Genotypic Variability among Diverse Red clover Cultivars for Nitrogen Fixation and Transfer

by

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I would like to dedicate my thesis to my loving

parents, wife and daughter.

Thank you for always being there for me, supporting me and encouraging me.

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Abstract

Legumes fix atmospheric nitrogen (N) via symbiotic biological N fixation where part of the N fixed by legumes can be transferred to non-legumes. Identification of genotypic variability for N transfer among different legume cultivars enables improving N transfer to non-legumes under mixed stands.

Six diverse red clover (RC) cultivars which include three diploid (AC Christie, Tapani and CRS15) and three tetraploid (Tempus, CRS18, CRS39) were selected to evaluate genotypic variability for N transfer. The above RC cultivars were characterized for root hair deformation, nodulation, growth, and N uptake under different levels of N supply during the growing period and for starter N supply under *in vitro* conditions. Significant genotypic differences among the RC cultivars were found for the above attributes where the cultivars responded differently to N applications during early growth.

The above RC cultivars were also evaluated for root exudate N content in the form of NO_3^--N, NH_4^+-N and dissolved organic N (DON) during early growth under *in vitro* conditions. Significant genotypic differences were found for root exudate inorganic and organic N content. In general, root exudate DON content was greater than the inorganic N content and positively correlated with average nodule dry weight and shoot N concentration. The NH_4^+-N and NO_3^--N content in root exudates were positively correlated with active nodule number and root growth parameters respectively.

Nitrogen fixation, N transfer ability and soil N profiles of the above six RC cultivars were evaluated with bluegrass under field conditions for two post establishment years. Significant genotypic differences were found for N fixation and transfer but, these attributes were not associated with the ploidy nature of the selected RC cultivars. Generally, N transfer increased as the season and production year advanced. Soil mineral N and potential N leaching were affected differently by the RC cultivars included in this study under mixed stands, thus showing genotypic differences for soil N cycling. The results of investigations in this thesis highlight the dynamics of N flow between legumes and companion grasses and may assist in developing management protocols and plant breeding strategies to identify genotypes with efficient N cycling profiles.

List of Abbreviations and Symbols Used

AAFC	Agriculture and Agri-Food Canada
ABC	ATP-Binding Cassette
ACC	AC Christie
AM	Arbuscular Mycorrhizae
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
Bg	Pure Bluegrass
BNF	Biological Nitrogen Fixation
C	Carbon
c	Cut
C15	CRS 15
C18	CRS 18
C39	CRS 39
CEC	Cation Exchange Capacity
Cv	Cultivar
d	Days
Dip	Diploid
DM	Dry Matter
DMY	Dry Matter Yield
DON	Dissolved Organic Nitrogen
EM	Ectomycorrhizae
F-prob	F-probability
GOGAT	Oxoglutarate Aminotransferase
GS	Glutamine Synthetase
HPLC	High-Performance Liquid Chromatography
K_2O	Potassium Oxide
KCl	Potassium chloride
lin	Linear

MS	Murashige and Skoog
Mono	Monoculture
N	Nitrogen
n	Number of Replicates
N_2O	Nitrous Oxide
Ndfa	Nitrogen Derived from Atmosphere
NH_3	Ammonia
NH_4^+	Ammonium
NH_4NO_3	Ammonium Nitrate
NIN	Nodule Inception
NO_3^-	Nitrate
Nod	Nodulation
ns	Not Significant
OD	Optical Density
OM	Organic Matter
P_2O_5	Diphosphorus Pentoxide
PBM	Peribacteroid Membrane
PC	Principal Component
PCA	Principal Component Analysis
Ploid	Ploidy
Quad	Quadratic
RC	Red clover
SEM	Standard Error of the Mean
Tap	Tapani
TDN	Total Dissolved Nitrogen
Tem	Tempus
Tetra	Tetraploid
Y	Production Year
wk	Week

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Chapter 1

Introduction

1.1 Studying the Nitrogen Transfer from Legumes to Non-legumes

Effective management of nitrogen (N) in agricultural cropping systems is becoming an important strategy throughout the world. Currently the majority of world crop production systems depend on cultivars developed for high yield under high input systems (Fees et al. 2011). However, increasing interest in low-input sustainable agriculture and the concerns about possible environmental pollution associated with synthetic N fertilizer usage especially ground water pollution by NO_3^- leaching, N_2O emission and soil acidification (Camargo and Alonso 2006; Davidson 2009) have led to a greater interest in using legumes as a source of biological nitrogen fixation (BNF). Legumes maintain symbiotic association with N fixing rhizobia, supplying N input to the ecosystem and thereby becoming a key factor in increasing sustainability of agricultural systems. Annually 50-70 Tg of N can be derived from BNF in agricultural systems (Herridge et al. 2008).

Part of the legumes' fixed N can be conveyed to non-legumes by means of N transfer (Fustec et al. 2010). Nitrogen transfer is the movement of N from one plant to another under mixed stand of plant community, predominantly from legumes to non-legumes during the growth of the legume plant (Brophy et al. 1987). The major mechanisms involved in belowground N transfer are N release through decomposition of belowground legume tissues and uptake by neighboring plants (Wichern et al. 2008; Fustec et al. 2010), plant root exudates (Paynel et al. 2008; Jalonen et al. 2009a, b) and mycorrhizal mediated transfer (Haystead et al. 1988; Høgh-Jensen 2006). With the increased focus for environmentally friendly sustainable agriculture, more attention has been paid to belowground N transfer during the last few decades.

Pasture and fodder legumes contribute 12-25 Tg N annually through symbiotic BNF (Herridge et al. 2008). Therefore research into the dynamics of N flow between legumes and companion grasses may provide the needed information to develop management and plant breeding strategies for designing efficient cycling of N, reduce losses of this

essential nutrient and improve profitability of forage-based production systems. Red clover is one of the most important forage legumes grown in northern latitudes (Taylor and Quesenberry 1996; Martin et al. 1999), where it is commonly grown as a mixed stand with grasses. Red clover is a good candidate for N fixation among forage legumes (Warembourg et al. 1997) where it has the ability to transfer fixed N to neighboring grass as well (Pirhofer-Walzl et al. 2012; Thilakarathna et al. 2012b). Therefore, part of the N demand of the grass can be met via the clover N transfer.

Quantification and identification of genotypes with improoved N transfer to associated grasses may become an attainable challenge for future agriculture sector. On the other hand, part of the N released by red clover can be lost from the system especially through nitrate leaching (MacPherson 2010). Identification of red clover cultivars with high N transfer capacity while minimizing N leaching is important to increase the N use efficiency within agricultural systems. Therefore it is important to study the genotypic variability among red clover cultivars for N transfer along with N leaching and soil N cycling under field conditions to understand the broad picture.

Among the three major mechanisms, N containing legume root exudates plays a key role in transferring N to non-legumes (Fustec et al. 2010). Under field conditions it is difficult to measure the N transfer from root exudates directly. Therefore *In vitro* studies with controlled environmental conditions need to be employed to study root exudates profiles of the different red clover cultivars for N. This also enables measurement of nodulation, root and shoot morphological characters of different red clover cultivars which can be used to understand the interrelationship between N transfer and morphological traits.

Different red clover cultivars including diploid and tetraploid have been bred and commonly used in legume-based forage mixtures in temperate regions, but information on the N transfer to associated grasses is lacking. Since each cultivar has a unique genetic makeup, N transfer can vary among the different red clover cultivars. The current research study mainly focused on evaluating the genotypic variability among diverse red clover cultivars for N transfer during the growing season. *In vitro* assays and field studies were used to understand the genetic variability among diverse red clover cultivars for N transfer and how it affects on N cycling under field conditions.

1.2 Research Hypothesis

There is genotypic variability among different red clover cultivars for nitrogen transferring capacity during growth.

1.3 Research Objectives

The main objective of this research was to investigate the genotypic variability among adapted red clover cultivars for N transfer to companion bluegrass.

The specific objectives of this research were to:

1. Evaluate the genotypic variability of nitrogen containing root exudates during early stages of the red clover growth.
2. Characterize nitrogen transfer from diverse red clover cultivars to companion bluegrass during the growing season under field conditions.
3. Investigate the genotypic variability of long-term nitrogen transfer from diverse red clover cultivars to companion bluegrass under field condition.
4. Estimate the genotypic variability of potential nitrogen leaching and impact on soil mineral nitrogen of different red clover cultivars in perennial forage stands.

1.4 Research Approach

To understand how different red clover cultivars vary in terms of N transfer, six diverse red clover cultivars were selected for my PhD study which include three diploid (AC Christie, Tapani and CRS 15) and three tetraploid (Tempus, CRS 18 and CRS 39) cultivars. Specific detail description of each cultivar is listed in the Table 1.1.

Table 1.1 Genetic population source of the selected six red clover cultivars.

Ploidy	Cultivar or experimental population	Description
Diploid	AC Christie	Recommended in Atlantic Canada (Martin et al. 1999). Early flowering, very winter hardy and no pubescence on stems. Produced more forage than the check cultivars in the second and third harvest years in Atlantic Canada.
	Tapani	New cultivar developed for Atlantic Canada (Papadopoulos et al. 2008). Early flowering, winter hardy and superior re-growth potential with high second-cut herbage yield. Produced more forage than the check cultivars over three production years in Atlantic Canada.
	CRS 15	Experimental synthetic; parent population selected in growth pouches for high nitrogenase activity from the cultivar 'Arlington' (Papadopoulos, unpublished data)
Tetraploid	Tempus,	Check Cultivar; recommended in Atlantic Canada-known to be high yielding with good persistence.
	CRS 18	Experimental synthetic; parent population selected for vigor following three winters from the cultivar 'Hungaropoli'. Selected plants were then subjected to two cycles of recurrent selection for superior dry weight, plant height, relative maturity, number of stems per plant and seed yield under simulated high density sward conditions. Superior plants from the above recurrent selection were intercrossed and the new experimental line demonstrated superior persistence and vigor under field conditions (Papadopoulos, unpublished data).
	CRS 39	Experimental synthetic; parental population selected from 1997 regional trials for vigor (Roots and top growth) and persistence following three winters post establishment (Papadopoulos, unpublished data).

Two preliminary studies were conducted to characterize the selected red clover cultivars for nodulation, growth and plant growth response to available N where the results of these studies aided to explain the findings of the major N transfer experiments in the study (Figure 1.1). The first preliminary study was conducted to characterize the selected six red clover cultivars for nodulation, growth, and nitrogen uptake under

different levels of N fertilization during the growth period using a growth chamber trial (Chapter 2). The second preliminary *In vitro* study was initiated to evaluate root hair deformation of the six red clover cultivars and subsequent nodulation in two red clover cultivars representing diploid and tetraploid under different additions of starter nitrogen (Chapter 3).

A detailed *In vitro* study was conducted using six diverse red clover cultivars to fulfill the specific objective 1 (Chapter 4). The first field experiment was conducted with two diverse red clover cultivars to achieve the specific objective 2 (Chapter 5). The second and third specific objectives were achieved through the second and third field experiments (Chapter 6).

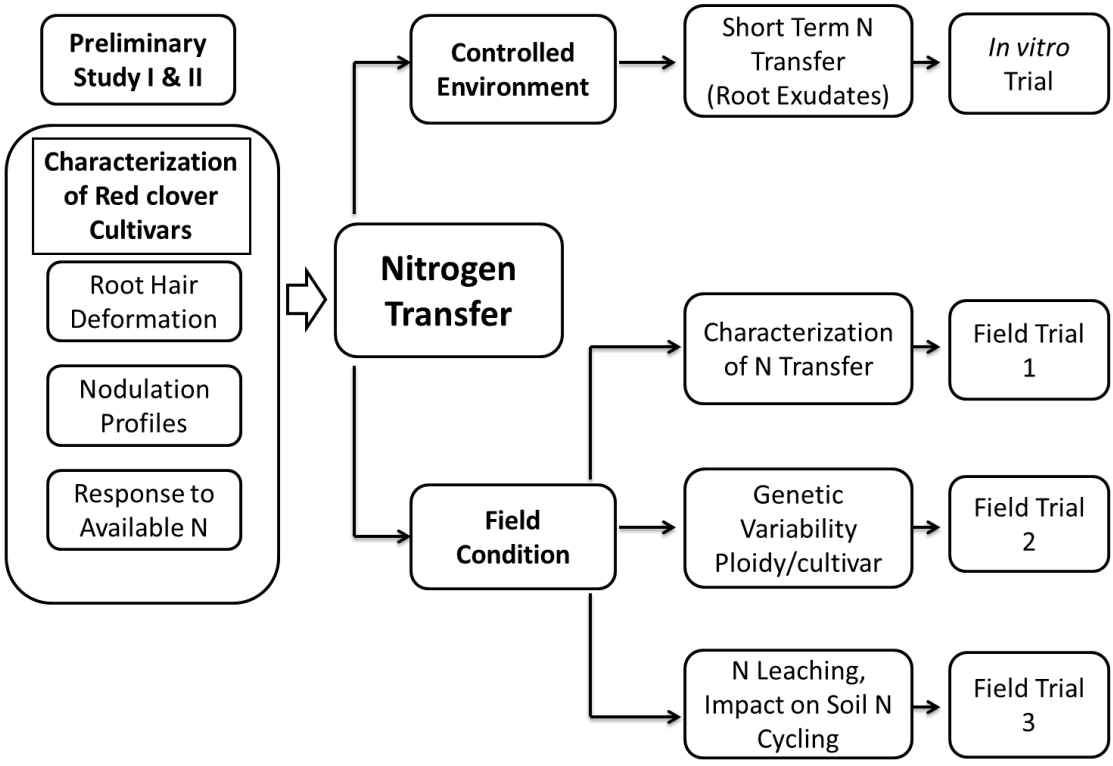

Figure 1.1 A schematic diagram of the overall research approach.

1.5 Red Clover

Red clover (*Trifolium pratense* L.) belongs to family fabaceae, which is one of the few agriculturally important species in the genus Trifolium (Taylor and Quesenberry 1996). Red clover is cross-pollinated and diploid with 14 chromosomes in the nuclei of their somatic cells. Tetraploid cultivars have also been artificially produced by chemical means. Red clover requires long days to initiate stems and flowers and does not have a vernalization requirement. Shade tolerance, superior light interception and N fixation capability allowed red clover to compete successfully with grasses (Taylor and Quesenberry 1996). Red clover usually lives 3 to 4 years in most clover growing regions, but generally conceded to be lacking persistence (Christie and Martin 1999; Montardo et al. 2003). Adequate time must be allowed after the last defoliation in the fall to facilitate ample accumulation of root carbohydrate storage reserves (Taylor and Quesenberry 1996).

Red clover is a productive legume species which is grown as a forage crop throughout most temperate regions of the world (Taylor and Quesenberry 1996; Leto et al. 2004). It is also commonly used in Atlantic Canada in crop rotations and the livestock industry for grazing, hay and silage production (Martin et al. 1999). Incorporation of red clover in the diet of the dairy cows increases animal intake and milk production (Moorby et al. 2009). Average red clover yield under maritime conditions is around 8 t ha^{-1} yr^{-1} under pure stand (Martin et al. 1999; Papadopoulos et al. 2008). The red clover yield and quality varies due to climate characteristics, ploidity level, cultivars and growth pattern (Leto et al. 2004; Drobná and Jančovič 2006). Different factors affect BNF in red clover, mainly it correlates with seasonal variations in clover biomass (Nesheim and Øyen 1994). Red clover often obtains more than 90% of its N from BNF (Thilakarathna et al. 2012b). Average total N fixation in grazed permanent clover/grass pasture in temperate regions of the world is approximately 80-100 kg N $ha^{-1}year^{-1}$ (Ledgard 2001). Part of the N fixed by red clover also transfers to neighboring grass under legume-grass mixed stands (Pirhofer-Walzl et al. 2012; Thilakarathna et al. 2012b) which highlight it is an important forage legume crop to study N transfer in depth. Different red clover cultivars are commonly used in legume based pasture mixtures in North America but their genetic variability for N transfer have not bveen understood in details.

1.6 Background - Biological Nitrogen Fixation (BNF)

1.6.1 What is Biological Nitrogen Fixation?

Nitrogen is one of the major macronutrients needed by plants to complete their growth and development. Although 78% of the atmosphere of the earth consists of N_2 gas, it is the most limiting nutrient available in the soil for plants (Valentine et al. 2010), because of the strong triple bond between the two N atoms. However, some prokaryotes are able to fix the atmospheric N_2 (diazotrophs) with the presence of the enzyme complex called nitrogenase where they reduced atmospheric N_2 into ammonia (NH_3) (Halbleib and Ludden 2000). Nitrogenase is composed of two parts; the iron (Fe) protein (dinitrogenase reductase) and the molybdenum-iron (MoFe) protein (dinitrogenase) (Dixon and Kahn 2004; Seefeldt et al. 2009). Biological N fixation is an energy intensive process where more reducing power and high energy adenosine triphosphate (ATP) are required than N assimilation through roots (Halbleib and Ludden 2000). Stoichiometry of BNF under optimum conditions can be described as;

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

(Dixon and Kahn 2004).

1.6.2 Biological Nitrogen Fixation Systems

Nitrogen fixing systems can be mainly categorized as free-living (non-symbiotic) and symbiotic BNF (Reed et al. 2011). Common free-living N fixing bacteria include cyanobacteria (*Anabaena* and *Nostoc*), *Azotobacter, Acetobacter, Azospirillum, Beijerinckia,* and *Clostridium*. The common and most studied plant associated N fixing symbiotic relationship is the legume-rhizobia symbiosis. In addition to legume plants, the only woody plant that can form a symbiotic relationship with rhizobia is the *Parasponia* sp. (Pawlowski and Bisseling 1996). Actinorhizal plants form endosymbiotic associations with N-fixing *Frankia* (actinomycete), where they form root nodules and fixed atmospheric N_2 (Franche et al. 2009; Santi et al. 2013). Other N fixing associations are cyanobacteria, endophytic diazotrophs and rhizospheric diazotrophs (Franche et al. 2009). Cyanobacteria can develop symbiotic relationships with different groups of organisms which include plants, fungi, animals and eukaryotic algae (Adams and Duggan

2008). Among all the N fixing symbiotic systems, legume-rhizobia symbiotic BNF plays a very important role in agricultural cropping systems in terms of N input (Bohlool et al. 1992; Herridge et al. 2008).

1.6.3 Legume-rhizobia Symbiotic Biological Nitrogen Fixation

1.6.3.1 Signal Exchange and Nodulation

Establishment of the symbiotic relationship between the legume and rhizobia starts with the signal exchange (cross talk) between both rhizobia and host plant (Mylona et al. 1995; Broughton et al. 2000). First a legume-to-rhizobia signal exchange takes place where legume plants release plant specific flavonoids through their root system (Zhang et al. 2008). Flavonoids bind to the rhizobia NodD protein and induce the nodulation genes (*nod* genes) (Mulligan and Long 1989; Jones et al. 2007). Once the *nod* genes are activated, rhizobia-to-plant signal exchange occurs by the release of lipo-chito-oligosaccharides which are referred as nodulation factors (*nod* factors) (Dénarié et al. 1996; D'Haeze and Holsters 2002). Nod factors consist of a backbone of three to five *N*-acetyl-D-glucosamine units with a fatty acyl group on the non-reducing sugar (Lerouge et al. 1990). Host specificity between rhizobia and legume depends on the structure of the Nod factors. The common nod genes (*nodABC*) are required for synthesizing the backbone of the nod factor, where host specific nod genes (*nod, nol, noe*) impact the modifications for the reducing and non-reducing ends of the nod factor (Wais et al. 2002; Masson-Boivin et al. 2009), and thus forming different nod factors. Nod factors have multiple functions mainly related to the rhizobia invasion and nodule formation (Geurts et al. 2005). Once plants receive the nod factors, it causes the root hair deformation where root hairs trap the rhizobia between the cell walls (Stougaard 2000; Gage et al. 2004). Rhizobia enter into the plant roots with the initiation of an infection thread which grows and directs bacteria towards the inner cortex of the roots (Jones et al. 2007). In the meantime nod factors stimulate root cortical cell division and initiation of nodule primordium (Gage et al. 2004). The infection thread releases bacteria into the cortical cells of the nodule primodium enveloped by the plant derived membrane called the peribacteroid membrane (PBM) (Mylona et al. 1995; Jones et al. 2007). Released bacteria

differentiate into bacteroids and this basic N fixing unit enclosed by the PBM is termed symbiosome (Udvardi and Day 1997; Day et al. 2001).

1.6.3.2 Nodule Structure and Types of Nodules

Nodules consist of three major tissues: a central infection zone (N fixing zone), inner cortex with vascular bundles, and outer cortex (Serraj et al. 1999; Sprent 2008). The type of nodules in legumes can be either determinate or indeterminate depending on the legume species (Gage 2004; Ferguson et al. 2010). Generally temperate legumes have indeterminate nodules which have a persistence meristem and are cylindrical in shape. The apical meristem develops new cells and infected by the bacteroids resulting gradient of bacteroids at different maturity stages from nodule apex towards the root (Ferguson et al. 2010). Determinate nodules lack persistence meristematic tissues and contain a homogeneous population of bacteroids (Gage 2004). They are spherical in shape and typically found in tropical legumes. There are some differences in the bacteroids of the determinate and indeterminate nodules. Bacteroids of the indeterminate type nodules are large, have low viability and contain one bacteroid per symbiosome whereas in determinate nodules bacteroids are normal rod size, highly viable and contain many bacteroids per symbiosome (Ferguson et al. 2010).

1.6.3.3 Functioning of Nodules

Host legumes supply reduced carbon mainly in the form of sucrose to fuel the N fixation in the bacteroids (Gordon et al. 1999) in exchange for fixed N which is transferred to the plant through the PBM (Udvardi and Day 1997). Fixed N needs to be taken away from the symbiosome in order to continue the N fixation process. Therefore, fixed NH_3 is assimilated by glutamine synthetase (GS) into glutamine, and then glutamine is converted into glutamate by the enzyme glutamine oxoglutarate aminotransferase (GOGAT) (Gordon et al. 1999; Patriarca et al. 2002). Before long-distance transport of fixed N occurs, N is converted into different N forms which depend on the legume type (tropical/temperate) (Udvardi and Day 1997; White et al. 2007). Temperate legumes with indeterminate nodules export fixed N from nodules to shoots in

the form of amides (asparagine) where tropical legumes with determinate nodules export N as the ureids. Synthesis of amides in indeterminate nodules takes place inside the rhizobia infected cells itself. However, in determinate nodules ureid synthesis takes place in uninfected cells attached to the infected cells (White et al. 2007; Franche et al. 2009). Root nodules are highly specialized structures facilitated with low oxygen environment since nitrogenase is highly susceptible to oxygen (Dixon and Kahn 2004; Jones et al. 2007). However, high oxygen demand of the bacteroids will be supply through high concentration of leghaemoglobin (Dixon and Kahn 2004). Active nodules are often characterized by their pink color, due to this oxygen-carrying leghaemoglobin (Sprent 2008).

1.6.3.4 Factors Affecting Legume-rhizobia Symbiotic Nitrogen Fixation

The symbiotic BNF in legumes can be affected by a wide range of factors including soil properties (pH, salinity, nutrient availability, moisture), soil biology (bacteria genetics), environmental conditions (rainfall, temperature, light) and other biotic and abiotic stress factors (pest and diseases, defoliation, mineral toxicity) (Walsh et al. 1995; Zahran 1999; Mohammadi et al. 2012). These factors have an impact on rhizobia, host legume and different steps in the nodulation process, such as attachment of rhizobia to host plants, infection and nodule organogenesis. Because of the interactions of different factors, it is hard to isolate the effect of the above factors on Rhizobium symbiosis and N fixation.

Soil moisture: Under soil moisture deficit conditions poor nodulation results from restrictions in migrating rhizobia in the soil, morphological changes in rhizobia, and reductions in infection thread formation (Zahran 1999; Mohammadi et al. 2012). Also N fixation is affected under drought conditions due to reduced nitrogenase activity (Pedersen et al. 1996; Pimratch et al. 2008). Drought conditions induce synthesis of abscisic acid in legumes where it reduces the leghaemoglobin level and increases oxygen diffusion resistance to nodules (González et al. 2001). Major physiological mechanisms involved in response to reduced symbiotic BNF under drought are C shortage, oxygen limitation, and feedback regulation by the accumulated fixed N (Serraj 2003).

Temperature: Different legume species have different optimum temperature ranges where maximum N fixation takes place (Bordeleau and Prévost 1994; Zahran 1999; Mohammadi et al. 2012). Both sub-optimum and supra-optimum temperature conditions cause delays in root hair infection and nodule initiation (Kumarasinghe and Nutman 1979; Peltzer et al. 2002; Maekawa-Yoshikawa et al. 2009), low nodulation (Mohammadi et al. 2012), small nodules (Zhang et al. 1996) and negative effects on nitrogen fixation activity (Kuzma and Layzell 1994; Bordeleau and Prévost 1994). Elevated temperature mainly interrupts nodule initiation, nodule development and functions of temperate legumes whereas in tropical legumes it mainly affects N fixation activity (Bordeleau and Prévost 1994).

Soil pH: Low soil pH reduces the number of nodules, nodule dry weight, nodule structure and nitrogenase activity (Vassileva et al. 1997) especially pH below 5.0 (Zahran 1999). Inhibition of nodulation under acidic conditions occurs in the early stages of the nodule ontogeny (Lin et al. 2012). Acidic conditions suppress the nodule gene expression (Richardson et al. 1988) and nod factor production (Morón et al. 2005) which interrupt signal exchange between rhizobia and legume resulting in low nodule numbers. Reduction in rhizobia number under acidic soil conditions also cause poor nodulation in legumes (Ibekwe et al. 1997). Rhizobia attachment to legume root hair is a Ca^{2+}-binding step (Rodríguez-Navarro et al. 2007). Low Ca^{2+} availability in soil is an indirect effect of low soil pH where reduction in rhizobia attachment and root hair deformation can result. On the other hand high soil pH also negatively affects rhizobia growth and nodulation in legumes (Bordeleau and Prévost 1994; Tang et al. 2006).

Salinity: Salinity negatively affects both the legume and rhizobia but the impact is more severe on the former than the latter (Bordeleau and Prévost 1994; Swaraj and Bishnoi 1999). Salinity reduces root hair deformation (Tu 1981), which reduces nodulation in legumes. The N fixation process is also reduced under salt stress due to direct effect on nitrogenase activity (Swaraj and Bishnoi 1999) or indirect effects reducing nodule respiration (Serraj et al. 1994), leghaemoglobin content (Nandwal et al. 2000), photosynthesis (Garg and Singla 2004) and disturbances in nodule structure (Redondo et al. 2012).

Soil available N: Nodulation and N fixation are negatively affected by high mineral N availability (Clayton et al. 2004; Naudin et al. 2011; Namvar et al. 2011). Signal exchange between legume and rhizobia can be negatively affected by high N levels where it reduces the expression of *nodD* and *nodABC* in rhizobia (Wang and Stacey 1990). Also high mineral N prevents the induction of the NIN gene by Nod factors (Barbulova et al. 2007), which is required for infection thread formation and initiation of nodule primodia (Schauser et al. 1999). High nitrate levels increase the barrier resistance of gas diffusion in nodule cortex resulting in low bacteroidal respiration and N fixation (reviewed by Luciñski et al. 2002). On the other hand nodulation is positively affected by application of starter N (Fei and Vessey 2003, 2004, 2009; Erman et al. 2009; Namvar et al. 2011; Thilakarathna et al. 2012a) and low soil mineral N levels (Gulden and Vessey 1997; Gan et al. 2004).

Phosphorus: Phosphorus has direct impact on both nodulation and N fixation in legumes (Hellsten and Huss-Danell 2000; Tang et al. 2001; Valverde et al. 2002; Gentili and Huss-Danell 2003). Application of P stimulates cell division in the cortex, increases number of pre nodules and nodule primodia (Gentili et al. 2006), increases nodule number (Hellsten and Huss-Danell 2000; Pramanik et al. 2009), nodule dry weight (Sanginga et al. 1996), specific nitrogenase activity (Tang et al. 2001) and counteracts the negative effect of high concentrations of N on nodulation (Gentili and Huss-Danell 2003).

Mo and Fe: Molybdenum and iron are important elements of the nitrogenase enzyme (FeMo-cofactor) (Abd-Alla 1999; Rubio and Ludden 2008) where nitrogenase reduces the atmospheric N_2 into NH_3 (Hu and Ribbe 2011). Iron is also needed for synthesis of leghaemoglobin in legumes (Kaiser et al. 2003) which carry the oxygen to rhizobia. External application of Mo and Fe induce the nitrogenase activity and nitrogen fixation (O'Hara et al. 1993; Abd-Alla 1999; Kaiser et al. 2005; Bambara and Ndakidemi 2010).

Heavy metals: Heavy metals negatively affect nodulation and nitrogen fixation in legumes (Manier and Deram 2009; Mandal et al. 2011). High concentrations of heavy metals reduce root hair deformation (Kopittke et al. 2007), number of nodules (Pastor et al. 2003) and effective N fixing area (due to changes in nodule ultrastructure) (Chen et al.

2003). Complete inhibition of nodulation at early seedling stages also results from heavy metals (Chen et al. 2003). Reduction in N fixation due to heavy metals is also associated with a reduction in nitrogenase activity (Younis et al. 2007; Banerjee et al. 2004).

Light: Photosynthesis provides both energy and C skeletons required for the synthesis of different organic N compounds from the reduced N (Kirizii et al. 2007). Nodules act as strong sinks for photosynthates (Aleman et al. 2010). Prolonged dark conditions reduced photosynthesis and C supply to the bacteroids which resulted in low nitrogenase activity and N fixation (Matamoros et al. 1999; Tsikou et al. 2013).

Defoliation: Defoliation temporary reduces both nodule biomass (Quinn and Hall 1996) and nitrogenase activity in legumes (Nygren et al. 2000). It is suggested that defoliation induces oxygen resistance to nodules which reduces nitrogenase activity (Hartwig and Nösberger 1994). However, defoliation increase the percentage N derived from atmosphere (%Ndfa) compared to uncut stands, but yield similar total N content by compensating for the reduced yield (Dahlin and Mårtensson 2008).

1.6.4 Importance of Legume Symbiotic Nitrogen Fixation

With the invention of Haber-Bosch process of NH_3 synthesis from N_2, resulted in increased usage of chemical N fertilizers which accelerated environmental pollution through N_2O emission, water eutrophication and soil acidification (Camargo and Alonso 2006; Davidson 2009). Synthetic N fertilizers are expensive due to high energy requirements for the manufacturing process. Biological N fixation provides an alternative to inorganic N fertilizer since legumes provide significant and sustainable N input via BNF (Ledgard 2001), especially under N limited conditions (Fujita et al. 1992). It is estimated that annually 50-70 Tg of N can be derived from BNF in agricultural systems where pasture and fodder legumes alone contribute 12-25 Tg N annually (Herridge et al. 2008). Biologically fixed N is less prone to leaching and volatilization (Dixon and Kahn 2004), because biologically fixed N is incorporated into organic forms. Since N fixed by legumes is slowly released to the environment, mainly through microbial decomposition, it reduces the environmental pollution associated with mineral N.

1.7 Nitrogen Transfer from Legume to Non-legumes

Nitrogen transfer is the movement of N from one plant to another under mixed plant stands predominantly from legume to non-legume during the growth of the legume plant (Brophy et al. 1987; San-nai and Ming-pu 2000). Nitrogen transfer is important in agricultural cropping systems to meet the N demand of non N_2-fixing plants while minimizing synthetic fertilizer application and the potential ground water pollution caused by nitrate leaching (Paynel et al. 2008; Fustec et al. 2010).

1.7.1 Nitrogen Transfer Mechanisms

Nitrogen transfer mechanisms can be categorized mainly as aboveground and belowground transfer (Ledgard 1991; Ledgard and Steele 1992; Høgh-Jensen and Schjoerring 2000; Ledgard et al. 2001; Rouquette Jr and Smith 2010). Aboveground transfer of N occurs through litter decomposition and animal excreta (Ledgard 1991, 2001; Fujita et al. 1992, Warembourge et al. 1997; Dahlin and Stenberg 2010b; Rouquette Jr and Smith 2010). However this dissertation focuses on the belowground N transfer mechanisms. The most common belowground N transfer mechanisms are decomposition of belowground legume tissues and uptake of released N by neighboring plants, N containing plant root exudates and mycorrhizal meadiated N transfer (Figure 1.2) (Xiao et al. 2004; Gylfadóttir et al. 2007; Wichern et al. 2008; Fustec et al. 2010; Dahlin and Stenberg 2010a; Schenck zu Schweinsberg-Mickan et al. 2010). Belowground N transfer can be categorized as direct or indirect. Nitrogen can be directly transferred from the donor plant to the receiver through common mycelial networks formed by mycorrhizal fungi interconnecting the root systems of both species (Haystead et al. 1988; Dubach and Russelle 1994; He et al. 2003). Indirect N transfer occurs through the soil compartment where N released into the soil as root exudates and decaying plant tissues are taken up by neighboring non legume plants (Paynel et al. 2008). However, Jalonen et al. (2009b) consider the root exudates as a direct N transfer mechanism since they can be directly absorbed by neighboring non-legumes without undergoing mineralization. Due to high competition for available N in the soil system by soil microbes, part of the rhizodeposited N can be immobilized as microbial residues and later contribute to the soil

N economy through microbial turnover (Mayer et al. 2004). Therefore N containing root exudates serve as both direct and indirect N transfer based on whether they are readily absorbed by the plants or undergo microbial immobilization and turnover.

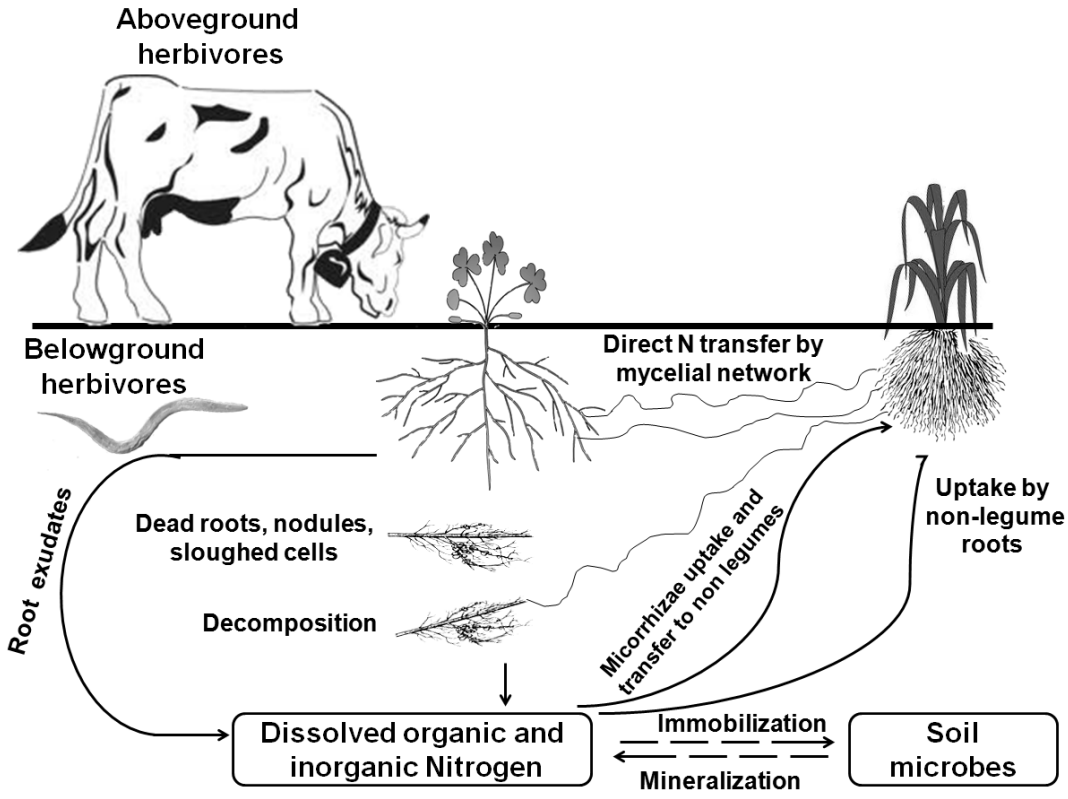

Figure 1.2 Possible nitrogen transfer mechanisms from legumes to non-legumes. (Nitrogen containing root exudates, N released by decaying roots, nodules and sloughed cells, mycorrhizae mediated N transfer).

1.7.1.1 Nitrogen Transfer through Senescence and Decomposition of Roots and Nodules

Nitrogen derived from turnover of roots, nodules, root caps, root border cells, sloughed cells and epidermal (water insoluble materials) significantly contribute to the belowground N transfer (Wichern et al. 2008; Fustec et al. 2010). It has been estimated that 3 to 102 kg N ha^{-1} yr^{-1} of N is transferred via decomposition of roots and nodules in legumes which is equal to 2 to 26% of the biologically fixed N in legumes (Ledgard and Steele 1992). Although, different belowground plant tissues are involved in N transfer, contribution of the different tissues to N transfer varies among the legume

species. Dubach and Russelle (1994) found that decomposing roots release more N than the nodules in alfalfa (*Medicago sativa* L.), whereas in birdsfoot trefoil (*Lotus corniculatus* L.) the opposite trend was found. Alfalfa nodules are not susceptible to defoliation stress and remained intact after harvesting compared to birdsfoot trefoil (Vance et al. 1979). Rapid nodule turnover can significantly contribute to N transfer since they maintain low C:N ratio (Warembourge et al. 1997). Nodule mineralization also depends on the form of the N (NH_4-N and hexosamine-N) present in nodules (Wardle and Greenfield 1991). This highlights the fact that nodule turnover depends on plant factors besides environmental factors. When comparing different mechanisms of N transfer, nodule and root turnover are the major N sources for N transfer (Trannin et al. 2000; Sierra et al. 2007) compared to root exudates or mycorrhizae-mediated N transfer, they depends on legume species as well (Ta and Faris 1987). Thilakarathna et al. (2012a) found that different legume cultivars have distinct rooting and nodulation profiles, which highlight the importance of considering genotypic differences for N transfer traits.

Although decomposition of belowground plant tissues and cells contribute significant amount of N for transfer, it is a slow process compared to the N transfer that occurs through root exudates and mycorrhizae. It involves decomposition and cycling of complex organic compounds rather than simple inorganic transfer (Goodman 1988). It takes about 20 days to release significant amounts of N from detached red clover roots under sand culture (Bingham and Rees 2008). Therefore, they mostly contribute to the N transfer in the later stages of plant growth or during the subsequent production years (Burity et al. 1989; Jørgensen et al. 1999).

1.7.1.2 Nitrogen Transfer as Root Exudates

Compounds released by the root system into the surrounding soil ("rhizosphere") are referred as root exudates (Walker et al. 2003). They can be either low molecular weight compound (amino acids, organic acids, sugars, phenolics, and various other secondary metabolites) or high molecular weight compounds (proteins) (Walker et al. 2003; Prithiviraj et al. 2007; Badri and Vivanco 2009). Root exudation is believed to be a passive process and includes diffusion, ion channels and vesicle transport (Badri and Vivanco 2009). In addition specific transporters also are involved in releasing root

exudates, mainly ATP-binding cassette (ABC) transporters and multidrug and toxic compound extrusion (MATE) families (Badri and Vivanco 2009, Weston et al. 2012; Sugiyama and Yazaki 2012). Anion channels facilitate NO_3^- efflux, whereas aquaporins provide routes for NH_4^+ efflux (Miller and Cramer 2004), but less attention has been paid on N efflux mechanisms than the influx mechanisms. Among the vast array of functions of root exudates (Dakora and Phillips 2002; Walker et al. 2003; Bais et al. 2006; Badri and Vivanco 2009) legume root exudates also act as a source for transferring N to neighboring non-legumes (Paynel and Cliquet 2003; Paynel et al. 2008; Jalonen et al. 2009a, b) especially for short-term N transfer (Paynel and Cliquet 2003; Gylfadóttir et al. 2007). At early growth stages of legumes, N is transferred via root exudates rather than predominates from decomposition of roots and nodule debris (Burity et al. 1989). Plants are able to absorb N in the form of dissolved organic N (amino acids, peptides and proteins) (Näsholm et al. 2009; Tegeder and Rentsch 2010) facilitating direct N transfer to non-legumes. On the other hand dissolved organic N can be rapidly turnedover by soil microbes (Owen and Jones 2001; Van Kessel et al. 2009) due to low C:N ratio (Uselman et al. 2000), and low diffusion coefficient in soil compared to NO_3^- (Jones et al. 2005), thus source of N to neighboring non-legumesafter mineralization (Jalonen et al. 2009b).

Ammonium and amino acids are the major N forms exuded by clover, but the former is exuded in much higher amounts than the later (Brophy and Heichel 1989; Paynel et al. 2001, 2008; Paynel and Cliquet 2003; Lesuffleur and Cliquet 2010). Among the different amino acids, glycine and serine are the dominant forms occurring in clover root exudates (Lesuffleur et al. 2007; Paynel et al. 2008), but the following amino acids are also found; glutamate, aspartate, tyrosine, asparagines, alanine, valine, arginine, glutamine, methionine, phenylalanine, leucine, isoleucine and lysine (Paynel et al. 2008). Although it was believed that amino acid exudation is a passive process (Bertin et al. 2003), using metabolic inhibitors Lesuffleur and Cliquet (2010) suggest that it is a plant controlled process. Main sites for amino acids exudation are nodules and root tips (White et al. 2007; Lesuffleur and Cliquet 2010) where most of the root-borne N compounds are present close to the roots and decrease with distance from root vicinity (Merbach et al. 1999). Therefore close root contact of the legume and non-legume is important for efficient N transfer. The form of N compounds exuded by legumes depends on the type

of legume, temperate legumes (eg: alfalfa) release most of the N as amino-N or NH_4-N whereas in tropical legumes (eg: soybean) it is other forms of N (Brophy and Heichel 1989). The reason is that temperate legumes transport fixed N through xylem in the form of amides (asparagine and glutamine) where tropical legumes, fixed N is transported mainly as ureids (Pélissier et al. 2004). Different plant factors affect exudation of N compounds by legumes where N_2 fixation (Paynel et al. 2008), root N concentration (Jalonen et al. 2009b) and total plant N content (Mahieu et al. 2009) positively impact this.

Other than the root exudate mediated direct N transfer, there are indirect effects of root exudates on N transfer including priming affect on organic matter where rapid mineralization results in more available N in soil (Kuzyakov 2010), enhanced plant-fungal interactions (mycorrhizae), which help to improve nutrient scavenging capacity of plants, and changes in soil pH improve some nutrient availability in soil (Dakora and Phillips 2002). Therefore it is very important to consider the indirect effect of root exudates on soil N availability when considering the N transfer from legumes to non-legumes.

1.7.1.3 Mycorrhizae Mediated Nitrogen Transfer

Arbuscular mycorrhizae (AM) and ectomycorrhizae (EM) are the most common and important mycorrhizae in agricultural ecosystems. Since mycorrhizae exhibits less host specificity than rhizobia (Albrecht et al. 1999; Shukla et al. 2012), around 70-90% of the terrestrial plant species form symbiotic associations with AM fungi (Parniske 2008). As legume-rhizobia symbiosis is initiated through plant-derived flavonoids, strigolactones found in root exudates acts as signal molecules to stimulate hyphal branching in mycorrhizae (Sugiyama and Yazaki 2012). One of the mechanisms of mycorrhizal-mediated N transfer is direct transfer of N by connecting donor and receiver root systems through common mycelia hyphae without entering N into the soil system (Haystead et al. 1988; San-nai and Ming-pu 2000; Høgh-Jensen 2006). Another mechanism is N released into the soil by legumes is takenup and transfer by mycorrhizal hyphae attached to the receiver roots (San-nai and Ming-pu 2000; Høgh-Jensen 2006). On the other hand, mycorrhizae increase the volume of soil which nutrints can be

extracted by colonizing the roots and this helps to reduce distance for nutrient diffusion and increase the soil volume that plants can explore for available N. Since AM fungi are able to obtain significant amount of N from decomposing plant materials (Hodge and Fitter 2010; Müller et al. 2013), decomposing roots and legume nodules serve as sources of N for transfer to non-legumes through mycorrhizae (Hamel et al. 1991). Furthermore plants colonized with AM are efficient in intercepting N from the soil system (Cavagnaro et al. 2012; Jannoura et al. 2012) and reduce N losses from the system (van der Heijden 2010; Asghari and Cavagnaro 2012), suggesting that under legume - non-legume mixed stands, N released by legume can be efficiently taken up by the mycorrhizae and subsequently transfer to the neighboring non-legume. Both AM and EM can uptake NH_4^+, NO_3^- and organic N but preferentially taken up NH_4^+ over NO_3^- (He et al. 2003). Govindarajulu et al. (2005) found that inorganic N taken up by the mycorrhizae is incorporated into amino acids, translocated from the extraradical to the intraradical mycelium in the form of arginine, then broken down to release NH_4^+. Finally the absorbed N by mycorrhizae is transferred to the plant in the form of NH_4^+.

Belowground N transfer through mycorhizae can be bidirectional; from legumes to non-legume as well as from non-legume to legume (He et al. 2003, 2009). However, the majority of the mycorrhizal mediated N transfer occurs from N_2-fixing legumes to non-N_2-fixing plants (up to 80%) whereas less than 10% of N transfer from non-N_2-fixing plants to legumes (He et al. 2009). Nitrogen can even be transferred between two plants through the mycorrhizal hyphae when the root system of one of the plants is decomposing (Johansen and Jensen 1996). According to the Jalonen et al. (2009b) mycorrhizae-mediated N transfer is driven by a source-sink relationship, where N transfer takes place from plants with high N concentration to low N. A number of studies have been carried out on the beneficial effects of N transfer from legumes to non-legumes through mycorrhizae (reviewed by He et al. 2003, 2009). Presence of mycorrhizal fungus increased the transfer of symbiotically fixed N from white clover to perennial ryegrass (Haystead et al. 1988), berseem clover to maize (Frey and Schüepp 1992), pea to barley (Johansen and Jensen 1996), soybean to maize (Van Kessel et al. 1985; Hamel et al. 1991), mung bean to rice (Li et al. 2009) and Gliricidia to angleton grass (Jalonen et al. 2009b). According to Haystead et al. (1988), up to 85% of the white clover roots (% of

root length) can be colonized by AM fungi. We found that on average 90% of the red clover roots (% of root length) were colonized by AM fungi under field conditions and red clover roots colonized by AM fungi are shown in Figure 1.3.

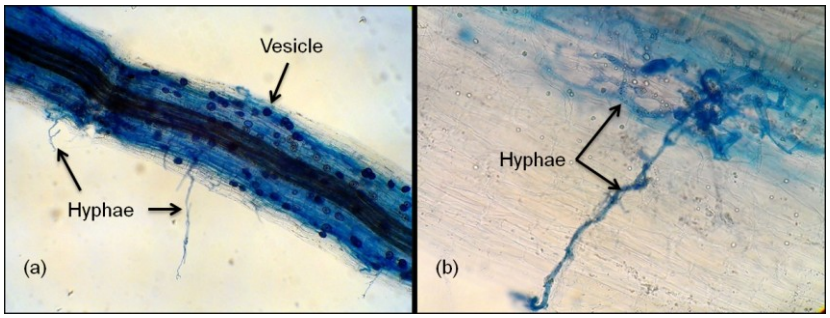

Figure 1.3 Arbuscular mycorrhizal root colonization in red clover roots.
(a) hyphae on root surface and vesicles inside the root, 40× magnified; (b) hyphae inside the roots, 150× magnified. Photo credit: Abhinandan Kumar.

There are some controversies regarding N transfer from legumes to non-legumes through mycorrhizal fungi. Although positive role of AM mediated N transfer in legume-grass mixtures is well documented (Haystead et al. 1988 and Zhu et al. 2000), there are reports that suggest N transfer is independent of AM fungi (Trannin et al. 2000; Rogers 2001).

1.7.2 Factors Affecting Nitrogen Transfer

Nitrogen transfer from legume to non-legume can range from 0 to 80% of the non-legume total N (Moyer-Henry et al. 2006; He et al. 2009). This broad range of N transfer is due to various biotic (Daudin and Sierra 2008; Jalonen et al. 2009a, b; Pirhofer-Walzl et al. 2012) and abiotic (Ta and Faris 1988; Brophy and Heichel 1989; Daudin and Sierra 2008; Mahieu et al. 2009) factors (Figure 1.4). Therefore it is important to consider all factors when evaluating the findings of N transfer from different studies.

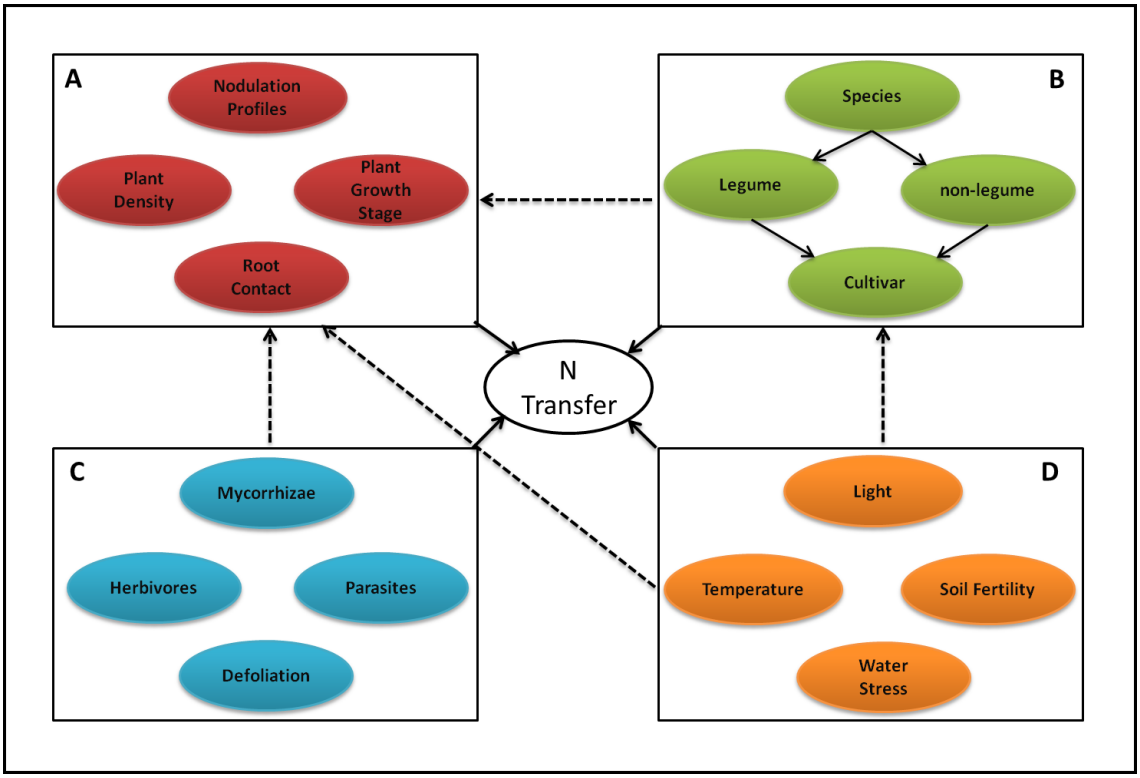

Figure 1.4 Major biotic and abiotic factors affect on N transfer from legume to non-legumes and their interaction with other component.
A: plant factors, B: genetic factors, C: other biotic factors, D: soil and environmental factors.

1.7.2.1 Water Stress

Water deficits affect N transfer from legume to non-legume directly or indirectly. Rapid loss of N and changes in the composition of the root exuded N by legumes under water deficit conditions (Brophy and Heichel 1989) can be considered a direct effect. Root cell walls can be damaged by drought releasing N into the rhizosphere. An example of an indirect effect is drought stress conditions which leads to root and nodule senescence in legumes. Subsequent mineralization of these belowground legume tissues facilitate N for non-legumes under mixed stand. Ledgard (1991) also found that N transfer from white clover to perennial ryegrass was favored by dry summer conditions. Contradictory to the above findings, a reduction in total N release by pea under water deficit was found by Mahieu et al. (2009), mainly by depleting the total plant N. Total plant N was reduced under water deficit, thus the subsequent reduction in N release is an

indirect effect since the proportion of total plant N released into the soil remains unchanged. This agrees with the findings of Goergen et al. (2009). Daudin and Sierra (2008) found that a 10% increase in the daily mean rainfall caused 14% increase in N transfer in a tree legume -grass system, where water availability may facilitate accessibility of released N to the neighboring grass. On the other hand legume N fixation also depends on water availability (Zahran 1999), where N fixation has a positive correlation with N transfer (Snoeck et al. 2000). Therefore it is clear that water stress directly as well as indirectly affects N transfer from legumes to non-legumes.

1.7.2.2 Temperature and Light Conditions

High temperature (35 ^{o}C) conditions stimulate N release from legume root systems (Fujita et al. 1992), thereby facilitating direct routes for N transfer to neighboring non-legumes. High soil temperature negatively affects longevity of fine roots due to an increase in maintenance respiration (Eissenstat et al. 2000), whereas turnover of these roots indirectly facilitates additional N transfer. Stimulation of C exudation into the soil under high temperature followed by its priming effect on soil organic matter decomposition (Uselman et al. 2000; Bengtson et al. 2012) also indirectly increases plant available N in cropping systems. Contradictory findings by Ta and Faris (1988) found that cool temperatures (20/16 ^{o}C day/night), high light intensity and long days increased N transfer from alfalfa to timothy (*Phleum pretense* L.), possibly due to increased N_2 fixation in the legume (Snoeck et al. 2000). Prolonged dark conditions affect nodule function and induce rapid nodule senescence (Hernández-Jiménez et al. 2002; Guerra et al. 2010). Dense mixed stands (eg: forage-grass mixed stand) shade conditions can lead to nodule senescence thereby increasing N transfer.

1.7.2.3 Soil Fertility and Application of Nitrogen Fertilizer

Soil N availability is a highly variable factor both spatially and temporal. Reduction in N transfer under high soil mineral N availability was reported in legume – non-legume mixtures; peanut - rice (Chu et al. 2004), white clover - turf grass (Sincik and Acikgoz 2007) and white clover - perennial ryegrass (Rasmussen et al. 2013). This could be

explained by a reduction in legume N fixation under high mineral N levels (Naudin et al. 2010) where N transfer is also directly proportional to legume N_2 fixation (Snoeck et al. 2000). Contrary to these findings, some reports have shown that N transfer is greater under high inorganic N availability compared to the low levels (Ofosu-Budu et al. 1995; Høgh-Jensen and Schjoerring 1997; Elgersma et al. 2000). A possible explanation for increasing N transfer under high N levels is the increased growth of grass roots growth which helps to compete and explore the available N in the soil system (Ofosu-budu et al. 1995; Paynel et al. 2008). On the other hand application of inorganic N fertilizer can have a priming effect where rapid mineralization of organic matter and N release takes place (Kuzyakov et al. 2000). Decomposition rate of roots and rhizodeposits is higher under N rich systems compared to the N deficient systems (Van der Krift et al. 2001). Released N from legumes can be temporary immobilized by the soil microbes (Mayer et al. 2004), but application of inorganic N accelerates the rapid turnover of the microbial N which were originally derived from legumes. Reports of both positive and negative impacts on N transfer due to high levels of inorganic N stresses the importance of further experimentation to understand exact mechanisms governing these processes.

1.7.2.4 Root Contacts

Spatial arrangement/proximity of the root systems of associated legume-non legume is important in N transfer, N transfer is high close to the legume root system and it reduces as distances from the legume root system increases (Fujita et al. 1990; Ofosu-budu et al. 1995; Daudin and Sierra 2008; Jalonen et al. 2009a). Xiao et al. (2004) found that N transferred from faba bean to the associated wheat increased when the roots of the both species are in contact. Close root contact of legume and non-legume permits common mycorrhizae contacts, reduces the distance to move N through mass flow and diffusion for plant uptake and finally increase use of legume derived N by neighboring non-legumes. Rhizodeposited N is high close to the root rhizosphere and decreases rapidly with increasing distance (Merbach et al. 1999; Schenck zu Schweinsberg-Mickan et al. 2010; Rasmussen et al. 2013). Rhizodeposited N concentration in legumes also decrease with soil depth where the majority of the rhizodeposited N (95%) was located in the top soil layer (0-15 cm) (Høgh-Jensen and Schjoerring 2001; Laberge et al. 2011).

Root architecture of the legumes also impact efficient root contacts with neighboring non-legumes and subsequent N transfer. Although, alfalfa has a high N_2 fixation capability compared to other forage legumes, the lower associated N transfer with alfalfa maybe due to its root architecture (Pirhofer-Walzl et al. 2012). Alfalfa has a deep taproot and less secondary roots which limit the close root contact with neighboring plants. These findings suggest the importance of close physical root contact between legumes and non-legume for efficient N transfer.

1.7.2.5 Legume – Non Legume Plant Density

Composition of legume: non-legume affects the efficiency of N transfer from legumes to non-legumes (Brophy et al. 1987; Viera-Vargas et al. 1995; Høgh-Jensen and Schjoerring 1997; Snoeck et al. 2000). Legume: non-legume plant density affects intermingling of plant roots, mycorrhizal connections and the distance for N movement. Generally N transfer is high in legume – non-legume mixed stands when the legume proportion is high compared to the non-legume. Maximum N transfer was found under high legume stands in different legume-grass mixtures; alfalfa, birdsfoot trefoil and reed canarygrass (Brophy et al. 1987), white clover, red clover, birdsfoot trefoil and tall fescue (Mallarino et al. 1990), seven tropical forage legumes and *Brachiaria brizantha* (Viera-Vargas et al. 1995). Different legume plant densities can be achieved through altering seeding rates where a high legume seeding ratio followed by better legume stands results in high N transfer compared to the lower seeding rates (Høgh-Jensen and Schjoerring 1997). Positive correlation between root density and legume N rhizodeposition (Mahieu et al. 2009) may explain a possible mechanism for high N transfer under dense legume mix sward. Reduction in N transfer during later production years in some field experiments was due to the loss of legume component in the sward compared to the grass component (Heichel and Henjum 1991). Non-legume component in legume mixed stand also play a key role where they rapidly depletes the available N in soil, resulting high N_2 fixation by legumes (Viera-Vargas et al. 1995). Overall high sward N yield results from the stimulatory interactions between these two functional groups of grasses and legumes (Nyfeler et al. 2011).

1.7.2.6 Plant Growth Stages and Maturity

Growth stage and the maturity of the legume plants affect N transfer (Ofosu-Budu et al. 1990, Heichel and Henjum 1991; Jensen 1996; Høgh-Jensen and Schjoerring 2000). A soil incubation study with pea roots conducted by Jensen (1996) found that rhizodeposited N at early growth stages (7 weeks) was more labile (mineralizable) compared to the rhizodeposits at maturity (14 weeks). Therefore, N released at the early growth stages of the legumes may result in short term N transfer. Growth patterns of the grass can also affect N transfer. Grass species with early maturity and rapid growth will compete vigorously for the N released by the legume and enhance N transfer (Ta and Faris 1987). When forage legumes age with increasing production years, N transfer also increases (Mallarino et al. 1990; Høgh-Jensen and Schjoerring 1997; Jørgensen et al. 1999; Elgersma et al. 2000). Jørgensen et al. (1999) found that apparent transfer of clover N to grass was negligible in the seeding year, but increased to 19 and 28 kg N ha^{-1} in the first and second production years, respectively. Similar findings by Høgh-Jensen and Schjoerring (1997) have shown that N transfer from clover to ryegrass was equivalent to 3, 16 and 31% of the N accumulated in the grass during the first, second and third production years, respectively. Nitrogen transfer also increased during the growing season as the season advanced (Dahlin and Stenberg 2010b; Thilakarathna et al. 2012b; Rasmussen et al. 2013). The proportion of grass N derived from N transfer by red clover increased from first harvest to third harvest in perennial ryegrass by 10.1, 11.7 and 22.7% (Dahlin and Stenberg 2010b) and in bluegrass by 7, 11, and 26% respectively (Thilakarathna et al. 2012b). Increases in N released by the legume root systems as plants mature are concomitant with increases in root mass and surface area (Brophy and Heichel 1989), increase in root exudates (Jalonen et al. 2009b) and senescence and decay of roots and nodules (Fustec et al. 2010).

1.7.2.7 Defoliation Stress

Defoliation of legumes enhances direct N transfer to neighboring non-legumes (Ayres et al. 2007). Defoliation removes the primary sink for N, which can lead to the release of N compounds to the rhizosphere (Hamilton et al. 2008; Carrillo et al. 2011).

Recently accumulated N in legume nodules can be released by passive leakage after shoot harvest (Brophy and Heichel 1989) facilitating short-term N transfer. On the other hand roots and nodule senescence due to defoliation stress (Chesney and Nygren 2002) facilitate long-term N transfer through turnover of above pant tissues. Root derived N has been found in the soil even eight months after defoliation (Carrillo et al. 2011), which can be available for plants in the following growing season thus facilitating long-term N transfer. In addition to direct effects, N can be indirectly available for N transfer through the C supplied from defoliated legumes into rhizosphere and increased microbial mediated N mineralization (Ayres et al. 2007). However Saj et al. (2008) suggests that under legume-grass mixtures defoliation mainly affects direct N transfer from legume to grass instead of altering available soil organic N. Defoliation frequency have positively related to the N transfer from legume to grass (Høgh-Jensen and Schjoerring 1994). Contrary to the above findings, there are some studies showing that defoliation frequency had no effect on N transfer from legume to non-legumes (Dahlin and Mårtensson 2008; Dahlin and Stenberg 2010a).

1.7.2.8 Parasites Induce Nitrogen Transfer

Above- and below-ground herbivory impacts N transfer from legumes to non-legumes (Hatch and Murray 1994; Murray and Clements 1998; Ayres et al. 2004; Dromph et al. 2006). Based on simulated aboveground herbivory study, increases the N transfer from white clover to perennial ryegrass (Ayres et al. 2007) and indirectly through increases in soil inorganic N (Ayres et al. 2004). Nitrogen transfer from legumes to the neighboring non-legume depended on root parasitic density of the infected legume roots (Bardgett et al. 1999; Dromph et al. 2006) where root infestation at low densities increased the leakage of N into the rhizosphere in white clover compared to uninfected plants (Bardgett et al. 1999). Plant roots can also be damaged by belowground insect herbivory and damaged roots can lead to significant N transfer through rapid mineralization (Hatch and Murray 1994; Murray and Clements 1998). Significant increase (37%) in N content of the perennial ryegrass was found due to root herbivore induced N transfer in white clover (Hatch and Murray 1994). Contrarily, reduced N transfer (13%) due to root herbivore (*Heterodera trifolii*) was found by Ayres et al.

(2007) using white clover and perennial ryegrass system. They suggest that root herbivory mediated N transfer may be apparent only after a few weeks of damage. Based on ^{15}N labeling Schmidt and Curry (1999) also showed that, endogeic earthworms significantly reduced the amounts of N transfer from living or decomposing clover roots to neighboring wheat plants. This reduction may be due to interruption of root contact of legume and non-legumes or disruption of mycorrhizal hyphal links by earthworms.

1.7.3 Genotypic Variability of Nitrogen Transfer

Biologically fixed N by legumes can be transferred to different classes of plants (grass, herbs, and trees) and impact their growth and development (Snoeck et al. 2000; Spehn et al. 2002; Pirhofer-Walzl et al. 2012). The extent of N transfer depends on the legume N donor component as well as the recipient non-legume component. Genetic variability of the above two components can be have a significant influence on N transfer from legume to non-legume.

1.7.3.1 Legume Species Effect

Nitrogen transfer from legume to non-legume is affected by the N donor legume species as well as the recipient non-legume species (Table 1.2). Species differences in N transfer in pasture legumes was previously reported by Ta and Faris (1987), Mallarino et al. (1990), Heichel and Henjum (1991), Pirhofer-Walzl et al. (2012) using alfalfa, white clover, red clover and birdsfoot trefoil. Pirhofer-Walzl et al. (2012) found that N transferred to the neighboring plants were highest in white clover (4.8 g m^{-2}) compared to the red clover (2.2 g m^{-2}) and alfalfa (1.1 g m^{-2}). Higher N transfer from white clover to tall fescue was also found by Mallarino et al. (1990) compared to the birdsfoot trefoil and red clover. It was reported that the higher percentage of grass N was derived from birdsfoot trefoil (47%), compared to alfalfa (29%) during the second year, which had the highest overall N transfer (Heichel and Henjum 1991). Based on above studies it is clear that generally white clover had higher N transfer compared to other pasture legume species while alfalfa had the lowest. Harvesting accelerates stolon death of white clover (Sturite et al. 2007), whereas stolon turnover can result in high N transfer. Low N transfer

from alfalfa may be due to storage of N by its root system to ensure regrowth after defoliation and lack of close root-root contact due to small number of secondary roots (Pirhofer-Walzl et al. 2012). Since white clover can multiply by their stolons, it is not necessary to store N within the root system. Therefore, more N can be derived from white clover roots for N transfer. Differences in the root characteristics (biomass, length, and surface area), nodulation profiles, root exudation profiles and mycorrhizal associations of the different legume species may result in different amount of N transfer to non-legumes.

1.7.3.2 Legume Cultivar Effect

Nitrogen transfer from legumes to non-legumes can also be affected by the cultivars within a species (Table 1.3). Based on four-year field experiment along with three white clover cultivars having different leaf sizes, Laidlaw et al. (1996) found cultivar differences in their ability in transfer N to perennial ryegrass. The large-leaved cultivar (Aran) transferred less N (15%) compared to the small-leaved (Kent Wild) cultivar (34%). Similarly, using two white clover cultivars with different leaf sizes Elgersma et al. (2000) found a significant legume cultivar effect on N transfer where the small-leaved cultivar transferred more N (115 kg N ha^{-1}) compared to the large-leaved cultivar (87 kg N ha^{-1}) during the second year. The suggested mechanisms for this genetic variability were either slow decay of roots and stolons in the large-leaved cultivars or competition from the large-leaved cultivars on grass to reclaim the available N (Laidlaw et al. 1996). Larged-leaved white clover cultivars have high herbage-to-root ratio (Seker et al. 2003) where they direct more N to the aboveground herbage, resulting in less N available for belowground N transfer. Other than the above possibilities, different root and nodulation profiles of the different cultivars within a species (Thilakarathna et al. 2012a) can lead to genotypic variability for N transfer. However, some studies have shown that there were no cultivar differences for belowground N transfer using different legume – non-legume stands; white clover - perennial ryegrass (Ledgard 1991), red clover - orchardgrass (Farnham and George 1993), red clover – bluegrass (Thilakarathna et al. 2012b) and faba bean – oat (Purnamawati and Schmidtke 2003).

1.7.3.3 Non-legume Species Effect

In addition to differences among legume types, difference among non-legume species also affects N transfer in legume – non-legume mixtures (Spehn et al. 2002; Sincik and Acikgoz 2007; Marty et al. 2009; Pirhofer-Walzl et al. 2012) (Table 1.2). Marty et al. (2009) have shown genotypic differences between two grass species (*Festuca eskia* and *Nardus stricta*) for receiving N from *Trifolium alpinum* (legume) under a mixed stands where 15% N transferred to *F. eskia* while 1% was transferred to *N. stricta*. Sincik and Acikgoz (2007) found differences in the amount of N transferred to perennial ryegrass, Kentucky bluegrass, and creeping bentgrass by white clover; 72.7, 50.4, and 48.6% of grass N was derived from N transfer, respectively. Burity et al. (1989) has shown that bromegrass was able to take up more transferred N from alfalfa compared to timothy grass under field condition. Grasses are better competitors for acquiring N from legumes compared to the dicotyledonous plants with tap root system, due to their fibrous root system (Pirhofer-Walzl et al. 2012). Therefore different plant factors associated with non-legumes such as root architecture, plant growth rate and mycorrhizal associated with roots may result in different quantities of N acquired through legume N transfer.

Table 1.2 Effect of plant species on nitrogen transfer.

Reference	Legume species	Grass species	N transferred (% non-legume total N)	Amount of N transferred	Remarks
Legume species effect					
Ta and Faris (1987)	*Medicago sativa* L.	*Phleum pratense* L.	5.08 - 36.43%	-	Pot experiment under greenhouse condition with five cuts. N transfer increased from cut 1 to cut 5. Method: ^{15}N dilution technique
		Bromus inermis	4.67 - 33.68%	-	
		Festuca rubra L.	3.92 - 28.37%	-	
		Festuca arundinaceae L.	5.92 - 35.14%	-	
		Dactylis glomerata L.	4.78 - 33.99%	-	
	Trifolium pratense L.	*Phleum pratense* L.	4.81 – 32.42%	-	
		Bromus inermis	4.28 – 31.86%	-	
		Festuca rubra L.	3.47 – 26.93%	-	
		Festuca arundinaceae L.	5.03 – 30.98%	-	
		Dactylis glomerata L.	4.27 – 35.30%	-	
	Lotus corniculatus L.	*Phleum pratense* L.	4.84 – 29.57%	-	
		Bromus inermis	4.36 – 30.71%	-	
		Festuca rubra L.	3.68 – 26.63%	-	
		Festuca arundinaceae L.	5.66 – 32.18%	-	
		Dactylis glomerata L.	5.04 – 30.04% (range c1- c5)	-	
Mallarino et al. (1990)	*Trifolium repens* L.	*Festuca arundinaceae* L.	Y1: 29% Y2: 60%	Y1: 24.7 Y2: 50.9	Two year field experiment with two locations. Method: ^{15}N dilution technique
	Trifolium pratense L.		Y1: 27% Y2: 54%	Y1: 21.7 Y2: 31.8	
	Lotus corniculatus L.		Y1: 29% Y2: 55% (mean N transfer)	Y1: 21.6 Y2: 47 (kg N ha^{-1} $season^{-1}$)	
Heichel and Henjum (1991)	*Medicago sativa* L.	*Phalaris arundinacea* L.	Y2: 29%	-	Four year field study. Highest N transfer found during the second year. Method: ^{15}N dilution technique
	Lotus corniculatus L.		Y2: 47%	-	
	Trifolium repens L.		-	-	
	Trifolium pratense L.		-	-	

Reference	Legume species	Grass species	N transferred (% non-legume total N)	Amount of N transferred	Remarks
Pirhofer-Walzl et al. (2012)	*Trifolium repens* L. *Trifolium pratense* L. *Medicago sativa* L.	Grass and herbs multi-species	- - -	4.8 2.2 1.1 (g N m^{-2})	Field study with grass-legume-herb multi-species mixtures. Method: ^{15}N leaf labeling technique
Grass species effect					
Burity et al. (1989)	*Medicago sativa* L.	*Phleum pratense* L. (Cv Climax) *Phleum pratense* L. (Cv Salvo) *Bromus inermis*	Y1c2: 31% Y2c2: 44% Y1c2: 33% Y2c2: 55% Y1c2: 47% Y2c2: 57% (Only significant results are listed)	Y1: 3.46 Y2: 13.73 Y3: 14.90 Y1: 3.51 Y2: 19.64 Y3: 15.53 Y1: 6.66 Y2: 27.12 Y3: 25.49 (kg N ha^{-1} $season^{-1}$)	Three year field study with two Timothy cultivars and bromegrass. Two cuts were taken during first two years and three cuts for third year. No cultivar differences during cut 1 at first two years and three cuts at third year. Method: ^{15}N dilution technique.
Sincik and Acikgoz (2007)	*Trifolium repens* L.	*Lolium perenne* L. *Poa pratensis* L. *Agrostis stolonifera* L.	73% 50% 49% (3 year average under 0N fertilization)	4.1 1.6 2.3 (g N m^{-2} $year^{-1}$)	Three year field study with four N levels. Method: N difference method
Marty et al. (2009)	*Trifolium alpinum* L. (Alpine clover)	*Festuca eskia* *Nardus stricta*	15% 1%	- -	Pot Experiment under greenhouse conditions. Method: ^{15}N leaf labeling technique

c; cut number, Y; Production year

Table 1.3 Effect of legume cultivar type on nitrogen transfer.

Reference	Legume species	legume cultivars	Grass species	N transferred (% grass total N)	Amount of N transferred	Remarks
Ledgard (1991)	*Trifolium repens* L.	Grasslands Huia Grasslands Pitau Grasslands Kopu Aran	*Lolium perenne* L.	No cultivar differences	Up to 70 kg N ha^{-1}	One year field study with dairy cow grazing. Method: ^{15}N dilution technique
Farnham and George (1993)	*Trifolium pratense* L.	APR-E701	*Dactylis glomerata* L.	Y1: 44.3% Y2: 70.5%	Y1: 15.7 Y2: 59.2	Two year field study with three harvests in each year. Method: ^{15}N dilution technique
		Arlington		Y1: 45.4% Y2: 68.6%	Y1: 16.3 Y2: 57	
		Mammoth		Y1: 43.7% Y2: 65.9%	Y1: 17.6 Y2: 53	
		Redland II		Y1: 48.9% Y2: 69.6% (seasonal mean)	Y1: 17.6 Y2: 62 (kg N ha^{-1})	
Laidlaw et al. (1996)	*Trifolium repens* L.	Aran	*Lolium perenne* L.	-	Y1: 26.0 Y2: 78.7 Y3: 49.2 Y4: 32.3	Four year field experiment with three white clover cultivars with different leaf sizes. Method: ^{15}N dilution technique
		Grassland Huia		-	Y1: 40.3 Y2: 83.8 Y3: 74.9 Y4: 52.2	
		Kent Wild White		-	Y1: 44.3 Y2: 75.8 Y3: 74.1 Y4: 57.9 (kg N ha^{-1})	

Reference	Legume species	legume cultivars	Grass species	N transferred (% grass total N)	Amount of N transferred	Remarks
Elgersma et al. (2000)	*Trifolium repens* L.	Alice	*Lolium perenne* L.	-	Y1: 64 Y2: 87	Two year field study with 0N and two N levels (150, 180 kg N ha^{-1}). Method: N difference method
		Gwenda		-	Y1: 61 Y2: 115 (kg N ha^{-1})	
Purnamawati and Schmidtke (2003)	*Vicia Faba* L.	Minica	*Avena sativa* L.	2.51%	1.280	Greenhouse Pot experiment Method: Split root technique using ^{15}N as labeled N
		Scirocco		1.62%	0.927 (mg N $plant^{-1}$)	
Thilakarathna et al. (2012b)	*Trifolium pratense* L.	AC Christie	*Poa pratensis* L.	c1: 5.5% c2: 10.5% c3: 23.6%	c1: 31 c2: 38 c3: 65	One year field study with three cuts, using two red clover cultivars. Method: ^{15}N dilution technique
		Tempus		c1: 7.6% c2: 11.6% c3: 28%	c1: 36 c2: 45 c3: 69 (mg N $plant^{-1}$)	

c; cut number, Y; Production year

Chapter 2

Genotypic Differences in Nodulation, Growth, and Nitrogen Uptake of Diverse Red Clover Cultivars under Different Levels of Nitrogen Fertilization

Thilakarathna, R. M. M. S., McElroy, M. S., Papadopoulos, Y. A., Fillmore, S. A. E., Prithiviraj, B. 2013. Genotypic differences in nodulation, growth, and nitrogen uptake of diverse red clover cultivars under different levels of nitrogen fertilization.

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2.1 Abstract

Plants respond differently to applied nitrogen (N) with respect to both species and cultivar. Genetic variability may exist among red clover (*Trifolium pratense* L.) cultivars for nodulation under different N treatments. Nodule formation and growth of three diploid red clover cultivars (AC Christie, Tapani, CRS 15) and three tetraploid cultivars (Tempus, CRS 18, CRS 39) were compared under controlled environmental conditions with four N applications (0, 0.5, 1.0, and 2.5 mg N plant^{-1} week $^{-1}$). As expected, there was a negative response to increasing N applications for the number of nodules plant^{-1} at harvest, with a significant interaction between red clover cultivar and N treatment. At 0 and 0.5 N, Tapani had the greatest nodulation (56 and 41 nodules plant^{-1}, respectively), whereas at 1.0 N and 2.5 N, AC Christie had the greatest nodulation (39 and 12 nodules plant^{-1}, respectively). Among the six red clover cultivars, CRS 15 and Tapani were the most sensitive to high N applications; active nodule numbers were reduced by more than 90% at 2.5 N compared with no N applied. A negative response to N fertilizer application

was evident for plant growth (shoot, root, total dry weight, and total leaf area) in all cultivars. There was an inverse relationship between tissue N concentration and biomass yield in response to N fertilization. Nitrogen concentration in plant tissues (shoot and root) was unrelated to total plant N, and plants treated with different N applications yielded similar levels of total plant N content. This study clearly shows that for nodulation, red clover cultivars respond differently to increasing N applications, which indicates this trait has genetic variability.

2.2 Introduction

Nitrogen (N) is the most important but limiting nutrient for crop growth worldwide. Application of synthetic N fertilizer has increased world food production but often with negative environmental effects. Not all crops respond similarly to soil mineral N, especially legume crops. High mineral N concentrations negatively affect legume nodulation, and N fixation (Naisbitt and Sprent, 1993; Hellsten and Huss-Danell, 2000; Voisin et al. 2002; Clayton et al. 2004; Naudin et al. 2011; Namvar et al. 2011). In addition to N concentration, N source also affects nodulation and N fixation (Abdel Wahab and Abd-Alla, 1996; Gan et al. 2004; Fei and Vessey, 2009). Nitrate, ammonium, and amino acids are the major sources of N available in the soil for plant uptake and metabolism (Miller and Cramer, 2004; Mokhele et al. 2012). Exposure to high nitrate applications reduces nodulation and nodule activity in legumes (Fujikake et al. 2003; Naudin et al. 2010, 2011). High ammonium applications also adversely affect nodulation (Nod) genes, legume nodulation, and growth (Rys and Phung, 1984; Wang and Stacey, 1990; Gulden and Vessey, 1997; Britto and Kronzucker, 2002). In contrast, some reports show that applications of small amounts of N increased the N_2 fixation in legumes by stimulating nodule formation and nitrogenase activity (Tsai et al. 1993; Gulden and Vessey, 1997; Gan et al. 2004; Thilakarathna et al. 2012a). Applications of starter N at low concentrations have been shown to improve the growth, nodulation, and subsequent N_2 fixation of legumes (Namvar et al. 2011; Naudin et al. 2011; Thilakarathna et al. 2012a). Plants not only show diverse adaptive responses to N application for individual species, but also for cultivars within a species (Schortemeyer et al. 1997; Cruz et al. 2011; Thilakarathna et al. 2012a). Characterizing the existing genetic variability for this

trait will allow farmers to select different genotypes according to available soil mineral N while maximizing N input through biological N_2 fixation (Zahran, 1999). Red clover is a major forage legume crop in North America, especially for pasture-based livestock production and within crop rotations for soil improvement. Although several red clover cultivars are commonly used in legume-based hay and pasture mixtures, information on how available N affects nodulation and growth is lacking. Therefore, this study was conducted to investigate the genotypic differences of red clover cultivars for nodulation, growth, and plant N under different levels of N fertilization during the early stages of plant growth.

2.3 Materials and Methods

2.3.1 Genetic Population and Plant Growth Conditions

Seeds of six diverse red clover cultivars, three diploid (AC Christie, Martin et al. 1999; Tapani, Papadopoulos et al. 2008; CRS 15, Y.A. Papadopoulos, AAFC, Personal communication) and three tetraploid (CRS 18 and CRS 39, Y.A. Papadopoulos, AAFC, personal communication; Tempus, http://www.inspection.gc.ca/english/plaveg/variet/regvare.shtml), 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 papers in the dark for 2 d before transferring them into plant growing containers. Washed sand (N free) was used as the growing media and Ray Leach plastic cone-tainers (125 ml volume size $cell^{-1}$) were used as the plant growing containers (Stuewe & Sons Inc., Tangent, Oregon USA). Cotton plugs were used at the bottom of each cell to prevent loss of sand. Using a randomized resolvable row-columns design (main plot, N fertility treatment and sub-plot, red clover cultivar), three pre-germinated seedlings were transferred into each cell followed by inoculation with 1 ml suspension of *Rhizobium leguminosarum* biovar *trifolii* ATCC 14480 during the same day. The rhizobia was grown on Trypton Yeast agar medium (Zigma Aldrich, Okville) for 3 d at 30 ^{o}C. To prepare the suspension, cell density was adjusted to 10^8 cells ml^{-1} (OD_{600}=0.1) with autoclaved distilled water (Thilakarathna et al. 2012a). Plants were thinned 7 d after seeds were transferred, leaving

one plant per cell that was re-inoculated with rhizobia, as described above, to ensure nodulation. Plants were grown in a growth room with supplemental lighting maintained for a photoperiod of 16 hr of daylight at 425 µmol m^{-2} s^{-1} and 8 hr of dark (16 D: 8 N) at 23 ± 2 ^{o}C.

2.3.2 Nitrogen Treatments

Plants received one of four N treatments (0, 0.5, 1.0, and 2.5 N mg $plant^{-1}$ $week^{-1}$) in the form of NH_4SO_4 (Sigma Aldrich, Okville) during the 8 wk growth period. Different N solution concentrations were obtained by dissolving an appropriate amount of ammonium sulfate in N-free Hoagland's nutrient solution (http://www.caissonlabs.com/product.php?id=313) and applied at 5 ml each week. The pH of the Hoagland's solution with the different N treatments was adjusted to pH 5.8 before application. Deionized water was supplied according to the plant water demand during the growing period.

2.3.3 Harvesting and Data Collection

Plants were harvested 8 wk after growth. Roots were carefully washed with distilled water to remove sand. Active nodules were counted based on the pink coloration of the nodules. Total leaf area was measured using Winfolia (Regents Instruments Inc., Quebec City) software system. Shoot and root dry weight (DW) were determined after drying the plant materials in a hot air oven set at 65 ºC for 3 d. Dry 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. Total N content was determined using the combustion method on a LECO protein/ N determinator FP-528 according to the Dumas method (Williams et al. 1998).

2.3.4 Statistical Analysis

The experimental design was a resolvable row-column design with the four nitrogen treatments randomized on the rows and the six red clover cultivars randomized on the

columns. There were four blocks of the resolvable four by six row-column design. Each attribute was analyzed using the row-column model with ANOVA, and the results were expressed at significant levels of $P < 0.10$ with blocks, rows and columns as random effects and nitrogen treatment by red clover cultivars as fixed effects. Polynomial contrasts were applied to assess the linear and quadratic relationships of responses to N additions and their interaction with cultivars. Orthogonal contrasts were used to evaluate differences between red clover cultivars. Responses that were significant at $P < 0.10$ were used in a principal component analysis (PCA) to explore the relationship between N treatments, cultivars, and selected responses. A biplot was used to overlay attributes on the scores for treatment combinations. Two variables (active nodule number and specific nodulation rate) were square-root transformed to adhere and meet assumptions of normality in the analysis. The statistical analysis of data was conducted with GenStat® (VSN International, 2011).

2.4 Results

2.4.1 Nodulation Response to Nitrogen Application

There was a negative response to increasing additions of N for the number of nodules per plant and for specific nodulation (active nodules g^{-1} root DW) at harvest, with a significant interaction between red clover cultivar and N treatment ($P < 0.10$) (Table 2.1). For 0 and 0.5 N applications, Tapani had the greatest nodulation, whereas for 1.0 N and 2.5 N applications, AC Christie had the greatest nodulation. In general nodule number was drastically reduced in the diploids compared with tetraploid cultivars in response to increasing N applications ($P = 0.02$). Nodulation was severely affected at the highest N application (2.5 mg N $plant^{-1}$ $week^{-1}$) compared with lower N applications; the active nodule number was reduced by more than 70% for all six red clover cultivars at 2.5 N compared with no N applied. Among the six red clover cultivars, CRS 15 and Tapani were most affected by high N concentration and active nodule number was reduced by more than 90% at 2.5 N compared with no N applied.

Table 2.1 Active nodule number, specific nodulation, and root N concentration of six red clover cultivars after 8 weeks of growth under four levels of nitrogen applications (0, 0.5, 1.0, and 2.5 N mg plant^{-1} week $^{-1}$).

Nitrogen application (mg plant^{-1} week $^{-1}$)	Red clover cultivar	Active nodule number§ (nodules plant^{-1})	Specific nodulation§ (number of nodules g^{-1} root DW)	Root N (%)
0	AC Christie	6.50 (42)	13.85 (192)	2.36
	Tapani	7.47 (56)	16.88 (285)	2.39
	CRS 15	5.97 (36)	14.58 (212)	2.44
	Tempus	5.29 (28)	11.55 (133)	2.11
	CRS 18	4.59 (21)	9.42 (89)	2.30
	CRS 39	5.10 (26)	11.38 (129)	2.16
0.5	AC Christie	5.54 (31)	12.19 (148)	2.34
	Tapani	6.39 (41)	15.27 (233)	2.50
	CRS 15	6.07 (37)	13.42 (180)	2.08
	Tempus	6.01 (36)	14.78 (218)	2.37
	CRS 18	5.56 (31)	11.70 (137)	2.48
	CRS 39	5.40 (29)	11.28 (127)	2.26
1.0	AC Christie	6.27 (39)	13.75 (189)	1.86
	Tapani	6.03 (36)	14.69 (216)	2.01
	CRS 15	5.03 (25)	12.60 (159)	1.84
	Tempus	5.22 (27)	11.55 (133)	2.16
	CRS 18	4.23 (18)	9.31 (87)	1.93
	CRS 39	4.92 (24)	10.79 (116)	2.30
2.5	AC Christie	3.46 (12)	9.19 (84)	2.41
	Tapani	1.76 (3)	4.66 (22)	2.47
	CRS 15	1.73 (3)	4.02 (16)	2.55
	Tempus	2.45 (6)	6.39 (41)	3.16
	CRS 18	1.69 (3)	4.33 (19)	2.76
	CRS 39	2.62 (7)	6.63(44)	2.88
Grand mean		4.80 (23)	11.01 (121)	2.34
SEM (N×Cultivar; n=4)		0.575	1.382	0.229
F-probability				
N×Cultivar		0.08	0.07	0.04
N_{lin}‡ (Diploid vs Tetraploid)		0.02	0.02	<0.01
….N_{lin} (Tem vs C18 & C39)		ns	ns	0.07
….N_{lin} (C15 vs ACC &Tap)		ns	0.08	0.01

ns = not significant ($P > 0.10$); SEM = standard error mean; ‡N_{lin} = linear relationship for N. Active nodule number§ and Specific nodulation§: square-root transformed values used in ANOVA are presented, corresponding detransformed values are within the brackets. Tem; Tempus, ACC; AC Christie, Tap, Tapani, C15; CRS 15, C18; CRS 18 and C39; CRS 39.

2.4.2 Plant Growth Response to Nitrogen Application

A negative linear response to N application was evident for shoot DW, root DW, total plant DW (shoot + root), and total leaf area for all six red clover cultivars ($P < 0.01$) (Table 2.2). Shoot DW, root DW, and total DW were reduced by 36%, 30%, and 34%, respectively, at the 2.5 N application compared with no N applied. Total leaf area of the red clover cultivars was severely affected at 2.5 N (mg plant^{-1} week $^{-1}$); total leaf area was reduced by 41% at 2.5 N compared with no N applied. There was no significant difference among the six red clover cultivars for shoot DW, total plant DW (shoot and root), root:shoot ratio, and total leaf area (Appendix II). Root DW was different among the six red clover cultivars where CRS 18 had the greatest root DW (203 mg plant^{-1}) and Tapani had the least (168 mg plant^{-1}) ($P = 0.028$) (Appendix II). In general, tetraploid red clover cultivars had slightly greater root DW (198 mg plant^{-1}) compared with the diploid cultivars (178 mg plant^{-1}) ($P = 0.01$).

Table 2.2 Shoot, root, and total dry weight, root:shoot ratio, and total leaf area of six red clover cultivars after 8 weeks of growth under four levels of nitrogen applications (0, 0.5, 1.0, and 2.5 N mg plant^{-1} week $^{-1}$).

Nitrogen application (mg plant^{-1} week $^{-1}$)	Shoot DW (mg plant^{-1})	Root DW (mg plant^{-1})	Total DW (shoot + root) (mg plant^{-1})	Root:shoot ratio	Total leaf area (cm^2)
0	418	210	628	0.524	48.2
0.5	361	203	564	0.583	44.8
1.0	337	193	538	0.605	41.2
2.5	269	147	416	0.591	28.4
Grand mean	346	188	536	0.576	40.6
SEM (n=24)	19	8	26	0.024	2.6
F-probability					
N	<0.01	<0.01	<0.01	ns	<0.01
N_{lin} ‡	<0.01	<0.01	<0.01	ns	<0.01
N_{quad} §	ns	ns	ns	0.06	ns

ns = P value greater than 0.10; SEM = standard error mean
‡N_{lin} = linear relationship for nitrogen; §N_{quad} = quadratic relationship for nitrogen.

2.4.3 Plant Nitrogen Content

Shoot N concentration (% N DW) increased linearly in response to N application; red clover plants supplied with 2.5 N had a 1.5 times higher shoot N concentration

compared with no N applied (Table 2.3). An interaction was found between red clover cultivars and N applications for root N concentration ($P = 0.04$) (Table 2.1). Maximum root N concentration (3.16%) occurred in Tempus gown in the 2.5 N treatment, but this cultivar had the lowest root N concentration when no N was applied. At 0.5, 1.0, and 2.5 N applications, CRS 15 had the lowest root N concentration. Interestingly, CRS 15 had the highest root N concentration when no N was applied. Shoot N content (mg plant^{-1}), root N content (mg plant^{-1}) and total plant N content (mg plant^{-1}) were not affected significantly by N application. Shoot N concentration, shoot N content, and total plant N content were not significantly different among the six red clover cultivars under different N applications (Appendix III). Root N content was different among the red clover cultivars, whereas CRS 39 had the highest root N content (4.97 mg plant^{-1}) and CRS 15 the lowest (3.84 mg plant^{-1}). In general, tetraploid cultivars had a significantly higher root N content (4.7 mg plant^{-1}) compared with the diploid cultivars (4.1 mg plant^{-1}) ($P = 0.006$) (Appendix III). According to the contrasts, CRS 15 had lower root N content compared with the other two diploid cultivars, whereas Tempus had lower root N content compared with the other two tetraploid cultivars ($P < 0.10$).

Table 2.3 Percentage of shoot and root nitrogen, and shoot, root, and total plant nitrogen, of six red clover cultivars after 8 weeks of growth under four levels of nitrogen applications (0, 0.5, 1.0, and 2.5 N mg plant^{-1} week^{-1}).

Nitrogen application (mg plant^{-1} week^{-1})	Shoot N (% DW)	Root N (% DW)	Shoot N content (mg plant^{-1})	Root N content (mg plant^{-1})	Total plant N (mg plant^{-1})
0	2.18	2.29	9.12	4.76	13.80
0.5	2.63	2.34	9.41	4.73	14.14
1.0	2.58	2.02	8.56	4.03	12.87
2.5	3.30	2.71	7.91	4.09	12.41
Grand mean	2.67	2.34	8.75	4.40	13.30
SEM (n=24)	0.208	0.183	0.53	0.388	0.772
F-probability					
N	0.02	ns	ns	ns	ns
N_{lin}‡	<0.01	ns	0.08	ns	ns
N_{quad}§	ns	ns	ns	ns	ns

†*F*-prob = F probability; ns = *P* value greater than 0.10; SEM = standard error mean;
‡N_{lin} = linear relationship for nitrogen; §N_{quad} = quadratic relationship for nitrogen.

2.4.4 Principal Component Analysis

Principal component analysis was performed using active nodule number, shoot DW, root DW, total plant DW, shoot N concentration, root N concentration, shoot N content, root N content, and total plant N content. The analysis captured 80% of the total variation with the first two principal components, with 59% explained for score 1 and 21% for score 2 (Figure 2.1). Score 1 correlates strongly with yield measurements (shoot DW, root DW, and total plant DW), and appears to discriminate between low- and high-N applications. Plants without N, or treated with low N applications, had greater plant biomass, whereas plants receiving the greatest N application had lower plant biomass. Score 2 is explained mostly by the root N percentage, root N content, and total plant N content. The cultivars Tapani, AC Christie and CRS 15 have lower N concentration in roots, root N content and total plant N content as the N level increases. It is interesting to note that plants with higher tissue N concentration are associated with low-biomass plants. In the PCA, biplot attribute points with angles < 90° to each other are positively correlated, whereas those with angles > 90° are negatively correlated. Angles of 0° or 180° indicate a perfect positive or negative relationship, respectively. In the two-dimensional array, attributes that are closest are those most closely related to each other. Root DW, shoot DW, total plant DW, shoot N content, and active nodule numbers were highly correlated to each other. Shoot N concentration and root N concentration were positively correlated. Total plant N content and root N content were also positively correlated.

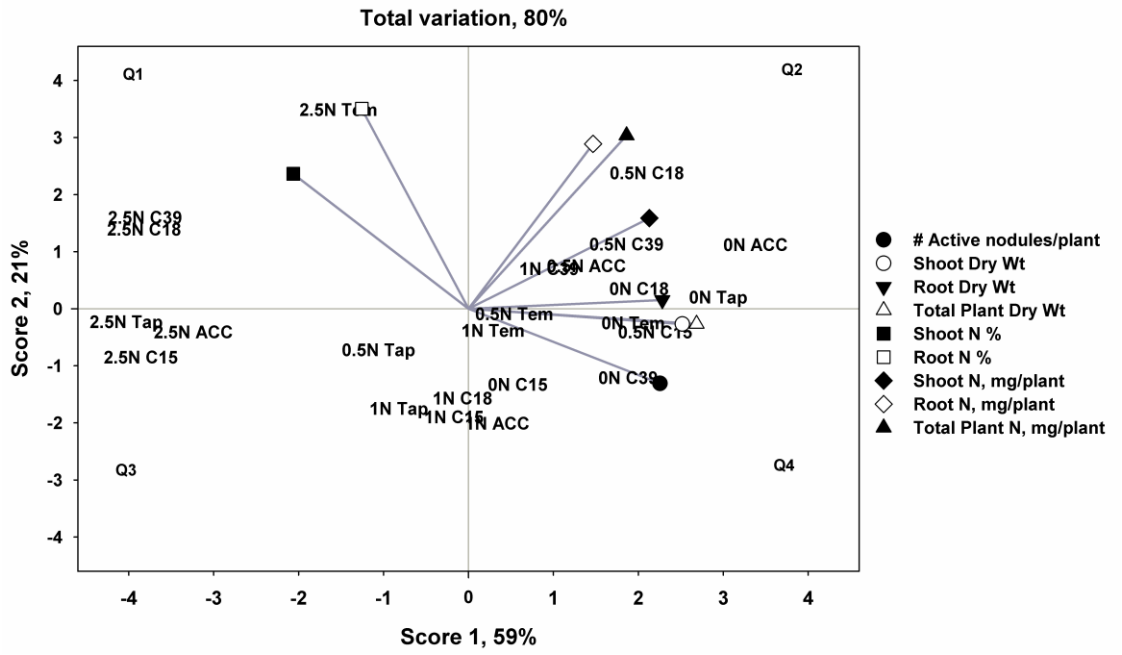

Figure 2.1 Principal component analysis of different attributes (active nodule number, shoot dry weight, root dry weight, total plant dry weight, percentage of shoot nitrogen, percentage of root nitrogen, shoot nitrogen content, root nitrogen content, and total plant nitrogen content) of six red clover cultivars [AC Christie (ACC), Tapani (Tap), CRS 15 (C15), Tempus (Tem), CRS 18 (C18), and CRS 39 (C39)] under four N applications [0 (0N), 0.5 (0.5N), 1.0 (1N), and 2.5 (2.5N mg plant^{-1} week $^{-1}$].

2.5 Discussion

As expected, red clover cultivars nodulate more at low N applications; nodulation (active nodule number and specific nodulation) was negatively affected by high N. Different phases of nodulation and biological N_2 fixation can be negatively affected by high external N, which includes the number of infection sites in the root, nodule development, nodule growth, O_2 diffusion, and nitrogenase activity (Abdel Wahab et al. 1996; Neo and Layzell, 1997; Gulden and Vessey, 1997; Zahran, 1999; Hellsten and Huss-Danell, 2000; Luciński et al. 2002; Barbulova et al. 2007). Nodulation in legumes is activated by response to rhizobial signaling molecules, called Nod factors (Esseling et al. 2003). Nodule inception protein (NIN) is important for infection thread formation and initiation of nodule primodia (Schauser et al. 1999). The presence of nitrate, or ammonium at high concentrations, prevents the induction of the NIN gene by Nod factors when compared with plants grown without N (Barbulova et al. 2007). Therefore, nodule

organogenesis is reduced by the lack of NIN induction (reviewed by Kraiser et al. 2011). Wang and Stacey (1990) also report that the expression of the *nodD* and *nodYABC* operons of *Bradyrhizobium japonicum* was repressed by the addition of ammonium, which can lead to poor nodulation. Nodulation is also reportedly affected by plant-N status (reviewed in Ruffel et al. 2008), whereby high levels of N in the tissues can repress the expression of the genes responsible for nodule formation and N_2 fixation. This result may help to explain the opposing N concentrations in shoot tissue and active nodule vectors in the PCA.

There was an N × cultivar interaction for nodulation for different red clover cultivars under different N applications. AC Christie and Tapani had their greatest nodulation with no N applied whereas the other four cultivars (Tempus, CRS 18, CRS 39, and CRS 15) had their greatest nodulation at 0.5 N (mg N plant^{-1} week $^{-1}$). Genotypic differences for nodulation are also found under different N applications (Abdel Wahab and Abd-Alla, 1996; Thilakarathna et al. 2012a). Nodulation and N_2 fixation are stimulated under low N conditions (Yinbo et al. 1997; Daba and Haile, 2002; Fei and Vessey, 2003, 2004, 2009; Indieka and Odee, 2005), but this trend is not consistent for all cultivars and also varies among cultivars. Our findings also corroborate earlier results on genotypic differences among red clover cultivars for nodulation under different starter N applications (Thilakarathna et al. 2012a). Nodule DM and nodule number can be negatively affected by high N concentrations and can result in smaller individual nodules (Abdel Wahab et al. 1996; Hellsten and Huss-Danell, 2000; Clayton et al. 2004). Applying N early when it overlaps with rhizobial inoculation leads to fewer nodules (Abdel Wahab et al. 1996). Since we applied N from the first day of seed transfer until 8 wk of growth, our findings explain the overall effect of N applied from germination to the initial vegetative stage.

Nodulation in legumes is an energy intensive process and nodules act as an important sink with a high demand for C from legumes (Voisin et al. 2003a, 2003b, 2007; Bourion et al. 2007). Voisin et al. (2007) report that genotypes with high nodule DM accumulate low levels of DM in shoots. However, our findings indicate that plants with more nodules had greater biomass and vice versa. This result may be due to the toxic effect created under high N levels that suppresses nodulation and plant growth. Unlike

NO_3^-, NH_4^+ does not need to be reduced in order to be assimilated from the soil, but it can be toxic to plants at a high concentration. Ammonium toxicity in plants is associated mainly with the accumulation of free NH_4^+ in plant tissues (Li et al. 2011) or the acidification of the rhizosphere by proton efflux by the root system (Rys and Phung, 1984; Britto and Kronzucker, 2002). Since we used NH_4^+ as the sole N source, plants could be affected by high N applications as described above. Carbon skeleton availability is very important to minimize NH_4^+ toxicity mainly by N assimilation, regulating internal NH_4^+ content (influx/efflux), and cell ionic balance (Britto and Kronzucker, 2002; Li et al. 2011; Ariz et al. 2011).

Shoot DW, root DW, total plant DW, and leaf area were affected by high N concentrations and decreased linearly for the different N sources. Growth reduction with high NH_4^+ levels is a common symptom reported previously (Schortemeyer et al. 1997; Britto and Kronzucker, 2002; Li et al. 2011). Ammonium assimilation and plant growth compete for available photosynthates (Schortemeyer et al. 1997). Reduced plant biomass under high NH_4^+ supply can be due to limited carbon/energy supply at the expense of ammonium assimilation (Schortemeyer et al. 1997), and high root respiration may be related to increases in N efflux to maintain low NH_4^+ in cytosol (reviewed by Jackson et al. 2008). Plants also show developmental plasticity for external N availability by modulating their root architecture to ensure adequate N is taken up by plants (Kraiser et al. 2011). Development of greater root length density is key to capturing immobile NH_4^+ at low concentrations (Jackson et al. 2008). We also observed that plants treated with low N concentration had denser roots compared with plants receiving greater N applications. A reduction in root size has been observed as being symptomatic of ammonium toxicity (Miller and Cramer, 2004; Ariz et al. 2011), such as stunted growth in general and the development of dark green leaves with necrotic spots (Hellsten and Huss-Danell, 2000), both of which were observed in plants treated with the greatest N application. No significant cultivar differences were observed for shoot DW, total plant DW, root:shoot ratio, and leaf area. However, root DW differed among the red clover cultivars and, interestingly, tetraploid cultivars had greater root DW compared with the diploids under different N applications. This result corroborates our previous findings for diploid and tetraploid cultivars under different starter N applications (Thilakarathna et al. 2012a).

Although shoot N concentration increased in plants grown in the 0 N to 2.5 N, root N concentration was not significantly influenced by N application. In contrast Gulden and Vessey (1998) reported total N concentration of both roots and shoots increased with increasing NH_4^+ concentration in soybean. Insufficient supply of photosynthates to root systems can limit ammonia assimilation in root systems while increasing NH_4^+ transport to the shoots (Schortemeyer et al. 1997). This phenomenon can lead to increased availability of NH_4^+ in shoots, and thereby high N concentrations in shoots under high N supply. Shoot and root N content were calculated by multiplying tissue N concentration by their DW. Root and shoot N content were not significantly affected by N applications. Red clover plants treated with different N sources accumulated a similar amount of total plant N (shoot and root N content). This result clearly demonstrates that greater tissue N concentration is associated with low plant biomass and vice versa, resulting in similar N contents for plants under different N applications. Red clover plants, therefore, can offset N fixation with mineral N uptake and viceversa under different N regimens. Nitrogen in red clover plants is derived from two mechanisms, soil N absorption, and biological N_2 fixation. Since nodulation was low at the greater N concentrations, plants possibly derived the majority of their N through root N uptake.

From the PCA, plants treated with low N applications produced more nodules compared to plants treated with higher N applications, and the nodule number was positively correlated to plant biomass. Therefore, the selected six red clover cultivars performed better under low N conditions. When plants have free access to N, they tend to absorb more from the growing media than through biological N_2 fixation. It is interesting to note that N concentration in plant tissues (shoot and root) is unrelated to total plant N, and plants treated with different N applications had similar levels of total plant N. Nodulation and subsequent N fixation is controlled by both soil N availability and plant auto-regulation processes (Reid et al. 2011). Our results show that nodulation in red clover depends on both external mineral N concentrations and a cultivar's ability to autoregulate nodule organogensis.

In conclusion, we found high N applications reduced nodulation and plant size, but increased shoot N concentration in red clover cultivars. Plants had similar total plant N content irrespective of N application. This study also shows that for nodulation, red

clover cultivars respond differently to N applications, suggesting genetic variability for this trait. Nitrogen × cultivar interactions may indicate that some cultivars are better adapted to low available N while other cultivars are better adapted to high available N levels.

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Chapter 3

Genotypic Differences in Root Hair Deformation and Subsequent Nodulation for Red Clover under Different Additions of Starter N Fertilization

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Contribution to writing: Written by R.M.M.S. Thilakarathna and reviewed by the other co-authors.

3.1 Abstract

Red clover cultivars, including diploid and tetraploid, are commonly used in legume-based pasture mixtures. However, information on nodulation under different starter N regimens is limited. We hypothesized that there is genetic variability among different red clover cultivars for nodulation. A root hair deformation assay was conducted using three diploid (AC Christie, Tapani, and CRS15) and three tetraploid (Tempus, CRS18, and CRS39) red clover cultivars by inoculating them with *Rhizobium leguminosarum* biovar *trifolii*. Nodulation and morphological characteristics of two selected red clover cultivars, AC Christie and Tempus, were determined under five starter N concentrations (0, 0.2, 0.4, 0.8, and 1.6 mg plant^{-1}). Inoculation with rhizobia increased root hair deformation with significant interaction across cultivars. Nodulation was delayed under high starter N concentrations, and genotypic differences were evident for days-to-nodule initiation. There was a positive quadratic response to starter N for AC Christie and a negative quadratic response for Tempus for nodulation. Tempus had more

active nodules (92 %) than AC Christie (73 %). The genetic variability of red clover cultivars should be considered in N fixation studies and their response to availability of initial N.

3.2 Introduction

An essential element of sustainable agriculture is the effective management of N fertilizer. Due to new trends in low-input agriculture and concern over the negative effects of high N inputs, many farmers consider using pasture legumes as a source of biological N fixation. Perennial forage legumes have great potential to enhance the sustainability of grassland farming systems (Carlsson and Huss-Danell 2003; Paynel et al. 2008). Red clover (*Trifolium pratense* L.) is an important legume in northern latitudes where much of agriculture is based on livestock production. It is an excellent forage legume for hay making, silage, and grazing, and it can be used in rotation to maintain soil fertility. Red clover is one of the few agriculturally important species in the genus *Trifolium* (Taylor and Quesenberry 1996) wherein symbiotic biological N fixation plays a crucial role.

Symbiotic N fixation involving legumes and rhizobia depends upon a complex series of processes; successful colonization of effective Rhizobium strains in the soil, infection of the host plant, and nodule initiation and development (Fisher and Long 1992; Ledgard and Steele 1992). Nodulation in legumes is activated in response to rhizobial signaling molecules, called nodulation (Nod) factors, which induce root hair deformation (curling) (Esseling et al. 2003). Bacterial infection generally occurs through root hair cells curling around the rhizobia, entrapping attached bacteria. These bacteria grow and form infection foci from which infection threads are initiated. Infection threads have a plant origin structure and can permeate cell boundaries, which enables bacteria to invade the cortical cells (reviewed by Oldroyd and Downie 2008).

When considering N dynamics in pasture fields, it is important to understand how soil N is affected by environmental change and microbial activity. In the long-term, symbiotic N fixation can lead to soil N accumulation and reduced BNF (Ledgard and Steele 1992). Nitrogen availability affects different phases of nodule formation and subsequent N fixation (Hellsten and Huss-Danell 2000; Ledgarad 2001; Fei and Vessey

2003, 2004; Erman et al. 2009). Besides N concentration, N type (nitrate or ammonium) also affects nodulation and N fixation (Wahab et al. 1996; Fei and Vessey 2009). Many reports show that additions of starter N support nodulation and subsequent N fixation (Namvar et al. 2011; Fei and Vessey 2003, 2004, 2009; Erman et al. 2009; Gulden and Vessey 1997; Wahab and Abd-Alla 1996). On the other hand, high N availability negatively affects nodulation and N fixation in legumes (Naudin et al. 2010, 2011; Datta et al. 2011; Namvar et al. 2011). Therefore, it is important to consider nodulation and N fixation in N deficient systems, as well as in systems having elevated supplies of starter N.

As the use of different red clover cultivars in agricultural systems increases, nodulation processes and subsequent growth under different starter N regimens needs to be better understood. The rapid establishment of nodulation and subsequent N fixation is particularly important under N deficient field conditions. Since root hair deformation varies, this study evaluated root hair deformation activity for several diploid and tetraploid red clover cultivars. We also evaluated genotypic differences between two red clover cultivars for nodulation and subsequent growth at different starter N levels.

3.3 Materials and Methods

3.3.1 Root Hair Deformation Assay

A root hair deformation assay was used following a method described earlier by Prithiviraj et al. (2000). *Rhizobium leguminosarum* biovar *trifolii* ATCC 14480 was obtained from the Danielle Prévost Research Center, Agriculture and Agri-food Canada, Quebec. Briefly, seeds of six diverse red clover cultivars; three diploid (AC Christie, Martin et al. 1999; Tapani, Papadopoulos et al. 2008; CRS 15, Papadopoulos, Personal communication) and three tetraploid (CRS 18 and CRS 39, Papadopoulos, Personal communication; Tempus, http://www.inspection.gc.ca/english/plaveg/variet/regvare.shtml), were surface sterilized with 2 % sodium hypochlorite for three minutes and washed with three changes of sterile distilled water. The seeds were then placed on half-strength MS (Murashige and Skoog Basal Medium) agar medium (Sigma Aldrich, Okville) in 9-cm diameter Petri plates

(three seeds per plate). The Petri plates were placed in an incubator set at 25 ºC in complete darkness for 7 days. Seeds germinated and developed tap roots on the agar surface. Roots with abundant root hairs (fluffy growth) were excised with a sterile scalpel[1]. These roots were placed on sterile grease-free glass slides and immediately treated with 200 µl *Rhizobium leguminosarum* biovar *trifolii* (OD_{600} 0.1). Control roots were treated with 200 µl of sterilized distilled water. Slides that contained roots were then placed in a closed moist chamber and incubated for different periods of 4, 12, and 24 hours in the dark. Root hair deformation was observed under a light microscope at the end of the incubation period[2]. Each treatment had at least three sampled roots, and a minimum of 100 root hairs were observed from each sample. This experiment was repeated three times.

3.3.2 Nodulation Assay

Seeds of two red clover cultivars, AC Christie (diploid) and Tempus (tetraploid), were surface sterilized with 2 % sodium hypochlorite for three minutes and washed with three changes of sterile distilled water. Seeds were germinated in plastic growth pouches (Mega International, Minneapolis, MN, USA) containing distilled water. After one week of germination, the seedlings were thinned to one seedling per growth pouch and the pouches were filled with 50 ml half-strength Hoagland's N-free nutrient solution (Bender et al. 1985). The pH of the Hoagland's solution was adjusted to 5.8. In this experiment we added five N treatments (0, 0.2, 0.4, 0.8, and 1.6 N mg $plant^{-1}$) in the form of ammonium nitrate two times over the course of the experiment. The first treatment was applied two days prior to the rhizobial inoculation. The plants were raised in an N deprived medium. As the objective of the experiment was to study the effects of different N additions on the rhizobia-red clover genotype interaction, this treatment ensured the presence of N in the medium at the time of inoculation. Plants tend to absorb N more rapidly when raised on N deprived medium, and this was the case in our experiment. Therefore, a second application of N was critical to ensure the required N was present in

[1, 2] Wording of the two sentences were modified without changing their meaning of the original sentences in the manuscript.

the system. One week after germination, each plant was inoculated with 1 ml suspension of *Rhizobium leguminosarum* biovar *trifolii* ATCC 14480 which had been grown on Trypton Yeast agar medium (Zigma Aldrich, Okville) for 3 days at 30 ^{o}C. To prepare the suspension, cell density was adjusted to 10^8 cells ml^{-1} (OD_{600}=0.1) with distilled water. During the rest of the growth period, plants were supplied with N-free Hoagland's nutrient solution. The plants were grown in a growth room with supplemental lighting maintaining a photoperiod of 16 hours of daylight at 125 µmol m^{-2} s^{-1} and 8 hours of dark (16 D: 8 N) at 23 ± 2 ^{o}C.

Attributes determined were: days-to-nodule initiation, number of nodules after four weeks following inoculation with rhizobia and at harvest (eight weeks), shoot dry weight, and root dry weight at harvest. Roots were observed daily after inoculation with rhizobia to detect days-to-initiation of nodule primodia. Outgrowths from the root's surface were designated as being developing nodules (to distinguish them from emerging lateral roots) when: 1) the angle of the junction between the perimeter of the outgrowth and the subtending root surface was less than or equal to 90º, and 2) the distal end of the outgrowth had a 'bulbous' morphology but not an acute shape which is characteristic of a lateral root primodium (Fei and Vessey 2003). Plants were harvested eight weeks after rhizobial inoculation. Total leaf area was measured by scanning using the WinFOLIA (Regents Instruments Inc., Quebec City) software system. A detailed root morphological analysis, including volume, total length, surface area, diameter, and projected area, was determined using WinRHIZO system (Regents Instruments Inc., Quebec City). Plants were evaluated for vigor (range from 0–10) at harvest based on visual growth characteristics. Shoot and root dry weight were determined after drying plant materials in a hot air oven set at 65 ºC for 3 days.

3.3.3 Statistical Analysis

3.3.3.1 Root Hair Deformation Assay

For the root hair deformation trial, three replicates of a split plot were run. The main plot consisted of six uninoculated cultivars and six cultivars inoculated with rhizobia. The subplot was the sample time of 4, 12, and 24 hours. Data were analyzed with the split plot

model in an ANOVA. A polynomial contrast was evaluated for linear and quadratic response across the three sampling times.

3.3.3.2 Nodulation and Growth Experiment

The experimental design is a randomized complete block with red clover cultivars as the main treatments (main plot: Tempus, AC Christie), and N additions (sub-plots: 0, 0.2, 0.4, 0.8, 1.6 mg N plant^{-1}) as split-plot treatments. Each variate was analyzed using the split plot model with ANOVA and results were expressed at a significance level of ($P < 0.05$) and ($P < 0.10$). Data were analyzed using GenStat® (VSN International, 2011). A polynomial contrast was used to evaluate the linear and quadratic relationships between the response variates with N additions, and their interaction with cultivars. Variates that were significant ($P < 0.10$) were used in a principal component analysis to assess the relationship between N additions and cultivars as explained by the co-variation among selected variates. A biplot was used to overlay the variates on the scores for the treatment combinations; this procedure was used to provide information on the relationship of the variates to the treatments as well as their correlation to each other.

3.4 Results

3.4.1 Root Hair Deformation

Root hair deformation (%) in the six red clover cultivars after rhizobia inoculation was compared with a control treatment consisting of autoclaved distilled water (Figure 3.1)[4]. Comparison of deformed root hairs and normal root hairs are provided in the Appendix IV[5]. The presence of rhizobia increased root hair deformation; there was a significant interaction across all cultivars and times. Not all cultivars responded similarly with a quadratic response differing among cultivars. After 4 and 12 hours of inoculation, the highest root hair deformation was reported by Tapani, which is a diploid. CRS 39,

[4] Fig. has been replaced with Figure throughout the manuscript in order to be consistent throughout this dissertation.

[5] This figure is an addition to the manuscript published in Journal of Agronomy and Crop Science and aimed to clarify difference between normal and deformed root hairs.

which is a tetraploid had the lowest root hair deformation percentage across the three time points selected. This study shows that although genetic variability may explain response differences among cultivars to rhizobia, these differences are not associated with ploidy (i.e., diploid vs tetraploid).

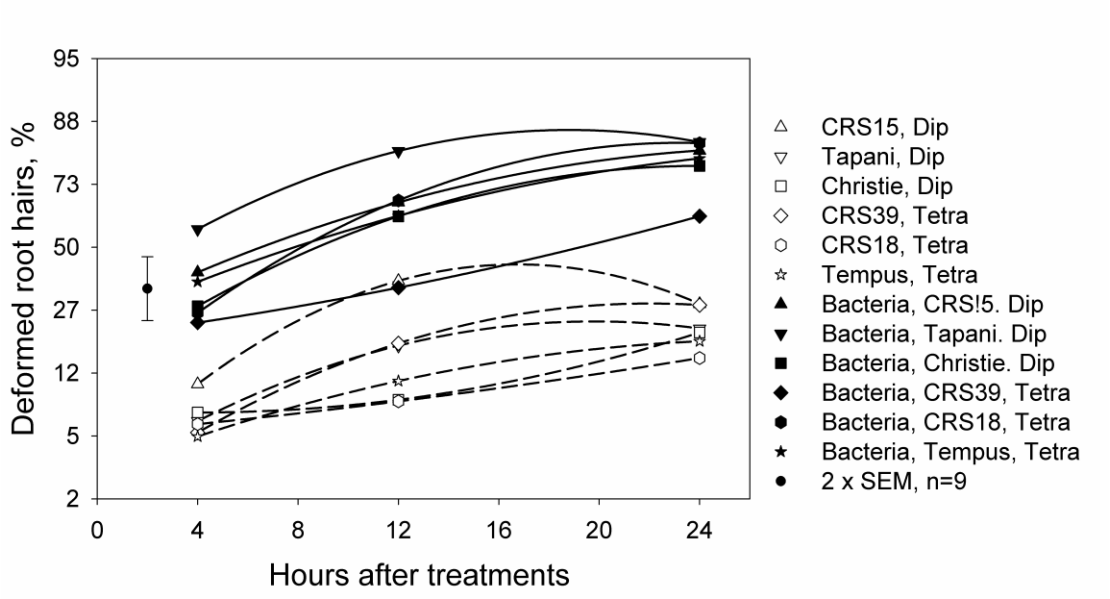

Figure 3.1 Root hair deformation (%) for six red clover cultivars after roots were treated with 200 µl of *Rhizobium leguminosarum* (OD_{600} 0.1) and distilled water as the control. Number of replicates (n) = 9, vertical bar represents two standard errors of the mean (SEM), Dip = diploid; Tetra = tetraploid. [6]Significant interaction for bacteria inoculation × ploidy/cultivar × hours, with quadratic response along the time; $P < 0.001$.

3.4.2 Number of Days to Nodule Initiation

The days-to-nodule initiation in legumes is particularly important when soil N availability is limited and also for small seed legumes which have limited N reserves for early establishment. Days-to-nodule initiation was different ($P = 0.002$) between AC Christie (6.0 days) and Tempus (4.6 days) (Figure 3.2). A positive linear relationship was observed between starter N concentration and days-to-nodule initiation ($P = 0.035$). There was no significant interaction between cultivars and N concentrations.

[5] Additional caption was included to explain the interaction and trends showed in the Figure 3.1.

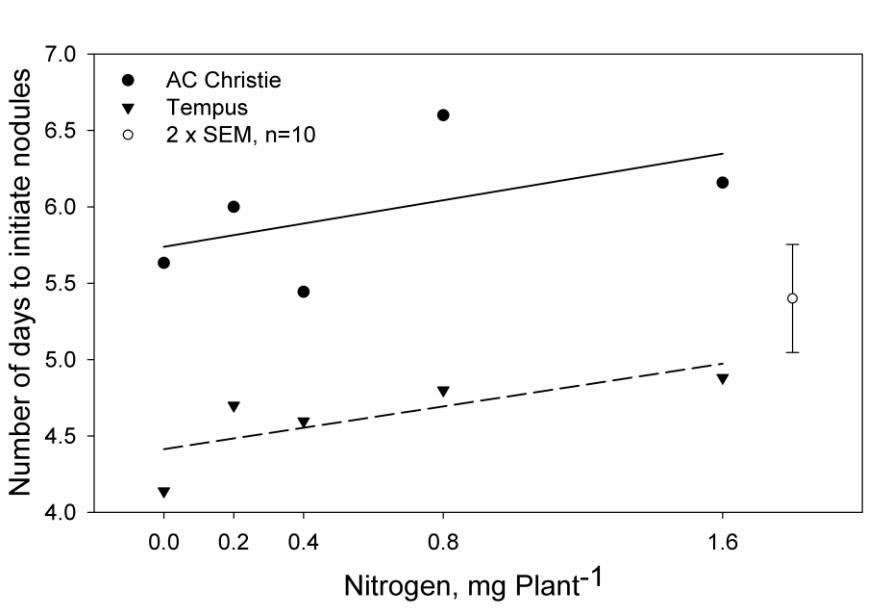

Figure 3.2 Number of days-to-initiate nodules for two red clover cultivars, AC Christie and Tempus, regressed on starter N concentration.
Number of replicates (n) = 10, vertical bar represents two standard errors of the mean (SEM). [6]Significant cultivar effect for days-to-nodule initiation ($P = 0.002$), with positive linear response for N addition ($P = 0.035$).

3.4.3 Nodulation Profiles

An interaction was found between red clover cultivars and N additions for active and total nodule number at harvest (Figure 3.3 and 3.4), with a quadratic relationship evident with the starter N profile ($P < 0.001$). A positive quadratic response to starter N fertilizer was evident in AC Christie while a negative quadratic response was evident in Tempus for both attributes. For the 0.8 mg plant^{-1} starter N concentration, AC Christie had the greatest active nodule number while Tempus had the least. For total nodule number at 0.4 mg plant^{-1} N, AC Christie had the greatest and Tempus the least. Initial nodulation as a response to starter N at four weeks after inoculation differed between AC Christie and Tempus. A strong linear relationship was evident for starter N levels and active nodule numbers; a positive response to starter N was observed in AC Christie while a negative response was evident in Tempus (Appendix V).

[6] Additional caption was included to explain the cultivar effect and trends showed in the Figure 3.2.

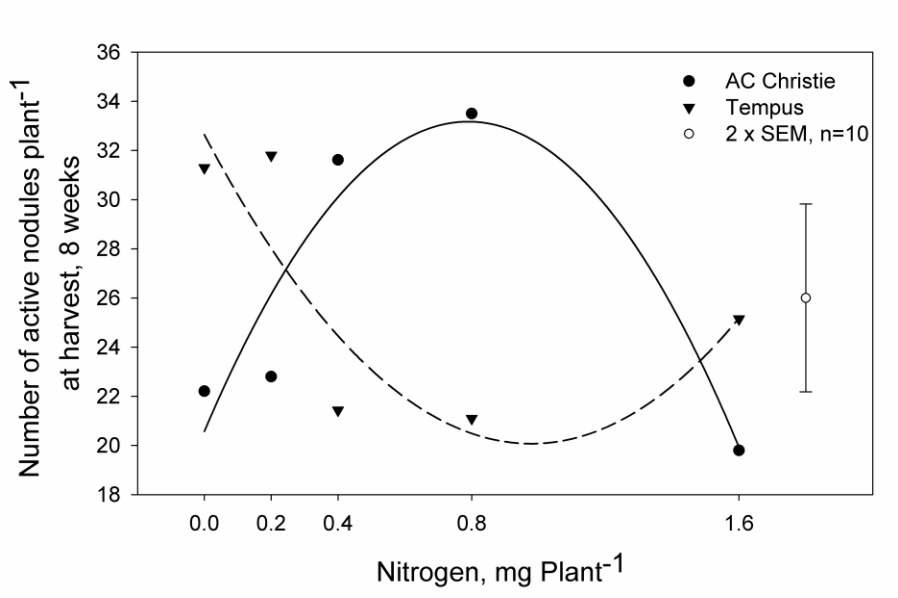

Figure 3.3 Active nodules per plant in response to starter N for two red clover cultivars, AC Christie and Tempus, eight weeks after inoculation with rhizobia.
Number of replicates (n) = 10, vertical bar represents two standard errors of the mean (SEM). [7]Significant cultivar effect for number of active nodules at harvest ($P = 0.035$), with quadractic responses for N addition ($P < 0.001$).

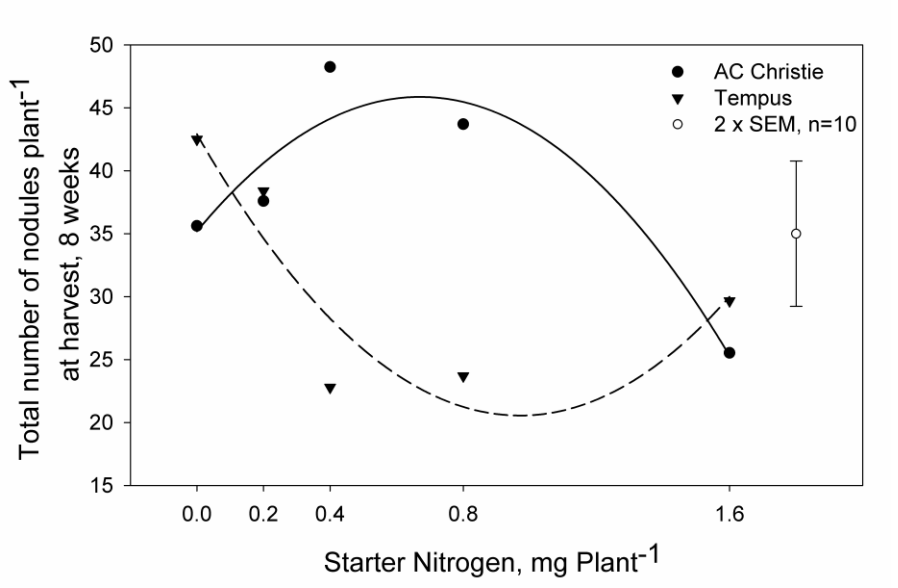

Figure 3.4 Total nodules per plant in response to starter N for two red clover cultivars, AC Christie and Tempus, eight weeks after inoculation with rhizobia.
Number of replicates (n) = 10, vertical bar represents two standard errors of the mean (SEM). [8]Significant cultivar effect for total number of nodules at harvest ($P < 0.001$), with quadractic responses for N addition ($P = 0.001$).

[7,8] Extra captions were included to explain the cultivar effect and trends showed in the Figure 3.3 and 3.4.

Active nodule numbers (%) at harvest was calculated as the number of active nodules compared with total nodule numbers. This attribute was greater in Tempus (92 %) than AC Christie (73 %, $P = 0.002$). There was a general quadratic trend between active nodule numbers at harvest and N additions ($P = 0.065$) (Figure 3.5). Active nodule numbers gradually increased from 0 to 0.8 mg N plant^{-1} but decreased at 1.6 mg N plant^{-1}.

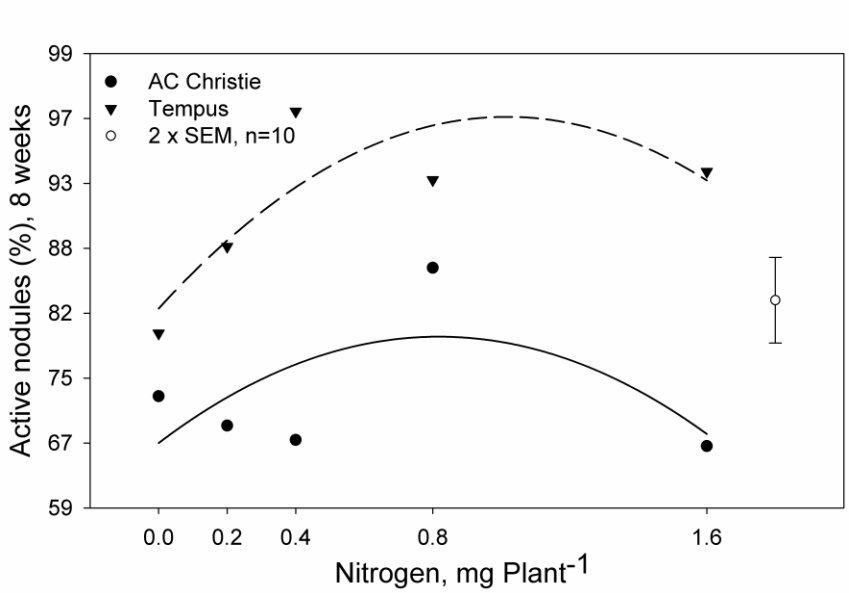

Figure 3.5 Active nodules per plant (%) in response to starter N for two red clover cultivars, AC Christie and Tempus, eight weeks after inoculation with rhizobia. Number of replicates (n) = 10, vertical bar represents two standard errors of the mean (SEM). [9]Significant cultivar effect for active nodules (%) at harvest ($P = 0.002$), with quadractic responses for N addition ($P = 0.065$).

Shoot and root dry weight was greater for Tempus than AC Christie ($P < 0.05$), which reflects genotypic differences between the two cultivars for plant growth (Table 3.1). The shoot dry weight of Tempus (263 mg plant^{-1}) at harvest was 44 % greater than AC Christie (182 mg plant^{-1}). Similarly, the root dry weight of Tempus (232 mg plant^{-1}) was 52 % greater than AC Christie (153 mg plant^{-1}). Plant leaf area also indicates plant growth and external nutrient supply, especially for N. Tempus had a greater leaf area (45.4 cm^2 plant^{-1}) compared with AC Christie (34.5 cm^2 plant^{-1}) (Table 3.1). Starter N additions did not significantly affect leaf area, shoot dry weight, and root dry weight at $P < 0.05$ (Table 3.1).

[7,8] Extra caption was included to explain the cultivar effect and trends showed in the Figure 3.5.

Total root length, root surface area, average root diameter, and root volume at harvest were used to interpret the root morphology of the two red clover cultivars receiving starter N. No significant differences were found between the two cultivars for total root length and root volume at harvest, but root diameter was greater for Tempus than AC Christie (Table 3.1). Starter N affected total root length ($P = 0.065$) with the greatest root length found at 0.2 mg N plant^{-1} concentration (Table 3.1). The root surface area for AC Christie (108.2 cm^2) was greatest at the starter N concentration of 0.4 mg N plant^{-1} but for Tempus (123 cm^2) was at 0.2 mg N plant^{-1}. The results indicate that both cultivars have greater root diameters at the starter N concentration of 0.4 mg N plant^{-1}.

Table 3.1 Different shoot (shoot dry weight, leaf area) and root traits (root dry weight, total root length, root surface area, root diameter, and root volume) at harvest for two red clover cultivars, AC Christie and Tempus, under five starter N concentrations (0, 0.2, 0.4, 0.8, and 1.6 mg N plant^{-1})

Red clover cultivars	N levels (mg)	Shoot dry weight (mg)	Root dry weight (mg)	Leaf area (cm^2)	Total root length (cm)	Root surface area (cm^2)	Root diameter (mm)	Root volume (cm^3)
AC Christie (n = 10)	0	189	151	38.5	606	81.7	0.459	257
	0.2	149	136	29.5	617	70.9	0.389	246
	0.4	194	168	35.4	680	108.2	0.489	295
	0.8	213	178	39.6	574	76.6	0.434	239
	1.6	167	134	29.4	616	80.2	0.398	262
Tempus (n = 10)	0	215	197	40.6	671	103.6	0.510	286
	0.2	288	263	48.4	773	123.0	0.522	330
	0.4	280	245	45.8	654	116.5	0.560	287
	0.8	249	216	45.2	562	99.8	0.515	263
	1.6	284	241	47.2	727	114.7	0.517	309
Grand mean		223	193	40.0	648	97.5	0.479	277
SEM (Cv × N)		25	23	4.0	61	8.3	0.022	26
F-prob (N)		ns	ns	ns	*	**	***	ns
F-prob (Cv)		***	***	***	ns	***	***	ns
F-prob (Cv × N)		*	ns	ns	ns	**	ns	ns

All the units are per plant basis. SEM = standard error of mean; F-prob = F-probability where ns indicates (P > 0.10); Cv = cultivar; Nit = nitrogen; Cv × N = cultivar by nitrogen interaction. *, **, *** Significant at the 0.10, 0.05, and 0.01 probability levels respectively.

Nit has been replaced with N in the Table 3.1 in order to be consistent throughout this dissertation.

3.4.4 Principal Component Analysis (PCA)

In the PCA biplot (Figure 3.6) attribute points with angles < 90° to each other are positively correlated, whereas those with angles > 90° are negatively correlated. Angles of 0° or 180° indicate a perfect positive or negative relationship, respectively. As a two-dimensional array, attributes that are closest are the ones most closely related to each other. The first two principal components (PCs) explain 91 % of the total variation for the 10 attributes (Figure 3.6); the PC1 and PC2 account for 77 % and 14 % of the total variation, respectively. The PC1 distinguishes differences between the two red clover cultivars with a weighted average of the variates. Tempus obtained positive PC1 scores, while AC Christie obtained negative PC1 scores. Since shoot dry weight, root dry weight, and root diameter were close on the positive side of PC1, they were the most influential for distinguishing cultivar differences. Plant vigor, leaf area, root surface area, and projected root area had the next highest degree of influence in distinguishing Tempus and AC Christie. Most of the variates tested were highly correlated and overlapped or were very close to each other on the biplot. Root diameter was highly correlated to shoot dry weight and root dry weight. Plant vigor and leaf area were also highly correlated, whereas projected root area and root surface area were also positively correlated. Active nodules at four weeks after inoculation (%) were negatively correlated with active nodules at harvest (%). Total root length was strongly correlated with active nodules one month after inoculation, but negatively correlated with the number of active nodules at harvest. Separation among N additions was measured by PC2. At the starter N concentration of 0.8 mg N $plant^{-1}$, both cultivars had high values for active nodules at harvest (%), plant vigor, and leaf area.

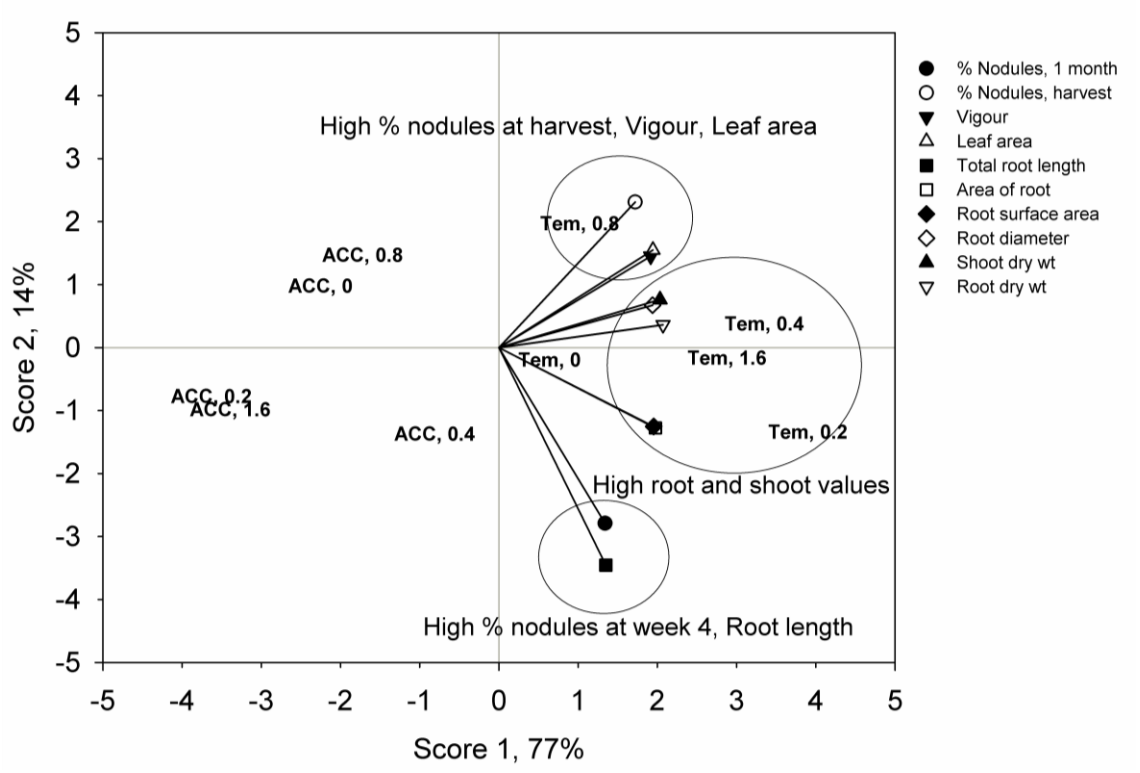

Figure 3.6 Principal component analysis of different variates (active nodules (%) after four weeks and harvest, plant vigor, leaf area, total root length, projected area of roots, root surface area, root diameter, shoot dry weight, and root dry weight) for two red clover cultivars, AC Christie and Tempus, under five starter N concentration (0, 0.2, 0.4, 0.8, and 1.6 mg N plant^{-1}).
T; Tempus, ACC; AC Christie

3.5 Discussion

Infection of legume roots with appropriate rhizobia is essential for successful nodulation. Rhizobia enter plant roots mainly via root hairs or through cracks in root epidermal tissue (reviewed by Oldroyd and Downie 2008). Root hair infection is common and leads to the development of infection threads. Different types of root hair deformation have been reported, i.e., curling, wigging, bulging of root hair tips, and bulging of root hair base (Prithiviraj et al. 2000). Our results for red clover corroborate earlier research on soybean; genotypic differences were identified in the root hair

T and C have been replaced with Tem and ACC respectively in the Figure 3.6 in order to be consistent throughout this dissertation.

deformation of soybean cultivars in response to Lipo-chito-oligosaccharide application (Prithiviraj et al. 2000). Cultivars that produced curling roots had more nodules while cultivars that produced bulging roots had fewer. This result shows that the type of root hair deformation is also important for successful nodulation (Prithiviraj et al. 2000).

The positive linear relationship between starter N concentration and days-to-nodule initiation shows that N availability or application during the early growth stages of red clover delays nodule initiation. Interestingly, the existence of genotypic differences for nodule initiation in response to starter N is advantageous for selecting suitable red clover cultivars for soils differing in N status. Delayed nodulation is a disadvantage for forage legumes competing for resources, especially in mixed crops with grasses. Schomberg and Weaver (1992) report delayed nodulation in arrowleaf clover with high N applications (10 mg plant^{-1}). High N levels inhibit early cell division in the cortex, which develop into nodule primordia (Gentili et al. 2006). Since seeds have limited nutrient reserves, plants with early nodulation establish successfully. Early nodulation in Tempus may facilitate early establishment and the onset of biological N fixation. When initial N concentration increases gradually, it delays nodule initiation which can delay the onset of biological N fixation.

For the active nodules, Tempus was more sensitive to high starter N concentrations during early nodulation (at four weeks) which reduced the number of initial active nodules. In contrast, AC Christie responded positively to starter N during early nodulation. The same trends existed for active nodule number at harvest (eight weeks after rhizobia inoculation), but the trends were quadratic (Figure 3.3). Clearly, the nodulation profile of these two cultivars differs in response to starter N. Genotypic differences have also been identified in the nodulation of soybean cultivars receiving different N fertilization under field conditions (Wahab and Abd-Alla 1996). This result corroborates our identification of genotypic differences in red clover cultivars for nodulation in response to different starter N. Many studies report that nodulation and N fixation are stimulated under low N conditions and suppressed at high N concentrations (Gulden and Vassey 1997; Yinbo et al. 1997; Daba and Haile 2002; Fei and Vessey 2003, 2004, 2009; Clayton et al. 2004; Indieka and Odee 2005). Based on our results, however, it is clear that this trend is not consistent among cultivars within a species. Under field

conditions, soil is diverse in terms of N availability, ranging from N deficient to N excess systems. Identifying genotypic differences in terms of nodulation with respect to available N, therefore, will help farmers to select cultivars based on the availability of soil N.

The number of active nodules at harvest (%) shows a quadratic increase with the starter N concentration for both red clover cultivars (Figure 3.5). Although legume plants can produce both active nodules and pseudo nodules, atmospheric N can only be fixed by active nodules due to the presence of oxygen-carrying leghemoglobin (Sprent 2008). Tempus has an advantage over AC Christie in that its higher percentage of active nodules leads to greater biological N fixation while minimizing the energy allocation needed to maintain pseudo nodules. Leps et al. (1980) reports that tetraploid alfalfa plants had higher N fixation activity during early growth (in terms of acetylene reduction assay) compared with diploid plants. The stimulation of greater nodulation can be associated with higher levels of cytokinins and may not affect auxin levels in the roots (Fei and Vessey 2004).

Legumes develop either determinate or indeterminate nodules; red clover develops indeterminate nodules. Fei and Vessey (2004, 2009) suggest that low levels of ammonium may only stimulate nodulation in legumes with indeterminate nodule development. Since we used ammonium nitrate (NH_4NO_3) as the starter N, our results cannot confirm those of Fei and Vessey (2004, 2009). Nitrogen concentration can also affect nodule size with low and medium N levels inducing larger nodules in red clover (Hellsten and Huss-Danell 2000). Soil inorganic N content is the main determinant of N fixation. In legume pasture mixtures with low soil N, legumes dominate and derive most N from N_2 fixation; grasses dominate and have a greater advantage over legumes under high soil inorganic N due to a high energy equivalent for biological N fixation (Ledgard 2001).

Greater leaf area increases the interception of incident radiation which results in greater dry matter accumulation and yield. Since carbon is essential for efficient biological N fixation, more photosynthetically fixed carbon is directed to efficient N fixation. Tempus, with a greater leaf area and root system than AC Christie, is more competitive under field conditions. Red clover has a thick taproot from which lateral

roots develop and branch. The uppermost part of the root system has most of the dry matter (Hellsten and Huss-Danell 2000). According to the traits we evaluated, it is clear that AC Christie and Tempus have different root structures.

The positive PC1 scores for Tempus and the negative PC1 scores for AC Christie confirm distinct differences between the two cultivars. According to PCA results, the percentage of active nodules at week four was in contrast to the percentage of active nodules at harvest. In general, cultivars had a higher percentage of active nodules at week four, and a lower percentage of active nodules at week eight. This response appears to be affected by the starter N level and cultivar. Supplementing plants with high N early in the establishment phase appears to initially interfere with nodule development, but this effect eventually diminished. The PC2 scores show different responses to N for each cultivar. At the starter N concentration of 0.8 mg N plant^{-1}, both red clover cultivars had a high percentage of active nodules and a greater leaf area at harvest. Having more active nodules at harvest and a greater leaf area can be explained in two ways using a sink-source relationship: more active nodules help plants to fix more N and translocate this fixed N for greater shoot growth. On the other hand, active nodules also act as a strong sink for energy (carbon). Therefore, plants need to develop a greater canopy to intercept incoming radiation and produce more photosynthates that are required to maintain active nodules.

In conclusion, we found genetic differences between red clover cultivars for root hair deformation. Days-to-nodule initiation depends on the cultivar, and a high concentration of starter N delays nodulation. The different nodulation patterns of the cultivars in response to starter N highlight the importance of matching starter N levels to the nodulation profiles of red clover cultivars under various cropping systems.

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Chapter 4

Nodulation, Plant Growth, Nitrogen Fixation, and Nitrogen Exudation of Diverse Red clover (*Trifolium pratense* L.) Cultivars during the Early Stages of Seedling Development

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4.1 Abstract

Plant and environmental factors affect root nitrogen (N) exudation in legumes, but research on the effects of plant genotypes is limited. Nodulation, plant growth, tissue N content, and root N exudation of six diverse (three diploid and three tetraploid) red clover cultivars were compared under controlled environmental conditions during the first eight weeks of plant growth. Significant genotypic differences were found for nodulation (active nodule number, nodule size, days to nodule initiation), shoot, root, and total plant dry weight, leaf area, root attributes (root length, surface area, volume, and diameter), shoot and root N concentration, and N content. On average, tetraploid cultivars had greater means for most attributes tested than the diploids. Significant genotypic differences were found for root exudate N content in terms of NO_3^--N, NH_4^+-N, and dissolved organic N (DON). In general, root exudate inorganic N content was greater in tetraploid cultivars than in the diploids throughout the growth period. Root exudate DON content was greater than the inorganic N content whereas AC Christie had the highest

DON among the cultivars. Root exudate NO_3-N content was positively correlated with root growth attributes and root N concentration while, NH_4^+-N content positively correlated with nodule number. Root exudate DON positively correlated with shoot N concentration and average nodule dry weight. The results of this study indicate that genotypic differences among red clover cultivars affect the quantities of root N release during the early stages of plant development.

4.2 Introduction

Forage legumes are an important component of legume/grass pasture systems worldwide mainly due to the role in providing significant N input through the symbiotic biological N fixation and for enhancing the nutritional quality of the grazed swards. According to Ledgard and Steele (1992), the amount of N fixed by forage legumes under legume/grass systems ranges from 13 to 682 kg N ha^{-1} yr^{-1}. Part of the N fixed by legumes can be released to neighboring non-legumes during their growth, which is referred to as N transfer (Brophy et al. 1987; San-nai and Ming-pu 2000). Nitrogen can be transferred belowground from legumes to non-legumes through different mechanisms: decomposition of belowground legume tissues including roots, nodules, root caps, root border cells, and sloughed cells wth the resulting N taken up by neighboring plants (Wichern et al. 2008; Fustec et al. 2010), plant root exudates (Paynel et al. 2008; Lesuffleur and Cliquet 2010; Fustec et al. 2010), and mycorrhizal-mediated N transfer (Haystead et al. 1988; San-nai and Ming-pu 2000; Høgh-Jensen 2006). Legume root exudates contain both low and high molecular weight N compounds (Badri and Vivanco 2009) and play a large role in transferring N to non-legumes (Paynel and Cliquet 2003; Paynel et al. 2008; Jalonen et al. 2009a, 2009b).

There are many N-containing root exudates, but ammonium and amino acids are the major forms of N exuded by clover, with ammonium contributing the most (Paynel et al. 2001, 2008; Lesuffleur and Cliquet 2010). In clover root exudates, glycine and serine are the dominant forms of amino acids (Lesuffleur et al. 2007; Paynel et al. 2008), but many others have been reported (Paynel et al. 2008). Nodules and root tips are considered to be the main sites of amino acids exudation (White et al. 2007; Lesuffleur and Cliquet 2010). Although exudation of amino acid is reported as being a passive process (Bertin et al.

2003), work by Lesuffleur and Cliquet (2010) using metabolic inhibitors indicates that root exudation may be active and under plant control. Since plants uptake N in both inorganic (NO_3^- and NH_4^+) and organic (amino acids, peptides, proteins) forms (Näsholm et al. 2009; Richardson et al. 2009), different types of N compounds in the legume root exudates can act as potential N transfer sources to non-legumes.

Plant and environmental factors clearly affect root exudation of N compounds by legumes (Paynel et al. 2008; Goergen et al. 2009; Jalonen et al. 2009b; Van Kessel et al. 2009; Mahieu et al. 2009). However, there is limited research on the genotypic variability of legume cultivars to exude different N compounds as root exudates. Previous results show that there is genetic variability among red clover cultivars for nodulaton and their root growth characteristics (Thilakarathna et al. 2012a), which may have an impact on their root exudation profiles. Findings by Paynel and Cliquet (2003) and Gylfadóttir et al (2007) showed that early during the growing season, N seems to be transferred from legumes to non-legumes as root exudates rather than through decomposing roots and nodule debris. Identification of genotypic variability among legume cultivars for N exudation and understand the relationship between N exudation and plant growth characteristics during early growth stages of the legumes will help in the development of management strategy to improve the N transfer from legumes to non-legumes.

The objectives of the present study were: 1) to evaluate the genetic variability among red clover cultivars for N exudation, and 2) to quantify the net N exuded by roots during the early stages of seedling development in red clover cultivars in terms of NO_3^--N, NH_4^+-N, and dissolved organic N.

4.3 Materials and Methods

4.3.1 Plant Materials, Rhizobia Inoculationn, and Growing Conditions

Seeds of six diverse red clover cultivars, three diploid (AC Christie, Martin et al. 1999; Tapani, Papadopoulos et al. 2008; CRS 15, Y.A. Papadopoulos, AAFC, personal communication) and three tetraploid (CRS 18 and CRS 39, Y.A. Papadopoulos, AAFC, personal communication; Tempus, http://www.inspection.gc.ca/english/plaveg/variet/regvare.shtml), 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 papers in the dark and three germinating seeds were transferred into plastic growth pouches (Mega International, Minneapolis, MN, USA) containing deionized water. One week after germination, the seedlings were thinned to one seedling per growth pouch and the plants were inoculated with a 1-ml suspension of *Rhizobium leguminosarum* biovar *trifolii* ATCC 14480, which had been grown on Trypton Yeast agar medium (Sigma Aldrich) for three days at 30 ºC. To prepare the suspension, cell density was adjusted to 10^8 cells ml^{-1} (OD_{600} = 0.1) with autoclaved distilled water.

One week following the transfer of germinating seedlings to the plastic growth pouches, plants were supplied with quarter-strength Hoagland's N-free nutrient solution (http://www.caissonlabs.com/catalog.php) where the pH of the Hoagland's solution was adjusted to 5.8. Volume of the plant growing solution in each growth pouch was maintained at approximately 25 ml during the trial. Plants were grown in a growth room with supplemental lighting maintained with a photoperiod of 16 hours of daylight at 125 µmol m^{-2} s^{-1} and 8 hours of dark (16 D: 8 N) at 23 ± 2 ºC.

4.3.2 Collection of Root Exudates and Harvesting of Plants

Plant-growing solutions which contained root exudates were transferred into 50 ml eppendorf tubes at 4, 6, and 8 weeks after inoculation with rhizobia. During the collection of the root exudates containing solution from the growing solution within individual growth pouches, the final volume was adjusted to 25 ml with deionized water. The exudate solutions were filtered through 0.45-µm micro filters and preserved at -20 ºC for detailed N analysis. Each growth pouch with red clover plant was immediately replaced with 25 ml of quarter-strength N-free Hoagland's nutrient solution.

Plants were harvested 8 weeks after rhizobia inoculation. The attributes determined were: days-to-nodule initiation, number of active nodules after 4, 6, and 8 weeks following inoculation with rhizobia, shoot dry weight (DW), root DW, and average nodule DW at harvest. Leaves were scanned using Epson Expression 1000X (Epson Canada Ltd., Markham, ON, Canada) and total leaf area was measured using the WinFOLIA (Regents Instruments Inc., Québec City, QC, Canada) software system. A

detailed root morphological analysis, including root volume, total length, surface area, and average diameter was determined using a WinRHIZO system (Regents Instruments Inc., Québec City, QC, Canada). Shoot and root DW were determined after drying the plant materials in a hot air oven set at 65 ºC for 3 days. Dry 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.

4.3.3 Plant Tissue and Root Exudate Analyses

Total N and carbon (C) content of the roots and shoots were analyzed by dry combustion at 1000 °C followed by combustion gas stream analysis of N_2 and CO_2 using a Elementar Vario MAX CN analyzer (Elementar Americas Inc., Mt. Laurel, NJ). Root exudates contained media in the growth pouches were analyzed for NO_3^--N and NH_4^+-N with flow injection 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 No 2 basal salt mix solution (http://www.caissonlabs.com/catalog.php). Total dissolved N (TDN) was determined similarily following potassium persulfate digestion at 121 °C and subsequent NO_3^--N analysis with matrix matched standards on the above described flow injection analyzer.

4.3.4 Statistical Analysis

The experimental design was a 6 × 6 Latin square design with six red clover cultivars repeated 10 times. Each attribute was analyzed using the Latin square model with row, and column as random effects and red clover cultivars as the fixed effect within the ANOVA. The results were expressed at a significance level of $P < 0.05$. Orthogonal contrasts were used to assess differences between the red clover cultivars for the different attributes. Using ANOVA, active nodule number and root exudates N were analyzed across three time points (4, 6, and 8 weeks) with repeated measurements expressed as the mean, linear, and quadratic coefficients across the growing period. Principal component analysis (PCA) was used to assess the relationship between different plant growth factors

and the different N compounds exudated by clover root systems during the growth. Following variables were log10 transformed to adhere and meet assumptions of normality in the analysis; active nodule number, number of days-to-nodule initiate, average nodule size, shoot and root C and N concentrations, N content of the root exudates containing medium in the form of NH_4^+-N, NO_3^--N and dissolved organic N. The statistical analyses of data were conducted with the software GenStat® (VSN International 2011).

4.4 Results

4.4.1 Nodulation Profiles

Active nodule numbers differed among the red clover cultivars during plant growth ($P < 0.001$, Table 4.1). The diploid cultivar CRS 15 had the greatest mean nodule number compared with the other diploid cultivars ($P = 0.002$). Number of active nodules on the red clover cultivars increased linearly during the 8 week of growth period ($P < 0.001$), but were not significantly affected by ploidy level. In general, diploid cultivars had greater specific nodulation (number of nodules g^{-1} root DW) than tetraploid cultivars ($P < 0.001$). In terms of specific nodulation, CRS 15 had greater nodulation than the other selected cultivars ($P < 0.001$). Days-to-nodule initiation depended on red clover cultivar ($P = 0.05$). In general, tetraploid cultivars had earlier nodulation (4.7 d) than diploid cultivars (4.9 d) ($P < 0.05$) (Table 4.1). Cultivars also differed for average nodule size; CRS 15 had smaller nodules than the other cultivars ($P < 0.05$, Table 4.1). Generally, average nodule size was bigger for tetraploid cultivars (0.604 mg $nodule^{-1}$) than for the diploids (0.352 mg $nodule^{-1}$, $P < 0.001$).

Table 4.1 Mean active nodules, specific nodulation at harvest, days-to-nodule initiation and average nodule dry weight of six red clover cultivars.

Red clover cultivars	Mean active nodules (# plant^{-1})		Specific nodulation (# nodules g^{-1} root DW)		Days-to-nodule initiation		Average nodule DW (mg nodule^{-1})	
AC Christie	1.171	(14.8)	2.619	(416)	0.69	(4.9)	-0.461	(0.346)
Tapani	1.085	(12.2)	2.545	(351)	0.71	(5.1)	-0.229	(0.590)
CRS 15	1.224	(16.8)	2.860	(724)	0.69	(4.9)	-0.670	(0.214)
Tempus	1.166	(14.7)	2.265	(184)	0.66	(4.5)	-0.229	(0.591)
CRS 39	1.199	(15.8)	2.498	(315)	0.68	(4.8)	-0.337	(0.460)
CRS 18	1.088	(12.2)	2.361	(229)	0.69	(4.9)	-0.091	(0.812)
Mean by ploidy level								
..Diploid	1.160	(14.5)	2.675	(473)	0.69	(4.9)	-0.453	(0.352)
..Tetraploid	1.151	(14.2)	2.375	(237)	0.68	(4.7)	-0.219	(0.604)
Grand mean	1.156	(14.3)	2.525	(335)	0.68	(4.8)	-0.336	(0.461)
SEM	0.025		0.037		0.01		0.080	
F-probability								
Ploidy	ns		<0.001		0.013		<0.001	
Cultivar	<0.001		<0.001		0.051		0.002	
..C15 vs ACC, Tap	0.002		<0.001		ns		0.002	
..Tem vs C39, C18	ns		<0.001		0.014		ns	
..C15 vs Tem	0.030		<0.001		ns		0.016	

Values in parentheses are de-transformed values.
SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18

4.4.2 Yield, Leaf Area, and Root Profiles

Genotypic differences were found for shoot DW, root DW, total DW (shoot and root), and total leaf area among the six red clover cultivars at harvest ($P < 0.001$, Table 4.2). Among the tetraploid cultivars, Tempus had the greatest means for the above-mentioned four attributes, whereas CRS 15 had the least dry matter yield and leaf area compared with the other two diploid cultivars ($P < 0.001$). Generally, tetraploid cultivars had greater means than diploid cultivars for the following attributes: shoot DW (118 vs 78 mg plant^{-1}), root DW (88 vs 52 mg plant^{-1}), total DW (206 vs 130 mg plant^{-1}), and leaf area (24 vs 17 cm^2 plant^{-1}) ($P < 0.001$).

The root growth indicators assessed in this study (rot length, surface area, volume and, average diameter) varied among the six red clover cultivars at harvest ($P < 0.05$, Table 4.2). Among the tetraploid cultivars, root surface area, average root diameter, and

root volume were greater for Tempus than for CRS 18 and CRS 39 ($P < 0.01$). CRS 15 had narrower root diameter and less root volume than the other two diploid cultivars ($P < 0.05$). Generally, tetraploid cultivars had greater means than the diploids for root growth indicators of the following attributes: root length (475 vs 402 cm), root surface area (56 vs 39 cm^2), root diameter (381 vs 305 mm), and root volume (545 vs 305 cm^3) ($P < 0.001$).

Table 4.2 Shoot dry weight, root dry weight, total dry weight (shoot and root), total leaf area, root length, root surface area, root volume and average root diameter of the six red clover cultivars after 8 weeks of seedling growth.

Red clover cultivars	Shoot DW[z] (mg)	Root DW (mg)	Total DW (mg)	Total leaf area (cm^2)	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Average root diameter (mm)
AC Christie	88.8	59.6	148	19.5	408	40.5	0.335	0.317
Tapani	85.1	54.7	139	18.8	384	38.2	0.312	0.314
CRS 15	59.8	42.7	103	13.5	414	37.7	0.266	0.284
Tempus	141.4	107.1	248	26.8	460	60.0	0.628	0.412
CRS 39	105.7	74.0	180	22.5	516	55.6	0.494	0.343
CRS 18	107.1	82.7	190	22.7	449	52.5	0.518	0.386
Mean by ploidy level								
..Diploid	77.9	52.3	130	17.2	402	38.8	0.305	0.305
..Tetraploid	118.1	87.9	206	24.0	475	56.0	0.546	0.381
Grand mean	98.0	70.1	168	20.6	438	47.4	0.426	0.343
SEM	5.1	3.5	8	1.0	14	1.8	0.023	0.007
F-probability								
Ploidy	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cultivar (n = 60)	<0.001	<0.001	<0.001	<0.001	0.005	0.050	<0.001	<0.001
..C15 vs ACC, Tap	<0.001	<0.001	<0.001	<0.001	ns	ns	<0.001	0.041
..Tem vs C39, C18	<0.001	<0.001	<0.001	<0.001	ns	0.009	<0.001	<0.001
..C15 vs Tem	<0.001	<0.001	<0.001	<0.001	ns	0.085	<0.001	<0.001

[z]All the measurements are per plant basis.
SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

4.4.3 Plant Nitrogen and Carbon Profiles

Cultivars differed in their tissue N and C profiles (Table 4.3). Among the diploid cultivars, CRS 15 had lesser shoot N concentration, tissue N content (shoot, root and total), and shoot and root C concentration than AC Christie and Tapani ($P < 0.01$). Tempus had a greater root C concentration and tissue N content (shoot, root, and total) among the tetraploid cultivars ($P < 0.001$). Generally, N and C profiles of the tetraploid cultivars were greater than the diploids for the following attributes: shoot N concentration (2.56 vs 2.26 %), root N concentration (2.83 vs 2.47 %), shoot C concentration (39.6 vs 38.6 %), root C concentration (41.5 vs 40.7 %), shoot N content (3.08 vs 1.84 mg plant^{-1}), root N content (2.51 vs 1.31 mg plant^{-1}), and total plant N content (5.59 vs 3.15 mg plant^{-1}) ($P < 0.001$). Cultivar differences were found for plant C:N ratio with CRS 15 having a greater C:N ratio than the other cultivars ($P < 0.001$, Table 4.3). Generally, the diploid cultivars had a greater C:N ratio (16.8) compared with the tetraploid cultivars (15.1) ($P < 0.001$).

Table 4.3 Nitrogen and carbon concentration (dry weight basis) of shoot and root, N content of the shoot, root and total plant (shoot + root) and C:N ratio of the six red clover cultivars at harvest.

Red clover cultivars	Plant N (%)				Plant C (%)				N content (mg plant^{-1})			C:N
	Shoot		Root		Shoot		Root		Shoot	Root	Total	ratio
AC Christie	0.391	(2.46)	0.391	(2.46)	1.59	(39.0)	1.61	(40.8)	2.21	1.47	3.68	16.2
Tapani	0.375	(2.37)	0.403	(2.53)	1.59	(38.8)	1.61	(41.2)	2.06	1.40	3.44	16.4
CRS 15	0.294	(1.97)	0.383	(2.42)	1.58	(38.0)	1.61	(40.2)	1.26	1.05	2.31	17.9
Tempus	0.416	(2.61)	0.458	(2.87)	1.60	(39.8)	1.62	(42.0)	3.71	3.07	6.78	15.0
CRS 39	0.408	(2.56)	0.463	(2.90)	1.60	(39.5)	1.61	(41.1)	2.77	2.18	4.94	15.0
CRS 18	0.410	(2.57)	0.436	(2.73)	1.60	(39.5)	1.62	(41.4)	2.77	2.27	5.04	15.4
Mean by ploidy level												
..Diploid	0.353	(2.26)	0.392	(2.47)	1.59	(38.6)	1.61	(40.7)	1.84	1.31	3.15	16.8
..Tetraploid	0.412	(2.58)	0.452	(2.83)	1.60	(39.6)	1.62	(41.5)	3.08	2.51	5.59	15.1
Grand mean	0.382	(2.41)	0.422	(2.64)	1.59	(39.1)	1.61	(41.1)	2.46	1.91	4.37	15.9
SEM	0.016		0.008		0.002		0.002		0.14	0.10	0.22	0.16
F-probability												
Ploidy	<0.001		<0.001		<0.001		<0.001		<0.001	<0.001	<0.001	<0.001
Cultivar (n = 10)	0.001		0.090		0.007		<0.001		<0.001	<0.001	<0.001	<0.001
..C15 vs ACC, Tap	<0.001		ns		<0.001		<0.001		<0.001	0.002	<0.001	<0.001
..Tem vs C39, C18	ns		ns		ns		<0.001		<0.001	<0.001	<0.001	ns
..C15 vs Tem	ns		ns		ns		ns		<0.001	<0.001	<0.001	<0.001

Values in parentheses are de-transformed values.
SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Temp; Tempus, C39; CRS 39 and C18; CRS 18.

4.4.4 Ammonium, Nitrate, and Dissolved Organic N in Root Exudates

Root exudates NO_3^--N, NH_4^+-N, and dissolved organic N (DON) differed among red clover cultivars during the 8 weeks of growth ($P < 0.05$, Table 4.4). Among the diploid cultivars, root exudates of CRS 15 had greater mean NH_4^+-N content but the opposite trend was found for DON; CRS 15 had less DON than AC Christie and Tapani ($P < 0.001$). Root exudates of Tempus had greater amount of NO_3^--N, and NH_4^+-N than CRS 18 and CRS 39 ($P < 0.05$). Generally, root exudates of the tetraploid cultivars had greater inorganic N content than the diploids: NH_4^+-N (0.408 vs 0.366 μg plant^{-1}), and NO_3^--N (0.547 vs 0.412 μg plant^{-1}). Along the red clover age, NH_4^+-N content of the root exudates increased linearly whereas DON content increased quadratically ($P \leq 0.001$).

Table 4.4 Mean NH_4^+-N, NO_3^--N, and dissolved organic N content in the root exudates containing growing solution of the six red clover cultivars, collected at 4, 6 and 8 weeks of plant growth.

Red clover cultivars	NH_4^+-N (μg plant^{-1})		NO_3^--N (μg plant^{-1})		Dissolved organic N[a] (μg plant^{-1})	
AC Christie	-0.471	(0.338)	-0.369	(0.428)	0.471	(2.96)
Tapani	-0.466	(0.342)	-0.425	(0.376)	0.391	(2.46)
CRS 15	-0.371	(0.426)	-0.361	(0.436)	0.271	(1.87)
Tempus	-0.352	(0.444)	-0.214	(0.610)	0.422	(2.64)
CRS 39	-0.413	(0.386)	-0.300	(0.502)	0.445	(2.79)
CRS 18	-0.404	(0.394)	-0.272	(0.535)	0.387	(2.44)
Mean by ploidy level						
..Diploid	-0.436	(0.366)	-0.385	(0.413)	0.378	(2.39)
..Tetraploid	-0.390	(0.408)	-0.262	(0.547)	0.018	(2.62)
Grand mean	-0.413	(0.386)	-0.323	(0.475)	0.398	(2.50)
SEM	0.013		0.021		0.030	
F-probability						
Ploidy	<0.001		<0.001		ns	
Cultivar	<0.001		0.012		0.001	
..C15 vs ACC, Tap	<0.001		ns		<0.001	
..Tem vs C39, C18	<0.001		0.007		ns	
..C15 vs Tem	ns		ns		ns	
Cultivar quadratic	ns		ns		<0.001	
Cultivar linear	0.001		0.004		0.004	

[a] Calculated as the difference from total dissolved N and NO_3^- -N and NH_4^+ -N
Values in parenthesis are de-transformed values.
SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

4.4.5 Principal Component Analysis

The first two principal components explain 88% of the total variation for the 15 attributes evaluated in this study (Figure 4.1); score 1 and score 2 account for 70% and 18% of total variation, respectively. Score 1 distinguishes differences between the red clover cultivars; all the tetraploid cultivars were positive for score 1 while the diploids were negative. Differences between cultivars were driven by total plant N and total plant DW, which were strongly correlated. Root diameter, root volume, root % N, total leaf area, and root surface area were correlated with total plant N and total plant DW. On score 2, differences between cultivars were due to the contrast between the number of active nodules and NH_4^+-N in exudates versus nodule size and DON in exudates. The cultivar CRS 15 had the most nodules but they were smaller in size than the other cultivars. Tempus, CRS 18, and Tapani had larger nodules but they were few in number.

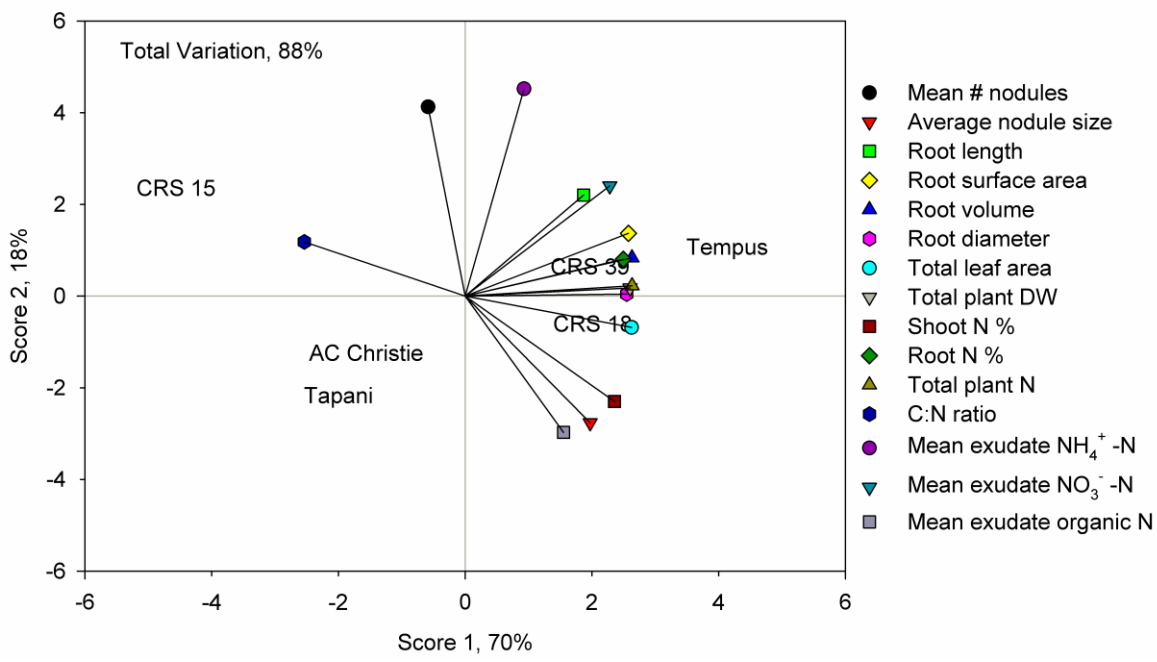

Figure 4.1 Principal component analysis of nodulation (number and size), root attributes (length, surface area, volume, diameter), leaf area, total plant DW, shoot and root N concentration, total plant N content, C:N ratio and N exuded (NH_4^+-N, NO_3^--N, and DON) during 8 weeks of growth in six red clover cultivars.

4.5 Discussion

Significant differences among the red clover cultivars for the nodulation profiles (active nodule number, average nodule size, and days-to-nodule initiation) highlight the genetic variability among cultivars for these attributes, which corroborates previous findings (Thilakarathna et al. 2012a). The cultivar CRS 15 had the most active nodules, although their average dry weight was smaller than the other cultivars. Since the area infected by rhizobia is smaller on smaller nodules (King and Purcell 2001), the efficiency of N fixation is limited. On the other hand, nodule maintenance is energy intensive (Rainbird et al. 1984), as having many nodules utilizes photosynthetically fixed carbon. These factors may have contributed to the lesser biomass of CRS 15 compared with other cultivars (similar results were found in our field studies, see chapter 6). Generally, tetraploid cultivars formed larger nodules and had greater N fixation which indicates that large nodules are better for N fixation (Vikman and Vessey 1993). Early nodulation is important when available soil N is limited and competition is intense. Some of the tetraploid red clover cultivars used in this study may be better suited than the diploids under these growing conditions.

Genotypic variability among the six red clover cultivars was also found for yield (shoot, root, and total plant DW) and plant morphological characteristics including leaf area, root length, root surface area, average root diameter, and root volume (Table 4.2), which is in agreement with previous findings (Thilakarathna et al. 2012a). Tetraploid cultivars produced greater shoot and root biomass compared with the diploids, yielding an average of 1.6 times the total plant DM. Photosynthetic rate is positively related to whole plant leaf area (Koyama and Kikuzawa 2009) and leaf N content (Reich et al. 1998). Therefore, the greater yields of tetraploid cultivars may be due to greater photosynthetic capacity (greater leaf area and leaf N content) and greater efficiency of N fixation (large nodules). Because N fixation is an energy intensive process (Halbleib and Ludden 2000), photosynthetically-assimilated C needs to be directed towards nodules to supply energy and for N assimilation. Since tetraploid red clover cultivars produced 40% more leaf area than the diploids, they may supply more C to their nodules for higher N fixation. Furthermore, their more extensive root systems may obtain more macro and micro-nutrients from the soil for better plant growth (Table 4.2).

Significant cultivar differences for tissue C and N profiles, plant N content, and C:N ratio validate genetic variability for the above attributes (Table 4.3). Compared with the diploid cultivars, tetraploids had 17% greater shoot N concentration and 78% more total plant N content at harvest, which also highlights their greater N fixation capacity. Since we did not supply any external N for plant growth, total plant N equates with total fixed N. Although CRS 15 had the most nodules, tissue N concentration and plant N content were less, which confirms that having many nodules does not always result in higher N fixation, possibly due to high C cost for nodulation (Bourion et al. 2007).

Generally, net exudation of inorganic N was greater for tetraploid red clover cultivars than for diploids in terms of NH_4^+-N, and NO_3^--N during their growth (Table 4.4). The net release of NH_4^+-N and NO_3^--N by red clover cultivars during growth was minor compared with DON. Total dissolved N is the combination of dissolved inorganic N (NH_4^+-N and NO_3^--N) and dissolved organic N (amino acids, peptides, and proteins). Therefore, it is clear that most of the N present in the red clover root exudates was in the form of dissolved organic N. In general, 74% of TDN in the red clover root exudates during the first 8 weeks of plant growth was dissolved organic N. Recent findings by Arcand et al. (2013) show that most of the rhizo-deposited N in peas and canola was in the organic N fraction. Legumes tend to increase root exudation of N as plants mature (Jensen 1996; Jalonen et al. 2009b). Similarly, as the plants matured, NH_4^+-N and DON in the red clover root exudates containing media increased linearly and quadratically over time, respectively. It is also possible that most of the released NH_4^+-N and NO_3^--N were taken-up again by the red clover plants since they would be readily available for plant uptake. Further, plants are also able to recapture released amino acids (Jones et al. 2005). Under liquid culture, there is greater uptake of amino acids than from a soil medium (Badalucco and Nannipieri 2007) mainly due to the readily-available amino acids not binding to the soil nor being immobilized by soil microbes. Therefore, we cannot directly conclude that most of the N released by red clover cultivars was dissolved organic N, without considering the plant efflux-influx mechanisms for N.

Root exudates are one of the major mechanisms for transferring N to neighboring non-legumes (Paynel et al. 2008; Jalonen et al. 2009a). Plants uptake N mainly as dissolved inorganic N as well as dissolved organic N (Näsholm et al. 2009; Tegeder and

Rentsch 2010). In general, AC Christie, CRS 39, and Tempus are considered better candidates for root exudate mediated N transfer among the selected cultivars based on root exudates DON. However, under soil conditions, dissolved organic N can be rapidly immobilized by soil microbes without plant uptake (Owen and Jones 2001; Van Kessel et al. 2009) due to a low C:N ratio (Uselman et al. 2000) and a low diffusion coefficient in soil (Jones et al. 2005), thus facilitating N transfer indirectly after microbial turnover (Jalonen et al. 2009b).

For most of the variates tested, overall performance of the different red clover cultivars can be generally ranked from high to low for root parameters, plant N content, and plant DM: Tempus > CRS 18 = CRS 39 > AC Christie = Tapani > CRS 15. This ranking is to be expected due to the different physical characteristics of tetraploids and diploids. Tetraploids have a large root structure (length, diameter, volume) which produces a larger plant (yield). Nitrogen exudation is positively correlated with root N concentration (Jalonen et al. 2009b) and total plant N content (Mahieu et al. 2009), which corroborates our PCA results. Exudate NH_4^+-N content was positively correlated with nodule number, whereas NO_3^--N content was positively correlated with root length and surface area. Despite having a smaller root structure, CRS 15 produced a greater mean NH_4^+-N content in root exudates, possibly due to its greater nodule numbers. However, exudate DON content was positively correlated with average nodule dry weight and shoot N concentration, which explain having lower DON in CRS 15 among the cultivars.

In summary, we found genetic variability among different red clover cultivars for nodulation, plant growth, plant N content, and root exudate N content. Root exudate NO_3^--N was positively correlated with root growth attributes and root N concentration while, NH_4^+-N positively correlated with nodule number. Dissolved organic N content of the root exudates positively correlated with shoot N concentration and average nodule dry weight. Understanding the genotypic variability among legume cultivars for N exudation, and its related attributes, provides valuable tools for selecting efficient cultivars to improve N transfer.

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Chapter 5

Characterizing Nitrogen Transfer from Red clover Populations to Companion Bluegrass under Field Conditions

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5.1 Abstract

The ability of two red clover (*Trifolium pratense* L.) cultivars, AC Christie (diploid) and Tempus (tetraploid), to transfer fixed nitrogen to companion bluegrass (*Poa pratensis* L.) was evaluated under field conditions. Plant samples were harvested three times during the 2009 growing season and N transfer from the red clover cultivars to bluegrass was determined using the natural abundance method for first harvest and ^{15}N dilution techniques for second and third harvests. Soil and soil water samples were used to evaluate cultivar effects on soil N conditions. Both red clover cultivars derived more than 90% of their N from biological N fixation. The proportion of bluegrass N derived from interplant N transfer was 7, 11, and 26% for the first, second, and third harvests, respectively. Soil KCl extractable nitrate increased along the three cuts for Tempus in the 0-15 cm soil zone. Soil-water nitrate content increased periodically for AC Christie and

remained constant for Tempus throughout the growing season. This result indicates that the two cultivars have distinctly different N cycling patterns.

5.2 Introduction

Red clover is one of the most important forage legumes grown in Canada, especially for livestock production and as a rotation crop. Its high biological N fixing ability and compatibility with different grasses makes it an ideal companion legume in forage mixtures (Carlsson et al. 2009). Red clover can also transfer fixed N to companion non-legumes (Ta and Faris 1987; Høgh-Jensen and Schjoerring 1994, 2000; Pirhofer-Walzl et al. 2011), which helps farmers to reduce inorganic N fertilizer applications and lessens subsequent nitrate leaching into groundwater. Transfer of N is the "movement of N from a legume to another plant, either during growth of an interplant associated with a legume component or as residual N for the benefit to a succeeding plant" (San-nai and Ming-pu 2000). Belowground N transfer can be categorized as being direct or indirect. Direct N transfer results from mycelia networks formed by arbuscular mycorrhizal fungi and directly supplies N from the donor plant to the companion plant by interconnecting the root systems of both species (Haystead et al. 1988; McNeill and Wood 1990; Dubach and Russelle 1994; He et al. 2003). Indirect N transfer occurs through the soil compartment. This takes place through the rhizodeposition of N into the soil followed by uptake by grass (Paynel et al. 2008). Proposed sources of rhizodeposition include: death and decay of nodules and roots (Ta et al. 1986; Dubach and Russelle 1994; Trannin et al. 2000; Sierra and Desfontaines 2009) and exudates from legume roots (Paynel et al. 2001, 2008; Jalonen et al. 2009a, 2009b; Sierra and Desfontaines 2009). Among the different N compounds exuded by legume roots and nodules are ammonium, amino acids, ureides, peptides, and proteins which have been identified in leachates of legumes (Ta et al. 1986; Wacquant et al. 1989; Paynel and Cliquet 2003; Paynel et al. 2008; Fustec et al. 2010). The most abundant amino acids found in clover root exudates are serine and glycine (Paynel and Cliquet 2003).

The N requirement of grasses grown in legume-grass mixtures can be met, in part, via transfer of symbiotically-fixed N from legumes to non-legumes (Walley et al. 1996). When considering the transfer distance of N from legumes to adjacent non-legumes,

Gebhart et al. (1993) report that N transfer occurred over a distance of at least 25 cm, and that N transfer increased as the proportion of legumes increased in the mixture. Also, Paynel et al. (2008) report that N fertilizer increase N transfer between legumes and grasses because the resultant increase in soil exploration by grasses provides greater access to available N sources, including the N compounds exuded by clover.

It is important to investigate the major aspects associated with N transfer during the growth of pasture stands. The objectives of this field study were to: 1) quantify N transfer during the growing season, 2) evaluate the effect of cultivar and legume/grass mixture on N transfer, and 3) determine the effect of N transfer on soil mineral N. To our knowledge, this is the first field research study to evaluate the N transfer ability of a pasture legume on an individual plant basis.

5.3 Materials and Methods

5.3.1 Site Description

The experimental area was located at the Agriculture and Agri-Food Canada research farm in Nappan, NS (45°N; 64°W, 20 m above mean sea level). Mean monthly temperatures during the June, July, and August 2009 growing periods were 15.4, 17.5, and 18.6 ^{o}C, respectively. At the experimental site, total precipitation during the study period (June to August 2009) was 355 mm; 81.4, 141.8, and 131.4 mm during the months of June, July, and August, respectively. The experiment was conducted on a Gleyed Brunisolic Gray Luvisol fine sandy loam of the Queens series (Webb and Langille 1995). From 2003 to 2006, the field was cropped to corn and was not treated with fertilizer (organic or inorganic). Oats and peas were grown for silage in 2007, followed by fall rye.

5.3.2 Plant Establishment

A pure bluegrass stand was established in May 2008 (variety Ginger, seeded at 14 kg ha^{-1}). Two red clover cultivars were selected based on different ploidy levels; diploid (AC Christie, Martin et al.1999) and tetraploid (Tempus, http://www.inspection.gc.ca/english/plaveg/variet/regvare.shtml). The red clover plants were grown in a greenhouse at the Nova Scotia Agricultural College for 90 days in

plastic rootrainer trays (Beaver Plastics Ltd., Alberta, Canada). Pro-mix was used as the growing media, and plants were inoculated with *Rhizobium leguminosarum* biovar *trifolii*. Three weeks before transplanting, the red clover plants were clipped, leaving 5-cm aboveground growth to induce the growth of vigorous plants. The two red clover cultivars were transplanted individually to the field on July 15, 2008. The experimental layout was a completely randomized design with 12 field replicates per red clover cultivar.

5.3.3 ^{15}N Labelling to Determine N_2 Fixation and Transfer

Nitrogen fixation and transfer were determined using a ^{15}N isotope dilution technique as well as natural abundance methods during the second year after red clover establishment. During the 2009 growing season, individual red clover plants surrounded by bluegrass were permanently marked with 45-cm diameter plastic rings in the field. The pure bluegrass control was also permanently marked with 45-cm diameter plastic rings. Both the red clover and bluegrass inside the plastic rings were uniformly labeled with 10 atom% ^{15}N-ammonium sulphate (SIGMA-ALDRICH, Oakville, Canada) on June 10, 2009 (just after the first harvest in the first production year) according to the ^{15}N dilution technique (Mallarino et al. 1990). The ^{15}N labeled ammonium sulfate (10 atom%^{15}N) was applied at 1 kg ^{15}N ha^{-1}. A water-based solution of ^{15}N-labeled fertilizer was sprayed on the marked areas (10 mm m^{-2}) and was carefully watered down with approximately 10 mm $m^{-2}$of water. The plot outside of the ^{15}N-labelled area received an equivalent top-dressing of ammonium sulfate.

5.3.4 Harvest and Analysis

Plants were harvested when 50% of the red clover plants reached the early blooming stage. The red clover plants, the bluegrass surrounding each red clover plant, and the pure bluegrass (control) were harvested 5 cm above the soil surface with scissors. One harvest was taken during the establishment year (late September 2008) and three harvests were taken during the growing season, on June 08, July 14 and Aug. 14, 2009. Herbage was harvested from the 0.159 m^2 area enclosed by the plastic rings. Herbage

from the bluegrass/red clover stand was separated by species. All the harvested plant material was dried individually at 65 ^{o}C for 48 h in a forced-air oven to measure dry matter yield (DMY). Dry plant samples were ground using a Wiley mill, standard model 3 (Arthur H Thomas Co., Philadelphia, USA), to pass through a 1-mm sieve followed by a mixer mill (Retsch Germany). The plant materials were analyzed for ^{15}N and total N using a mass spectrometer (Costech ECS4010 Elemental Analyzer coupled to a Delta V mass spectrometer).

Since the plant materials from the first harvest (2009) were not labeled with enriched ^{15}N, the proportion of N derived from the atmosphere (% Ndfa) of the two red clover cultivars was calculated using the following formula, according to the natural abundance technique (Høgh-Jensen and Schjoerring 1994),

$$\%\,Ndfa = \left(\frac{\delta\ ^{15}N\ grass\,mono - \delta\ ^{15}N\ clover}{\delta\ ^{15}N\ grass\ pure - B} \right) \times 100$$

Where $\delta^{15}N$ is the ^{15}N enrichment relative to atmospheric N. The B value is the ^{15}N enrichment relative to atmospheric N, for the clover grown solely on atmospheric N. A B value of - 0.76 δ^{15}N was used (Nimmo 2011).

The *% Ndfa* of the two red clover cultivars for the second and third harvests was calculated using the following formula, according to the isotope dilution technique (Jørgensen et al. 1999),

$$\%\,Ndfa = \left(1 - \frac{atom\%\ ^{15}N\ excess_{\ (clover)}}{atom\%\ ^{15}N\ excess_{\ (grass\ pure)}} \right) \times 100$$

Where atom% ^{15}N excess = atom% ^{15}N $_{\text{(clover or grass)}}$ – 0.3663.

The ^{15}N natural abundance of 0.3663 was used to adjust for the ^{15}N abundance of plant samples from the background contribution.

The amount of N fixed (g) by different red clover cultivars was determined by

$$N\ Fixed\ =\ Red\ clover\ DMY\ \times\ \frac{\%\ \ N\ in\ Red\ clover}{100}\ \times\ \frac{\%\ \ Ndfa}{100}$$

.

The apparent transfer of N from clover to grass during the first harvest was calculated using the method cited by Høgh-Jensen and Schjoerring (1994):

$$\%\ N\ Transfer\ =\ \left(\frac{\delta\ ^{15}N\ grass\ mono-\delta\ ^{15}N\ grass\ mix}{\delta\ ^{15}N\ grass\ mono}\right)\times 100$$

.

The apparent transfer of N from clover to grass during the second and third harvests was calculated using methods cited by Jørgensen et al. (1999); the amount of biologically fixed N transferred from red clover to bluegrass was calculated, and the ^{15}N enrichment for bluegrass in pure stand was compared with that from a mixed stand:

$$\%\ N\ Transfer\ =\ \left(1-\frac{atom\%\ ^{15}N\ excess_{\ (grass\ mix)}}{atom\%\ ^{15}N\ excess_{\ (grass\ pure)}}\right)\times 100$$

.

Total N yield of the red clover and bluegrass was calculated by multiplying their dry mass by their tissue N concentration.

5.3.5 Soil Mineral Nitrogen Study

During the 2009 growing season, the same set of plants was used to evaluate the impact of two red clover cultivars (AC Christie and Tempus) under a mixed stand and a pure bluegrass stand on soil N conditions. In early spring, ceramic suction lysimeters, consisting of round-bottom porous ceramic cups (Hoskins Scientific, Ontario, Canada) with polyvinyl chloride rubber tubing attached, were permanently inserted into the soil 5 cm from the base of the red clover plant within each ring to a depth of 15 cm. As a reference control, a second set of ceramic suction lysimeters was installed outside the plot area in a pure bluegrass stand. After each rainfall that exceeded 10 mm, soil water samples were collected from the lysimeters by applying a vacuum of 80 kPa using a mobile vacuum pump (Bouman et al. 2010). Soil water samples were collected six times

over the 2009 growing season. The accumulated soil water from the ceramic cups was vacuumed up through the rubber tubing into an outside collecting amber glass bottle. Samples were immediately stored at -20 ºC until the nitrate analysis. The nitrate concentration of the samples was analyzed using a Waters Ion Chromatography System (Waters Canada Ltd.) which comprised of a Waters Model 1525 Binary HPLC Pump, a Waters Model 717-Plus Autosampler, and a Waters Model 432 Conductivity Detector. Soil solution samples were syringe-filtered with 0.45-µm nitrocellulose membrane filters in preparation for single-column ion chromatography with direct conductivity detection (Eaton et al. 2005). A Waters IC-PAK Anion HC 4.6 × 150 mm was the anion-exchange column used. The detection limit of NO_3^- analysis was 0.08 mg L^{-1}. For the soil water analysis, samples with concentrations below the detection limit were assigned a value of 0.04 mg L^{-1}, as per an option outlined in McBean and Rovers (1998).

Soil samples were collected in the establishment year (2008) and on three harvest dates of the first production year. Soil cores were obtained randomly within each ring from soil depths of 0-15 and 15-30 cm. These soil samples were extracted using 2.0 *M* KCl according to Maynard et al. (2008) and analyzed for available N (NO_3^- and NH_4^+) using Technicon Auto Analyzer II (Tarrytown, NY, USA). Total N content was also determined from the 0-15 cm soil-depth samples using the combustion method on a LECO protein/ N determinator FP-528 according to the Dumas method (Williams et al. 1998).

Initial chemical properties of the soil (pH, organic matter, CEC, P, K, Ca, Mg, Na, S, Fe, Mn, Cu, Zn, and B) during the establishment year (2008) were analyzed (Table 5.1). Soil samples were dried at 35 °C and ground to pass through a 2-mm sieve. Soil pH was determined on a 1:1 ratio of soil to distilled water (Schofield and Taylor 1955). Soil organic matter was determined by loss on ignition according to Donald and Harnish (1993). Phosphorus, K, Ca, Mg, Na, S, Fe, Mn, Cu, Zn, and B was extracted by a Mehlich 3 extractant solution and were analyzed using Jarell-Ash ICAP-9000 Plasma Spectrometer (Mehlich 1984).

Table 5.1 Chemical properties of the soil during the establishment year (2008) at two soil depths (0-15 and 15-30 cm).

	Soil depth	
Chemical properties[z]	0-15 cm	15-30 cm
Organic matter (g kg^{-1})	29.1	25.5
Soil pH	6.79	6.81
CEC (meq $100g^{-1}$)	10.56	9.89
Total N concentration (g kg^{-1})	0.52	
P_2O_5 (kg ha^{-1})	498	437
K_2O (kg ha^{-1})	398	385
Ca (kg ha^{-1})	2774	2555
Mg (kg ha^{-1})	477	464
Na (kg ha^{-1})	54	50
S (kg ha^{-1})	17	13
Fe (mg kg^{-1})	318	295
Mn (mg kg^{-1})	152	138
Cu (mg kg^{-1})	1.47	1.19
Zn (mg kg^{-1})	1.52	1.17
B (mg kg^{-1})	0.53	0.45
Base saturation (g kg^{-1})		
K	3.99	4.09
Ca	65.37	64.11
Mg	18.78	19.46
Na	1.12	1.08
H	10.77	11.26

[z]Soil organic matter was determined by loss on ignition according to Donald and Harnish (1993). Soil pH was determined on a 1:1 ratio of soil to distilled water (Schofield and Taylor 1955). Total N content was determined from the 0-15 cm soil-depth samples using the combustion method on a LECO protein/ N determinator FP-528 according to the Dumas method (Williams et al. 1998). The P, K, Ca, Mg, Na, S, Fe, Mn, Cu, Zn, and B was extracted by a Mehlich 3 extractant solution and were analyzed using Jarell-Ash ICAP-9000 Plasma Spectrometer (Mehlich 1984).

5.3.6 Statistical Analysis

A completely randomized study of two red clovers (AC Christie and Tempus) in mixture with bluegrass was established with 12 field replicates to evaluate N transfer. Yield, shoot total N, and ^{15}N were collected across three harvests of the red clover. Data were analyzed by ANOVA with repeated measurements across harvests expressed as linear and quadratic trends across the growing season. Pure stands of bluegrass were

selected to compare soil mineral N status. Orthogonal contrasts, within an ANOVA for a completely randomized design, were used to compare the pure bluegrass stand with the red clover cultivars in mixed stands with bluegrass, and between the two red clovers at a significance level of $P < 0.05$. Principal component analysis was used to compare soil N measurements and soil water nitrate response to the treatments (AC Christie, Tempus, and pure bluegrass). Data were analyzed using GenStat® (VSN International 2011).

5.4 Results

5.4.1 Forage Yield

During the 2008 establishment year, the yield of the two red clover cultivars and their associated bluegrass yield under a mixed stand were not significantly different (Appendix VII). On average, the dry matter yield of AC Christie was 7.8 g plant^{-1} whereas Tempus was 8.6 g plant^{-1} during the establishment year. During the 2009 growing season, cumulative yield of the AC Christie and Tempus cultivars across the three harvests was 36 g and 41g plant^{-1}, respectively (Table 5.2). The dry weight of the two red clover cultivars at the first, second, and third harvests, as well as total seasonal yield, were not significantly different. However the first red clover harvest in June had greater yields (mean of 15.7 g plant^{-1}) for both red clover cultivars than the July and August harvests. Similarly, mixed stand bluegrass yield was greatest for the first harvest and decreased for the second and third harvests. However, this trend was neither linear nor quadratic. Bluegrass yield in mixed stands for the three harvests, and for the cumulative seasonal yield, was not significantly different between the red clover cultivars.

Table 5.2 Yield of two red clover cultivars (AC Christie and Tempus) grown in mixed swards with bluegrass across three harvests during the 2009 growing season.

Red clover cultivars	Red clover yield (g plant^{-1})[z]				Bluegrass yield (g m^{-2})			
	Harvest				Harvest			
	1	2	3	Total	1	2	3	Total
AC Christie	15.7	8.2	11.7	35.5	223	126	78	427
Tempus	15.8	9.9	11.8	41.2	204	126	74	404
SEM[y] (n=12)	2.69	1.76	2.28	6.36	8.8	5.7	4.2	16.6
F-prob[x]	ns[w]	ns	ns	ns	ns	ns	ns	ns

[z]All measurements are on a dry weight basis.
[y]SEM = standard error mean.
[x]F-prob = F probability.
[w]ns = *P* value greater than 0.05.

NS has been replaced with ns in all the tables in the manuscript in order to be consistent throughout this dissertation.

5.4.2 Plant Nitrogen Content

Tissue N concentration of AC Christie and Tempus was not significantly different for each harvest during the 2009 production year (Table 5.3) nor in the 2008 establishment year (Appendix VII). Mean N concentration of the two red clover cultivars ranged from 3.18% to 3.54% in 2009. The greatest mean N yield per plant was from the first harvest at 0.58 g per plant. The N yield at three harvests and the cumulative total seasonal N yield were not significantly different between the two red clover cultivars. Bluegrass N from the mixed stand increased from 1.42, 1.78, and 2.06% for the first, second, and third harvests, respectively. However, this trend was neither linear nor quadratic. Total N yield for the bluegrass in mixed stands decreased from first harvest to third harvest.

Table 5.3 Nitrogen concentration and nitrogen content of the two red clover cultivars (AC Christie and Tempus) under mixed stands with bluegrass across three harvests during the 2009 growing season.

Red clover cultivars	Red clover N[z] Concentration (%)			Bluegrass N Concentration (%)			Red clover N (g plant^{-1})			Bluegrass N (g m^{-2})		
	Harvest			Harvest			Harvest			Harvest		
	1	2	3	1	2	3	1	2	3	1	2	3
AC Christie	3.18	3.51	3.38	1.40	1.80	2.05	0.51	0.28	0.41	3.10	2.28	1.59
Tempus	3.18	3.58	3.44	1.44	1.75	2.08	0.65	0.36	0.43	2.94	2.22	1.53
SEM[y](n=12)	0.14	0.06	0.13	0.04	0.03	0.03	0.14	0.07	0.09	0.13	0.10	0.08
F-prob[x]	ns[w]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

[z]All measurements are on a dry weight basis.
[y]SEM = standard error mean.
[x]F-prob = F probability.
[w]ns = *P* value greater than 0.05.

5.4.3 Nitrogen Fixation and Transfer

The N fixing capacity (%) and total N fixation for the two red clover cultivars during the 2009 production year were not significantly different across the first, second, and third harvests (Table 5.4). However, both cultivars reported high N fixing capacity across the three harvests with more than 90% of the red clover N being derived from biological N fixation. Nitrogen fixation for both red clover cultivars increased from the first to third harvest, but the increase was not statistically significant. The greatest N fixing capacity was found during the third harvest at 98.7%.

Table 5.4 Percentage (%) nitrogen derived from atmosphere (%Ndfa) by two red clover cultivars (AC Christie and Tempus) and the amount of N fixed under mixed stand with bluegrass at three harvests during the 2009 growing season.

Red clover cultivars	Ndfa by red clover[z] (%)			N fixed (g plant^{-1})			
	Harvest			Harvest			
	1	2	3	1	2	3	Total
AC Christie	90.3	97.6	98.8	0.46	0.28	0.41	1.14
Tempus	91.6	97.7	98.7	0.59	0.35	0.42	1.37
SEM[y](n=12)	0.92	0.32	0.20	0.12	0.06	0.09	0.24
F-prob[x]	ns[w]	ns	ns	ns	ns	ns	ns

[z]All measurements are on a dry weight basis.
[y]SEM = standard error mean.
[x]F-prob = F probability.
[w]ns = *P* value greater than 0.05.

Nitrogen in bluegrass derived from red clover N-transfer increased gradually over the season; mean N was 7, 11, and 26% for the first, second, and third harvests of 2009 respectively (Figure 5.1). The quantity of N transferred from red clover to bluegrass on a per-plant basis was 33, 41, and 67 mg per red clover plant for the first, second, and third harvests, respectively (Appendix VIII). There were no significant differences between the N transfer ability of the diploid AC Christie and that of the tetraploid Tempus cultivar. The seasonal total of N transferred to bluegrass was 140 mg per red clover plant with no significant difference between the two red clover cultivars (Appendix VIII).

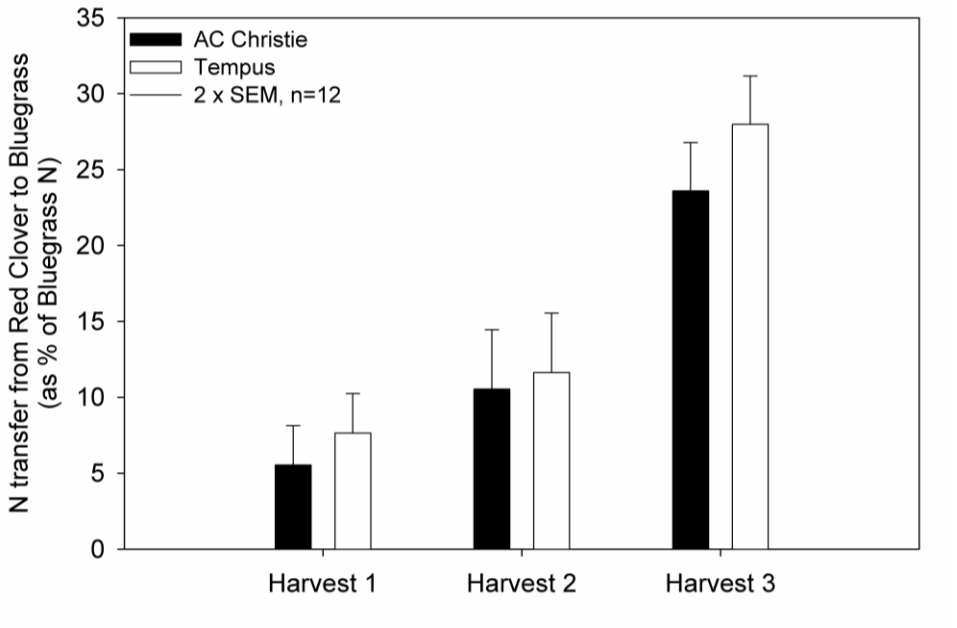

Figure 5.1 Nitrogen (%) in bluegrass transferred from AC Christie and Tempus red clover cultivars throughout the 2009 growing season.
Vertical bars indicate standard error of the mean.

5.4.4 Soil Mineral Nitrogen Study

Based on soil N results for the 2008 establishment year, the soil nitrate and ammonium content of AC Christie and Tempus under mixed stands of bluegrass and pure bluegrass at a 0-15 cm depth were not significantly different (Table 5.5). However, at a depth of 15-30 cm, the soil nitrate content associated with the pure bluegrass sward was significantly greater compared with the mixed stands which contained either Tempus or AC Christie ($P = 0.006$).

Soil extractable nitrate content of the 0-15 and 15-30 cm soil depth were not significantly different between the two red clover cultivars (under mixed stand with bluegrass) and pure blue grass over three harvests during the 2009 production year (Table 5.5). At 0-15 cm, soil nitrate content below Tempus-bluegrass mixed stand increased along the three different cuts (4.75, 9.60, and 12.04 mg kg^{-1} dry soil) but the nitrate content under AC Christie-bluegrass and pure bluegrass did not follow the same trend. Soil extractable ammonium content of the 0-15 and 15-30 cm soil was also not significantly different under the two red clover/bluegrass mixed stands and the pure stand

of bluegrass at the three harvests during the 2009 production year. Based on the total soil N data at the top 0-15cm soil depth, total soil N content was not significantly different between AC Christie, Tempus (mixed stand with bluegrass), and pure bluegrass at three harvesting points (Table 5.5). The mean total N concentration of the top 15 cm soil was 0.070, 0.064, and 0.064 % with respect to first, second, and third harvests.

Table 5.5 Soil extractable nitrate, ammonium, and total nitrogen concentration of the 0-15 cm and 15-30 cm soil depths during the establishment year (2008) followed by the production year (2009) for AC Christie and Tempus under mixed stand with bluegrass compared to pure bluegrass stand.

Year	Harvest	Plant type	Nitrate[z] (mg kg^{-1} dry soil)		Ammonium[z] (mg kg^{-1} dry soil)		Total N^z (%)
			0-15 cm	15-30 cm	0-15 cm	15-30 cm	0-15 cm
2008		AC Christie	0.613	0.458	0.109	0.002	
		Tempus	0.065	0.142	0.172	0.394	
		Pure bluegrass	0.533	1.608	0.020	0.000	
		SEM[x]	0.316	0.006	0.066	0.221	
2009	1 (June 8)	AC Christie	2.697	5.680	0.515	0.121	0.072
		Tempus	4.750	4.880	0.372	0.073	0.078
		Pure bluegrass	3.050	6.770	0.359	0.198	0.052
		SEM	0.841	0.770	0.068	0.040	0.015
	2 July 14	AC Christie	9.170	6.400	0.333	0.056	0.067
		Tempus	9.600	6.660	0.447	0.117	0.063
		Pure bluegrass	4.105	6.160	0.318	0.222	0.059
		SEM	2.600	0.814	0.068	0.060	0.005
	3 Aug 14	AC Christie	2.440	4.510	0.646	0.211	0.064
		Tempus	12.04	7.220	0.729	0.224	0.066
		Pure bluegrass	1.346	5.340	0.643	0.197	0.061
		SEM	4.590	0.088	0.106	0.040	0.011

[z]All measurements are on a dry weight basis.
[x]SEM = standard error mean.

Soil water samples collected from 15-cm deep lysimeters were analyzed for nitrate and plotted as cumulative nitrate values (Figure 5.2). Tempus showed the lowest soil water nitrate throughout the growing season. Soil water nitrate concentration under AC Christie mixed stand fluctuated over the season compared with Tempus under mixed

stand and pure bluegrass. Soil water nitrate increased at the end of July (July 21 and 31) for AC Christie in mixed stands but remained constant for Tempus in mixed stands. In pure bluegrass stands, soil water nitrate levels were comparatively higher in late June and remained constant throughout the growing period.

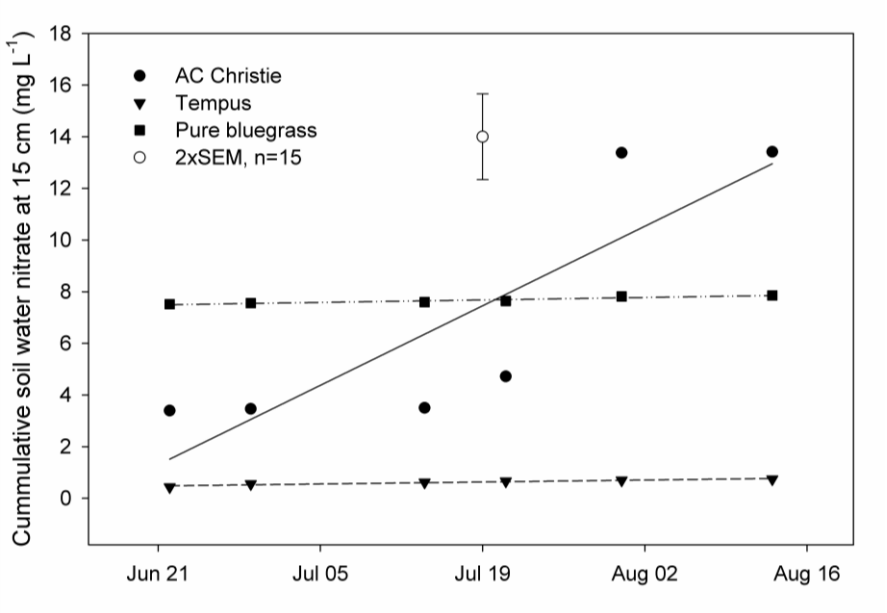

Figure 5.2 Variation of cumulative soil water nitrate-nitrogen in top 15 cm of two red clover cultivars (AC Christie and Tempus) vs pure bluegrass during the 2009 growing season.
The regression line for each species was fitted using linear regression analysis (n = 15). *P* = 0.002, r^2 = 0.85

In the principal component analysis biplot, the first two principal components (PC) explained 76% of total variation for the five quantitative traits studied (Figure 5.3). The PC1 and PC2 accounted for 47 and 29% of the total variation, respectively. Score 1 depicts a contrast between soil-water nitrate at 15 cm and for soil nitrate at the two sampling depths (0-15 and 15-30 cm). At the third harvest, Tempus had higher soil nitrate concentration at both sampling depths (Figure 5.3). AC Christie released more N into the soil water later in the season than Tempus. Score 2 is a weighted average dominated by soil ammonium at 0-15 and 15-30 cm depths. Tempus had a substantial increase in soil ammonium over the season, while bluegrass and AC Christie were not significantly affected by the score 2 soil ammonium response. Principal component analysis depicts a positive correlation between soil ammonium for both sampling depths. There was also a

positive correlation between the two depths for nitrate but a negative correlation between soil nitrate and soil-water nitrate. Since soil nitrate and ammonium are dominated on two different scores, it is unlikely that they have any relationship to each other.

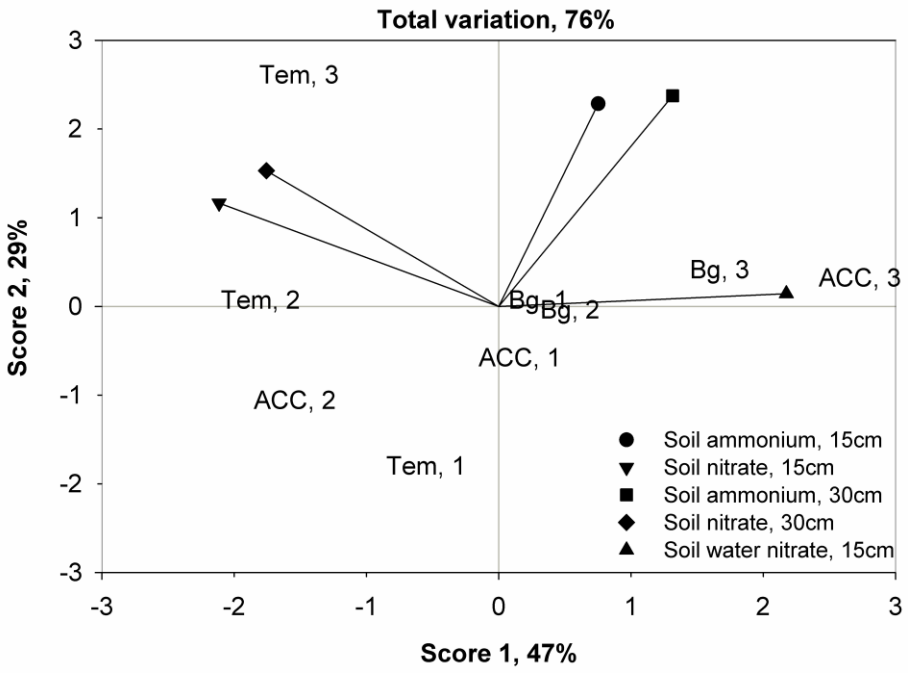

Figure 5.3 Principal component analysis of soil extractable nitrate and ammonium at two soil depths (first 15 cm and 15-30 cm) representing two red clover cultivars (AC Christie and Tempus) and pure bluegrass.
Tem, Tempus; ACC, AC Christie; Bg, pure bluegrass; 1, first harvest; 2, second harvest; 3, third harvest.

5.5 Discussion

Although AC Christie and Tempus have different ploidy levels (diploid vs tetraploid), yield differences were not significant for the three harvests during the 2009 production year. Similarly, two red clover cultivars associated with bluegrass did not show any yield differences under a mixed stand. Greater nutrient availability and more favourable growing conditions in early spring (May-June) may have stimulated plant

T, C and B have been replaced with Tem, ACC and Bg respectively in the Figure 5.3 in order to be consistent throughout this dissertation.

growth, and may explain the higher yields for the first harvest of red clover and bluegrass. Repeated defoliation reduces root mass (Carrillo et al. 2011) which can reduce

yield in later harvests. There were no significant differences between the two red clover cultivars for N concentration and no change over the growing period. However, bluegrass N concentration in the mixed swards did increase from the first harvest to the third, which may indicate N transfer. Total N content (g) of the bluegrass in the red clover mixed sward decreased over the growing season.

Both red clover cultivars showed high biological N fixing capacity under bluegrass mixed stands across the three harvests, which highlights the importance of using red clover in forage mixtures. High biological N fixation is especially valuable because biologically fixed N is less susceptible to volatilization, denitrification, and leaching. Our findings corroborate results from Nyfeler et al. (2011) and Dahlin and Stenberg (2010a); red clover can derive 90-98% of their own N from BNF under mixed stand with ryegrass. The intensity of BNF by pasture legumes can vary widely according to soil properties and environmental conditions (Hardarson 1993). Ta and Faris (1988) report that high light intensity, long days, and cool temperatures (20/16 ^{o}C day/night) are optimal for N fixation in alfalfa. Low soil mineral N also promotes high N fixation in legumes (Ledgard 2001). Low total N concentration (0.05-0.08 DW basis) in the top 15 cm of soil in our experimental site may have contributed to the high BNF for the two red clover cultivars during the 2009 growing season. Cutting regimens also induce N fixation (Dahlin and Mårtensson, 2008; Dahlin and Stenberg 2010a) and our results show an increase in Ndfa (%) over the growing season. Cutting helps keep plants at a vegetative stage which may contribute to a continuous demand for N.

Based on the natural abundance method, 0 to 17% of N in grasses may be derived from clover N transfer (Høgh-Jensen and Schjoerring 1994). According to our results, 7% of N in bluegrass derived from red clover N transfer during the first harvest. Our results also show that bluegrass N derived up to 11 and 26% from clover N transfer during the second and third harvests, respectively. These results are in agreement with those of Høgh-Jensen and Schjoerring (2000).

Although there was no significant difference in N transfer ability between the two red clover cultivars, N transfer from clover to bluegrass increased over the season. Apparent N transfer tends to increase with sward age in legume sward mixtures (Jørgensen et al. 1999; Høgh-Jensen and Schjoerring 1994, 2000; Dahlin and Stenberg

2010b). Grasses prefer to uptake N released by legumes, possibly due to a lower energy cost compared with soil N absorption (Daudin and Sierra 2008). This N transfer could result from the release of N from the breakdown and decomposition of dead tissues and direct excretion from living root systems (Fustec et al. 2010). Shoot harvesting may also increase belowground N release (Trannin et al. 2000; Ayres et al. 2007; Carrillo et al. 2011). Also, defoliation affects rhizosphere respiration, stimulation of root exudates followed by rhizosphere priming affect the decomposition of soil organic matter (Fu and Cheng 2004; Hamilton et al. 2008). However, positive effects of legume defoliation on grass N nutrition mainly occurs by direct transfer of fixed N rather than from changes in the availability of soil organic matter N (Ayres et al. 2007; Saj et al. 2008).

Chu et al. (2004) report that N transfer from legume to non-legume was high under low soil-N availability using a peanut-rice model. However, Paynel et al. (2008) found more N transferred at high N levels because the increase in soil exploration by grasses provided greater access to other N sources, including N compounds exuded from clover. In contrast, Høgh-Jensen and Schjoerring (1994) report that N application does not impact N transfer from clover to associated grasses. Since we applied ^{15}N ammonium sulfate to label the plants with ^{15}N after the first harvest, N transfer from clover to bluegrass during the second and third harvests may have been affected. Since total N and ammonium content in the top soil at each harvest were low, application of ^{15}N fertilizer should not have impacted the N transfer.

Ta and Faris (1988) report that high light intensity, long days, and cool temperatures (20/16 ^{o}C day/night) were optimal for high N fixation-transfer in an alfalfa-timothy stand. Nitrogen transfer also depends on the root density of legume plants (Sierra and Nygren 2006). Since we analyzed N transfer based on an individual plant basis, our results might differ because dense clover populations affect root density and their interaction with grass roots. Therefore, it is important to consider legume plant density when evaluating N transfer data from different studies. Interestingly, Nyfeler et al. (2011) report that an increase in the percentage of grasses in a sward also increased the apparent N transfers from legumes to grasses. Therefore, belowground N transfer depends not only on the N donor plant, but also on the plant receiving the N (Pirhofer-Walzl et al. 2011).

Under mixed swards, soil extractable nitrate was greater than ammonium at both soil depths (0-15 cm and 15-30 cm) during the 2009 growing season. Based on a micro-lysimeter study, using sand as the growing medium, Paynel and Cliquet (2003) report that clover releases a large amount of ammonium compared to nitrate from root exudates. However, based on our results, nitrate was the dominant N source in the soil extracts compared to ammonium. This result is particularly relevant for producers. Even though the majority of N exuded by clover is ammonium, it rapidly converts to a nitrate form in the soil. Soil extractable nitrate levels in deep soil (15-30 cm) were consistently greater than in shallow soil (0-15 cm) for pure bluegrass stands, and were greater than in the mixed stands, which included either the red clover cultivars Tempus and AC Christie in 2008 and for the first cut of 2009. These results may have implications for nitrate leaching since further movement of nitrate in the profile will be out of the active root zone and be lost to the environment.

Tempus showed increasing soil nitrate availability over the growing season. This result strongly supports our finding of increasing N transfer from red clover to bluegrass over the growing season. Once plants mature, they may release more N from the root system into the rhizosphere that can be used by neighbouring plants. Also, nodule senescence and subsequent decomposition may enhance the N transfer capacity of red clover over the growing season. The soil N behaviour of the two red clover cultivars differed during the growing season, with Tempus contributing more N in the top 15 cm of soil. In our *in vitro* study using the same two red clover cultivars, we found that Tempus had a greater fraction of active nodules out of total nodules, and a greater root dry weight and root surface area, compared with AC Christie (Thilakarathna et al. 2012a). Having more root mass supported by a greater fraction of active nodules enhances biological N fixation and N release from belowground parts.

The PCA shows that the soil nitrate associated with Tempus increased over the three harvests while soil water nitrate levels remained low. In contrast, AC Christie had low soil nitrate late in the season. However, AC Christie released increasing amounts of nitrate (soil water nitrate) over the season, which was not retained in the soil for use by other species probably due to leaching. This result may indicate that the nitrate supplied

by AC Christie late in the season was not fully utilized by the companion bluegrass in this study.

In summary, this study quantifies and describes the cultivar effect on N transfer from two diverse red clover cultivars to companion bluegrass under field conditions. N transfer increased as the season advanced. The impact of the two red clover cultivars on soil mineral N status showed genetic variability between the two cultivars.

5.6 Acknowledgements

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Chapter 6

Red clover (*Trifolium pratense* L.) Genotype Affects Nitrogen Fixation and Transfer to Companion Kentucky Bluegrass (*Poa pratensis* L.) under Field Conditions

Thilakarathna, R. M. M. S., Papadopoulos, Y. A., Rodd, A. V., Grimmett, M., Fillmore, S. A. E., Prithiviraj, B. 2012. Red clover (*Trifolium pratense* L.) genotype affects nitrogen fixation and transfer to companion Kentucky bluegrass (*Poa pratensis* L.) under field conditions. (in process).

Publication status: In process
Contribution to research: R.M.M.S. Thilakarathna conducted the experiment.
Contribution to writing: Written by R.M.M.S. Thilakarathna and reviewed by the other co-authors.

6.1 Abstract

In the current study, the nitrogen (N) transfer from six (three diploid and three tetraploid) red clover cultivars to companion bluegrass was evaluated under field conditions for two consecutive production years. Three harvests were collected during two post establishment years (2010 and 2011) to determine N fixation and N transfer capacity from the red clover cultivars using the ^{15}N dilution technique. The effects of the various red clover cultivars on the potential for N leaching and soil N cycling were also assessed during the growing periods by analyzing soil-water samples for NO_3^- and NH_4^+, and soil samples for NO_3^-, NH_4^+, and total N, under a bluegrass mixed stand. All six red clover cultivars derived more than 92% of their shoot N from biological N fixation with significant differences among cultivars for the amount of N fixed during the growing periods. Genotypic differences among the red clover cultivars were associated with the extent of N transfer to companion bluegrass in the sward. The proportion of bluegrass N derived from interplant N transfer increased over the growing season and with each

production year. Nitrogen transfer was positively correlated with red clover yield and N fixation. Soil NO_3^- and NH_4^+ content were high under red clover cultivars in bluegrass mixed stand compared with the pure bluegrass stand. Significant cultivar differences were also found for potential NO_3^- leaching among red clover cultivars studied during the second production year only. Diverse N transfer and cycling patterns indicate genetic variability among the red clover populations assessed in this study. These results indicate the potential for developing red clover cultivars for mixed stands to improve N transfer to companion non-legume plants while minimizing N losses through leaching.

6.2 Introduction

Nitrogen is the major mineral element required by plants for growth and development, but is also the most limiting available nutrient for plant growth (Valentine et al. 2010). With the invention of the Harber-Bosh process, inorganic N fertilizers were used on a mass scale to boost world food production. However, due to the increasing cost of inorganic N fertilizers and concerns about environmental pollution (Good and Beatty 2011), attention has shifted to more environmentally friendly and sustainable methods of crop production. Legumes are capable of fixing atmospheric N_2 through symbiotic biological N fixation (BNF) which plays a major role in supplying N to cropping systems. Nitrogen fixed by legumes is also available to non-legumes by means of N transfer (Paynel et al. 2008; Fustec et al. 2010). Nitrogen transfer is the movement of N from one plant to another (Brophy et al. 1987; San-nai and Ming-pu 2000), primarily from legumes to non-legumes during the growth period of the legume plant.

Possible belowground N transfer mechanisms from legumes to non-legumes include: N released through decomposition of belowground legume tissues, N-containing root exudates, and mycorrhizal mediated N transfer (Gylfadóttir et al. 2007; Wichern et al. 2008; Dahlin and Stenberg 2010a; Fustec et al. 2010; Schenck zu Schweinsberg-Mickan et al. 2010). Nitrogen derived from decomposition of roots, nodules, root caps, root border cells, and sloughed cells can substantially contribute to belowground N transfer (Wichern et al. 2008; Fustec et al. 2010), but this is generally a slow process compared with the other mechanisms. Root exudates play a major role in short-term N transfer (Paynel and Cliquet 2003; Gylfadóttir et al. 2007); ammonium and amino acids

are the major forms of N exuded by legumes (Paynel et al. 2008; Lesuffleur and Cliquet 2010). Potential pathways of mycorrhizae-mediated N transfer from legumes to neighboring non-legumes include: uptake of N released by legumes into the soil, transfer by mycorrhizal hyphae attached to receiver roots, and direct N transfer if legume and non-legume root systems connect through common hyphae (San-nai and Ming-pu 2000; Høgh-Jensen 2006).

The extent and type of N transfer from legumes to non-legumes depends on the species (Mallarino et al. 1990; Heichel and Henjum 1991; Pirhofer-Walzl et al. 2012) and on the cultivar within a species (Laidlaw et al. 1996; Elgersma et al. 2000). Red clover is one of the most important forage legumes grown in North America. It is commonly used in clover-grass mixed stands due to its high N fixation (Nyfeler et al. 2011) and high N transfer to neighboring non-legumes (Dahlin and Stenberg 2010a; Thilakarathna et al. 2012b). Kentucky bluegrass is an important forage grass species commonly used in legume-grass mixtures in eastern Canada due to its persistence and compatibility with other forage legumes (Dürr et al. 2005). In order to maximize the N transfer, it is important to understand the genetic variability associated with different red clover cultivars for N transfer, as well as, the N cycling patterns under clover-grass mixed stands. Previous findings show that red clover cultivars differ in terms of nodulation, root characteristics, and plant growth and that these differences may have a significant impact on N transfer (Thilakarathna et al. 2012a). The main objectives of current study was to evaluate the genotypic variability of different red clover cultivars for N transfer to neighboring grasses and assess the associated N cycling patterns under field conditions for two production years.

6.3 Materials and Methods

6.3.1 Site Description and Experimental Design

The study was conducted in Nova Scotia, Canada; Agriculture and Agri-Food Canada research farm in Nappan (45°N; 64°W, 19.8 m above mean sea level) from 2009 to 2011. Monthly averages of daily mean temperature and monthly precipitation during the 2009, 2010, and 2011 growing seasons for the experimental site are shown in Figure

6.1. The soil was an imperfectly drained Gleyed Eluviated Sombric Brunisols moderately to highly permeable sandy loam of the Debert series (Webb and Langille 1995). From 2005 to 2006, the field was cropped with barley. Red clover was grown for silage during the 2007 and 2008 growing seasons.

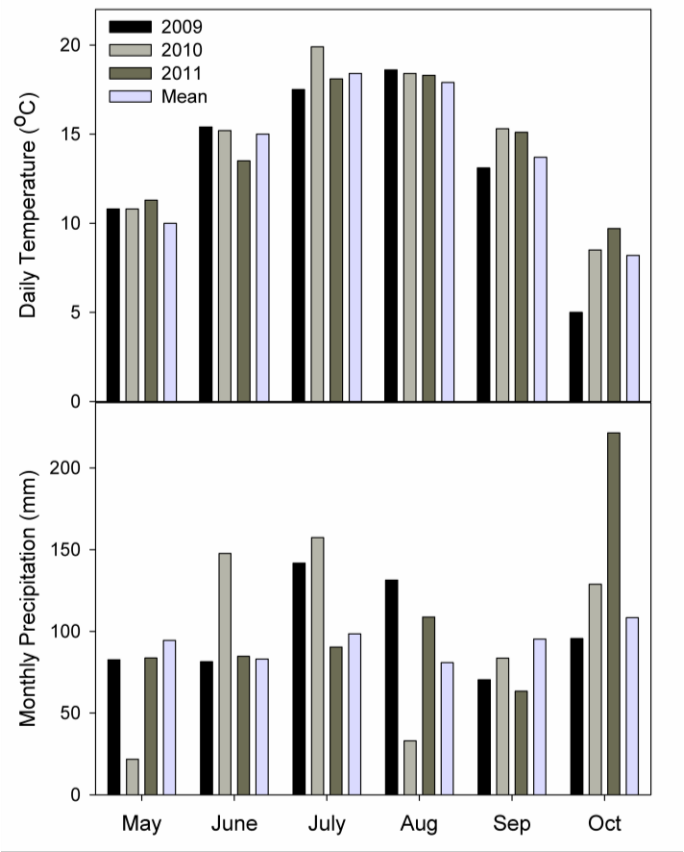

Figure 6.1 Daily temperature and monthly precipitation during the 2009, 2010, and 2011 growing seasons.

6.3.2 Plant Establishment

A pure bluegrass stand (variety Ginger, seeded at 14 kg ha^{-1}) was established at the Nappan Agriculture and Agri-Food Canada research farm on 10 June 2009. Six red clover cultivars were selected for the study based on different ploidy levels: three diploid (AC Christie, Martin et al. 1999; Tapani, Papadopoulos et al. 2008; CRS 15, Y.A. Papadopoulos, AAFC, personal communication) and three tetraploid (CRS 18 and CRS 39, Y.A. Papadopoulos, AAFC, personal communication; Tempus,

http://www.inspection.gc.ca/english/plaveg/variet/regvare.shtml accessed 05 March 2013) cultivars. Red clover plants were grown for 90 days in plastic root trainers (Beaver Plastics Ltd., Alberta, Canada) using pro-mix as the growing media under greenhouse conditions at the Atlantic Food and Horticulture Research Centre, Kentville. Plants were inoculated with *Rhizobium leguminosarum* biovar *trifolii.* Three weeks before transplanting, red clover plants were clipped leaving 5 cm above ground to induce vigorous growth. Plants of six red clover cultivars were transplanted individually into a pure bluegrass stand on 26 August 2009. Plants were spaced 60 cm apart in rows, with 1 m between rows. The experimental layout was a Latin square design (6 × 6) with each experimental unit consisting of 10 plants per row.

6.3.3 ^{15}N Labelling to Determine N_2 Fixation and Transfer

Nitrogen fixation and transfer of the six red clover cultivars were determined using the ^{15}N dilution technique. During the first production year (2010), three individual red clover plants were randomly selected from each experimental unit (10 plants), representing each of the six cultivars, and resulting in 18 red clover plants under bluegrass mixed stand receiving ^{15}N labeling. Individual red clover plants surrounded by bluegrass were permanently marked with 45 cm diameter plastic rings in the field for ^{15}N fertilizer application. As a control, 36 areas of pure bluegrass were permanently marked with similar plastic rings adjacent to each red clover experimental unit. Both red clover under bluegrass mixed stand and pure bluegrass inside the plastic rings were uniformly labeled with 10% ^{15}N ammonium sulfate (SIGMA-ALDRICH, Oakville, ON) on 13 May 2010 according to the ^{15}N dilution technique (Mallarino et al. 1990; Thilakarathna et al. 2012b). The ^{15}N-labeled ammonium sulfate was sprayed on at 1 kg ^{15}N ha^{-1} as water-based solution in 10 mm m^{-2}. Post ^{15}N application, an additional 10 mm m^{-2} of water was added. The plot outside the ^{15}N-labelled area received a top-dressing of ammonium sulfate fertilizer with an equivalent rate. The same procedure was followed during the second production year (2011) where previously ^{15}N unlabeled (from 2010) red clover plants (under bluegrass mixed stand) and pure bluegrass were selected and marked in the same manner with plastic rings to avoid a residual ^{15}N effect from the previous year. The

selected plants were labeled with 10% ^{15}N ammonium sulfate on 31 May 2011 according to the technique described above.

6.3.4 Harvest and Analysis

Red clover plants were harvested when 50% of the plants reached the early blooming stage at each harvest. The red clover plants, bluegrass surrounding each red clover plant (mixed stand), and pure bluegrass (control) were harvested 5 cm above the soil surface with scissors. Three harvests were collected from each production year: in 2010 on June 16, July 19, and August 20; in 2011 on June 29, August 10, and September 20. Herbage was harvested from the 0.159 m^2 area enclosed by the plastic rings. Herbage from red clover/bluegrass (mixed stand) was separated into bluegrass and red clover. All harvested plant materials were dried separately at 65 ^oC for three days in a forced air oven to determine dry matter yield (DMY). Dry plant samples were ground using the Wiley mill, standard model 3 (Arthur H. Thomas Co., Philadelphia, USA), to pass through a 1-mm sieve followed by a bead mill (Retsch, Germany). The plant materials were analyzed for ^{15}N and total N using a mass spectrometer (Costech ECS4010 Elemental Analyzer coupled to a Delta V mass spectrometer).

The proportion of red clover N derived from atmosphere (% Ndfa) was calculated as described by Thilakarathna et al. (2012b) according to the ^{15}N dilution method:

$$\%\,Ndfa = \left(1 - \frac{atom\%\ ^{15}N\ excess_{(clover)}}{atom\%\ ^{15}N\ excess_{(grass\ pure)}}\right) \times 100$$

where atom% ^{15}N excess = atom% $^{15}N_{(clover\ or\ grass)} - 0.3663$.

The ^{15}N natural abundance of 0.3663 was used to adjust for the ^{15}N abundance of plant samples from the background contribution. The amount of N fixed (g) by different red clover cultivars was determined by,

$$N\ Fixed = Red\ clover\ DMY \times \frac{\%\ N\ in\ Red\ clover}{100} \times \frac{\%\ Ndfa}{100}$$

The apparent transfer of N from red clover to grass was calculated by comparing ^{15}N enrichment in bluegrass under a pure stand versus the mixed stand with red clover (Thilakarathna et al. 2012b) as,

$$\%\, N\, Transfer = \left(1 - \frac{atom\%\ ^{15}N\ excess_{\,(grass\ mix)}}{atom\%\ ^{15}N\ excess_{\,(grass\ pure)}} \right) \times 100$$

6.3.5 Soil-Water Sampling and Analysis

The same experimental setup described in section 6.3.2 was used to evaluate the impact of six red clover cultivars (under mixed stand with bluegrass) and pure bluegrass stand on potential N leaching. In early spring, round-bottom porous ceramic suction cups (Hoskins Scientific, Ontario), with polyvinyl chloride tubing attached, were permanently inserted into the soil 5 cm from the base of the red clover plant, within each ring, to a depth of 45 cm. As a control, a second set of suction cups was installed in the pure bluegrass stand parallel to the red clover. Three suction cups were installed representing each red clover cultivar under mixed stand with bluegrass. After each rainfall event (greater than 10 mm), soil-water samples were collected from the suction cups by applying a vacuum of 80 kPa using a mobile vacuum pump (Thilakarathna et al. 2012b). The soil-water from the ceramic cups was vacuumed up through the rubber tubing and collected into an outside amber glass bottle. Samples were immediately stored at -20 ºC until they were analyzed for NO_3^--N and NH_4^+-N using a Technicon Auto Analyzer II (Tarrytown, NY, USA). For the soil-water analysis, samples with concentrations below the minimum detection limit were assigned a value of half the minimum detectable limit of 0.0125 mg L^{-1}, an option outlined in McBean and Rovers (1998).

6.3.6 Soil Sampling and Analysis

Soil samples were collected during the establishment year (2009), first (2010), and second (2011) production years on the harvest dates of each production year. Soil cores were sampled randomly within each plastic ring from soil depths of 0–15 cm and 15–30 cm, representing the six red clover cultivars under mixed stand with bluegrass and pure bluegrass stand. Six soil cores were collected from each experimental unit representing

red clover under mixed stand and pure bluegrass stand. The soil samples were extracted using 2 *M* KCl, according to Maynard et al. (2008), and analyzed for NO_3^- and NH_4^+ using Technicon Auto Analyzer II (Tarrytown, NY, USA). Total N and carbon (C) content from the soil collected at a 0–15 cm soil depth were determined using the combustion method on a LECO protein/ N determinator FP-528 according to the Dumas method (Williams et al. 1998). Initial chemical properties of the soil during the establishment year (2009) were analyzed (Appendix IX).

6.3.7 Statistical Analysis

Yield, tissue N content, N fixation, and N transfer data were analyzed using a Latin square model with ANOVA; rows and columns were considered to be random effects and cultivar differences to be fixed effects. Results were expressed at significant levels of $P < 0.05$. Differences between cultivars were defined using a set of orthogonal contrasts. Pure stands of bluegrass were selected parallel to the red clover experimental unit in the same field to compare soil mineral N status and soil-water NO_3^- and NH_4^+. Due to the different collection times of soil-water samples, the data were interpolated on the same timescale. Cumulative values were analyzed using a repeated measure ANOVA where the mean, linear, and quadratic coefficients over time were evaluated, with rows and columns as random effects and cultivars as fixed effects. Orthogonal contrasts between cultivars were used to assess differences between cultivars. Principal component analysis was used to explore the relationships between data from different sources. The red clover yield, N fixation, N transfer from the aboveground plant data, soil mineral N parameters from the soil data, and soil-water nitrate and ammonium from the soil-water data sets were combined in response to the six red clover cultivars under the bluegrass mixed stand. The software used for all analysis was GenStat® (VSN International 2011).

6.4 Results

6.4.1 Forage Dry Matter Yield

There were significant yield differences for the six red clover cultivars under the mixed stand with bluegrass during the first (third harvest and total seasonal yield) and

second production years (first harvest and total seasonal yield) ($P < 0.05$, Table 6.1). AC Christie and Tapani had significantly greater yield compared with CRS 15 for both production years. Dry matter yield of Tempus was greater compared with the other two tetraploid cultivars for total seasonal yield during the first production year, and for the first harvest of the second production year. Red clover yield was greater in the first harvest than subsequent harvests for both 2010 and 2011. Bluegrass yield in the mixed stand for the three harvests in both production years was not significantly different among the red clover cultivars (Table 6.1). The greatest bluegrass yield under mixed stand was for the first harvests of 2010 and 2011.

Table 6.1 Yield (dry weight basis) of six red clover cultivars grown in mixed stands with bluegrass across three harvests during the 2010 and 2011 growing seasons.

Year	Red clover cultivars	Red clover yield (g plant^{-1})				Bluegrass yield (g m^{-2})			
		Cut 1	Cut 2	Cut 3	Total	Cut 1	Cut 2	Cut 3	Total
2010	AC Christie	42.28	28.44	13.92	78.91	223.77	79.75	77.99	381.51
	Tapani	47.62	36.83	11.33	95.79	244.84	76.79	73.96	390.94
	CRS 15	32.29	26.05	7.87	66.21	196.16	72.89	70.50	339.56
	Tempus	49.29	43.93	12.01	105.20	207.48	73.46	77.04	357.99
	CRS 39	35.44	36.91	9.53	81.89	220.06	76.23	77.17	375.47
	CRS 18	36.54	36.03	10.06	82.64	205.72	73.02	69.06	347.80
	Grand mean	40.58	34.70	10.79	85.11	216.35	75.35	74.28	365.53
	SEM	4.76	3.34	1.08	7.86	18.29	3.95	4.43	24.24
	F-probability								
	Ploidy	ns	0.005	ns	ns	ns	ns	ns	ns
	Cultivar (n = 18)	0.064	0.097	0.008	0.034	ns	ns	ns	ns
	..C15 vs ACC, Tap	0.042	ns	0.002	0.040	ns	ns	ns	ns
	..Tem vs C39, C18	0.034	0.083	ns	0.027	ns	ns	ns	ns
	..C15 vs Tem	0.057	ns	0.015	0.049	ns	ns	ns	ns
2011	AC Christie	59.54	33.22	14.06	106.80	488.18	86.67	86.60	661.64
	Tapani	43.07	21.96	13.23	78.26	561.95	86.98	95.28	744.03
	CRS 15	32.47	18.65	9.37	62.70	503.02	86.73	89.69	675.47
	Tempus	58.98	30.83	10.45	100.10	519.62	97.36	92.96	710.06
	CRS 39	37.55	23.71	11.42	74.22	564.34	95.53	95.60	764.78
	CRS 18	50.95	24.70	12.58	89.58	505.85	92.26	84.84	676.10
	Grand mean	47.09	25.51	11.85	85.28	523.84	90.94	90.82	705.03
	SEM	5.72	4.31	1.67	9.12	28.71	5.01	4.92	32.67
	F-probability								
	Ploidy	ns	ns	ns	ns	ns	0.057	ns	ns
	Cultivar (n = 18)	0.008	ns	ns	0.015	ns	ns	ns	ns
	..C15 vs ACC, Tap	0.014	ns	ns	0.015	ns	ns	ns	ns
	..Tem vs C39, C18	0.048	ns	ns	ns	ns	ns	ns	ns
	..C15 vs Tem	0.038	ns	ns	0.059	ns	ns	ns	ns

SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

6.4.2 Plant Nitrogen Content

Shoot N content (g N plant^{-1}) differed among red clover cultivars for the first (third harvest and total seasonal yield) and second production years (first harvest and total seasonal yield) ($P < 0.05$, Table 6.2). Tempus had the highest shoot N content among the tetraploid cultivars for total seasonal N during 2010 and for the first harvest of the 2011 season ($P < 0.05$). Among the diploid cultivars, AC Christie and Tapani had a greater shoot N content compared with CRS 15 for total seasonal N yield in both production years ($P < 0.05$), and for the third harvest of the 2010 and first harvest of the 2011 season ($P < 0.01$). Shoot N concentration (g N per 100 g of DM) was not significantly different for the six red clover cultivars during both production years (Appendix X). Average shoot N concentration of the tetraploid cultivars was greater than the diploid cultivars at first harvest (3.04 vs 2.86%) and at second harvest (2.93 vs 2.77%) of the first production year ($P < 0.01$). No cultivar differences were found for bluegrass shoot N concentration (%) (Appendix X) and shoot N content (Appendix XI) under mixed stands for the three harvests of both production years.

Table 6.2 Shoot nitrogen content (g) of the six red clover cultivars (dry weight basis) under mixed stands with bluegrass across three harvests during the 2010 and 2011 growing seasons.

Red clover cultivars	Red clover shoots N content (g plant^{-1})							
	2010				2011			
	Cut 1	Cut 2	Cut 3	Total	Cut 1	Cut 2	Cut 3	Total
AC Christie	1.19	0.82	0.47	2.48	1.31	0.89	0.51	2.72
Tapani	1.38	1.02	0.41	2.81	1.00	0.61	0.47	2.08
CRS 15	0.91	0.70	0.26	1.87	0.76	0.49	0.31	1.62
Tempus	1.47	1.29	0.42	3.17	1.31	0.79	0.38	2.65
CRS 39	1.07	1.08	0.32	2.46	0.88	0.67	0.42	2.00
CRS 18	1.13	1.04	0.36	2.53	1.12	0.68	0.44	2.28
Grand mean	1.19	0.99	0.37	2.56	1.06	0.69	0.42	2.22
SEM	0.15	0.10	0.04	0.23	0.12	0.10	0.06	0.22
F-probability								
Ploidy	ns	0.001	ns	0.095	ns	ns	ns	ns
Cultivar (n = 18)	0.095	0.079	0.005	0.027	0.008	0.065	ns	0.011
..C15 vs ACC, Tap	0.052	0.071	<0.001	0.014	0.012	0.037	0.018	0.009
..Tem vs C39, C18	0.058	0.067	ns	0.030	0.040	ns	ns	0.070
..C15 vs Tem	0.087	ns	0.008	0.029	0.031	ns	ns	0.033

SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

6.4.3 Nitrogen Fixation

All red clover cultivars had high N fixation capacity (% Ndfa) during both production years with more than 92% of the red clover N derived from BNF (Appendix XII). Significant cultivar differences for % Ndfa were found only during the first harvest of 2011. Diploids AC Christie and Tapani had a greater N fixation capacity compared with CRS 15, but the difference was less than 1% ($P < 0.05$). Although % Ndfa was unchanged across three harvests of 2010 (97–98%), it was slightly lower (3.6%) in the third harvest (93.6%) compared with first harvest (97.2%) during 2011. Significant cultivar differences among the diploid cultivars for the amount of N fixed (g N plant^{-1}) was found for the third harvest of 2010 and for the first two harvests of 2011; AC Christie and Tapani had greater N fixation compared with CRS 15 ($P < 0.05$, Table 6.3). Among the tetraploids, the amount of N fixed by Tempus was greater than that fixed by CRS 18 or CRS 39 in the first harvest of 2011 ($P < 0.01$). A significant ploidy effect was found for the amount of N fixed during the second harvest of 2010 where tetraploid cultivars fixed more N compared with the diploids ($P < 0.01$).

6.4.4 Nitrogen Transfer

The amount of N transferred (mg N plant^{-1}) by the red clover cultivars was calculated using % N in bluegrass derived from red clover N transfer (% N transfer) and bluegrass shoot N content within each plastic ring under mixed stand (Table 6.3). The amount of N transferred at each harvest of both production years (except the first harvest of 2010) and seasonal N transfer were significantly different for red clover cultivars during both production years ($P < 0.001$) (Table 6.3). Seasonal N transfer was greatest in CRS 15 compared with AC Christie and Tapani during 2010 ($P < 0.001$), but the opposite trend was found during 2011. Among the selected tetraploid cultivars, seasonal N transfer was greatest in Tempus during both production years ($P < 0.001$). In the second harvest of 2010 and first two harvests of 2011, Tempus also transferred more N compared with the other two tetraploids ($P < 0.001$). No positive/net N transfer from the six red clover cultivars to companion bluegrass was found during the first harvest of 2010, but N transfer generally increased over the second and third harvests. Interestingly, no net N

was transferred by Tapani and CRS 18 during the first and second production years, respectively. In terms of % N in bluegrass derived from clover N transfer (% N transfer), the greatest N transfer was with Tempus (16.4%) for the second harvest and with CRS 15 (18.8%) for the third harvest of 2010 (Appendix XIII). During 2011, bluegrass N derived from N transfer was greatest in Tempus at first (3.7%) and second (8.8%) harvests, whereas CRS 39 (10%) transferred more during the third harvest.

Table 6.3 The amount of N fixed (g plant^{-1}) and amount of N transferred (mg N plant^{-1}) by six red clover cultivars (dry weight basis) under mixed stands with bluegrass across three harvests during the 2010 and 2011 growing seasons.

Year	Red clover cultivars	N fixed (g plant^{-1})			N transfer (mg N plant^{-1})			
		Cut 1	Cut 2	Cut 3	Cut 1	Cut 2	Cut 3	Total
2010	AC Christie	1.16	0.80	0.46	0.0	16.2	13.5	29.7
	Tapani	1.33	1.00	0.40	0.0	0.0	0.0	0.0
	CRS 15	0.88	0.68	0.26	0.0	8.4	38.8	46.9
	Tempus	1.43	1.27	0.41	0.0	36.4	8.6	44.7
	CRS 39	1.04	1.06	0.31	0.0	0.2	11.8	11.9
	CRS 18	1.10	1.02	0.35	0.0	0.0	4.0	4.0
	Grand mean	1.16	0.97	0.36	0.0	10.2	12.9	22.9
	SEM	0.14	0.09	0.04	0.0	1.0	1.8	2.2
	F-probability							
	Ploidy	ns	0.001	ns	ns	<0.001	<0.001	<0.001
	Cultivar (n = 18)	ns	0.086	0.008	ns	<0.001	<0.001	<0.001
	..C15 vs ACC, Tap	ns	0.076	0.001	ns	ns	<0.001	<0.001
	..Tem vs C39, C18	ns	0.067	ns	ns	<0.001	ns	<0.001
	..C15 vs Tem	ns	ns	0.011	ns	<0.001	<0.001	<0.001
2011	AC Christie	1.28	0.87	0.47	5.6	18.8	35.6	58.8
	Tapani	0.97	0.59	0.45	0.0	12.7	23.7	36.4
	CRS 15	0.74	0.47	0.29	0.0	0.0	18.9	18.9
	Tempus	1.56	0.76	0.35	31.5	25.4	34.0	90.9
	CRS 39	0.85	0.64	0.39	15.1	18.6	39.8	73.0
	CRS 18	1.08	0.65	0.41	0.0	0.0	0.0	0.0
	Grand mean	1.08	0.66	0.39	8.7	12.6	25.3	46.4
	SEM	0.14	0.09	0.05	0.5	0.8	1.8	2.4
	F-probability							
	Ploidy	ns	ns	ns	<0.001	<0.001	ns	<0.001
	Cultivar (n = 18)	0.004	0.061	ns	<0.001	<0.001	<0.001	<0.001
	..C15 vs ACC, Tap	0.032	0.034	ns	<0.001	<0.001	<0.001	<0.001
	..Tem vs C39, C18	0.002	ns	ns	<0.001	<0.001	<0.001	<0.001
	..C15 vs Tem	0.010	ns	ns	<0.001	<0.001	<0.001	<0.001

SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

6.4.5 Soil Mineral Nitrogen

During 2010 and 2011, no significant cultivar differences were found for the six red clover cultivars under mixed stand with bluegrass for soil extractable nitrate at 0–15 cm and 15–30 cm soil depths (Table 6.4). During 2011, soil nitrate content was generally greatest in soils that contained tetraploid red clover cultivars compared with diploid cultivars under bluegrass mixed stand at 0–15 cm soil depths during first harvest ($P \leq 0.01$), but the opposite trend was found at the 15–30 cm soil depth at third harvest ($P < 0.05$). As expected, soil under the pure bluegrass stand had less soil extractable nitrate compared with the red clover-bluegrass mixed stand at both soil depths during 2010 ($P < 0.05$) and 2011 ($P < 0.01$) (Table 6.4).

Soil extractable ammonium content at two soil depths differed for cultivars during both production years ($P < 0.05$, Table 6.4). During the 2010 season, AC Christie and Tapani had more soil ammonium compared with CRS 15 at 0–15 cm (third harvest) and 15–30 cm soil depths (first harvest) ($P < 0.05$). Tempus had less soil ammonium than CRS 18 and CRS 39 at 0–15 cm (2011 first harvest) and 15–30 cm soil depths (2010 first harvest) ($P < 0.05$). Compared with pure bluegrass stand, red clover under the bluegrass mixed stand had greater soil ammonium at a 0–15 cm soil depth at the third harvest for both years ($P < 0.05$). Similar trends were found for this attribute at the 15–30 cm soil depth only during the 2010 season (first and third harvests, $P < 0.05$).

Table 6.4 Soil extractable nitrate and ammonium concentration (mg kg^{-1} dry soil) of the 0 – 15 cm and 15 – 30 cm soil depths during the 2010 and 2011 production years for six red clover cultivars under mixed stand with bluegrass compared to pure bluegrass stand.

Year	Plant type	Nitrate-N (mg kg^{-1} dry soil)						Ammonium-N (mg kg^{-1} dry soil)					
		Cut 1		Cut 2		Cut 3		Cut 1		Cut 2		Cut 3	
		0-15 cm	15-30 cm	0-15 cm	15-30 cm	0-15 cm	15-30 cm	0-15 cm	15-30 cm	0-15 cm	15-30 cm	0-15 cm	15-30 cm
2010	AC Christie	12.70	7.68	16.55	10.56	6.00	6.41	1.75	1.27	2.06	1.04	2.42	1.35
	Tapani	10.08	12.00	22.10	13.53	20.30	16.44	1.71	1.40	2.05	1.29	2.41	1.63
	CRS 15	11.30	17.60	28.79	12.43	7.38	23.55	1.72	1.01	1.79	1.28	1.91	1.63
	Tempus	11.64	9.80	12.13	13.88	32.19	19.61	1.63	1.02	1.98	1.24	2.06	1.62
	CRS 39	13.03	9.94	12.07	10.05	12.27	8.92	1.64	1.27	2.16	1.17	2.34	1.64
	CRS 18	12.34	12.69	22.82	16.65	4.21	8.89	1.64	1.35	1.93	1.32	2.11	1.64
	Pure bluegrass	10.83	8.08	6.13	9.03	1.91	4.15	1.53	0.95	1.96	1.41	1.78	1.32
	Grand mean	11.60	10.73	15.84	11.90	10.77	11.51	1.64	1.15	1.99	1.27	2.10	1.52
	SEM	1.38	2.32	3.96	1.74	5.65	3.54	0.10	0.07	0.10	0.09	0.09	0.08
	F-probability												
	..Bg vs RC	ns	ns	0.012	0.075	0.088	0.031	ns	0.007	ns	0.075	0.001	0.015
	..Tetra vs Dip	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Cultivar (n = 3)	ns	ns	ns	ns	ns	ns	ns	0.044	ns	ns	0.041	ns
	..C15 vs ACC, Tap	ns	ns	ns	ns	ns	ns	ns	0.022	ns	ns	0.006	ns
	..Tem vs C39, C18	ns	ns	ns	ns	ns	ns	ns	0.041	ns	ns	ns	ns
	..C15 vs Tem	ns	ns	ns	ns	ns	ns	ns	0.044	ns	ns	0.045	ns
2011	AC Christie	20.51	11.62	32.83	14.57	23.97	17.95	0.36	0.64	0.42	0.21	0.25	0.16
	Tapani	11.09	16.18	32.04	19.00	28.07	29.18	0.06	0.11	0.46	0.24	0.26	0.24
	CRS 15	10.85	22.45	26.76	23.82	20.64	35.18	0.47	0.52	0.36	0.17	0.23	0.25
	Tempus	27.45	17.01	23.41	20.04	17.12	14.16	0.28	0.23	0.35	0.23	0.23	0.19
	CRS 39	17.99	27.89	18.87	21.71	16.24	23.22	1.62	0.14	0.35	0.14	0.26	0.22
	CRS 18	20.06	13.26	27.86	14.37	20.27	16.63	0.29	0.14	0.36	0.20	0.27	0.19
	Pure bluegrass	7.12	7.744	15.17	12.44	11.48	20.07	0.23	0.17	0.31	0.15	0.21	0.20
	Grand mean	15.27	15.49	24.01	17.30	18.66	22.06	0.44	0.27	0.37	0.18	0.24	0.21
	SEM	2.27	3.01	3.42	1.78	4.31	3.06	0.12	0.11	0.03	0.03	0.02	0.02
	F-probability												
	..Bg vs RC	<0.001	0.009	0.009	0.006	0.072	ns	0.059	ns	0.074	ns	0.043	ns
	..Tetra vs Dip	0.010	ns	0.089	ns	ns	0.016	0.006	0.052	ns	ns	ns	ns
	Cultivar (n = 3)	0.073	0.092	ns	0.057	ns	0.071	<0.001	ns	ns	ns	ns	0.047
	..C15 vs ACC, Tap	ns	ns	ns	0.036	ns	0.042	ns	ns	ns	ns	ns	0.064

Year	Plant type	Nitrate-N (mg kg^{-1} dry soil)						Ammonium-N (mg kg^{-1} dry soil)					
		Cut 1		Cut 2		Cut 3		Cut 1		Cut 2		Cut 3	
		0-15 cm	15-30 cm	0-15 cm	15-30 cm	0-15 cm	15-30 cm	0-15 cm	15-30 cm	0-15 cm	15-30 cm	0-15 cm	15-30 cm
2011	..Tem vs C39, C18	0.049	ns	ns	ns	ns	ns	0.005	ns	ns	ns	ns	ns
	..C15 vs Tem	ns	ns	ns	ns	ns	ns	0.035	ns	ns	ns	ns	ns

SEM; standard error mean, ns; not significant.
Bg; pure bluegrass stand, RC; Red clover under bluegrass mixed stand, Tetra; Tetraploid, Dip; Diploid, C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

6.4.6 Total Soil Nitrogen and Carbon

Total soil N (%) was not significantly different for red clover cultivars under the bluegrass mixed stand during the first production year (Appendix XIV). Total soil carbon (C %) was analyzed only during the 2011 season. Cultivar effects were found during the second production year for total soil N and C ($P < 0.05$, Table 6.5). Soils under Tempus had greater total N and C compared with soils under the other tetraploid cultivars at second and third harvests ($P < 0.05$). Soil under the diploid cultivar, CRS 15, had less total soil N and C compared with soils under AC Christie and Tapani for the second harvest of 2011 ($P < 0.05$).

Table 6.5 Soil total nitrogen and carbon (as % of dry matter) of the 0 – 15 cm soil depths during the 2011 production year for six red clover cultivars under mixed stand with bluegrass compared to pure bluegrass stand.

Plant type	Total N (%)			Total C (%)		
	Cut 1	Cut 2	Cut 3	Cut 1	Cut 2	Cut 3
AC Christie	0.142	0.146	0.146	1.647	1.725	1.643
Tapani	0.141	0.141	0.154	1.670	1.653	1.681
CRS 15	0.145	0.131	0.147	1.666	1.509	1.627
Tempus	0.143	0.145	0.161	1.644	1.675	1.748
CRS 39	0.141	0.136	0.147	1.626	1.587	1.628
CRS 18	0.142	0.127	0.139	1.643	1.447	1.613
Pure bluegrass	0.136	0.145	0.143	1.584	1.667	1.640
Grand mean	0.141	0.140	0.148	1.633	1.616	1.653
SEM[y]	0.004	0.003	0.003	0.044	0.034	0.019
F-probability						
..Bg vs RC	ns	0.036	0.057	ns	ns	ns
..Tetra vs Dip	ns	ns	ns	ns	ns	ns
Cultivar (n = 3)	ns	0.017	0.009	ns	0.005	0.014
..C15 vs ACC, Tap	ns	0.024	ns	ns	0.007	ns
..Tem vs C39, C18	ns	0.018	0.001	ns	0.015	0.001
..C15 vs Tem	ns	0.031	0.020	ns	0.014	0.015

SEM; standard error mean, ns; not significant.
Bg; pure bluegrass stand, RC; Red clover under bluegrass mixed stand, Tetra; Tetraploid, Dip; Diploid, C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

6.4.7 Soil-Water Nitrate and Ammonium Content

Soil-water samples collected from 45-cm deep ceramic suction cups were analyzed for both nitrate and ammonium concentration and plotted as cumulative values through the growing season (Figure 6.2). No cultivar differences were found for either soil-water

nitrate or ammonium during the 2010 growing season (Appendix XV). During the 2011 growing season, cumulative soil-water nitrate content increased quadratically with a significant difference for red clover cultivars under mixed stand with bluegrass ($P < 0.10$, Figure 6.2a). Among the tetraploid cultivars, Tempus had greater soil-water nitrate content compared with CRS 18 and CRS 39 ($P < 0.05$). In general, CRS 18, AC Christie, CRS 15 (under mixed stand with bluegrass), and pure bluegrass had low soil-water nitrate content compared with the other red clover cultivars during the 2011 growing season. No significant cultivar differences were found among red clover cultivars for soil-water ammonium content during the 2011 growing season (Figure 6.2b). Soil-water nitrate content rapidly increased towards the latter part of the 2011 growing season in Tempus, Tapani, and CRS 39; the same trend was found for soil-water ammonium in CRS 39 and Tempus.

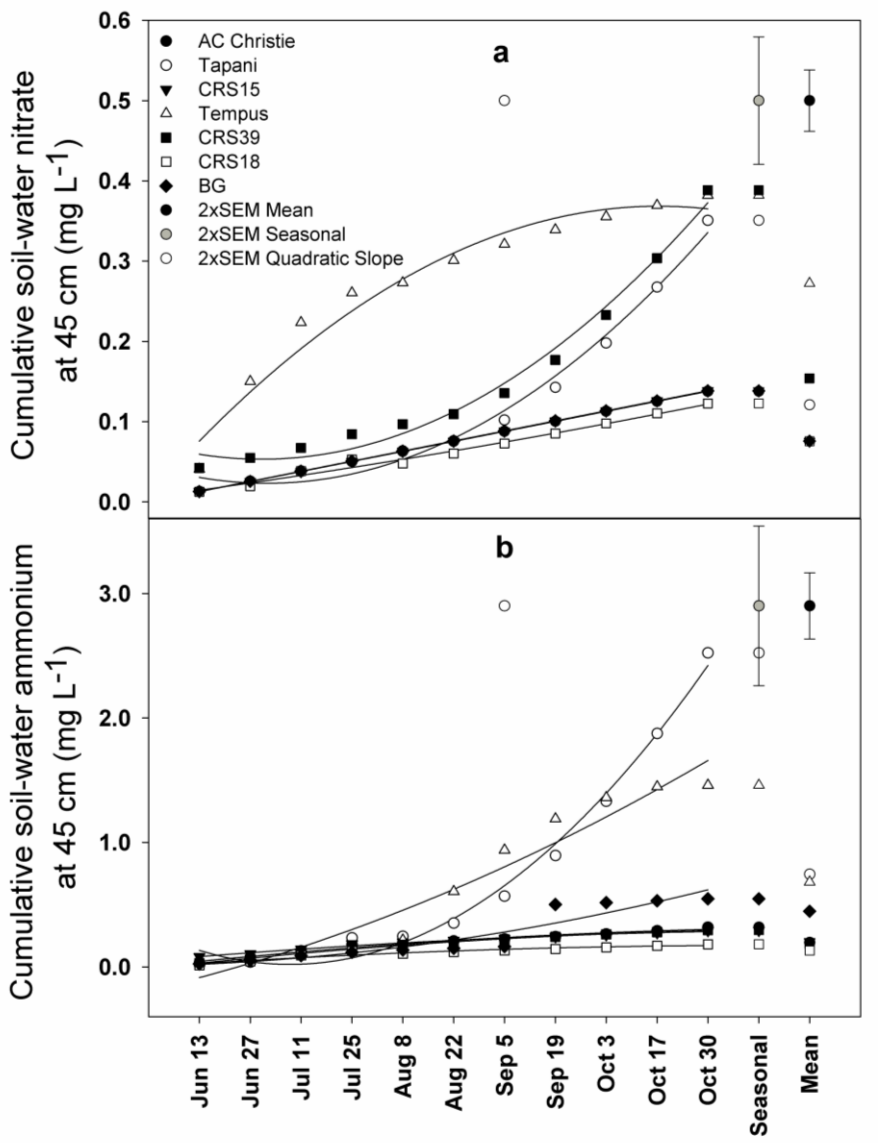

Figure 6.2 Variation of cumulative soil-water nitrate and ammonium content at 45-cm soil depths for six red clover cultivars under mixed stand with bluegrass verses a pure bluegrass stand during the 2011 production year.

6.4.8 Principal Component Analysis (PCA)

In the PCA biplots, the first two principal components explain 73.8% in 2010 PCA (Figure 6.3a) and 75.7% in 2011 PCA (Figure 6.3b) of the total variation for the nine attributes considered. Red clover cultivars were mainly separated by score 1 in both PCAs. Shoot N concentration, red clover yield, and total N fixed (seasonal) were correlated on the positive side of score 1 during 2010 and in contrast to soil-water NH_4^+.

The cultivar differences are spread along these variates (Figure 6.3a). Tempus dominated the cultivars followed by CRS 18, Tapani, and CRS 39, which had positive scores during 2010. The % Ndfa, N transfer, and the seasonal soil-water ammonium content were correlated on the positive side of score 1 for 2011. Cultivar differences are based on the spread across score 1 (Figure 6.3b). Total N fixed, red clover yield, and soil-water nitrate content are correlated, in contrast to soil NH_4^+. This relationship explains the cultivar separation on score 2. As well, during the second production year, Tempus dominated the cultivars while Tapani and AC Christie were positive for score 1. Although CRS 18 was on the positive side of score 1during the first production year, it did not perform as well during the second production year. The cultivar CRS 15 had a negative score 1 for both production years, which indicates it is stable across both years even though it may not be the greatest yielding cultivar nor have the greatest total N fixed.

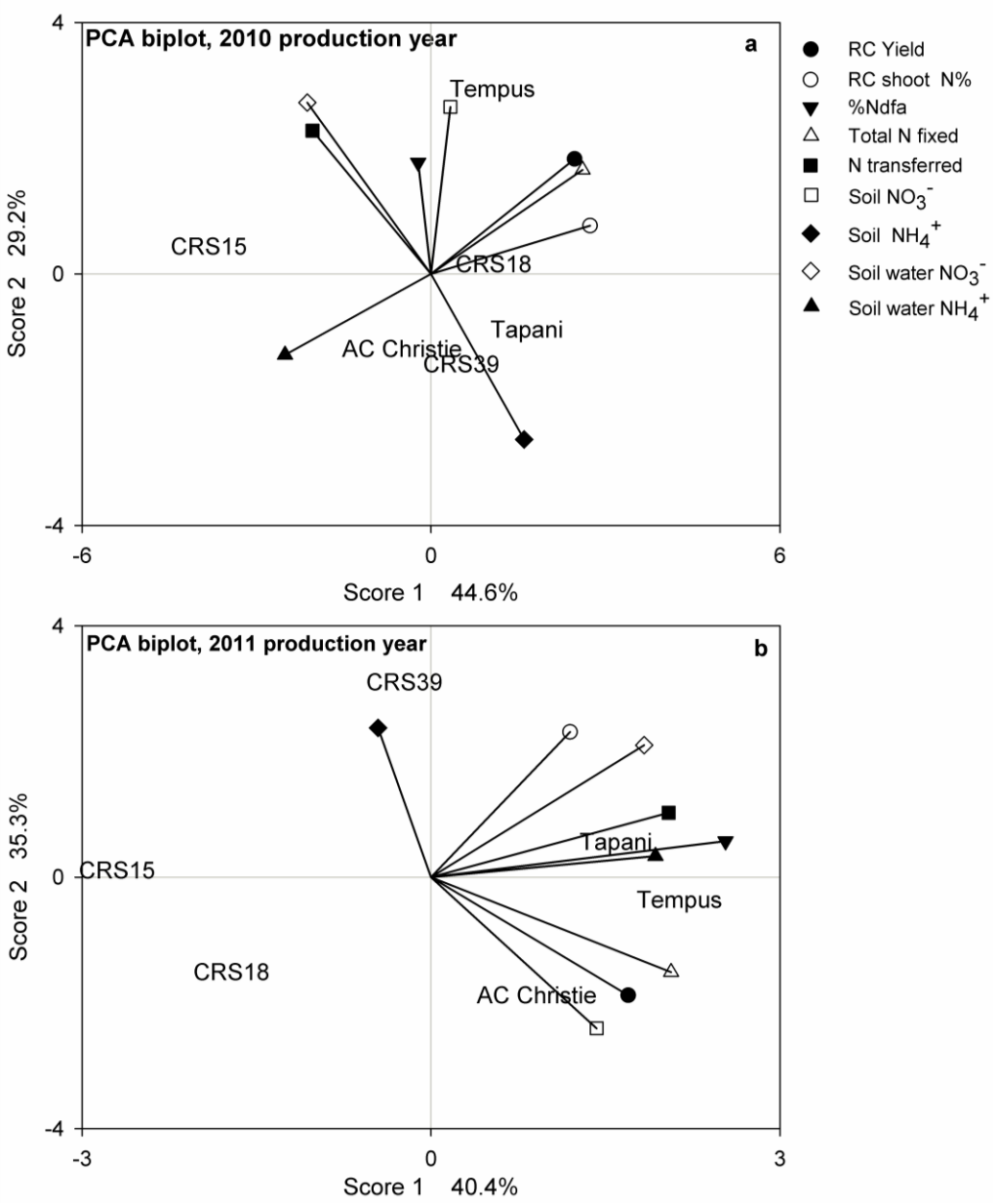

Figure 6.3 Principal component analysis of yield, shoot N concentration, N fixation, N transfer, soil N parameters (NO_3^- and NH_4^+), and soil-water N parameters (NO_3^- and NH_4^+) for six red clover cultivars under bluegrass mixed stand during the 2010 and 2011 production years.

6.5 Discussion

In general, ploidy level had a limited effect on herbage yield in this study. The ranking of cultivars for herbage yield differed between the two production years. Over the two years, Tempus was the best performing cultivar in terms of DM yield. AC

Christie had below average DM yield in the first production year but the greatest yield in the second production year (Table 6.1). The effect of red clover cultivars on bluegrass yield in mixed stand for three harvests, and on the cumulative seasonal yield for two production years, was not significantly different among cultivars (Table 6.1). Average cumulative seasonal yield of the bluegrass under the mixed stand was greater during the second production year (705 g m^{-2}) than the first (366 g m^{-2}). This greater seasonal yield was due mainly to differences in first harvest (524 g m^{-2} in 2011 vs 216 g m^{-2} in 2010) and lack of precipitation early in the spring of 2010 may have been a factor (Figure 6.1). However, compared with the pure bluegrass stand, cumulative seasonal bluegrass yield during the second production year was still 9% greater under mixed stand (Appendix XVI). The greater biomass in bluegrass under mixed stand was probably due, in part, to additional N derived from red clover N transfer.

Significant genotypic differences among the red clover cultivars were found for shoot N content (mg N $plant^{-1}$) in both production years. However, ploidy level had a limited effect on plant N content. Generally, high shoot N content was associated with Tempus (of the tetraploids) and AC Christie and Tapani (of the diploids) (Table 6.2). Shoot N content of red clover declined from first harvest to third harvest by 69% and 60% for 2010 and 2011, respectively. On the other hand, shoot N concentration of the red clover cultivars increased from first harvest to third harvest by 18% and 56% for 2010 and 2011, respectively (Appendix X). Similar trends were found in bluegrass under mixed stands where shoot N concentration increased by 63% and 167% for 2010 and 2011, respectively, which corroborates previous findings (Thilakarathna et al. 2012b). Bluegrass shoot N concentration at different harvests and seasonal N content under mixed stand were slightly greater compared with the pure bluegrass stand (Appendix XVI). This finding also indicates potential N transfer from red clover to bluegrass under the mixed stand.

The high N fixation capacity (% Ndfa) associated with the six red clover cultivars under mixed stand agrees with previous findings by Dahlin and Stenberg (2010a), Nyfeler et al. (2011), and Thilakarathna et al. (2012b); red clover can derive up to 99% of its own N through BNF under grass-clover mixed stands. Nitrogen fixation by forage legumes is greater in mixed stands with grass compared with pure legume stands

(Jørgensen et al. 1999); competition for available soil N by grasses in mixed stands stimulates N fixation in legumes. Tempus consistently had high N fixation (g N plant^{-1}) among the tetraploid cultivars in both production years (Table 6.3). Among the diploid cultivars, AC Christie and Tapani fixed greater amounts of N than CRS 15 in both production years. Among the six red clover cultivars, CRS 15 fixed the least amount of N mainly due to its low yield. The amount of N fixed by legumes is positively correlated with legume DM yield (Gierus et al. 2012), which is also evident from our PCA results (Figure 6.3). Furthermore, in keeping with yield reduction, the amount of N fixed by red clover cultivars declined from first harvest to third harvest by 69% and 64% for 2010 and 2011, respectively. There was little change in % Ndfa over the growing seasons for all cultivars in both production years. Therefore, it is clear that the reduction in the amount of N fixed (g N plant^{-1}) is mainly associated with reduced yields for red clover cultivars (Carlsson and Huss-Danell 2003).

The great variation in N transfer for the selected red clover cultivars does not appear to be related to ploidy levels. Among the tetraploid cultivars, Tempus transferred more N to companion bluegrass during the two production years (Table 6.3), which positively correlates with its high yield and N fixation capacity (Figure 6.3). Diploid AC Christie was consistent in transferring N to companion bluegrass during both production years, whereas CRS 15 had high N transfer only during the first production year. Nitrogen transfer increased over the season for both production years, which agrees with previous findings (Høgh-Jensen and Schjoerring 2000; Dahlin and Stenberg 2010b; Thilakarathna et al. 2012b; Rasmussen et al. 2013). On average, seasonal apparent N transfer doubled in the second production year compared with the first. Increased N release by legume root systems with plant age can be attributed to increases in root mass, surface area, root exudates (Brophy and Heichel 1989; Jalonen et al. 2009), and senescence and decay of roots and nodules (Fustec et al. 2010). Defoliation also increased the amount of N released by legume root systems (Ayres et al. 2007; Hamilton et al. 2008; Carrillo et al. 2011), thus creating more N to transfer which agrees with increased N transfer during second and third harvests following defoliation. On the other hand, the experimental site was cropped with red clover in 2007 and 2008, which may have contributed organic N to the soil. Potential contribution by red clover residues to

soil mineral N pool is high in the first year after planting and dropping during subsequent year (Christie et al. 1992). Turnover of red clover residuals in 2009 and 2010 may have increased soil N availability for bluegrass, resulting in low apparent N transfer for the first production year.

In comparison with pure bluegrass stands, inclusion of red clover cultivars increased soil N (nitrate and ammonium) availability under mixed stands, possibly due to the N derived from decomposing legume roots and nodules and N-containing root exudates (Table 6.4). Soil nitrate levels under red clover mixed stand were greater during the second production year compared with the first, which strongly supports our finding of increased N transfer during the second production year. Greater soil nitrate during the second production year (2011) compared with the first (2010) is probably due to a higher turnover of red clover dead roots and nodules (Rasmussen et al. 2008). Also, an increase in exudation of N-containing root exudates with plant age (Chapter 4) can result in greater soil nitrate during the second production year. Opposite trends, however, were found for available soil ammonium, which was lesser during the second production year due to greater nitrification which converted ammonium into nitrate (Norton and Stark 2011). High precipitation over the 2010 growing season (especially June and July) compared to 2011 (Figure 6.1) possibly created anaerobic conditions in the soil, thereby reducing the nitrification rate.

Significant cultivar effects were found for total soil N and C during the second production year, but these effects were not related to ploidy level. Among the tetraploids, soils under Tempus had greater total soil N and C, whereas soils under CRS 15 had the lowest levels among the diploid cultivars under bluegrass mixed stand. High soil C content supports rapid mineralization of legume dead roots and nodules which facilitates greater N transfer to neighboring non-legumes. Under legume-grass mixed stands, more C may be accumulated in the soil mainly due to a greater root biomass than with legume and grass monoculture stands (Fornara and Tilman 2008). However, in terms of soil C, we did not find any difference between red clover-bluegrass mixed stands and pure bluegrass stands during the second production year. Since soil C accumulation is a slow process, significant differences between monoculture and mixed stands may not be apparent for several years (Fornara and Tilman 2008).

In general, ploidy level did not affect the potential nitrate leaching in this study. From the beginning of the season to mid-October, the potential for nitrate leaching, as determined by soil-water from suction cups, increased for Tempus (tetraploid), and Tapani and CRS 39 (diploid), under the mixed stand (Figure 6.2a). Similar to nitrate, soil-water ammonium content was also greater for Tempus and Tapani in late fall (Figure 6.2b). The rapid increase in the potential for nitrate leaching in the late fall of 2011 may be due to N released through root and nodule senescence and turnover, coupled with high rainfall in October (221.5 mm). However, potential nitrate leaching values were low compared with previous results from the same region (Bouman et al. 2010; MacPherson 2010). In the current study, nitrate leaching estimates were based on an individual red clover plant basis under bluegrass mixed stands whereas other studies were conducted under dense and complex mixed stands.

The diverse performance (DM yield, N transfer, and N cycling) of the six red clover cultivars evaluated in this study shows genetic variability for traits that affect N transfer potential. Some cultivars, i.e., Tempus and AC Christie, consistently sustained N transfer capability while other cultivars, i.e., CRS 15, had greater N transfer capability in 2010 but lesser rates in 2011 (Figure 6.3). Differentiating genotypes on such attributes may help plant breeders identify new cultivars that are capable of transferring significant amounts of N to companion grasses, thereby improving forage performance under reduced input production systems. A major mechanism of N transfer is N-containing root exudates; exudation of N compounds by legumes is positively correlated with N fixation (Paynel et al. 2008), root N concentration (Jalonen et al. 2009), and total plant N content (Mahieu et al. 2009). Similarly, we also found that N transfer was positively correlated with red clover yield, N fixation, and shoot N concentration (especially during the second production year). Furthermore, the results of this study show that total N fixed by clover was positively correlated with soil nitrate at the 0–15 cm soil depth. Therefore, red clover cultivars with high N fixation improved soil N availability, thus facilitating N transfer to companion non-legumes (Figure 6.3). On the other hand, we found that N transfer positively correlated with soil-water nitrate and ammonium content. This implies that cultivars with high N transfer also have a risk for potential N leaching. However, the risk

of N leaching may be better managed by using forage mixtures that contain grass species with significant root biomass to occupy deeper soil zones than bluegrass roots.

In summary, this study highlights the existence of significant genotypic differences among the currently available red clover cultivars for N transfer to the companion bluegrass under field conditions. However, this genetic variability does not appear to be related to ploidy level. Generally, N transfer increased over seasons and production years. Plant attributes that are positively associated with N transfer can guide the selection of cultivars to enhance N transfer under legume/non-legume mixed stands. Soil N conditions were affected differently by the selected red clover cultivars under mixed stands, thus showing genotypic differences for soil N traits. Research into the dynamics of N flow between legume and companion grasses may assist in developing management and plant breeding strategies with efficient N cycling profiles. These strategies would reduce N losses and improve the profitability of forage based production systems.

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Chapter 7

Conclusion

This study identified differences among selected red clover cultivars for the amount of starter N required to maximize nodulation. Agricultural soils are diverse in terms of available N, ranging from N deficient to N excessive systems. Therefore, the existence of genotypic variability highlights the importance of selecting genotypes according to available soil N to optimize the nodulation, growth and N fixation of red clover under field conditions. The red clover cultivars evaluated in the current study were efficient in N fixation under local soil and climatic conditions and derived more than 90% of their N from BNF. Therefore, these red clover cultivars are recommended to provide high N fixation in forage mixed stands. The amount of N fixed varied according to genotype mainly due to their yield potential. Red clover cultivars with high yield potential will likely contribute more fixed N to cropping systems; cultivars with less yield potential may contribute less.

In addition to nodulation and N fixation, significant genotypic variability was also evident for N transfer potential among red clover cultivars in terms of root exudate N content, available soil N and amount of fixed N transferred to bluegrass. During early growth, N transfer in legumes is mainly mediated through N-containing root exudates rather than through decomposing roots and nodules (Paynel and Cliquet 2003; Gylfadóttir et al. 2007). In general, during the initial growth period, inorganic N content in the root exudates was greater for tetraploid cultivars than diploids. However, in the longer term and under field conditions, this genotypic variability for N transfer did not appear to be related to ploidy level. Overall, decomposing roots and nodules appear to transfer more N than other mechanisms of N transfer. In the current field studies, most of the N transferred from red clover cultivars to bluegrass may have derived from decomposing legume tissues belowground rather than from N-containing root exudates.

Different biotic (genotype) and abiotic (growing season, production year and available soil N) factors affect N transfer from legumes to non-legumes. In this evaluation, the amount of N transferred by red clover cultivars to companion bluegrass

was negligible during early growth of the first production year. Generally, the proportion of bluegrass N derived from interplant N transfer increased over the growing season and with stand maturity. Nitrogen release through decomposing legume roots and nodules is one of the major mechanisms of N transfer (Wichern et al. 2008; Fustec et al. 2010), with the rate of decomposition mainly depending on soil temperature. Due to lower temperatures, less N is derived from decomposing belowground legume tissues during early spring than later in the season. On the other hand, defoliation leads to N release through legume root systems (Hamilton et al. 2008), thereby inducing root and nodule senescence (Chesney and Nygren 2002). For the *in vitro* study of this evaluation, the N content of red clover root exudates increased as the plant grew during early stages of plant establishment (Chapter 4). Therefore, we conclude that temperature, defoliation and plant age are the major abiotic factors responsible for increasing N transfer during the growing season.

Our results also indicate that turning over more legume roots and nodules would release significant amounts of N as the stand matures (post establishment year of legume/grass stands), resulting in increased N transfer to neighbouring non-legumes. Therefore, the beneficial effects of N transfer to neighbouring non-legumes under intercropping would be clearly evident as newly established red clover/grass stands mature and higher amounts of N transfer occur as the growing season advances. Soil N availability (through residual soil N or N fertilization) also affects the amount of N transfer from red clover to companion bluegrass. Generally, N transfer from red clover to bluegrass was higher in the first field study (Chapter 5) than in the second field study (Chapter 6). Since the soil of the first field study was lower in N, this result highlights that N transfer from red clover to bluegrass is higher on soils with low residual N than on soils rich in N.

Red clover genotypes play an important role in transferring N to non-legume companion grasses. Only a few of the cultivars we evaluated, such as AC Christie and Tempus, were consistent in transferring significant amounts of N to companion bluegrass for two years following stand establishment. On the other hand, some cultivars, such as CRS 18, had a consistently lower N transfer capacity to companion bluegrass within the growing season for both production years. Red clover cultivars with consistently high N

transfer capacity are more suitable for inclusion in forage mixtures; they provide a sustainable N benefit to neighbouring non-legumes while minimizing external N fertilizer inputs. Cultivars with low N transfer may be more effective in crop rotations, where fixed N may be released slowly for the following crop. Interestingly, some red clover cultivars were not consistent in N transfer to companion bluegrass during the two production years. For example, CRS 15 had a significantly high N transfer only during the first production year, while CRS 39 had a significantly high N transfer only during the second production year. These results indicate there is likely a significant red clover genotype × year interaction for N transfer as well.

In comparison with pure bluegrass stands, inclusion of red clover cultivars increased available soil N under mixed stands. Furthermore, total N fixed by red clover was positively correlated with soil nitrate at a 0–15-cm soil depth highlighting the fact that red clover cultivars with high N fixation enrich the soil with available N. Significant cultivar differences were also found among red clover cultivars for potential nitrate leaching. This result underscores the importance of currently available red clover cultivars. AC Christie, for example, is characterized by its ability to transfer a significant amount of N to companion grasses while minimizing N leaching during the growing season. These types of cultivars can use fixed N efficiently in forage-grass mixtures while minimizing negative environmental effects from nitrate leaching.

This dissertation evaluated how red clover cultivars affect different components of soil N cycling, including fixation, transfer and leaching. The information provided on the dynamics of N flow between legumes and companion grasses will help in the development of management and plant breeding strategies that improve the efficiency of N cycling, thereby reducing N losses in forage based production systems.

Larger field trials conducted over multiple years are needed to validate results under practical field conditions, and to evaluate N transfer in older stands under variable environmental conditions. Evaluating more cultivars, including those that are less efficient at nodulation, will help to confirm whether N transfer is a function of nodulation and N fixation. Future studies should also evaluate the use of deeply rooted forage grasses in legume-grass mixed stands to determine whether different root profiles can recover N lost through leaching.

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Appendix I

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Appendix I.A Copyright Permission for Chapter 3

Publication

Thilakarathna, R. M. M. S., Papadopoulos, Y. A., Fillmore, S. A. E., Prithiviraj, B. 2012. Genotypic differences in root hair deformation and subsequent nodulation for red clover under different additions of starter N fertilization. Journal of Agronomy and Crop Science. 198: 295-303.

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Appendix I.B Copyright Permission for Chapter 5

Publication

Thilakarathna, R. M. M. S., Papadopoulos, Y. A., Rodd, A. V., Gunawardena, A. N., Fillmore, S. A. E. and Prithiviraj, B. 2012. Characterizing nitrogen transfer from red clover populations to companion bluegrass under field conditions. Canadian Journal of Plant Science. 92: 1163-1173.

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Appendix II

Table: Shoot DW, total DW (shoot and root), root/shoot ratio and total leaf area in six red clover cultivars grown under four different concentrations of nitrogen (0, 0.5, 1.0 and 2.5 N mg plant^{-1} week $^{-1}$) for 8 weeks.

Cultivar	Shoot DW (mg)	Root DW (mg)	Total DW (mg)	Root/Shoot ratio	Total leaf area (cm^2)
AC Christie	353	196	549	0.581	41.5
Tapani	328	168	507	0.567	41.6
CRS 15	361	171	533	0.498	44.8
Tempus	362	184	546	0.540	39.4
CRS 18	337	207	544	0.637	37.5
CRS 39	336	203	539	0.631	39
Grand mean	346	188	536	0.576	40.6
SEM	29.4	6.3	35.9	0.038	3.01
F-Probability					
Ploidy	ns	0.012	ns	ns	ns
Cultivar	ns	0.028	ns	ns	ns
…Tem vs C18, C39	ns	ns	ns	ns	ns
…C15 vs ACC, Tap	ns	ns	ns	ns	ns

ns = not significant ($P > 0.10$); SEM = standard error mean.
Tem, Tempus; 18, CRS 18; 39, CRS 39; 15, CRS 15; ACC, AC Christie; Tap, Tapani. n = 16

Appendix III

Table: Shoot N%, root N%, total shoot N, total root N and total plant N in six red clover cultivars grown under four different concentrations of nitrogen (0, 0.5, 1.0 and 2.5 N mg plant^{-1} week $^{-1}$) for 8 weeks.

Cultivar	Shoot N (%)	Root N (%)	Total shoot N (mg plant^{-1})	Total root N (mg plant^{-1})	Total plant N (mg plant^{-1})
AC Christie	2.72	2.24	9.04	4.36	13.40
Tapani	2.66	2.34	8.38	4.13	12.51
CRS 15	2.42	2.23	8.49	3.84	12.58
Tempus	2.78	2.45	9.24	4.32	14.33
CRS 18	2.84	2.37	8.73	4.80	13.52
CRS 39	2.64	2.40	8.63	4.97	13.48
Grand mean	2.67	2.34	8.75	4.40	13.30
SEM	0.143	0.080	0.597	0.158	0.499
F-Probability					
Ploidy	ns	0.095	ns	0.006	0.068
Cultivar	ns	ns	ns	0.026	ns
…Tem vs C18, C39	ns	ns	ns	0.088	ns
…C15 vs ACC, Tap	ns	ns	ns	0.088	0.056

ns = not significant ($P > 0.10$); SEM = standard error mean.
Tem, Tempus; 18, CRS 18; 39, CRS 39; 15, CRS 15; Chr, AC Christie; Tap, Tapani.

Appendix IV

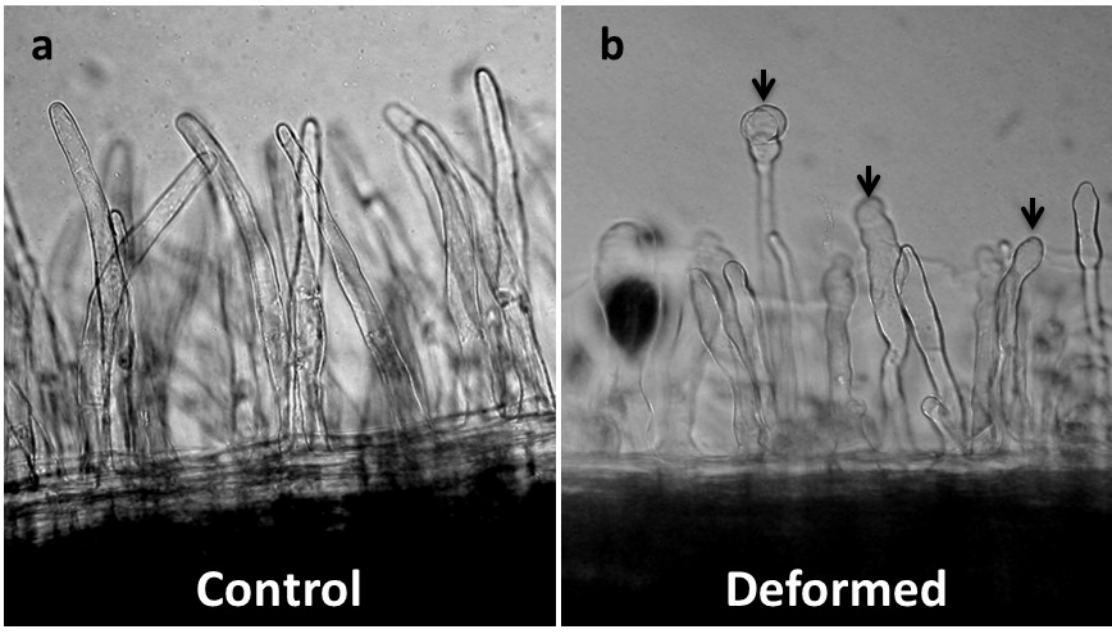

Figure: Comparison of normal root hairs and deformed root hairs in red clover. Root hairs in control (a) were treated with 200 µl of sterilized distilled water where root hairs in (b) were treated with 200 µl *Rhizobium leguminosarum* biovar *trifolii* (OD_{600} 0.1).

Appendix V

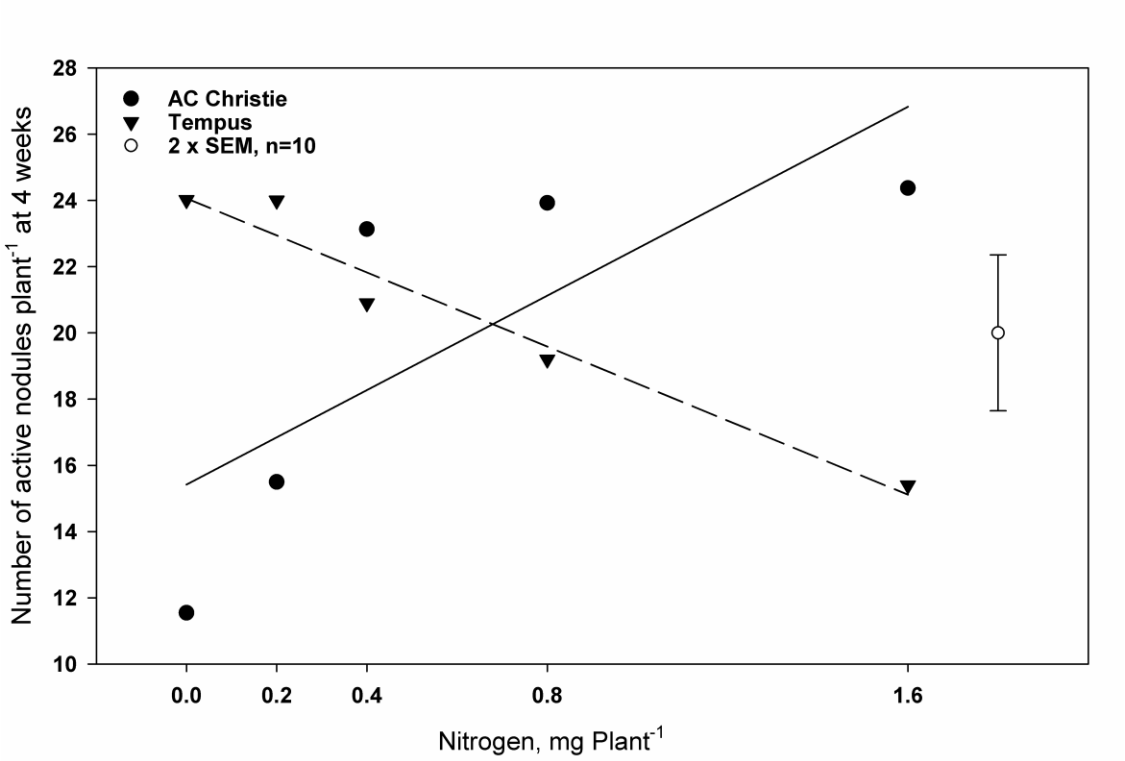

Figure: Active nodules per plant in response to starter N for two red clover cultivars, AC Christie and Tempus, four weeks after inoculation with rhizobia.
Number of replicates (n) = 10, vertical bar represents two standard errors of the mean (SEM). Cv x Linear N; $P < 0.001$.

Appendix VI

Root Exudates as N source for Perennial Ryegrass Growth

Materials and Method

Seeds of perennial ryegrass (*Lolium perenne* L.) were surface sterilized with 2% sodium hypochlorite for 30 seconds and washed with three changes of sterile distilled water. Seeds were pre-germinated on wet sterile filter papers in the dark for 1 week. Individual pre-germinated seedlings were transferred into sterile 10 ml plastic tubes which contained 5 ml of root exudates collected from different red clover cultivars at different growth stages (4, 6, and 8 weeks after inoculation with rhizobia). Plants were grown in a growth room with supplemental lighting maintained with a photoperiod of 16 hours of daylight at 125 μmol m^{-2} s^{-1} and 8 hours of dark (16 D: 8 N) at 23 ± 2 ^{o}C. Plants were harvested 2 weeks after growth and scanned using Epson Expression 1000X. A detailed root morphological analysis, including volume, total length, surface area, and diameter was determined using WinRHIZO system (Regents Instruments Inc.). Maximum shoot height was measured using Image J software (Abràmoff et al. 2004).

Results

Root exudates of red clover cultivars collected at 8 weeks affected ryegrass root growth (root length, root surface area, and root volume) (Table 4.5, $P < 0.001$). Exudates from CRS 15 resulted in perennial ryegrass having greater root length, surface area, and volume than the other two diploid cultivars (AC Christie and Tapani) ($P < 0.001$). Generally, ryegrass grown on root exudates of the diploid cultivars had better root growth than those grown on exudates of the tetraploids, especially at 6 and 8 weeks of red clover growth ($P < 0.05$). However, shoot length of the ryegrass was not significantly affected by root exudates except at 4 weeks where ryegrass grown in root exudates of diploid cultivars had greater shoot length than the tetraploids.

Table: Root length, root surface area and root volume of the perennial ryegrass grown for two weeks in root exudates collected from six red clover cultivars at 4, 6, and 8 weeks after inoculation with rhizobia.

Red clover cultivars	Root length (cm)			Root surface area (cm^2)			Root volume (cm^3)		
	4-week	6-week	8-week	4-week	6-week	8-week	4-week	6-week	8-week
AC Christie	44.6	31.0	18.4	3.79	2.65	1.78	0.026	0.018	0.014
Tapani	46.4	30.9	15.3	3.76	2.66	1.59	0.024	0.019	0.014
CRS 15	45.7	25.8	29.9	3.72	2.43	2.83	0.024	0.018	0.021
Tempus	39.4	21.3	14.5	3.05	2.09	1.54	0.019	0.016	0.014
CRS 39	43.7	25.2	16.1	3.33	2.23	1.61	0.020	0.016	0.013
CRS 18	45.7	23.0	15.7	3.72	2.15	1.58	0.024	0.017	0.013
Grand mean	44.2	26.2	18.3	3.56	2.37	1.82	0.023	0.017	0.015
SEM	4.0	2.6	2.2	0.33	0.19	0.18	0.002	0.001	0.001
F-probability									
Ploidy	ns	0.007	0.003	ns	0.011	0.002	0.062	0.058	0.005
Cultivar	ns	ns	<0.001	ns	ns	<0.001	ns	ns	<0.001
..C15 vs ACC, Tap	ns	ns	<0.001	ns	ns	<0.001	ns	ns	<0.001
..Tem vs C39, C18	ns	ns	ns	ns	ns	ns	ns	ns	ns
..C15 vs Tem	ns	ns	0.001	ns	ns	<0.001	ns	ns	0.001

SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Temp; Tempus, C39; CRS 39 and C18; CRS 18.

Table: Shoot length of the perennial ryegrass grown for two weeks in root exudates collected from six red clover cultivars at 4, 6 and 8 weeks after inoculation with rhizobia.

Red clover cultivars	Shoot length (cm)		
	4-week	6-week	8-week
AC Christie	8.52	8.01	8.53
Tapani	8.06	8.32	8.86
CRS 15	7.28	8.94	8.99
Tempus	6.51	7.68	9.06
CRS 39	6.68	8.23	7.58
CRS 18	7.64	9.07	8.12
Grand mean	7.45	8.38	8.52
SEM	0.44	0.52	0.53
F-probability			
Ploidy	0.007	ns	ns
Cultivar	ns	ns	ns

SEM; standard error mean, ns; not significant.

Appendix VII

Table: Yield and shoot nitrogen concentration of two red clover cultivars (AC Christie and Tempus) grown in mixed swards with bluegrass during the 2008 establishment year.

Red clover cultivars	Red clover yield (g plant^{-1})^{z}	Bluegrass yield (g m^{-2})	Red clover N (%)	Bluegrass N (%)
AC Christie	7.77	11.61	2.04	3.26
Tempus	8.61	11.81	2.04	3.26
SEM^{y} (n=12)	0.624	0.397	0	0
F-probability	ns^{w}	ns	ns	ns

^{z}All measurements are on a dry weight basis.
^{y}SEM = standard error mean.
^{w}ns = *P* value greater than 0.05.

Appendix VIII

Table: The amount of N transferred (mg N plant^{-1}) by two red clover cultivars (AC Christie and Tempus) at three harvests during the 2009 growing season.

Red clover cultivars	Harvest[z]			
	1	2	3	Total
AC Christie	31	38	65	134
Tempus	36	45	69	146
Mean	33	41	67	140
SEM[y](n=12)	13.1	14.1	10.6	20.2
F-probability	ns[w]	ns	ns	ns

[z]All measurements are on a dry weight basis.
[y]SEM = standard error mean.
[w]ns = *P* value greater than 0.05.

Appendix IX

Table: Chemical properties of the soil during the establishment year (2009) at 0-15 cm soil depth.

Chemical properties	0-15 cm
Organic Matter (g kg^{-1})	30.9
Soil pH	6.4
CEC (meq $100g^{-1}$)	9.67
Total N (g N kg^{-1})	0.66
P_2O_5 (kg ha^{-1})	675
K_2O (kg ha^{-1})	185
Ca (kg ha^{-1})	2441
Mg (kg ha^{-1})	426
Na (kg ha^{-1})	48
S (kg ha^{-1})	28
Fe (mg kg-1)	218
Al (mg kg-1)	1228
Mn (mg kg-1)	122
Cu (mg kg-1)	1.38
Zn (mg kg-1)	1.83
B (mg kg-1)	0.80
Base saturation (%)	
K	2.03
Ca	63.1
Mg	18.4
Na	1.07
H	15.4

Appendix X

Table: Nitrogen concentration (%) of the six red clover cultivars under mixed stands with bluegrass across three harvests during the 2010 and 2011 growing seasons.

Year	Red clover cultivars	Red clover shoots N concentration (%)			Bluegrass shoots N concentration (%)		
		Cut 1	Cut 2	Cut 3	Cut 1	Cut 2	Cut 3
2010	AC Christie	2.83	2.86	3.38	1.15	1.82	1.88
	Tapani	2.90	2.79	3.62	1.14	1.82	1.96
	CRS 15	2.86	2.65	3.30	1.20	1.80	1.84
	Tempus	2.97	2.95	3.55	1.23	1.90	1.97
	CRS 39	3.08	2.93	3.39	1.13	1.80	1.91
	CRS 18	3.07	2.91	3.58	1.19	1.83	1.88
	Grand mean	2.95	2.85	3.47	1.17	1.83	1.91
	SEM	0.064	0.047	0.103	0.034	0.034	0.048
	F-probability						
	Ploidy	0.003	<0.001	ns	ns	ns	ns
	Cultivar	ns	0.074	ns	ns	ns	ns
	..C15 vs ACC, Tap	ns	0.009	ns	ns	ns	ns
	..Temp vs C39, C18	ns	ns	ns	ns	ns	ns
	..C15 vs Tem	ns	0.076	ns	ns	ns	ns
2011	AC Christie	2.22	2.72	3.64	1.03	1.82	2.59
	Tapani	2.33	2.89	3.57	0.99	1.89	2.55
	CRS 15	2.35	2.75	3.38	0.95	1.76	2.83
	Tempus	2.26	2.70	3.64	1.02	1.82	2.61
	CRS 39	2.36	2.85	3.66	0.98	1.78	2.61
	CRS 18	2.23	2.78	3.54	0.98	1.80	2.66
	Grand mean	2.29	2.78	3.57	0.99	1.81	2.64
	SEM	0.052	0.111	0.077	0.024	0.040	0.080
	F-probability						
	Ploidy	ns	ns	ns	ns	ns	ns
	Cultivar	ns	ns	ns	ns	ns	ns
	..C15 vs ACC, Tap	ns	ns	ns	ns	ns	ns
	..Tem vs C39, C18	ns	ns	ns	ns	ns	ns
	..C15 vs Tem	ns	ns	ns	ns	ns	ns

SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

Appendix XI

Table: Shoot nitrogen content (g m^{-2}) of the six red clover cultivars associated bluegrass (dry weight basis) under mixed stands across three harvests during the 2010 and 2011 growing seasons.

Red clover cultivars	Bluegrass shoots N content (g m^{-2})					
	2010			2011		
	Cut 1	Cut 2	Cut 3	Cut 1	Cut 2	Cut 3
AC Christie	2.54	1.44	1.47	5.02	1.51	2.24
Tapani	2.76	1.40	1.45	5.58	1.65	2.44
CRS 15	2.31	1.31	1.26	4.78	1.51	2.55
Tempus	2.47	1.38	1.51	5.29	1.77	2.43
CRS 39	2.38	1.36	1.47	5.50	1.65	2.50
CRS 18	2.42	1.33	1.31	4.94	1.63	2.25
Grand mean	2.48	1.37	1.41	5.19	1.62	2.40
SEM	0.167	0.061	0.085	0.286	0.087	0.150
F-probability						
Ploidy	ns	ns	ns	ns	0.084	ns
Cultivar	ns	ns	ns	ns	ns	ns
..C15 vs ACC, Tap	ns	ns	ns	ns	ns	ns
..Tem vs C39, C18	ns	ns	ns	ns	ns	ns
..C15 vs Tem	ns	ns	ns	ns	ns	ns

SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

Appendix XII

Table: Percentage (%) nitrogen derived from atmosphere (%Ndfa) by six red clover cultivars under mixed stand with bluegrass at three harvests during the 2010 and 2011 growing seasons.

Red clover cultivars	Nitrogen derived from atmosphere (%)					
	2010			2011		
	Cut 1	Cut 2	Cut 3	Cut 1	Cut 2	Cut 3
AC Christie	96.81	97.79	98.12	97.09	96.73	93.86
Tapani	96.37	97.29	97.55	97.64	96.74	94.74
CRS 15	96.61	97.89	97.99	96.58	95.93	93.18
Tempus	97.34	97.99	97.88	97.73	96.26	94.11
CRS 39	97.26	97.82	97.61	97.28	96.37	93.42
CRS 18	97.16	98.04	98.06	96.75	95.69	92.54
Grand mean	96.92	97.8	97.87	97.18	96.29	93.64
SEM	0.394	0.358	0.306	0.292	0.471	0.642
F-probability						
Ploidy	0.054	ns	ns	ns	ns	ns
Cultivar	ns	ns	ns	0.040	ns	ns
..C15 vs ACC, Tap	ns	ns	ns	0.039	ns	ns
..Tem vs C39, C18	ns	ns	ns	0.059	ns	ns
..C15 vs Tem	ns	ns	ns	0.075	ns	ns

SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

Appendix XIII

Table: Percentage of N (%) in bluegrass derived from N transferred by six red clover cultivars under mixed stand across the three harvests during 2010 and 2011 growing seasons.

Red clover cultivars	N transfer (%)					
	2010			2011		
	Cut 1	Cut 2	Cut 3	Cut 1	Cut 2	Cut 3
AC Christie	-15.56	7.09	5.80	0.69	7.83	9.99
Tapani	-5.32	-21.82	-5.36	-0.32	4.87	6.11
CRS 15	-15.05	4.08	18.84	-2.13	-1.49	4.68
Tempus	-8.704	16.44	3.59	3.66	8.83	8.79
CRS 39	-1.96	0.05	5.04	1.73	7.08	10.01
CRS 18	-7.67	-2.49	1.91	-11.16	-10.56	-6.79
Grand mean	-9.04	0.56	4.97	-1.26	2.76	5.47
SEM	6.01	9.39	8.55	5.92	5.11	4.22
F-probability						
Ploidy	ns	ns	ns	ns	ns	ns
Cultivar	ns	ns	ns	ns	0.065	0.061
..C15 vs ACC, Tap	ns	ns	ns	ns	ns	ns
..Tem vs C39, C18	ns	ns	ns	ns	ns	ns
..C15 vs Tem	ns	ns	ns	ns	ns	ns

SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

Appendix XIV

Table: Soil total nitrogen concentration of the 0 to 15-cm soil depths during the 2010 production years for six red clover cultivars under mixed stand with bluegrass compared to pure bluegrass stand.

Plant type	Cut 1	Cut 2	Cut 3
AC Christie	0.186	0.195	0.196
Tapani	0.195	0.202	0.187
CRS 15	0.198	0.195	0.191
Tempus	0.190	0.189	0.192
CRS 39	0.191	0.187	0.190
CRS 18	0.196	0.191	0.180
Pure bluegrass	0.191	0.191	0.187
Grand mean	0.192	0.193	0.189
SEM	0.006	0.004	0.005
F-probability			
Ploidy	ns	ns	ns
..Bg vs RC	ns	ns	ns
..Tetra vs Dip	ns	ns	ns
Cultivar	ns	ns	ns
..C15 vs ACC, Tap	ns	ns	ns
..Tem vs C39, C18	ns	ns	ns
..C15 vs Tem	ns	ns	ns

SEM; standard error mean, ns; not significant.
C15; CRS 15, ACC; AC Christie, Tap; Tapani, Tem; Tempus, C39; CRS 39 and C18; CRS 18.

Appendix XV

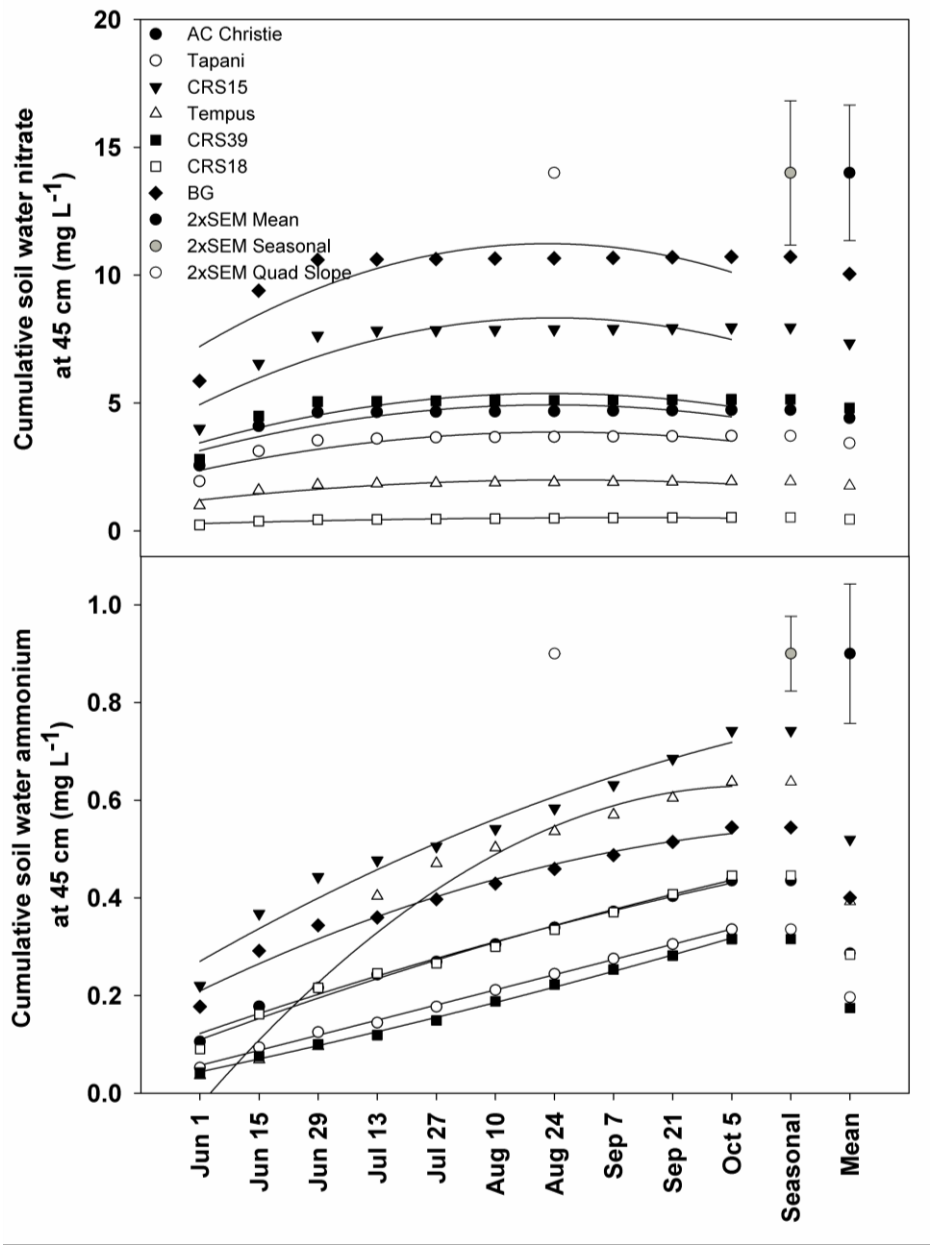

Figure: Variation of cumulative soil water nitrate and ammonium content at 45-cm soil depths of six red clover cultivars under mixed stand with bluegrass vs. pure bluegrass stand during the 2010 production year.

Appendix XVI

Table: Seasonal yield, seasonal N content and shoot N concentration across three harvests (dry weight basis) of the bluegrass under mixed stands and pure bluegrass stand during the 2010 and 2011 growing seasons.

Year	Forage stand	Seasonal Yield ($g\ m^{-2}$)	Seasonal N content ($g\ N\ m^{-2}$)	Shoot N concentration (%)		
				Cut 1	Cut 2	Cut 3
2010	Bluegrass (mixed stand)	365.53	5.25	1.17	1.83	1.91
	Pure Bluegrass	356.92	4.74	1.12	1.62	1.73
2011	Bluegrass (mixed stand)	705.03	9.18	0.99	1.81	2.64
	Pure Bluegrass	648.43	8.52	0.95	1.74	2.52

Appendix XVII

Table: Atom % ^{15}N Excess of the six red clover cultivars under mixed swards with bluegrass, bluegrass under red clover mixed stand and pure bluegrass stand across three harvests during the 2010 and 2011 growing seasons.

Year	Red clover cultivars	Atom % ^{15}N Excess								
		Red clover			Bluegrass (Mixed stand)			Pure Bluegrass		
		Cut 1	Cut 2	Cut 3	Cut 1	Cut 2	Cut 3	Cut 1	Cut 2	Cut 3
2010	AC Christie	0.0234	0.0075	0.0025	0.8072	0.2915	0.1256	0.7251	0.3527	0.1329
	Tapani	0.0249	0.0072	0.0029	0.7987	0.3146	0.1311	0.7146	0.2726	0.1264
	CRS 15	0.0270	0.0068	0.0028	0.8899	0.3307	0.1168	0.8050	0.3301	0.1417
	Tempus	0.0216	0.0069	0.0031	0.8386	0.2936	0.1291	0.8230	0.3513	0.1166
	CRS 39	0.0214	0.0059	0.0031	0.7261	0.2801	0.1142	0.7916	0.2993	0.1311
	CRS 18	0.0217	0.0065	0.0028	0.7463	0.2882	0.1237	0.7955	0.3439	0.1492
	Mean	0.0234	0.0068	0.0029	0.8011	0.2998	0.1234	0.7758	0.3250	0.1330
	SEM	0.0019	0.0006	0.0003	0.0500	0.0160	0.0104	0.0537	0.0289	0.0144
2011	AC Christie	0.0180	0.0086	0.0059	0.6180	0.2430	0.0889	0.6222	0.2644	0.0990
	Tapani	0.0144	0.0072	0.0049	0.6026	0.2260	0.0887	0.6075	0.2411	0.0958
	CRS 15	0.0213	0.0103	0.0066	0.6213	0.2604	0.0932	0.6152	0.2580	0.0988
	Tempus	0.0143	0.0099	0.0058	0.6065	0.2391	0.0911	0.6344	0.2649	0.1015
	CRS 39	0.0167	0.0090	0.0063	0.6120	0.2323	0.0867	0.6294	0.2519	0.0971
	CRS 18	0.0186	0.0098	0.0065	0.6375	0.2523	0.0938	0.5877	0.2310	0.0898
	Mean	0.0172	0.0091	0.0060	0.6163	0.2422	0.0904	0.6161	0.2519	0.0970
	SEM	0.0016	0.0009	0.0005	0.0209	0.0098	0.0037	0.0287	0.0120	0.0044

SEM; standard error mean. (n = 18)