefore, that our results underestimate the amount of certain amino acids excreted because of microbial uptake. However, several studies have noted no major differences in the composition of amino acids in exudates between sterile and non-sterile treatments (Ta et al. 1986; Paynel et al. 2001; Paynel and Cliquet 2003), so it is still likely representative of the N compounds available for uptake by non-fixing plants in similarly non-sterile conditions.

While ammonium and amino acids did make up a significant portion of the N found in leachates, the largest proportion by far was still dissolved organic N, comprised of protein and peptides. While this finding is hardly surprising, as studies have demonstrated that a significant portion of the N excreted by roots is in the form of dissolved organic N (Arcand et al. 2013), it does underscore that a large portion of the N supplied by legumes to the soil may not be immediately available for 'direct' transfer to non-fixers. While uptake of inorganic N and amino acids are well understood, uptake of peptides and protein do not seem to be common in plants (Kraiser et al. 2007), requiring catabolic

microbes to first break down these more complex compounds into more readily absorbed forms (Jackson et al. 2008), putting an intermediary between N-donor and N-receiver plants that might reduce its efficiency, depending on the N-demands of the micro-organisms themselves.

Results of our study indicate that there are likely genotypic factors in the exudation rate of nitrogenous compounds of pasture legumes, between species and cultivars, and that choice of N-donor identity may be critical when trying to facilitate direct N-transfer.. As largest provider of dissolved N in its leachates and the largest exuder of ammonium (a 'transferable' form of N), the red clover cultivar 'AC Christie' shows good potential to be an efficient N-donor in temperate legume-grass forage swards during the early establishment period.

3.6 Conclusion

Leachates collected from two cultivars each of red clover and alfalfa in microlysimeters revealed species and cultivar differences in the N released by root exudation. One particular cultivar of red clover, AC Christie, had the highest amount of ammonium and amino acids in its leachates, two components important in direct N transfer. This difference may be related to its high nodule number relative to its size, resulting in a larger difference between its rate of N-fixation versus its N-demand and, ultimately, a higher amount of excess N lost to the surrounding soil solution. Future research into the relationship between this trait and N-exudation could help in identifying forage legume populations with a higher potential for direct N-transfer to non-fixing species in the field.

Chapter 4: Interactions between cultivars of nitrogen-fixing legumes species (*Trifolium pratense* L., *Medicago sativa* L.) and grasses (*Phleum pratense* L., *Lolium perenne* L.) under different nitrogen levels

4.1 Abstract

Legumes and grasses co-exist in a relationship that ranges from competition to facilitation, influenced by environmental conditions, plant genetics and the availability of nitrogen (N). One of the more important relationships that these two types of plants have in forage production is the transfer of N fixed by legumes (N-donors) to non-fixing grasses (N-receivers). Evidence exists that species and populations of legumes and grasses can vary in their capacity to excrete and uptake N, respectively, suggesting that there may be certain legume-grass combinations that are more efficient at N transfer. This study examined what effects grasses and legumes had on each other's growth and N accumulation in a pot experiment under a range of nitrogen fertility levels to determine what effect donor and receiver species and cultivar play in N transfer. Two cultivars each of alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), perennial ryegrass (*Lolium perenne* L.) and timothy (*Phleum pratense* L.) were grown in all possible grass-legume combinations under one of three N-fertility treatments. Dry matter and N accumulation were compared between plants grown in combination with those grown alone to quantify the net benefits that legumes and grasses conferred on each other. Increased N had a positive effect on the total yield of grasses (from 144 to 269 mg DM) and total N (1.01 to 2.11 mg N plant^{-1}). Nitrogen additions reduced total yield of legumes at the highest treatment level (437 to 247 mg DM), as a result of higher grass growth. The presence of legumes had an overall negative effect on the growth of grasses (87%

compared to growing alone), but did improve tissue N content (129%) and total N (120%). Relative improvements of total N were highest in a single timothy cultivar (cv Champ; 169%), likely due to lower N uptake when grown alone compared to other grasses. Highest net total was found in a cultivar of perennial ryegrass (cv Bastion; 1.92 mg N).Total N was improved more in the presence of alfalfa then red clover, particularly at medium N additions. Results indicate that grass dry matter might be a limiting factor in potential N transfer, and that grass growth rate will depend on the competitiveness of grasses and legumes in mixture

4.2 Introduction

Pasture legumes and grasses provide an interesting example of the complex relationship between plants inhabiting a common space. 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), which reaches grasses through a process known as 'N transfer' (Paynel et al. 2008; Fustec et al. 2010; Thilakarathna et al. 2012). At an individual level, however, 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). Long-term transfer of N from legumes to grasses via the turnover of root litter (Fustec et al. 2010), can occur without the two plants having to share a common space simultaenously. However, short-term N transfer via root exudates, which has been identified as an important flow of N in cool grasslands (Gylfadóttir et al. 2007), requires

that plants grow in close spatial proximity and growing actively (Brophy et al. 1987), and thus likely to compete for space and resources.

Models exploring the legume-grass dynamic in pasture systems (Thornley 2001; Schwinning and Parsons 1996) predict that competition between the plants 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*) genets 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; Bathram 1997; Adams and Velland, 2011) confirmed that 'compatible' genotypes of legumes and grass were more productive than mismatched neighbours.

Besides general growing compatibility, there also exists the potential for legume and grass pairs to differ in their ability to efficiently transfer N. Evidence exists that species and genotypes release different forms of N via exudation at different rates. Grayston et al. (1997), for example, noted a wide variety of amino acid profiles among trees, even those closely related. Other studies using diverse collections of non-woody plants have noted different rates of influx and efflux of amino acids across roots, resulting in very different amino acid exudeate content between species (Phillips et al. 2004; Lesuffleur et al. 2007). Other studies comparing the exudates of crop cultivars with dissimilar

capacities to uptake metals (Mozafar et al. 1992; Rasouli-Sadaghiani et al. 2012) or suppress diseases (Li et al., 2009) have noted different amino acid contents. These studies suggest that certain species or populations of legumes could be more efficient 'N-donors' by virtue of the amount of N that they exude, and the form that it takes. On the other side, it has been demonstrated that temperate perennial grasses species differ both in their general nitrogen use efficiency (NUE; Vasquez de Aldana and Berendse, 1997) and their ability to take up specific forms of N (Wiegelt et al. 2005), with species found in nutrient rich environments (e.g. *Lolium perenne*) generally showing lower NUE and a preference for inorganic nitrogen, unlike those typical of nutrient-poor environments (e.g. *Anthoxanthum odoratum*). 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). By matching donors with high exudation rates with receivers capable of rapid, more efficient direct N transfer could be achieved.

To test if there is variation in the efficiency of N-transfer among different donor and receiver combinations, a pot experiment was devised to examine the interactions between cultivars of two species of N-fixing legumes (alfalfa, *Medicago sativa* L.; red clover, *Trifolium pratense* L.) and perennial forage grasses (perennial ryegrass, *Lolium perenne* L.; timothy, *Phleum pratense*). To reduce the impacts of competition, the experiment was deliberately made spare, with only a single plant of each type (N-donor and N-receiver) in each pot. The effect of pairings on N-donor and N-receiver dry matter yield and N accumulation was measured and compared to each other and to relative performance when grown alone. Our objective was to determine if the presence of legumes would have an effect on the N content and total N accumulation of grasses, and how this effect

would be changed as mineral N fertilization levels decreased. In addition to this, we wanted to assess if the species/cultivar identity of the N-donor (legume), the N-receiver (grass), or a combination of both, would impact on the transfer of N

4.3 Methods

4.3.1 Growing Conditions and Experimental Design

Species and cultivars chosen for the study were red clover, A.C. Christie, a diploid variety (Martin et al. 1999) and Tempus, a tetraploid,(Oseva Uni., Czech Republic, Breeding Station Domoradice, 1988; http://www.osevauni.cz/vlastni-odrudy), and alfalfa, Apica, a tap-rooted tetraploid (Michaud and Richard 1983) and CRS1001, a rhizomatous-rooted tetraploid (Y.A. Papadopoulos, AAFC, unpublished data); perennial ryegrass (*Lolium perenne* L.; 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); and timothy (*Phleum pratense* L.; Tm), Champ a mid-season hexaploid variety (Childers et al. 1978) and Richmond, a late season hexaploid variety (http://extension.psu.edu/plants/crops/forages/species /timothy). Seeds of all cultivars were surface sterilized for two minutes 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 and at 20 ºC for two days.

Once germinated, seedlings were transferred to standard 10.2 cm pots (approx. 700mL 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.

Pots were placed in a split-plot design consisting of replicated latinized arrays to account for environmental variation within growth chambers. Legumes (Af-Apica, Af-CRS1001, RC-Christie, RC-Tempus, and 'No Legume') were used as as columns and grasses (PR-Bastion, PR-Feeder, Tm-Champ, Tm-Richmond, 'No Grass') as rows. This five-by-five pot arrangement included all pair-wise 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 seperate growth chambers, which were used as blocks.

Growth chambers were maintained at a photoperiod of 16 hr of daylight at 425 μmol m^{-2} s^{-1} at 21 ± 2 °C and 8 hr 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 one week, 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}.

4.3.2 Nitrogen Treatments

After four weeks, 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 four weeks fertilized at the same rate with the same No-N Hoagland, adjusted with ammonium sulphate ($(NH_4)_2SO_4$) to give three different N fertility levels: 0.6 mg N pot^{-1} wk^{-1},same as in establishment phase ('Full N'); 0.3 mg N pot^{-1} wk^{-1}, half of what was supplied during establishment phase ('Half N'); and 0 mg N pot^{-1} wk^{-1} ('No N').

4.3.3. Harvesting and Data Collection

Pots were destructively harvested at four ('Baseline') or eight weeks ('No N', Half N, 'Full N'). Shoots were clipped at the soil level and leaf area was measured using a Canon LiDE 210 scanner and ImageJ software (Abramoff et al. 2004). Shoot tissue was then dried in a hot air oven at 65 ºC for three days and weighed to measure dry matter (DM) yield. Roots were gently washed of sand and then photographed using a 50cm x 35cm light table and a Carl Zeiss CyberShot 5.0 megapixel digital camera. Root images were analyzed using ImageJ (Abramoff et al. 2004) to obtain total root length. Due to difficulties in separating fine roots, only primary roots were measured for grasses. For legumes, nodule number was also recorded. Root tissue was then dried and weighed as above. Total plant yield (root and shoot combined) and root:shoot ratio were determined before shoot and root tissue of individual plants were recombined and ground to pass through a 1-mm sieve in a micro Wiley mill, standard model 3 (Arthur H Thomas Co.,

Philadelphia, 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).

4.3.4 Statistical Analysis

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

Likewise, 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 to 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. $CI=x_{combination}/x_{alone}$, where x is the variable being examinedA score of <1 indicates a

negative effect on growth in combination compared to growing alone in similar conditions, while a score of >1 indicates a positive effect.

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 factors. Orthogonal contrasts were used to determine differences within main effects.

4.4 Results

4.4.1 Grass Cultivar Response

The addition of N had an overall positive effect on the growth of grasses (Fig. 4.1), with a significant increase in accumulated yield over N treatments (Table 4.1). Similarly, grass total leaf area and root length increased significantly with increasing N fertilization levels, while the root:shoot ratios decreased (Table 4.3). Mean grass yield CI values remained similar over N treatment levels indicating no significant change in mean relative performance (Table 4.1). Nitrogen fertilization also affected plant N status with near-significant (P=0.08) and significant (P=0.01) positive responses in N concentration 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 only one mean value (N concentration at 'Half N' level) was significantly greater than 1.

Table 4.1: Grass mean total dry matter yield, nitrogen content and total nitrogen, and corresponding copatability indices (CI) under different N fertility treatments and in different legume-grass cultivar combinations

Treatments‡		Total Yield* mg DM	CI†	N content mg N g^{-1} DM	CI†	Total N mg N $plant^{-1}$	CI†
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
Bastion (PR)	Apica (Af)	294	1.02	7.8	1.20	2.22	1.36
	CRS1001 (Af)	269	0.94	7.6	1.17	1.99	1.23
	AC Chris.(RC)	264	0.96	7.3	1.12	1.90	1.17
	Tempus (RC)	218	**0.75**	7.1	1.09	1.59	0.93
	CV mean	261	0.92	7.5	1.15	1.92	1.17
Feeder (PR)	Apica (Af)	227	0.86	8.2	1.21	1.86	1.02
	CRS1001 (Af)	215	**0.78**	7.7	1.11	1.56	0.83
	AC Chris.(RC)	213	0.85	8.8	1.19	1.63	0.96
	Tempus (RC)	228	0.88	8.2	1.19	1.75	1.16
	CV mean	221	0.84	8.2	1.17	1.70	0.99
Champ (Tm)	Apica (Af)	162	0.94	8.3	**1.79**	1.39	**1.67**
	CRS1001 (Af)	171	0.92	8.2	**1.74**	1.40	**1.69**
	AC Chris.(RC)	165	0.93	7.8	**1.64**	1.29	**1.62**
	Tempus (RC)	142	0.94	7.9	**1.67**	1.10	**1.79**
	CV mean	160	0.93	8.0	1.71	1.29	1.69
Rich. (Tm)	Apica (Af)	152	0.80	7.7	1.17	1.19	0.87
	CRS1001 (Af)	183	0.90	7.9	1.05	1.33	1.04
	AC Chris.(RC)	167	0.89	8.2	1.15	1.35	0.95
	Tempus (RC)	150	0.80	8.3	1.12	1.20	0.89

	CV mean	163	0.84	8.0	1.12	1.26	0.94
	SEM	16.5	0.105	0.36	0.178	0.140	0.281
	Grand Mean	201	0.89	7.9	1.29	1.54	1.20
***F*-probabilities**[+]							
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**

* combined root and shoot DM

†CI= compatibility index, ($V_{combination}/V_{alone}$.). A score of <1 indicates a negative effect on growth in combination compared to 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

‡ N treatments are 0.0, 0.3, and 0.6 mg N $pot^{-1}wk^{-1}$ for 'No N', '1/2 N', and 'Full N' respectively; Af=alfalfa, RC=red clover, PR=perennial Ryegrass, Tm= timothy

+F-probabilities significant at the P<0.05 are given in bold

Table 4.2: Legume mean dry matter total yield, nitrogen content and total nitrogen, and corresponding compatability indices (CI), under under different N-fertility treatments and in different legume-grass cultivar combinations

Treaments‡		Total Yield* mg DM	CI†	N Content mg N g^{-1} D.M.	CI†	Total N mg N $plant^{-1}$	CI†
Nitrogen	No N	347	1.01	19.7	1.03	7.23	1.07
	Half N	346	0.97	19.4	1.00	6.82	1.07
	Full N	247	**0.64**	18.5	1.01	4.63	**0.65**
	SEM	23.2	0.136	0.43	0.049	0.597	0.179
Apica (Af)	Bastion (PR)	304	1.17	19.4	0.96	5.37	1.23
	Feeder (PR)	306	1.20	19.9	0.98	5.98	1.23
	Champ (Tm)	232	1.03	19.5	0.95	4.41	1.00
	Rich. (Tm)	274	1.09	19.8	0.96	5.46	1.16
	CV mean	279	1.12	19.7	0.96	5.30	1.16
CRS1001 (Af)	Bastion (PR)	250	0.72	21.3	**1.12**	5.24	0.79
	Feeder (PR)	303	0.92	20.1	1.09	6.66	1.02
	Champ (Tm)	298	0.93	20.2	1.06	6.06	0.99
	Rich.(Tm)	326	0.94	16.6	0.93	6.37	1.12
	CV mean	294	0.88	19.6	1.05	6.08	0.98
AC Chris.(RC)	Bastion (PR)	288	0.82	19.1	1.01	5.91	0.96
	Feeder (PR)	236	**0.61**	19.0	1.05	4.65	0.81
	Champ (Tm)	337	0.91	18.9	1.05	6.51	0.97
	Rich.(Tm)	257	0.72	18.2	1.01	4.72	0.72
	CV mean	279	0.76	18.8	1.03	5.44	0.87
Tempus (RC)	Bastion (PR)	361	0.66	18.3	0.97	7.13	0.66
	Feeder (PR)	358	**0.60**	19.3	1.04	6.99	**0.60**
	Champ (Tm)	451	0.83	19.2	1.03	9.97	0.87
	Rich.(Tm)	436	0.79	18.8	1.01	8.21	0.79

	CV mean	401	0.72	18.9	1.05	8.07	0.73
	SEM	44.9	0.187	0.074	0.058	0.905	0.200
	Grand Mean	313	0.87	1.92	1.01	6.23	0.93
***F*-probabilities**							
Nitrogen treatment		**0.03**	0.19	0.19	0.95	**0.04**	0.24
Legume cultivar		**0.01**	0.24	0.43	0.63	**0.01**	0.37
.. Af vs RC		0.06	0.09	0.10	0.45	0.08	0.13
Nitrogen × Legume		0.49	0.58	0.47	0.80	0.96	0.44
Grass Cultivar		0.70	0.73	0.26	0.30	0.62	0.96
.. Tm vs PR		0.25	0.30	0.19	0.24	0.32	0.61
Nitrogen × Grass		0.10	0.12	0.06	0.07	**0.03**	**0.03**
Legume × Grass		0.30	0.43	**<0.005**	**0.04**	0.06	0.13
Nitrogen × Legume × Grass		0.28	0.24	**<0.005**	0.05	0.14	**<0.001**

* combined root and shoot DM

†CI= compatibility index,($V_{combination}/V_{alone}$.). A score of <1 indicates a negative effect on growth in combination compared to 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

‡ N treatments are 0.0, 0.3, and 0.6 mg N $pot^{-1}wk^{-1}$ for 'No N', '1/2 N', and 'Full N' respectively; Af=alfalfa, RC=red clover, PR=perennial Ryegrass, Tm= timothy

+*F*-probabilities significant at the P<0.05 are given in bold

Table 4.3: Legume (Leg.) and grass (Gr.) morphology attributes under different N-fertility treatments and in different legume-grass cultivar combinations

Treaments‡		Root:Shoot Ratio		Total Leaf Area (cm^2 $plant^{-1}$)		Total Root Length (cm $plant^{-1}$)		Specific Nodulation (nod. g^{-1} root DM)
		Leg.	Gr.	Leg.	Gr.	Leg.	Gr.	
Nitrogen	No N	0.81	2.53	25.1	5.8	800	149	305
	Half N	0.79	2.16	23.2	8.5	744	170	236
	Full N	0.89	1.90	17.1	11.7	557	230	275
	SEM	0.036	0.152	2.25	0.73	86.8	15.9	38.9
Bastion (PR)	Apica (Af)	0.90	2.27	15.9	9.7	472	160	263
	CRS1001(Af)	0.94	2.20	19.5	8.4	512	228	268
	AC Chris. (RC)	0.68	1.92	26.8	9.5	720	213	365
	Tempus (RC)	0.76	1.91	24.9	8.0	749	185	197
	CV mean	0.82	2.08	21.8	8.9	613	197	273
Feeder (PR)	Apica (Af)	0.85	2.35	15.5	8.0	648	240	194
	CRS1001 (Af)	0.96	2.08	23.1	9.0	697	198	273
	AC Chris. (RC)	0.74	2.57	18.6	8.3	692	233	390
	Tempus (RC)	0.72	2.39	26.0	7.7	783	245	202
	CV mean	0.82	2.34	20.8	8.2	705	228	265
Champ (Tm)	Apica (Af)	1.02	2.22	12.1	8.3	447	166	290
	CRS1001 (Af)	0.99	2.18	19.6	9.9	633	155	198
	AC Chris. (RC)	0.69	2.26	26.7	8.9	846	169	479
	Tempus (RC)	0.68	2.21	33.1	7.4	1108	142	177
	CV mean	0.84	2.21	22.9	8.6	701	158	286
Richmond (Tm)	Apica (Af)	1.04	2.11	13.8	7.8	510	136	252
	CRS1001 (Af)	0.85	2.41	21.8	9.3	734	145	207
	AC Chris. (RC)	0.77	2.25	20.7	10.0	606	181	403
	Tempus (RC)	0.75	1.86	30.4	8.3	1052	123	195

	CV mean	0.85	2.16	21.7	8.9	725	143	264
	SEM	0.083	0.203	3.37	0.75	115.9	21.9	45.5
	Grand Mean	0.83	2.20	21.8	8.6	701	183	272
***F*-probabilities**[+]								
Nitrogen Treatment		0.23	0.07	0.10	**<0.005**	0.19	**0.03**	0.50
Legume Cultivar		**<0.005**	0.79	**0.01**	**0.03**	**0.04**	0.35	**<0.005**
.. Af vs RC		**<0.005**	0.64	**0.01**	0.40	**0.02**	0.50	0.11
Nitrogen × Legume		0.11	0.42	0.81	**0.03**	0.97	0.27	0.85
Grass Cultivar		0.95	0.41	0.79	0.77	0.24	**<0.005**	0.87
.. Tm vs PR		0.56	0.82	0.50	0.70	0.12	**<0.005**	0.78
Nitrogen × Grass		0.45	0.78	**<0.005**	0.90	**0.01**	0.95	0.84
Legume × Grass		0.27	0.25	**<0.005**	0.37	**<0.005**	**0.03**	**0.04**
Nitrogen × Legume × Grass		**<0.005**	0.12	**<0.005**	**0.04**	**0.03**	**0.01**	**<0.005**

‡ N treatments are 0.0, 0.3, and 0.6 mg N $pot^{-1}wk^{-1}$ for 'No N', '1/2 N', and 'Full N' respectively; Af=alfalfa, RC=red clover, PR=perennial Ryegrass, Tm= timothy

+*F*-probabilities significant at the $P<0.05$ are given in bold

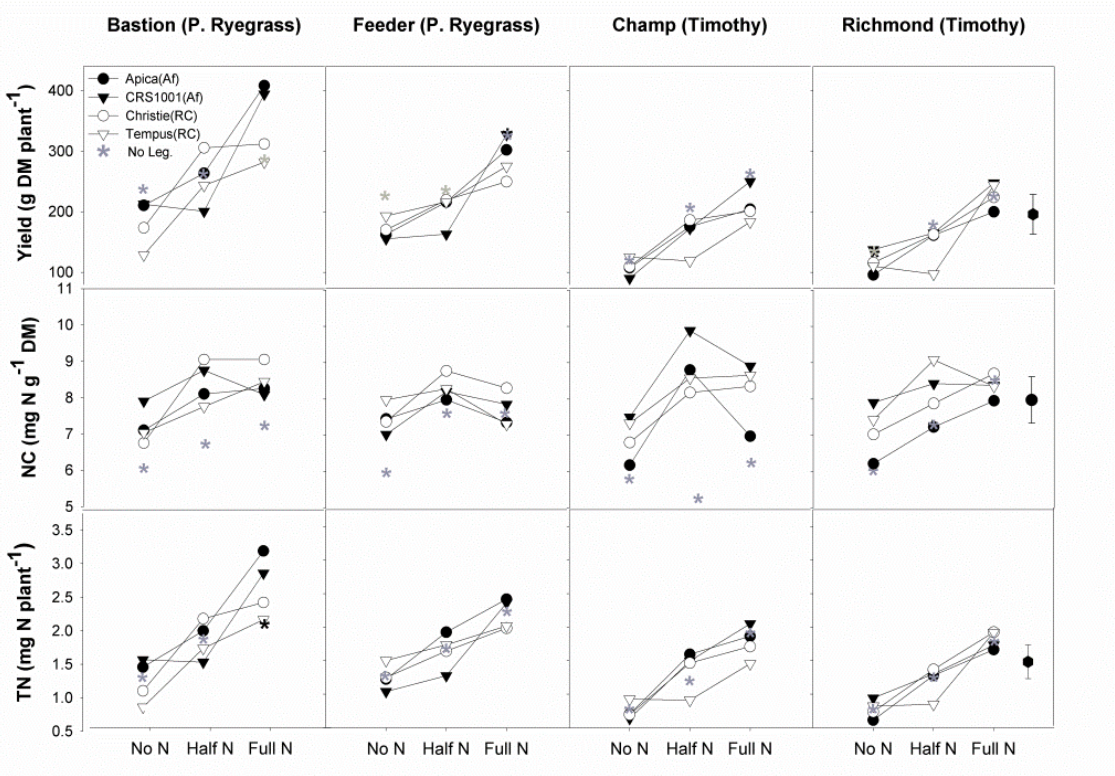

Figure 4.1: Grass total dry matter (DM) yield, nitrogen content (NC) and total plant nitrogen (TN) by grass cultivar, legume companion cultivar and nitrogen fertilization treatment**.** Hexagonal points in the far right column indicate the grand mean for each attribute; error bars represent the SEM for the three-way interaction of grass cultivar ×legume cultivar ×nitrogen.

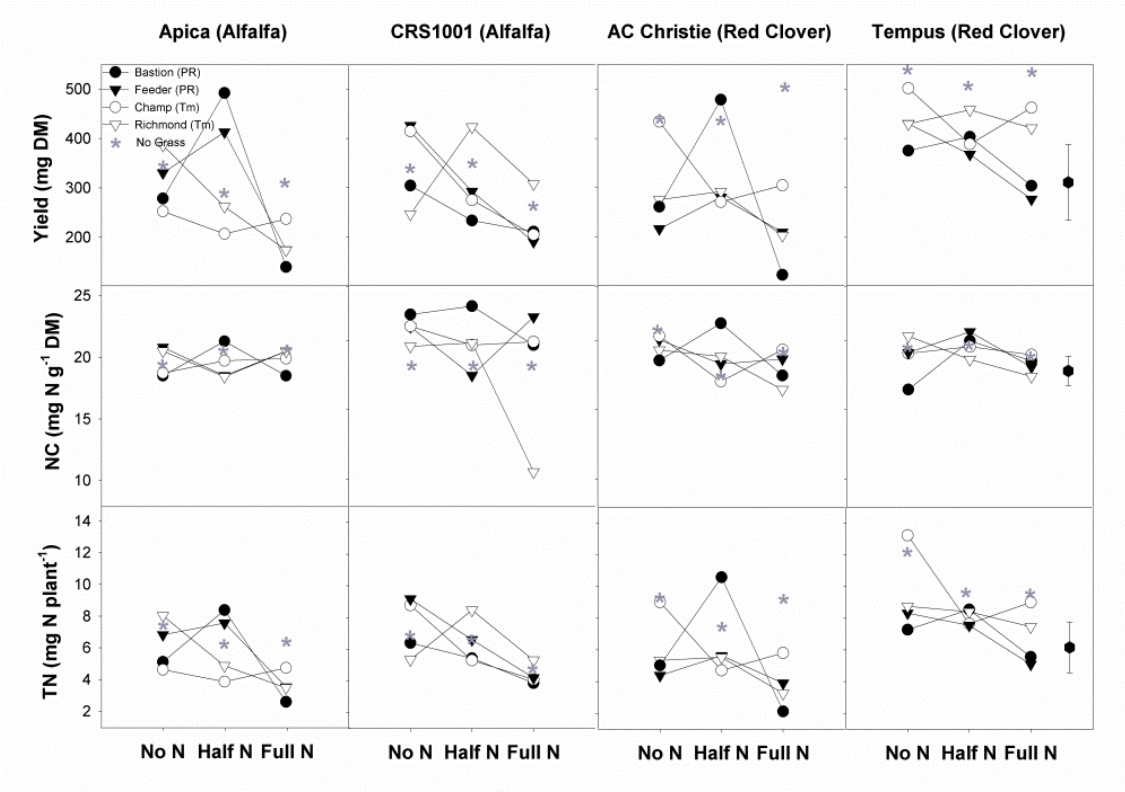

Figure 4.2: Legume total dry matter (DM) yield, N content (NC) and total plant nitrogen (TN) by legume cultivar, grass companion cultivar and nitrogen fertilization treatment**.** Hexagonal points in the far right column indicate the grand mean for each attribute; error bars represent the SEM for the three-way interaction of grass×legume×nitrogen treatment.

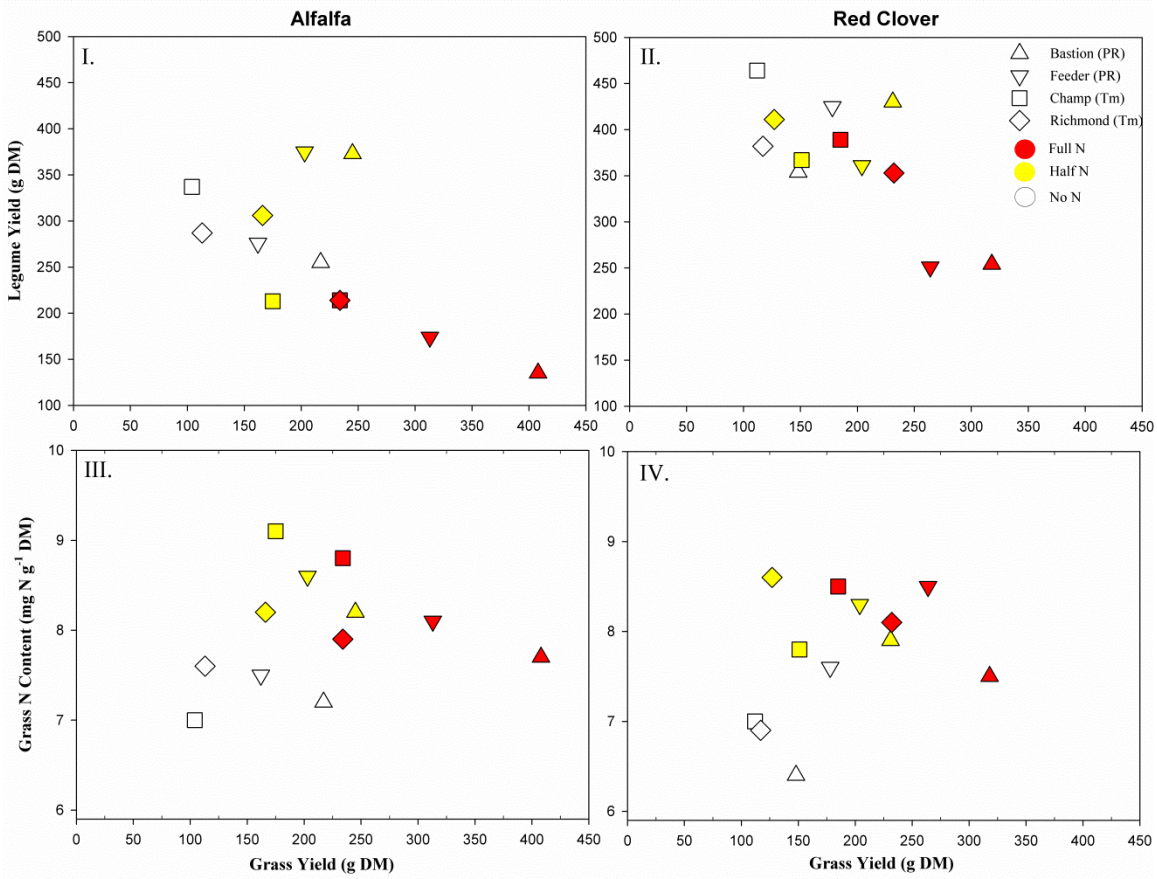

Figure 4.3: Relationship between grass and legume yield (mg DM; I, II) and grass nitrogen content (mg N g^{-1} DM; III and IV) of two cultivars of perennial ryegrass (PR) and timothy (Tm) paired with alfalfa (left) and red clover (right) under three nitrogen treatments.

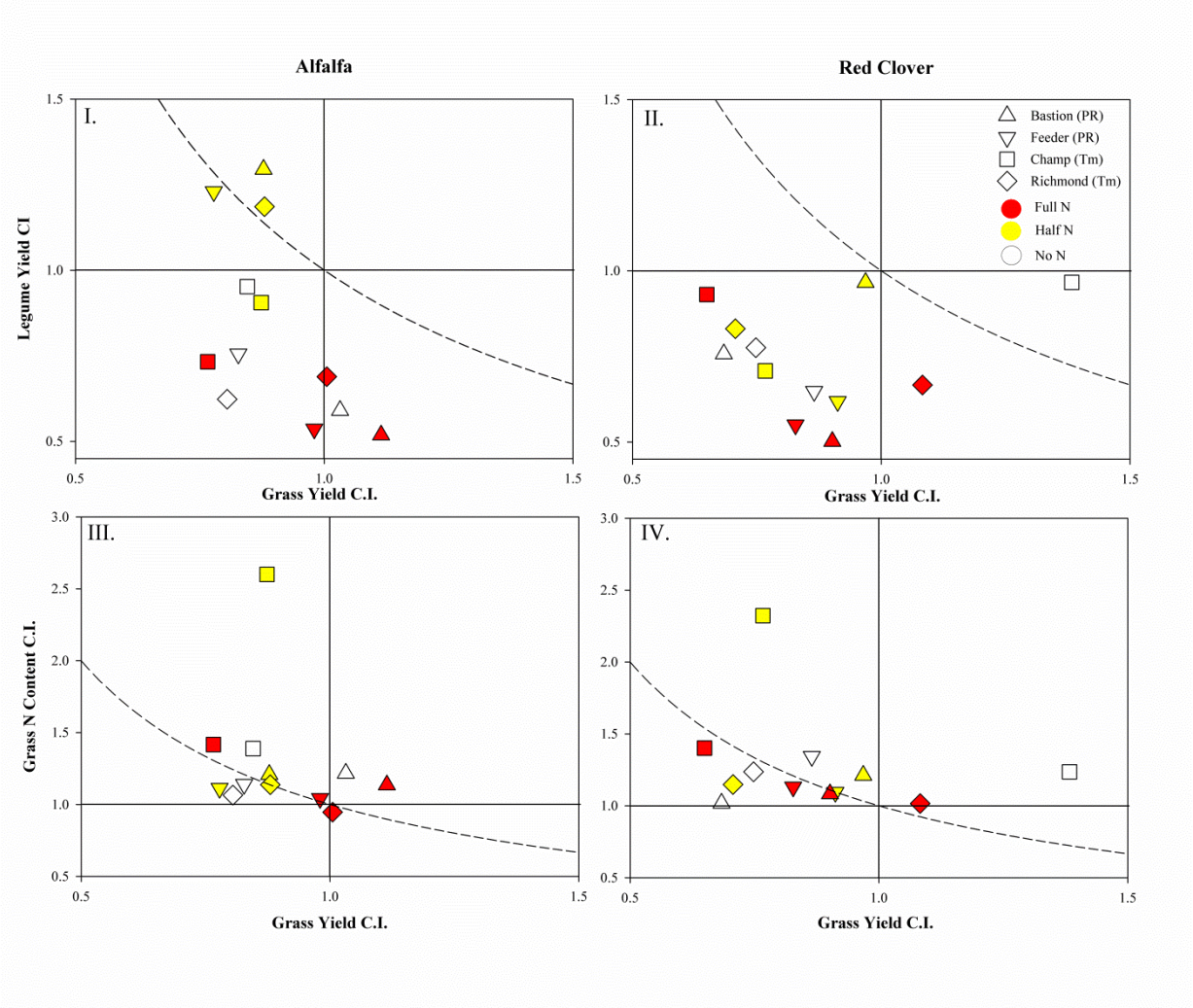

Figure 4.4: Relationship between relative grass yield (grass yield compatibility index; C.I.) and relative legume yield (Legume Yield C.I.; I and II) and relative grass nitrogen content (grass N C.I.; III and IV) of two cultivars of perennial ryegrass (P.R.) and timothy (Tm) paired with alfalfa (left) 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)

Grass species and cultivars varied in yield, total N and total root length, 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 4.1) and total root length (Table 4.3). Grass N concentration was near 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 4.1).

Grass N content CI value was near significant (P=0.065), with one grass cultivar (Champ) showing much higher values over the rest, all of which were found to be significantly higher than 1, a trend observed in total N as well (Table 4.1). 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 4.1), or any plant morphology metric, with the exception of grass leaf area, which was reduced in the presence of Tempus (Table 4.3) Interaction terms demonstrated a complex relationship between treatments with many attributes affected significantly by the combination of grass and companion legume cultivars in an N-dependent context. Grass yield and total N accumulation, and their respective CI values, were significantly affected by the three-way grass-legume-nitrogen interaction (Table 4.1). Patterns of 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 to clover (Fig. 4.1), while timothy cultivars show reduced growth in the presence of Tempus (RC) more than any other legume, particularly at the 'Half-N' treatment level. 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. 4.3).

4.4.2 Legume Cultivar Response

In contrast to grass cultivars, the addition of N had mainly negative effect on the yield and total N accumulation of legumes at the highest level, while CI values dropped to a point significantly below 1 (0.64, 0.65 respectively; Table 4.2). Legume N content, on the other hand, remained largely similar across N levels, while CI values showed little difference to plants grown alone.

Legume cultivars were similar in size and total N yield, with the exception of the red clover cultivar Tempus, which had higher DM yield and total N (Fig. 4.2). Legume morphology differed mainly between species, with alfalfa showing a greater root:shoot ratio, smaller leaf area, and shorter total root length than red clover (Table 4.3). Mean specific nodulation rate was highest in one clover cultivar (AC Christie; 409 nodules g^{-1} DM) and lowest in the other (Tempus; 198 nodules g^{-1} DM). Grass companion cultivar had limited effect on the growth and N status of legumes, although it is interesting to note that one alfalfa cultivar (Apica) had, on average, a positive interaction with grasses based on legume yield and TN CI values, even if the difference is not significant (Table 4.2).

As with grasses, legumes cultivars showed distinct patterns depending on the grass companions and the N provided. Legume N content showed a significant interaction of all three factors, which seems to reflect the large drop in N content of a single alfalfa cultivar (CRS 1001) paired with a single grass cultivar (Richmond) at a specific N-level ('Full N'). Legume total N seemed principally a response to nitrogen×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. 4.2).

4.4.3 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. 4.3) and relative to their performance alone (C.I. values; Fig 4.4). Because the influence of 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 CI graphs in figure 4.4.with the equation '$x \times y = 1$'. For the graphs showing the relationship between grass and legume yield CI values (Fig. 4.4 I, II), this line represents the point at which total relative growth is neutral compared to 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 and yield and N content CI values (Fig. 4.3 III, IV), 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.

Figure 4.3 shows a generally negative relationhip between the yield of grasses and legumes while demonstrating that the effect of the larger red clover plants on grass growth (I, II). As results above had 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 (Fig 4.3 III, IV) shows a positive influence of N additions from the 'No N' to 'Low N' treatments, but little change from the middle 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. 4.4 I) with most treatment groups falling well below the total relative yield isoclines. Alfalfa cultivars at '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 grass decreased theirs. Combinations of red clover and Bastion (fig 4.3, II) 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 (III, IV) 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', however, saw large gains in relative N content (CI= 2.60, 2.32 for alfalfa and clover combinations, respectively) that were larger in magnitude 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.

4.5 Discussion

The aim of this study was to gauge the relative benefits of N-donor legumes to N-receiver grasses via direct N transfer (Paynel et al. 2008) 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 certain cultivar pairs would be more efficient at N transfer.

As predicted, N status of grasses was improved with the addition of legumes, both in terms of N content and total N (Table 4.1). Legume N content stayed consistent throughout, meaning total N was a direct product of biomass accumulation (Table 4.2). 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 (Table 4.1). 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 (Table 4.1).

In terms of biomass yield, grasses and legumes respectively produced 89% and 87% when grown in combination than alone (Tables 4.1, 4.2). This was somewhat surprising, given that the non-N resources generally cited in grass-legume competitions (light, P; Haynes 1980; Marriott and Zuazua 1996) were given in ample supply given the density. As for N itself, it was definitely a limiting nutrient for grasses (see Table 4.1, Fig. 4.1), 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. (2013) as ideal conditions for pot-grown plants, but in the absence of competition for any specific resource, this seems to be a likely reason.

The relationship between grass and legume yield over N treatments showed a definite inverse trend in terms of actual DM produced per plant, with lower N treatments favouring legume growth and higher N treatments favouring grass (Fig 4.3) but relative rates of DM production suggest that this was not a perfect '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 position 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 8-plant per pot red clover-ryegrass study, found a similar reduction in biomass but increased tissue N content when compared to 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 8-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 the amount of N transferred via exudation. 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). Analysis of variance did reveal differences in grass total N, and grass total N CI (Table 4.1), 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 (Table 4.1), and largely when N was supplied at a moderate ('Half N' treatment) rate (Fig. 4.4). While the difference may appear to be 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 and well below the N accumulation seen by ryegrass varieties (Table 4.1, Fig. 4.3). This suggests that while Champ benefits relatively well from the presence of legumes, its performance alone is the major factor in its large CI score. On the other hand, alfalfa plants grown with Champ also showed a tendency towards lower relative growth compared to other grass cultivars (Fig. 4.3 I), 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). N-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' (Fig. 4.2, 3). Unlike Champ, however, these gains came largely from difference in yield, as Bastion was able to maintain relatively high levels of growth while maintaining similar improvements to N content (Fig. 4.3 III, IV). This may be due to the lower 'aggressiveness' of alfalfa, as compared to red clover, towards grasses reflected in the relative dry matter yield CI (Fig. 4.3 I, II).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 (Fig. 4.3, 4.4). These results are in agreement with several field studies that noted that greater soil N increased the amount of N transferred from legumes to grasses. 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. Pirhofer-Walzl et al. (2012), in a multi-species N transfer study, noted specific differences between the species used here, specifically that alfalfa doubled their transfer of N to grasses after fertilization, while red clover increased their transfer by less than half. In 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. Rate of growth of grasses therefore becomes an important factor in the 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.

Some differences in grass response to N and legume presence may be attributed to the different growth patterns and adaptations of the two species used. Typically, plants adapted to low- and high-fertility environments will differ in their N use efficiency (NUE) which can be broken down into the factors of N productivity (NP; high in high-fertility adapted plants) and mean residence time of N within the plant ('MRT'; high in low-fertility adapted plants; Berendse and Aerts, 1987). Ryegrass has been described as an archetypical high fertility adapted perennial grass (Wilkins 1991; Vasques de Aldana and Berendes 1997) with a less conservative growth habit than timothy (Jørgensen et al. 2010). In our experiment, however, NUE is unlikely to completely explain differences in grass growth, as mean N content (our best measure of NP) was mostly similar across grass cultivars in spite of large differences in DM yield production, while the short time-frame of the experiment made it unlikely that MRT played a major role. Instead, it seems that rapid growth of grasses was important to maximizing uptake of legume-derived N. Bastion, as an early-maturing tetraploid variety, would be most suited to this role while Feeder, a mid-season diploid, would be less so. It has been noted in previous studies that early-maturing varieties of ryegrass are more compatible with clover (Sanderson and Elwinger 1999).

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 4.2, Fig. 4.4), 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 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 to 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 important point: the role of competitiveness in the N transfer. While the probable factor responsible for reduced growth of plants in this experiment (pot space) 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 N

transfer can be limited by N-receiver size which means that excess exuded 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).

4.6 Conclusion

In this pot study of grass-legume combinations over a range of N fertility levels, perennial ryegrass and timothy cultivars accumulated less DM than alone, when paired with alfalfa and red clover cultivars, but showed higher tissue N content. Once particular cultivar of timothy ('Champ') had large improvements in total N when paired with alfalfa cultivars due to its low N uptake 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 may have traits that could increase the level of N transfer in early growth, and that net N transfer during this period may be limited as much by grass capacity as legume N exudation output.

Chapter 5: Literature Review: Why Lose Nitrogen? Plant Exudation of Low Molecular Weight Nitrogen

5.1 Abstract

Considerable attention has been paid recently to the characterization of low molecular weight (LMW) compounds in the exudates of plant roots and their role in rhizosphere processes. While the majority of the LMW fraction is composed of carbohydrates and organic acids, a major portion also consists of amino acids, ammonia and other nitrogen-rich compounds that are exported out of the root. As most terrestrial ecosystems are N-limited, the cost to an individual of losing this vital resource must be offset by benefits, but as yet these benefits are not well understood. This review outlines three major potential effects of LMW N exudates on plant-soil interactions, namely enhanced nutrient uptake, rhizosphere microbe selection and rhizosphere microbe stimulation. We consider the evidence for each and suggest future research to distinguish between the hypothesized benefits of N loss.

5.2 Introduction

The role of root exudates in plant-soil interactions has received considerable attention, revealing an increasing number of ways in which plants are able to influence biological and chemical interactions within the region of soil surrounding the roots known as the 'rhizosphere'. Many of the compounds exuded by roots are complex, high molecular weight (HMW) compounds with highly specific purposes, such as allelopathy (Bertin et al. 2003), or plant-microbe/plant-plant communication (Bais et al. 2006). A large fraction of root exudates, however, is made up of simpler low molecular weight (LMW) compounds, including sugars, organic acids/carboxylates and amino acids, which are

more likely to be metabolized quickly within the soil, and whose function can be more ambiguous. While the majority of this fraction is carbon, there are also significant amounts of nitrogen(N)-rich LMW compounds (amino acids, peptides, ammonia and ureides) present, which must be acquired by plants through root uptake or N-fixation, adding an additional metabolic 'cost' of up to 6 g of C for every gram of N acquired (Cannell and Thornley 2000). In N-limiting ecosystems, any loss of N for plants represents an opportunity cost for growth and thus competitive ability, making N lost via exudation more costly than carbohydrates and organic acids, in spite of the fact that they are lost in comparable amounts (Carvalhais et al. 2011). However, the ecological benefits of these compounds are perhaps the least well-understood of the LMW fraction of root exudates. If plants can control the release of these compounds, why lose nitrogen at all?

This review will explore the process of nitrogen exudation and consider three broad, and perhaps overlapping, potential benefits conferred to plants through the release of LMW N compounds: (1) improved nutrient uptake, (2) selection of microbes within the rhizosphere and (3) stimulation of rhizosphere microbial activity, illustrated in figure 1. It will then assess the evidence for each and propose future directions to help understand why LMW N loss via exudation occurs so widely in plants.

5.3 LMW N Exudation Mechanisms

Like many compounds traded at the root surface, LMW N compounds are actively moved into the plant via carrier proteins (Fisher et al. 2002; Ludewig et al. 2002; Segonzac et al. 2007) and lost, mainly passively, through transport channels (Taylor and Bloom 1998; Badri et al. 2009).

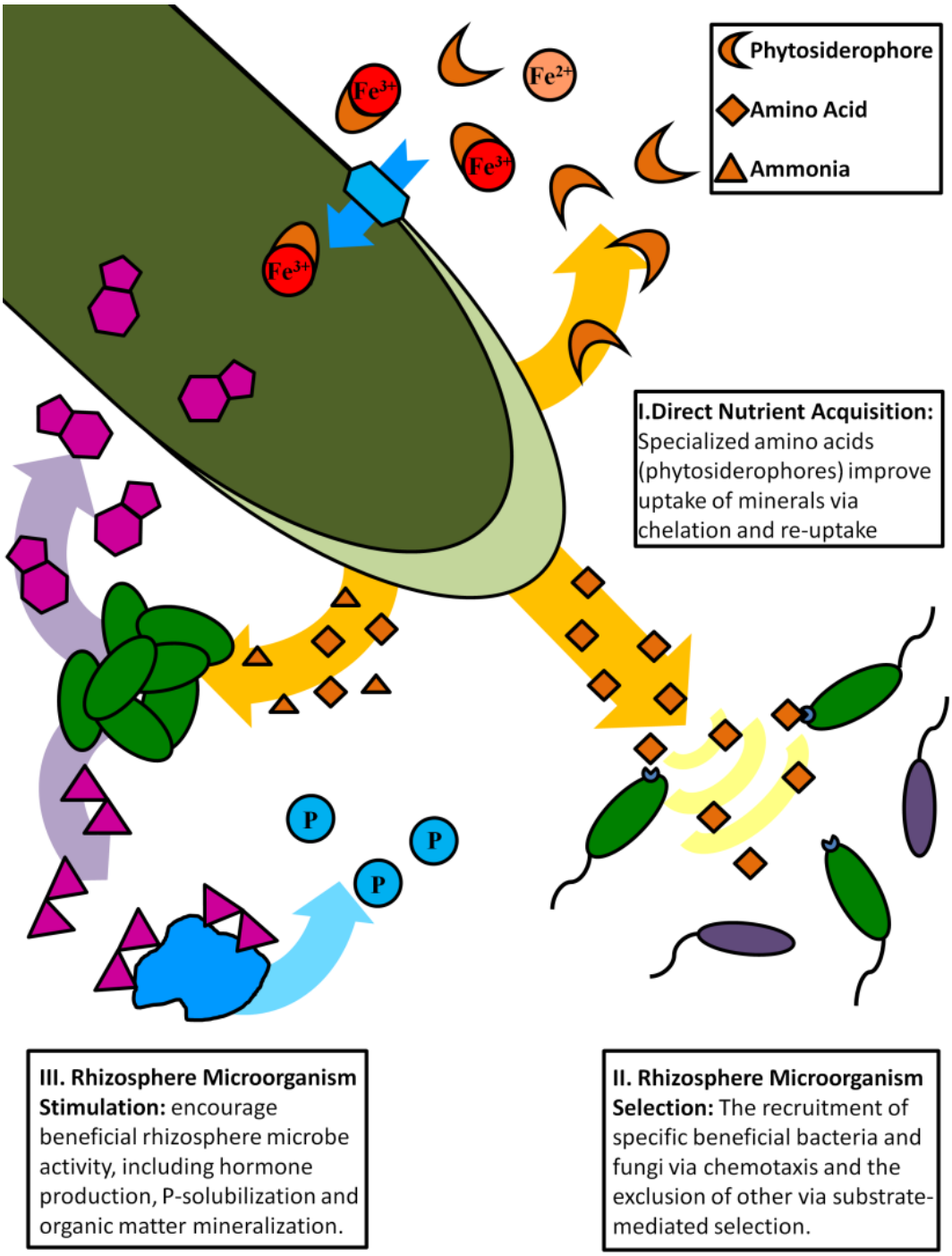

Figure 5.1: Schematic representation of three hypotheses on the major role of plant-exuded nitrogen

The difference between the rates of influx and efflux will determine the net movement of the compound in question across the root surface membrane, and has been shown to be under the control of the plant (Phillips et al. 2004). Although the process by which different LMW N compounds are released (or taken up) is similar, each form is derived from different metabolic processes. The organic fraction is thought to be derived from newly synthesized and recycled free amino acids and related compounds that make up the free amino-N pool cycled in the xylem and phloem (Cooper and Clarkson, 1989), while inorganic N ions are generally derived from recently acquired N that has not yet been incorporated into the organic pool, and thus is not a 'new' source of nitrogen in the soil. The exception is nitrogen-fixing plants such as legumes, which exude relatively high amounts of N into the soil as ammonium (Fustec et al. 2010) or ureides (Reynolds et al. 1982), as a result of N-fixation by symbiotic *Rhizobia* and related bacteria. Exudation by actinorhizal plants associated with N-fixing *Frankia* bacteria have not been studied as extensively, but as the process of fixation is similar (Huss-Daniel, 1997) it is likely that they exude ammonia in a similar fashion.

The exudation of LMW N compounds from the root is a dynamic process that can change rapidly in response to stimuli. Plants have been shown to modulate their LMW N exudation in response to a number of abiotic factors, but research has focused almost wholly on amino acids. For example, exudation of amino acids increases with higher light (Rovira 1959; Ofosu-Budu et al. 1992) and elevated CO_2 levels in a variety of plant species (Paterson et al. 2003; Phillips et al. 2006). Amino acid exudation has also been found to increase under conditions of macro- and micronutrient scarcity in sorghum (Graham 1981) and maize (Carvalhais et al. 2011). Interestingly, insufficient N does not

affect amino acid exudation in maize (Carvalhais et al. 2011). In fact, there is little evidence that LMW N exudation increases with N availability or supply, and net exudation of N including amino acids and ammonia has been found to be uncoupled from N root tissue levels and from the rate of N-fixation in white clover (Lesuffleur and Cliquet 2010).

On the biotic side, the presence of soil bacteria and fungi (Leyval and Berthelin 1993) or the compounds they produce (Phillips et al. 2004) have been shown to increase efflux of amino acids from a variety of plants. Herbivory, both of shoots (Ofosu-Budu et al. 1995; Paterson et al. 2003) and roots (Haase et al. 2007), has also been linked to increased amino acid exudation. Plant stage of development has also been shown to influence LMW N exudation patterns (Ofosu-Budu et al. 1990; Wichern et al. 2007 Chaparro et al. 2013) leading to speculation that exudation rate of specific compounds may be programmed into early plant development for rhizosphere promotion (Chaparro et al. 2013).

5.4 Challenge of Quantifying LMW N Exudation

Estimating N loss through exudation in a natural setting is challenging for a number of reasons. In both short-term and long-term studies, bacteria can rapidly absorb and mineralize a significant portion of exuded N compounds, causing an underestimate of total exudation (Fustec et al. 2010). In studies lasting more than a few days, N loss will include all components of rhizodeposition including not only exudates but also other labile organic material, including cell lysates, sloughed-off root cells and root hairs (Nguyen, 2003) and, depending on the rate of microbial mineraliztion, soon become

indistinguishable from the LMW N exuded by living roots (Cassman and Munns 1980). Nitrogen rhizodeposition has been estimated to account for 4-71% of total soil and plant N (Wichern et al. 2007), but the proportion of this total released from living cells as LMW compounds is not known. Controlled experiments conducted in hydroponic solutions or in inert growing media can give accurate measurements of influx, efflux and net movement of nitrogenous compounds, but do not replicate the chemical and biological conditions encountered by roots in the soil, leading to possible over- or underestimation of N loss in natural systems.

Jones and Darrah (1993) estimate that the amino acid and protein portion of root exudates is no more than 1-2% of the total carbon released, but this estimate is derived from a single species (*Zea mays*) under ideal conditions and may not represent typical root function in a dynamic environment. Klein et al. (1988), for instance, observed that the C:N ratio of exudates in temperate grasses ranged from 2.0-2.7, while more conservative model estimates range from 25-100 (Drake et al. 2013). Furthermore, under nutrient deficiency, exudation rates of amino acids are comparable to those of organic acids and sugars (Fan et al. 2001; El-baz et al. 2004; Carvalhais et al. 2011), implying that while N exudation might not be relatively high during normal plant function, it may be increased as a response to nutrient deficiencies or other stresses.

5.5 Potential Functions of LMW N Exudates

5.5.1 Nutrient acquisition

Nutrient acquisition is one of the root's primary functions, and the role of root exudates, and LMW exudates in particular, in enhancing nutrient uptake is well documented

(Neumann and Römheld 1999; Dakora and Phillips 2002). Because amino acids can form complexes with minerals required for plant growth, like phosphorus (P) and potassium (K), it has been proposed that they directly influence nutrient availability in the rhizosphere (Uren and Reisenaur 1988). The most well-studied are phytosiderophores, a class of non-proteinogenic amino acids that bind to and solubilise iron (Fe) and other micronutrient metals, such as manganese (Mn), zinc (Zn) and copper (Cu), making them available for uptake (Römheld and Marschner 1986).While these amino acids do contribute directly to plant mineral uptake, their use is limited to the Poaceae, which has evolved an efflux/influx system separate from that of the cellular transporters used for proteinogenic amino acids found more widely among plants (Dakora and Phillips 2002). In contrast, the role of proteinogenic amino acids in improving nutrient availability directly has been dismissed as largely inconsequential (Jones et al. 1994).

Conversely, the evidence for increased root exudation of both proteinogenic and non-proteinogenic amino acids under nutrient deficiencies has increased in recent years for grasses (Carvalhais et al. 2011; Rasouli-Sadaghiani et al. 2011; Tawaraya et al. 2013) and eudicots (Tawaraya et al. 2014). In a study of the exudation of LMW compounds in *Z. mays* under different nutrient deficiencies, Carvalhais et al. (2011) observed that exudation of amino acids, organic acids and carbohydrates increased under Fe- and P-deficient conditions but decreased under N- and K-deficient conditions. Because the trend was similar for all three classes of compound, the authors hypothesized that exudation served as a non-specific means of increasing the solubility of nutrients prior to the evolution of phytosiderophores in grasses and similar nutrient-acquisition strategies in eudicotelydons.

5.5.2 Rhizosphere microorganism selection

In spite of the fact that the rhizosphere contains much higher concentrations of bacteria than bulk soil, the diversity of such rhizobacteria is much lower, p ointing to strong selective forces (Matilla et al. 2007). High-molecular-weight antimicrobial compounds can exclude certain classes of microorganisms (Brigham et al. 1999; Bais et al. 2006), but simpler LMW compounds may serve to promote the recruitment of specific microbes (Dennis et al. 2010). LMW N compounds in particular may be important in two key aspects of rhizosphere recuitment: chemotaxis and selection by substrate.

Chemotaxis: Amino acids in root exudates have been associated with a chemotactic response in beneficial bacteria. Fluorescent Pseudomonads have been shown to be attracted to amino acids in root exudates (Kato et al. 1997; Weert et al. 2002; Badri et al. 2009). Oku *et al.* (2012) recently identified the surface sensory proteins that detect these amino acids, while Carvalhais *et al*. (2013) demonstrated that bacterial chemotaxis genes were downregulated in the presence of N-deprived maize. These beneficial rhizobacteria are considered to be critical for root development in many plant species, as they exclude many potential plant pathogens both by outcompeting them for nutrients (Kloepper et al. 1980; Fgaier and Eberl 2010) and by producing antimicrobials (Nóbrega et al. 2005). Exudates have also been found to attract endophytic bacteria, such as *Corynebacterium flavescens* and *Bacillus pumilus* in rice (Bacilia-Jiminez et al. 2004), and N-fixing *Rhizobium* and *Sinnorhizobium* in legumes (Götz et al. 1982; Miller et al. 2007; Webb et al. 2014). Despite these benefits, exudates might have associated risks because pathogens can exploit the same chemotactic signals to identify potential hosts (Yao and Allen 2006).

Selection by substrate: While much of the research on microbial selection within the rhizosphere focuses on carbon, labile N may also play a significant selective role. Rhizobacteria have been found to preferentially catabolize amino acids as a source of both C and N (Hoskisson et al. 2003; Hirsch and Valdes 2010), in contrast to most other soil bacteria, which favor ammonia (Geisseler et al. 2010). Dennis et al. (2010) argue that the importance of root exudates in shaping rhizosphere community structure is overstated, as the exudate components thought to be responsible for selecting microbes are released primarily at root apices, and not along older root sections where established rhizosphere communities exist. Nevertheless, rhizosphere microbe communities have been shown to vary significantly between root zones (Yang and Crowley 2000), so the fact that exuded LMW N might influence one community and not another does not necessarily diminish its importance.

5.5.3 Stimulation of rhizosphere activity

Root exudates might also function to stimulate rhizosphere microorganisms to perform functions beneficial to the host plant. This is especially true of LMW 'building block' compounds. Plants benefit from a variety of microbe-synthesized compounds in the rhizosphere, including plant growth hormones, antimicrobials and nutrient-uptake enhancers. In this way, export of carbon and nitrogen to the rhizosphere could be viewed as 'subcontracting' the work of producing certain compounds to beneficial microbes, collectively described as 'plant growth promoting rhizobacteria' (PGPR; Kloepper et al. 1980). Nitrogen has been shown to stimulate the production of hormones and nutrient-uptake enhancers in PGPR.

Hormones: Growth-promoting plant hormones are produced exogenously by a wide variety of rhizobacteria (Vessey 2003). A classic example of this plant-microbe interaction involves the amino acid tryptophan, which is used in the production of the auxin indole-3-acetic acid (IAA). Synthesized by an estimated 80% of rhizobacteria (Khalid et al. 2004) this phenomenon is part of the microbial loop in soil (Bonkowski, 2004), although there is still some question as to whether IAA production in the rhizosphere depends solely on plant-exuded tryptophan (Spaepen et al. 2009). Cytokinins are another class of phytohormone produced by rhizobacteria (de Garcïa Salamone et al. 2001) and their synthesis is stimulated by root exudates (Martinez-Toledo et al. 1988). While there is no direct evidence that plant-derived amino acids in particular are responsible for the production of these hormones, cytokinin-producing strains of *Bacillus subtilis* have the capacity to stimulate amino acid exudation, a trait not shared with their non-producing counterparts (Kudoyarov et al. 2014)

Nutrient Uptake Enhancers: Like many plants, rhizosphere microbes produce a variety of compounds that increase the bioavailability of minerals in the soil, including siderophores that chelate P (Vassilev et al. 2006) and Fe (Lemanceau et al. 2009). While it has been shown that mineral deficiencies will increase the exudation of most LMW compounds (Carvalhais et al. 2011), studies of fluorescent Pseudomonads under wheat determined that amino acids, but not carbohydrates or organic acids, stimulate the production of siderophores (Sayyed et al. 2005) increasing nutrient availability to both microbes and plants.

Evidence from rhizosphere models suggests that the relatively small amounts of N exuded into the soil can liberate much larger amounts of soil N available for plant uptake.

A model of nutrient flows in temperate forest soil by Drake et al. (2013) implies that a modest input of nitrogen via exudates is sufficient to stimulate the proliferation of soil bacteria and the production of exoenzymes used to mineralize soil organic matter (SOM), resulting in a net increase in soil nitrogen available for uptake by plants later on. This 'priming effect' has been described previously (Fontaine et al. 2003), but typically concentrates on the flow of labile carbon from plants to stimulate N mineralization. There is some debate as to whether low-N conditions ('microbial N-mining hypothesis'; Moorhead and Sinsabaugh 2006) or high-N conditions ('stoichiometric decomposition hypothesis'; Hessen et al. 2004) favour higher rates of plant-mediated organic matter decomposition, but it may be possible that N-addition favours the decomposition of certain types of organic matter substrate (Chen et al. 2014).

5.6 Future Directions

Finding a specific role for LMW N compounds in root exudates can be challenging, in large part because our understanding of rhizosphere function is limited. The identification of specific ways in which exudation of these compounds is controlled by the plant (Lesuffleur and Cliquet 2010; Carvalhais et al. 2011), coupled with knowledge of the importance of nitrogen to plant growth and function, strongly suggests that there is an important 'return' on this investment of lost N. The methodological challenges of observing rhizosphere functions *in vivo* hamper our ability to make a direct link between loss of N and plant benefit. Only a very small number of amino acids found in root exudates have been linked to specific functions (Vranova et al. 2011; Moe, 2013). However, many of the most commonly exuded amino acids (e.g., Gly, Ser; Lesuffleur et al. 2007), as well as other major LMW N compounds found in exudates (ammonia,

ureides), have no known function and seem to represent a more general export of labile N from the plant to the rhizosphere. From this, we can make some inferences about the function of this transfer.

Microbial respiration in the rhizosphere is often N-limited (Cheng et al. 1996; Phillips and Fahey 2006). Although it has been determined that N exuded by plants is probably insufficient to sustain the rhizosphere microbial community on its own (Simons et al. 1997), plant-controlled release of N is likely still an important determinant of rhizosphere metabolism, population size and composition. The selective exudation of specific amino acids might make this control even more precise, as bacterial species and strains will vary in their ability to utilize different classes of amino acids (Sonawane et al. 2003).

In this way, amino acid and other LMW N exudation can be seen as an important factor in the interaction of a plant's roots and its rhizosphere microbiome (Berendsen et al. 2012), by transferring a limiting nutrient to a select set of microbes that will use it not just for their own proliferation, but for functions beneficial to the plant as well. In a wider view, N exudation is not so much a transfer to the rhizosphere as a loan. Microbes exhibit a greater affinity than plants for most N sources, including amino acids (Harrison et al. 2007). Recent studies however, conclude that N dynamics in the rhizosphere ultimately favour plants, because N taken up by microbes is remineralized relatively quickly, making it available for plant re-uptake, and preventing it from being leached out of the root zone (Jones et al. 2013; Kuzyakov and Xu 2013). Furthermore, plant and microbe N-demand may vary over seasons, allowing for a temporal sharing of N resources (Kaštovská et al. 2015)

Periods of high LMW N exudation might offer insight into situations when controlled release of N via exudation can be beneficial. Observations of increased amino acid exudation under mineral deficiencies other than N (Carvalhais et al. 2011) present a good example of a situation when LMW N transfer to the rhizosphere may be most beneficial. If a plant is limited by nutrients other than N, it is best to allocate N that would be allocated to growth to root-associated microbes that are in a better position to improve access to the nutrients in demand. The observation that amino acid efflux varies over different developmental stages (Chaparro et al. 2013), suggests that the stimulation N-limited rhizosphere microbes may not only be in times of stress, but integrated into regular plant function.

In order to understand the role of LMW N in the rhizosphere the following questions must be addressed.

Where are the sites of LMW N exudation on the root? Since the important study of Jaeger et al. (1999) showing that the exudation of tryptophan occurred primarily at root tips in *Avena barbata*, little work has been done to determine either the sites of exudation or the utilization in the rhizosphere of other amino acids. If root exudation of all amino acids is confined to root tips, then we can assume that their function is primarily in selecting and stimulating the rhizosphere microbial community surrounding new roots (Dennis et al. 2010), and that microbial community in older root sections relies on decomposition for N, over which a plant has less control. If on the other hand significant exudation of certain amino acids, or other LMW N, occurs in other regions of the root, there may be cause to rethink plant-microbe interactions with respect to N in the rhizosphere. Mapping LMW N

loss under different conditions (e.g., nutrient deficiencies, defoliation, etc.) should therefore provide insight on this issue.

What is the role of non-amino acid exudation? Plants can release significant amounts of ammonia into the soil, providing an N-substrate that is the preferred form for most soil bacteria and much less selective than amino acids (Geisseler et al. 2010). In addition, sites of ammonia exudation are likely to be different from those of amino acids: around nodules, and in non-fixers, on older root sections, where nitrate is more readily absorbed and reduced (Taylor and Bloom 1998). Whether the exudation of ammonia is exclusively a response to its toxicity or serves other purposes, it represents a shift in the quality and quantity of N available in the rhizosphere, but so far its role in rhizosphere N availability has not been well studied. Investigations into the composition of microbiomes of nodulated and non-nodulated legumes could provide insight into the fate of nitrogen excreted from the *Rhizobium*-plant association, and what possible benefits this export could confer.

What role do LMW N exudates play in plant-plant interactions? While the majority of research into the role of root exudates in plant-plant interactions focuses on allelopathy (Bertin et al. 2003) and kin selection (Dudley and File 2007), there is solid evidence of direct plant-to-plant N-transfer via exudation as well (Paynel et al. 2001; Paynel et al. 2008). From a plant ecology standpoint, exporting a growth-limiting nutrient to a potential competitor does not seem to carry any clear advantage. While many instances of N-transfer have been noted in the field (Ta and Faris 1987; Høgh-Jensen and Schjoerring 1997; Thilakarathna et al. 2012), it is difficult to ascertain which pathway (root exudates or root turnover) is responsible for this transfer, but it would be assumed that exudation is

under tighter control than turnover. Microcosm experiments mimicking natural conditions may shed light on the importance of root exudation is in the plant-plant nitrogen transfers.

How do plant populations differ in their LMW N exudation? Perhaps the most promising avenue for understanding the role of LMW N exudation is the examination of nitrogen quantity and composition in the exudates of contrasting plant populations. With recent work demonstrating that the rhizosphere microbiomes of plants are heritable (Bouffaud et al. 2012; Peiffer et al. 2013) and that plant genotypes differ in their amino acid exudation (Rasouli-Sadaghiani et al. 2011), there exists the potential to relate how LMW N exudation composition correlates with rhizosphere microbe community composition and activity within plant species. Current work on the exudation patterns in agronomic species has already yielded some promising results, linking differences in amino acid exudation between cultivars with resistance to disease (Pan and Wu 2007; Li et al. 2009) and increased nutrient uptake (Mozafar et al. 1992; Rasouli-Sadaghiani et al. 2011), and making a case for the role of LMW N release as part of a microbially-mediated response to many different root-borne stresses. Work on N-fixing legumes has demonstrated similar differences in the quantity of ammonia in the exudates of different cultivars of red clover (*Trifolium pratense*; Thilakarathna et al. 2012), meaning that there is a significant genetic component to non-organic N release as well. Further research into contrasting the LMW N profile of cultivars differing in nutrient uptake, disease resistance and tolerance to adverse soil conditions (heavy metals, salinity, etc.) could contribute greatly to our understanding of how plants use nitrogen in their exudates to interact with their root microbiota.

5.7 Conclusion

Plant exudates contain a variety of LMW N compounds in both organic and inorganic forms that have been linked to a diversity of functions, including improved nutrient uptake, selection of root-associated microbes and the stimulation of rhizosphere microbial activity. While the export of this resource still likely represents a metabolic cost to the plant, the selective exudation of LMW N in situations when it is advantageous or in forms that most benefit plant growth or function likely offsets its loss. Further research on sites of exudation, the role of non-amino acid LMW N, effects on plant-plant interactions and the exudation profiles of plant genotypes and populations may help us better understand the role of this apparently universal plant process.

Chapter 6: Conclusion

This dissertation sought to identify potential genotypic variation in three key processes involved in direct N transfer: nodulation (and by extension, fixation) of legumes, the release of soluble N in the exudates of those legumes, and the uptake of N by grasses paired with them.

With respect to nodulation (Chapter 2), we were able to identify differences between our legume species in nodulation rate, and in their response in nodulation to additions of N. Alfalfa maintained a steady low level of nodulation over most N fertilization levels, while red clover nodulated much higher on average, but was more sensitive to N additions. These results are in line with previous studies comparing these species (Sherer and Lange 1996; Staley and Belesky 2004). Results of this study, coupled with other research (Chmelíková et al. 2015a; Chmelíková et al. 2015b) suggest that root morphology may play a role in this species difference, as plant with higher levels of root branching (i.e. red clover) tend to nodulate more than those with more prominent tap roots (aflafa), but more research would have to be done to confirm this trend. Within the context of this dissertation, results suggest that alfalfa, while not as prolific a nodulater as red clover, may offer certain advantages in that it is less sensitive to changes in external N, and could thus be a source of biologically-fixed N over a wide range of conditions.Exudation of soluble LMW N, the route by which direct N transfer occurs, has been shown to vary across both species (eg: Grayston et al. 1996; Fan et al. 2001; Klein et al. 1988; Ofusu-budsu et al. 1992) and cultivars (El-baz et al. 2004; Pan and Wu 2007; Huang et al. 2011; Thilikarathna 2013), While most of the N measured in the study was high molecular weight (HMW) organic N that is presumably unavailable for direct uptake by

neighbouring plants, differences in ammonium exudation rate between and within legume populations were found, with red clover exuding more N than alfalfa, and cv. AC Christie exuding more than cv.Tempus. As a form of N that is readily taken up by cultivated grasses (Weigelt et al. 2005), exudates ammonium content could be an important factor in determining the efficiency of N-transfer between legumes and grasses. It was noted that the cultivar with the highest N exudation rate (AC Christie) was also the plant with the highest mean specific nodulation rate. Given that ammonium production occurs in nodules, a high nodule to biomass ratio could be important trait for ammonium exudation, but further research with more cultivars would be necessary to confirm this.

As for the transfer of N between cultivars of grasses and legumes over different N levels observed in Chapter 4, results were not as straightforward as were predicted. It was assumed that grasses at the most deprived of mineral N would benefit the most from the presence of N-exuding legumes, and that the least benefit would be conferred at the highest level. While grass N content was consistently improved by the presence of legumes, regardless of cultivar/species, grass total N tended to be maximized at the highest N fertilization levels and seemed to be mostly a function of improved plant biomass rather than N content, and was most apparent when the more aggressiveearly-growing grass cultivars (cv. Bastion, perennial ryegrass) were planted with the least aggressive legumes (alfalfa). Even in the low-density potting design employed, it appears that plant competition may have played an important role in deciding the efficiency of N transfer among these grass-legume pairings. Futhermore, it suggests that the demand for N (i.e. uptake by grasses) may be just as critical as the supply (i.e. exudation by legumes) in N transfer. Our results also suggest that both legume and grass identity can have a role

in regulating grass demand by growth rate (grass) and competition (legume), and that the amount of N available can alter the competitive blance between the two species.

Overall, this dissertation provides evidence of some degree of genotypic variation affecting three components of N transfer investigated which could provide plant breeders avenues for improving an important aspect of forage production. Further research in two key areas could improve this even further. First, relationship between legume specific nodulation and ammonia exudation rate warrants further investigation, as it may be one of the more important aspects of the 'supply' side of N transfer. The association between high ratio of potential N production (i.e. high nodulation rate) to plant N demand (i.e. biomass) with N excretion may seem intuitive, but lacks more definitive proof in the scientific literature. If it could be demonstrated across different legume species, it could provide new applications for a trait already well understood. Second, the understanding of competition between legumes and grasses has been well covered on a small-scale basis in the ecology literature (e.g. Turkington and Harper 1979; Aarssen et al. 1985), but is often ignored agricultural studies, where measurements at the scale of hectares is more common. Chapter 4 demonstrates that even when resources are supplied in abundance, plant-plant competition may play an important role in N transfer efficiency. As the 'demand' portion of the N-transfer relationship, net N uptake by a grass may depend on its size, which will depend in part by competition from neighbouring legumes. Thus, compatibility in growth of species and cultivars in growth will have important consequences on the efficiency of N transfer, and this provides another avenue for further research when finding suitable cultivar pairs for forage production.

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Appendix I: Sample HPLC Chromatographs (Chapter 3)

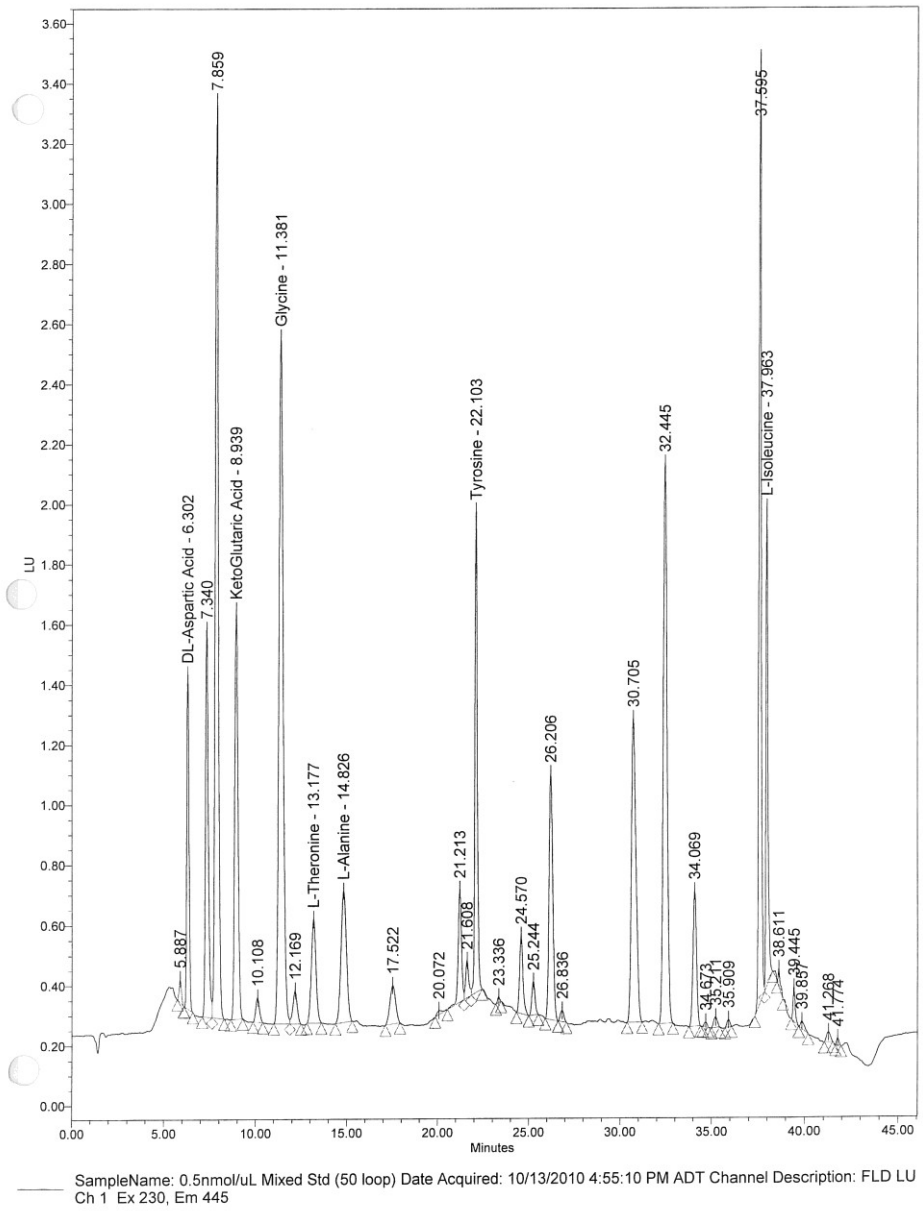

Fig AI.1: Complete amino acid standard chromatograph (no dilution)

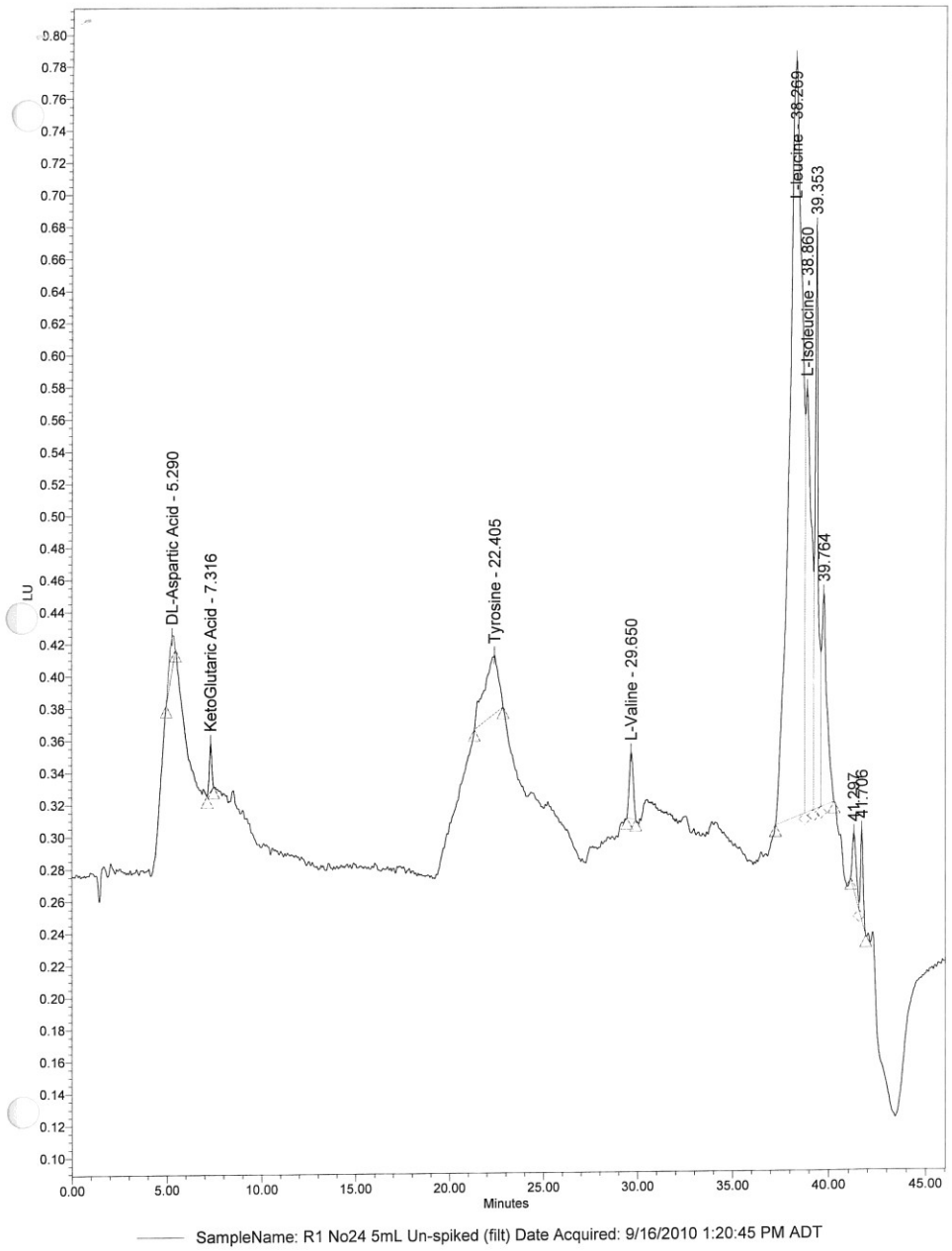

Fig AI.2: Sample leachate amino acid chromatograph (red clover cv 'Tempus')

Appendix II: Mean square tables for ANOVA analyses

Table II.i ANOVA mean squares for nodulation analysis (Table 2.1, 2.2)

Source	d.f.	Yield	R:S ratio	leaf area	Shoot N	Root N	Total N	Active Nod.	Spec. Nod.
Tray stratum	1	12377	0.013	12.18	1.78	2.95	0.62	0.628	0.500
Nit	3	23336	0.102	699.80	1.08	1.09	5.40	12.960	48.400
Residual	11	19792	0.108	168.60	0.48	1.35	21.07	0.346	2.452
Cultivar	3	19579	1.626	1117.00	0.13	0.17	23.62	9.621	88.290
.. Af vs Rc	1	18428	4.825	3314.00	0.10	0.15	14.35	24.940	251.70 0
.. Apica vs CRS1001	1	40257	0.038	2.30	0.10	0.01	50.20	0.008	2.108
.. Tempus vs Christie	1	52.53	0.013	35.49	0.17	0.35	6.32	3.918	11.050
Residual	3	10312	0.076	94.28	0.07	0.08	2.48	1.088	9.995
Nitrogen X Cultivar	9	9774	0.097	129.80	0.25	0.28	9.55	2.327	10.550
Residual	32	12756	0.061	37.79	0.16	0.11	9.62	0.903	4.239
Total	62								

Table II.ii ANOVA mean squares for exudation analysis (exudates components;Table 3.1)

term	d.f.	TN	Am	AA	DON	Sp. TN	Sp. Am	Sp. AA	Sp. DON
Cultivar	3	678	14.1	0.00017	487	4988	112.29	0.0009	3695
...Af vs RC	1	685	14.68	0.00053	447	6543	122.88	0.0002	4872
...within Af	1	712	0.05	0.00002	617	1856	0.38	0.0000	1803
...within RC	1	634	27.45	0.00001	398	6565	213.62	0.0004	4411
residual	9	229	3.29	0.00003	190	1305	22.32	0.0001	1070
total	15								

Table II.iii ANOVA mean squares for exudation analysis (Plant measurements; Table 3.3)

Term	d.f.	DM Yield	RSR	Shoot	Root	N Content	Total N	Total Nod	Sp. Nod
Cultivar	3	32559	0.079	11499	9175	0.011	12.85	231.7	5479
...Af vs RC	1	53361	0.166	31329	2916	0.014	27.06	125.6	104
...within Af	1	1830	0.003	648	300	0.009	0.05	40.5	405
...within RC	1	42486	0.067	2520	24310	0.011	11.42	528.1	15927
Residual	9	6714	0.038	2987	2527	0.001	2.14	88.1	1280
Total	15								

Table II.iv ANOVA mean square values for nitrogen transfer analysis (grass growth measurements; Table 4.1)

Term	d.f	gr. DM yield	gr DM CI	gr. N con.	gr NC CI	gr. TN	gr TN CI
Block	3	142209	0.389	0.999	2.41	1.64	1.805
Nitrogen level	2	256724	0.083	0.277	1.90	19.53	1.491
Residual	6	20464	1.132	0.071	1.19	1.57	5.512
Legume	3	6485	0.046	0.005	0.06	0.52	0.024
.. Af vs Rc	1	11915	0.021	0.000	0.05	0.92	0.041
Nit.Legume	6	7722	0.098	0.017	0.03	0.34	0.447
Residual	27	4900	0.089	0.014	0.04	0.42	0.242
Grass	3	113653	0.095	0.052	3.84	4.94	5.689
.. Tm vs Prg	1	301584	0.004	0.014	3.18	13.58	2.597
Nit.Grass	6	4492	0.662	0.008	1.86	0.23	3.102
Residual	27	4644	0.345	0.021	1.42	0.25	3.443
Legume.Grass	9	3682	0.062	0.015	0.03	0.27	0.234
Nit.Legume.Grass	18	3310	0.090	0.009	0.03	0.21	0.278
Residual	77	1783	0.047	0.014	0.03	0.14	0.057
Total	205						

Table II.v ANOVA mean square values for nitrogen transfer analysis (legume growth measurements; Table 4.2)

Term	d.f	leg. DM yield	leg. DM CI	leg. N con.	leg. NC CI	leg. Total N	leg. TN CI
Rep stratum	3	230447	1.357	0.769	0.335	107.20	3.504
Nitrogen	2	214880	2.571	0.258	0.008	125.10	3.787
Residual	6	34448	1.176	0.119	0.152	22.82	2.070
Legume	3	167091	1.531	0.094	0.068	78.19	1.586
.. Af vs Rc	1	138521	3.137	0.277	0.012	54.74	3.536
.. Deviations	2	181375	0.728	0.002	0.096	89.91	0.611
Nit.Legume	6	14045	1.111	0.095	0.059	3.94	1.479
Residual	27	36893	1.030	0.098	0.116	16.44	1.459
Grass	3	10994	0.084	0.162	0.033	6.19	0.025
.. Tm vs Prg	1	32060	0.216	0.206	0.038	10.76	0.071
.. Deviations	2	460.6	0.019	0.140	0.031	3.90	0.002
Nit.Grass	6	46957	0.362	0.270	0.059	28.26	0.758
Residual	27	23127	0.192	0.114	0.026	10.35	0.263
Legume.Grass	9	22413	0.153	0.128	0.022	12.09	0.231
Nit.Legume.Grass	18	22087	0.188	0.111	0.019	8.95	0.357
.. Deviations	12	30147	0.254	0.112	0.022	11.00	0.515
Residual	77	18431	0.150	0.027	0.011	6.26	0.143
Total	205						

Table II.vi ANOVA mean square values for nitrogen transfer analysis (grass/ legume morphology measurements; Table 4.3)

Term	d.f	Gr RSR	Leg RSR	Gr. TLA	Leg TLA	Gr. TRL	Leg TRL	Leg SNOD
Block	3	1.342	0.527	198.50	2261.00	26407	823863	264572
Nitrogen	2	6.454	0.161	551.30	1129.00	113648	1035525	76524
Residual	6	1.476	0.085	34.09	323.20	16163	470657	96865
Legume	3	0.252	0.783	19.54	1681.00	6335	1374009	429850
.. Af vs Rc	1	0.159	2.339	4.03	3260.00	2571	2716108	161594
Nit.Legume	6	0.728	0.233	15.00	123.70	7339	89733	25045
Residual	27	0.699	0.121	5.49	351.90	5391	428867	58096
Grass	3	0.607	0.016	4.43	36.18	66647	186256	4949
.. Tm vs Prg	1	0.031	0.044	1.73	48.90	172387	330913	1686
Nit.Grass	6	0.323	0.126	4.05	474.50	3088	451668	9396
Residual	27	0.603	0.126	11.57	102.00	11863	125594	20910
Legume.Grass	9	0.442	0.056	5.64	140.90	6339	182118	21174
Nit.Legume.Grass	18	0.505	0.115	9.13	132.50	6187	83889	27464
Residual	77	0.342	0.044	5.07	45.85	2897	45278	10139
Total	205							