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Optimizing Red Clover (*Trifolium pratense*) for soil fertility variety selection, management and cultivation

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**Optimizing Red Clover (*Trifolium pratense*) for Soil Fertility:
Variety Selection, Management and Cultivation**

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*A thesis submitted in partial fulfilment of the University's requirements for the
Degree of Doctor of Philosophy*

September 2017

Coventry University

In association with the Royal Agricultural University

Declaration

I declare this research is my own work and all written work and investigations are my own, except where stated and referenced otherwise. This work has not been accepted or submitted for an academic award elsewhere.

Patrick McKenna

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Abstract

Environmental and economic considerations are currently driving a resurgence in interest in fertility-building crops. A literature review indicated red clover (*Trifolium pratense*) has the capacity to provide a large amount of nitrogen to subsequent crops, as well as a suite of ecosystem services. Available red clover varieties can be split into categories of early/late flowering, diploid/tetraploid and erect/prostrate. Many viable management and cultivation practices can be applied. A field trial focused on arable farming of six red clover varieties was carried out on clay soil in Cirencester UK from 2015-2017. Fertility-building was documented using dry matter (DM) and nitrogen (N) yields, extent of nodulation, soil mineral nitrogen (SMN) accumulation and morphological variation over two years. Treatments of one and two cuts were applied and all cuttings were removed. The experimental design in the fertility-building phase was two factorial (red clover variety and number of cuts) in completely randomized blocks. Soil fertility was then assessed using wheat (*Triticum aestivum*) and mustard (*Sinapsis alba*) as bioassay crops. The experimental design in the bioassay phase was a split-plot factorial design in completely randomized blocks with preceding variety and number of cuts as whole plot treatments and inclusion or omission of herbicide as splits. The central hypothesis was that red clover variety selection, cutting management and use of herbicide significantly effect capacity to build soil-fertility.

SMN accumulation was high in the first year, in which DM and N yields were low, but low in the second, in which DM and N yields were high. One cut and varieties Astred and Ruby were less susceptible to winter leaching of SMN. The early diploids Ruby and Astred were more resistant to clover rot (*Sclerotinia* sp) and powdery mildew (*Erysiphe* sp) than other varieties and tetraploidy was not predictive of disease resistance. The late variety Amos provided most of its yield at the first cut and the early varieties the bulk of theirs at the second, but the intermediate variety Maro gave high yields at both cuts in both years. Morphology was highly variable, stem hair density may have effected disease resistance and mineralization rates and was correlated with growth habit. Nodulation and root biomass was assessed. All varieties were shown to be nodulated but Ruby had significantly fewer nodules than all other varieties and Ruby and Astred had significantly smaller root systems than all other varieties.

Herbicide (glyphosate) application and omission was included as a treatment in bioassay cultivation. The non-herbicide (NH) treatment failed to adequately terminate the clover crop and volunteering became problematic and hindered crop production in the herbicide (H) treatment. Number of cuts and variety selection were non-significant in almost all cases in

wheat production, but this may have been caused by the increased variability incurred by the clover volunteering. Variety Ruby and 1 cut treatment produced higher mustard DM and N yields but only in the H treatment. The interaction of variety and cut was seen most prominently in Ruby, in which one cut was consistently higher than two. Herbicide application following red clover cultivation did significantly effect subsequent crop performance, but this was likely only due to the elimination of volunteers. One cut yielded significantly more total mustard DM and N than two cut, whilst Ruby yielded significantly more total mustard DM and N than all other varieties.

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Abbreviations

AHDB	Agricultural and Horticultural Development Board
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
BNF	Biological Nitrogen Fixation
°C	Degree Celsius
C:N	Carbon:Nitrogen Ratio
C	Carbon
CaCO ₃	Calcium Carbonate
CVA	Canonical Variate Analysis
cm	Centimeter
CV	Coefficient of Variance
dS m ⁻¹	DeciSiemen per metre
DM	Dry Matter
DEFRA	Department for Environment, Farming and Rural Affairs
FAO	Food and Agricultural Organization of the United Nations
g	Gram
GS	Growth Stage
ha	Hectare
H	Herbicide
HGCA	Home-Grown Cereals Authority
HI	Harvest Index
HCl	Hydrochloric Acid
HP	Horse Power
H ₂ SO ₄	Sulphuric Acid
IBERS	Institute of Biological, Environmental and Rural Science
kg	Kilogram
K ₂ SO ₄	Potassium Sulphate
l	Liter
LSD	Fischer's Protected Least Significant Difference
M	Molar
m	Meter
m	Million
mg	Milligram
ml	Milliliter
mm	Millimeter
MPa	Megapascal
N	Nitrogen
NH	Non-Herbicide
NH ₄ ⁺	Ammonium
(NH ₄) ₂ MoO ₄	Ammonium Molybdate
NHI	Nitrogen Harvest Index
NO ₃ ⁻	Nitrate
NO _x	Nitrogen Oxides
N ₂ H ₄ O ₃	Ammonium Nitrate
NaHCO ₃	Sodium Bicarbonate
NaOH	Sodium Hydroxide
NIAB	National Institute of Agricultural Botany
nm	Nanometer

ns	Non-Significant
P	Phosphorus
PCA	Principal Component Analysis
ppm	Parts Per Million
RC	Red Clover
SED	Standard Error of the Difference
SMN	Soil Mineral Nitrogen
SOM	Soil Organic Matter
t	Metric Tone
TGW	Thousand Grain Weight
μg	Microgram
UK	United Kingdom
UN	United Nations
USA	United States of America
USDA	United States Department of Agriculture
WGD	Whole Genome Duplication

General Introduction

Clarion calls for an increase in food production to feed a rapidly growing population have been ubiquitous in the agricultural science literature for some time (FAO 2009b, Godfray *et al.* 2010, Beddington 2011, Conway *et al.* 2012, Godfray and Garnett 2014). An increase in productivity is certainly required, and this will need to be achieved using fewer inputs and more sustainable practices (Pretty 2008), but the discourse surrounding this issue has become more nuanced in recent years (Bernard and Lux 2017). Population growth remains a challenge which must be met, but we must accept that almost all of this growth will be in developing countries where productivity remains low, not developed countries where productivity is comparatively high (FAO 2015). Food is distributed throughout the world, but trade of often subsidized food from rich to poor countries can undermine smallholder farmers and damage local economies (Oxfam 2005). Non-emergency food aid programs may also exacerbate food insecurity in poor countries (Moyo 2010).

Goals for global agriculture must then be split into two categories. For the developing world, particularly Sub-Saharan Africa, the goal is simple. Increase food production using sustainable farming practices to fulfil the productivity component of food security (van Ittersum *et al.* 2016, Hall *et al.* 2017). Additional components of access, stability and utility must also be addressed, but these require mainly political change and therefore exist outside the remit of agriculture (FAO 2009a). Goals for agriculture in the developed world will, however, involve more nuanced considerations.

For example, although a significant increase in food production is a commonly cited goal for developed world agriculture, it remains unclear how this will address food security in a society where up to half of all food is wasted (FAO 2011). Increases in productivity based on current practices may also increase agricultural greenhouse gas emissions, which may further undermine the capacity of developing world farmers to increase productivity (Bennetzen *et al.* 2016). The large increase in livestock product consumption in the developed world over the last century has damaged environmental integrity and human health (Weis 2013), and a return to more plant-based diets would be preferable to further increase if this damage is to be limited. An increase in productivity is therefore only desirable if it is achieved using sustainable practices and runs concomitant with social and economic change.

Wheat (*Triticum aestivum*) accounts for 20% of global calories and its production is primarily associated with developed world agriculture (Shiferaw *et al.* 2013). Conventional wheat

production relies on mineral fertilizer to sustain yields, but agronomists and farmers are increasingly looking to leguminous crop rotations to reduce this dependency (Snyder *et al.* 2016, Tamm *et al.* 2016). The adoption of such rotations contributes a more sustainable Nitrogen (N) source through biofixation (Smil 1999) and a number of ecosystem services which contribute to overall system sustainability.

Purpose of Study

The benefits of leguminous crop rotations have been known for centuries (Galloway *et al.* 2004) but optimal management and cultivation strategies remain unclear. Agricultural legumes can be split into varying categories; green manures (e.g. *Crotalaria* sp) cultivated to be tilled into soil, forage legumes (e.g. red clover – *Trifolium pratense*) grown for forage but may also be green manured, grain legumes (e.g. soybean – *Glycine max*) grown for edible seed, and trees (e.g. *Inga* sp) grown in agroforestry systems (Giller 2001b). Cereals are often grown in rotation with both forage and grain legumes, but grain legumes are less associated with subsequent soil fertility and may require fertilizer applications themselves (Salvagiotti *et al.* 2008). Rotations with forage legumes may then be optimal for fertility-building and the reduction of mineral fertilizer dependency.

A number of forage legumes are currently used in temperate agriculture, notably red clover (*Trifolium pratense*), white clover (*Trifolium repens*) and lucerne (*Medicago sativa*). This study is focused on red clover for reasons reviewed in detail in Chapter 1. The overall aim of this study is to understand how RC variety selection, cutting management, termination with/without herbicide, and interactions between all three impact on soil fertility-building capacity. Increased understanding of these factors may allow farmers to optimize their RC rotations and increase the sustainability of their cereal production.

Means of Study

All results are obtained from a two year agronomic field trial conducted between April 2015 and February 2017. Experimental design is detailed in Chapter 2. Various statistical methods are employed to make accurate inferences, the details of which are given in the relevant chapters.

Aims and Objectives

The overall aim of this work is to select six different varieties of RC and trial them for soil-fertility building capacity using different management practices. Soil fertility-building capacity is assessed using the cultivation of subsequent bioassay crops, wheat and mustard (*Sinapsis alba*). Morphological variation among varieties is predicted, and this is also documented to determine its extent and relevance in fertility-building capacity.

The specific objectives are:

- To cultivate a diverse range of RC varieties over two years
- To apply treatments of one and two cuts and remove all cuttings
- To document dry matter (DM) and N yields and protein content
- To describe RC varietal morphological diversity
- To document the accumulation of soil mineral nitrogen (SMN)
- To assess nodulation and root biomass

These objectives are pursued with a view to assess their role in soil fertility-building capacity.

Additional objectives are:

- To cultivate wheat following one year of RC cultivation and apply treatments of inclusion and omission of herbicide prior to cultivation
- To cultivate three mustard crops following one year of RC cultivation and apply treatments of inclusion and omission of herbicide prior to cultivation
- To document DM and N yields of these crops

These objectives are pursued to assess the fertility-building capacity of the preceding clover cultivation and treatments applied (1 & 2 cuts) in arable systems exclusively. This research is carried out with conventional farming in mind, as applications of fungicides and herbicides were carried out. One and two years of monoculture red clover cultivation was employed, not undersowing in subsequent cereal crops.

Hypotheses

Fertility-Building Phase

- Red clover varieties are morphologically, agronomically and phenologically diverse as described by breeders and seed merchants
- This diversity significantly effects the capacity of the crop to build soil fertility
- Cutting management (1 or 2 cuts) significantly effects the subsequent soil fertility following one year of red clover cultivation
- The accumulation of SMN can be used as a proxy to assess subsequent soil fertility following cultivation of red clover under different management

Bioassay Phase

- The inclusion of a herbicide prior to cultivation will significantly effect yields of subsequent wheat and mustard crops
- Multiple crops of mustard will have a higher nutritional demand than a single wheat crop and will give a more accurate measure of soil fertility

Thesis Structure

Chapter 1. Literature Review.

Review of RC use in fertility-building. Topics included are rates of biofixation, mineralization of residues, associated ecosystem services, varietal differences, management practices and economic implications

Chapter 2. Materials and Methods

Summary of methodologies and field operations used to cultivate crops and collect data. Basic information on statistical procedures is included, further details are given prior to use in relevant chapters.

Chapter 3. Core Experiment 1

RC Cultivation 2015. DM/N yields, protein content, SMN accumulation and morphological diversity.

Chapter 4. Core Experiment 2

RC Cultivation 2016. DM/N yields, protein content, SMN accumulation, morphological diversity, nodulation and root biomass assessment.

Chapter 5. Core Experiment 3

Bioassay Cultivation 2016. Main effects and interactions between variety selection, cutting management and inclusion/omission of herbicide at termination on subsequent DM/N yields of wheat and mustard crops.

Chapter 6. Discussion and Conclusion

General discussion and conclusions. The effect and interactions of Chapter 5 are discussed, conclusions drawn and recommendations on how RC use in fertility-building can be optimized. Implications of the study and new areas for further research are also included.

CHAPTER 1 - Literature Review

1.1 Introduction

The human population has more than doubled in the past fifty years (UN 2015). This growth is predicted to reach 9.6 billion by 2050, and it is estimated this will necessitate a 70-100% rise in food production (Godfray, *et al.* 2010). The developing world must achieve this increase to ensure global food security, but Malthusian alarmism should not blind us from the crux of the issue. Overemphasising productivity can diminish the ecological and social elements of ensuring global food security (Swaminathan 2006), and this is perhaps the strongest criticism of previous agricultural innovation. Increases in productivity are required, but they must be appropriately contextualized and achieved with commensurate environmental goals (Hunter *et al.* 2017). They must also be coupled with the resolution of food access and distribution inequalities to be effective in achieving food security (Loos *et al.* 2014).

The conclusions of Beddington (2011) and the ‘100 questions’ of Pretty *et al.* (2010) lay out the complexity of achieving this goal. Innovation in a wide variety of agricultural systems from crop rotations, agroforestry, mixed crop-livestock and crop-aquaculture systems to minimum tillage and precision agriculture will be increasingly required. These strategies will need to be carefully designed, locally adapted and appropriate to specific agroecological/socioeconomic contexts. They will also need to be developed in a farmer-inclusive and participatory manner to ensure they can be successfully adopted. Wheat, rice and maize supply over 50% of global calories (Cassman 1999) and their future production must continue in a sustainable manner if they are to continue feeding the planet. Cereal production was increased in the 20th century using Green Revolution technologies, but this sometimes came with associated environmental damage, most prominently reported in Asia (Singh 2000, Pingali 2012). The Green Revolution agricultural model of modern crop variety adoption supported by intensive irrigation and substantial chemical-use may be increasingly incompatible with sustainable intensification. If food security is to be achieved in the future then cereals must be produced through greater optimization of external inputs, in systems that also prioritize environment and soil conservation (Pretty, *et al.* 2010).

This review will focus on the contribution that red clover (*Trifolium pratense*, L.) can make to sustainable agriculture. Historically red clover (RC) was traditionally cultivated in rotations

with other crops to build soil fertility, but the advent of nitrogen fertilizers in the 20th century has displaced much of this use. Clearly RC still has a role in many organic systems (Nykanen *et al.* 2000) but most of its modern usage now lies in grass/clover leys for forage. As a result there exists a rich literature on its role in stocked systems, for example persistence in swards (Frame *et al.* 1998, Abberton and Marshall 2005); the feed-quality of silage (Owens *et al.* 1999); the effect of polyphenol oxidase on protein digestibility (Sullivan and Hatfield 2006, Eickler *et al.* 2011) and the potential for bloating caused by phytoestrogen content (Moorby *et al.* 2004). The traditional role of RC as a fertility-building crop remains, however, underutilized and this review will focus solely on arable systems and omit both stocked and mixed systems.

1.2 Historical Perspective

Wild RC is thought to have originated in South East Eurasia and was first cultivated by farmers in Europe as early as the third century (Taylor and Quesenberry 1996). Cultivation and domestication proceeded over the centuries and landrace populations were established all over Europe. These landraces are now mostly conserved *ex situ* and are used as starting points by modern breeders (Boller *et al.* 2010). The use of RC in Europe became ubiquitous by the 16th century (Mousset-Declas 1992), mainly for its protein-rich fodder and nitrogen (N) contribution in crop rotations. This dual role is cited by some commentators as having more impact on European agriculture than the introduction of the potato (Fergus and Hollowell 1960).

Flemish farmers were first to replace the fallow periods in their rotations with RC and this significantly increased productivity (Rham 1860). The rotation of wheat-turnip-barley-RC was pioneered by Charles Townshend in Britain and became known as the Norfolk-4 Course Rotation (Knox *et al.* 2011), an important development in the Agricultural Revolution of the time. The capacity of RC to increase productivity was also recognised by Thomas Jefferson, who wrote in a letter to a friend;

‘Horizontal and deep ploughing, with the use of plaister and clover, which are but beginning to be used here will, as we believe, restore this part of our country to its original fertility’

(Jefferson 1817)

Of course, Jefferson wrote this letter a century before the Dust Bowl and was unaware of the effect ‘horizontal and deep ploughing’ would go on to have in parts of America (Baveye *et al.* 2011), but he was clearly ahead of his time in understanding the importance of leguminous

rotations in the maintenance of soil fertility. RC was instrumental in temperate arable rotations from here onwards (Chorley 1981) and is now found almost globally. Recommended growing conditions are summarized in Table 1.1, along with reported distribution and bioactive compounds.

Table 1.1 Summary of RC distribution, growth conditions and bioactive compounds

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1. Study cited took place on sandy loam

Biologically available N had previously been a limiting factor in food production for European farmers (Kitsikopoulos 2004), but clover leys allowed this to be overcome. RC was subsequently taken to the New World by European explorers, and its use became more and more widespread until 1909 when Fritz Haber and Carl Bosch first synthesized ammonia (Smil 1991). This watershed moment for agriculture allowed industrialists to manufacture nitrogenous fertilizer, marking the beginning of modern dependency on mineral N and the decline of clover cultivation (Jenkinson 2001). The post-war economic boom was marked by a significant increase in Haber-Bosch ammonia synthesis (Erisman *et al.* 2008). Global production rose from 3.7 Mt in 1950 to 85 Mt in 2000 (Smil 2011) and RC cultivation declined in tandem. Hectare losses ranging from 30% (Rochon *et al.* 2004) to 70% (Frame 1976) have been reported. Nitrogenous fertilizers became a hallmark of late 20th century agriculture, and

this reliance on mineral inputs has led to the commentary that modern agriculture has become ‘the art of turning oil into food’ (Foster 2010). RC is still used as a fertility-building crop in the organic sector, particularly in Europe (Nykanen, *et al.* 2000, Aamlid 2002, Cormack *et al.* 2003) but its overall use in global agriculture has markedly declined (Taylor 1985). Sustainable intensification will require sustainable N sources, and a revival of RC cultivation can assist in meeting this requirement (Beddington 2011)

1.3 Prospects for Red Clover in Soil Fertility-Building

Concerns over the environmental impact of mineral nitrogen fertilizers in cereal production have revived interest in fertility-building with forage legumes (Taylor 2008a). However, the high N demand of cereals makes their production challenging in lower-input systems (Gooding and Davies 1997). Timely residual N release (i.e. during the spring growth season) is also essential for cereal production. Doel (2013) investigated the impact of various fertility-building plants on the yield of subsequent winter and spring wheat in the UK, and reported significantly higher yields following RC cultivation. Moyo et al (2015) investigated how management practices and companion grasses can significantly impact on the cereal yield benefits associated with RC rotations. These studies are summarized in Figures 1.1 and 1.2.

Figure 1.1 Impact of various fertility-building crops on winter/spring wheat yields over 6 and 12 months with differing management strategies (Doel 2013)

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Figure 1.2 Impact of various RC combinations under differing management strategies on spring/winter wheat over 16 months (winter wheat) and 20 months (spring wheat) (Moyo et al. 2015)

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The data summarized in Figure 1.1 (Doel 2013) indicates RC significantly contributes to soil-fertility in short time periods. These significant yield contributions, combined with a reduction in mineral inputs, contribute to the environmental and economic sustainability of cropping systems, but readoption remains limited despite these benefits (Schipanski and Drinkwater 2011). This may be partly due to externalities like government policies, market dynamics or culture (Olmstead and Brummer 2008), but research into how the yield benefits of RC/cereal rotations can be further optimized could provide a more convincing case for their readoption. Management practices and companion grasses can significant impact on the cereal yield benefits associated with RC rotations (Moyo 2015), and further research into this area will allow farmers to tailor their use of RC to suit their site and climate.

The data summarized in Figure 1.2 (Moyo *et al.* 2015) indicates mixtures of RC and companion grasses result in higher subsequent cereal yields than pure swards. Legume/grass combinations can exploit nutrient sources more effectively than monocultures (Haynes 1980) and grasses can put an increased demand for fixed N on legumes by competing for rhizosphere N (Loges *et al.* 1999, Cuttle *et al.* 2003). However, these benefits may be cancelled by deleterious competition for water and light from grasses, which can reduce the legume-content and soil-fertility building capacity of the crop (Kleen *et al.* 2011). Both systems are however shown to be efficacious in building soil-fertility for cereal production. This flexibility in use can also allow farmers to tailor their use of RC to suit their own personal needs, i.e. in mixed systems farmers wishing to use above-ground residues for silage may use a grass mixture without compromising too much on N supply to subsequent cash crops such as cereals (Rochon, *et al.* 2004).

1.4 Nitrogen for Contemporary Agriculture

Crop production is the biggest human alteration to the global nitrogen cycle and the supply of N has historically been the most limiting nutrient in productivity (Smil 2002). Many historical agricultural developments have focused on overcoming this, for example the use of guano (Hadas and Rosenberg 1992); transportation of night soil (Petrik 1954); Chilean saltpetre (Vilches 2011); traditional recycling of organic wastes (Smil 2011) and leguminous crop rotations (McNeill and Winiwarter 2004). Ecologically-friendly farming practices such as organic farming prohibit the use of artificial fertilizers (IFOAM 2014), but organic manures can be even more susceptible to N losses than conventional fertilizers (Allingham *et al.* 2002)

Although modern conventional agriculture is most associated with Haber-Bosch N, it also utilizes a number of other sources. This N is applied to agricultural land in crop residues,

biofixation, lightening, irrigation and atmospheric deposition (Smil 1999). Quantification of all these inputs is beset by uncertainties and the only reliably accurate statistics for agricultural inputs are for mineral fertilizers, although some notable estimates have been calculated by Smil (1999) and Galloway *et al* (2004). More recently in the European Nitrogen Assessment Project, researchers have made estimates of N cycling in European agriculture (Leip 2011).

Knowledge of the various N sources in global agriculture helps to contextualize the contribution of legumes, but describing the flow of N in agriculture is difficult for a number of reasons. There are a myriad of uses for crop residues (various fuels, fodders and fibres) and no country keeps comprehensive statistics of their uses. This makes it difficult to assess their contribution to soil N. It is also difficult to calculate the N content of manure from stock under different systems of production, no less to determine what percentage of this is returned to the soil after manuring. Furthermore, as this review outlines, the numerous methods of measuring fixation in the nodules of legumes have produced widely varying accounts of the contribution of fixation to the overall N economy. These uncertainties make assessing the current and potential N contribution of RC and other legumes crops to global agriculture harder to quantify. Examples of estimated N inputs to global, regional and single-farm agriculture are given in Tables 1.2 and 1.3.

Table 1.2 Summary of estimated N inputs to global agricultural soils (Liu *et al.* 2010)

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Table 1.3 Summary of estimated N inputs to global agricultural soils (Allingham *et al.* 2002, Leip 2011, Smil 1999)

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These estimations include both natural and anthropogenic inputs. They are essential for productivity, but can also drive environmental pollution through eutrophication (Boesch and Brinsfield 2000, de Vries *et al.* 2011), water quality degradation (Liu *et al.* 2005, Bouraoui and Grizzetti 2014) and greenhouse gas emissions (Richardson *et al.* 2009). Nitrogen pollution occurs through loss of mineral N (leaching); losses from slurry and manure applications (run-off) and the conversion of mineral N to gaseous N by soil microbes (denitrification). The degree to which this occurs is variable, but it can be up to 70% of what is applied (Mulvaney *et al.* 2009). These losses can harm the environment whilst limiting the nitrogen-use efficiency of crop plants (Fageria and Baligar 2005), which means N supplies for sustainable intensification will need to be more efficient in their transfer to crops. RC cover crops may simultaneously fix N and mitigate leaching under optimal management strategies (Larsson *et al.* 2005), which can reduce reliance on inputs and improve nitrogen efficiency.

Understanding how sustainable sources of N can be optimized demands an understanding of the biogeochemical cycling of N in systems using legumes. This can be split into three component parts; biological fixation of atmospheric nitrogen by *Rhizobia* bacteria living in the root nodules, the subsequent return of organic N to the soil, and the uptake of this by subsequent crops (Cuttle, *et al.* 2003). Biological nitrogen fixation (BNF) in legumes can be limited or enhanced by soil N status; establishment/persistence, genotypic variation and stresses (Ledgard and Steele 1992, Cherr *et al.* 2006b), mineralization rates by the C:N ratio of crop residues, management strategies, climate and soil microbe activity (Groffman *et al.* 1987, Sarrantonio and Scott 1998). This means that a variety of agronomic factors must be considered to optimize N contributions from RC cultivation.

1.5 Biological Nitrogen Fixation

BNF occurs in specialized structures known as nodules in RC and other plants, particularly legumes. Nodules are formed following infection by soil-dwelling bacteria of varying genera (predominantly *Rhizobium* and *Sinorhizobium* (Sprent 2007)). Once formed the legume supplies the rhizobium with sugar for growth, and the rhizobium converts atmospheric nitrogen contained in the soil into ammonia.

A number of studies documenting the volume of nitrogen fixed by RC under varying management strategies have been conducted. Tables 1.4-1.7 summarize the results of these findings, along with the relevant crop, location, management, weather conditions and measurement methodology information

Table 1.4. Summary of nitrogen fixation under cut & mulch management using difference method

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*Hyphen indicates range documented, Forward slash indicates multiple years of data cited

*Wet refers to a year in which annual precipitation was higher than 550mm. Dry refers to years lower than 550mm.

* 'Root factor' principle outlined in Unkovich *et al* (2010) and calculated to be 1.72 for RC by Peoples *et al* (2012). Average fixation measurements are multiplied by 1.72 to include N contributions from above/below ground biomass.

Table 1.5 Summary of nitrogen fixation under cut & remove management using difference method

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* 'Root factor' principle outlined in Unkovich *et al* (2010) and calculated to be 1.72 for RC by Peoples *et al* (2012). Average fixation measurements are multiplied by 0.72 to include N contributions from root biomass and exclude removed cuttings

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* 'Root factor' principle outlined in Unkovich *et al* (2010) and calculated to be 1.72 by Peoples *et al* (2012). Average fixation measurements were multiplied by 0.72 to include N contributions from root biomass and exclude removed cutting

N fixation measurement methodologies vary in detail and accuracy. A detailed discussion of the technicalities, merits and limitations associated with each methodology can be found in Peoples *et al* (1989) and Ledgard and Steele (1992). The ^{15}N methods are considered more accurate and therefore more widely used than the difference method, however Peoples *et al* (2015) compared records of the ^{15}N abundance/dilution methods and found a probability of .54 that the methods provided estimates within $\pm 10\%$. Therefore all values reported should be considered estimations. Additionally, all tabulated studies only account for aboveground herbage N. To account for this the ‘root factor’ estimations of belowground contributions are also included, as outlined by Unkovich *et al* (2010). All reported estimations have been averaged and are summarized in Table 1.7.

Table 1.7 Summary of Means from Tables 1.4-1.6

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Measurements of soil N contribution from RC rotations allow farmers to empirically account for N legacies. Recorded ranges confirm that of other published ranges (Bowley *et al.* 1984, Taylor 1985, Carlsson and Huss-Danell 2003), which recorded a wide range from 25 to 373 kg $\text{ha}^{-1} \text{yr}^{-1}$ for RC. The broad range of Tables 1.4-1.6 precludes robust statistical analysis, however the key limitation of these studies is they only account for atmospheric nitrogen contained within the aboveground herbage. The belowground N remains unaccounted for in the majority of cases. This oversight is compounded by the usual RC management strategy, which as Tables 1.4-1.6 indicate, is mostly to harvest for silage or hay. Mulching is also practiced, but this can inhibit subsequent fixation and regrowth (Loges, *et al.* 1999, Moyo 2014). There are also concerns of N losses through leaching and denitrification with RC green manures (Stopes *et al.* 1996, Schmidt *et al.* 1999), but the use of companion grasses can mitigate this in leaching-prone sites and climates (Moyo, *et al.* 2015). If RC management is mostly cut and remove, then the challenge for agronomists is to translate the measurement of fixation obtained from these cuttings into a realistic estimate of the actual N contribution to the soil.

This contribution is made through the senescence of the root system (which contains a store of N) and deposition of N in the rhizosphere (Russell 1973). Difficulties in defining the extent of the rhizosphere, and in recovering entire root systems for analysis, make this contribution difficult to predict (Hogh-Jensen and Schjoerring 1997). Consequentially there is limited information on the belowground N contributions in RC leys and this may confound the applicability of N fixation measurements based on aboveground herbage (which is generally used for forage). RC is known to grow a large taproot which can grow to a depth of 1 meter in the soil (Boller and Nosberger 1987) which means estimates based on aboveground herbage may underestimate the whole-plant N contribution to the cropping system. An attempt to account for this has been made by Unkovich *et al* (2010), who proposed that knowledge of the above/below ground N partitioning can be used to develop a ‘root factor’ which can be applied to aboveground measurements to estimate N contributions from roots. Peoples *et al* (2012) calculated this to be 1.72 for RC (based on a recording of 42% of total plant N contained in the roots). This consideration is included in Tables 1.4-.1.6. Hogh-Jensen *et al* (2004) offers a more detailed empirical model for assessing N legacies in clovers, but this model requires soil N details that are often absent in studies documenting N fixation.

Table 1.7 shows what N contributions farmers can realistically expect from 1 year RC crops under differing management strategies. Nitrogen fixation in forage legumes is moisture-sensitive and will be reduced in times of drought (Ledgard *et al.* 1987), but sparsely available studies on dry years with RC make this difficult to assert. As a deep-rooted plant, fixation in RC may be less affected than more shallow-rooted forage legumes, like white clover (*Trifolium repens*). The literature on measurement methodologies also suggests the difference method tends to overestimate fixation (Ledgard and Steele 1992) and this tendency has been confirmed by the lower recorded measurements for the isotopic methods tabulated in Table 1.6. However, none of the studies reported in Tables 1.4-1.6 account for the rhizodeposition of N by RC. Some some authors cite RC as releasing more N in this way than that containing in the aboveground biomass (Hogh-Jensen and Schjoerring 2001). This means estimates of N contributions from RC cover crops omitting this may be in significant underestimations.

Given the varying crop management and field conditions in which these studies took place, it is difficult to draw definitive conclusions about the amount of N that may be fixed by RC in a given period. Agronomists should accordingly ask not how much N will RC fix, but what are the environmental conditions, genetic varieties and management strategies which increase fixation rates. Widely reported sub-optimal fixation in N-rich environments should be of

particular concern (Haynes 1980, Waterer and Vessey 1993, Goh *et al.* 1996, Stopes, *et al.* 1996) as many farmers will use fertilizers in tandem with RC cultivation. Further research into rhizobial interactions may also facilitate optimal fixation rates in RC cropping.

BNF is a two stage process encompassing nodulation followed by N fixation. This subject has been widely studied (Long 1989, Fisher and Long 1992, Oldroyd *et al.* 2011). Nodulation occurs following a successful exchange of signalling chemicals between the host and *Rhizobium*, and is dependent on the host plant genotype, the Rhizobia strain and their combined interactions with soil and climate (Bordeleau and Prevost 1994). Some rhizobia have a very broad host range, for example the NG345 strain is known to nodulate over 100 genera (Pueppke and Broughton 1999), whilst other rhizobia are much more promiscuous (Fisher and Long 1992).

Most legumes exhibit a degree of specificity in relation to the Rhizobia species with which they form symbiosis, and association with a non-specific strain can result in sub-optimal nodule formation and low levels of BNF. This issue must be considered when using legumes for soil improvement. RC exhibits a high degree of specificity for the Rhizobia species *Rhizobium leguminosarum* biovar *trifolii* (Taylor and Quesenberry 1996), which is known to only nodulate plants of the *Trifolium* genus (Denarie *et al.* 1992). Seed inoculation with this biovar can be used to ensure optimal nodulation. This is most important in regions without indigenous *Rhizobium leguminosarum* biovar *trifolii* populations like Latin America (Batista *et al.* 2015) and Australia, where the *Trifolium* genus is not naturally abundant (Brockwell *et al.* 1995). There is also evidence suggesting that within *Rhizobium leguminosarum* biovar *trifolii* there are variations in the strains, some of which will nodulate RC more effectively than others (Miller *et al.* 2007). Pre-treatment with Nod factors (Rhizobial signalling chemicals) may also enhance nodulation (Dominika *et al.* 2009). These findings indicate how the N contribution of RC can be further optimized through better understanding the nodulation process and chemistry involved, and improved inoculation where necessary.

1.6 Mineralization of Nitrogen

N contributions from RC cultivation can be split into two categories; that derived from rhizodeposition during the growth phase (Hogh-Jensen and Schjoerring 2001, Dahlin and Stenberg 2010b), and that released upon mineralization of plant residues following termination and cultivation of the subsequent bioassay (Kuo and Sainju 1998, Eriksen 2001). These

contributions may be further boosted by N derived from external sources, for example atmospheric deposition or mineralization of pre-existing plant residues (Briggs 2005). These contributions are likely to be small, although some authors documented an average of 30 kg ha⁻¹ yr⁻¹ NO_x rain pollution (Pretty 2015). The assessment of such contributions, however, exists outside of the scope of this review.

Rhizodeposition can be broadly defined as the process of release of organic and inorganic compounds from living plant roots (Gregory 2006) and is primarily studied in the context of carbon cycling (Hutsch *et al.* 2002, Butler *et al.* 2003). Nitrogenous compounds such as nitrate (Wacquant *et al.* 1989), ammonium (Brophy and Heichel 1989) and amino acids (Schilling *et al.* 1998) can also be deposited. Rhizodeposition of N is predicted to be higher in legumes than non-legumes because biofixation increases their overall N assimilation rate (Urbatzka *et al.* 2009), but accounting for this N in fertility-building studies is challenging. Wichern *et al.* (2008) reviewed studies including both grain and forage legumes and multiple measurement methodologies, and found legumes deposited a median of 16% of their total N content. The tendency for grain legumes to sequester N in the seed during grain-filling implies they may deposit less N. Other authors have also shown how measurement methodologies can overestimate N rhizodeposition and therefore the significance of rhizodeposition in soil fertility-building (Gasser *et al.* 2015).

Estimates of rhizodeposition rates associated with RC leys range from 5% of total plant N (Hammelehle *et al.* 2014) to over 100% of total plant N (Hogh-Jensen and Schjoerring 2001). They further range across different climates, soil types and measurement methodologies. Some are field-based, others are pot-based. This variation likely contributes to the large range observed, and any inferences regarding subsequent soil fertility that could be drawn from reviewing these studies are further compromised by considering the ultimate fate of this deposited N. Some will remain in the soil to be taken up by the subsequent crop. How much will be determined by the weather conditions and soil texture. For example, clay soils typically hold more ammonium ions than sandy soils due to the binding effect associated with smaller particles (Espinoza *et al.* 2005). Some rhizodeposited N will also be taken up by the crop itself (Janzen 1990), whilst some more will be lost, either through denitrification (Jensen 1996) or immobilization by microbial activity (Mayer *et al.* 2003). This evident lack of clarity on the amount of N actually deposited and utilized by subsequent crops means the rhizodeposition component of fertility-building with RC leys remains unclear.

Return of organic N to soil is the second component of N cycling in cover cropping. This is known as mineralization and is defined as the decomposition of plant residues into ammonium and nitrate. Traditional agricultural practice favours forage legumes like clovers over grain legumes for this purpose, because they decompose at a faster rate (Peoples *et al.* 1995). Within the forage legumes, RC has a relatively low C:N ratio range of 13.6-16.7 (Bruulsema and Christie 1987), which particularly lends itself to rapid decomposition. Grasses typically have higher C:N ratios which causes N immobilization. This reduces the availability of N to the subsequent crop, but is also the reason grasses are used to prevent leaching (Schroder *et al.* 1996, Kuo and Sainju 1998). Knowledge of the C:N ratio of cover crops is important when selecting candidate plants for specific purposes. Table 1.8 summarizes the C:N ratio of RC along with other common cover crops for reference

Table 1.8 Summary of C:N ratio of common cover crops (Bruulsema and Christie 1987, USDA 2011)

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The C:N ratio of cover crops may also change over the growing period. Increased lignification associated with maturity tends to raise the C:N ratio and further immobilize N, evidenced by a higher C:N ratio within wheat and oat straw compared to their leaves. A low C:N ratio is desirable for rapid mineralization, however this will not always guarantee N transference. Ensuring efficient N transfer in cover cropping with legumes is cited as one of the most important elements of N management (Crews and Peoples 2005, Snapp and Borden 2005). Favourable timing of tilling and sowing may optimize transfer of fixed N from legume residues to subsequent crops (Francis *et al.* 1995, Brandsaeter *et al.* 2008). Other factors include residual biochemical composition (Fox *et al.* 1990, Sarrantonio and Scott 1998), management strategies (Varco *et al.* 1989), climatic conditions (Jarvis *et al.* 1996) and soil microbial activity (Juma and McGill 1986). It is therefore difficult to draw direct 'one-for-one' comparisons between

RC systems and the mineral inputs of conventional agriculture, or the animal-origin inputs of organic agriculture (Cherr *et al.* 2006a).

1.7 Ecosystem Services

Agriculture both provides and relies upon ecosystem services (Zhang *et al.* 2004). If new cropping strategies are to achieve production of ‘two times more with two times less’ then they must generate as many ecosystem services as possible (Sibbing 2014). RC is known to contribute multiple ecosystem services, many of which enhance the sustainability of the overall system. These ecosystem services are summarized in Table 1.9.

Table 1.9. Summary of ecosystem services associated with RC rotations

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As Table 1.9 indicates, the ecosystem services associated with RC cropping are mostly soil improvements, although the flowers are also documented to benefit pollinator population and diversity. RC can increase soil organic matter (SOM) which in turn facilitates soil aggregation (FAO 2014) and water-use efficiency (Thierfelder and Wall 2009). SOM levels are indicative of soil health and are critical for soil quality and function (Varvel 1994). As a legume, RC is

most commonly associated with N contributions, but it is important to note it can also deliver multiple non-N benefits to the system in which it is cultivated.

1.8 Weed Control

RC cropping may also control weeds. Weed control will always be desired of cover crops (Liebman and Dyck 1993), particularly in the UK, where blackgrass (*Alopecurus myosuroides*) populations are becoming increasingly problematic and resistant to herbicides (Chauvel *et al.* 2001). Systems using conservation tillage also require optimal weed control (Bhaskar *et al.* 2013). Predicted DM yields of 10-15 t ha⁻¹ for RC crops will likely be capable of weed suppression (Elsaesser *et al.* 2016, Nagibin *et al.* 2016), but this can be enhanced by the exudation of phytotoxic chemicals released by the crop itself, a process known as allelopathy (Singh *et al.* 2003, Liebman and Sundberg 2006).

A number of studies have been carried out on allelopathic weed control by RC. These studies are summarized in Table 1.10. Most of these studies were field-based; however some were lab-based (Liebman and Sundberg 2006, Liu *et al.* 2013). Potential candidates for the hypothesized allelopathic chemicals were suggested, for examples phenols in Ohno *et al.* (2000, 2001) and isoflavonoids in Liu *et al.* (2013). Maiksteniene *et al.* (2009) was the only study to observe an increase in weed biomass following a RC/vetch mixture, but this was attributed to an increase in soil tilth and fertility. Although these studies suggest that RC has the capacity to control weeds with allelopathic chemicals, in field studies it still remains unknown if this control is caused by allelopathy or the physical presence of residues on the topsoil. However, efforts to understand the physiological basis for weed suppression and allelopathy in cover crops remain important. If allelopathic chemicals can be verified and identified then plant breeders can perhaps begin to increase their expression in breeding programs, and make contributions to sustainability by reducing the need for intensive tillage and/or herbicide applications.

Table 1.10 Summary of reported weed control by RC in field and laboratory conditions

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1.9 Variety Selection

RC is a bee-pollinated plant with a gametophytic self-incompatibility system (Taylor *et al.* 1995), a profile which typically caused intra-species diversity. This has been consistently observed in RC wild populations and ecotypes (Ulloa *et al.* 2003, Dias *et al.* 2008, Pagnotta *et al.* 2011) and confers diversity to breeding germplasm. Three classifications of RC exist, early or late flowering, erect or prostrate and diploid or tetraploid. Characteristics of these varieties and agronomic consequence are reviewed here.

1.9.1 Early and Late Flowering

The most well-known classification of RC varieties is early and late flowering (Frame, *et al.* 1998). Terms such as single/double cut and medium/mammoth are used in North America to describe the same classification (Taylor and Quesenberry 1996). Early varieties flower 1-2 weeks before late varieties and have the agronomic consequence of providing more vigorous regrowth than late varieties (Madill and Skepasts 1981). Late varieties give most of their annual yield at the first cut and will regrow with less vigour than early varieties. Some authors cite late varieties as storing more reserves in the root system (Vleugels 2013), which may also be of agronomic significance. Classification is more spectrum than dichotomy, and some varieties could be described as ‘intermediate’, i.e. falling somewhere between early and late classification. The literature comparing RC varieties is almost exclusively focused on disease resistance (reviewed here in Section 1.9.3.2), and the comparative performance of early and late varieties in soil-fertility building remains largely unstudied.

1.9.2. Erect and Prostrate

RC naturally exhibits an erect growth habit (Bowley, *et al.* 1984), but advances in plant breeding have created prostrate varieties which produce stolons and can root at auxiliary nodes under optimal conditions (Rumball *et al.* 2003). These varieties were initially created in Australia and New Zealand by breeders seeking to increase the persistence of RC in response to grazing (Wrightson 2015). Prostrate varieties can persist in the sward like white clover (Abberton and Marshall 2005) but also deliver the high-protein forage RC is known for (Lee 2014). Prostrate varieties are not commonly grown outside of Australia and New Zealand, although European breeders have created one prostrate variety called ‘Pastor’ (Boller *et al.* 2012). A stoloniferous growth habit may predict increased persistence, but public sector research confirming this is scarce. The behaviour of prostrate varieties in response to cutting, as opposed to grazing also remains unknown. Vleugels (2013) investigated whether a prostrate

growth habit was correlated with resistance to clover rot (*Sclerotinia* sp) using the prostrate varieties Astred, Crossway and Broadway, but found no correlation was observed.

1.9.3 Diploid and Tetraploid

RC is a natural diploid ($2n=14$), but artificial tetraploid varieties ($4n=28$) have been created by breeders (Evans 1954). Doubling of chromosomes in RC can occur through application of colchicine, nitrous oxide or other chemicals that interfere with chromosome segregation, and create tetraploid varieties (Taylor and Quesenberry 1996). Improved agronomic performance in RC tetraploids is predicted by some because natural tetraploidy in angiosperms is often associated with adaptation to adverse environmental conditions (Fawcett and Peer 2010). Polyploidy occurs naturally in angiosperms through a process known as whole genome duplication (WGD), in which unreduced gametes are produced due to errors in meiosis. Why this happens is unclear, but the subsequent evolutionary success of polyploid populations indicates polyploidy provides a mechanism for speciation and adaption to new environments (Thompson and Lumaret 1992, Ramsey and Schemske 2002). The potential adaptive advantages associated with polyploidy are thought to be the driving forces underpinning their evolutionary success (Soltis *et al.* 2009, Renny-Byfield and Wendel 2014, Tang *et al.* 2014).

These advantages include higher levels of biomass accumulation due to relatively larger polyploid cells (Stebbins 1971, Knight and Beaulieu 2008), hardiness due to their evolutionary connection to adverse environments (Thompson and Lumaret 1992), and increased pest resistance (Nuismer and Thompson 2001). The breeding of tetraploid plants has been an effort to input these potential advantages into new varieties for agriculture.. This raises the question; given some of the observed consequences of polyploidy in angiosperms, could tetraploid varieties of RC exhibit desirable morphology and performance? If tetraploidy is found to impart traits that enhance sustainability, then this could open up new avenues to plant breeders developing new cultivars, as well as further options for farmers deciding which varieties to use in fertility-building.

1.9.3.1 Cell Size, Biomass Accumulation and Agronomic Consequence

Tetraploid varieties produce significantly heavier seeds than diploids (Taylor and Quesenberry 1996). Heavier seeds produce seedlings with more energy reserves, which means that tetraploid RC varieties may grow more vigorously and establish better than diploid varieties. It also means tetraploids may be preferable for farmers sowing directly into mulched residues. However, it must be noted that correspondingly higher sowing rates may also be necessary, which may offset a favourable emergence rate (Taylor and Quesenberry 1996). This disadvantage can be compounded by poor seed yield associated with tetraploid varieties (Boller, *et al.* 2010), and this adds to financial costs.

The seed yield deficiency of tetraploid RC is a major constraint in breeding, and if it could be overcome the higher-yielding and more disease resistant varieties could be more successfully adopted. Documented attempts to increase seed yield in RC have included boron and cobalt applications (Stoltz and Wallenhammar 2014, Tomic *et al.* 2014) and the use of marker assisted selection and quantitative trait loci analysis (Herrmann *et al.* 2006, Vleugels *et al.* 2014) have also been cited as potential tools for identifying methods to improve seed yield in breeding. Investigations into the molecular basis of seed yield in tetraploid RC remain ongoing at the time of writing, and may yet facilitate breeders in increasing seed yield and making it more economically viable (Amdahl *et al.* 2017, Kovi *et al.* 2017)

The increase in biomass in tetraploid varieties is a hindrance in seed yield, but it can be advantageous if it also occurs in the vegetation of RC tetraploids (Anderson 1971). Tetraploid varieties have been shown to exhibit higher above-ground biomass yields than diploids. For example, McBratney (1980) reported that examined tetraploid cultivars had larger petioles and leaf-areas than diploids across a sample of three varieties at both ploidy levels. Similarly, tetraploid varieties have been shown to give significantly higher DM yield than diploids (Zuk-Golaszewska *et al.* 2010), but it was observed that these increases were marred by a reduction in mineral content of P and Ca. This indicates that a trade-off exists between the yield and nutritive value of different varieties, and knowledge such as this is useful in developing site-specific strategies. For example, high-yielding tetraploids may contribute more to soil fertility through increased SOM contributions, whilst high-nutritive diploids may be preferable for stocked systems that use residues for fodder.

These studies indicate tetraploids provide greater yields than diploids, but a broader study involving 93 RC varieties concluded tetraploidy only resulted in a 6.5% average increase in

dry matter yield compared to diploids (Liatukas and Bukauskaite 2012). In another study documenting location/cultivar interaction in RC, a 10-60% increase in dry matter accumulation was observed in tetraploid cultivars in humid mountain regions (Leto *et al.* 2004), but reported as dependent on environmental factors, as performance was not matched in dry lowland areas. The effect of tetraploidy on RC yield may then be dependent on genotype/environment interactions and difficult to ascribe to polyploidy alone.

1.9.3.2 Pest and Disease Resistance

Directly correlating polyploidy with disease and pest resistance is difficult, but Madlung (2013) suggested polyploidy may contribute to a functional increase in response options to a spectrum of stressful environmental conditions, including disease and pest attack. This can occur through increased allelic diversity, as higher diversity in immunity genes can boost recognition of pathogens (Spurgin and Richardson 2010), or through supplementary expression of genes related to immunity through multiple genomes (King *et al.* 2012). Vleugels (2013) proposed that polyploidy may contribute to resistance through a broader range of resistance occurrence. RC, as an obligate out-crosser, has mostly heterozygous genotypes. At 50% frequency of a dominant resistant allele, only 75% of a diploid population will contain at least one resistance allele (25% RR, 50% Rr and 25% rr). If this population were tetraploid the number of individuals with a least one resistance allele would increase to 93.75% (as only 6.25% of individuals occur as rrrr). This implies that chromosome doubling in breeding may enhance and strengthen pre-existing resistance in naturally occurring diploid populations. The main diseases and pests of RC are summarized in Table 1.11, with information about resistant varieties and their ploidy.

Table 1.11 Summary of pests and diseases of RC (main appearing in bold) with reported resistant variety

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Some pest and disease problems with RC can be managed agronomically. For example clover rot can be mitigated by spring sowing (Jones *et al.* 2003) or a prophylactic bacterial biocontrol application (Ohberg and Bang 2010). Crop rotations are also advised, particularly in root rot (Peters *et al.* 2003). However much of the literature suggests breeding for resistance is the most effective strategy (Skipp and Christensen 1990, Pokorny *et al.* 1995, Annicchiarico *et al.* 2015, Jacob *et al.* 2015). Improved varieties can vastly improve resistance, as seen in the closely related white clover (*Trifolium repens*), which has undergone significant improvement in modern molecular plant-breeding (Abberton *et al.* 2015). Sequencing work shown RC to be a suitable candidate for association studies and genomic selection (De Vega *et al.* 2015). There are also large numbers of RC varieties, cultivars and landraces available for study. The USDA alone holds an estimated 1367 RC accessions (Smykal *et al.* 2015), whilst the estimate for *Trifolium* accessions globally is about 74,000 (FAO 2010). New varieties with improved disease/pest resistance could vastly improve persistence, which could contribute greatly to the overall sustainability of the cropping systems in which RC is used.

1.10 Management

1.10.1 Grass Mixtures & Monocultures

RC in fertility building can either be grown in monoculture or with companion grasses. It can also be grown in mixture with other legumes such as sainfoin and lucerne, a strategy which has become popular in the UK (Wilkinson 2011). Monocultures and mixtures have their own advantages and disadvantages, but these are complicated by environmental interactions. For example, higher DM yields are predicted for mixtures (Frame *et al.* 1985), but if these increased yields deplete soil N then monocultures may be preferable. Monocultures may not take up the same volume of N as mixtures, but more N may be lost through increased leaching over the winter period (IBERS 2014), which may offset the initial benefit. The advantages and disadvantages associated with mixtures and monocultures are summarized in Table 1.12.

Table 1.12 Advantages and disadvantages of RC-grass mixtures

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RC is most commonly grown with grasses in the UK (Rasmussen, *et al.* 2012). Here RC is primarily associated with forage production, at least in the conventional sector, and mixtures are more common than monocultures as grasses tend to improve the overall forage quality (Sturludottir *et al.* 2014). Given how the advantages and disadvantages of mixing with grasses are not common to all climates and soil types, and may have complex interactions with each other, it remains unclear whether mixtures or monocultures are optimal for fertility-building. Farmers should then make the decision to include or omit a grass based on their climate, soil type and nutrition requirement of the desired subsequent crop.

1.10.2 Cutting

As Tables 1.4-1.6 indicate, RC crops can be cut and removed or cut and mulched. Cut and remove is primarily associated with forage production (Cassida *et al.* 2000) and mulching is primarily associated with fertility-building in the organic sector (Dahlin *et al.* 2011), although both can be practiced in either. Cutting is generally advised before the crop reaches the full

bloom stage (Wiersma *et al.* 1998), as the transition to flowering is associated with a decrease in biomass accumulation and biofixation (Voisin *et al.* 2002). Early varieties will generally require two cuts, whereas late varieties may only require one. Mulching could be superficially viewed as best practice for fertility-building, as it returns N to the system, but mulching can limit subsequent regrowth (Moyo *et al.* 2011), limiting future N contributions from biofixation. Mulches themselves are also subject to leaching (Nelson and King 1996, Bergström and Kirchmann 2004), which is undesirable both for subsequent soil fertility and environmental integrity. Both cut and remove and cut and mulch are practiced by farmers in fertility-building in RC, but the decision over which to employ is more likely to be determined by individual needs rather than what is optimal, e.g. if forage is required then cuttings must be removed, if not they may be mulched etc.

1.10.3 Cultivation

The termination of RC crops and soil preparation for subsequent cultivation is known as green manuring (Repsiene and Skuodiene 2008). Green manuring is an important component of fertility-building with RC, as it contributes N-rich aboveground biomass and maintains SOM levels (Bath and Elfstrand 2007). The effect green manuring has on subsequent crop production is widely variable, as the mineralization required for nutrient release is heavily dependent on plant residue quality (C:N ratio, lignin/polyphenol content etc) and soil/weather conditions (Kumar and Goh 2000). Depth of tillage will also influence mineralization rates, as deep tillage is associated with greater and more rapid mineralization rates and shallow tillage with smaller and slower mineralization rates (Al-Kaisi *et al.* 2005).

Herbicides can also be included in RC termination, either instead of or in tandem with tillage. This is only permissible in the conventional sector and mostly associated with farmers practicing conservation agriculture (Bajwa 2014). Herbicide use may even be essential in systems utilizing reduced tillage as some authors describe the long RC taproot as difficult to terminate without deep tillage (Curell 2011). More rapid mineralization rates would be predicted for systems using herbicide application, and this may have significant effects on the expected yields of subsequent crops, but given how these effects are influenced by soil, climate, plant quality and complex interactions between all, they are difficult to predict.

1.11 Economic Considerations

Economic considerations can be the dominant factor influencing the adoption of new technologies and management practices. If agriculture is to be truly sustainable then productivity and environmental integrity must be maintained without compromising on profits. Legumes can offer farmers substantial economic returns and there has been international documentation of this; for example India (Ghosh 2004), Kenya (Rao and Mathuva 2000), Spain (Sanchez-Giron *et al.* 2004) and the USA (Biermacher *et al.* 2012). These returns are generally conceived of in direct ways such as the value of silage, reductions in fertilizer/fodder costs and yield increases. The non-marketable impacts of improved soil fertility/structure and various ecosystem services (weed control and pollinator attraction etc) are much harder to quantify (Swinton *et al.* 2007). The primary use of RC as forage for grazing animals in Europe means that researchers in this region have recognized its value more as silage (Doyle and Topp 2002). Table 1.12 summarises their findings on the value difference from growing and feeding a RC instead of grass silage in four European countries. High-legume silages indicate a 70:30 legume:grass ratio and low-legume silages 40:60. Other forages white clover and lucerne are included for comparison. RC is consistently shown to be the most economically viable.

Table 1.13 Value of RC silage in four European countries (Doyle and Topp 2002)

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Difficulties in determining the economic efficacy of RC rotations may restrict their adoption. External political factors may impose further restrictions. This review has argued that increasing availability of cheap N fertilizer in the post-war era favoured agricultural practices based on mineral input, but other factors have also contributed. Most significant is the replacement of these traditional rotations by more intensive production systems (Rochon, *et al.* 2004). These replacements are often soy and maize rotations, grown to produce fodder for industrial livestock production (Weis 2013) and biofuels (Lal 2005). This is seen most strikingly in North America, where the political economy of grain/industrial-livestock production provides subsidies and greater profits to farmers willing to participate (Reganold *et al.* 1990). Conventional wisdom is that these agricultural models are probably environmentally damaging and unsustainable, but there is less financial incentive for farmers to develop more sustainable practices more generally (unless they embrace a greener philosophy).

Contributions to soil fertility and crop resilience made by RC can be difficult to predict. These predictions are even more challenging to translate into financial returns, but fortunately the cost of business as usual is much easier to quantify. The price of fertilizer is linked to the price of fossil fuel energy, which is subject to volatile market forces and may ultimately be unsustainable. This cost is compounded by potential environmental and ecological damage associated with fertilizer-dependent industrial agriculture. In the UK alone it is estimated that the total financial cost of nitrogen leaching to health and the environment was £16m over a six year period (Pretty *et al.* 2000). In order for the economic sustainability of RC rotations to be fully realised therefore, farmers and governments will need to prioritize longer-term sustainability more over short-term returns with respect to agricultural practices

1.12 Summary of Main Knowledge Gaps

The literature on fertility-building with forage legumes is extensive. The majority of research carried out in North American is focused on alfalfa, and in Europe on white clover, as these crops are the main fertility-builders used in these regions. Advancements in plant breeding and recognition of potential contributions to livestock production have increased interest in cultivating RC for soil fertility in both regions (Annicchiarico *et al.* 2015), but optimal use remains unknown. As outlined in this review, the main knowledge gaps are:

1. What management optimizes BNF?
2. What management maximizes transfer of fixed N to subsequent crops?
3. What management most enhances the delivery of ecosystem services?
4. What management is best for enhancing allelopathic weed control?
5. Which varieties are optimal for which agronomic property (yield, nodulation, disease resistance etc) and how does this impact on capacity to build soil fertility?
6. What contributes most to soil fertility, monocultures or clover/grass mixtures?
7. How can RC leys be profitably reintegrated into arable rotations?

Time and financial constraints preclude all knowledge gaps from being examined in this thesis. The gaps to be tested are numbers 1, 3 and 5. The main hypotheses derived from these knowledge gaps are:

- Red clover varieties are morphologically, agronomically and phenologically diverse as described by breeders and seed merchants
- This diversity significantly effects the capacity of the crop to build soil fertility
- Cutting management (1 or 2 cuts) significantly effects the subsequent soil fertility following one year of red clover cultivation
- The accumulation of SMN can be used as a proxy to assess subsequent soil fertility following cultivation of red clover under different management
- The inclusion of a herbicide prior to cultivation will significantly increase yields of wheat and mustard
- Multiple crops of mustard will have a higher nutritional demand than a single wheat crop and will give a more accurate measure of soil fertility

CHAPTER 2 - Materials and Methods

Materials and methods for all field cultivations and lab procedures are included in this chapter. Additional information concerning particular statistical issues is given in the relevant chapter.

2.1 Experimental Site

Core Experiment 1 was initiated in March 2015. The site selected was also used for Core Experiments 2 and 3, which were undertaken in 2016. Cranfield University (2015) indicated the soil type was a shallow, lime-rich soil over limestone. Cranfield University (2016) showed the site soil was of the Sherborne series. This series is characterized by a calcareous topsoil of 250-350mm depth over a thin subsoil that quickly passes to oolitic limestone (Avery *et al.* 1980). It is typical of the area and commonly referred to as 'Cotswold Brash'. Cotswold Brash occurs as either clay or clay-loam texture. The soil texture analysis undertaken for this research confirmed the trial soil was clay. The field selected for the trial had previously been seeded to become a red clover/grass ley, but this was terminated with glyphosate before any significant growth had taken place. The site was located east of the Royal Agricultural University, UK at 51° 71' N latitude and 01° 99' W longitude, at an altitude of 135m above sea level. A chemical and physical characterization of the site soil was undertaken before the trial began, the results of which are given in Table 2.4 following the summary of how this characterization was carried out.

2.2 Meteorological conditions

Meteorological conditions for 2015 and 2016 are given in Figures 2.1-2.3. Data for 2015 and 2016 is taken from the weather station at the Royal Agricultural University and long term average data is taken from the Met Office data for Cirencester (2010).

2015

The maximum and minimum mean monthly air temperatures recorded throughout the cropping season were 21.8°C and 11°C. Maximum and minimum mean monthly precipitation recorded were throughout the cropping season were 71mm and 13mm. Spring and winter air temperatures were higher than the long-term average but comparable to the long term average throughout the cropping season. Precipitation was higher than the long-term average in the winter months but comparable to the long term average throughout the cropping season.

2016

The maximum and minimum mean monthly air temperatures recorded throughout the cropping season were 9.6°C and 23.2°C. Maximum and minimum mean monthly precipitation recorded were 109mm and 14mm. Air temperatures were comparable to the long term average. Total precipitation throughout the cropping season was comparable to the long term average, but it was sporadic. June was twice as wet as the long term average and July was twice as dry.

Figure 2.1 Total precipitation (mm) for both years of cultivation with a long-term average

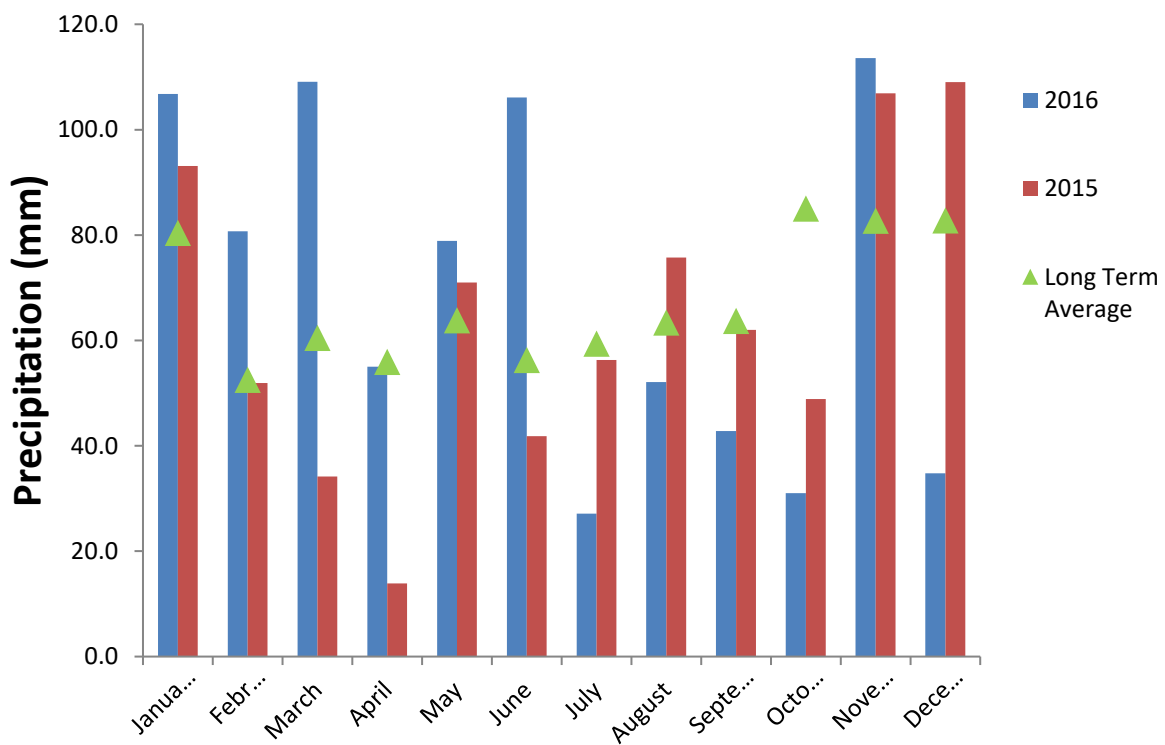


Figure 2.2 Mean temperature (°C) for both years of cultivation with long term average

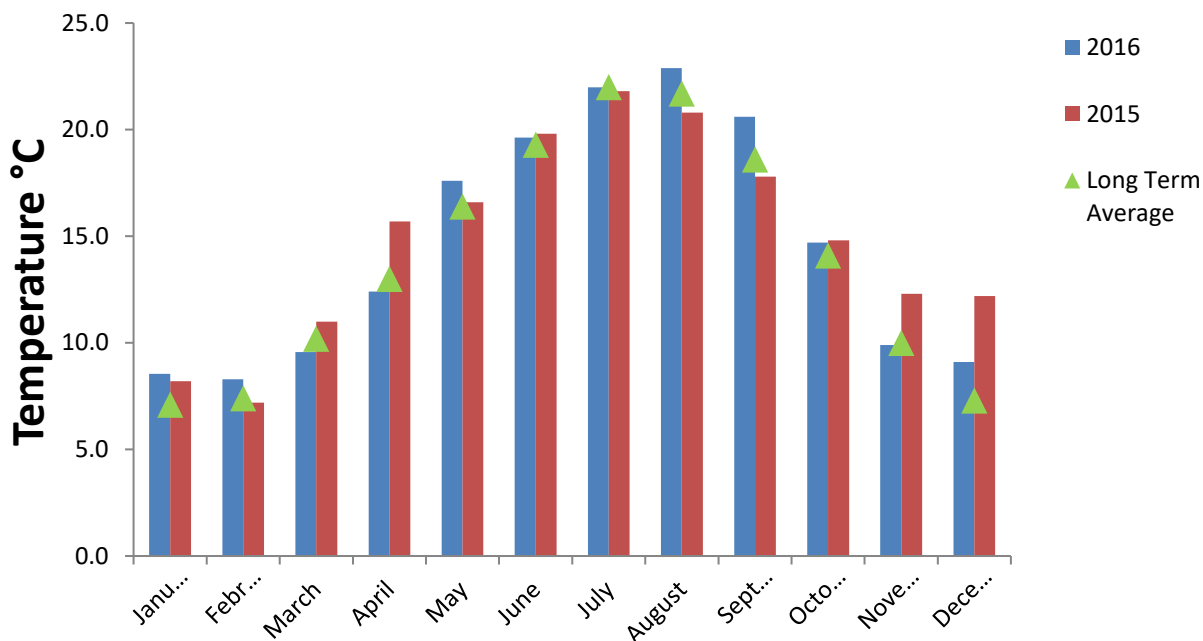
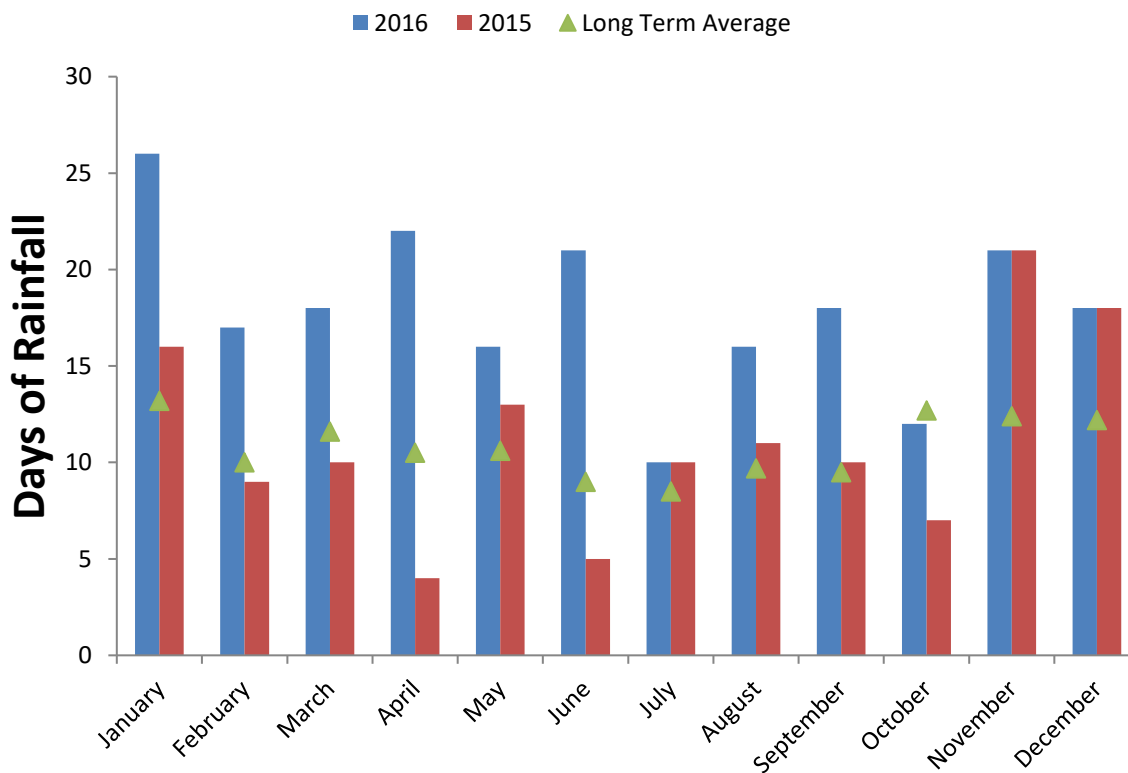


Figure 2.3 Days of rainfall for both years of cultivation with long term average



2.3 Experimental design and treatments

2015 – Core Experiment 1

The field trial for Core Experiment 1 was a completely randomized block design with four replications. Blocks and plots were demarcated using flexicanes in March prior to planting. Each block measured 6 x 36m and was composed of 24 9m² plots (3x3m). A path of 1m was left between each block for ease of access. Each plot was assigned two treatments of RC variety (Amos, Astred, Claret, Maro, Milvus, Ruby) and cutting management (1/2 cuts). This was a factorial experimental design, with 6 x 2 = 12 treatments applied, one combination applied to each plot (e.g. Plot 4 Amos 1 cut, Plot 25 Claret 2 cut etc). Descriptions of varieties and reasons for selection are given in Chapter 3. This site design and layout is illustrated in Figure 2.4

2016 – Core Experiments 2 and 3

The field trial for Core Experiments 2 and 3 took place on the same site as Core Experiment 1 in 2015, and followed the same completely randomized block design. Plots assigned for two year RC cultivation were left to grow, whilst all plots assigned for Core Experiments 2 and 3 were terminated and new crops of wheat and mustard were cultivated. This was done by splitting each individual plot into 4 subplots. The experiment then became a split-plot design with prior treatments of RC variety and cutting management, and splits of herbicide and non-herbicide application. This site design and layout is illustrated in Figure 2.5

Figure 2.4 Diagram of field trial design for Core Experiment 1.

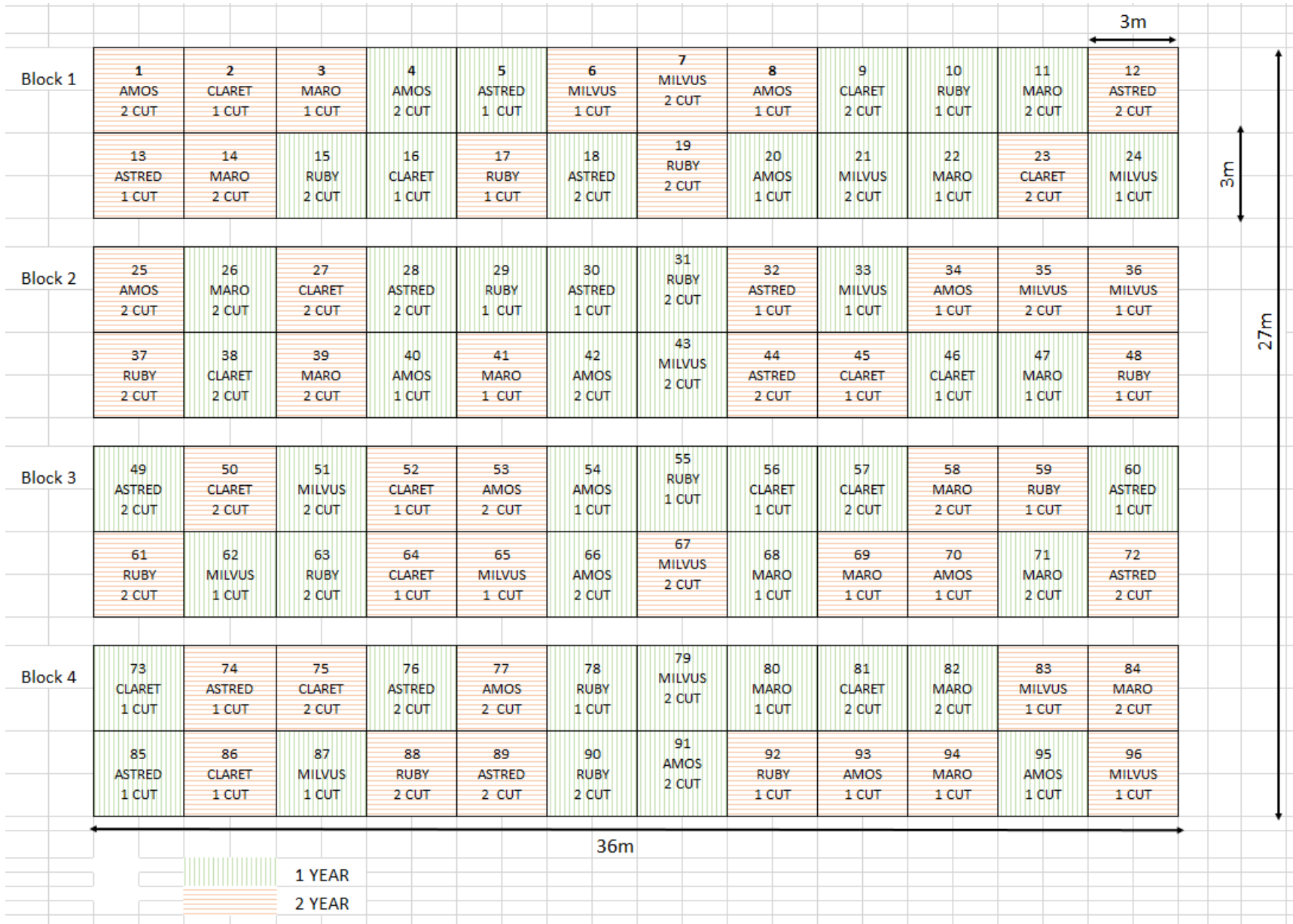


Figure 2.5 Diagram of field trial design for Core Experiments 2 and 3.



2.4 Cultivation Details

2.4.1 Core Experiment 1

Field Cultivations

The field selected for trial was a grass ley up until 2015. In February of 2015 it was tilled and a red clover/timothy ley was planted, but the site area was sprayed with glyphosate on 18th March before any significant growth had taken place. Time was then given for the area to clear and the site was lightly harrowed using a disc harrow (Simba 2B) on April 5th to incorporate any remaining biomass into the soil. This also ensured a level seedbed to aid crop establishment. An electric fence was erected following demarcation to minimize rabbit predation.

The trial was sown on the 29th of April, when weather data indicated the soil temperature was above 8°C, as RC requires surface soil temperatures of at least this for successful germination (Bowley, *et al.* 1984). Seeds were sown by hand at the advised rate of 15 kg ha⁻¹ (Frame, *et al.* 1998, Teagasc 2016), which equated to 13.5 g of seed for every 9 m² plot. The field trial soil was known to contain *Rhizobium leguminosarum* biovar *trifolii* and therefore no prior inoculation was required. Following sowing the whole site was rolled with a mini-roller to ensure good seed/soil contact for optimal germination. A dry period followed immediately afterwards and germination did not occur until May 6th (following a spell of rain). This delay allowed weeds to establish but the plots were left to establish without manual weeding. This was done to allow assessment for weed competitiveness and to make the research commercially applicable.

The first cut began when the earliest flowering varieties were deemed to be between early and half bloom. This is the generally recommended cutting time for RC (Hall and Eckert 1992). Varieties were cut one by one in the order in which they flowered (Ruby – Milvus – Claret – Astred – Maro – Amos). All cutting was done by hand with a hedge clippers and all cuttings were bagged and taken to the lab for drying and analysis. Care was taken to cut at 5cm aboveground to avoid damaging the crowns. The second cut began on at the same stage of flowering as the first cut and proceeded in the same fashion as the first. Incidences of diseases were also observed and scored. Infected plant tissues were removed using a rake and a fungicide (Iprodione) was applied at a rate of 2 kg ha⁻¹. A diary of field trial operations is given in Table 2.1. Photographs of the field trial are given in Figures 2.6-2.8.

Table 2.1 Diary of field operations for Core Experiment 1

Date	Field Operation	Date	Field Operation
April 14-29	Site Preparation	Oct 12-26	Second Cut & Second Morphology Measurement
May 6	Germination	Oct 14	Clover Rot Observed and Scored ¹
July 12-23	Flowering	Nov 1	Soil Samples taken for SMN
July 30-Aug 28	First Cut & First Morphology Measurement	Nov 11	Fungicide (Iprodione) Applied
Aug 7	Powdery Mildew Observed and Scored ¹	Nov 14-17	Infected Plant Tissue Removed

1. Further information on disease biology, and scoring methods are contained in Section 3.4.5

Figure 2.6 Demarcated plots following preparation



Figure 2.7 Established plots with paths demarcating blocks (left) and single plot following cutting (right)



RC plots



Plot following cutting

Assessments

Germination Test

100 seeds from each variety were counted out and placed in a petri dish of sterilized water. The dishes were incubated at 25 °C for 72 hours and the number of germinated seedlings was counted. Percentage germination was calculated and this procedure was repeated three times. Percentage germination and thousand seed weight were investigated following sowing after field observations indicated establishment in some varieties was lower than others.

Establishment

A 25 cm quadrat was randomly placed within plots and all individual plants were counted. This was in all plots and expressed as plants m⁻².

Dry Matter

All cutting was done by hand using a garden clippers. All plots were cut at a height of 5 cm above the crown (to avoid damaging it) and all cuttings were raked off and bagged. A sub-sample of 1 kg was taken from every plot and weeds were separated from clover by hand. A clover:weeds ratio was determined and this ratio was used to estimate the clover:weed ratio in the whole plot. Cuttings were then quickly taken to the lab where they were weighed and dried for 24 hours in an oven at 105 °C. Dried plant samples were retained for further analysis.

Morphological Measurements

Three individual plants were randomly selected from each plot at the early-mid flowering stage and cut just above the crown. Samples were then taken to the lab where morphological variation was recorded. Further information concerning which traits were measured, how and why these measurements were taken is given in Chapter 3 prior to results.

Soil Mineral Nitrogen (SMN)

SMN assessments were carried out in following the growing season (November 1-11) and repeated at the beginning of the 2016 season (February 22nd – March 10th) to determine SMN build-up and winter leaching. Methodologies are listed in Section 2.5.1

2.4.2 Core Experiment 2

Field Cultivations

As Core Experiment 2 was a continuation from Core Experiment 1, no further cultivation was necessary and the crop was left to grow again in the spring of 2016. A diary of field trial operations is given in Table 2.2. A photograph of the trial site in spring 2016 is given in Figure 2.8.

Table 2.2 Diary of field operations for Core Experiment 2

Date	Field Operation	Date	Field Operation
June 14 th - 26 th ¹	First Cut, morphology assessment and soil samples taken for SMN	July 27 th – August 9 th	Nodulation Assessment
August 10 th – 18 th ¹	Second Cut, morphology assessment and soil samples taken for SMN	December 1 st	Clover Rot observed and scored

1. The clover was determined to be between the early and mid bloom stages at these dates, the preferred cutting time for RC to avoid the reduction in growth associated with flowering (Wiersma *et al.* 1998)

Figure 2.8 RC plots spring 2016 showing new season growth



Assessments

Dry Matter

A 25cm quadrat was placed within all plots and a sample was cut. These samples were transported to the lab, weighed and dried at 105°C for 24 hours and DM was recorded. Dried plant samples were retained for further analysis.

SMN

SMN assessments were carried out following cutting in 2016. These dates can be found in Table 2.3. Methodologies are listed in Section 2.5.1.

Nodulation and Root DM

Individual plants were excavated from all plots to assess nodulation. This was done by gently inserting the tines of a garden fork underneath individual plants and gently prising upwards. This was done following a period of rain to ensure soil moisture levels were favourable for ease of excavation. Excavated plants with the clay still attached were then left to dry in the sun for several hours. This allowed the clay to be easily removed without also removing the nodulated adventitious roots. The clay was gently crumbed away by hand and the number of nodules were counted and recorded. The roots were then oven-dried at 105°C for 24 hours for root DM assessment. Difficulties in determining exactly how many plants occurred per unit

area as stands were crowded and telling individual crowns apart was impossible without excavation. Root DM is therefore expressed as g plant^{-1} rather than g ha^{-1} .

2.4.3 Core Experiment 3

Field Cultivations

All plots allocated for bioassay cultivation were split into 4 sub-plots. Glyphosate was applied at 40 g L^{-1} to all herbicide allocated plots using a knapsack sprayer (Cooper Pegler) on April 13th. One week was allowed for the herbicide to take effect and all bioassay plots were then tilled to a depth of 8-10cm using a mechanical rotavator (8HP Ermin Machinery). One pass of the machine was applied and care was taken to ensure each plot received the same treatment. The tilled soil was then gently raked to smooth out the surface.

Spring wheat (*Triticum aestivum*) and mustard (*Sinapsis alba*) were then planted on the 21st-23rd April. Varieties selected were 'Paragon' for wheat and 'Rumba' for mustard. Each sub-plot was further cultivated with a hand cultivator until a tilth was obtained. Wheat was planted at a rate of 250 kg ha^{-1} and mustard at 25 kg ha^{-1} . These rates are both 25% higher than that recommended by the seed merchants, as an increase in seeding rates may offset limitations in germination or establishment associated with hand broadcasting and manual seedbed preparation. The seeds were gently raked into the soil and manual pressure was applied using a length of plastic piping. This was done to ensure good seed to soil contact for optimal germination. A diary of field trial operations in wheat is given in Table 2.3 and cultivation procedures are illustrated in Figures 2.9-2.11. Mustard was harvested and replanted three times. Harvesting took place when the crop reached the early-bloom stage. The first two assessments took place in tandem with the wheat biomass assessments and began on May 29th and July 15th. The third and final assessment began on August 19th.

Table 2.3 Diary of field operations for Core Experiment 3

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1 Growth stages according to Zadoks et al (1974)

Figure 2.9 Clover plot showing herbicide (left) and non-herbicide (right) treatments prior to rotavating. Black arrows indicate split-plot demarcations



Figure 2.10 Tilled plot and rotavator used with established plot showing wheat and mustard growth



Midway through tilling RC plot prior to bioassay cultivation



Bioassay plot following establishment of wheat and mustard

Figure 2.11 Bioassay plot showing wheat and mustard under herbicide and non-herbicide treatments



Assessments

Establishment

A 25 cm quadrat was randomly placed within each sub-plot and all seedlings were counted. This was replicated three times in each sub-plot.

Tiller & Stem Count

A 25 cm quadrat was randomly placed within each sub-plot and all tillers or stems were counted. This was replicated three times within each sub-plot.

Dry matter

A 25 cm quadrat was placed within all plots and a sample was cut. These samples were transported to the lab, weighed and dried at 105°C for 24 hours. DM was recorded, converted to $t\ ha^{-1}$ and subjected to statistical analysis. Dried plant samples were retained for further analysis. This was undertaken three times for both wheat and mustard.

Plant Height

Height of the tallest wheat shoot in a randomly assigned 25 cm quadrat was measured using a length of bamboo with 10 cm increments marked. The height was taken as the distance from the ground to the peduncle when the stem was held at 90° to the ground.

Final Biological Harvest (wheat)

Final harvest was undertaken using the same protocol as the DM assessments. Before drying the ears were removed and numbers recorded. Ears and straw were then dried separately. Ears were then threshed by hand using a cloth bag and the grain was weighed for total grain weight and grain yield, which was corrected to 15% moisture content. Thousand grain weight (TGW) was recorded with the threshed grain using an automatic counter (Farmtec). Harvest index was calculated as the ratio of grain weights to total biomass.

2.5 Lab Procedures

Applicable to Core Experiments 1, 2 and 3

Soil Characterization

Eight soil samples were taken from each block to assess the initial chemical composition of the site soil. These were taken using a 25 cm augur and were sampled in a zig-zag pattern to ensure

they were representative of the whole block. Subsamples for assessments requiring dry soil were then hand-crumbed, thoroughly mixed and left to dry in an airing cupboard. Fresh samples were withheld for assessments requiring fresh soil.

2.5.1 Soil Chemical Analysis

Soil pH

Three 20 g samples of sieved (2mm) dry soil from each block were weighed into individual 150 ml shaking bottles and 50 ml deionised water was added. Bottles were shaken at 120 oscillations per minute for 30 minutes using a shaker unit (Gerhardt, Germany) and allowed to settle. A pH electrode was then calibrated using buffers of pH 4, 7 and 10, and immersed in each soil solution. The electrode was then swirled and the measured pH was allowed to stabilize and then recorded. Average values were taken for each block.

Soil Potassium

The ammonium nitrate extraction (Faithfull 2002) was used to determine soil potassium. Three 10 g samples of sieved (2mm) dry soil from each block were inserted into individual 150 ml shaking bottles and 50 ml ammonium nitrate ($\text{N}_2\text{H}_4\text{O}_3$) solution was dispensed into each. Bottles were then shaken at 120 oscillations per minute for 30 minutes using a shaker unit and allowed to stand for a further 15 minutes. Solutions were then filtered through Whatman No 4 filter papers with the initial 2-3 drops discarded. Each filtrate was then diluted by a factor of 50 in deionised water using volumetric flasks.

Seven known standard K solutions (0, 0.1, 0.25, 0.5, 1.0, 2.5 and 5 $\mu\text{g K ml}^{-1}$) were then prepared and concentrations were confirmed using a spectrophotometer (Cecil Instruments Lt., UK). This produced a standard curve, which was used to determine the $\mu\text{g K ml}^{-1}$ in the filtrates. Three blanks were also included to account for lab contamination. The $\mu\text{g K ml}^{-1}$ was converted to mg L^{-1} by subtracting the blank and accounting for the initial dilution by multiplying by 50.

Soil Phosphorus

The Olsen Method (Olsen *et al.* 1954) was used to determine soil P content. Three 5 g samples of sieved (2mm) dry soil from each block were inserted into individual 150ml shaking bottles. 1g of powdered charcoal and 100ml sodium bicarbonate (NaHCO_3) were then added, and the pH was adjusted to 8.5. Bottles were then shaken in a shaker unit at 120 oscillations per minute for 30 minutes and allowed to stand for a further 15 minutes. Solutions were then filtered

through Whatman No 4 filter paper with the initial 2-3 drops discarded. 5ml of the filtrate was then pipetted into 100 ml conical flasks and 1 ml of 1.5M sulphuric acid (H₂SO₄) was added. 20 ml ammonium molybdate (NH₄)₂MoO₄ (in 1.2% m v⁻¹ ascorbic acid solution) was then added and solutions were allowed to stand for 30 minutes.

Seven known standard P solutions (0, 0.1, 0.25, 0.5, 1.0, 2.5 and 5 µg K ml⁻¹) and two blanks were then determined using a spectrophotometer (Cecil Instruments Lt., UK) at 880nm wavelengths. This produced a standard curve, which was used to determine the µg P ml⁻¹ of the filtrates.

Soil Mineral Nitrogen

Soil mineral nitrogen (SMN) was determined using the potassium sulphate (K₂SO₄) extraction method. Soil samples were taken from a 25 cm depth using a 25 cm augur. These samples were then immediately taken to the lab for extraction, to prevent any subsequent mineralization creating inaccurate measurements. Soil samples were frozen for future analysis when time constraints occurred.

Prior to extraction the samples were thoroughly hand-mixed, crumbed and passed through a 6.7mm sieve. Stones and plant material were removed. Three 12.5 g of each sample were weighed out and transferred to individual 150ml extraction bottles. 50ml of 0.5M K₂SO₄ was then added to each bottle. Three blanks were included in every extraction batch. The bottles were then shaken at 120 oscillations per minute in a shaker unit for 30 minutes, and then allowed to stand for 15 minutes. The suspensions were then filtered through Whatman No 4 filter paper and the filtrate was collected in a 60 ml bottle. The initial 2-3 drops were discarded to prevent contamination. The filtrates were then labelled and stored in a freezer for subsequent analysis using the FIAstar 500 system. As the SMN calculations require a dry weight parameter, dry matter was also calculated for 3 samples within each extraction batch to confirm soil moisture percentage at time of sampling.

The SMN kg ha⁻¹ was calculated using the formula:

$$\text{SMN (mg ha}^{-1}\text{)} = \left[\frac{\text{Extraction Volume (ml)} \times \text{Extract mineral N (mg l}^{-1}\text{)}}{\text{Soil Dry Weight (g)}} \right]$$

$$\text{SMN (kg ha}^{-1}\text{)} = \left[\text{SMN (mg kg}^{-1}\text{)} \times \text{bulk density (g cm}^{-3}\text{)} \times \frac{\text{sample depth (cm)}}{10} \right]$$

These formulas are taken from Unkovich et al (2008). Extract mineral N was calculated as the sum of the nitrate and ammonium readings taken from the FIAstar analysis. The initial bulk density measurement (Table 2.5) was used for all samples and the sample depth was the length of the augur used for sampling (25cm).

Soil Inorganic Carbon

The calcimeter method was used to determine the inorganic carbon content. An initial investigation was undertaken to determine the optimal soil volumes required for accurate analysis. Five drops of 1M HCl were added to 2.5 g samples of sieved (2mm) dry soil from each block and the time taken for effervescence to stop was noted. Effervescence was strong and persisted for 10-15 seconds, which indicated samples were highly calcareous. A small sample size of 1.25 g was accordingly selected for analysis.

The calcimeter (Eijkelkamp) was calibrated the day before the analysis. 0.2 g and 0.4 g samples of calcium carbonate (CaCO_3) were inserted into individual reaction vessels (conical flasks). 20 ml deionized water was added and the solution was stirred. 7 ml 1M HCl was then pipetted into individual aliquots and carefully inserted into each reaction vessel. The vessels were connected to the calcimeter, sealed, and the reaction was initiated by tipping the acid into the soil solution. The CO_2 omitted was allowed to bubble through each measurement cylinder for 24 hours to precipitate any dissolved CO_2 , which can create inaccurate readings. This was repeated for all five measurement cylinders.

The following day five 1.25 g subsamples from each block was then weighed into individual reaction vessels and 20 ml water was added. The suspensions were then stirred. 7 ml 1M HCl was then pipetted into individual aliquots and carefully inserted into the reaction vessels using tweezers. The conical flasks were then connected to the calcimeter and properly sealed using the attached bungs. When secure, the reaction was initiated by tipping the acid over into the soil suspension. Two blanks with starting values of 20 ml and 80 ml CO_2 to provide an average blank determination were included in the assessment procedure. The CaCO_3 content of the samples was then determined using the formula:

$$w(\text{CaCO}_3) = 1000 \left[\frac{m_2(V_1 - V_3)}{m_1(V_2 - V_3)} \right] \left[\frac{100 + w(\text{H}_2\text{O})}{100} \right]$$

Where:

M_1 = Mass of soil sample (g)

M_2 = Mass of calcium carbonate standards (g)

V_1 = Volume of CO₂ produced by soil sample (ml)

V_2 = Volume of CO₂ produced by calcium carbonate standards (ml)

V_3 = Volume change in blank determinations (ml)

W (H₂O) = Water content of dried sample (%)

Soil Organic Carbon

Soil samples were oven dried at 105 °C for 24 hours and then coarse milled using a Fritsch P19 mill (5 mm gauze). The samples were then further micro-milled using a 1093 Foss Cyclotech mill (0.5 mm gauze). 50mg of soil was then encapsulated in a foil weighing boat with 0.25 mg tungsten oxide added (to aid combustion). Soil organic carbon was calculated by subtracting the percentage inorganic carbon from the percentage total carbon obtained via the Elementar Cube analyser.

Soil Organic Matter

Percentage soil organic matter was estimated by multiplying the percentage organic carbon by 1.72, a commonly used technique (Grewal *et al.* 1991).

2.5.2 *Soil Physical Analysis*

Soil Texture

Soil texture was assessed using the by dispersing soil solutions using sodium hexametaphosphate and a hydrometer to determine the different speeds at which sand, silt and clay fall through water suspension. Three 50 g samples were weighed into individual 250ml shaking bottles. A 1.5 L ‘Calgon’ solution of 30 g sodium hexametaphosphate and 3 g anhydrous sodium carbonate solution was then prepared, and 100 ml was added to each shaking bottle. The solutions were then transferred to a shaker unit and shaken at 120 oscillations for 7 hours.

The next day the bottles were shaken again for one hour. The contents of each bottle were then transferred to individual 1 L measuring cylinders, rinsing the each bottle with deionized water.

The volume was made up to 1 L and the cylinder was sealed with parafilm and until a soil slurry was produced (~20 times). Three drops of amyl alcohol was added to disperse any foam produced.

A hydrometer was quickly inserted into the cylinder and a reading was recorded. This time was dubbed Time A, and subsequent recordings were taken at intervals of 4 (B), 37 (C) and 120 (D) minutes. The readings were staggered to ensure all recordings could be made with the same hydrometer. A blank was included to ensure quality control, and these values were subtracted from the recorded values. The recorded values were then used to determine the percentage content of sand, silt and clay according to the calculations shown below:

Total Sand % (particle size > 60 μm) = $2(50-A)$

Total Clay % (particle size < 2 μm) = $2(D)$

Total Silt % (particle size 2-60 μm) = $50-(\text{Sand \%} + \text{Clay \%})$

These results were then plotted on a standard soil texture triangle and the texture was determined.

Soil Moisture

50 g fresh soil from each block (three replications) was dried at 105°C for 24 hours and the weights were recorded again. Soil moisture percentage was then calculated by dividing the difference by the dry weight and multiplying by 100.

Soil Bulk Density

Six 225 cm³ soil cores were taken from across the four blocks. The cores were then dried at 105 °C and the resulting dry mass was divided by the volume of the core to give the soil bulk density (g cm⁻³).

2.5.3 Plant Tissue Analysis

Plant Tissue Carbon and Nitrogen

Clover and weed samples from each plot were analysed for total C and N. This was repeated at both cuttings. Samples were oven dried at 105 °C for 24 hours and then coarse milled using a Fritsch P19 mill (5 mm gauze). The samples were then further micro-milled using a 1093 Foss Cyclotech mill (0.5 mm gauze). Both mills were vacuum cleaned between samples to avoid cross-contamination. 0.25mg of the micro-milled samples were then encapsulated in a

foil weighing boat with 0.25 mg tungsten oxide (to aid combustion) and analysed for C, N and S content using an Elementar Cube (Elementar Analysensysteme GmnH) auto analyser.

Plant Tissue Crude Protein Content

Crude Protein for all RC samples was calculated using the Kjeldahl digestion method. Plant samples were dried and milled as described for CNS analysis. 0.45 g was then weighed out onto a Whatman No. 2 filter paper and placed inside a labelled glass digestion tube. Two blanks and two known standards (powdered milk containing 35.5% crude protein) were also weighed out for every sixteen samples. Two 5 g Kjeldahl tablets ($\text{NA}_2\text{S}_2\text{O}_8 \cdot \text{CuSO}_4$) and 12 ml of 2M sulfuric acid were then added to each tube and left to sit overnight. All tubes were then placed on a thermostatically controlled heating block. The block was incrementally heated up to 400 °C until all samples had turned a clear, blue-green colour. The samples were then allowed to cool and 40 ml deionized water was added.

All tubes were then individually placed in a distillation apparatus which first dispensed 40 ml 40% sodium hydroxide (NaOH) and then steam-distilled the sample for 40 minutes. The distillate was transferred to a conical flask containing 25 ml boric acid & indicator solution using a delivery tube. This distillate was then back-titrated using 0.1 M hydrochloric acid. The HCL volume at which the distillate changed from blue to grey was recorded, and this titre was used to calculate the total N g kg⁻¹ DM. This figure was then multiplied by a conversion factor of 6 to convert to crude protein, a conversion factor described for clover by the FAO (1996).

Grain protein was calculated by multiplying grain N by 5.7 as described by (Delmolino 1991).

Plant N Content

Plant N was determined according to the following formulae:

$$\text{Grain N (kg ha}^{-1}\text{)} = \left(\frac{\text{Grain Yield (t ha}^{-1}\text{)}}{100} \times \text{grain N\%} \right) \times 1000$$

$$\text{Plant N (kg ha}^{-1}\text{)} = \left(\frac{\text{DM t ha}^{-1}}{100} \times \text{N\%} \right) \times 1000$$

$$\text{N Harvest Index \%} = \left(\frac{\text{N\% in grains}}{\text{N\% in grains} + \text{N\% in straw}} \right) \times 100$$

Table 2.4 Soil parameters at field trial initiation (March 2015)

Parameter	Block 1	Block 2	Block 3	Block 4
% Moisture	26.4	26.5	27.2	28.4
pH	7.3	7.2	7.1	7.1
Bulk Density (g cm ⁻³)	1.2	1	1.1	1.1
Sand %	26	28	31	27.3
Silt %	27	25.7	22	26.3
Clay %	47	46.3	47	46.4
Total Carbon %	3.7	3.6	3.9	3.9
Inorganic Carbon %	1.8	1.7	1.9	1.9
Organic Carbon %	1.9	1.9	2	2
Organic Matter %	3.3	3.3	3.4	3.4
Soil Mineral Nitrogen (kg ha ⁻¹)	11.1	12.5	13.1	13.9
Phosphorus (mg L ⁻¹)	7.6	11.4	12.4	13.3
Potassium (mg L ⁻¹)	250.4	233.4	232.2	220.5

2.6 Statistical Analyses

All data collected were inputted into the Genstat (18th Edition, VSNI Ltd) software package for analysis. Analysis of Variance (ANOVA) was used to analyse the differences among treatment means and interactions in all chapters, whilst Analysis of Covariance (ANCOVA) was additionally included in Chapter 5 to improve quality of analysis. Further information on why and how ANCOVA was utilized is contained prior to its use in Chapter 5. ANOVA and ANCOVA assumptions (independence of observations, normality of residual distribution,

homoscedasticity of variance and linearity of relationship) were satisfied before reporting results. Appropriate transformations were undertaken where assumptions were not satisfied. ANOVA results are reported with treatment means, degrees of freedom (df), standard error of difference (S.E.D.), details of any performed transformation and the *P* value. Four levels of significance were decided upon; non-significant, 0.05, 0.01 and 0.001. Fischer's Protected Least Significant Difference (LSD) was used to compare means of main treatment effects. Interactions are reported using bar charts and means plots. Non significant interactions are included in tables in the Appendices.

Canonical Variate Analysis (CVA) was used to determine the relationships among morphological variates in Chapters 3 and 4. Further information on why and how CVA was utilized is contained prior to its use in Chapter 3.

CHAPTER 3 - Core Experiment 1 – Red Clover Cultivation 2015

3.1 Introduction

Contemporary agriculture is increasingly looking to forage legumes, such as RC, to sustain cereal yields whilst reducing reliance on mineral fertilizer (Iannetta *et al.* 2016, Snyder, *et al.* 2016, Tamm, *et al.* 2016). These crops provide a suite of ecosystem services and provide a high-protein forage for livestock when not mulched. The production of this forage is particularly pressing, as European agriculture remains reliant on imported soy for livestock production (de Visser *et al.* 2014). The inclusion of forage legumes in arable rotations also increases system diversity, which may increase overall yields (Prieto *et al.* 2015), reduce weeds (Ratnadass *et al.* 2012), break pest cycles (Nicholls and Altieri 2004) and build resilience to climate stresses (Picasso *et al.* 2008).

The contribution RC can make to productivity and sustainability is apparent. What remains unclear is how its cultivation can be further optimized through management and variety selection. As reviewed in Chapter 2, management of RC leys can vary between grazing, cutting for hay or silage and mulching. This generally varies with the individual requirements of the farm and farmer. Different management strategies will influence the growth of the crop, its chemical composition, and the amount of biomass removed/returned to the system. Management strategies, therefore, may impact the crop's capacity to build soil fertility.

As RC is an important temperate forage crop, a large number of varieties are commercially available. The plant has an obligate out-crossing reproductive strategy (Townsend and Taylor 1985), which creates significant intra-species diversity in wild populations. This in turn increases the diversity existing within the genetic resources available for breeders, and currently there are three distinct categories of RC available to farmers. These can be broadly split into three types; early and late flowering (Frame, *et al.* 1998), erect and prostrate (Taylor and Quesenberry 1996) and diploid and tetraploid (Vleugels *et al.* 2013c). These types incur phenological, morphological and genetic variability, all of which may impact upon agronomic performance and fertility-building capacity.

3.2 Objectives & Hypotheses

Different RC varieties, under different management, may contribute more or less to soil fertility than others. If certain varieties and management strategies are optimal, this knowledge could help farmers receive the maximum benefit from their RC rotations. Inferences about why certain varieties and management strategies are optimal may also further our general understanding of soil fertility-building with legumes. The objectives for Core Experiment 1 were:

- To trial a selection of RC varieties for soil fertility-building capacity over 1 year
- To assess the influence of 1 and 2 cut management strategies on soil fertility-building capacity
- To document DM and N yields of RC varieties
- To assess RC varietal morphological diversity

The hypotheses for Core Experiment 1 were:

- Cutting management significantly effects DM and N yields of selected varieties
- RC varieties are morphologically diverse, as breeders describe
- Variety selection, cutting management and interactions between the two significantly effects the accumulation of soil mineral nitrogen

3.3 Materials and Methods

Materials and methods for all field operations and lab procedures are given in Chapter 2. Additional information concerning the RC varieties selected, morphological measurements taken, and reasons for these selections and measurements is contained in this section.

3.3.1 RC Varieties Selected

Six varieties of RC were selected to be evaluated. Effort was made to select varieties representative of all available categories, but as large treatment numbers would require much replication and reduce statistical power (Clewer and Scarisbrick 2001), a limit was set at six. A balance was struck between fully representing all categories and selecting varieties likely to be used by British farmers. For example, as early varieties are more commonly cultivated in Britain, more early varieties were selected. Varieties included on the British Grassland Society Varieties list (2014) were given preference, as this list is regarded highly by British farmers.

The varieties selected and their description is given in Table 3.1. Further details and reasons for selecting these varieties are given in the proceeding section. Efforts were made to gather all available information, but this was sometimes limited due to commercial sensitivity.

Table 3.1 Description of RC varieties selected for trial

Variety	Flowering	Ploidy	Growth Habit	Seed Merchant
AberRuby	Early	Diploid (2n)	Erect	IBERS (UK)
AberClaret	Early	Diploid (2n)	Erect	IBERS (UK)
Astred	Early	Diploid (2n)	Prostrate	PGG Wrightson (Australia)
Amos	Late	Tetraploid (4n)	Erect	DLF Trifolium (Denmark)
Maro	Late	Tetraploid (4n)	Erect	DLF Trifolium (Denmark)
Milvus	Early	Diploid (2n)	Erect	DSV (Germany)

AberRuby

Ruby is an early diploid with an erect growth habit. It is the only British variety to have been bred from a British landrace. It was bred from a Welsh landrace named ‘Glamorgan Red’, which was collected in Wales in the 1940s by breeders working at the former Welsh Plant Breeding Centre (Lloyd 2015). The IBERS seed catalogue describes Ruby as exceptionally high-yielding (IBERS 2011). Marley et al (2011) reported Ruby was low in phytoestrogens, which may make it more likely to be used by farmers interested in using their RC cuttings for forage. As a relatively new release, little work has been published on Ruby in the formal scientific literature, but it has been a staple on the British Grasslands Society list since its release (2016).

AberClaret

Claret is an early diploid with an erect growth habit. It was bred from an older variety known as ‘Formica’ in Wales and was selected for hardier and more developed crowns, as well as high

yield (Lloyd 2015). The IBERS seed catalogue describes Claret as high-yielding with stronger crown growth, resulting in vastly improved persistence (IBERS 2011). It also claims Claret can achieve a yield of 17 t ha⁻¹ in the second year of cultivation when combined with a companion grass.

Astred

Astred is an early diploid with a prostrate growth habit. It is described by its seed merchant as having low phytoestrogen levels and high persistence due to its prostrate growth habit (Wrightson 2015). Astred is described as stoloniferous by some authors (Taylor and Quesenberry 1996), but this may be somewhat misleading. The seed merchants of Astred describe it as ‘weak-stoloniferous’ (Stewart 2015), meaning the erect stem grows secondary branches which are very prostrate and will root at the nodes under optimal conditions (i.e. adequate moisture and proximity to the ground). This is not strictly stoloniferous growth, such as that of white clover (*Trifolium repens*), which does not grow an erect stem at all (Cain *et al.* 1995). As RC cultivation in Australia is primarily associated with livestock production, Astred was bred to persist longer in the sward in response to grazing, whilst providing a high-protein and low phytoestrogen forage.

Concerns were initially raised about the suitability of an Australian plant variety for British soil and climate. However, as Astred is bred from a wild European variety collected in Portugal named CPI 48825 (Taylor and Quesenberry 1996), it was decided it would be suitable for growth in the UK. Breeders describe it as having a longer flowering period than most RCs, with prostrate secondary branches which root at the nodes and permit vegetal reproduction, a trait which may improve persistence, particularly in response to grazing (Smith and Bishop 1998). This growth habit may also increase resistance to diseases, particularly *Sclerotinia* or clover rot, an important disease of RC, as less dependence on a single infected taproot may improve the survival chances of infected plants. This hypothesis has, however, been challenged by Vleugels (2013), who investigated this along with two other prostrate varieties Crossway and Broadway, and found no correlation between their prostrate growth habit and resistance to clover rot.

Amos

Amos is a late tetraploid with an erect growth habit. It is described by seed merchants as high-yielding with vigorous growth, low phytoestrogen content and high protein. It was the latest flowering variety on the British Grassland Society list (Society 2014), and therefore was

selected as the most appropriate late flowering variety for the trial. Hejduk et al (2010) found Amos to be the second highest yielding variety in a trial of 21 varieties in the Czech Republic.

Maro

Maro is an intermediate flowering tetraploid variety with an erect growth habit. Intermediate in this sense means it is neither early nor late, but flowers at some point in the middle of the 10-14 day span of flowering. It is described by seed merchants as high-yielding and possessing a higher leaf to stem ratio than diploids (Seeds 2002). Other reports describe Maro as highly persistent, with a possible lifespan of five or more years under optimal conditions (Porter 2010). Vleugels et al (2013c) found Maro to be highly resistant to clover rot. Maro was also the highest rated (in terms of yield and protein content) on the British Grassland Society list (Society 2014). Most available RC varieties are also discretely classified into early or late, and as Maro is the only highly regarded intermediate variety, it was selected for the trial.

Milvus

Milvus is an early diploid with an erect growth habit. It is advertised as high-yielding with strong autumnal growth and resistance to anthracnose and powdery mildew (DSV 2017). Milvus was bred in Switzerland from a particular collection of landraces called the 'Mattenklee' landraces (Boller 2000). These were reportedly bred over centuries by farmers who consciously cultivated RC leys for three or more years before harvesting seed. This process increased their persistency, and they remain among the foundational germplasm for European RC breeding programs (Boller *et al.* 2004). Milvus was not included on the British Grasslands Society list at the time of sowing, but given its long-standing reputation as a high-yielding and persistent variety, it was included in this trial.

The available literature on Milvus confirms it is high-yielding and persistent. Marshall et al (2012) found it to be the highest yielding variety in a trial of 12 British varieties. Hejduk et al (2010) found it to be the third highest yielding variety in a trial of 21 Czech varieties. A trial of 30 varieties over 2 years in Croatia cited Milvus as the second-most persistent variety with a 90.9% plant survival rate over both years (Tucak *et al.* 2013).



3.3.2 Morphological Measurements

As outlined in Chapter 1 and illustrated by the above descriptions, RC varieties are morphologically diverse. RC architectural characteristics have been related to biomass yield and regrowth capacity (Van Minnebruggen *et al.* 2015), which can, in turn, relate to nitrogen fixation and therefore soil fertility-building capacity (Becker and Johnson 1999). Previous studies have observed relationships between morphology and biomass yield for other legumes, for example cowpea (Nelson 1994) and alfalfa (Shen *et al.* 2013). Studies in RC specifically have also described this relationship. For example, Drobna *et al.* (2006) positively correlated plant height with yield. Morphology may also change in response to cutting (Farnham and George 1994) and some varieties may regrow differently to others. Stem number and plant height may increase or decrease, and prostrate varieties may become more or less prostrate, etc.

These changes may impact positively or negatively on biomass yield and persistence. Documentation of the morphological variation exhibited by the RC varieties in this trial, initially, in response to cutting, and over multiple years, may assist in explaining differences in biomass yield, biochemical composition (protein, C:N ratio etc) and soil fertility-building capacity. The morphological characteristics recorded are given in Table 3.2 All measurements were taken prior to cutting at the early-mid bloom stage. The methodologies of how they were measured and reasons why they were selected for assessment are given in the subsequent section. The International Union for the Protection of New Plant Varieties (known by their French acronym UPOV) provide the internationally recognized protocols for descriptions of agricultural plant varieties, and their descriptions for RC (2001) provide most of the methodologies for this study. However, some adjustments have been made and these are detailed as follows

All measurements were taken from whole individual plants with three replications per plot. Measurements were taken before both cuts were applied, which gave two data sets; ‘Cut 1’ and ‘Cut 2’.

Table 3.2 Morphological traits recorded during trial

Plant Height (cm)	Number of Nodes (n)
Density of Hairs (n)	Number of Stem Leaves (n)
Growth Habit (n)	Number of Crown Leaves (n)
Number of Stems (n)	

Plant Height

Plant Height was measured as the distances in cm from the cut base of the plant to the tip of the highest apical inflorescence. This measurement may have slightly underestimated the true value, as all plants were cut at 5cm above the crown, but this was done uniformly across varieties and considered to be a negligible difference. Centimetres were chosen over the conventional UPOV method of allocating 1-9 values for a (very short – very long) range (UPOV 2001). This was done to assist non-specialists in conceiving of how tall the varieties actually grew, an idea previously described by Tucak et al (2013). Plant height was measured because it is considered a rough indicator of performance by agronomists (Mourtzinis *et al.* 2013), and is commonly measured for this purpose. It has also previously been positively correlated with DM yield in a trial of different RC varieties (Zoran *et al.* 2010).

Density of Hairs

RC can be pubescent or glabrous (Taylor and Quesenberry 1996). These terms refers to the presence or absence of trichomes (hair like structures) on the stems. Density of these hairs is commonly used to distinguish between varieties. It was measured here in accordance with UPOV (2001), on a scale of 1-9 on the 3rd internode of a fully expanded flower head on the same stem on which the length of stem was measured. Details of this scale are given in Table 3.3.

Table 3.3 Scoring system for hair density trait

Very Low	1
Low	3
Medium	5
High	7
Very High	9

Some authors cite European cultivars as mainly hairless and American cultivars as hairy (Taylor and Quesenberry 1996). Density of hairs was selected for two reasons. The first was to

investigate if a relationship, if any, existed between density of hairs and resistance to certain pests. Stem hairs are generally associated with defences against herbivorous insects (Levin 1973), for example the clover weevil *Hyperapunctata fabricius*. Stem hairs can limit insect mobility and secrete insecticidal chemicals, but this has not been demonstrated for RC specifically. Hair density may, therefore, impact upon plant productivity and persistence and was considered appropriate to measure.

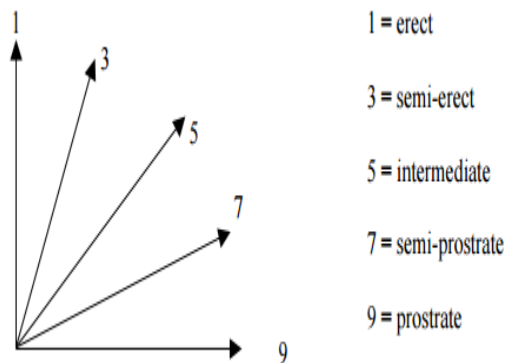
Mulching is commonly associated with RC fertility-building, but cutting for forage is also undertaken, particularly the first cut, as mulching this can inhibit subsequent crop growth and biofixation (Loges, *et al.* 1999, Moyo 2014). In both management systems the quality of the forage remains relevant, and this can also be influenced by hair density. Hairier stems are associated with longer drying times in RC hay production (Lenssen *et al.* 2001), and longer drying times are undesirable for farmers, as untimely rainfall may then degrade forage quality.

These hairier stems, then, may also take longer to mineralize when the crop is mulched or used for green manure, as hairs can be composed of proteins and secrete chemicals resistant to microbial digestion (Schillmiller *et al.* 2008). It is important to note that forage qualities such as stems hairs, N, lignin and polyphenol contents etc, are effectively the same as those which determine rates of mineralization and immobilization, and therefore the utility of the crop in green manuring

Growth Habit

Growth Habit was measured in accordance with UPOV (2001), on a scale of 1-9. 1 meant completely erect and 9 meant completely prostrate. A value was assigned by examining the angle at which the secondary branches emitted from the main stem. A value of 1 meant the branches were almost erect (i.e. an angle $>20^\circ$) whilst a value of 9 meant the branches were prostrate (i.e. an angle of 90°). This information is graphically described in Figure 3.1

Figure 3.1 Diagram of scoring system for growth habit morphological trait



Number of Stems and Nodes

The number of stems on the individual plant was recorded as the stem number. The number of nodes was recorded as the number of nodes on the tallest stem (the same used for plant height and hair density). These traits were selected because stem and node number have been correlated with biomass yield with other RC varieties (Chmelikova *et al.* 2015).

Number of Stem and Crown Leaves

The total number of stem and crown leaves were recorded. Crown leaves were defined as those growing directly from the crown, stem leaves from the stems. These traits were selected because fixed N concentrations have been shown to be highest in RC leaves (Huss-Danell and Chaia 2005), and therefore higher numbers of leaves may relate to N accumulation and rates of biofixation.

3.3.3 Statistical Analysis

All recorded results were subjected to statistical analysis using GENTSTAT® 18th Edition. The design was completely randomized block and RC variety was inputted as a treatment. All results were subjected to ANOVA and are presented with the standard error of the difference between the means and the degrees of freedom. Fischer's LSD was used to determine differences among means where significant F values (<0.05) were calculated. The assumptions of ANOVA were satisfied initially by using the summary statistics function to view the data distribution histogram and skewness coefficient. Assumptions were further satisfied by

ensuring normal distribution of residuals and homogeneity of variance in the residual fitted values plot following the ANOVA. Where assumptions were not satisfied, appropriate transformations were undertaken and the analysis was repeated. Further descriptions of specificities and transformations are given in the appropriate results section.

3.4 Results

3.4.1 Germination Test, 1000 Seed Weight and Establishment of RC Varieties

Significant differences were observed in the initial establishment counts, which were conducted following germination in early May. Ruby established at a significantly lower density than all other varieties and Claret at a higher. A lab germination test was then conducted to investigate if this was caused by seed quality or field conditions. This confirmed Ruby and Astred germinated at a significantly lower percentage than all other varieties. The 1000 Seed Weight assessment showed the tetraploid varieties had significantly heavier seeds than the diploids. This information is summarized in Table 3.4.

Table 3.4 Germination test, 1000 seed weight and establishment of RC varieties

Variety	Germination %	1000 Seed Weight g	Establishment plants m ⁻²
Amos	94.7a	2.72a	181b
Astred	53.0b	2.09b	165bc
Claret	96.0a	1.93b	247a
Maro	90.0a	2.67a	180b
Milvus	87.4a	1.84b	191b
Ruby	52.7b	1.99b	145c
S.E.D	4.23	0.28	17.56
df	12	12	87
P	<0.001	<0.001	<0.001

Values followed by the same letter do not differ significantly at cited P value

3.4.2 DM t ha⁻¹ and N kg ha⁻¹ (First Cut)

The intermediate and late varieties Maro and Amos yielded significantly more DM than the early varieties at cut 1. Milvus yielded significantly lower DM than the other early varieties. Weed problems following slow establishment due to the dry April and May of 2015 (see Figure 2.1) were also documented by assessing weed DM. Ruby and Amos yielded significantly less weeds, whilst Claret, Maro and Milvus yielded the most. Maro and Amos also yielded

significantly more N than the early varieties and Astred yielded the lowest N content. This information is summarized in Table 3.5

Table 3.5 DM and N yield at Cut 1

Variety	Clover DM (t ha ⁻¹)	Weeds DM (t ha ⁻¹)	Clover N (kg ha ⁻¹)
Amos	0.75a	0.64c	24.4ab
Astred	0.56bc	0.65bc	15.8c
Claret	0.61b	0.68abc	19.3bc
Maro	0.85a	0.79a	28.1a
Milvus	0.49c	0.76ab	19.3bc
Ruby	0.56bc	0.64c	19.2bc
S.E.D.	0.056	0.056	3.16
df	87	87	39
P	<0.001	0.021	<0.001

Values followed by the same letter do not differ significantly at cited P value

3.4.3 DM t ha⁻¹ and N kg ha⁻¹ (Second Cut and Total)

The early diploids yielded significantly more DM and N than the late tetraploid Amos at cut 2. However, the intermediate variety Maro maintained good growth and yielded the same DM and N as the early varieties. Amos yielded significantly less DM and N than all other varieties when both cuts were summed. No significant differences were observed for all other varieties when both cuts were summed. This information is summarized in Table 3.6

Table 3.6 DM and N yield at Cut 2 and total (sum of both cuts)

Variety	Clover DM t ha ⁻¹	Clover N kg ha ⁻¹	Total DM t ha ⁻¹	Total N kg ha ⁻¹
Amos	0.82c	30.0c	1.58b	54.4b
Astred	1.57a	67.0a	2.11a	82.8a
Claret	1.64a	61.9ab	2.20a	81.1a
Maro	1.51ab	56.6b	2.39a	83.2a
Milvus	1.60a	61.8ab	2.17a	80.9a
Ruby	1.35b	55.9b	1.95ab	75.6a
S.E.D.	0.11	4.69	0.15	6.27
df	39	39	39	39
P	<0.001	<0.001	<0.001	<0.001

Values followed by the same letter do not differ significantly at cited P value

3.4.5 Protein Content of Cuttings

Early varieties tended to contain significantly more protein than intermediate and late varieties at cut 1, although Astred (an early variety) yielded significantly less protein than the other early

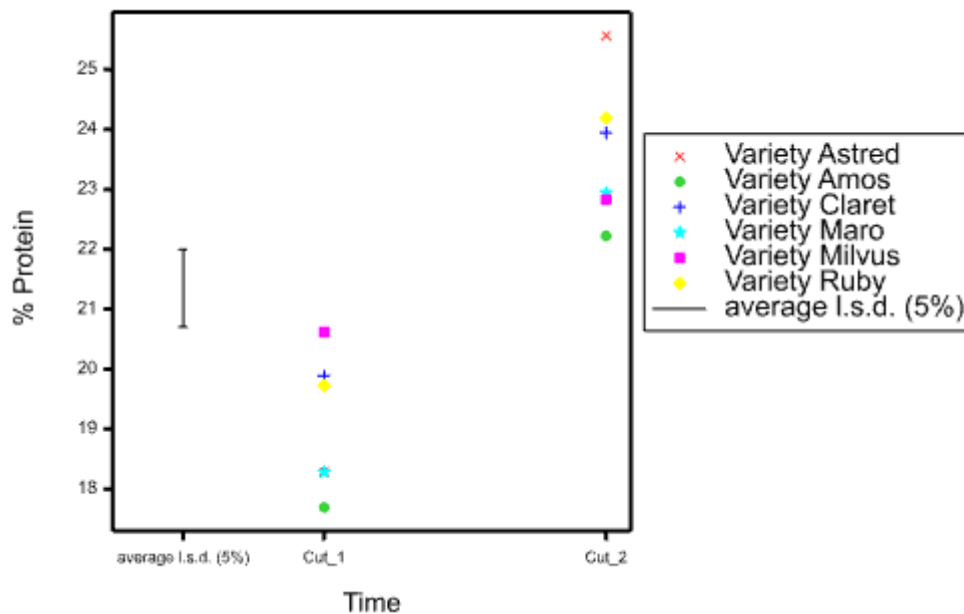
varieties. Tetraploid varieties contained significantly less protein than diploids at both cuts. Further analysis was undertaken using the repeated measure ANOVA function to incorporate the temporal element. This was done using Cut 1 and Cut 2 as the times at which the repeated measurements were taken. Time was shown to be a highly significant factor ($P < 0.001$) and all varieties significantly increased their protein content. At the second cut Astred contained more protein than all other varieties. This information is summarized in Table 3.7 and Figure 3.2.

Table 3.7 Protein content of RC cuttings (both cuts)

Variety	Cut 1 Protein (%)	Cut 2 Protein (%)
Amos	17.7b	22.3d
Astred	18.3b	25.6a
Claret	19.9a	24.0bc
Maro	18.3b	22.9cd
Milvus	20.6a	22.9cd
Ruby	19.7a	24.2b
S.E.D. (39 df)	0.65	0.61
P	<0.001	<0.001

Values followed by the same letter do not differ significantly at cited P value

Figure 3.2 Effect of interaction between time of cut and variety on protein content



3.4.5 Disease Assessment

3.4.5.1 Powdery Mildew (*Erysiphe sp*)

Incidence of powdery mildew was scored according to Singh et al (2013a). This key refers to powdery mildew incidence in lentils, but as its methodology focused on leaf infection observations, it was considered appropriate. Powdery mildew is not considered a serious concern for RC leys (Mosjidis 2011), and as a result, no standard assessment key is available. Ten individual plants were randomly selected from each plot and disease severity was recorded on the upper, middle and lower leaves on the basis of leaf area covered by infection. As the infection was first observed mid-cut (Aug 7 2015, see Table 2.1), only '1 cut' plots were scored to avoid any non-varietal variance in infection caused by the cut. The infected leaf area was scored according to the descriptions given in the key, which are summarized in Table 3.8. Figure 3.3 illustrates the incidence of powdery mildew.

Table 3.8 Description of scoring system for powdery mildew – taken from Singh et al (2013a)

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Figure 3.3 Powdery mildew infection (Variety Maro)



A percentage disease index for each plot was then calculated according to the formula:

$$\text{Percent Disease Index} = \left[\frac{\text{Sum of All Numerical Rating}}{(\text{Total Leaves Observed})(\text{Max Rating})} \right] 100$$

Astred, Claret, Milvus and Amos were the most resistant varieties, whilst Maro was significantly less resistant than these. Ruby was significantly less resistant than all varieties. The results for each variety are given in Table 3.9

Table 3.9 Percentage disease incidence of powdery mildew

Variety	Percentage Disease Index*
Amos	24.4bc
Astred	19.3c
Claret	20.0c
Maro	28.9b
Milvus	20.0c
Ruby	36.9a
S.E.D. (39 df)	3.03
P	<0.001

*Higher scores indicate higher infection

Values followed by the same letter do not differ significantly at cited P value

3.4.5.2 Clover Rot (*Sclerotinia* sp)

Incidence of clover rot was scored according to Dixon and Doodson (1974). This key was developed by scientists at NIAB. As clover rot is a much more serious and prevalent disease of RC leys (Taylor and Quesenberry 1996), such a key exists for RC specifically. Dixon and Doodson (1974) has been since cited by subsequent researchers more than any other (Delclos *et al.* 1997, Ohberg *et al.* 2008, Vleugels *et al.* 2013b, Mikaliuniene *et al.* 2015), and can therefore be considered the standard assessment key.

Ten individual plants were randomly selected in each plot. As the *Sclerotinia* infected occurred as the second cut was about to take place (Oct 14 2015, see Table 2.1), only '2 cut' plots were scored to avoid any non-varietal variance in infection incurred by the cut. Each plant was divided into four categories of symptom expression. These categories are given in Table 3.10. A fungicide was applied on November 1 using a knapsack sprayer to control this disease. The

fungicide was Iprodione (a hydantoin fungicide) at an application rate of 2 kg ha⁻¹. Figure 3.4 illustrates the incidence of clover rot.

Table 3.10 Description of scoring system for clover rot – taken from Dixon and Doodson (1974)

Some materials have been removed from this thesis due to Third Party Copyright. The unabridged version of the thesis can be viewed at the Lanchester Library, Coventry University.

Figure 3.4 Initial and late stages of clover rot infection



RC Leaf showing acscospores indicating initial Sclerotinia infection



RC plot indicating late stage of infection with large patches of necrotic plant tissue

A disease index for each plot was then calculated according to the following formula:

$$Disease\ Index = 10(x + 2y + 3z)$$

In this equation x, y and z are the numbers of plants rated 1, 2 and 3 respectively.

Ruby and Astred were found to be significantly more resistant to clover rot than Claret and Maro, and these four varieties in turn were more resistant than Amos and Milvus. This information is summarized in Table 3.11

Table 3.11 Disease index of clover rot

Variety	Disease Index*
Amos	258.8a
Astred	136.2c
Claret	225.0b
Maro	213.8b
Milvus	245.0a
Ruby	136.2c
S.E.D. (39 df)	9.44
P	<0.001

*Higher scores indicate higher infection

Values followed by the same letter do not differ significantly at cited P value

3.4.6 Soil Mineral Nitrogen (SMN)

The initial characterization of the field trial soil showed the SMN prior to RC cultivation was 11-13 kg ha⁻¹ (Table 2.5). Soil structural properties such as bulk density and texture can affect permeability and therefore susceptibility to SMN leaching (Anei *et al.* 2013), but little difference was observed between blocks for these parameters. The completely randomized block design also accounts for other differences in soil structure, such as soil aggregation and porosity, which were not measured at the beginning of the trial.

SMN was assessed again following the growing season in Core Experiment 1 (Nov 1 2015) and no significant effects of variety or cut were observed. However, SMN levels had increased to ~40 kg ha⁻¹, indicating a net contribution of ~25 kg ha⁻¹ was made throughout Core Experiment 1. SMN was calculated again before Core Experiment 2 began (Feb 22 – Mar 10 2016). This was done to document any losses that may have occurred over the winter period. Significant differences were observed for both variety and cut at this measurement. The treatment of 1 cut was significantly higher than 2 (P<0.001) and Astred and Ruby had higher levels of SMN than all other varieties. The Leached SMN data showed 1 cut was significantly less susceptible than 2, and Astred and Ruby were less susceptible than all other varieties. This data is summarized in Table 3.12.

Table 3.12 Soil mineral nitrogen accumulation Core Experiment 1

Variety	Winter 2015 kg ha ⁻¹	Spring 2016 kg ha ⁻¹	Leached N kg ha ⁻¹
Amos	39.4ns	16.7c	22.3a
Astred	38.9ns	26.2a	12.3d
Claret	39.3ns	21.6b	17.3bc
Maro	40.6ns	17.9bc	22.3a
Milvus	40.5ns	18.7bc	21.4ab
Ruby	39.9ns	25.9a	13.6cd
S.E.D. (273 df)	1.06	1.16	1.48
P	0.543	<0.001	<0.001
1 Cut	40.1ns	22.6a	17.1b
2 Cut	39.4ns	19.8b	19.2a
S.E.D. (273 df)	0.61	0.67	0.85
P	0.273	<0.001	<0.001

Values followed by the same letter do not differ significantly at cited P value

Further analysis was undertaken using the repeated measure ANOVA function to incorporate the temporal element. This was done using winter and spring as the times at which the repeated measurements were taken. Time was shown to be a highly significant factor ($P < 0.001$). The interaction between time and variety was highly significant ($P < 0.001$) (Figure 3.5). The interaction between time and cut was significant as well (Figure 3.6). The interaction between variety, cut and time was also significant (Figure 3.7). These interactions showed Astred and Ruby to leach significantly less SMN than all other varieties throughout the winter period. They also showed the treatment of 2 cut leached significantly more SMN than 1 cut

Figure 3.5 Effect of interaction between variety and time on SMN accumulation before and after winter leaching (Repeated ANOVA)

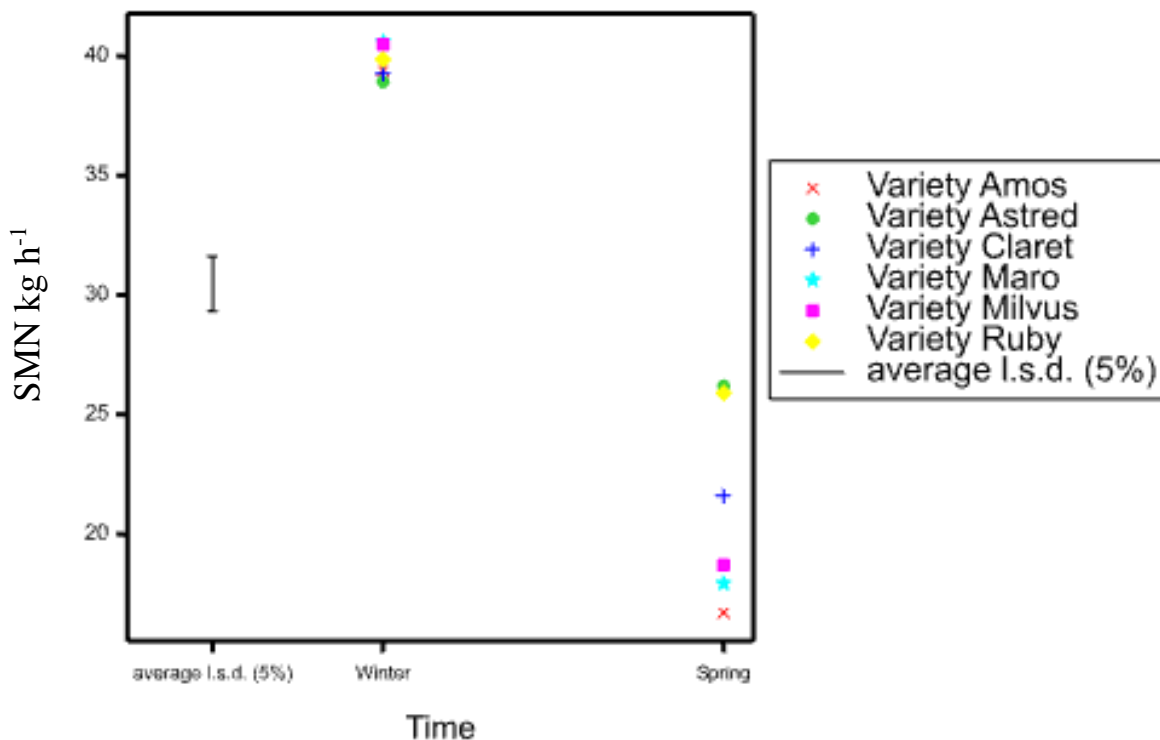


Figure 3.6 Effect of interaction between cut and time on SMN accumulation before and after winter leaching (Repeated ANOVA)

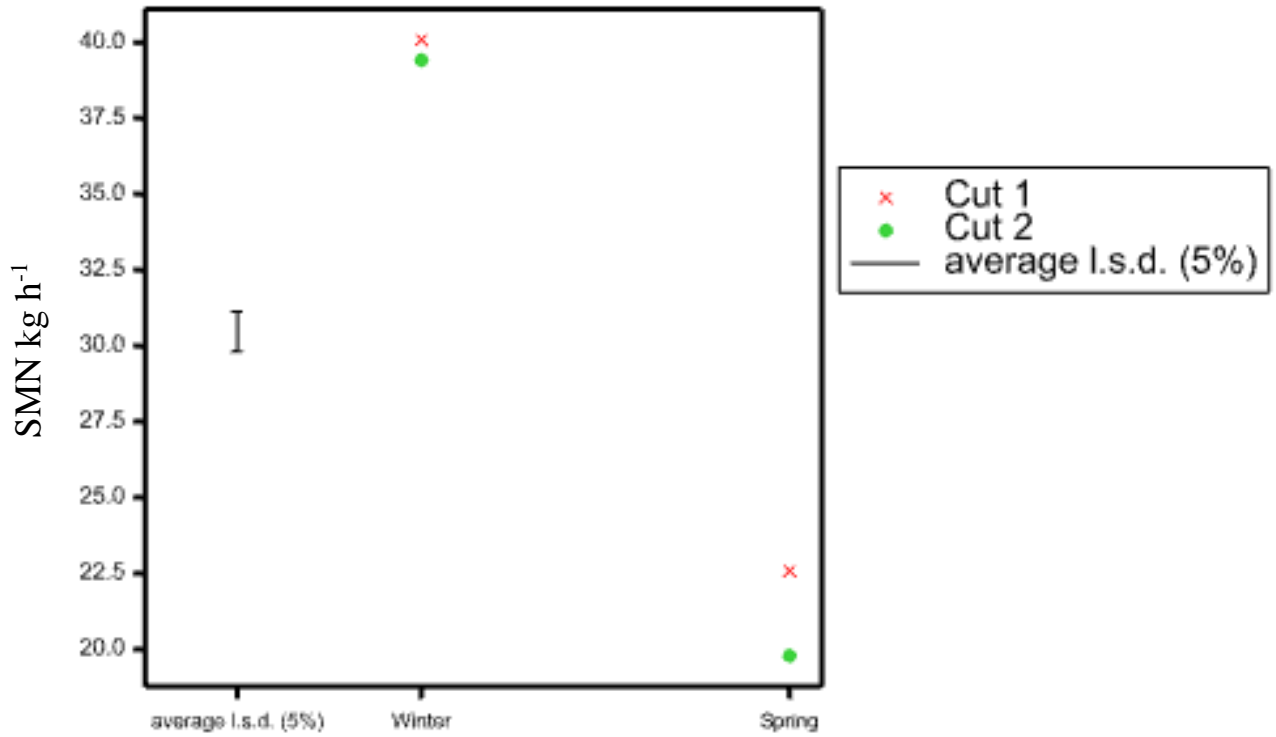
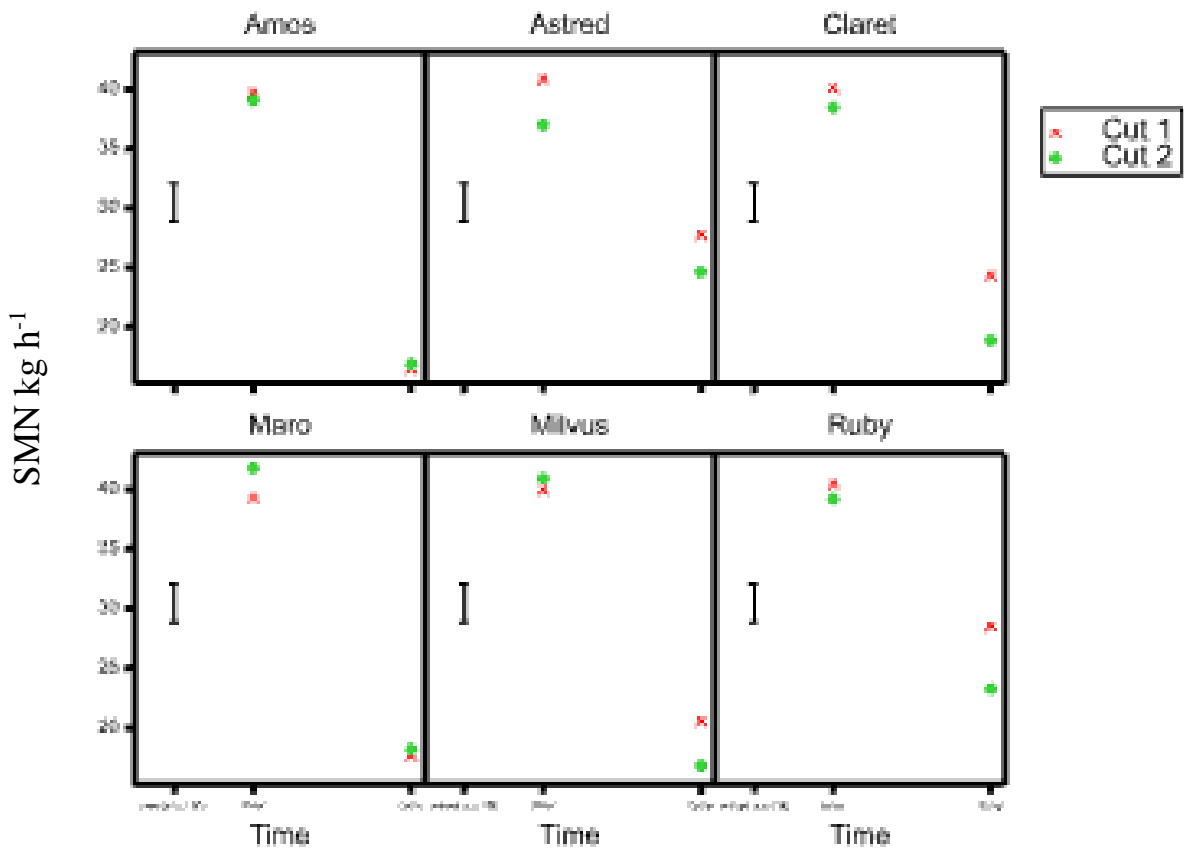


Figure 3.7 Effect of interaction between variety, cut and time on SMN accumulation (Repeated ANOVA)



3.4.7 Morphological Assessments

3.4.7.1 ANOVA

One-way ANOVAs were used to assess the variation for each trait measured. Initial investigations using the summary statistics function revealed some data sets were not normally distributed (i.e. the characteristic of growth habit was positively skewed, given only one prostrate variety was being trialled). Appropriate transformations were made to reduce heterogeneity of variance and satisfy the ANOVA assumptions, followed by an examination of the residual plots to confirm homogeneity of variance. As Amos failed to regrow stems following cutting, only crown leaves values were obtained for this variety at Cut 2. Details of transformations are given beneath the relevant Tables.

Significant differences between varieties were observed for all traits at both cuts. Astred was not observed to be the most prostrate variety at cut 1, but regrew to be the most prostrate variety at cut 2. Ruby was the shortest variety with the least stem leaves at the first cut, but regrew to be one of the tallest with the most stem leaves. Astred and Ruby were significantly hairier than the others at both cuts. Claret, Maro and Milvus scored the same values for most traits across most cuts. The latest variety (Amos) failed to produce stems following cutting. This information is summarized in Tables 3.13 and 3.14

Table 3.13. Summary of morphological variation (Cut 1)

Variety	Hair Density (n) ¹	Growth Habit (n) ¹	Height (cm)	Stems (n) ²	Nodes (n)	Stem Leaves (n) ²	Crown Leaves (n)
Amos	1.4c	1.6c	54.2a	3.1ab	4.6a	16.4b	15.6a
Astred	3.3a	1.8bc	45.5b	3.4a	4.6a	24.6a	11.1b
Claret	1.9bc	1.4c	46.0b	2.9b	4.4a	16.6ab	11.3b
Maro	1.9bc	2.2ab	41.5b	2.8b	3.7b	10.9c	10.0bc
Milvus	1.4c	2.0ab	36.4c	3.1ab	3.5b	12.9bc	9.9bc
Ruby	2.4b	2.3a	35.0c	3.4a	3.3b	15.4bc	7.4c
SED (282 df)	0.07	0.04	1.5	1.1	0.16	1.1	0.8
P	<0.001	<0.001	<0.001	0.015	<0.001	<0.001	<0.001

Values followed by the same letter do not differ significantly at cited P value

1. Data has been log transformed to reduce heterogeneity of variance. An arbitrary constant of 1 was added to avoid zeros (values of 1 were commonly reported and $\log 1=0$). Reported data has been back-transformed using the antilog function.

2. Data has been log transformed to reduce heterogeneity of variance. Reported data has been back-transformed using the antilog function.

Table 3.14. Summary of morphological variation (Cut 2)

Variety	Hair Density (n) ¹	Growth Habit (n)	Height (cm)	Stems (n) ²	Nodes (n)	Stem Leaves (n) ²	Crown Leaves (n)
Amos	N/A	N/A	N/A	N/A	N/A	N/A	11.3b
Astred	4.3a	3.8a	40.5c	3.3b	4.4ab	22.6b	18.9a
Claret	2.2c	1.3c	41.0c	3.3b	4.0b	16.2b	15.7ab
Maro	2.9c	2.0c	44.0bc	3.0b	4.0b	17.6b	17.2ab
Milvus	2.3c	1.4c	48.6ab	3.3b	4.6ab	22.7b	11.4b
Ruby	3.7a	2.7b	52.0a	5.5a	5.1a	51.9a	11.3b
SED	1.1	0.35	3.3	1.1	0.35	1.2	1.8
df	115	115	115	115	115	115	138
P	<0.001	<0.001	0.002	<0.001	0.01	<0.001	<0.001

Values followed by the same letter do not differ significantly at cited P value

1. Data has been log transformed to reduce heterogeneity of variance. An arbitrary constant of 1 was added to avoid zeros (values of 1 were commonly reported and $\log 1=0$). Reported data has been back-transformed using the antilog function.

2. Data has been log transformed to reduce heterogeneity of variance. Reported data has been back-transformed using the antilog function.

Tables 3.13 and 3.14 describe the variation existing among different traits for the trialled varieties. However, from this data it is difficult to determine the relationship that exists among the traits. Understanding this relationship may assist in determining which varieties are optimal for use in forage production and fertility-building. If, for example, hairier varieties produce shorter stems and fewer leaves, this trait may limit regrowth and yield. Prostrate varieties may produce less leaves, which may increase persistence but limit yield. A multivariate analysis is required to investigate if such relationships exist.

3.4.7.2 Canonical Variate Analysis

Four such multivariate analyses are described in a review of research methodologies concerning morphological variation in biological studies (Klingenberg 2010). These are principal component analysis (PCA), canonical variate analysis (CVA), multivariate regression and partial least squares analysis. Each of these methods has its own assumptions, advantages and limitations. CVA was found to be the most appropriate analysis for this research, for two reasons. The first reason was utilitarian; unlike the other analyses CVA does not assume multivariate normality (Tabachnick and Fidell 2006). As shown in Tables 3.13 and 3.14, some traits were normally distributed, whilst others were not and required transformation. Multivariate normality also requires normality all of linear combinations, which would be difficult to show and would likely require complicated transformations. The use of CVA to

describe the relationship between variates avoided this problem. The second reason for selecting CVA was its suitability for this particular task. CVA is most appropriate when the variates are already classified into groups (Harding and Payne 2015), in this case varieties. This contrasts with the similar PCA, in which individual units are described in relationship to each other, rather as members of a group.

CVA belongs to the regression family of analyses and finds linear combinations of the data variates that represent most of the variation between groups. These combinations are known as canonical variates. Canonical variates are then used to describe the total variation existing between populations. As morphological metrics will generally relate to each other (i.e. big teeth require big jaws, large seeds require large pods, small trees will have thin trunks etc), CVA on such data will usually generate two canonical variates which describe the majority of the variation. In certain very variable data sets three may be necessary. When two canonical variates describe the majority the variation, all data points can then be graphed as multivariate analogues of simple scatter plots using the first canonical variate as the x axis and the second as the y (Gower *et al.* 2014).

These graphs can be used by researchers to examine the total variation existing for all varieties, and be viewed as a more easily interpreted summary of the data contained in Tables 3.13 and 3.14 respectively. They can be further used to understand how the traits relate to each other through conversion into biplots. CVA biplots provide a visual means of assessing the relationship between individual observations (in this case the varieties) and their characteristics (plant height, growth habit etc). This is achieved by plotting individual vectors corresponding to each variate. These vectors define where each individual data point relates to each variate in question. Furthermore, the angle created between vectors represent correlations between variates (small angles indicate high correlation and large angles indicate low) and vector direction also indicates correlation (opposite directions indicate negative correlation and vice versa) (Harding and Payne 2015). All variates in the morphological assessment were subjected to CVA using variety as the group factor. This was done individually for cuts 1 and 2. Results are presented graphically in Figures 3.8 – 3.11.

Cut 1

The CVA on all morphological variates in cut 1 determined 94.03% of the variation could be explained by the first two canonical variates. This analysis was then determined appropriate for visual representation in scatterplot form. These graphs are given in Figures 3.8 and 3.9

Figure 3.8 Scatterplot of CVA of First Cut

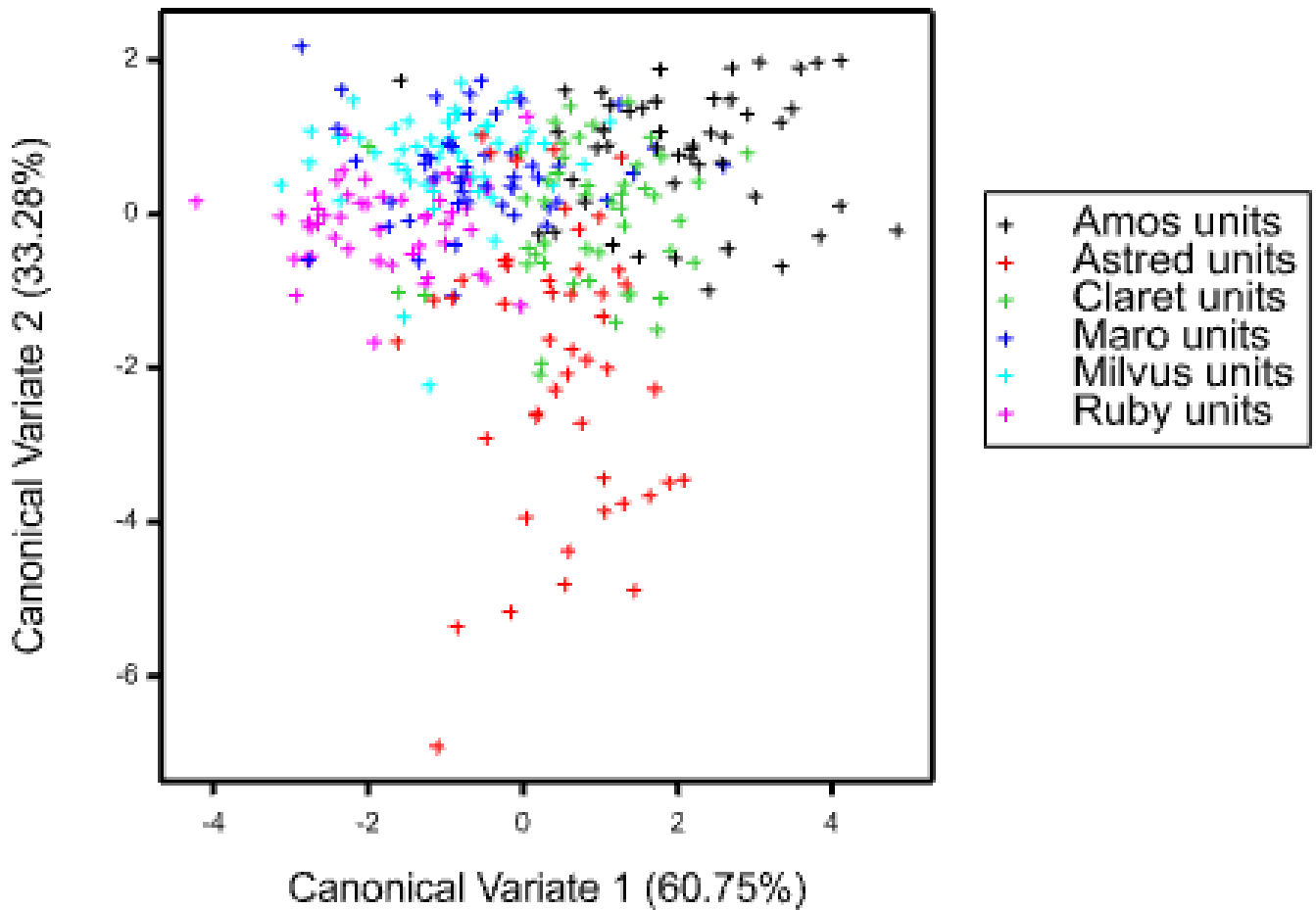


Figure 3.8 illustrates the total variation described by the first two canonical variates. Here Astred is shown to be the most variable and most distinct variety, as it occupies the most space and extends into sections of the graph which contain no other data points. Little variation is shown for Maro, Milvus and Claret, but Ruby and Amos are shown to be more distinct from the other varieties. A biplot version of Figure 3.8 is given in Figure 3.9. Vector direction is indicated by arrow symbols and always direct toward the vector label.

Figure 3.9 CVA Biplot of First Cut

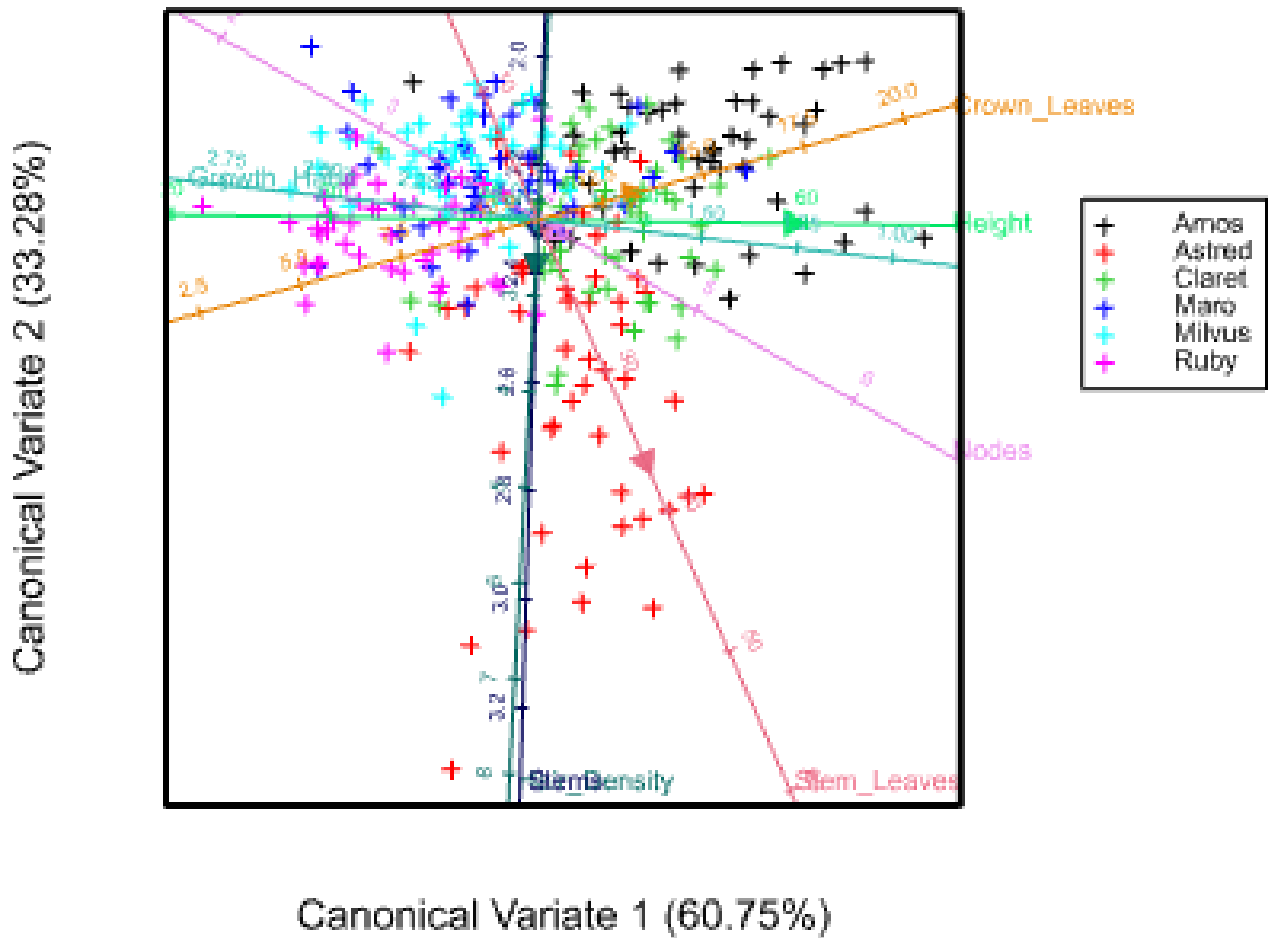


Figure 3.9 shows the variation observed in Astred was caused primarily by stem, stem leaf number and hair density (the individual points are most distributed along these vectors). Small angles between stem number and hair density indicate these traits were well-correlated. The opposing directions of the height and growth habit vectors indicate these traits were negatively correlated. Most of the variation for Ruby, Maro and Milvus was occurred along the height, growth habit and crown leaves vectors, and little variation was observed for stem number, hair density and stem leaves.

Cut 2

The CVA on all morphological variates in cut 2 determined 94.37% of the variation could be explained by the first two canonical variates. This analysis was then determined appropriate for visual representation in scatterplot form. As Amos failed to regrow stems in the regrowth phase (because the required photoperiod had already passed) this variety has been removed from this analysis. These graphs are given in Figures 3.10 and 3.11.

Figure 3.10 Scatterplot of CVA of Second Cut

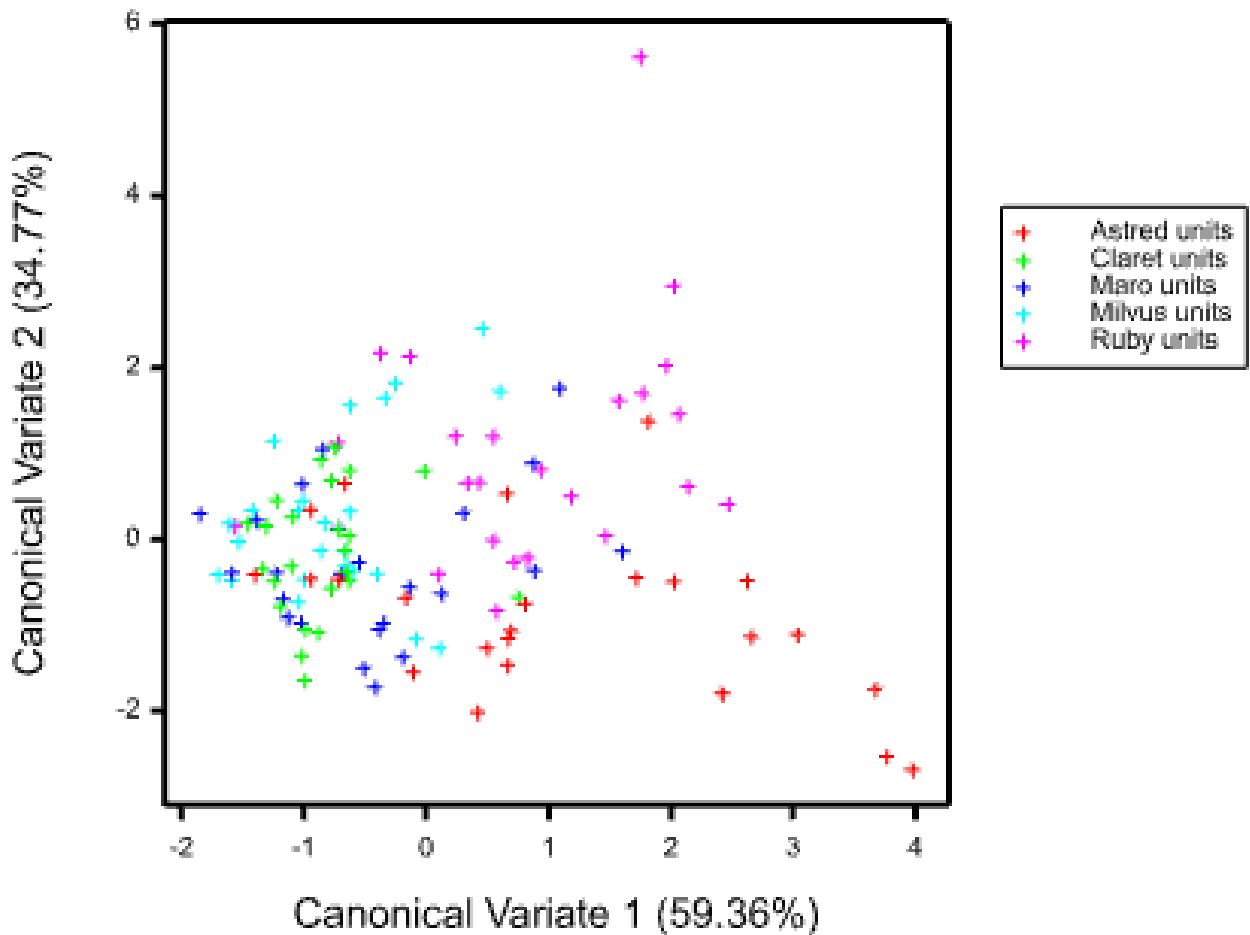
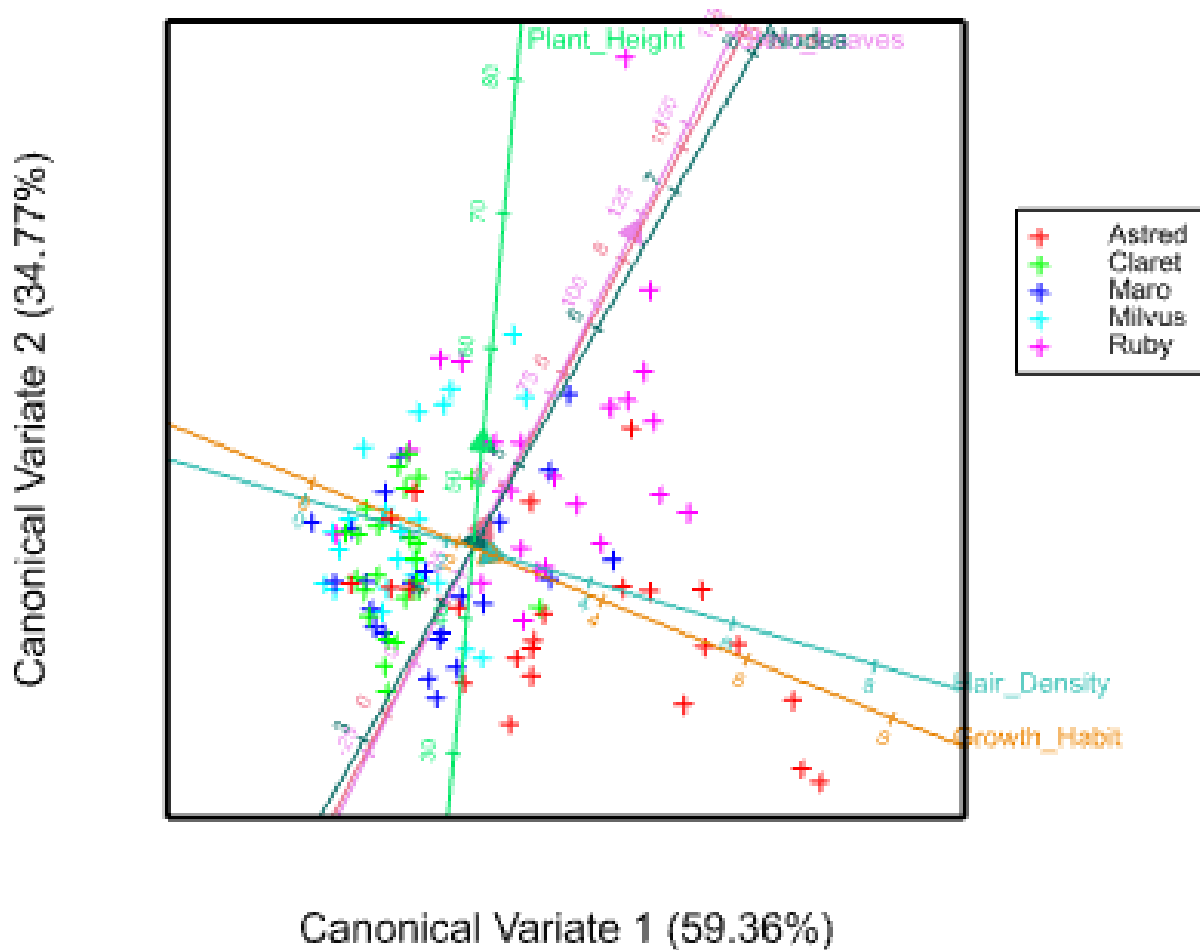


Figure 3.10 illustrates the total variation described by the first two canonical variates. As in cut 1, Astred and Ruby take up the most area on the graph and are therefore the most morphologically variable. Less variation is shown for Claret, Milvus and Maro, but the space taken up by these varieties seems larger than that observed at cut 1, indicating these varieties may have become more morphologically variable in response to cutting. The scale of the canonical variates is similar to that of cut 1, meaning general comparisons could be made between both graphs, but as the canonical variates are themselves different, conclusions regarding how distribution of variance has changed in response to cutting cannot be robustly made.

Figure 3.11 Biplot of CVA Cut 2



Nodes, stems and stem leaves vector all converge at the same point at the top of the graph

Figure 3.11 shows how the variation observed in Astred was mostly explained by hair density and growth habit (Astred takes up the most area along these vectors). Variation in Ruby was mostly along the height and number of nodes, stems and stem leaves vectors. Hair density and growth habit were shown to be well-correlated. As this correlation was not observed at cut 1, this implies this traits may correlate in the regrowth period following cutting. No correlation between number of crown leaves and growth habit was observed. Number of nodes, stems and stem leaves were all strongly correlated (all vectors have tight angles).

3.5 Discussion

3.5.1 Germination Test, 1000 Seed Weight and Establishment

Results of the germination test indicated the seed of both Astred and Ruby was of significantly poorer quality than the other varieties trialled ($P < 0.001$). Low germination rates can be an issue with small-seeded legumes following a natural hardening of the seed coat following storage (Skogen *et al.* 2010), and this may have happened with Astred and Ruby. These differences in germination rates did appear to impact upon establishment rates, as Ruby established at a significantly lower density than all other varieties. Astred did, however, establish at the same density as varieties with high germination rates ($>90\%$). Seedling populations are also self-thinning as crop growth proceeds, and visual field observations indicated little difference lay between all varieties once this process had taken place. Establishment of 200 plants m^{-2} is desirable for RC leys (Grass 2014) and all varieties established within 25% of this range. Furthermore, no significant differences were observed for DM yield at both cuts between Astred and Ruby and other varieties with higher rates of germination and establishment (Claret and Maro).

The 1000 seed weight assessment confirmed the findings of Sevecka (1986), tetraploid varieties had significantly heavier seeds than diploids ($\sim 2.7g$ to $\sim 2.0g$). This extra seed mass may give tetraploid varieties greater seedling emergence than diploids, but as the tetraploids trialled in this experiment established at the same rate as the diploids (and significantly lower than one diploid, Claret), it is unlikely this difference in seed mass had significant impact on establishment. Some authors suggest higher sowing rates for tetraploids, as the lower number of seeds per unit weight may result in lower seedling emergence (Taylor and Quesenberry 1996), but again this effect was not observed in this experiment, in which all varieties were sown at 15 kg ha^{-1} .

3.5.2 DM, N and Weeds (Cut 1)

The literature on RC varietal differences suggests early varieties reach the point of flowering quickly, thus limiting the yields of the first cut, as the conversion from vegetative to reproductive growth effectively stops biomass accumulation (Frame, *et al.* 1998). Conversely, the delayed flowering of the later varieties allows for higher DM yields at cut 1. Tetraploid varieties are also cited as higher-yielding than diploids (Frame 1976, Vleugels 2013, Amdahl *et al.* 2016). These varietal effects were clearly shown at the first cut, as the later tetraploids yielded significantly more DM than the diploids. This does, however, make it difficult to assert

which characteristic was most operable, lateness or tetraploidy. Ruby, Claret and Milvus all flowered on the same day, whilst Astred flowered two days later. Maro then flowered 5 days after Astred, and Amos 3 days after Maro. These small differences did not result in significantly different DM yields among early and late varieties, only between both groups. This implies the minutiae of phenological differences are not of great significance for DM production of the cut 1, and tetraploidy may be more influential. This is, however, difficult to say as the presence of weed populations at the first cut were also likely an influence on clover DM production.

RC is a slow-establishing crop (Verhallen *et al.* 2003, Iepema *et al.* 2006, Bjorkman and Shail 2010). This is likely caused by its need to establish a taproot and sufficient crown strength to produce aboveground biomass. Some authors suggest combining RC with other, faster-establishing legumes such as crimson clover, to offset this problem and reduce weed pressure (Rosenfeld and Francis 2015). The weed DM assessment at the first cut indicated weeds had been a problem, but whether this was caused by inherently slow RC establishment or unfavourably dry conditions following sowing remains unclear. Weed pressure during the initial growth period was likely partially responsible for the small DM yields observed for all varieties ($>1 \text{ t ha}^{-1}$), but the weed DM recorded for each variety did not appear to correlate well with clover DM, as the highest yielding variety (Maro), also yielded significantly higher weed DM than all other varieties.

N yields appeared to be correlated with DM yield. Despite yielding more DM than all the diploids, Amos yielded the same amount of N as all but one diploid, Astred. This property of high dry matter but low N content properties has previously been shown for other tetraploid varieties (Zuk-Golaszewska, *et al.* 2010). This property was, however not shown for Maro, the other tetraploid, which yielded more N than all other varieties. The longer vegetative growth period of Amos (compared to Maro) may have caused greater lignification of plant tissues, which would reduce the overall N content, but given the difference in flowering time was negligible (3 days), this seems unlikely. N yields across all varieties were on the smaller end of that predicted by the literature (Carlsson and Huss-Danell 2003, Huss-Danell, *et al.* 2007), indicating weed pressure and dry conditions at establishment may have been more of an influence than RC variety.

3.5.3 DM and N of Second Cut and Total (Sum of Both Cuts)

Weed biomass observed for all plots at cut 2 was low (>5% total biomass). This was deemed insignificant and not recorded. This supports the conclusions of Bilalis *et al* (2009) and Anderson (2015) that RC has a capacity to reduce weed populations during the regrowth period in response to cutting. This is likely caused by rapid regrowth and the exudation of allelopathic chemicals (Ohno, *et al.* 2000, Ohno and Doolan 2001), although it remains unclear which is most influential. The absence of weed pressure allowed most varieties to give higher yields of DM and N at the second cut than the first.

Amos was the only variety that failed to increase its yield of DM and N from the first to second cut. Field observations and morphological assessments indicated Amos failed to produce any stems following cut 1, which was likely the main factor unpinning its poor performance at the second. Amos, being the latest variety, was the last variety cut at cut 1. This meant the cut took place in the third week of August, a time of a 14 hour photoperiod in the UK. RC is generally thought of as a long day plant, requiring a photoperiod of >12 hours to switch from vegetative to reproductive growth (Julen 1977), however the key difference between early and late varieties is sensitivity to this requirement. Early varieties require less daylight and late varieties require more.

The apparent >14 hour photoperiodic requirement of Amos to initiate stem growth was notable. Similar results were found by Tanasch (1979), who described a critical photoperiod of 14 hours for late RC varieties. Maro was cut right before Amos at cut 1, meaning its regrowth period was marked by almost the same photoperiod. Maro gave the same yield of DM as the other highest yielding early varieties (Astred, Claret and Milvus), and subsequent morphological measurements indicated it also grew the same number of stems as these varieties. Although Maro is described as an intermediate variety, and observed as such in this experiment, it required the same short photoperiod to produce stems following cutting as the early varieties. This photoperiod has been documented as being ~10 hours by Puai *et al* (1982). Intermediate varieties, such as Maro, may then be the optimal choice for DM accumulation, as they may deliver higher initial yields at the first cut and maintain this during regrowth. The role of tetraploidy on photoperiodic sensitivity was investigated by Julen (1977) and found to be non-significant, indicating the differences observed in this experiment between early diploids and late tetraploids was more likely caused by phenology than tetraploidy.

Despite yielding the same amount of DM at cutting, Maro yielded significantly less N than the highest yielding diploids, but no significant differences were observed between Maro and the diploids for N when both cuts were summed. The total N accumulated was a statistically insignificant range of 75-83 kg ha⁻¹ for all varieties except Amos, which yielded a significantly lower 54 kg ha⁻¹. This was likely caused by its inhibited growth following cutting. These values of N accumulation in the aboveground biomass would be considered low when compared to the range documented in Tables 1.4-1.6. Total N values are also limited estimations of nitrogenase activity, as some of the N in the plant tissues will be derived from sources other than biofixation. The documented range of %N derived from atmosphere for forage legumes when other nutrient sources are removed is, however, 80-95% (Vallis *et al.* 1977, Cadisch *et al.* 1989). This range has also been reported for RC specifically (Thilakarathna *et al.* 2012b, Anglade *et al.* 2015a), but given cuttings were removed from the system, it is best to make inferences about contributions to soil fertility from the SMN accumulation data than that of the aboveground N accumulation.

3.5.4 Protein Content

Protein content in RC can be used to predict both forage value and crop performance. It is known to be influenced by environment, particularly soil type. For example, Jaksic *et al.* (2015) found RC protein is highest in soils with high levels of SOM, total N and phosphorus. Unfavourable soil pH can also affect nitrogenase activity in RC (Ferreira *et al.* 2012), which can reduce protein content. However, in soils where pH is neutral and nutrients are not strongly facilitative or limiting, growth stage is known to be the most important factor (Drobna and Jancovic 2006, Markovic *et al.* 2008, Grabber 2009, Vasiljevic *et al.* 2009). In the early stages of growth RC has a high proportion of leaves and high content of protein and moisture. This peaks at the budding stage, when growth shifts from vegetative to reproductive. Flowering causes morphophysiological changes including stem elongation, thickening, lignification and a halt in production of vegetative organs. This occurs concomitant with a reduction in protein content (Wiersma, *et al.* 1998). Optimal management for forage quality will, then, be to cut as the crop enters early bloom. Knowledge of this may also be useful in predicting optimal management in fertility-building leys, as the chemical parameters governing forage quality are also those governing green manure quality.

High protein percentage is correlated with low fibre proportion, which means more rapid mineralization when green manured. Conversely, low protein percentage is correlated with high fibre proportion and slower mineralization rates. The results from the protein content analysis indicated all varieties significantly increased their protein content ($P < 0.001$) from cut 1 to cut 2 (despite samples being taken at the same growth stage at both cuts). Vasiljevic *et al* (2009) and Azevedo *et al* (2013) also observed this temporal effect. This may have been caused by the more developed root system at cut, which allowed the crop to accumulate more N in the aboveground biomass and facilitate more protein synthesis. The value of RC cuttings as green manures may then increase in response to cutting, if of course a rapid rate of mineralization is considered valuable. Indeed, if a green manure were required for a slow growing or long season crop, like winter wheat, the green manure provided for the first cut may be more appropriate.

Varietal differences were also observed at both cuts, but as the literature tends to focus on age of stand rather than variety, there is little literature with which to compare these differences. Amos and Maro, the late and intermediate tetraploid varieties contained significantly less protein than the early diploids at both cuts. This contrasts with Bender (2012), who observed more protein in late varieties than early, albeit with different varieties trialled. Zuk-Golaszweski *et al* (2010) also contradicted these findings, observing higher protein contents for tetraploids than diploids. The influence of tetraploidy on increased protein content is well documented (Savatti *et al.* 1978, Krasnaya and Drobets 1979, Bender 2001, Kaldmäe *et al.* 2002), meaning the results observed here stand in contrast to the bulk of the literature. However, given how the varieties trialled in these studies are not specifically Amos or Maro, it may be their lower protein contents observed in this experiment result from physiological or climatic, rather than genetic differences.

3.5.5 Disease Assessment

The role of plant establishment was initially postulated as influential in determining severity of powdery mildew incidence, as high plant densities can exacerbate fungal diseases, particularly in monocultures (Jaccoud Filho *et al.* 2016). Astred and Ruby were the varieties which established at a lower plant density due to seed quality issues. Astred was among the least affected varieties whilst Ruby was the most infected. The variety with the highest density of establishment (Claret), was, along with Astred, among the least affected varieties. This

implies density of establishment was not influential in incidence of powdery mildew, and the differences observed for each variety were caused by only varietal differences.

With the exception of Ruby, tetraploids were shown to be significantly more susceptible to powdery mildew infection than diploids. Some researchers have also documented this (Pall *et al.* 1981) but others have found tetraploid varieties to be more resistant (Nagovitsyna 1989) to powdery mildew. As with protein content, it remains challenging to draw substantial conclusions about the role of ploidy in disease resistance because studies typically work with different RC varieties in different field and climate conditions, possibly even with different strains of the disease in question. Although some reports indicate the pathogenicity of powdery mildew has been increasing (Vincelli and Taylor 2003), it is still considered unserious by most authors (Mosjidis 2011). The characteristic downy covering of leaf surface area may decrease photosynthetic capacity and reduce yield, but as the fungus only occurs in the late summer, when the growing season finishes, its impact on yield is considered minimal.

Clover rot (*Sclerotinia* sp) is, however, considered a serious disease of RC (Taylor and Quesenberry 1996). Clover rot resistance is considered a key target in breeding programs (Abberton and Marshall 2005, Annicchiarico, *et al.* 2015), as crop losses to clover rot have been cited as a major factor in the decline of red clover cultivation following the Second World War (Vleugels *et al.* 2012). Its life cycle is given in Figure 3.12 As two distinct species of *Sclerotinia* infect RC, *Sclerotinia sclerotiorum* and *Sclerotinia trifoliorum*, and the appropriate molecular work to distinguish which one occurred in this trial was not undertaken, the disease is referred to here as *Sclerotinia* sp or clover rot.

Figure 3.12 Lifecycle of *Sclerotinia trifoliorum* (taken from Ohberg et al (2008))

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As Figure 3.12 indicates, the pathogenicity of clover rot is exacerbated by the production of sclerotia, small fungal bodies capable of overwintering and prolonging infection into the next growth season. The sclerotia population increases each year, meaning infections will become progressively worse if appropriate rotations are not applied. Some authors claim a 7 year rotation of non-sensitive plants is required to break the disease cycle (Williams and Spooner 1991). Significant differences in susceptibility may then be useful in predicting performance of two year leys following infection in the first year. Ruby and Astred were shown to be significantly more resistant to clover rot than all other varieties, but this may have been caused by their lower establishment density than varietal difference. This effect was not observed with powdery mildew, but the pathology of dry and wet fungal diseases may be influenced by population density in different ways. However, Claret was shown to be more resistant than Amos and Milvus, both of which established at lower densities, indicating density of establishment may not have been consequential. Tetraploids were shown to be more susceptible than the diploids Ruby and Astred, but not Milvus. This stands in contrast to most of the literature, which predicts tetraploidy incurred increased resistance to clover rot (see Table

1.11). Another valid measurement of resistance for clover rot is capacity to survive the winter period and produce a crop in the second year, but this can only be accounted for in the performance data of Core Experiment 2.

3.5.6 Soil Mineral Nitrogen

The vast majority of studies concerning the soil fertility-building of RC leys focus on fixed N accumulated in the aboveground biomass. Various methodologies exist for measuring this and a wide range of capacities have been reported. This range has been summarized in detail in Tables 1.4-1.6, along with the relevant weather and cropping information. The relevance of this data for antecedent soil fertility, particularly if uncoupled with SMN measurement and based on cuttings removed from the system, is questionable. N losses through denitrification and leaching may also reduce contributions to soil from cuttings even when mulched. Aboveground N measurements also do not account for belowground N contributions, i.e. the senescence of the RC taproot following termination, which can also be significant (Unkovich, *et al.* 2008).

Initial investigations into SMN accumulation following the growing season indicated variety and number of cuts had insignificant effects on SMN accumulation. An increase of 25-30 kg ha⁻¹ on the preliminary SMN measurement was observed, indicating RC cultivation did make a significant contribution to SMN. Although previous research found correlation between SMN accumulation and DM yield for RC (Halling and Scholefield 2001), this was not observed in this trial. The recorded SMN of ~40 kg ha⁻¹ is lower than that of Kayser *et al.* (2010), who recorded an accumulation of 60 kg ha⁻¹ for one year of RC cultivation on a similar clay soil. However, a DM yield of 3.3 t ha⁻¹ was recorded for this study. This smaller rate of accumulation in this trial was concomitant with lower yields (1.6 – 2.2 t ha⁻¹), which implies SMN accumulation may be correlated to aboveground DM yields.

The Repeated Measurement ANOVA indicated highly significant losses ($P < 0.001$) occurred over the winter period. This was likely exacerbated by the unusually warm and wet December weather (110mm precipitation and mean temperature 11°C – see Figures 2.1 and 2.2). This capacity for high levels of winter losses has been previously demonstrated for RC monocultures (Scholefield *et al.* 2002), even on clay soils, which are generally regarded to be less susceptible to nitrate leaching (Gaines and Gaines 1994). Treatments of variety and cut incurred highly

significant losses ($P < 0.001$). Ruby and Astred lost significantly less SMN than other varieties and the treatment of 1 cut lost significantly less SMN than 2 cut.

The varietal differences in SMN loss were most likely caused by differences in clover rot susceptibility. Astred and Ruby were significantly less susceptible to clover rot than all other varieties. This meant when the treatment of removing infected plant tissues (applied in December as a means to control the disease), there was far less infected tissue to be removed from these varieties. Field notes indicated Astred and Ruby exhibited mild incidence of clover rot, whilst other varieties were severely infected, and the aboveground biomass was reduced to a mass of black, necrotic plant tissues. The idea in removing these tissues was to prevent the disease spreading to the taproot and killing the plant (Davies 2015, Wilkinson 2015). More biomass was removed from Amos, Claret, Milvus and Maro, as more of it was infected. This removal meant less mineralized N released from senescing plant tissues could be returned to the soil, which meant less N was added to the soil over the winter period in these varieties.

This is, however, hard to say. The fate of mineralized N from these necrotic tissues would have been uncertain. A certain amount may have entered the soil as ammonium and nitrate, but some may also have been lost from the system through leaching or denitrification. Some more would have been lost in the metabolism of the fungus. These intricacies made assessment of N contributions from this source beyond the scope of this study, and so it remains uncertain if the removal of necrotic tissues had significant impact on SMN throughout the winter. The removal did result in a more exposed soil surface. This may have permitted higher rates of N losses, as the physical presence of a cover crop can mitigate such losses, particularly during wet winter periods (Tosti *et al.* 2014, Teixeira *et al.* 2016, Cooper *et al.* 2017). This effect is shown in the interaction between variety, cut and time.

No significant differences for cut were observed for Amos, Maro and Milvus at the Spring Assessment. The 1 cut treatment for Claret was significantly higher than the 2 cut, but was statistically the same as the treatments which lost the most (Amos and Maro at both cuts). The 1 cut value for Astred and Ruby was higher than the two cut, and higher than the 1 cut for all other varieties (this information can be viewed in Figure 3.7).

This means the effect of cutting on SMN retention was greater in Astred and Ruby than all other varieties. The effect of cutting was likely reduced in the other varieties through the removal of necrotic tissues, i.e. once the removal was complete very little biomass was left, regardless of whether the plot had been cut once or twice. Conversely, in Astred and Ruby,

healthy biomass remained following removal of necrotic tissue, and *more* of this biomass remained in the 1 cut plots, given these plots had received more regrowth time. It is likely this extra biomass provided more N through senescing plant residues and fortified the capacity of the cover crop to mitigate leaching through its physical presence on the soil surface. The significant differences in susceptibility to clover rot required different control strategies, which in turn impacted on the effect of both variety and cut on reductions in SMN over a warm and wet winter period.

The reduction in SMN following the winter period was apparent across both treatments. The range ran from 35% reduction (in Ruby 1 cut) to 50% reduction (in Amos 2 cut). This problem has been previously associated with legume monocultures (Campbell *et al.* 1994, Odhiambo and Bomke 2008) and with RC monocultures specifically (White *et al.* 2014). Companionship with a grass is seen as the appropriate means to mitigate this, as the winter growth and N demand can sequester mineral N and reduce losses from leaching (Mroczkowski *et al.* 1996). This may involve a trade-off, because although companion grasses can reduce vulnerability to winter leaching, their high N demand during the growing season may cause less SMN to accumulate in the first place. Which cropping strategy is optimal for SMN accumulation may then be context specific. For example in areas with soil or climate conditions prone to significant leaching mixtures may best, but monocultures may be preferable for areas without these concerns. Management of cut and mulch may be optimal for mixtures and cut and remove for monocultures. Areas in which disease pressure is not expected may be more suited for monocultures, but sites known to be at risk from disease incidence may require grass companions, as mixing clovers with grasses can reduce disease incidence and pressure (Korteweg and S'Jacob 1980)

3.5.7 Morphology

Variation among morphological traits for all varieties was observed at both cuts. Most traits appeared to change between cuts, and the CVA indicated relationships between the traits changed as well. Many studies documenting morphological variation in RC have observed a similar trend, albeit mainly in landrace and ecotype populations (Pagnotta, *et al.* 2011, Aygün and Olgun 2015, Chmelikova, *et al.* 2015, Meng and Zhao 2015). As many of these studies were undertaken in the fields of plant genetic resources and breeding, they mainly attempt to describe the variation among individuals, in order to classify them into groups. Studies concerning individuals already classified into groups are less common, but some are available (Tucak, *et al.* 2013, Van Minnebruggen, *et al.* 2015).

Despite being advertised as prostrate, Astred exhibited a growth habit that was significantly less prostrate than Maro, Milvus and Ruby at the cut 1. It then regrew to be significantly more prostrate than all other varieties at cut 2. This effect was also observed for Crossway, a different prostrate variety in a study documenting the relationship between architectural traits and regrowth in RC varieties (Van Minnebruggen, *et al.* 2015). Crossway is a variety produced by the same breeders and is considered to express a growth habit similar to Astred (Rumball, *et al.* 2003). Although significantly more prostrate than other varieties at the second cut, Astred was itself not very prostrate (a rating of 3.8 out of 9 was recorded). This confirmed the breeder's description; Astred will require a period of grazing to grow fully prostrate branches and root at the nodes (Stewart 2015), but regrowth following cutting in forage management can produce a weaker variant of this response.

Ruby produced the tallest plants and the most stems at cut 2. This did not seem to correlate with DM and N yields however, as here Ruby produced significantly less DM and N. Ruby, along with Astred, also regrew stems which were significantly hairier at cut 2. Some authors have also described positive correlations between hair density and powdery mildew resistance in RC (Naydenova and Georgiev 2013), but as Ruby and Astred had the same hair density but completely different rates of powdery mildew resistance, this research did not support this correlation.

The CVA for cut 1 indicated little variation among Maro, Milvus and Claret. Astred was shown to be the most variable, primarily because of higher values for hair density and stem number. Amos and Ruby differed from other erect varieties mostly on height and crown leaves; Ruby grew shorter with less crown leaves and Amos taller with more. Height and growth habit were negatively correlated, i.e. more prostrate plants grew shorter and vice versa. This trade-off has been previously documented by Aygun *et al.* (2015), and should be useful to breeders, as plant height has previously been used as a means to select good accessions in RC breeding programs. Plant height may not be a suitable means to select promising accessions in the breeding of prostrate varieties.

The CVA for the second cut also showed little variation among Maro, Milvus and Claret. Astred and Ruby were shown to be the most variable, and Amos was omitted due to limited regrowth. A positive correlation between growth habit and hair density, i.e. more prostrate plants were hairier and vice versa, was observed. The American variety 'Freedom!' is reported to grow erectly with hairless stems (Taylor 2008b), which gives support to the positive

correlation observed. But as it was not observed in the first cut for Astred, here it may be a response to cutting rather than an intrinsic genotypic trait. As stem hairs on forage legumes are associated with slower drying times and mineralization rates, these may have contributed to slower decomposition over the winter period and contributed to the significantly lower rates of leaching observed for Astred. Ruby also experienced lower rates of leaching, and was documented to be as hairy as Astred, which supports this hypothesis. Plant height and stem number are most commonly associated with DM yields (Pagnotta, *et al.* 2011, Chmelikova, *et al.* 2015), likely because the stems are where most of the DM lies for forage legumes. These traits were not good predictors for DM yield in this trial, particularly in the second cut, in which Ruby grew significantly taller with more numerous stems than all other varieties, but yielded less DM

3.6 Conclusions

- RC cultivation net accumulation $\sim 25 \text{ kg ha}^{-1}$ SMN in the first year of cultivation
- Variety and number of cuts were non-significant factors in this accumulation
- Astred and Ruby were less susceptible to winter leaching than all other varieties, but this was more likely caused by disease management than varietal difference
- 1 cut resulted in less SMN losses than 2 cuts
- RC monocultures are vulnerable to weed infestation at establishment, but are capable of suppressing these weeds in response to cutting
- Significant differences were observed for DM and N yields for RC varieties. Late tetraploids yielded more at the first cut, but early diploids yielded more at the second
- The intermediate tetraploid (Maro) yielded among the highest for DM and N at both cuts
- The regrowth of the latest variety (Amos) was significant following cutting by the unfavourable photoperiod
- RC varieties were highly morphological variable for all traits measured
- Plant height and stem number were not shown to be good predictors of DM and N yields
- Hair density may have contributed to resistance to leaching in some varieties
- Both positive and negative correlations were observed for some traits, but these changed in response to cutting

CHAPTER 4 - Core Experiment 2 – Red Clover Cultivation 2016

4.1 Introduction

RC is generally thought to be a short-lived, tap-rooted perennial with minimal capacity for vegetative reproduction (Taylor and Quesenberry 1996, Sturite and Lunnan 2015), although this may not always hold true for prostrate varieties and certain weather/soil conditions. The taproot can enter senescence after one growing season (Kendall and Stringer 1985) which may limit regrowth in the second year of cultivation. Breeding for improved persistence has been a longstanding target of all major RC breeding programs (Marshall, *et al.* 2012, Ortega *et al.* 2014, Suter *et al.* 2014, Annicchiarico, *et al.* 2015) and significant progress has been made, but knowledge of which varieties yield optimally in the second year will still be useful for farmers deciding which ones to grow. Additionally, as Chapter 3 has shown, yields of RC can also be limited by adverse weather conditions and weed pressure in the first year. Knowledge of which varieties respond best to these constraints in the second year may further optimize RC use.

Further investigation into root-soil dynamics may also further explain why different varieties, under different cutting management, yield more or less aboveground biomass, or contribute more to soil fertility. The number, position, colour and size of nodules growing on RC roots can be used to describe nodulation in RC (Rice, *et al.* 1977), and this can be measured by researchers as field trials proceed. Nodulation can be used to predict fertility-building capacity (Njira *et al.* 2013). Root biomass may also predict fertility-building capacity, as the senescence and mineralization of roots during subsequent cereal cultivation is an important component of the N contribution from RC cropping (Li *et al.* 1996, Unkovich, *et al.* 2010). These predictions may then be combined with rhizodeposited N (accounted for as the ammonium and nitrate values given in the SMN analyses) and the aboveground yields, to give an overall view of the performance and N contribution of the varieties trialled.

4.2 Objectives & Hypotheses

Core Experiment 2 sought to repeat the investigations of Core Experiment 1, with the added component of assessing nodulation and persistence. The objectives for Core Experiment 2 were:

- To trial a selection of RC varieties for soil fertility-building capacity
- To assess the influence of 1 and 2 cut management strategies on soil fertility-building capacity
- To document nodulation and root biomass of RC varieties
- To document DM and N yields of RC varieties
- To assess RC varietal morphological diversity

All objectives refer to RC in its second year of cultivation

The hypotheses for Core Experiment 2 were:

- Cutting management significantly effects DM and N yields of selected varieties
- RC varieties are morphologically diverse, as breeders describe
- Variety selection, cutting management and interactions between the two significantly effects the accumulation of soil mineral nitrogen
- RC varieties are diverse in capacity to nodulate

All hypotheses refer to RC in its second year of cultivation

4.3 Materials and Methods

Core Experiment 2 was a continuation of Core Experiment 1 and the same experimental design and plots were used. This was a completely randomized block design with four replications. Half of the clover plots from Core Experiment 1 were assigned to bioassay cultivation (see Chapter 5) and the remaining half were allowed to regrow and be studied for Core Experiment 2. Materials, methods and a field operations diary for Core Experiment 2 are listed in Section 2.4.2. A diagram of the field trial is given in Figure 2.5. Further information concerning the varieties trialled and reasons why these particular varieties were selected is given in Section 3.3.1.

The effect of the number of cuts from Core Experiment 1 was initially investigated in all statistical analyses of Core Experiment 2. This was done by including it as second factor and inputting it through the ANOVA. Number of cuts was found to be highly non-significant in all cases ($P > 0.4$) and results for this treatment have accordingly been omitted from results reported here.

The nodulation assessment was undertaken on individual plants gently excavated with a garden fork. The field trial site was clay and the soil surrounding the roots of the individual plants was initially difficult to remove without damaging the root system. To address this all individual plants were left to dry in the sun for a few hours prior to soil removal. This allowed the soil to gently crumb away and expose the root system so nodules could be examined. Nodules were counted and root DM was calculated as the weight of the individual root systems following cleaning and drying at 105°C for 24 hours.

4.4 Results

4.4.1 DM t ha⁻¹ and N kg ha⁻¹ (First and Second Cut)

Significant differences for both DM and N were observed at both cuts. The later varieties yielded significantly more than the early varieties at the first cut (with the intermediate flowering Maro yielding significantly more than the late Amos). Ruby yielded significantly less DM than the other early varieties, for which no differences were observed. A similar pattern was observed for N at the first cut, although no difference was observed between Amos and Maro.

Ruby gave the highest yield at the second cut, but this was equal to that of Milvus and Claret. Amos, Astred and Maro yielded the same as Milvus and Claret, but lower than Ruby. Ruby yielded significantly more N at the second cut than all other varieties, for which no differences were observed. Visual observations indicated weeds composed <5% of all biomass in all plots at both cuts. This was considered negligible and omitted from the yield assessments. This data is summarized in Table 4.1

Table 4.1 DM and N yield (Cuts 1 and 2)

Variety	First Cut		Second Cut	
	Clover DM t ha ⁻¹	Clover N kg ha ⁻¹	Clover DM t ha ⁻¹	Clover N kg ha ⁻¹
Amos	8.70b	330.0a	4.06b	159.7b
Astred	6.07c	236.7b	4.51b	183.0b
Claret	6.28c	217.5bc	4.93ab	184.0b
Maro	9.74a	351.3a	4.32b	177.1b
Milvus	6.40c	232.5b	5.22ab	179.5b
Ruby	5.00d	191.6c	6.37a	231.9a
<i>S.E.D.</i>	0.48	17.22	0.49	19.81
<i>df</i>	135	135	63	63
<i>P</i>	<0.001	<0.001	<0.001	0.017

Values followed by the same letter do not differ significantly at cited P value

4.4.2 DM t ha⁻¹ and N kg ha⁻¹ (Total)

Maro and Amos yielded significantly more DM and N than all other varieties when both cuts were summed. No significant differences were observed between Amos and Maro for both DM and N. Amos yielded the same as all other varieties. A range of 399-544 kg ha⁻¹ of N was reported and the significant differences between varieties were the same as that of DM yield. This data is summarized in Table 4.2

Table 4.2 DM and N yield (Total)

Variety	Total (Sum of Both Cuts)	
	Clover DM t ha ⁻¹	Clover N kg ha ⁻¹
Amos	12.24ab	472.0ab
Astred	10.17b	403.3b
Claret	11.61b	399.5b
Maro	14.66a	544.1a
Milvus	11.59b	405.5b
Ruby	11.37b	414.7b
<i>S.E.D. (63 df)</i>	0.74	27.12
<i>P</i>	<0.001	<0.001

Values followed by the same letter do not differ significantly at cited P value

4.4.3 Protein Content

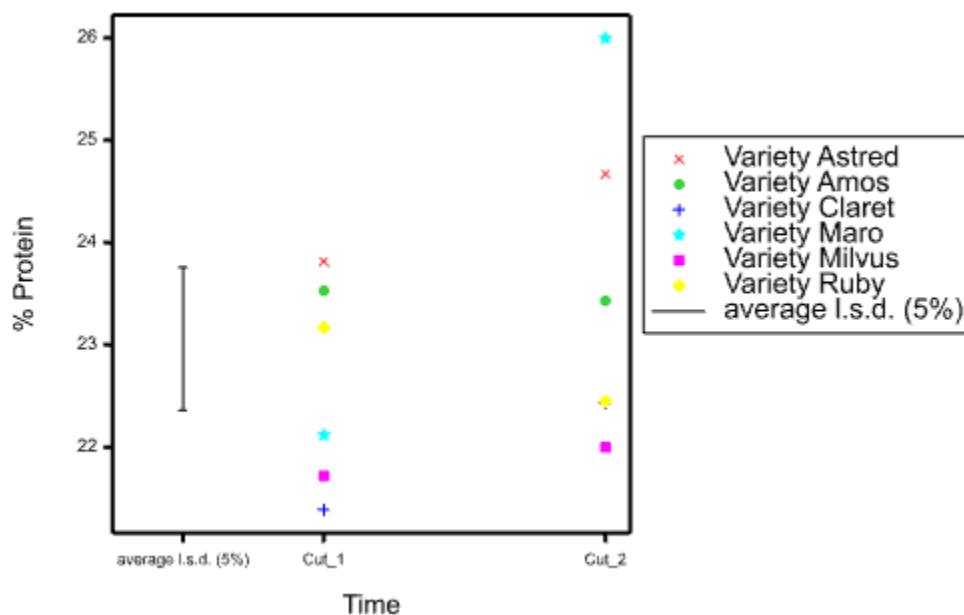
The late tetraploids Amos and Maro, along with Ruby and Astred, contained the most protein at the cut 1. This general trend was also observed at the second cut, except for Ruby, which contained significantly less protein here. This data is summarized in Table 4.3. Further analysis was undertaken using the Repeated ANOVA function to incorporate the temporal element. This was done using Cut 1 and Cut 2 as the times the repeated measurements were taken. Maro was the only variety to significantly increase its protein content between cuts. Astred and Maro at the second cut contained significantly more protein than Claret and Milvus at the first cut. These data is summarized in Figure 4.1

Table 4.3 Protein content of RC cuttings (both cuts)

Variety	Cut 1 Protein (%)	Cut 2 Protein (%)
Amos	23.5ab	23.4ab
Astred	23.8a	24.7ab
Claret	21.4c	22.4b
Maro	22.1abc	26.0a
Milvus	21.7bc	22.0b
Ruby	23.2abc	22.5b
<i>S.E.D. (39 df)</i>	0.51	0.85
<i>P</i>	<0.001	<0.001

Values followed by the same letter do not differ significantly at cited P value

Figure 4.1 Effect of interaction between time and variety on protein content (Repeated ANOVA)



4.4.4 Root Biomass and Nodule Count

Root Biomass and nodulation was measured in early August, just before the second cut. Amos, Maro, Claret and Milvus grew significantly more nodules than Ruby, whilst Ruby and Astred had significantly smaller root systems than all other varieties, for which no significant differences were observed. This data is summarized in Table 4.4. A photograph of a RC taproot showing nodules on adventitious roots growing from the crown region is given in Figure 4.2.

Table 4.4 Nodule number and root biomass

Variety	Nodule Number ¹	Root Biomass (DM g) ¹
Amos	64.7ab	3.8a
Astred	45.6b	2.0b
Claret	57.9ab	4.1a
Maro	86.2a	4.6a
Milvus	70.3ab	5.8a
Ruby	20.4c	1.8b
<i>S.E.D. (87 df)</i>	1.19	1.2
<i>P</i>	<0.001	<0.001

1. Data has been log transformed to reduce heterogeneity of variance. Reported data has been back-transformed using the antilog function.

Values followed by the same letter do not differ significantly at cited P value

Figure 4.2 RC taproot showing nodulation on adventitious roots (Variety Maro)



4.4.5 Disease Assessment

No incidence of powdery mildew was observed during Core Experiment 2. An incidence of clover rot was observed during Core Experiment 2, and this was recorded according to the same protocol as Core Experiment 1 (see Section 3.4.5.2). Following infection it was hypothesized that the number of cuts may be a significant factor in incidence of clover rot. Scoring was carried out six weeks after the second cut had finished in order to accommodate the number of cuts as a factor in the analysis. A two factor ANOVA in completely randomized blocks was then carried out, with variety and cut as factors. No significant differences were observed between varieties for infection, but 2 cut plots had significantly less infection than 1 cut plots ($P < 0.001$). No significant interaction was observed between variety and cut. This data is summarized in Table 4.5

Table 4.5 Effect of variety and cut on *Sclerotinia* disease index

Variety	Disease Index*
Amos	177.5ns
Astred	178.8ns
Claret	167.5ns
Maro	168.8ns
Milvus	175.0ns
Ruby	202.5ns
<i>S.E.D. (33 df)</i>	16.81
<i>P</i>	0.359
Cut 1	229.2a
Cut 2	127.5b
<i>S.E.D. (33 df)</i>	9.71
<i>P</i>	<0.001

*Higher score indicates higher infection

Values followed by the same letter do not differ significantly at cited P value

4.4.6 Soil Mineral Nitrogen

No significant differences among varieties were observed for SMN accumulation across all measurements throughout the growing season (June 2016-February 2017). Variety was also shown to be non-significant before and after the winter period (August 2016-February 2016). Number of cuts was also non-significant at June and August assessments, but 1 cut accumulated

more SMN at the February 2017 assessment. Leached SMN was also shown to be significantly higher for 2 cut than 1 cut at this assessment. This data is summarized in Table 4.6.

Table 4.6 Soil Mineral Nitrogen Accumulation Core Experiment 2

Variety	June 2016	August 2016	February 2017	Leached ¹
Amos	18.7ns	23.6ns	17.6ns	5.8ns
Astred	17.7ns	20.4ns	17.3ns	2.5ns
Claret	18.9ns	20.8ns	18.8ns	2.0ns
Maro	20.3ns	21.6ns	17.7ns	2.6ns
Milvus	17.2ns	23.0ns	19.6ns	3.3ns
Ruby	16.7ns	21.7ns	16.7ns	5.0ns
<i>S.E.D. (33 df)</i>	1.95	3.10	1.60	2.53
<i>P</i>	0.295	0.657	0.361	0.411
1 Cut	18.2ns	21.0ns	18.9a	1.4b
2 Cut	18.3ns	22.7ns	17.0b	5.6a
<i>S.E.D. (33 df)</i>	1.06	1.8	0.9	1.46
<i>P</i>	ns	0.392	<0.001	<0.001

1. Leached SMN calculated as the difference between August 2016 and February 2017

Values followed by the same letter do not differ significantly at cited P value

Further analysis was undertaken using the Repeated ANOVA function to incorporate the temporal element. This was done using the times at which the repeated measurements were taken. Time was shown to be a highly significant factor ($P < 0.001$). Interactions between time and variety and time and cut were also highly significant, and the interaction between all three was significant ($P < 0.05$) as well. These interactions are illustrated in Figures 4.3 - 4.5.

Varieties with the lowest SMN levels in June (Milvus and Ruby) were the only varieties to significantly increase SMN between the June and August assessments (Figure 4.3). Maro was the only variety not subject to significant losses over the winter period (Figure 4.3). The treatment of cut was non-significant throughout the growing season, but 1 cut had significantly more SMN than 2 cut at the February assessment (Figure 4.4). This implies 1 cut was less susceptible to losses than 2 cut. The influence of cutting management on susceptibility to winter losses was seen most prominently in Astred, Milvus and Maro (Figure 4.5).

Figure 4.3 Effect of interaction between time and variety on SMN accumulation (June 2016 – Feb 2017 measurements from Repeated ANOVA)

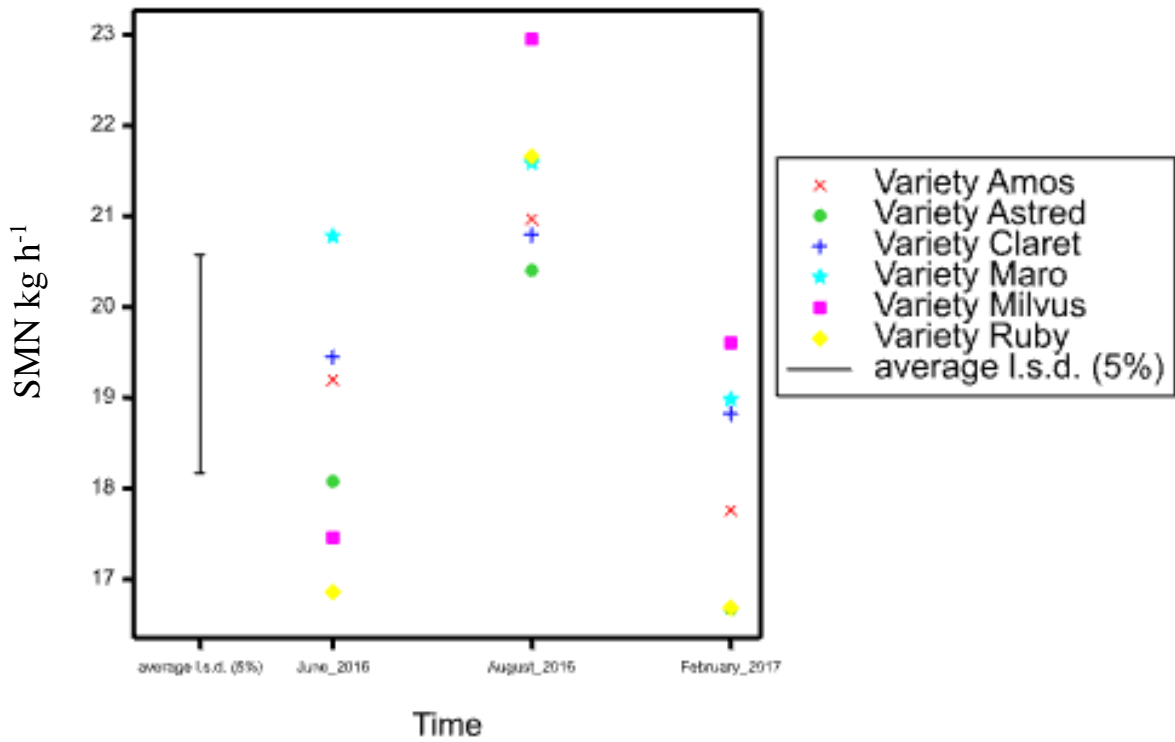


Figure 4.4 Effect of interaction between cut and time no SMN accumulation (June 2016- Feb 2017 measurements from Repeated ANOVA)

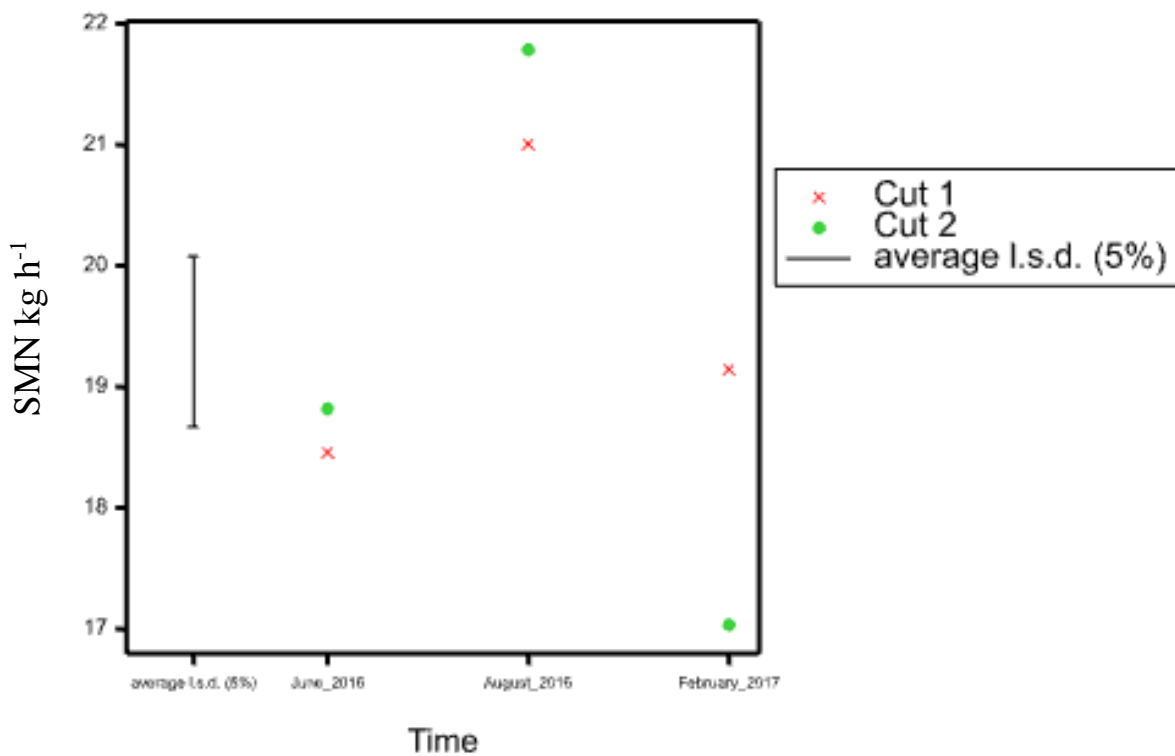
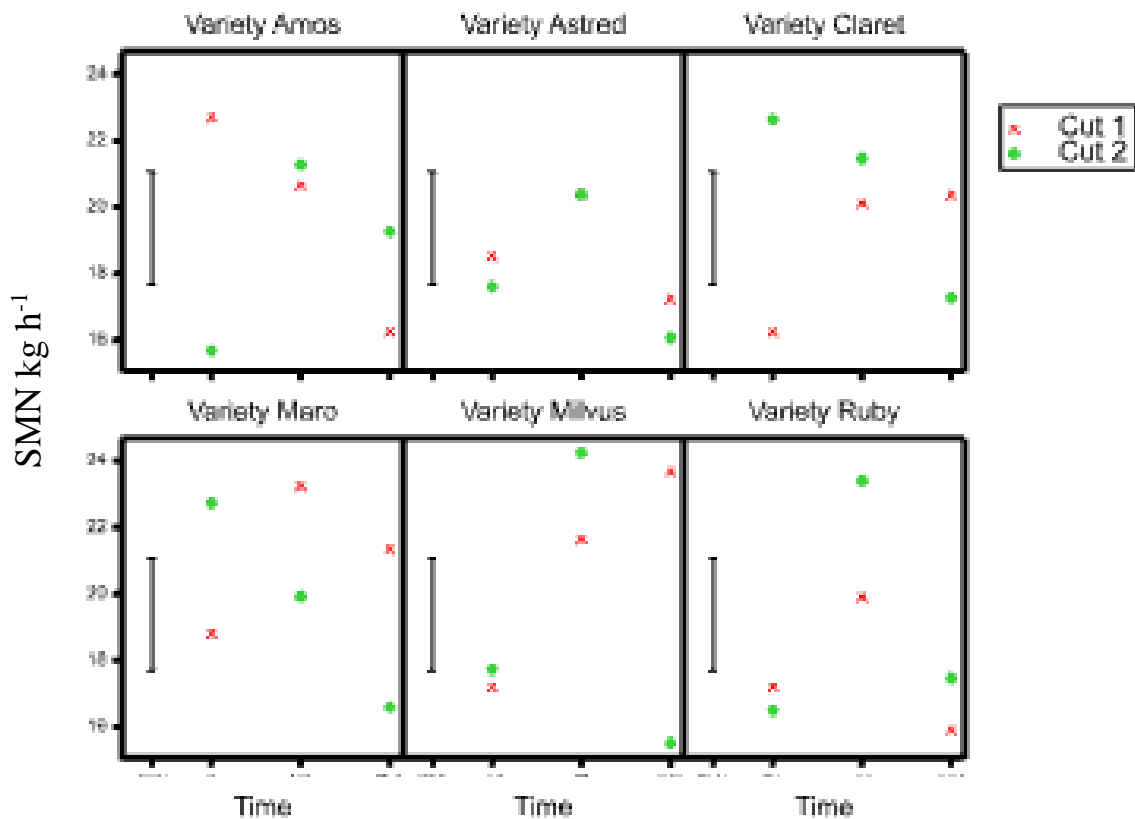


Figure 4.5 Effect of interaction between variety, cut and time on SMN accumulation (June 2016 – Feb 2017 measurements from Repeated ANOVA)



4.4.7 Morphology

4.4.7.1 ANOVA

Cut 1

Significant differences were observed for all morphological traits at the first cut, apart from crown leaves. Astred was significantly more prostrate than all other varieties and, along with Ruby was significantly hairier. The tetraploid varieties Amos and Maro grew significantly taller than the diploids, but produced significantly less stem leaves. Significant differences were also observed for stems and nodes. This data is summarized in Table 4.7.

Cut 2

Cutting dates were earlier in Core Experiment 2 than in Core Experiment 1. This allowed Amos to regrow stems in Core Experiment 2 and its morphological variation is described along with all other varieties at cut 2 in Table 4.8. Astred was shown to be significantly hairier and more prostrate than all other varieties, for which no significant differences were observed in growth habit or stem hairs. As in cut 1, Astred and Ruby produced significantly more stems than all other varieties, but no significant differences were observed in number of nodes. Amos and

Maro again produced the tallest plants, but no significant differences were observed between them and Claret. Amos produced less stem leaves than all other varieties and no significant differences were observed in number of crown leaves. This data is summarized in Table 4.8

Table 4.7 Summary of morphological variation (Cut 1)

Variety	Hair Density (n) ¹	Growth Habit (n) ¹	Height (cm)	Stems (n)	Nodes (n)	Stem Leaves (n) ²	Crown Leaves (n)
Amos	2.0c	1.1b	90.3a	3.9ab	4.6ab	25.5b	5.0ns
Astred	3.7a	4.8a	73.8bc	5.4a	5.2a	46.9a	5.6ns
Claret	2.1c	1.1b	74.9bc	4.4ab	4.6ab	32.6ab	6.5ns
Maro	2.3bc	1.1b	85.3ab	3.4b	4.3bc	22.1b	5.9ns
Milvus	2.4bc	1.3b	71.1c	4.6a	4.0bc	30.2b	6.6ns
Ruby	3.2ab	2.2b	56.4d	4.9a	3.7c	30.6ab	7.0ns
<i>S.E.D.</i> (135 df)	1.1	0.33	3.3	1.1	0.25	1.1	1.0
<i>P</i>	<0.001	<0.001	<0.001	0.006	<0.001	<0.001	0.179

1. Data has been log transformed to reduce heterogeneity of variance. An arbitrary constant of 1 was added to avoid zeros (values of 1 were commonly reported and $\log 1=0$). Reported data has been back-transformed using the antilog function.

2. Data has been log transformed to reduce heterogeneity of variance. Reported data has been back-transformed using the antilog function.

Values followed by the same letter do not differ significantly at; * = $P<0.05$, *** = $P<0.001$, ns = non-significant

Table 4.8 Summary of morphological variation (Cut 2)

Variety	Hair Density (n) ¹	Growth Habit (n) ¹	Height (cm)	Stems (n)	Nodes (n)	Stem Leaves (n) ²	Crown Leaves (n)
Amos	2.6b	2.0b	76.9ab	2.9b	4.5ns	28.6b	3.8ns
Astred	4.4a	4.3a	56.8d	4.0ab	4.7ns	40.4ab	3.4ns
Claret	2.5b	2.0b	78.1a	3.4b	5.0ns	34.3ab	2.6ns
Maro	2.8b	2.0b	77.3ab	3.1b	4.5ns	29.1ab	4.2ns
Milvus	2.2b	2.1b	68.4bc	3.3b	4.9ns	26.1b	3.5ns
Ruby	2.7b	2.3b	64.1cd	5.3a	4.4ns	46.4a	2.7ns
<i>SED</i> (135 df)	1.1	1.1	2.7	1.1	1.0	1.2	1.3
<i>P</i>	<0.001	<0.001	<0.001	<0.001	0.166	<0.001	0.216

1. Data has been log transformed to reduce heterogeneity of variance. An arbitrary constant of 1 was added to avoid zeros (values of 1 were commonly reported and $\log 1=0$). Reported data has been back-transformed using the antilog function.

2. Data has been log transformed to reduce heterogeneity of variance. Reported data has been back-transformed using the antilog function.

Values followed by the same letter do not differ significantly at; * = $P<0.05$, *** = $P<0.001$, ns = non-significant

4.4.7.2 Canonical Variate Analysis

All variates in the morphological assessment were subjected to CVA using variety as the group factor. More information on this multivariate analysis technique can be found in Section 3.4.7.2. This analysis was done individually for cuts 1 and 2. Results are presented graphically here in Figures 4.6 - 4.9.

Cut 1

The CVA on all morphological variates in Cut 1 determined 94.8% of the variation could be explained by the first two canonical variates. This analysis was then determined appropriate for visual representation in scatterplot form. These graphs are given in Figures 4.6 - 4.7

Figure 4.6 Scatterplot of CVA of First Cut

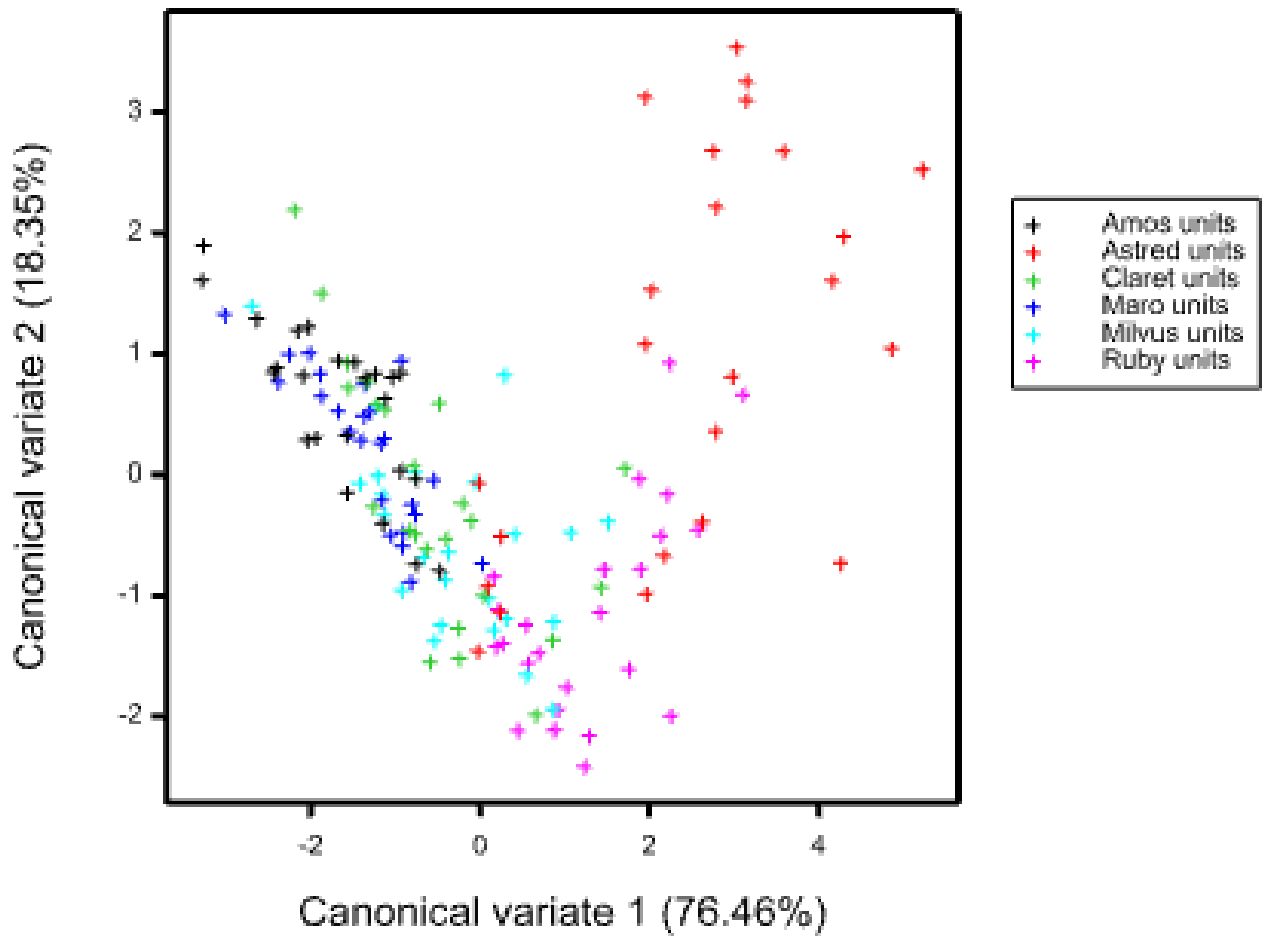


Figure 4.6 illustrates the total variation described by the first two canonical variates for cut 1. Astred and Ruby are shown to be the most variable, as they occupy the most space and extend into sections of the graph which contain no other data points. Little variation exists for Amos, Maro, Claret and Milvus. A biplot version is given in Figure 4.7.

Figure 4.7 Biplot of CVA of First Cut

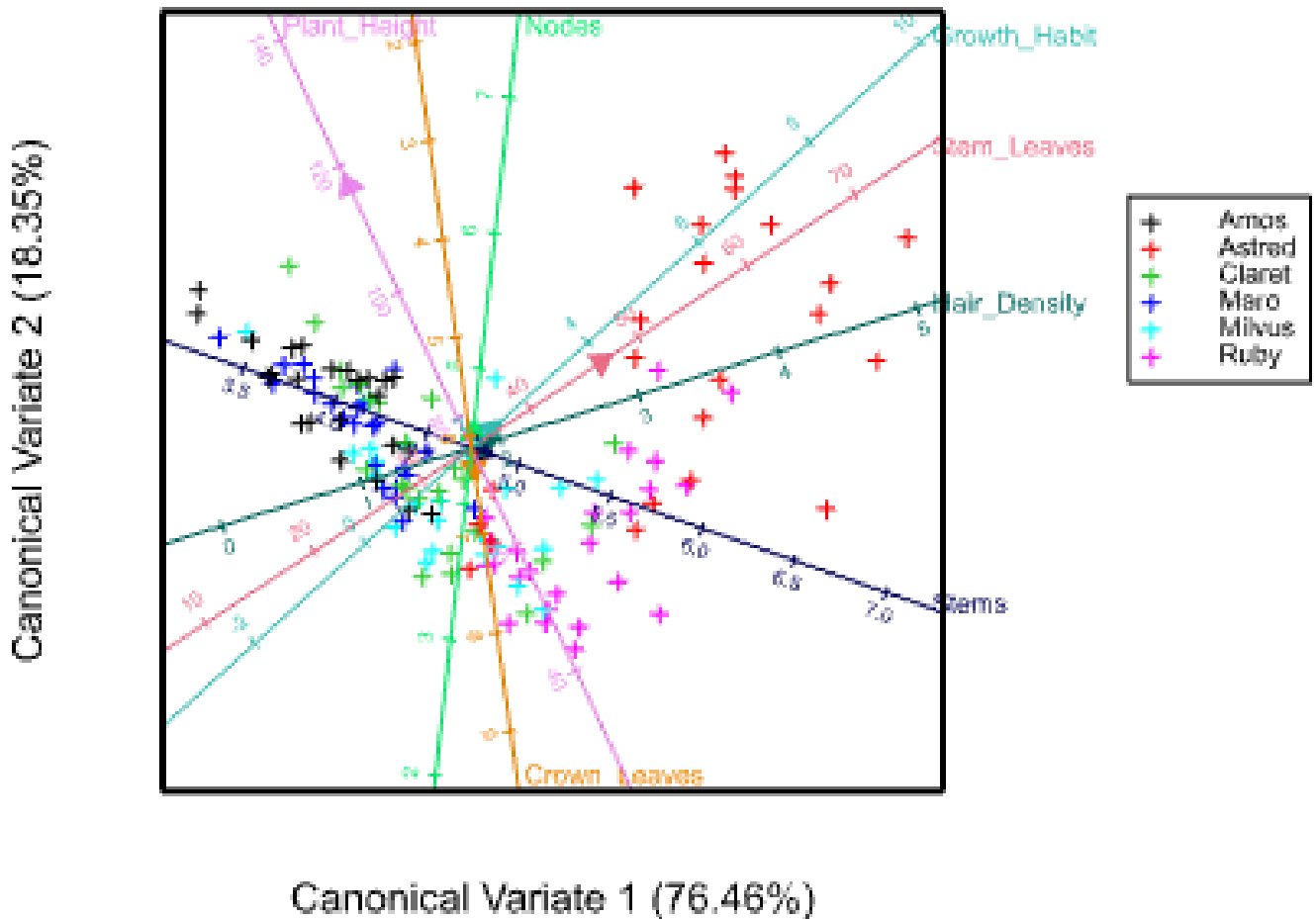


Figure 4.7 shows Astred and Ruby were much more variable in growth habit, stem leaves and hair density than Amos, Maro Milvus and Claret. The biplot shows variation among these varieties was caused by differences in stem number and plant height, although these differences are smaller than those of Astred and Ruby. Small vector angles indicate correlations between growth habit, stem leaves and hair density. The vector directions of crown leaves and plant height/number of nodes indicate these traits were negatively correlated.

Figure 4.8 Scatterplot of CVA Second Cut

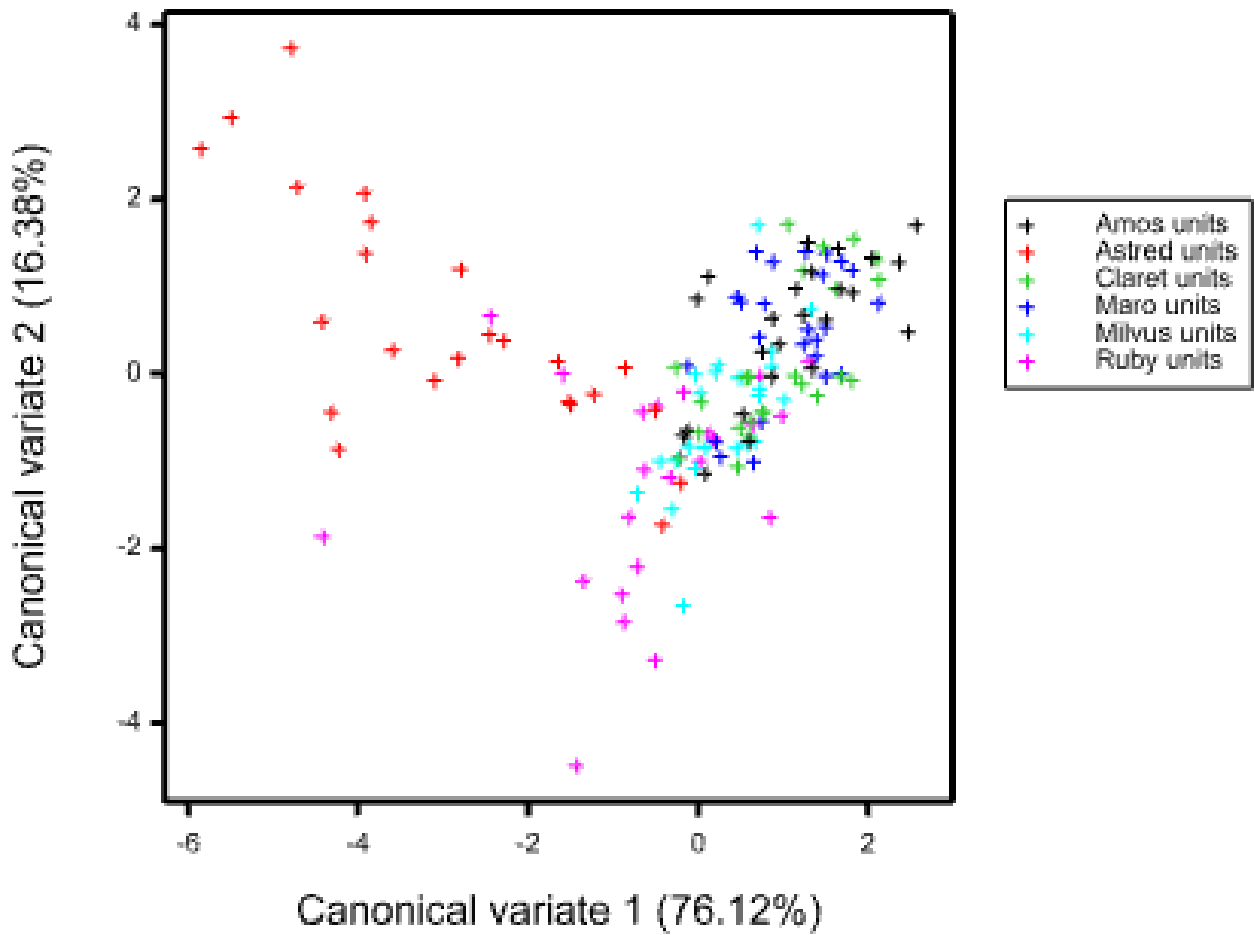


Figure 4.8 illustrates the total variation described by the first two canonical variates at cut 2. Astred and Ruby are again shown to be the most variable. The cluster of Amos, Claret, Maro and Milvus in the same section of the graph indicates little variation exists among these varieties. A biplot version of this graph is given in Figure 4.9.

Figure 4.9 Biplot of CVA Second Cut

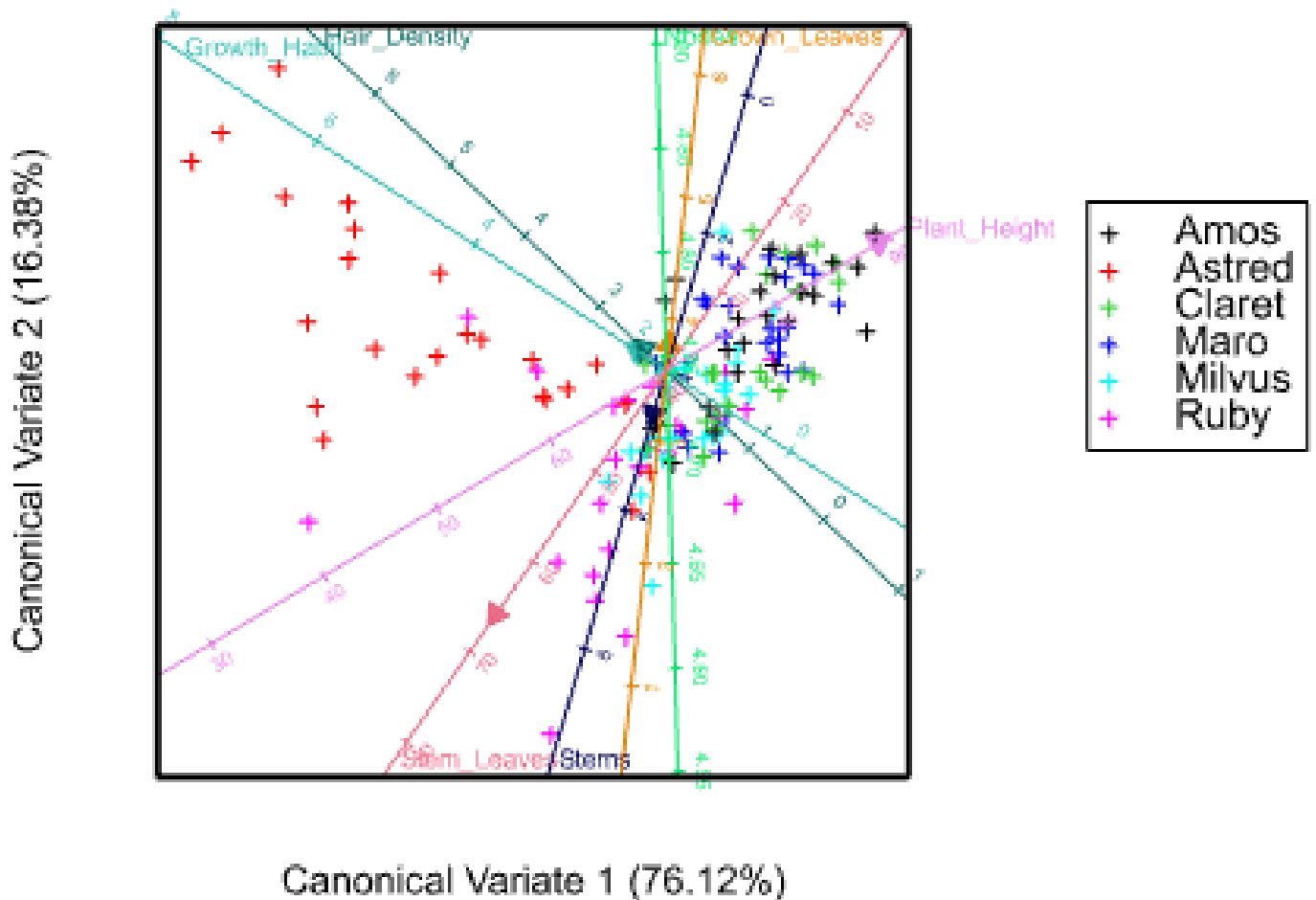


Figure 4.9 shows Astred and Ruby were much more variable in growth habit, stem leaves and hair density than Amos, Maro Milvus and Claret, as was the case at cut 1. At cut 2, plant height was also a cause of significant variation in Astred. Growth habit and hair density were again shown to be correlated. Crown leaves and nodes were positively correlated with each other and negatively correlated with number of stems and stem leaves. Again little variation was observed among Amos, Maro, Milvus and Ruby, but what variation did occur was mostly caused by plant height.

4.5 Discussion

4.5.1 DM and N yield (Cuts 1 and 2)

DM and N yields at cut 1 were higher than that of Core Experiment 1. The absence of weed pressure, prior root establishment and longer growth period were likely the main contributors to this improvement. As with Core Experiment 1, later tetraploids outperformed early diploids for both DM and N yields. However, Maro yielded significantly more DM and N than Amos at cut 1 ($P < 0.001$), whereas no significant differences were observed for Maro and Amos the preceding year. Amos crowns may have been more significantly damaged by the clover rot infection of the previous season, as this variety was significantly more susceptible to clover rot infection (see Table 3.11). Severe crown infection is capable of progressing to the taproot (Ohberg, *et al.* 2008, Lowe *et al.* 2014), which can damage capacity for regrowth the following season.

Disease incidence in perennial crops is complicated and severity of infection could equally be determined by regrowth capacity, or reduction thereof, during the subsequent years. This concept is known as ‘The Ghost of Disease Past’ by some researchers (2005) and is known to be problematic RC leys infected with clover rot (Yli-Mattila *et al.* 2010). Monocultures are also known to be more vulnerable to this disease than grass mixtures (Harper 1990). Amos was the most susceptible to clover rot in Core Experiment 1 but its DM and N yields were significantly higher than that of more resistant varieties at cut 1 in Core Experiment 2, which implies the Ghost was not influential in the regrowth of the following year. This is corroborated by the yields of Ruby in Core Experiment 2, which were lower than all other varieties despite being scored as the most clover rot resistant variety the previous year.

Prior establishment and faster growth in Core Experiment 2 caused earlier flowering dates, which in turn required earlier cutting dates. The earlier date of cut 1 (Mid-June instead of Late July) allowed for more significant regrowth. This was particularly important for Amos, the latest flowering variety, as it granted the required photoperiod for stem formation in the regrowth. This allowed it to achieve the same DM and N yields as the early varieties Astred, Claret and Milvus at cut 2. Claret, Milvus and Ruby were the highest DM yields, and Ruby yielded more N than all other varieties ($P < 0.001$).

When the total DM was calculated Maro yielded 14.6 t ha^{-1} , significantly more than all other varieties, which all yielded a statistically non-significant range of $10\text{-}11 \text{ t ha}^{-1}$. This was likely caused by its capacity to yield more than early varieties at cut 1, but then regrow with similar

vigour to early varieties, whose yields at cut 1 are limited by more rapid onset of flowering. Total DM yields were comparable to the findings of Marley *et al* (2013a) and Elsaesser *et al* (2016), who both documented DM yields of 10 t ha⁻¹ in the second year of cultivation, and Nagibin *et al* (2016) who documented 15 t ha⁻¹. However, disease issues may still have restrained total DM yields, as some authors have reported significantly higher figures for total yields in the second year. For example, Alibegovic *et al* (2010) reported yields of 19 t ha⁻¹ and Tucak *et al* (2013) reported a range of 18-25 t ha⁻¹. Marshall *et al* (2012) reported 21 t ha⁻¹, all in the second year of cultivation. These higher yields may still not be indicative of the true potential, as RC breeding programs are less established than cereals, its role in global agriculture is less significant, and there exists no theoretical yield ceiling data in the literature.

N yields for both cuts were comparable to the higher end of range reported in the literature (summarized in Tables 1.4-1.6). No significant differences were observed between Amos and Maro, but these varieties remained the highest yielding. From this data it is not possible to state what percentage of this N was derived from biofixation and what percentage from the soil. The SMN data from February (before spring growth had begun) and June (when cut 1 was carried out) showed SMN decrease in some varieties, particularly in Astred and Ruby (see Tables 3.12 and 4.6). These decreases were, however, very small (<8 kg ha⁻¹) when compared to the total N yields (>300 kg ha⁻¹). The SMN available for plant growth was consistently far less than the N yields observed at both cuts, indicating most of the N yielded in the aboveground biomass was derived from biofixation.

4.5.2 Protein Content

Protein content at cut 1 was higher than that of Core Experiment 1. This was likely caused by prior establishment and a more favourable spring growing season (February and March received significantly more rainfall than the long term average and no dry periods occurred in April or May (see Figures 2.1-2.2)). Late tetraploids had among the highest protein contents at both cuts, and Maro was the only variety to significantly increase its protein content from cut 1 to cut 2. This contrasts with the results of Core Experiment 1, in which the late tetraploids had significantly lower protein contents at both cuts, but corroborates the findings of Bender (2001, 2012), Savatti *et al* (1978) and Kaldmae *et al* (2002), who described tetraploid varieties as containing more protein. More favourable growing conditions may then be required for tetraploidy to incur increased protein content. The significant increase in protein content in

Maro implies this variety would provide a more nutritive forage than the early diploids at cut 2. The green manure properties of Maro may also differ from that of the early diploids in the second year of cultivation. The higher protein content can result in more rapid mineralization, meaning the green manure of Maro may be more appropriate for winter-sown preceding crop, whereas the green manure of Ruby or Milvus, with lower protein (and therefore slower mineralization) may be more appropriate for a spring-sown preceding crop.

4.5.3 Root Biomass, Nodule Count

Nodulation assessments can be used to evaluate nitrogen fixation by nodulated legumes under field conditions (Peoples 2015) and are useful tools in assessing productivity and fertility-building in legume cropping. The research of Rice (1977) on nodulation in RC and alfalfa provided the framework for the nodulation assessment in Core Experiment 2. This paper outlines four key areas of interest; nodule number, size, colour and position. Various scores are then attributed to each parameter and a total score is granted. The logic underpinning such a system is well documented. For example, RC and the entire *Trifolium* genus nodulate indeterminately (Blauenfeldt *et al.* 1994). This means nodules occur with meristematic tissue at the tip and continuously grow out from roots in a cylindrical shape until senescence takes place. The size of the nodule will then impact upon rates of fixation, as longer nodules typically harden and become sub-optimal fixers (Melino *et al.* 2012). The optimal size for RC nodules is described by Rice (1977) as 3-10mm in length.

Nodule colour is also an important consideration. The nitrogenase enzyme operates best in anaerobic conditions, which are achieved via leghemoglobin-mediated buffering within nodules (Ampomah *et al.* 2012). Much like haemoglobin, leghemoglobin appears red in colour when bound with oxygen. Pinkish or red nodules are therefore associated with higher rates of fixation than green, white or brown ones, as the pinkish colour indicates leghemoglobin has reduced the oxygen levels so fixation may proceed (Lynd and Ansman 1991). Rice (1977) describes pink nodules as optimal, followed by white, then green and finally brown.

Nodule position is also considered important for RC specifically. The crown region (<5cm from the crown) is described as the optimal position (Rice, *et al.* 1977), as here fixed N can be more readily absorbed. N fixed in nodules located deeper than this may be more prone to losses via leaching. The taproot itself typically does not nodulate, probably because it is suberized and impervious to *Rhizobia* infection. This effect was clearly seen in the nodulation assessment

in this trial, in which nodules were found exclusively on adventitious roots. Rice (1977) described ‘crown’ nodules as optimal and non-crown as suboptimal. Nodule number is also important, as ‘the more the better’ is the general consensus within studies documenting nodulation (Unkovich, *et al.* 2008, Peoples, *et al.* 2012).

The assessment undertaken for this trial however found little use for many of these parameters. Nodules were exclusively between 3 and 10mm in length. Nodules occurring outside the crown region were so few they were considered negligible (1 or 2 nodules per individual plant assessed). A subtle range of colour was observed, from pinkish-white to pink. A negligible amount of nodules were white and none were green or brown. Significant differences were observed for nodule number, but as Rice (1977) describes >20 as optimal, this scale was deemed insufficient. Nodule counts were almost all >20. Some were in excess of 100. Recording the precise figure and subjecting results to ANOVA was considered the best way to report the differences in nodule number among varieties.

The Rice (1977) methodology was designed to document the effect of soil acidity in nodulation on RC and alfalfa, as increasing soil acidity can impact negatively on *Rhizobia*'s capacity to nodulate (Ferreira, *et al.* 2012), but the soil pH for this trial was determined to be near neutral at the beginning of the trial (see Table 2.5). This implies the nuance of the methodology described by Rice (1977) in assessing nodulation may be unnecessary when soil pH is not a limiting factor. The insufficiency of Rice's nodule number in this trial (almost all plants examined were well in excess of the nodule number considered optimal) is also notable. Dating from 1977, and lacking in varietal information, this study was at best conducted with old landrace populations, or even wild ecotypes. Nodulation on these older varieties may have been more limited than that of modern varieties. This means modern high-yielding varieties may have more developed root systems than older ones, and may be more effective in engaging the rhizobial relationships required for nodulation and biofixation.

The lack of significant differences in capacity to fix nitrogen (uniformity of active, pink nodules) was also notable. RC is known for its specificity in rhizobial relationships and will only form effective nodules with a specific species (Denarie, *et al.* 1992). It was initially postulated Astrad may better utilize an Australian subspecies, as the *Trifolium* genus is not found naturally in Australia (Brockwell, *et al.* 1995) and the *Rhizobia leguminosarum biovar trifolii* populations were introduced along with the genus in the 18th century. It is possible these populations changed over the centuries as adaptation to new soils and climate took place. RC

varieties bred in Australia may then be more effectively nodulated by Australian *Rhizobia* populations, but this effect was not observed in this trial, as Astred was found to nodulate to the same degree as several European varieties. Initial concerns about the capacity of Astred to form effective nodules therefore proved unfounded.

Nodule number and root biomass did appear to correlate with aboveground DM and N yields. The highest yielding variety for both of these assessments, Maro, also had the largest root biomass and highest number of nodules. Here, a more developed root system may have enhanced crop productivity and additional nodules may have provided additional fixed N. Maro also ranked among the highest protein percentages at both cuts, and was the only variety shown to significantly increase its protein content between cuts. This was also likely facilitated by its root biomass and nodule number, as these were measured right before cut 2. Ruby and Astred were also shown to have significantly less nodules and smaller root systems than other varieties, with Ruby growing fewer nodules than all others. The lower scores for these varieties, under these parameters, may explain their reduced yields of DM and N at cut 1.

This explanation could not be offered for cut 2. Despite growing the least nodules and possessing the smallest root system, Ruby and Astred yielded among the highest yielding varieties for DM, and Ruby yielded more N than all other varieties. Smaller root systems are predicted for prostrate varieties like Astred (Taylor and Quesenberry 1996), and smaller root systems will likely grow fewer nodules, but this does not explain how Ruby, an erect variety, could produce a significantly higher yield than all other varieties, with fewer nodules and a smaller root system. Cutting in forage legumes is known to incur relocation of root carbon stores to newly growing leaves and shoots (Volenc *et al.* 1996, Erice *et al.* 2011) and a senescence of nodules (Jarvis, *et al.* 1996), responses which may have occurred here. Marshall (2004) showed cutting in RC specifically can cause nodule senescence and reduction in root biomass. However, as all varieties in this trial were cut prior to the nodulation assessment, and this effect was not observed in Amos, Claret, Maro and Milvus, it seems unlikely this was the cause of the growth limitation in Astred and Ruby.

As the earliest variety, Ruby was the first to be cut at both cuts. Each variety cut took 3-4 days in Core Experiment 2. Ruby then received slightly more time to regrow than others, but the staggering of the flowering times over the early-late flowering spectrum meant all varieties were cut at roughly the same growth phase, which served to ameliorate the variation incurred by the time-consuming cut. Despite this, these few extra days of growth may have compensated

for Ruby's reduced root system (the difference in regrowth time for Ruby and Maro for example would have been 9-12 days).

Weather conditions during the regrowth period may also have played a role. Figure 2.1 indicates rainfall for August 2016 was comparable to the long term average (slightly dryer at 51mm compared to 62mm), but this rainfall was not evenly distributed throughout the month. Daily readings indicated 21.3mm fell on August 2nd, 3.1mm on August 3rd and then none until August 18th. Additionally, the heavy rainfall on the 2nd was preceded by an exceptionally dry July (27mm compared to long term average of 60mm) with the latter half of July having only three days of rainfall, all >1.5mm). The heavy rainfall at the beginning of August was perfectly timed for the regrowth of Ruby, and water availability at the initial stage of regrowth is crucial for productivity (Hofer *et al.* 2017). Water availability for the regrowth of the other varieties may then have been reduced (i.e. the heavy rainfall in early August may have been taken up by the crop prior to cut 2, leaving less water available for regrowth). This was not the case with Ruby, which was cut prior to the rainfall, allowing this water to be used for regrowth. This extra water availability may have compensated for the smaller root system and fewer nodules observed in Ruby.

4.5.4 Disease Assessment

No recurrence of powdery mildew was observed in Core Experiment 2. This may have been caused by weather conditions, as July and August were significantly drier, and this was the time at which powdery mildew had occurred the previous year.

Cutting is often performed to control disease outbreaks in forage cropping on organic farms (Caldwell *et al.* 2013). Following the outbreak of clover rot in Core Experiment 1, it was hypothesized that cutting would significantly impact on susceptibility to the disease (removal of the infection biomass breaks the disease cycle and new, uninfected tissue is allowed to grow). Assessment of the impact of the cut on disease susceptibility was however unsuitable for Core Experiment 1, as the disease was at a late stage when cut 2 was completed. No assessment could then be made for the 2 cut treatment and the disease assessment focused only on the 1 cut treatment. Although cutting in Core Experiment 2 occurred at the same phenological stage as Core Experiment 1, the calendar dates differed due to the faster growth rate caused by the prior establishment of Core Experiment 2. Cut 2 finished at an earlier date in Core Experiment 2 and six weeks was left for regrowth in order to include the cut treatment

as a factor in the clover rot assessment. Cutting was shown to be highly effective in controlling the disease as the 1 cut treatment gave a disease index which was significantly higher than 2 cut ($P < 0.001$).

Clover rot is considered a serious disease by most authors and significant yield reductions are expected in the subsequent year of cultivation, as the disease can damage taproots and curtail regrowth the following year (Vleugels *et al.* 2013a, Mikaliuniene, *et al.* 2015). This was not observed in Core Experiment 2. Yields significantly increased and were on par with those expected for the second year of cultivation in the literature. The disease management put in place (i.e. the fungicide application and removal of necrotic tissues) may have reduced the capacity of the disease to recur the following year. Modern plant breeding may also have been successful in creating modern varieties which are less susceptible to overwinter damage caused by clover rot, as resistance to this disease is cited as integral in ongoing breeding programs (Abberton and Marshall 2005, Annicchiarico and Pagnotta 2012).

In contrast to Core Experiment 1, variety was not significant factor in clover rot resistance. Ruby and Astred appeared to score a higher disease index in the second year of cultivation, which may have been caused by a reduction in capacity to resist the disease following infection in the previous year. This progression of worsening pathogenicity is cited in the literature (Bardin and Huang 2001) and is likely the reason clover rot incidence is so severe in RC cropping, but does not explain why the disease index for other varieties did not get significantly worse as well. It may be that only varieties with some degree of resistance suffer this worsening of disease incidence in the second year (Ruby and Astred), whilst varieties with severe infection in the first year do not worsen in the second.

4.5.5 SMN Accumulation

Variety and cut were non-significant factors in SMN accumulation throughout the growing season of Core Experiment 2. Ruby and Milvus were the only varieties to significantly increase SMN levels from June to August, and were then the most susceptible to winter losses (see Figure 4.3). The accumulation of $\sim 30 \text{ kg ha}^{-1}$ throughout the growing season of Core Experiment 1 was not observed and marginal increase ($\sim 5 \text{ kg ha}^{-1}$) were instead the case for all treatments. This effect was previously described by Bell (2017), who observed no significant increases in SMN during cultivation of a selection of forage legumes (closest to RC being lucerne) in soils where forage legumes had been previously cultivated. This was thought to be

caused by the high N demand of the forages trialled and the high levels of SMN which preceded cultivation. It was concluded forage legume cropping for the accumulation of SMN was optimal and most effective in soils deficient in SMN.

N yields were higher in Core Experiment 2 than Core Experiment 1 (range of 405-544 kg ha⁻¹ compared to 54-80 kg ha⁻¹, see Tables 4.2 and 3.6). Higher N yields in the Core Experiment 2 were more likely caused by the higher DM yields (range 10.17-14.66 t ha⁻¹ compared to 1.58-2.39 t ha⁻¹, see Tables 4.2 and 3.6) than N concentration, as observations from the CNS analysis indicated N concentrations were consistently ~4% across both years of cultivation. These high yields placed higher demand for mineral N from biofixation, but through facilitation by a larger root system, may have also taken up additional mineral N from the soil. The capacity for legume crops to contribute little or no SMN following cultivation is well understood and is commonly the case in grain legume cultivation (Giller 2001a). This is most associated with soybean cultivation, in which the crop receives 50-70% of N requirements from biofixation and the remainder from SMN (Dukic' *et al.* 2014). The high N demand of soybeans can even leave an SMN deficit, unless the stover is mulched. The lower N demand of forage legume seed means these crops are generally not associated with these effects, and they are reported as accumulating SMN throughout the cropping system (Anglade *et al.* 2015b).

However this was not observed in Core Experiment 2. N offtake in the aboveground biomass was high in all varieties and therefore SMN did not significantly increase as the growing season proceeded. The only varieties to significantly increase SMN between June and August were Ruby and Milvus, both among the lowest yielding varieties for total N. RC breeding programs tend to cite yield as a primary objective (Abberton and Marshall 2005, Annicchiarico, *et al.* 2015), but this may not be desirable for RC's soil fertility-building capacity if high N yields can *deplete* SMN. Of course, subsequent soil fertility should not be reduced to SMN levels, as many components of soil fertility and enhanced by legume cropping (improvements in soil structural properties, contributions to SOM etc) are non-N in nature. These components are generally enhanced by higher rates of root growth and may offset these reductions in SMN, but as N will always be the most limiting factor in agricultural productivity, management of legume crops grown for soil fertility should adjust accordingly.

If high N yields in the second year of cultivation are associated with no significant increases in SMN levels, it is clear optimal management should be to mulch at least some of these cuttings. This is further corroborated by the effect of cutting on susceptibility to losses in the winter

period. The treatment of 2 cuts lost significantly more SMN than 1, an effect was also seen in Core Experiment 1. No significant differences were observed for variety, which contrasted with Core Experiment 1, but this was likely caused by the lack of disease control treatment applied in Core Experiment 2. Less susceptibility to SMN losses was likely caused by the larger amount of soil-covering biomass present in the cut 1 treatment, a presence which can reduce leaching susceptibility (Ward *et al.* 2012). This effect may be further enhanced by mulching, which would further increase soil cover, but the mineralization of this mulch can also itself be susceptible to winter leaching (Loges *et al.* 2010). The use of RC in soil fertility-building in the second year of cultivation may be further optimized by mulching cuttings, but from these results it is difficult to assert which cut should be mulched, or even if both would be best. The increase in plant available N resulting from mid-season mulching can limit biofixation rates in the regrowth (Danso *et al.* 1991, Pietsch *et al.* 2009, Moyo 2014), as the crop limits the supply of sugars to rhizobia in response to the 'free' source of N. The extent to which this limitation on biofixation occurs however remains unknown, and it is therefore difficult to say if removing or mulching cut 1 would be best practice in RC cropping for soil fertility.

4.5.6 Morphology

Most variates assessed showed highly significant differences ($P < 0.001$). No differences were found for crown leaves at either cut and the number of crown leaves recorded in all cases appeared smaller than that of Core Experiment 1. This may be undesirable, as some authors cite the retaining of crown leaves over multiple years as a breeding target in lucerne (*Medicago sativa*) (Bristar 1991). This appears unlikely to have been the case here, given how the DM and N yields of Core Experiment 2 were so much higher than that of Core Experiment 1.

Astred continued to be significantly hairier and more prostrate than other varieties ($P < 0.001$) although no significant differences were observed for hairiness between Astred and Ruby at cut 1. These traits were shown to be positively correlated by the biplots of the CVAs for both cuts, correlations also observed for cut 2 of Core Experiment 1, but not cut 1. Astred and other prostrate RC varieties are known to become more prostrate in response to cutting and grazing (Hyslop *et al.* 1999) and it may be this response also causes an increase in expression of stem hairs. The increased expression of stem hairs in response to defoliation from either grazing or cutting would be a natural response for RC varieties, as stem hairs are generally associated with defence against insects (Schillmiller, *et al.* 2008). Some authors have demonstrated this

effect in lucerne (Elden and McCaslin 1997) but it remains unclear if RC does this as well. Stem hair density did not increase in other varieties, implying certain genes for stem hairs may be required for this progression to occur.

Amos produced stems at the morphology assessment at cut 2, indicating it had received the required photoperiod for stem initiation and concomitant regrowth. The significance of this was clearly shown in the DM and N yields of cut 2, in which Amos yielded the same as some early varieties (Astred, Claret and Milvus). Good regrowth in Amos was facilitated by the earlier date of cutting in Core Experiment 2 (Mid-June instead of Mid-August). This gave a photoperiod of 16 hours, well above the 14 documented by Tanasch (1979) as essential for stem initiation in late RC varieties. The biplot for cut 2 showed the regrowth of Amos to show very similar morphological variation to Maro, the other later-flowering variety. This comparison was not possible in the morphological assessment of Core Experiment 1.

4.6 Conclusions

- DM and N yields of all varieties greatly exceeded that of the previous year
- Late tetraploids yielded more DM and N at the first cut but early diploids yielded more at the second
- The intermediate variety Maro yielded the most DM and N when both cuts were summed. It was also the only variety to significantly increase protein content between cuts
- All varieties trialled successfully formed many effective nodules
- Ruby had less nodules and a smaller root system than all other varieties but this did not appear to impact on its DM and N yields at the second cut
- More favourable weather conditions prevented the powdery mildew infection from recurring
- No variety was more susceptible to clover rot but the 2 cut treatment was more effective at controlling the disease than 1 cut
- Contributions to SMN throughout the growing season were negligible compared to the previous year
- Only Ruby and Milvus significantly increased SMN between June and August
- The 1 cut treatment was less susceptible to leaching than the 2 cut treatment
- Varieties were morphologically diverse in all variates except for crown leaves
- Astred was consistently hairier and more prostrate than all other varieties
- Amos received the required photoperiod for stem initiation and yielded the same DM and N as other varieties as a result

CHAPTER 5 - Core Experiment 3 – Bioassay Cultivation 2016

5.1 Introduction

The accumulation of SMN was significant in Core Experiment 1, but negligible in Core Experiment 2 (see Tables 3.12 and 4.6). This implies these crops can make different contributions to subsequent soil fertility when cultivated for more than one year. These are important contributions to note, as available N will generally be the most limiting factor in subsequent crop production (Smil 2002). However, they do not account for the mineralization of the root system or spring-grown aboveground biomass. Nor do they account for increases in SOM, microbial activity, aggregation and porosity, all of which contribute to overall soil fertility and are associated with RC cultivation (see Table 1.9).

Contributions to soil fertility from these varieties may be best described in this overall sense, but this makes ‘soil fertility’ a broad term. Its meaning can also differ between the scientific and farming communities, which may cause confusion (Patzel *et al.* 2000). Strict definitions usually describe it as a synthesis of physical, chemical and biological processes which contribute to crop productivity (Stockdale *et al.* 2002), but modern definitions further stress the roles of SOM and microbial activity (Piutti *et al.* 2015). Soil fertility may also be described using management histories (Vanlauwe *et al.* 2010).

Cooke (1975) proposed a practical definition of soil fertility as its capacity to produce and sustain a desired crop. This definition may be most useful to both agronomists and farmers. Using it, it is clear SMN documentation alone cannot assess the fertility-building capacity of the RC varieties trialled. This assessment is best achieved through the cultivation of subsequent crops (known as bioassays), as the yields and N uptakes of these crops can then be used to account for the multidimensional nature of fertility-building with legumes. This a common strategy in the current literature on fertility-building (Alam *et al.* 2016, Hossain *et al.* 2016, Rose *et al.* 2016, St Luce *et al.* 2016, Plaza-Bonilla *et al.* 2017).

Different bioassay crops can fulfil different purposes in the assessment of subsequent soil fertility following legume cover crops. Cereal bioassays give N dependent parameters such as grain yield and protein, which are influenced by soil fertility. Cereals are also important economically in the UK. Over 16m tonnes of wheat (*Triticum aestivum*) were produced in 2015 (DEFRA 2016b). This allows for financial interpretation of the fertility legacy associated with

legume cultivation, but given how only one cereal crop can be grown per season, they may not fully exhaust soil fertility and may, therefore, give an incomplete picture of true fertility. This concern can be addressed by utilizing a bioassay crop with a high N demand and short growth period such as mustard (*Sinapsis alba*). Mustard can be grown three times in one season and is better equipped to fully determine the fertility of the soil.

Cultivation techniques such as tillage and chemical use can also impact upon rates of mineralization for legume residues (Ledgard 2001), which can introduce a temporal parameter to subsequent soil fertility. Herbicide application can rapidly increase residue mineralization and make SMN available more quickly (Singh *et al.* 2012). Herbicide omission allows mineralization to occur more slowly. Legume green manures are most commonly associated with organic farming, in which herbicide use is not permitted, and as a result there exists few studies on the impact of herbicide use on subsequent soil fertility. Conventional farmers may however be interested in using herbicides to terminate their clover leys, particularly those practicing conservation tillage.

5.2 Objectives & Hypotheses

A trial of different bioassays, using different cultivation procedures, may facilitate a full assessment of the fertility-building capacity of the preceding RC variety and management strategy. The purpose of Core Experiment 3 was to cultivate two different bioassay crops (spring wheat and mustard), using two cultivation techniques (application and omission of herbicide), to assess the fertility-building capacity of the preceding RC varieties and management strategies. The objectives for Core Experiment 3 were then:

- To cultivate spring wheat under treatments of herbicide and non-herbicide application
- To cultivate three mustard crops under treatments of herbicide and non-herbicide application
- To use the yield and N uptake data of these crops to determine the soil fertility-building capacity of the preceding RC variety, management strategy and cultivation procedure (herbicide and non-herbicide)

The hypotheses for Core Experiment 3 were:

- Application of herbicide prior to tillage significantly increases the yields of subsequent crops in RC cropping

- The higher N demand of multiple mustard crops better indicates the subsequent soil fertility following RC cultivation than cereal crops

5.3 Materials and Methods

Details of all field cultivations and lab procedures are given in Sections 2.3 and 2.4. A diagram of the field trial design is given in Figure 2.5. Further information concerning the bioassay crops selected, cultivation procedures employed and statistical procedures for Core Experiment 3 is given here.

5.3.1 Bioassay Crops

Spring wheat was selected as a bioassay crop to give economic relevance to the project and to allow for a ‘real world’ interpretation of subsequent soil-fertility following RC cultivation. Wheat is the most widely grown arable crop in the UK (DEFRA 2015), which means information concerning wheat yields following RC cultivation will be most meaningful to farmers. The vast majority of wheat grown in the UK (>95%) is winter wheat (DEFRA 2016b). Winter wheat production can be limited by winter leaching following sowing, particularly in wet years (HGCA 2009) and blackgrass (*Alopecurus myosuroides*) infestation (AHDB 2014). This is further exacerbated by increasing herbicide resistance in blackgrass populations (Chauvel, *et al.* 2001), a problem which clearly creates an agronomic and economic incentive to switch to spring cultivation. Spring wheat cultivation also permitted assessment of winter leaching susceptibility in Core Experiment 1.

The variety selected was ‘Paragon’. This variety was chosen for its good resistance to rust and long, sturdy straw (HCGA 2014). Paragon is also documented to grow well under low nutrient (Gaju *et al.* 2016) and drought conditions (Sikder *et al.* 2015), making it a suitable candidate for this trial.

Mustard was selected as the other bioassay for its high N demand and rapid growth rate (Wang *et al.* 2008). Although mustard is traditionally known as an arable crop, grown for seed oil and in some cases young greens (Boland 2013), it is also used as a cover crop and green manure. Its function in the rotation is to limit winter leaching through N uptake associated with winter growth (Jackson *et al.* 1993) and to suppress weeds through its physical presence and exudation of allelopathic chemicals (Haramoto and Gallandt 2004, Pii 928025275 Tesio and Ferrero 2010). Core Experiment 3 sought to utilize these agronomic properties for a different purpose; to use its high N demand and rapid growth to determine the subsequent soil fertility associated

with the preceding RC cultivation and management. The variety 'Rumba' was selected for its particularly high N demand and capacity to suppress weeds (Seeds 2017).

5.3.2 Cultivation

Termination of perennial crops such as RC will present management challenges. It can be carried out with tillage (most associated with the organic sector), herbicide application (most associated with the conventional sector) or some combination of both. Different depths/types of tillage and different spectrum herbicides add a further layer of complexity. The nature of this project (based on individual plots of 9m² split into plots of 2.25m²) made tillage an impractical aspect of cultivation. All plots were instead rotavated using a motorized mechanical rotavator (8HP – Ermin Machinery) to an estimated depth of 8-10cm, because some degree of tillage was essential for successful wheat and mustard establishment. As all plots were rotavated equally, this was not considered a treatment within the experiment.

Crop performance will always be determined by genotype, environment, management and interactions between all three (Montesino-San Martin *et al.* 2014). Understanding this is particularly pressing in systems using legume cultivation for crop nutrition, as the N release from mineralizing plant residues will be more influenced by management and environment than systems using mineral fertilizers. Tillage was unfortunately not appropriate for trial in Core Experiment 3, but the lack of logistical constraints involving the application or omission of herbicide meant it could be included as a treatment.

This treatment was selected for its impact on mineralization rates. The incorporation of aboveground biomass of leguminous green manures through tillage will generally terminate the crop. The green manure will then senesce and mineralize in response to soil microbial activity and weather conditions. The living nature of the manure in these practices can, however, cause it to compete with the subsequent crop for water and nutrients, and act as an inhibitor of growth at the initial growth stages (Kolota and Adamezewska-Sowinska 2013). This stands in contrast to the inclusion of herbicide application prior to tillage, in which the green manure is already dead, the rate of mineralization is expected to be more rapid (Burgess *et al.* 2014), and no competition effects would be expected. To assess the impact of the treatments of herbicide and non-herbicide, herbicide (40 g L⁻¹ glyphosate from a knapsack sprayer) was applied to half of all plots 1 week prior to rotavating in Core Experiment 3.

5.4 Statistical Analysis

5.4.1 Experimental Design

Core Experiment 3 was a continuation of Core Experiment 1 and the same completely randomized block design with 4 replications was used. The statistical design for Core Experiment 3 then became a two-factor split-plot design. The two factors were preceding RC variety and number of cuts (1 or 2) and the split of herbicide (H) and non-herbicide (NH) was allocated to each plot. This allowed for the outline of Core Experiment 1 to become the basis of both components of Core Experiment 3, the cultivation of wheat and mustard under the treatments of herbicide and non-herbicide. All collected data was analysed using the ‘Split-Plot’ function in the ANOVA menu of the Genstat® 18th Edition statistical software package. The design was set up using RC variety and management as whole plot and cultivation (H and NH) as split plots. This information is illustrated in Figure 5.1 and summarized in Table 5.1 using the Genstat input language.

The Variety*Cut*Cultivation treatment structure can be expanded as:

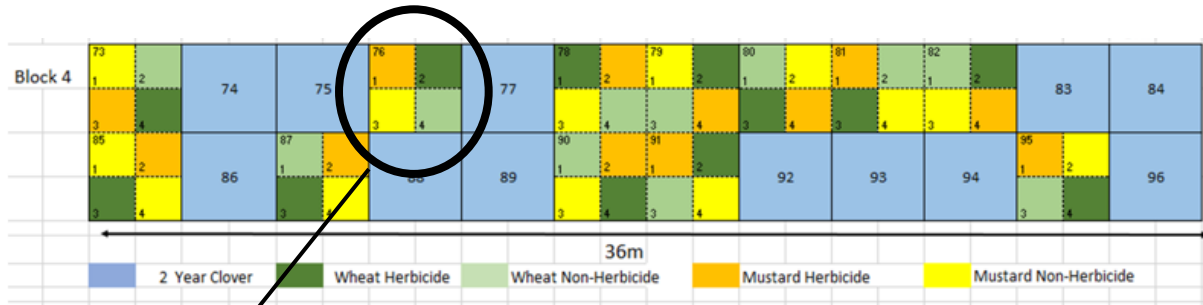
Variety + Cut + Cultivation + Variety.Cut + Variety.Cultivation + Cut.Cultivation + Variety.Cut.Cultivation

Where the plus (+) symbol indicates the main effects and the dot (.) symbol indicates interactions between main effects.

Table 5.1 Statistical design for Core Experiment 3

	Wheat		Mustard
Treatment Structure	Variety*Cut*Cultivation	Treatment Structure	Variety*Cut*Cultivation
Block	Block	Block	Block
Whole Plot	Variety & Cut	Whole Plot	Variety & Cut
Sub Plot	Cultivation	Sub Plot	Cultivation

Figure 5.1. Diagram and illustration of statistical design for Core Experiment 3



76	
Mustard H	Wheat H
1	2
Mustard NH	Wheat NH
3	4

All ANOVA assumptions were checked using residuals plots and skewness test statistics. Appropriate transformations were undertaken when assumptions could not be satisfied and reported results were back transformed.

5.4.2 Statistical Issues

Further statistical methods were required in Core Experiment 3. A problem of RC volunteering became apparent following the establishment of the wheat and mustard crops in early May. The RC taproot is documented to extend into soil for up to 1m (Boller and Nosberger 1987) and the rotavator used to terminate the crop failed to do so in some instances. Volunteering occurred almost entirely in the NH plots (as the H plots contained plants that were almost uniformly dead following the herbicide application). The volunteer clover then began to compete with the mustard and wheat crops for light, water and nutrients (effectively acting like a weed) and suppressing growth.

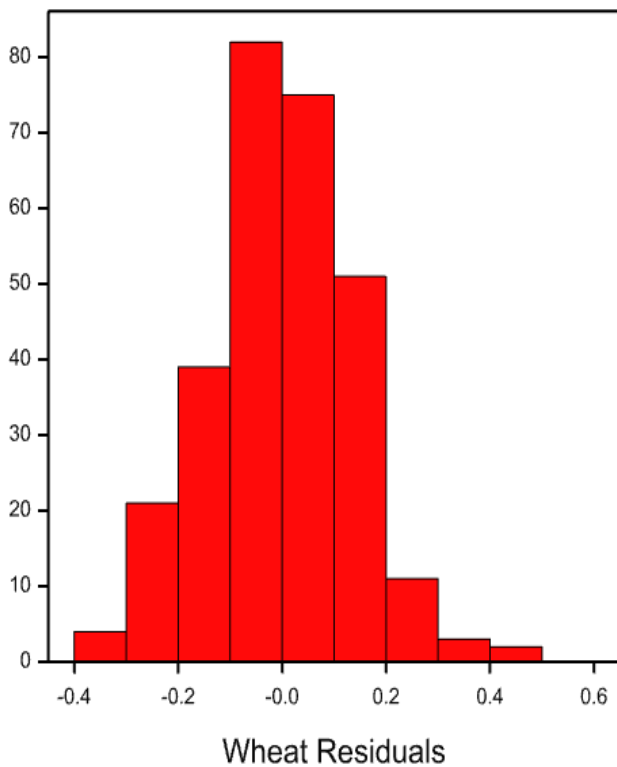
This issue was addressed by proceeding as planned, but when taking assessments of plots containing volunteer clover, both clover and bioassay were cut, bagged and separated by hand in the lab prior to drying for DM analysis. This gave two data sets, one for bioassay yield and one for RC yield. It seemed reasonable to expect the yields to be related (i.e. low volunteer

clover yields would result in high bioassay yields and vice versa). This expectation was initially investigated by running both data sets separately through ANOVA using the design previously outlined. However, as volunteering was only a major issue in half of the trial plots (the NH plots) difficulties in satisfying the assumptions of ANOVA become apparent. This issue is illustrated by viewing the histograms and test statistics of the residuals for the separate ANOVAs of both wheat and volunteer clover at the first bioassay assessment. These histograms are given in Figures 5.2 and 5.3. The test statistic is calculated as:

$$\frac{\text{Skewness}}{\text{Standard Error of Skewness}}$$

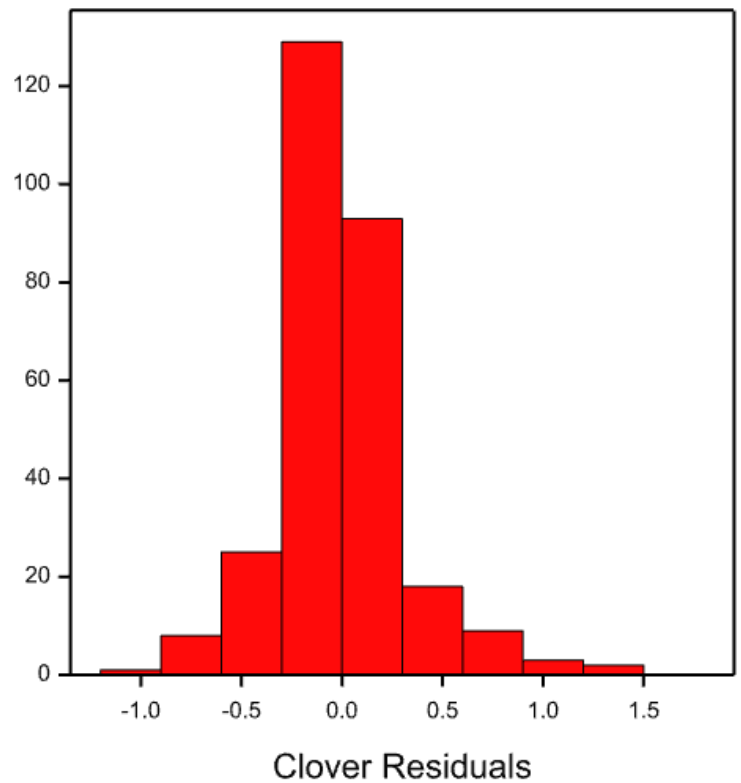
Where skewness and standard error of skewness are given by the Genstat summary statistics function. This formula is given by George and Mallery (2010) to assess skewness in distributions. A test statistic of ± 2 indicates normality, >2 indicates positive skew and <2 indicates negative skew.

Figure 5.2 Histogram of wheat DM residuals (First Assessment)



Test Statistic: 0.75

Figure 5.3 Histogram of clover DM residuals (First Assessment)



Test Statistic: 6.59

Distribution of the wheat DM residuals (Figure 5.2) indicates normality. This is confirmed by the calculated test statistic of 0.75 (slightly positively skewed but in the acceptable ± 2 range).

Distribution of the clover DM residuals (Figure 5.3) indicates stronger positive skew. This is confirmed by the calculated test statistic of 6.59 (considerably outside the acceptable range for normality).

Lack of normality of residual distribution does not invalidate ANOVA so much as weaken it (Tabachnick and Fidell 2006), but in this instance a test statistic of 10.51 (5 times outside the accepted range) was considered too strong for the F values calculated by ANOVA to be reliable and robust. Standard transformations (log, square etc) were subsequently applied in attempt to reduce skewness in the clover DM data, but the large number of zero values for the H plots resulted in the residuals distribution remaining highly skewed. This issue became the case for all volunteer clover biomass assessments. The data for volunteer clover DM could therefore not be subjected to ANOVA and reported in this way. The normally distributed residuals of the bioassay data meant this data was suitable for ANOVA, but this analysis would not account for the expected suppressive influence of the volunteer clover in the NH plots.

5.4.3 The Use of Analysis of Covariance (ANCOVA)

Analysis of covariance (ANCOVA) is a statistical method used in agriculture to account for variation caused by unforeseen but measureable factors, for example soil heterogeneity, stand irregularities caused by weeds, and non-uniformity in pest incidence (Gomez and Gomez A 1984). It operates by including the values of the nuisance variate as a covariate (in this case the volunteer clover DM) in the ANOVA for the dependent variate (in this case the wheat DM). This design is then inputted into Genstat and is illustrated in Figure 5.4.

Figure 5.4. Genstat command for ANCOVA

Some materials have been removed from this thesis due to Third Party Copyright. The unabridged version of the thesis can be viewed at the Lanchester Library, Coventry University.

ANCOVA is best described by its architect as a combination of ANOVA and linear regression (Fischer 1934). It calculates whether population means of the dependent variate (wheat DM) are equal across all levels of its factor (treatments of variety, cut and cultivation), whilst controlling for variation incurred by a covariate (clover DM). This control is achieved by

combining a linear regression between the covariate (clover DM) and the dependent variate (wheat DM) with the standard ANOVA (Tabachnick and Fidell 2006). Means are then adjusted to what they would be have been if the values of the covariate had been equal across all assessments; essentially a mathematical estimation of what would have happened had there been no covariate present in the first place. ANCOVA was considered suitable for the statistical procedures of Core Experiment 3 because although it is predicated on normally distributed dependent variates, it is not predicated on normality of distribution of the covariate. The large amount of zero values for the volunteer clover, which skewed the distribution and made it unsuitable for ANOVA, could then be included as a covariate and handled in this way.

However, as ANCOVA contains a regression element it assumes linearity of relationship between covariate and dependent variates. This assumption was satisfied in all cases by running separate ANOVAs of both the dependent variate and the covariate, saving the residuals and plotting each against each other in a standard scatterplot. This process is illustrated in Figure 5.5. When no strong evidence of non-linearity was observed (i.e. no significant curvature) the assumption of linearity of relationship was deemed satisfied. Further assumptions (i.e. normality of residuals of dependent variables and homoscedasticity of variance) were confirmed using the usual post-ANOVA residual plots).

Figure 5.5. Scatterplot of clover and wheat DM residuals (First Biomass Assessment)

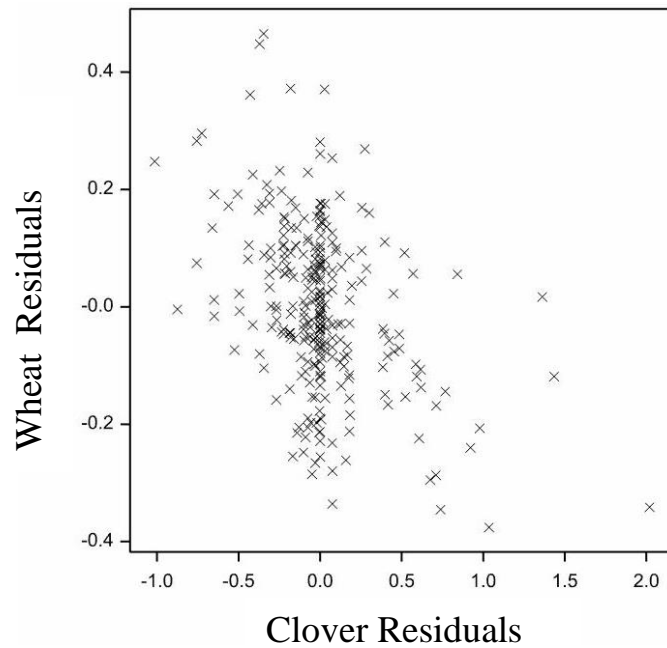


Figure 5.5 indicates no strong curvature exists between the graphed variates. It could be argued the distribution is Poisson or quadratic, in which case a different regression model would be required, but ANCOVA is cited as appropriate in all cases where evidence of non-linearity is not strong (Payne 2016) and was therefore deemed appropriate. Scatterplots such as these were used in Core Experiment 3 to confirm linearity of relationship between covariates and variates, so that ANCOVA could be employed to assess the impact the volunteer clover had on bioassay performance. This process was repeated wherever ANCOVA was used. Both ANOVA and ANCOVA are reported for comparison.

Both P values obtained from the ANCOVA are reported in all tables. The 'Adjusted' P value refers to the significance value calculated for each treatment effect. The 'Covariate' P value refers to the value quoted for the significance of the covariate itself. A sample ANCOVA table is given in Figure 5.6 to illustrate this point.

Figure 5.6 Sample ANCOVA table of First Assessment (wheat and clover DM)

Analysis of variance (adjusted for covariate)

Variate: Biomass_1_t_h
Covariate: Vol_Clover_1

Source of variation	d.f.	s.s.	m.s.	v.r.	cov.ef.	F pr.
Block stratum						
Covariate	1	9.56422	9.56422	29.56		0.032
Residual	2	0.64718	0.32359	8.22	10.52	
Block.Variety stratum						
Variety	5	0.68641	0.13728	3.49	0.86	0.030
Covariate	1	0.34842	0.34842	8.86		0.010
Residual	14	0.55082	0.03934	1.05	1.52	
Block.Variety.Cultivation stratum						
Cultivation	1	0.01986	0.01986	0.53	0.98	0.477
Variety.Cultivation	5	0.38326	0.07665	2.04	0.92	0.123
Covariate	1	1.72610	1.72610	46.05		<.001
Residual	17	0.63727	0.03749	1.84	3.50	
Block.Variety.Cultivation.*Units* stratum						
Cut	1	0.04786	0.04786	2.35	1.00	0.127
Variety.Cut	5	0.08495	0.01699	0.83	0.99	0.527
Cultivation.Cut	1	0.04892	0.04892	2.40	0.96	0.123
Variety.Cultivation.Cut	5	0.07188	0.01438	0.70	0.99	0.620
Covariate	1	1.06843	1.06843	52.38		<.001
Residual	227	4.63012	0.02040		1.23	
Total	287	21.04542				

In Figure 5.6 the F Pr values following treatments and interactions describe where significance differences lie between means following adjustment by the covariate. The F Pr values given following the covariate *itself* at each stratum describe how significant the impact of the covariate was. For example, the covariate in Figure 5.6 had a highly significant impact on the cultivation treatment ($P < 0.001$) but no significant differences were observed between cultivation treatments following covariate adjustment ($P = 0.477$). The values are referred to as ‘Adjusted’ P and ‘Covariate’ P and are quoted in all further ANCOVA results. ANCOVA results are only reported in cases where the Covariate P was significant for at least one effect or interaction. ANOVA is also reported for comparison. Results are reported in two sections, 5.5 for wheat performance and 5.6 for mustard performance.

5.5 Wheat Results

5.5.1 Establishment and Number of Tillers

Establishment and tiller assessments took place before RC volunteering became an issue and are therefore reported without the use of ANCOVA. The herbicide treatment resulted in significantly higher wheat establishment than the non-herbicide treatment. No significant differences were observed for cut or variety. No significant differences were observed in number of tillers for any treatments. Significant interactions were observed in establishment between variety and cultivation, and variety and cut. Astred established at a higher density in the H treatment but this interaction was not observed for other varieties (Figure 5.7). Astred and Milvus established at higher densities in the 2 cut treatment but Maro established at a higher density under 1 cut (Figure 5.8). Although these differences were observed, all treatments gave an establishment of ~260 plant/m², the density described as ideal by AHDB (2015). This data is summarized in Table 5.2

Table 5.2 Main Effects on wheat establishment and tiller number following different RC varieties

Treatment	Establishment plants m⁻²	Number of Tillers m⁻²
Herbicide	267.3a	262.8ns
Non-Herbicide	239.1b	256.7ns
<i>S.E.D. (18 df)</i>	12.96	10.6
<i>P</i>	0.043	0.572
1 Cut	258.3ns	254.9ns
2 Cut	248.1ns	264.6ns
<i>S.E.D. (228 df)</i>	14.11	6.01
<i>P</i>	0.469	0.109
Amos	240.7ns	252.0ns
Astred	258.0ns	268.3ns
Claret	268.7ns	248.3ns
Maro	255.7ns	268.7ns
Milvus	267.0ns	288.7ns
Ruby	229.3ns	232.3ns
<i>S.E.D. (15 df)</i>	27.43	21.77
<i>P</i>	0.68	0.212

Values followed by the same letter do not differ significantly at cited P value

Figure 5.7 Effect of interaction between variety and cultivation on wheat establishment

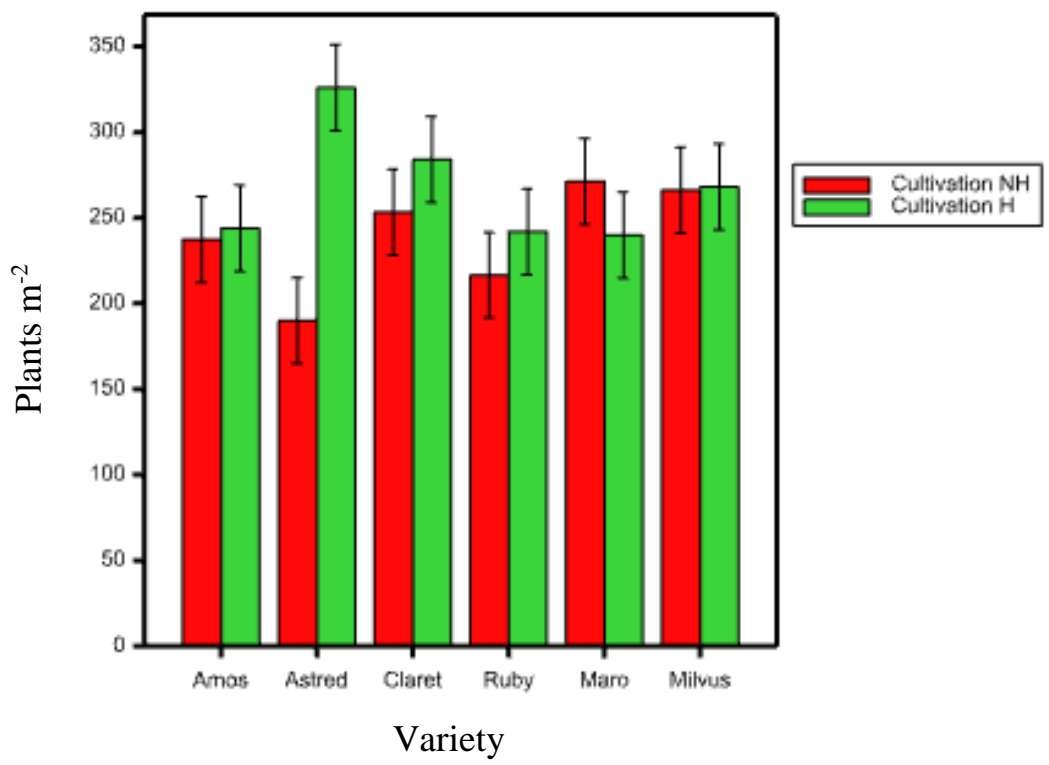
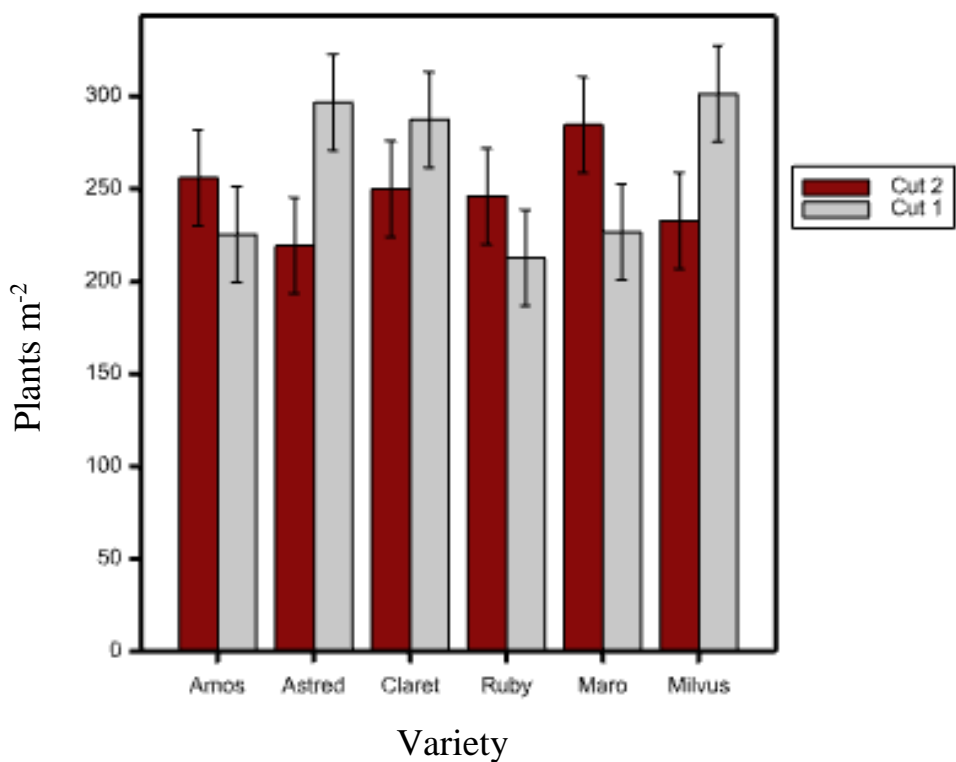


Figure 5.8 Effect of interaction between variety and cut on wheat establishment



5.5.2 First Biomass Assessment (Wheat DM and N)

RC volunteering had occurred (almost exclusively in NH plots) when the First Biomass Assessment was taken. ANCOVA was undertaken to investigate the impact of this volunteering on wheat growth. The assumptions were satisfied using the methodology described in Section 5.4.3. This is illustrated in Figures 5.9 and 5.10

Figure 5.9 Scatterplot of clover & wheat DM residuals

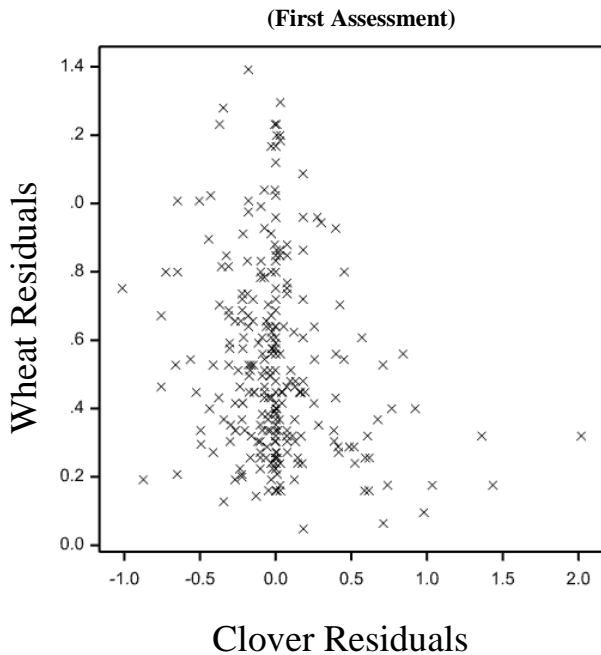
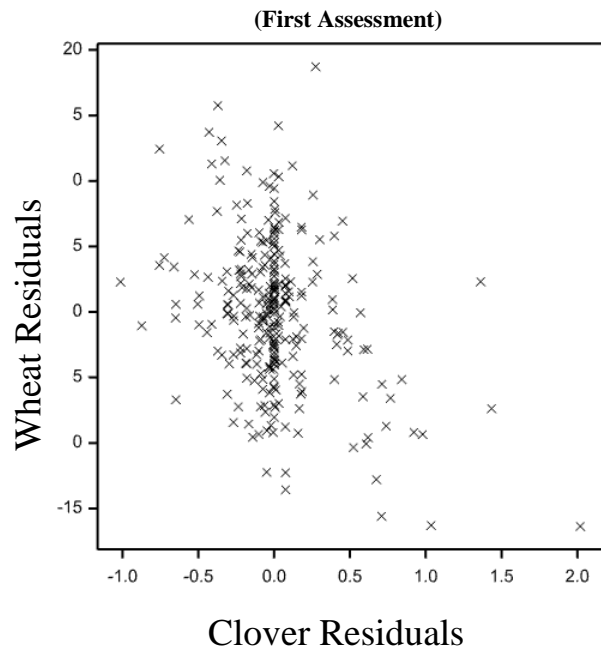


Figure 5.10 Scatterplot of clover DM and wheat N residuals



Figures 5.9 and 5.10 indicate linearity of relationship between the desired covariate (clover) and dependent variate (wheat on DM and N yields), satisfying the assumptions of ANCOVA. Further assumptions were satisfied by examining the residual plots following both ANCOVAs. These showed normality of distribution and homogeneity of variance, indicating accurate inferences could be made about the differences among groups following adjustment from the covariate. The results of the ANCOVA were found to be significant at all treatment and interaction levels for both components of the First Biomass Assessment (wheat DM and N). F values ranged from $P < 0.001$ to $P < 0.05$. In the further ANCOVA no significant differences were observed in the cultivation or cut treatments for both DM and N, but the effect of variety was significant. Amos, Claret, Maro and Milvus yielded significantly more DM than Ruby. Amos and Milvus yielded significantly more DM than Astred and Ruby. N yields generally followed the same pattern as DM yields, with Amos, Claret and Milvus yielding significantly more than Ruby and Astred. No significant interactions were observed between any treatments

for both DM and N. This data is summarized in Table 5.3. No significant treatment effects or interactions were observed in the initial ANOVA or the subsequent ANCOVA.

Table 5.3 Main Effects on wheat DM and N following different RC varieties (ANOVA and ANCOVA) – First Assessment

Treatment	ANOVA		ANCOVA	
	Wheat DM t ha ⁻¹	Wheat N kg ha ⁻¹	Wheat DM t ha ⁻¹	Wheat N kg ha ⁻¹
Herbicide	0.55ns	21.0ns	0.52ns	21.5ns
Non-Herbicide	0.51ns	22.6ns	0.54ns	22.1ns
<i>S.E.D. (18 df)</i>	0.04	1.85		
<i>P</i>	0.187	0.229		
<i>Covariate S.E.D. (17 df)</i>			0.02	1.74
<i>Adjustment P</i>			0.013	0.076
<i>Covariate P</i>			<0.001	<0.001
1 Cut	0.54ns	22.2ns	0.54ns	22.3ns
2 Cut	0.52ns	21.5ns	0.52ns	21.3ns
<i>S.E.D. (228 df)</i>	0.02	0.74		
<i>P</i>	0.352	0.553		
<i>Covariate S.E.D. (227 df)</i>			0.017	0.67
<i>Adjustment P</i>			0.136	0.128
<i>Covariate P</i>			<0.001	<0.001
Amos	0.57ns	23.5ns	0.59a	23.9a
Astred	0.50ns	20.0ns	0.49bc	19.5bc
Claret	0.51ns	22.5ns	0.56ab	24.0a
Maro	0.51ns	21.9ns	0.53ab	22.4ab
Milvus	0.59ns	23.3ns	0.58a	23.3a
Ruby	0.48ns	19.6ns	0.43c	17.8c
<i>S.E.D. (18 df)</i>	0.05	1.73		
<i>P</i>	0.293	0.259		
<i>Covariate S.E.D. (17 df)</i>			0.04	1.6
<i>Adjustment P</i>			0.03	0.022
<i>Covariate P</i>			0.01	0.022

Values followed by the same letter do not differ significantly at cited P value

5.5.3 Second Biomass Assessment (Number of Stems and Wheat DM and N)

RC volunteering continued to be a problem throughout the growing season. ANCOVA was undertaken to assess the impact of this volunteering on wheat growth, using the same methodology as in Biomass Assessment 1. The assumptions were first satisfied using the same scatterplot method previously outlined. This is illustrated in Figure 5.11-5.13.

Figure 5.11 Scatterplot of clover & stem number residuals

(Second Assessment)

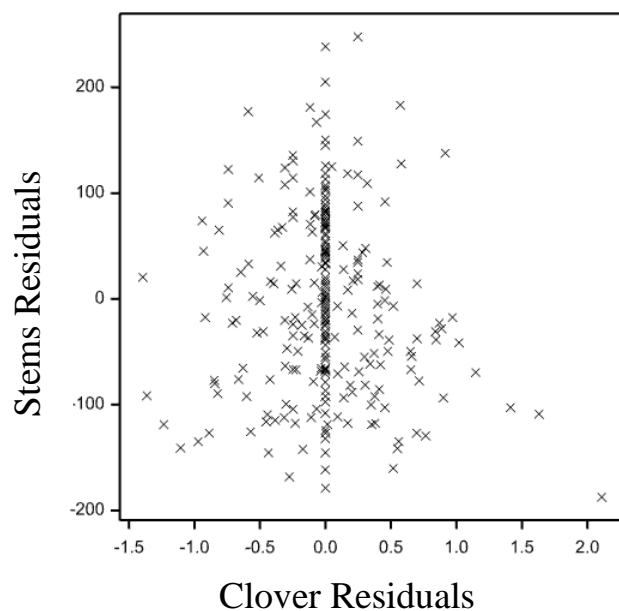


Figure 5.11 indicates linearity of relationship exists between the covariate and number of stems and therefore this variate was deemed acceptable for ANCOVA. This process was repeated for wheat DM and N yields and is illustrated in Figures 5.12 and 5.13. No transformations were required for wheat DM (Figure 5.12), but log transformation was required to establish linearity of relationship between covariate and N yields (Figure 5.13). Both scatterplots show linearity of relationship and deemed acceptable for ANCOVA.

Figure 5.12 Scatterplot of clover & wheat DM residuals

(Second Assessment)

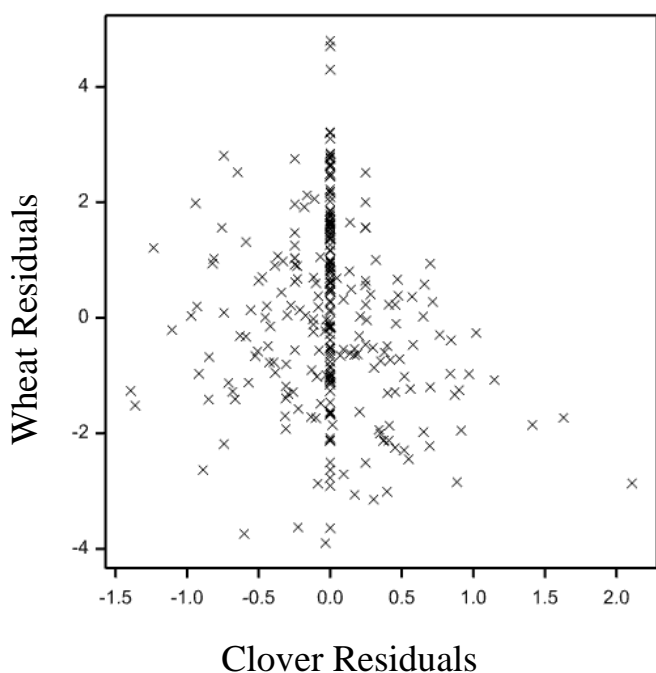
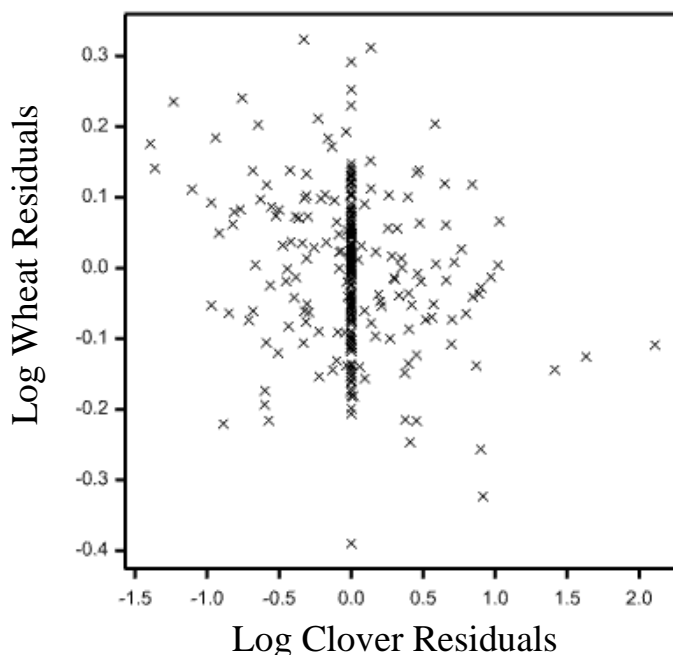


Figure 5.13 Scatterplot of log clover & log wheat N residuals

(Second Assessment)



The results of the initial ANOVA indicated the H treatment yielded significantly ($P < 0.001$) more stems, DM and N than the NH treatment. 1 cut yielded significantly more DM and N ($P < 0.05$) than 2 cut, but this effect was not observed in stem number. No significant differences were observed for variety in any treatment. Significant interactions were also observed between cut and variety in both DM and N yields. Ruby yielded significantly less DM and N under 2 cut but this interaction was not observed in other varieties. These interactions are summarized in Figures 5.14-5.15.

The ANCOVA gave highly significant ($P < 0.001$) Covariate P values for cultivation and cut in number of stems and DM yield. Significant ($P < 0.05$) Covariate P values for cultivation and cut were also observed in N yield. No significant Adjustment P values were observed in any variate and therefore no significant differences were described between treatment means following ANCOVA means adjustment. No significant interactions were observed in the ANCOVA. These results are summarized in Table 5.4.

Table 5.4 Main Effects on number of stems and wheat DM and N following different RC varieties (ANOVA and ANCOVA) – Second Assessment

Treatment	ANOVA			ANCOVA		
	Stems m ²	Wheat DM t ha ⁻¹	Wheat N kg ha ⁻¹	Stems m ²	Wheat DM t ha ⁻¹	Wheat N kg ha ⁻¹ (1)
Herbicide	436.2a	6.67a	77.6a	303.8ns	4.97ns	61.1ns
Non-Herbicide	263.2b	3.58b	43.3b	331.7ns	5.28ns	52.7ns
<i>S.E.D. (18 df)</i>	17.6	0.35	6.2			
<i>P</i>	<0.001	<0.001	<0.001			
<i>Covariate S.E.D. (17 df)</i>				23.0	0.51	8.4
<i>Adjusted P</i>				0.053	0.444	0.293
<i>Covariate P</i>				<0.001	<0.001	0.027
1 Cut	356.8ns	5.29a	62.3a	320.6ns	5.27ns	63.4ns
2 Cut	342.7ns	4.96b	57.6b	314.4ns	4.98ns	58.9ns
<i>S.E.D. (228 df)</i>	10.9	0.16	1.9			
<i>P</i>	0.203	0.031	<0.001			
<i>Covariate S.E.D. (227 df)</i>				9.9	0.25	4.3
<i>Adjusted P</i>				0.226	0.35	ns
<i>Covariate P</i>				<0.001	<0.001	<0.001
Amos	366.3ns	5.22ns	59.5ns	358.7ns	5.09ns	58.6ns
Astred	318.3ns	4.92ns	58.6ns	315.3ns	4.88ns	59.8ns
Claret	361.0ns	5.31ns	60.5ns	357.8ns	5.34ns	59.5ns
Maro	354.7ns	5.64ns	65.7ns	343.6ns	5.59ns	63.4ns
Milvus	374.0ns	5.08ns	60.1ns	359.0ns	5.16ns	61.8ns
Ruby	324.0ns	4.59ns	55.6ns	322.3ns	4.70ns	58.6ns
<i>S.E.D. (18 df)</i>	22.7	0.55	6.21			
<i>P</i>	0.127	0.527	0.697			
<i>Covariate S.E.D. (17 df)</i>				24.3	0.55	7.9
<i>Adjusted P</i>				0.148	0.639	0.765
<i>Covariate P</i>				0.52	0.247	0.355

1. Data has been log transformed to reduce heterogeneity of variance and ensure linearity of relationship with covariate. Reported results have been back transformed using the antilog function.

Values followed by the same letter do not differ significantly at cited P value

Figure 5.14 Effect of interaction between variety and cut on wheat DM (ANOVA)

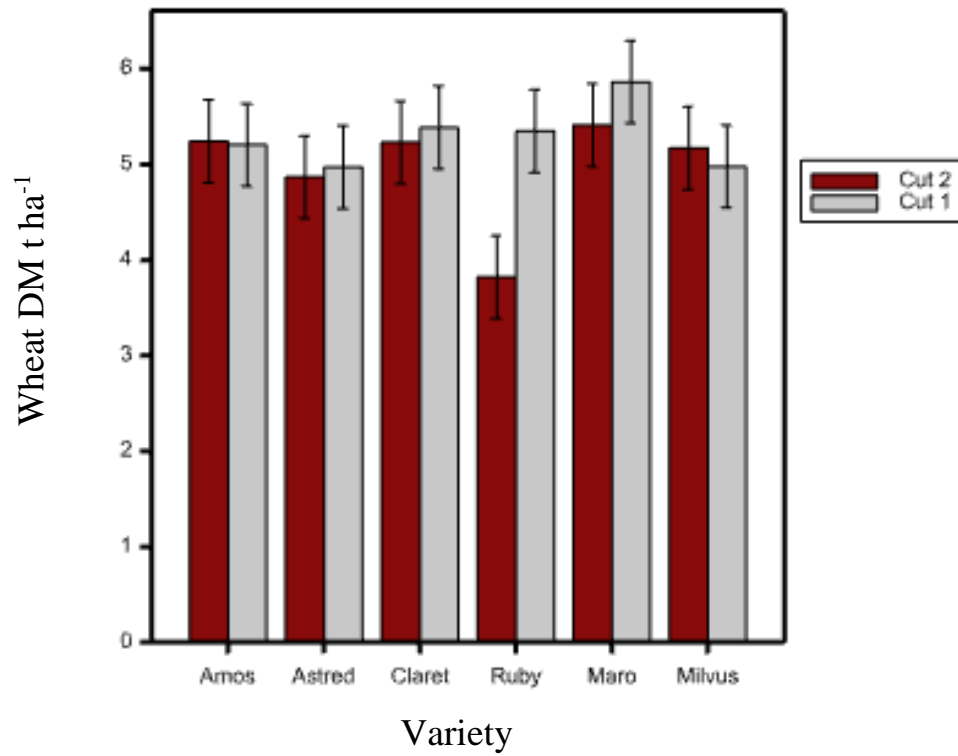
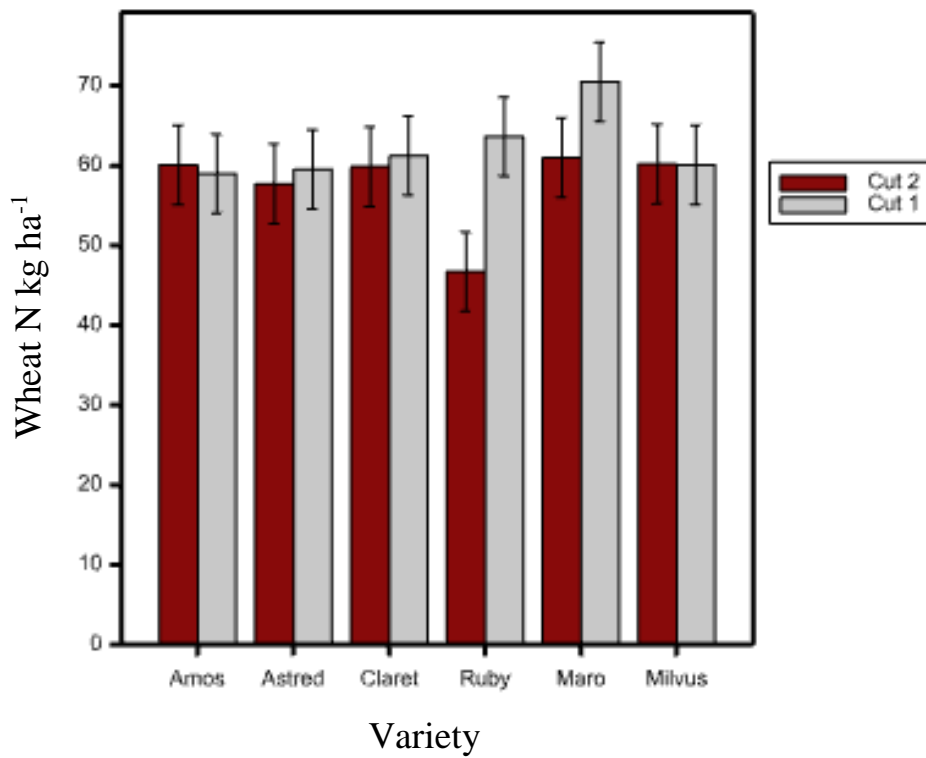


Figure 5.15 Effect of interaction between variety and cut on wheat N (ANOVA)



5.5.4 Final Biological Harvest

Final Biological Harvest took place in early September. Visual observations at this stage indicated the volunteer clover had had a suppressive effect on wheat growth in the NH plots, but the volunteer clover had flowered in early June and entered senescence by mid-July. The Final Biological Harvest assessment did harvest a negligible amount of volunteer clover, but the vast majority of it had died away. This negligible amount was still dried for DM as in Biomass Assessments 1 and 2, and the results were taken as a covariate to assess the impact it may have had on wheat production. However, the resulting ANCOVAs gave no significant covariate values for all treatments and interactions across all variates measured (plant height, ears DM, harvest index etc.).

The ANCOVA procedure was then eliminated for all variates accounted for in Final Biological Harvest. This is not to say the volunteer clover did not impact on wheat production, but rather its impact could not be accounted for at this assessment. Significant differences ($P < 0.001$) were observed for cultivation in many variates assessed at Final Biological Harvest, but these differences were more likely caused by suppressive volunteering in the NH plots than differences in mineralization rates associated with different cultivation treatments.

Recorded values for all variates were significantly higher in H treatments than NH ($P < 0.001$). Cut treatments were insignificant for all variates except grain protein, in which 1 cut accumulated significantly more grain protein than 2 cuts. Varietal differences were minimal. Maro and Milvus yielded significantly shorter wheat than other varieties. Ruby, Claret and Amos yielded grains with significantly higher TGW and Claret and Maro yielded grains with significantly higher protein percentage. These results are summarized in Tables 5.5 – 5.7.

Significant interactions between variates were also observed at Final Biological Harvest. NH treatment yielded significantly more ears m^{-2} than H treatment when the clover was cut twice, but this effect was not observed in the 1 cut treatment (Figure 5.16). Amos was the only variety to give higher grain yield in the 2 cut treatment, and Ruby yielded higher grain yield than that of Maro and Astred when 2 cuts were applied (Figure 5.17). N yields had similar interactions to grain yield. Amos was the only variety which yielded significant differences between cuts and Ruby yielded higher than some other varieties in the 2 cut treatment for both grain and total N yields (Figures 5.18 and 5.19).

Table 5.5 Main Effects on wheat height, number of ears and ears, straw and total DM following different RC varieties

Treatment	Plant Height cm	Ears m ⁻²	DM t ha ⁻¹		
			Ears DM	Straw DM	Total DM
Herbicide	78.0ns	270.6ns	5.71a	3.91a	9.62a
Non-Herbicide	77.9ns	305.0ns	2.53b	1.91b	4.45b
<i>S.E.D. (18 df)</i>	1.05	34.3	0.40	0.13	0.39
<i>P</i>	0.099	0.733	<0.001	<0.001	<0.001
1 Cut	77.8ns	286.0ns	4.14ns	2.93ns	7.07ns
2 Cut	78.1ns	289.6ns	4.10ns	2.90ns	7.00ns
<i>S.E.D. (228 df)</i>	0.80	12.8	0.22	0.14	0.26
<i>P</i>	0.684	0.773	0.846	0.807	0.812
Amos	78.1ab	257.3ns	3.82ns	2.69ns	6.51ns
Astred	79.2a	292.0ns	3.87ns	2.93ns	6.80ns
Claret	79.8a	287.0ns	4.20ns	3.04ns	7.25ns
Maro	74.9c	265.7ns	4.06ns	2.88ns	6.95ns
Milvus	76.9bc	283.2ns	3.99ns	2.67ns	6.66ns
Ruby	78.8ab	341.5ns	4.77ns	3.26ns	8.04ns
<i>S.E.D. (18 df)</i>	0.97	84.0	0.55	0.46	0.96
<i>P</i>	0.002	0.302	0.249	0.099	0.179

Values followed by the same letter do not differ significantly at cited P value

Figure 5.16 Effect of interaction between cut and cultivation on number of ears m⁻²

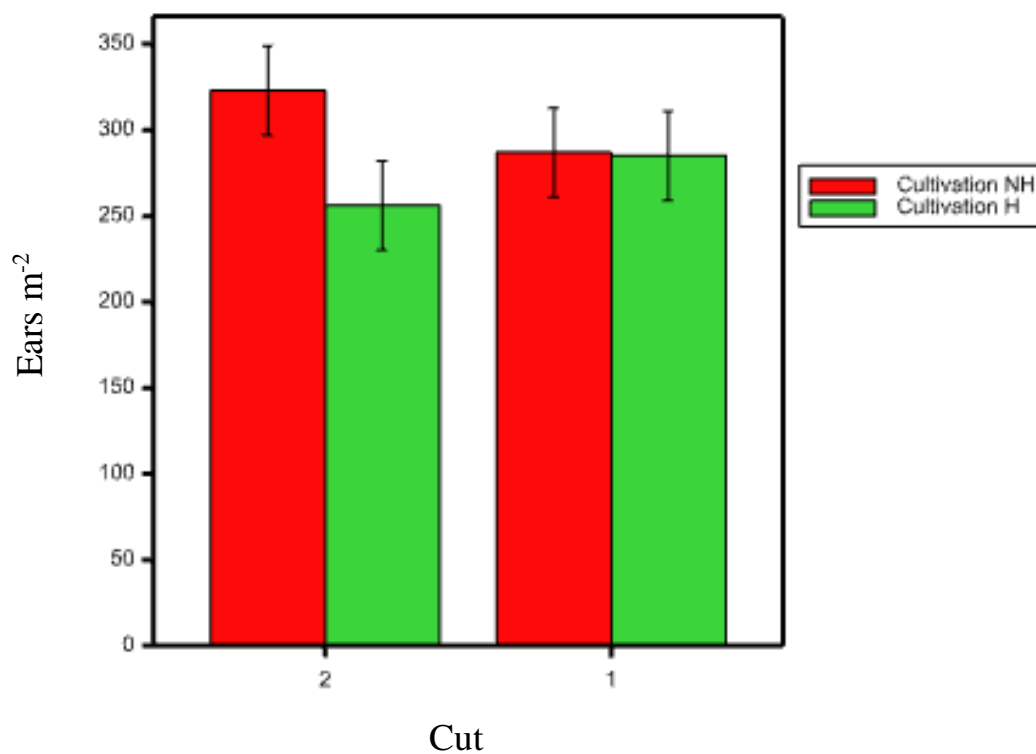


Table 5.6 Main Effects on grain yield, thousand grain weight (TGW) and harvest index (HI) following different RC varieties

Treatment	Grain Yield t ha ⁻¹	TGW	HI
Herbicide	4.23a	37.26a	44.5a
Non-Herbicide	1.60b	35.20b	36.0b
<i>S.E.D. (18 df)</i>	0.22	0.4	2.25
<i>P</i>	<0.001	<0.001	<0.001
1 Cut	2.91ns	36.1ns	40.6ns
2 Cut	2.91ns	36.3ns	39.8ns
<i>S.E.D. (228 df)</i>	0.13	0.23	1.20
<i>P</i>	0.567	0.405	0.394
Amos	2.64ns	36.83ab	39.0ns
Astred	2.73ns	35.03c	39.3ns
Claret	2.99ns	37.35a	40.2ns
Maro	2.73ns	35.69bc	38.2ns
Milvus	2.88ns	35.24c	41.2ns
Ruby	3.51ns	37.25a	43.4ns
<i>S.E.D. (18 df)</i>	0.54	1.13	3.84
<i>P</i>	0.631	0.017	0.582

Values followed by the same letter do not differ significantly at cited P value

Figure 5.17 Effect of interaction between variety and cut on grain yield

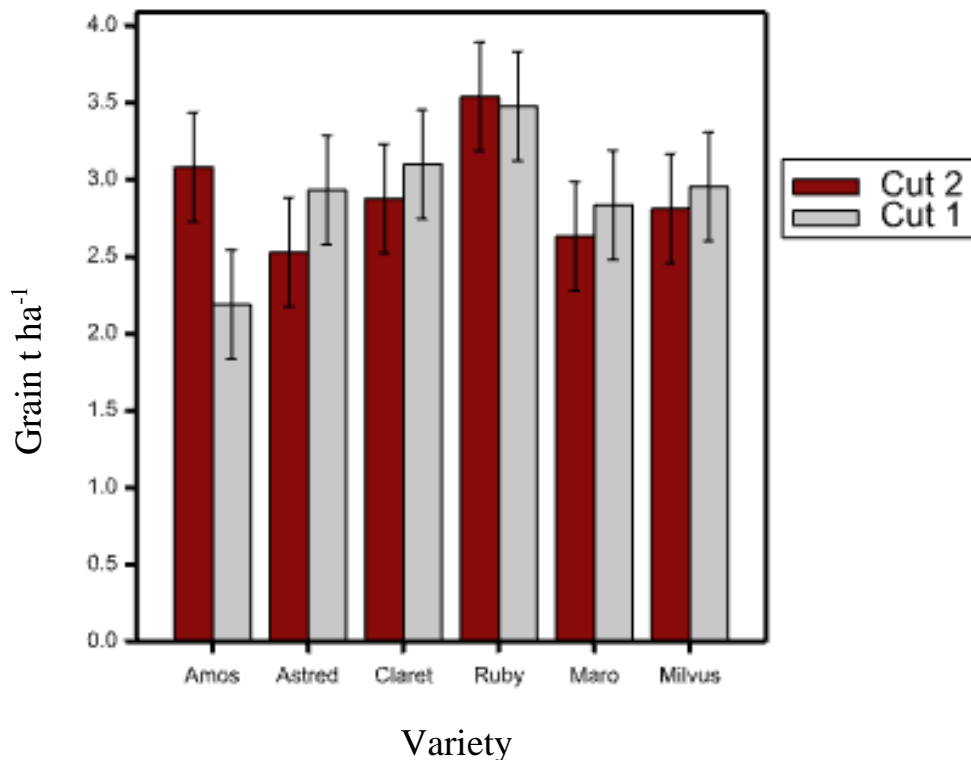


Table 5.7 Main Effects on grain N, total N, N harvest index (N HI) and grain protein following different RC varieties

Treatment	Grain N kg ha ⁻¹	Total N kg ha ⁻¹	N HI	Grain Protein (%)
Herbicide	79.5a	99.7a	79.1a	11.8a
Non-Herbicide	32.5b	44.7b	71.7b	10.8b
<i>S.E.D. (18 df)</i>	4.09	4.70	1.51	0.16
<i>P</i>	<0.001	<0.001	<0.001	<0.001
1 Cut	57.2ns	73.7ns	75.7ns	11.4a
2 Cut	54.8ns	70.7ns	75.0ns	11.1b
<i>S.E.D. (228 df)</i>	2.41	2.86	0.91	0.07
<i>P</i>	0.353	ns	0.295	<0.001
Amos	49.9ns	64.5ns	75.3ns	11.2bc
Astred	52.0ns	69.1ns	74.0ns	11.2bc
Claret	60.0ns	77.2ns	75.5ns	11.9a
Maro	54.1ns	69.7ns	75.1ns	11.6ab
Milvus	54.9ns	69.5ns	76.2ns	11.0cd
Ruby	65.2ns	83.0ns	76.1ns	10.7d
<i>S.E.D. (18 df)</i>	10.0	11.50	3.7	0.4
<i>P</i>	0.441	ns	0.374	<0.001

Values followed by the same letter do not differ significantly at cited P value

Figure 5.18 Effect of interaction between variety and cut on grain N yield

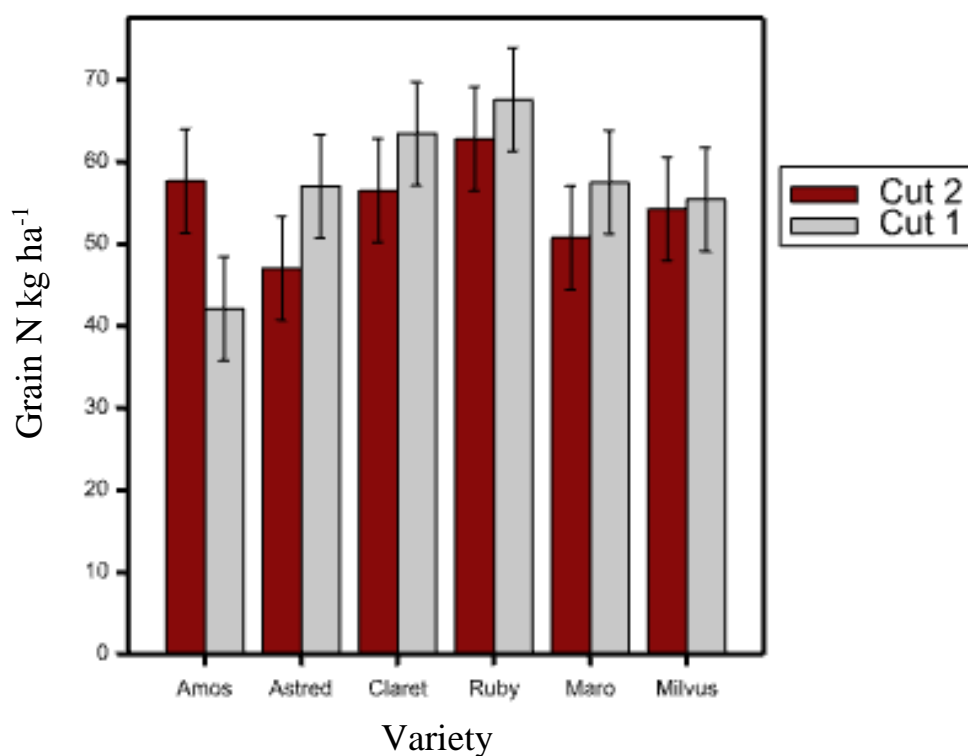
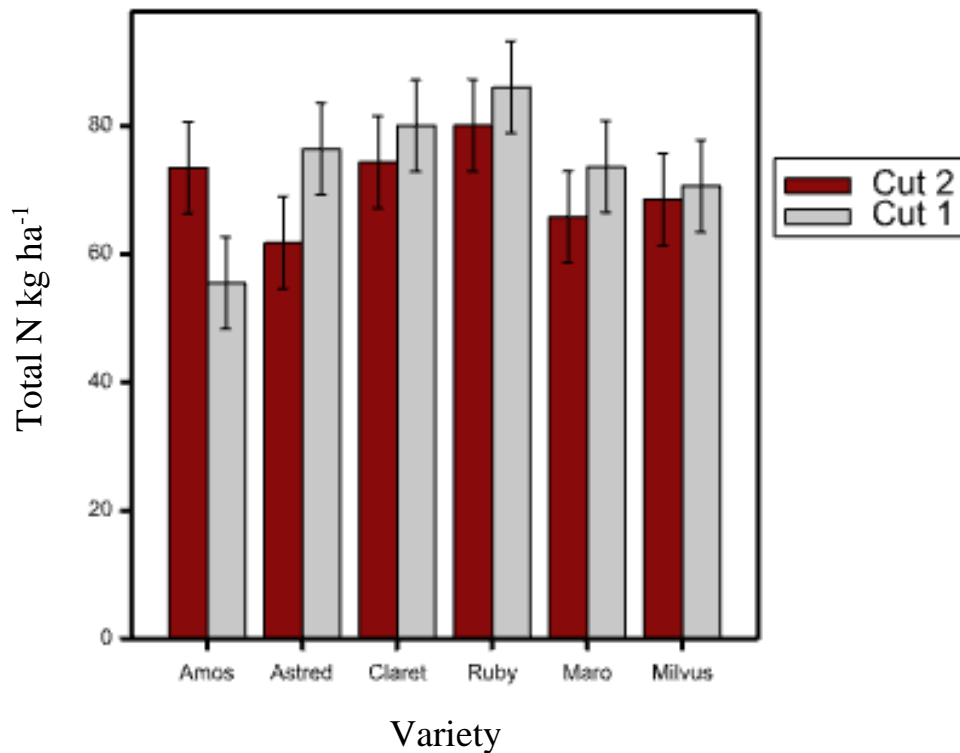


Figure 5.19 Effect of interaction between variety and cut on total N yield



5.6 Mustard Results

5.6.1 First Biomass Assessment (Mustard DM and N)

Volunteer clover was also a problem in the mustard cultivation, but as mustard exhibits faster growth than spring wheat, it was less problematic at Biomass Assessment 1. Any clover found growing in assessment quadrats was harvested and handled in the same fashion as the spring wheat. Linearity of relationship was established using the scatterplot method previously described and this is illustrated in Figures 5.20-5.21. Linearity of relationship could not be established for mustard N and this variate was log transformed to address this (Figure 5.21).

In the initial ANOVA highly significant ($P < 0.001$) differences were observed for cultivation (H yielded more DM and N). 1 cut also yielded more DM ($P < 0.05$) and more N ($P < 0.001$). No varietal differences were observed in DM but Ruby accumulated significantly more N than all other varieties and Amos significantly less ($P < 0.05$). The ANCOVA showed the covariate to be non-significant for cultivation and variety, but significant in cut for DM ($P < 0.001$) and N yields ($P < 0.01$).

Significant interactions were observed in the initial ANOVA between cut and variety for DM and N. 1 cut yielded significantly more DM than 2 in Ruby, but no differences were observed for cut treatment in all other varieties (Figure 5.22). 1 cut yielded significantly more N in Ruby, Astred and Claret, but no differences were observed the for cut treatment in all other varieties. 1 cut treatment in Ruby yielded more N than all other varieties at both treatments (Figure 5.23). The Covariate P of the subsequent ANCOVA was insignificant for DM and N yields in both cultivation and variety, implying volunteering did not significantly impact on these variates. The Covariate P was significant in cut for DM ($P < 0.001$) and N ($P < 0.01$). Means adjustmetns however appeared negligible and the effect of cut did not change between ANOVA and ANCOVA. No interactions were observed in the ANCOVA. This data is summarized in Table 5.8.

Figure 5.20 Scatterplot of log clover & mustard DM residuals

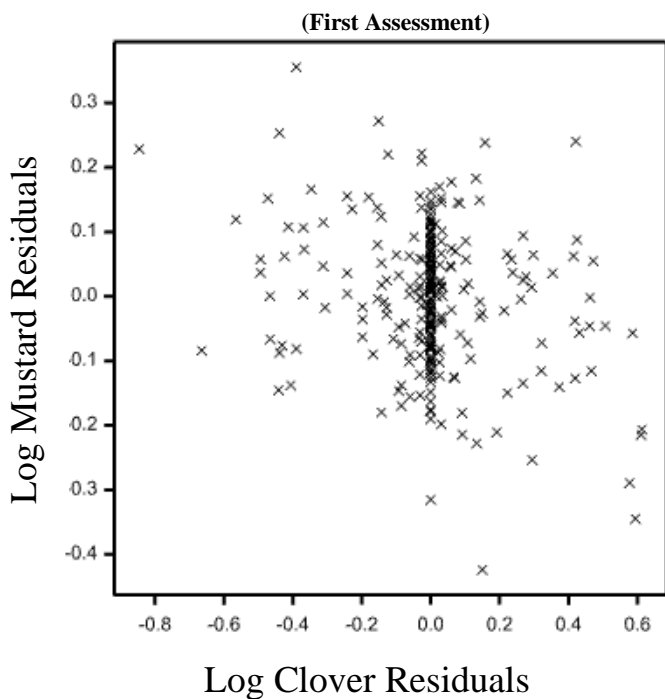


Figure 5.21 Scatterplot of clover DM & mustard N residuals

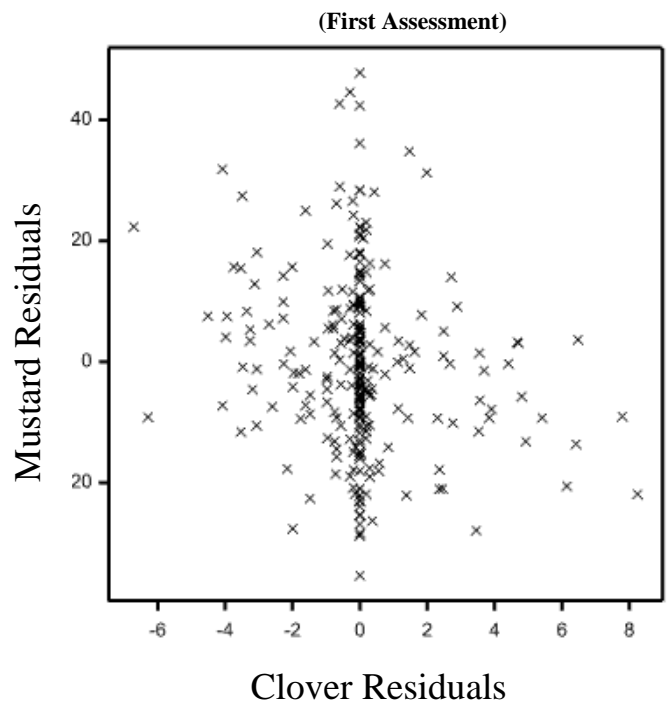


Table 5.8 Main treatment effects on mustard DM and N following different RC varieties – First Biomass Assessment

Treatment	ANOVA		ANCOVA	
	Mustard DM t ha ⁻¹	Mustard N kg ha ⁻¹	Mustard DM t ha ⁻¹	Mustard N kg ha ⁻¹
Herbicide	1.82a	65.4a	1.70ns	61.0ns
Non-Herbicide	1.45b	50.2b	1.53ns	54.6ns
<i>S.E.D. (18 df)</i>	0.07	3.14		
<i>P</i>	<0.001	<0.001		
<i>Covariate S.E.D. (17 df)</i>			0.14	6.94
<i>Adjusted P</i>			0.22	0.268
<i>Covariate P</i>			0.442	0.176
1 Cut	1.7a	61.3a	1.70a	61.3a
2 Cut	1.57b	54.2b	1.56b	54.3b
<i>S.E.D. (228 df)</i>	0.05	1.88		
<i>P</i>	0.004	<0.001		
<i>Covariate S.E.D. (227 df)</i>			0.05	1.85
<i>Adjusted P</i>			0.004	<0.001
<i>Covariate P</i>			<0.001	0.006
Amos	1.49ns	47.7c	1.63ns	53.3ns
Astred	1.91ns	64.3ab	1.90ns	63.8ns
Claret	1.45ns	52.3bc	1.56ns	56.8ns
Maro	1.55ns	52.8bc	1.55ns	52.4ns
Milvus	1.55ns	57.8bc	1.45ns	53.7ns
Ruby	1.84ns	71.7a	1.72ns	66.7ns
<i>S.E.D. (18 df)</i>	0.17	7.7		
<i>P</i>	0.068	0.01		
<i>Covariate S.E.D. (17 df)</i>			0.19	6.25
<i>Adjusted P</i>			0.159	0.116
<i>Covariate P</i>			0.137	0.075

Values followed by the same letter do not differ significantly at cited P value

Figure 5.22 Effect of interaction between variety and cut on mustard DM (Biomass Assessment 1)

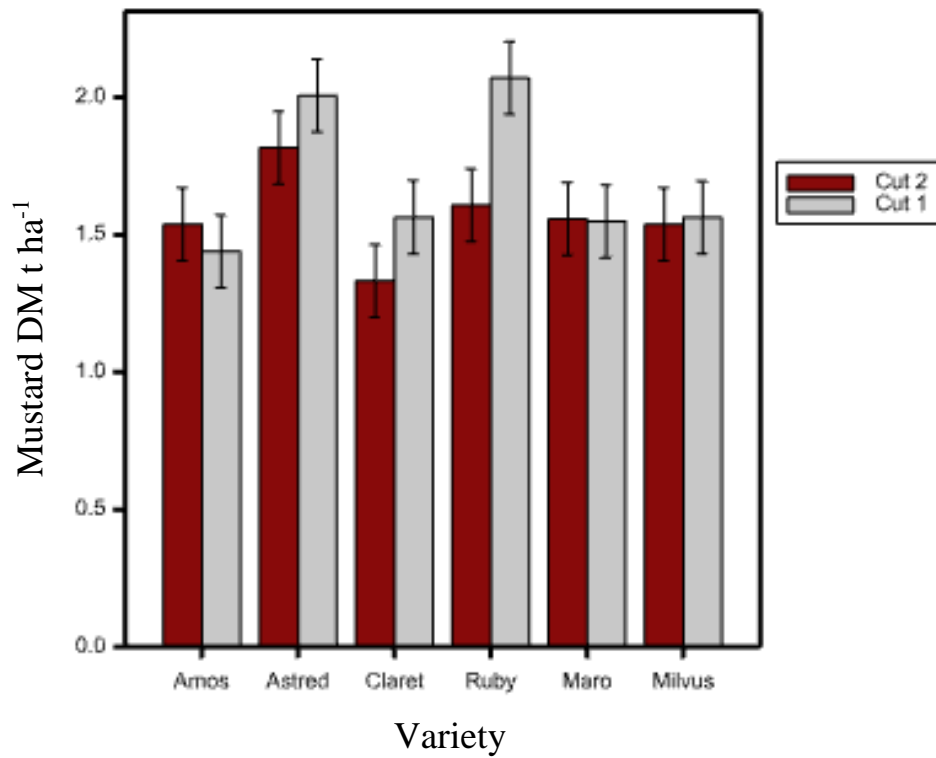
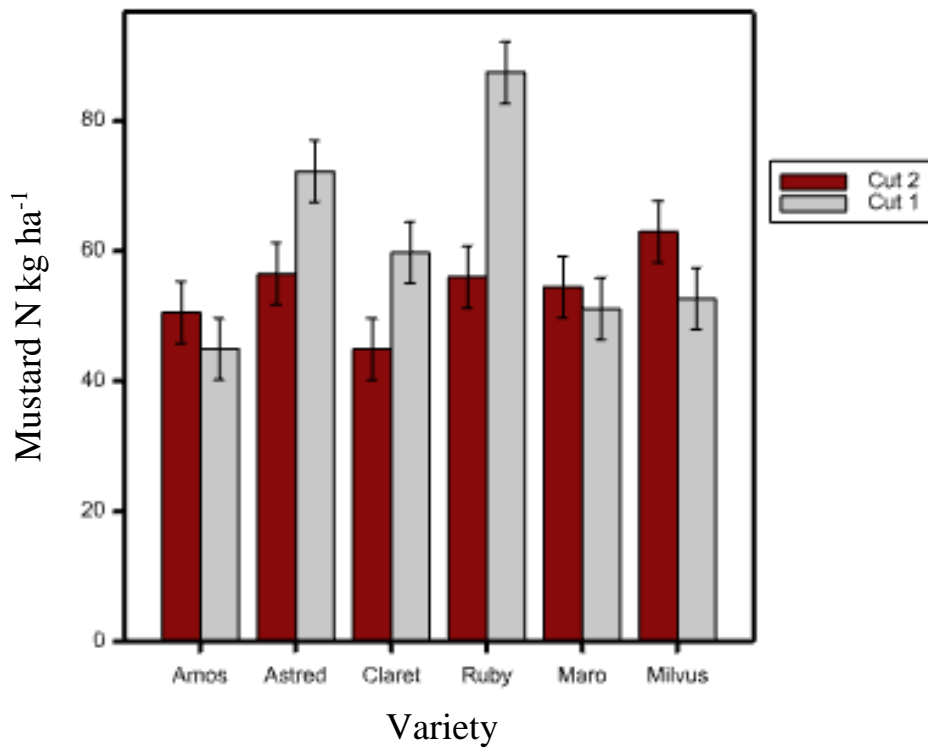


Figure 5.23 Effect of interaction between variety and cut on mustard N (Biomass Assessment 1)



5.6.2 Second Biomass Assessment (Mustard DM and N)

Although volunteer clover in the NH mustard plots was cut back before the second mustard crop was planted, it regrew quickly and completely choked the mustard crop. This resulted in no data for the NH treatment at the Second Biomass Assessment. This growth also likely altered the properties of the soil beyond the treatments of the preceding year of cultivation. The NH treatment was, therefore, eliminated from the second and third mustard biomass assessment. The data was treated as a 2 factor ANOVA (variety and cut) in completely randomized blocks for the rest of the experiment.

Weed pressure was also observed throughout the Second Biomass Assessment. The species involved was almost exclusively *Cirsium arvense* (creeping thistle), but a small presence of *Atriplex patula* (orache) was also observed. The effect of weed establishment on mustard growth was determined using the ANCOVA method as previously described. Linearity of relationship was first determined, again as previously described, and is illustrated in Figures 5.24 and 5.25.

Figure 5.25 Scatterplot of mustard N and weed DM residuals

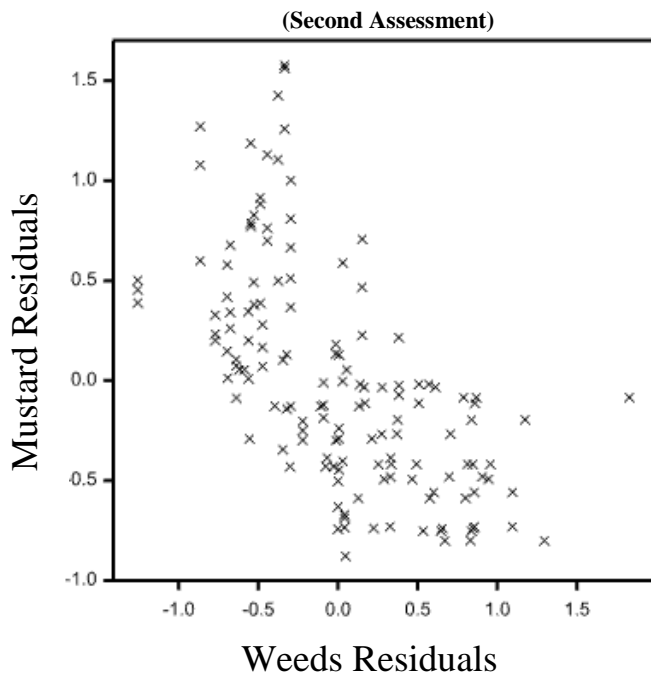
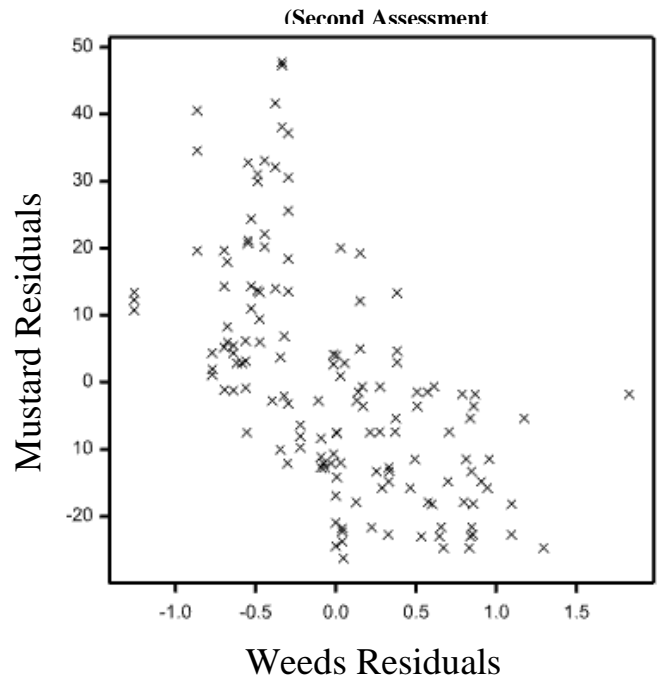


Figure 5.24 Scatterplot of mustard and weed DM residuals



Initial observations indicated weed populations were better established in blocks 1 and 4. This was likely caused by the greater exposure of these blocks to the trial margins, where populations of these weeds had established throughout the growing season. The timing of the second mustard sowing (30th June) also coincided with the seed-setting of the adjacent weed

populations. Weed pressure resulted in lower yields than the First Biomass Assessment, in which weeds were not an issue. These reductions were also more dramatic in the blocks most affected (1 & 4), but this difference in variability is accounted for by the completely randomized block design (Payne 2007). The initial ANOVA showed the 1 cut treatment yielded significantly more DM and N than 2 cut ($P < 0.001$). Amos yielded significantly less DM than all other varieties and some differences were observed among the others ($P < 0.05$). Minor varietal differences were observed for N accumulation ($P < 0.01$), with Amos yielding significantly less N than Claret, Maro and Ruby. No significant interactions were observed in the ANOVA.

When weed DM was taken as a covariate the 'Covariate P' was highly significant for both mustard DM and N. Means were adjusted accordingly, but differences were found to be almost exactly the same of that of the ANOVA, apart from some minor changes to the significance lying between the varieties Claret and Ruby. No significant interactions were observed in the ANCOVA. These results are summarized in Table 5.9.

Table 5.9 Cut and variety treatment effects on mustard DM and N following different RC varieties – Second Biomass Assessment

Treatment	ANOVA		ANCOVA	
	Mustard DM t ha ⁻¹	Mustard N kg ha ⁻¹	Mustard DM t ha ⁻¹	Mustard N kg ha ⁻¹
1 Cut	0.82a	25.4a	0.71a	22.1a
2 Cut	0.40b	12.3b	0.50b	15.5b
<i>S.E.D. (129 df)</i>	0.09	3.03		
<i>P</i>	<0.001	<0.001		
<i>Covariate S.E.D (128 df)</i>			0.08	2.55
<i>Adjusted P</i>			0.007	0.009
<i>Covariate P</i>			<0.001	<0.001
Amos	0.21c	6.6b	0.37c	10.6c
Astred	0.52bc	16.5ab	0.48bc	15.3bc
Claret	0.79ab	24.8a	0.75a	23.4ab
Maro	0.87a	27.2a	0.77a	24.1a
Milvus	0.56abc	16.8ab	0.62abc	18.7abc
Ruby	0.66ab	21.0a	0.66ab	20.9ab
<i>S.E.D. (129 df)</i>	0.17	5.24		
<i>P</i>	0.005	0.002		
<i>Covariate S.E.D. (128 df)</i>			0.13	4.25
<i>Adjusted P</i>			0.004	0.012
<i>Covariate P</i>			<0.001	<0.001

Values followed by the same letter do not differ significantly at cited P value

5.6.3 Third Biomass Assessment (Mustard DM and N)

Weed biomass was found to be <5% of overall biomass in all plots in the Third Biomass Assessment. This was considered negligible, removed from the analysis and only ANOVA was applied. No significant differences were observed for DM in either treatment. Cut was found to be insignificant for N yield but differences were observed for variety ($P < 0.05$). Amos, Astred and Maro had among the lowest N yields and Ruby, Milvus and Claret among the highest. No significant interactions were observed. These results are summarized in Table 5.10.

Table 5.10 Cut and variety treatment effects on mustard DM and N following different RC varieties

Treatment	Mustard DM t ha⁻¹	Mustard N kg ha⁻¹
1 Cut	0.63ns	18.0ns
2 Cut	0.56ns	18.8ns
<i>S.E.D. (129 df)</i>	0.05	1.9
<i>P</i>	0.962	0.680
Amos	0.46ns	12.3b
Astred	0.58ns	17.7ab
Claret	0.64ns	24.0a
Maro	0.60ns	18.2ab
Milvus	0.59ns	18.9a
Ruby	0.69ns	19.3a
<i>S.E.D. (129 df)</i>	0.09	3.25
<i>P</i>	0.051	0.028

Values followed by the same letter do not differ significantly at cited P value

5.6.4 Total Mustard Yield (DM and N)

Total mustard yield was calculated as the sum of the DM and N yields for Biomass Assessments 1, 2 and 3. Only the H treatment was calculated, as volunteering constraints meant NH values could not be obtained at Biomass Assessments 2 and 3. DM and N yields were significantly higher in the 1 cut treatment ($P < 0.001$). Amos yielded the lowest DM and Ruby the highest, although the Ruby yielded the same as Astred, Claret and Maro. Ruby yielded more N than all other varieties, for which no significant differences were observed, except for Amos, which yielded less than all other varieties. These results are summarized in Table 5.11.

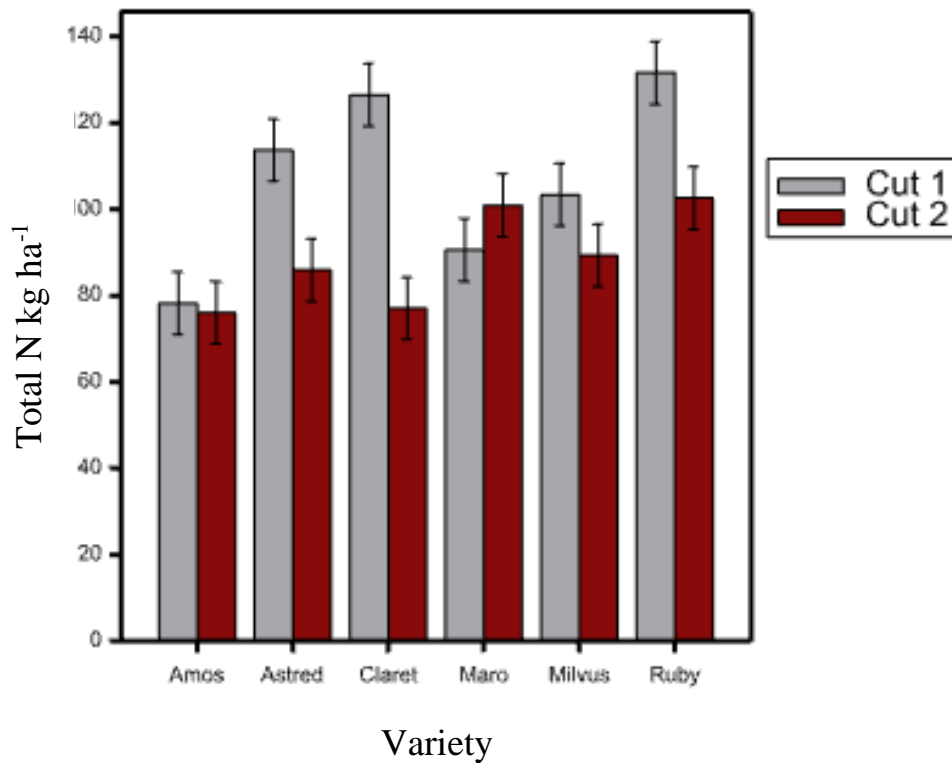
A significant interaction was observed between variety and cut in N accumulation. 1 cut resulted in more N than 2 cut in Astred, Claret and Ruby, but the treatment of cut was insignificant in Amos, Maro and Milvus. This interaction is summarized in Figure 5.26.

Table 5.11 Cut and variety treatment effects on total mustard DM and N in H treatment following different RC varieties (Total)

Treatment	Total DM t ha ⁻¹	Total N kg ha ⁻¹
1 Cut	3.33a	107.3a
2 Cut	2.71b	88.7b
<i>S.E.D. (129 df)</i>	0.14	4.19
<i>P</i>	<0.001	<0.001
Amos	2.48c	77.2c
Astred	3.12ab	99.8b
Claret	3.20ab	101.8b
Maro	3.13ab	95.7b
Milvus	2.77bc	96.4b
Ruby	3.41a	117.1a
<i>S.E.D. (129 df)</i>	0.24	7.25
<i>P</i>	0.008	0.023

Values followed by the same letter do not differ significantly at; * P<0.05, ** P<0.01, *** P<0.001

Figure 5.26 Effect of interaction between variety and cut on mustard N (Total)



5.7 Discussion (Wheat)

5.7.1 Establishment and Tillers

Different types of tillage can change soil physical properties and cause different establishment densities in wheat cultivation (Bhaskar *et al.* 2013, Naresh *et al.* 2013, Li *et al.* 2016). This is caused by differing degrees of change in soil aeration, porosity, aggregation etc. The wheat cultivation of Core Experiment 3 was subject to the same degree of tillage in all cases, meaning significant differences in establishment densities were not expected. Increased presence of RC residues were however expected in the NH treatment, as the H treatment rapidly terminated the RC crop, which could be more easily incorporated into the soil. The physical presence of living residues can hinder subsequent crop establishment (Känkänen *et al.* 2001), an issue cited as problematic for farmers in organic and CA systems (Oxner *et al.* 1997) and this effect may have caused the significantly lower establishment density observed in the NH treatment.

The RC incorporated in the NH plots was, as further assessments would indicate, ineffectively terminated and remained alive whilst buried under the surface of the tilled soil. The regrowth which then became the volunteering issue may have competed under the soil for water and nutrients, effectively acting as a weed. Herbicide applications are commonly cited as contributing to favourable establishment in wheat cultivation (Davis 1989, Charles *et al.* 1992, Cassal *et al.* 2013), but it is not clear if this results from weed control or the creation of more favourable conditions (i.e. rapid mineralization of residues causing increased nutrient availability for good seedling growth). If the lower rate of establishment in the NH treatment was indeed caused by competition from the unterminated clover, then the impact of herbicide was more likely weed control than creation of favourable growth conditions.

The differences in soil conditions and volunteering caused by the cultivation treatments did not significantly impact on tiller production. No significant treatment effects or interactions were observed. Some research predicts higher rates of tillering in response to weed pressures (Lemerle *et al.* 1996), but this effect was not observed in the NH treatment, indicating weed pressure at this growth stage may not yet have been problematic. Other research suggests lower establishment densities result in higher rates of tillering (Darwinkel 1978), but this was also not observed. Tiller initiation in spring wheat is also documented to be unaffected by N deficiency (Longnecker *et al.* 1993), meaning cultivation, cut and variety treatments could have provided more or less N for growth at establishment, but this may not have been consequential at the tillering stage.

5.7.1.2 Interactions

Significant interactions between variety/cultivation and variety/cut were observed in establishment. Astred was the only variety to establish at a significantly lower density in the NH treatment, which may have been caused by an increased survival rate incurred by its prostrate growth habit. 1 cut gave higher plant establishment in Milvus and Astred but 2 cut gave higher establishment in Maro. No significant differences were caused by cut in other varieties, and no significant interactions were observed between cultivation, cut and variety in establishment. The main consequence of the cut treatment was the volume of biomass (and therefore N) removed from the system, but given how N supply is unlikely to be of serious consequence at this assessment, and how all treatments established at acceptable densities of ~ 260 plants m^{-2} , differences observed for the interaction between cut and variety may have been negligible.

5.7.2 First Biomass Assessment

Modern spring wheat varieties are advertised as growing vigorously following establishment, with lower N demand than their winter sown counterparts (KWS 2016). Visual observations indicated all plots were quick to establish and tiller. Weeds were unable to establish, except for some incidence on the margins. Volunteer clover was a problem in the NH plots, but this was treated as a special case of weeds and documented accordingly using ANCOVA. No significant differences were observed for any treatment effect or interaction in the initial ANOVA, first applied to both DM and N yield assessments. This implies the problem of RC volunteering had yet to significantly constrain wheat growth, as no significant differences were observed between cultivation treatments. When the volunteer clover DM was taken as a covariate, significant effects ($P < 0.05$) were observed for variety in both DM and N, and no significant effects were observed for cultivation or cut.

Variability within the initial ANOVA in wheat DM and N yields across all treatments may have been increased by the presence or absence of volunteers. This variability may have increased the S.E.D., reduced statistical precision and rendered the results insignificant. The ANCOVA was carried out as a means to address this problem, as ANCOVA offers a greater degree of statistical precision by incorporating the nuisance variate of the volunteer clover as a covariate. The efficacy of ANCOVA in achieving this can be assessed through examination of the coefficient of variance (CV) for both analyses. The CV is the ratio of the standard

deviation to the grand mean and can be used as a yardstick to determine the variability existing between two sets of data (Little and Hills 1978). It is commonly cited as a percentage. Gomez and Gomez (1984) describe how high CV values are associated with data sets with high variability and low CV values are associated with low variability.

The CV value for variety in the wheat DM ANOVA was 13.4%, and the same value for the ANCOVA was 10.8%, indicating the incorporation of the covariate reduced variability in the analysis by ~25%. This reduction may have increased the statistical precision and allowed significant differences for the variety treatment to be observed in the ANCOVA of wheat DM yield. CV values for N yield were similar, the ANOVA was 11.2% but the ANCOVA was 9.6%. The apparent capacity of ANCOVA to reduce variability and increase statistical precision in these instances gives support to the decision taken to include it in the results of Core Experiment 3. However, it only found significant differences for variety, not cultivation or cut. This makes it unclear if these differences were caused by differences in the fertility-building capacity of the preceding variety, or different rates of volunteering during Core Experiment 3.

5.7.3 Second Biomass Assessment

The Second Biomass Assessment was carried out when the crop had reached GS 60-69 (Zadoks 1974), the stage at which anthesis takes place. This growth stage was selected because it follows the canopy establishment phase, where nutrient uptake peaks (YARA 2017). This makes it the optimal time for assessing differences in soil fertility between treatments. Stem number was also included as an assessment as this is also influenced by soil nutrient availability (Kuneva *et al.* 2016) and can be used as a mid-season proxy to assess crop performance. Stem number was assessed at GS 50, the initiation of flowering stage.

The results of the initial ANOVA showed highly significant differences ($P < 0.001$) occurred in the cultivation treatment; H outperformed NH in all assessments. 1 cut yielded more DM ($P < 0.05$) and N ($P < 0.001$) than 2 cut and no significant differences were observed for variety. The cultivation treatment did not affect tiller number at the First Biomass Assessment, but the H treatment produced significantly ($P < 0.001$) more stems at the Second Biomass Assessment, indicating the growth of tillers into stems was inhibited in the NH treatment between May 24th and July 13th. This may have been caused by the suppressive effect of the volunteer clover or a reduction in N availability caused by the slower mineralization rates incurred by the

cultivation treatment. Weed pressure is known to reduce tiller fertility in spring wheat (Mason *et al.* 2007), but stem production is also influenced by fertility dynamics. This assessment must be coupled with the results of the ANCOVA in order to understand which factor was more optimal in the significantly lower values of all variates in the NH treatment.

The ANCOVA adjusted means showed no significant differences for all treatments. The ‘Covariate P’ for the variety treatment was found to be insignificant, indicating minimal differences lay in weed pressure among the different varieties. A highly significant ‘Covariate P’ for both stems and wheat DM ($P < 0.001$) in the cultivation and cut treatments, indicated volunteering had a highly significant impact for these treatments and means adjustments were made accordingly. Stem number, DM and N yield means were adjusted and no significant effects of cultivation were shown, indicating the differences observed in the initial ANOVA were more likely the result of inhibitive volunteering in the NH plots, not differences in soil fertility resulting from the cultivation treatment. Adjustments to the means of stems at the cut treatment appeared marginal, but in contrast to the initial ANOVA, no significant differences were observed. 2 cuts in the fertility-building phase may then have increased capacity for volunteering during Core Experiment 3. For DM however, again adjustments were made but a larger S.E.D. resulted in no significant differences observed, which contrasts with the initial ANOVA results, in which significant higher DM yields were observed in the 1 cut treatment. A similar effect was observed in N yield.

To view the results of both ANOVA and ANCOVA side by side in this fashion is an attempt to assess the impact of the covariate (volunteer clover) on the overall results. Despite the endorsement of ANCOVA by several authors (Gomez and Gomez A 1984, Yang and Juskiw 2011), studies actually using it for this purpose remain uncommon. Some authors cite the omission of ANCOVA in university statistics courses as a possible reason for this (Yang and Juskiw 2011), but the majority of agronomists may have planned and carried out their research with enough diligence to limit the need for an error-reducing statistical method. There is, however, some research in which authors found a similar use for ANCOVA.

For example, in a study on contrasting weed biodiversity in conventional and organic systems Hyvonen *et al* (2003) used individual plant number as a covariate, with species number as the main variate to adjust means of species number between systems, improving the precision of the analysis. Another study documenting the impact of N leaching on microflora in livestock-adjacent rivers used stream depth as a covariate and found the adjusted means improved the

model fit and allowed for assessment of this extra variable (Kyriakeas and Watzin 2006). The use of soil N heterogeneity as a covariate was also described by Thole *et al* (2013) as effective in increasing the accuracy of grain yield assessments. Given how ANCOVA is documented as increasing statistical precision by accounting for unforeseen variables, the question then remaining for the results of Core Experiment 3, is how effective was the clover covariate in determining the impact of the weeds growth on the overall experiment?

This is addressed through examination of the CV values and adjusted means. Unlike the First Biomass Assessment, the CV values at the Second did not appear to change much between ANOVA and ANCOVA (all analyses gave CVs of 23-25%). These CVs were also higher than that of the first, which ranged from 10-14%. Higher variability at the Second Biomass Assessment may have been incurred by more significant volunteering, but at this instance ANCOVA did not appear to reduce variability. High S.E.D. values concomitant with high variability may then have given insignificant differences between treatment means for cultivation and management (variety was found to be insignificant in the ANCOVA). However, critical value calculation is not the only component of ANCOVA adjustment, and the adjusted means themselves must also be taken into account when interpreting results.

Inferences regarding ANCOVA adjusted means and their interpretation are not straightforward. Some authors cite covariate-adjusted means as the most accurate interpretation of data when all assumptions are satisfied (Klockars and Beretvas 2001), whilst others warn of how treatment effects on the covariate itself can distort the adjustment process (Clason and Mundfrom 2012). This warning may be worthy of consideration for the results of this ANCOVA, as although interpretation up to this point has been premised on the impact the covariate had wheat yields, it is not impossible for the wheat growth to have had significant impact on the covariate itself (due to competition perhaps). If this did occur, and if it was highly significant, it may negate the ANCOVA results for the Second Biomass Assessment, and possibly even the First, if this occurred at this assessment as well.

This seems unlikely, given how field observations indicated volunteering in the NH treatment appeared vigorous and inhibitive of wheat growth in all cases. Impressions drawn from these observations are bolstered by the highly significant differences ($P < 0.001$) between H and NH treatments in the initial ANOVA for stems, DM and N (H yielded around 1.7 times more than NH in all three). The adjustment following the ANCOVA reduced these differences, which implies the main cause of the differences in the ANOVA was the unaccounted for covariate.

The ANCOVA adjusted means can be taken as a more accurate reflection of what would have happened without the issue of the volunteer clover.

5.7.3.1 Interactions

In the ANOVA, Ruby yielded significantly more DM and N when only 1 cut was applied. This interaction was only observed in Ruby and was also observed for wheat DM following ANCOVA adjustment. The results of Core Experiment 1 showed 1 cut was significantly less susceptible to SMN leaching than 2, which may have contributed to this difference, but one would expect this difference to occur in all varieties if 1 cut was indeed optimal. Ruby was documented in Core Experiment 1 to be more resistant to clover rot than most other varieties and was accordingly managed differently; the aboveground biomass was not removed by raking prior to winter because it was not heavily infected. This allowed Ruby, along with Astred (the other resistant variety), to retain significantly more SMN than other varieties throughout the winter period. The 2 cut treatment also likely resulted in less incorporated biomass than 1 cut, as biomass removal occurred twice and not once. These factors were more likely to be the cause of the significant interactions between cut and DM/N yields at the Second Biomass Assessment, although it does remain unclear why this interaction was observed in Ruby and not Astred, given how these varieties were similar in performance, management and SMN accumulation prior to Core Experiment 3.

5.7.4 Final Biological Harvest

The senescence of the volunteer clover prior to Final Biological Harvest meant ANCOVA could not be undertaken for all assessment at this stage, and only the results of ANOVA are discussed here.

5.7.4.1 Main Effects on Plant Height, Ear Number and DM yields

Taller wheat plants are associated with earlier flowering (Lin *et al.* 1995) which allows more time for grain filling. These plants may however be more at risk of lodging. Although rainfall in August was comparable to the long-term average, it occurred in sparse, heavy downpours (discussed previously in Chapter 4 Discussion Section and illustrated by Figures 2.1-2.3). Some authors warned of lodging risks in UK cereal production in response to these weather conditions (AHDB 2016a). Although not assessed in a scientific sense, lodging was not observed at any point throughout the growing season. Maro and Milvus produced the shortest wheat, whilst no significant differences were shown for the other four varieties. This was

thought to be insignificant for lodging risk. Height differences also appeared to have little impact on DM yields, as no significant effects of variety were observed for these variates. The role of wheat dwarfing in increasing yields historically is well documented (Law *et al.* 1978), but the relevance of small differences in height in modern varieties is likely to be less important. The difference between the shortest and tallest variety was ~5cm, a difference which may be trivial.

High ears m^{-2} is associated with increased yields in spring wheat (Zhang *et al.* 2007). Ear number is also known to be significantly limited by drought following anthesis (Weldearegay *et al.* 2012). Ear formation took place in early July, a time in which rainfall was below the long term average and this may have limited ear number across all treatments, as the range of 265-341 ears m^{-2} is below the optimal number of 460 described for UK growth conditions by AHDB (2015). No significant differences were observed for all treatments. The significantly higher number of stems documented for the H treatment at GS 50 failed to produce significantly more ears at the Final Biomass Assessment, indicating the limitation on growth associated with the NH treatment took place at this stage. This limitation was also more likely caused by volunteering than intrinsic differences in soil fertility.

Highly significant ($P < 0.001$) differences were shown for the cultivation treatment in ear DM, implying although neither cultivation treatment produced significantly more ears, the ears produced by the H treatment were significantly larger. Less than 20% of ear DM is thought to be chaff (AHDB 2015) and therefore most of the DM in the ears at Final Biological Harvest should be composed of grain. Grain filling is of course limited by soil N supply (Mathura *et al.* 2012), but the weed competition of the volunteer clover was also likely to be a significant factor in the reduction of ear DM in the NH treatment. Straw DM was also significantly lower in the NH treatment, indicating limitations on growth in this treatment were not confined to the post anthesis growth stage, but had begun early at stem formation. The sum of ear and straw DM (total DM) gave a highly significant ($P < 0.001$) two-fold difference between H and NH treatments, but no significant differences for cut and variety. 1 cut yielded significantly more DM than 2 at the Second Biomass Assessment but this was not repeated at Final Biological Harvest.

The increased statistical robustness of the split-plot design and the high variability associated with the inhibitive effect of volunteering, may have generated high S.E.D. values and curtailed the capacity of the analysis to describe significant differences within this treatment.

Considerable ranges lie between the highest and lowest means for some variates, notably number of ears (257 for Amos and 341 for Ruby) and total DM (6.51 for Amos and 8.04 for Ruby), but no significant differences were described. It is possible this was caused by the experimental design, as some authors cite the split-plot design as being prone to making Type 2 Errors (Jirangrat *et al.* 2014). Others describe how the precision for the measurement of the effects of the main-plot factor, in this case variety, is sacrificed to improve that of the subplot factor, in this case cultivation, (Gomez and Gomez A 1984). These attributes of the design, in combination with the high variability of the cultivation treatment, might have caused Type 2 errors in the variety assessment, but it is difficult to say for certain from the data.

Interactions

Two cuts resulted in significantly more ears m^{-2} when no herbicide was applied but this interaction was not observed for 1 cut, for which no significant differences were observed. This interaction was not observed at the Second Biomass Assessment under both ANOVA and ANCOVA and was likely caused by an increased rate of volunteering in the 1 cut/NH treatment, as no significant differences were observed for the H treatment under both 1 and 2 cuts. Forage legumes may have an optimal number of cuts to maximize yields in the subsequent years of cultivation (Cupina *et al.* 2017), and it may be the optimal number of cuts for RC is 1, as here it appears volunteering and therefore crop regrowth the following year was maximized in the 1 cut treatment.

5.7.4.2 Main Effects on Grain Yield, Thousand Grain Weight and Harvest Index

As with yield variates, highly significant differences were observed for grain variates ($P < 0.001$) for the cultivation treatment, but little difference for observed for other main treatments. Grain yield for all treatments was considerably lower than the reported range for wheat from other institutions in 2016, for example AHDB (2016b) reported a range of 7.8-9.6 t ha^{-1} for spring wheat, whilst DEFRA (2016a) reported yields as high as 14.6 t ha^{-1} , although this figure was likely based on winter wheat, for which higher grain yields are predicted. These figures reflect commercial production, in which mineral fertilizers are used to increase yields. Low-input systems are associated with yield gaps in wheat production (Aggarwal and Kalra 1994, Gooding and Davies 1997, Kravchenko *et al.* 2017) and as Core Experiment 3 could be considered low input, this effect may have caused the limitation in grain yield in all treatments.

As with DM yields, it is likely the highly significant differences in the cultivation treatment were more a result of volunteering in the NH plots and not intrinsic differences in soil fertility. TGW is associated with increased agronomic performance (Fuller 2007) and grain yield (Williams and Sorrells 2014) and this association was also observed in Core Experiment 3. However, as with grain yield, the significantly higher TGW of the H treatment was more likely due to the lack of weed pressure from clover volunteering. Weed pressure is also known to decrease HI in wheat (Dhirender *et al.* 2016) and the HI in the NH treatment would be considered very low by some reported ranges (Abdul *et al.* 2015, Anees *et al.* 2015). This implies the grains in the NH treatment failed to fill and mature to the same degree as those in the H treatment and grew into a smaller percentage of the overall harvested biomass.

Significant varietal effects were also observed for TGW. The highest TGW for variety appeared to correlate with the corresponding variety mean for grain yield (Ruby had the highest TWG and grain yield and Astred among the lowest in both), but no significant varietal differences were observed for grain yield. TGW may have had less variability, allowing for smaller S.E.D. values and a higher likelihood of finding significance in treatments, whereas the high variability of other variates may have lowered the chances of observing significant differences. This is evidenced by the CV values, which were 2.9% for TGW but 21.9% for grain weight, meaning grain weight was roughly seven times more variable than TGW. The increased variability further supports the idea that the experimental design and high variability associated with the volunteering may be incurring Type 2 Errors in the variates which were highly variable.

Interactions

Amos was the only variety to yield significantly more grain in the 2 cut treatment. No significance was observed for this reaction in all other varieties, although Ruby 2 cut yielded more than that of Astred and Maro. Amos had significantly curtailed regrowth in response to cutting in Core Experiment 1 due to its longer photoperiod requirement. Amos was among the highest for overwinter leaching in Core Experiment 1, but Maro and Milvus showed similar interactions between cut and variety for SMN losses (see Table 3.12 and Figures 3.5-3.7). The difference observed in grain yield for Amos in Core Experiment 3 could then not be explained by lowered availability of SMN in the 2 cut treatment alone. The reduced yields observed for Amos in Core Experiment 1 may however have been associated with increased root growth, or a reduced demand for non-N nutrients, such as P or K. These hypotheses are supported by

Volenc *et al* (1996) and Li *et al* (1996), who described how forage legumes transfer root reserves to newly growing shoots following cutting. This transfer may have occurred to a lesser extent in Amos, due to its reduced regrowth, and this may have left behind larger root systems for mineralization throughout Core Experiment 3.

Read *et al* (2014) described how forage legumes increase their phosphorus uptake with each consecutive cut, but this is contradicted by Wiersma *et al* (1998), who found the opposite for RC specifically. Forage phosphorus content is likely influenced by soil phosphorus levels and it may be unwise to draw conclusions from trials in different locations. However, given how low soil P was at the beginning of the trial (7-13ppm see Table 2.5), if increased phosphorus uptake was associated with higher yields in the second cut of Core Experiment 1, and Amos regrowth was associated with less phosphorus uptake, reduced phosphorus availability in the bioassay cultivation may then have caused this reduction in grain yield for the only variety with a significantly reduced second cut.

5.7.4.3 Main Effects on Grain N, NHI and Protein

Main effects on N variates generally mirrored those of grain variates. Highly significant differences were observed for cultivation in all variates and varietal differences were minimal, although they were observed in grain protein.

Grain N variates are key components of grain quality, particularly for the bread makers (Banziger *et al.* 1994). The total N uptake of the bioassay crop can also be used as a proxy to determine the N legacy of the preceding RC cultivation. Like most small grains, wheat is thought to take up the majority of its N prior to anthesis (Austin *et al.* 1977). This N is held in the leaves prior to flowering and is then transferred to the grain following pollination. The results of the ANOVAs from the Second and Third Biomass Assessment appear to show an increase in total N, which contradicts this conventional wisdom. Although total N did not increase between these assessments in the NH treatment, the H treatment saw an increase of ~20 kg ha⁻¹ or 25%, implying the significantly higher grain N of the Third Biomass Assessment was the cause of increased N uptake as well as more efficient N partitioning. This is uncommon, as many authors cite anthesis at the point at which >90% of N uptake has taken place (Loffler *et al.* 1985, Clarke *et al.* 1990), but some findings indicate N uptake may proceed beyond this stage if soil conditions are favourable, or if drought conditions impeded N uptake in the vegetative phase (Campbell *et al.* 1977, Heitholt *et al.* 1990). Although rapid mineralization of

herbaceous aboveground biomass would be expected in the H treatment, it is possible further mineralization of suberized taproots proceeded more slowly, and the N released from this mineralization may have been responsible for the post-anthesis N uptake observed.

Almost no change took place in the total N uptake in the NH treatment between Biomass Assessments 2 and 3. The reduction of total N uptake in the NH treatment resulted in significantly reduced grain N ($P < 0.001$), which in turn gave a significantly lower NHI and grain protein ($P < 0.001$). Total N uptake was clearly limited in the NH treatment. NHI can be used to assess the efficiency of N partitioning in the grain filling stage (Fageria and Baligar 2003), meaning these results indicate the NH treatment was limited by both N uptake in the canopy establishment phase and the grain filling, partitioning phase. Given the highly significant differences observed for this treatment, it seems likely the differences observed for the cultivation treatment were the result of competition from RC volunteers and not intrinsic soil fertility differences associated with the treatment. This is corroborated by Korres and Froud-Williams (2002) who demonstrated how weed pressure reduces N uptake in the canopy establishment phase, and Yin and Schapendonk (2004), who demonstrated how weed pressure limits the capacity of wheat crops to adequately transfer N from stem and leaf to grain.

Grain protein showed highly significant differences ($P < 0.001$) for all main treatments. The reported range was 10.7-11.9%, which is below the 13% desired by British bread makers (AHDB 2017), however low protein content is to be expected in low-input systems (Rieux *et al.* 2013). Higher total N uptake and more efficient partitioning in the H treatment likely resulted in the highly significant differences observed in cultivation. Higher grain protein in the 1 cut treatment may have been caused by higher rates of soil N resulting from the incorporation of more aboveground biomass to the soil following cultivation, or by increased volunteering incurred by the 2 cut treatment. The effect of variety on grain N was also significant, with wheat following Ruby and Milvus containing among the least protein and wheat following Maro and Claret containing among the most. Despite containing less protein than all other varieties, wheat following Ruby was consistently the highest yielding variety for other variates (e.g. total DM and N; albeit insignificantly, but as previously described high variability and experimental design may have incurred Type 2 Errors). Wheat following Ruby may have achieved these yields through increased N use efficiency, which in turn may have reduced grain protein content, as these traits are thought to be negatively correlated (Latshaw *et al.* 2016).

Interactions

Interactions between variety and cut on grain and total N were similar to that of grain yield. No significant differences were observed for all varieties except for Amos, which yielded significantly more grain and total N in the 2 cut treatment. This may have been caused by the same differences in Core Experiment 1 as previously described.

5.8 Discussion (Mustard)

Although mustard is not commonly grown with high rates of fertilizer applications, the importance of favourable soil N levels in achieving high yields is recognized (Roop *et al.* 2014). Mustard performance is best assessed through its seed yield, TGW and oil quantity/quality (Patel and Meisheri 1997), but for the purposes of Core Experiment 3 it was considered preferable to assess DM and N yields without taking the crop to full maturity. This was to allow for three individual crops, which would place more demand for N on the soil and give a more accurate assessment of the soils fertility following 1 year of clover cultivation under different management. Soil N levels are known to influence mustard vigour, branching and plant height (Keivanrad and Zandi 2014), which are good predictors of DM and N yield. These variates, over three crops, were then decided as the best means to assess the fertility of the soil following Core Experiment 1.

5.8.1 First Biomass Assessment

Significant differences were observed for most main treatments in the first mustard crop, and some significant interactions were also observed. As with the wheat cultivation, problems arose with volunteering in the NH plots. Visual observations indicated volunteering had been less severe in mustard than wheat. This may have been caused by the faster establishment and growth of the mustard. DM and N yields were then found to be significantly higher ($P < 0.001$) in the H treatment, indicating that although the volunteering appeared less severe, it may have restricted growth in the NH plots. Singh *et al* (2013b) describes mustard yield losses of 25-45% when weeds occur; the range depending on the intensity, timing and diversity. The difference between cultivation treatments in the first mustard crop appeared to fall within this range, implying these differences were the result of growth inhibition caused by volunteering in the NH plots. The finding of an insignificant Covariate P in the variety treatment contradicts this hypothesis, as this implies the volunteering did not have a significant impact on mustard

growth. However, the ANCOVA does not account for belowground weed competition, which may have been more operable in the early stages of growth and is known to significantly impact on crop production (Satorre and Snaydon 1992).

One cut yielded more DM ($P < 0.05$) and N ($P < 0.001$) than 2 cut. This was likely caused by the capacity of the 1 cut treatment to reduce winter N losses (documented in the SMN assessments of both Core Experiments 1 and 2). This treatment was also likely to contribute more aboveground biomass to the soil, as what became the removed second cut in the 2 cut treatment was simply left to senesce in the 1 cut. No significant differences were observed for DM in variety, but superficial differences did appear to lie between Ruby and Astred ($> 1.8 \text{ t ha}^{-1}$) and the other varieties ($< 1.55 \text{ t ha}^{-1}$). As with the wheat DM yields in some instances, variability associated with volunteering problem and the statistical rigour of the split-plot design within completely randomized blocks may have generated high S.E.D. values and incurred Type 2 Errors. The data from Core Experiment 1 regarding SMN accumulation and retention predicts higher yields of these varieties, as they were significantly less susceptible to winter leaching ($P < 0.001$) than all other varieties. However, although highly significant, the differences in leaching were numerically small (a range of 13kg for Ruby and 22kg for Amos). Differences in N yield for variety were comparatively small but significant, unlike variety. Ruby and Astred yielded among the highest ($P < 0.05$), indicating differences in available soil N following the winter period may have been influential in causing significant differences in N yields, but not in DM.

The ANCOVA showed the covariate to be non-significant for cultivation, implying the volunteering did not impact on mustard growth in the NH plots, but as previously mentioned belowground competition for water and nutrients may have reduced mustard growth and remained unaccounted for by the ANCOVA. A highly significant Covariate P was observed for cut, but means adjustments did not change the results of the initial ANOVA. This implies that volunteering had a significant impact on mustard growth in the cut treatment (1 cut yielded more DM and N than 2), but this significant impact was accounted for in the initial ANOVA, implying differences in means were significant but marginal.

5.8.1.1 Interactions

Significant interactions were observed between variety and cut in both DM and N yields. The 1 cut treatment yielded significantly higher than 2 cut in Ruby, but this interaction was not observed in all any other variety. The SMN accumulation data showed Ruby significantly

retained significantly more SMN under 1 cut than 2 following the winter, whereas for Astred this difference was insignificant (see Figure 3.7). This difference may then have been responsible for the lack of significance seen for mustard DM at the First Biomass Assessment in the combined Astred/1 cut treatment. A similar interaction was also seen for N yield, except here Astred and Claret also yielded more N under 1 cut. The difference was larger in Ruby and Astred than Claret, indicating the interaction was stronger in these varieties. Milvus yielded significantly more N under 2 cut, which may make the data underpinning this interaction inconclusive, but as Figure 3.7 indicates, the overall effect of cutting on N losses over the winter period was insignificant, implying the biomass retained in the 1 cut treatment was more influential than SMN in increasing yields of the subsequent bioassay crop.

5.8.2 Second Biomass Assessment

Cutting back the RC volunteers in the NH plots prior to planting the second mustard crop only stimulated vigorous growth and the mustard was unable to establish amid the newly growing volunteers. The only means to address this problem would have been a herbicide application (which would have negated one of the main treatments) or further rotavation (which would have been impractical given the size of the plots, and likely ineffective given it had already been shown to be so). Even if termination could be effectively applied, the volunteering throughout the growing season of Core Experiment 3 may have had some impact on soil fertility, thus negating the fertility-building treatments of variety and cut applied throughout Core Experiment 1. Accordingly, it was decided to abandon the NH treatment in both the second and third mustard crops and treat the remainder of the experiment as a two factor experiment (variety and cut), which would at least maximize precision for the measurement of the effects of the main factors from Core Experiment 1 (Gomez and Gomez A 1984).

Weed issues were also problematic throughout the second mustard crop cultivation. The establishment of *Cirsium* populations at the margins is a common problem in systems where herbicides are not routinely applied to control weeds (Lundkvist *et al.* 2008). Efforts were made throughout the trial to control such populations, but despite the creation of a weed-free border of >1m, weed populations were established in trial plots throughout the cultivation of the second mustard crop. Visual observations indicated weed populations had finished flowering and entered seed production, a stage at which invasion capacity in arable land can increase (Recasens *et al.* 2005).

The initial ANOVA showed 1 cut yielded significantly more DM and N than 2 ($P < 0.001$), but the yields were less than half that of the first mustard crop. This reduction was likely caused by weed pressure rather than declining soil fertility. When the weed DM was taken as a covariate it was found to be highly significant for both treatments (Covariate $P < 0.001$), but 1 cut was still found to yield significantly more than 2 for both DM and N. This implies although the weed DM was significant, the treatment effects would have been the same even if weed pressure had not been an issue. The ANCOVA had a similar effect on variety; adjustments were made but almost no changes occurred in the statistical significance between means.

5.8.3 Third Biomass Assessment

No significant differences in DM were observed for either treatment. Cut was insignificant in N yield and marginal differences were observed for variety (Amos yielded less N than Claret, Milvus and Ruby and no significant differences were observed for all other varieties). Growth conditions may have reduced yields, as planting occurred on August 22nd when the favourable photoperiod of the growing season was waning. However, mustard is typically grown as a cover crop because of its capacity to accumulate biomass in short day conditions (Jackson, *et al.* 1993) and strong limitations from environmental conditions were therefore not expected. Such conditions are thought only to severely limit seed yield (Rabbani *et al.* 1997), which was not relevant for the purposes of Core Experiment 3. No significant treatment effects were observed for DM yield, but significant effects were observed in N yield for variety. Amos yielded significantly less than Claret, Ruby and Milvus and no differences were observed for the other five varieties. Amos had consistently been the lowest yielding variety for N throughout Core Experiment 3, so it would be expected to yield the lowest amount of N at the Third Biomass Assessment. As N yields were the same for all other varieties at this assessment, their contributions to soil fertility are harder to determine.

5.8.4 Total Mustard Yield

Total mustard yields discussed here only refer to the H treatment, as the volunteering in the NH treatment in the First Biomass Assessment prevented all future planting within those plots. It is challenging to compare these results with other research, as mustard is most commonly grown as a winter cover crop (Singh *et al.* 2009, Kapila *et al.* 2016) and such cultivations generally take place following a harvest and do not require herbicide applications. The literature is also somewhat skewed toward yield data for this green manure use, which may not

be appropriate for comparison because it refers to winter rather than summer growth (Goffart *et al.* 1992, Bakšiene *et al.* 2009, Macák *et al.* 2012). Other research on mustard yields is carried out in breeding trials, but these studies tend to focus on individual plant gDM (Yesh *et al.* 2008, Singh *et al.* 2015), which also makes comparison with Core Experiment 3 difficult. Summer cultivation research also tends to concern mustard grown for seed or oil and therefore only involve the cultivation of one crop, not three (Bazewicz-Wozniak and Wach 2012, Hardev *et al.* 2013). The DM yields of these studies will also be influenced by the lignification process when the crop shifts from vegetative to reproductive phase, a process avoided in Core Experiment 3 by sampling biomass before the reproductive phase. For example, Bazewicz-Wozniak and Wach (2012) documented a DM yield of 5.2 t ha⁻¹ and an N yield of 113 kg ha⁻¹ for mustard in Lithuania, but it is difficult to compare this yield to that of Core Experiment 3 because it involved a crop taken to full maturity, where higher DM yields would be expected anyway.

Results may then be best viewed in isolation and in reference to the treatments applied. 1 cut yielded significantly ($P < 0.001$) more DM and N than 2 cut, a result predicted by the increased capacity of 1 cut to mitigate SMN leaching in the winter period (see Tables 3.12 and 4.6). The capacity to limit leaching is likely further enhanced by the increased presence of aboveground biomass, as the 2 cut treatment involved the removal of more biomass. This also meant that more biomass was available to be green manured prior to cultivation. The N released from mineralizing biomass was likely more important than the SMN accumulated, as the total N yield range was 77-117 kg ha⁻¹, considerably higher than that deposited in Core Experiment 1.

Varietal differences were minimal for total DM yields. Ruby was the highest yielding variety but was equal to that of Astred Claret and Maro. Amos yielded less DM than all other varieties, a result which may have been caused by its reduction in regrowth in response to cutting in Core Experiment 1. A high susceptibility to clover rot was also observed in Amos during Core Experiment 1 and the control treatment of removing the infected biomass may have reduced the amount of N that could be returned to the soil prior to the cultivation of the mustard crops, which also may have reduced yields. Total N yields tended to follow the same pattern except Ruby yielded 117 kg ha⁻¹, significantly higher ($P < 0.05$) than all other varieties and Amos yielded less than all other varieties.

5.8.4.1 Interactions

The significant interaction between variety and cut showed Ruby, Claret and Astred yielded significantly more N in the 1 cut treatment. Ruby and Astred were significantly more resistant to clover rot in Core Experiment 1, which meant the control treatment of removal the infected tissues was not applied to these varieties (as the aboveground biomass was not as infected as the other, less resistant varieties). The treatment of 1 cut in these varieties was then more likely to provide cover from the winter rain and retain N for incorporation into the soil in Core Experiment 3. This explanation however cannot suffice, as Claret also yielded significantly more N in the 1 cut treatment. Claret was not as resistant to clover rot as Ruby and Astred, but also not as susceptible as Milvus and Amos (Table 3.11). It is likely higher yields in the 1 cut treatment were caused by the larger amount of biomass this treatment retained for incorporation prior to Core Experiment 3, but here it remains unclear why this interaction was observed in Claret but not in Amos, Maro or Milvus, given how its performance in Core Experiment 1 was similar in most assessments.

5.9 Conclusions

- The RC taproot can present challenges in termination and if tillage cannot be applied to an adequate depth then herbicides should be applied
- ANCOVA can be used to supplement ANOVA in determining the effect RC volunteers had on a subsequent crop
- Volunteer RC is inhibitive of growth in both wheat and mustard cultivation, particularly in the stem formation stage of wheat
- Cutting strategy and variety selection had no significant effects on the Final Biological Harvest in wheat cultivation, but experimental design and variability caused by unforeseen problems may have caused this
- Cultivation had highly significant impact on almost all variates in the Final Biological Harvest of wheat cultivation, but this was more likely the result of inhibitive volunteering than intrinsic differences in soil fertility
- Cultivation had highly significant impact on mustard DM and N yields in the First Biomass Assessment, but again this was more likely caused by volunteering
- Mustard crops are prone to weed infestation in the mid-summer period, but when inputted as a covariate these weeds did not appear to alter the results
- 1 cut yielded significantly higher total mustard DM and N. Mustard following Ruby yielded significantly more total N than all other varieties. This was likely caused by the disease resistance and higher SMN associated with this variety in the fertility-building phase of Core Experiment 1

CHAPTER 6 - Discussion & Conclusion

6.1. Introduction

Mineral fertilizer synthesis is powered by fossil fuel energy (Woods *et al.* 2010) and its use can harm environmental integrity and human health (Galloway *et al.* 2008, Dungait *et al.* 2012). The role for legume cropping in providing more sustainable sources of N is apparent (Peoples, *et al.* 1995, Schmidt, *et al.* 1999), but what remains unclear is how this is most effectively realized. RC can provide a large amount of N to cropping systems, contribute a suite of ecosystem services (see Table 1.9) and provide a high protein forage for livestock (Eickler, *et al.* 2011). The cultivation of RC, particularly in rotations with high N-demand crops like cereals, can therefore contribute to sustainable intensification in a multidimensional manner; a quality described as desirable by many authors (Godfray, *et al.* 2010, Pretty, *et al.* 2010, BBRSC 2017).

The goal of the present study was to investigate the effects of variety selection, cutting management and cultivation techniques on the capacity of RC to contribute to soil fertility (Chapters 3 and 4). Six varieties were selected and 2 cut treatments were applied (1 and 2). Subsequent crops of mustard and wheat were used as bioassays to determine what effects, if any, these treatments had on soil fertility (Chapter 5). The inclusion or omission of herbicide prior to cultivation was also included as a treatment. This chapter will discuss the results of these experiments in two sections: the fertility-building phase and the bioassay cultivation phase. The effect of the main treatments and interactions will be described in both sections and conclusions and recommendations will be made on the how RC-use in fertility-building can be optimized based on the results of these experiments.

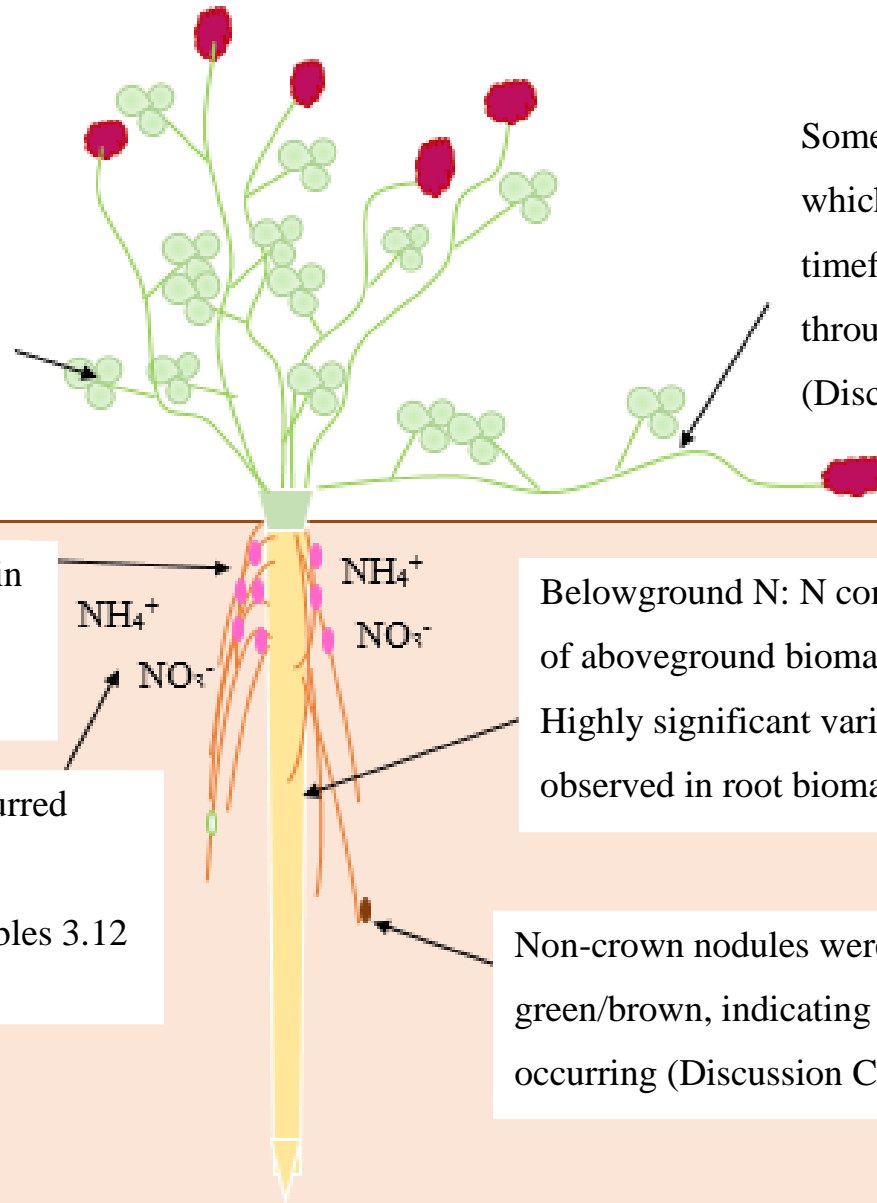
6.2 The Fertility Building Phase

The contribution of RC crops to soil N can be split into three components: aboveground biomass, belowground biomass and accumulated SMN. Variety selection, cutting management and subsequent cultivation may optimize or diminish these components. These components are illustrated in Figure 6.1 with reference to Chapters 3 and 4.

Figure 6.1 Diagram of fertility-building with RC (Core Experiments 3 and 4)

Aboveground N: Up to 83 kg ha⁻¹ in Year 1 (Table 3.6) and up to 544 kg ha⁻¹ in Year 2 (Table 4.2)

Some varieties are stoloniferous, which may extend mineralization timeframes and limit winter leaching through increased ground cover (Discussion Chapter 3)



Crown nodules were numerous and pinkish in colour, indicating fixation was taking place (Discussion Chapter 4)

SMN Accumulation: Deposition occurred over both years, increase were more significant in Year 1 than Year 2 (Tables 3.12 and 4.6)

Belowground N: N content thought to be 42% of aboveground biomass (Peoples *et al* 2012). Highly significant varietal differences were observed in root biomass in Year 2 (Table 4.4)

Non-crown nodules were sparse and green/brown, indicating no fixation was occurring (Discussion Chapter 4)

6.2.1 Aboveground Biomass

6.2.1.1 Management

This study sought to utilize N accumulation in the aboveground biomass as a proxy to assess the fertility-building capacity of the trialled varieties. This is a common strategy in the literature concerning fertility-building with RC (see studies documented in Tables 1.4-1.6) and other legumes such as white clover, lucerne and sainfoin (Hume *et al.* 1985, Gault *et al.* 1995, Dhamala *et al.* 2017). RC is cited as accumulating more N in the aboveground biomass than other forage legumes (Frame, *et al.* 1998), but only the N yields of Core Experiment 2 could be deemed high when compared to the literature; a range of 400-540 kg ha⁻¹ was reported whereas the range is typically 30-300 kg ha⁻¹ (Huss-Danell and Chaia 2005). It is however possible the recorded N yields of the present study, although statistically robust, were artificially raised by the small plot size, a documented problem in research station field trials (University of Reading 1998). N yields were clearly limited by weed pressure in the initial stages of Core Experiment 1, but the capacity of the crop to smother weeds in response to cutting was notable. This was particularly the case in Amos, which yielded significantly less N and DM ($P < 0.001$) than all other varieties at cut 2 due to its limited regrowth. The capacity to outperform weeds in response to cutting likely contributes to the high N yields associated with RC aboveground biomass.

The relevance of aboveground N yields for soil fertility is determined by management and the weather conditions in which management is applied. Management of cut and remove or cut and mulch will determine if the N accumulated is returned to the system. Weather conditions will determine how much is returned and how much is lost via leaching and denitrification. Soil conditions will also play a role in this (Gaines and Gaines 1994). For example, high bulk density may reduce leaching susceptibility and clay soils tend to be less prone to leaching but more prone to denitrification from the soil surface (Allingham, *et al.* 2002, Posmanik *et al.* 2017). This study applied 1 or 2 treatments of cut and remove in the understanding that the value of RC cuttings is most often realized as a high-protein forage in the real world, but these treatments were also selected with subsequent soil fertility in mind. The decision to remove cuttings may appear superficially counterintuitive, as this removes N from the system, but mulching cuttings can limit regrowth (Loges, *et al.* 1999, Moyo, *et al.* 2011) as the mulch effectively acts like a weed. Limitations in growth are undesirable as they decrease both DM/N yields and biofixation (Deprez, *et al.* 2004). Any growth that does place is also likely to use the senescing mulch as its N source; to limit the transfer of sugars to nodules and use the 'free'

source of N. This process is known as N recycling and has been shown to occur in mulched RC leys (Dahlin and Stenberg 2010a, Dahlin, *et al.* 2011).

Recycling is undesirable in fertility-building, at least throughout the growing season, in which the majority of annual biofixation takes place (Ledgard and Steele 1992), but this may not hold true for the winter period in which growth and fixation is limited. Documentation of SMN accumulation in Chapters 3 and 4 showed the 2 cut treatment was significantly ($P < 0.001$) more susceptible to N losses than 1 cut. This was likely caused by a combination of lower N addition (as more N was removed in the 2 cut treatment) and increased soil exposure (as more biomass removal resulted in more exposed soil in the 2 cut treatment). The N recycling caused by not performing a second cut may then be preferable to the increased leaching of the 2 cut treatment, but it would depend on the time at which the cut was to be applied.

Core Experiment 1 saw much later cutting dates than Core Experiment 2 (October 12th and August 10th respectively). The earlier flowering associated with the prior establishment in Core Experiment 2 required earlier cutting dates in order to offset the decrease in biofixation which occurs when the crop shifts from vegetative to reproductive growth (Voisin, *et al.* 2002). N recycling would likely have been minimal in Core Experiment 1, given how little growth would be expected from 12th October onwards, but it may have affected the regrowth of Core Experiment 2, given how some degree of regrowth would be expected in August. Mulching may then be preferable to removing when later cutting dates are required. N recycling was also found to be minimal in autumnal cuts compared to summer cuts by Dahlin *et al* (2011) and Rasmussen *et al* (2008), which indicates biofixation is less inhibited by mulching at this time and implies mulching may be optimal management for this cut. The volume of N contained in the biomass must also be considered, as N yields of both cuts were much higher in the second year of cultivation. Even if N recycling were to limit biofixation to some degree in an autumnal mulch, N yields in the range of 160-230 kg ha⁻¹ (Table 4.1) may be well in excess of what would be expected from biofixation at this stage in the growing season.

6.2.1.2 Variety Selection

The six varieties cultivated in this study proved to be phenologically and morphologically distinct. Tetraploid varieties also appeared to yield more DM and N than diploids in the second year of cultivation, although ascribing this to tetraploidy solely (and not phenology, as both were late flowering) is difficult. The relevance of this for fertility-building is obscure, as the highly significant differences in DM and N yields in Core Experiment 2 were not subjected to

bioassay cultivation. Although significant, differences in DM and N yields were small (a range of 1.6-2.4 t ha⁻¹ was recorded – Table 3.6). Despite yielding significantly less DM and N than all other varieties in Core Experiment 1, Amos did not yield less DM or N for wheat at all assessments. However, this may have been caused by the high degree of variability in wheat variates associated by the volunteering issue and split-plot design, as explained in Section 5.7.4. Amos did however yield significantly less total mustard DM and N than every variety apart from Milvus (Table 5.10). Total mustard yields only accounted for the H treatment, which eliminated the variability associated with the volunteering, and may then be a more accurate measurement of the fertility-building capacity of these particular varieties. This result may however be more related to the degree to which these varieties were affected by clover rot throughout Core Experiment 1, as they were both found to be more susceptible than all other varieties ($P < 0.05$ – Table 3.11). Clover rot infection can severely damage crowns and roots (Lowe, *et al.* 2014), which may have caused deterioration of root systems and untimely senescence and mineralization of belowground biomass in these varieties. These varieties also exhibited among the highest rates of winter leaching (Table 3.12), which corroborates this idea. Varietal differences in this instance are however only related to disease resistance and not phenological or genotypic characteristics particular to Amos or Milvus.

Morphological differences among varieties may also have played a minor role in their capacity to contribute to soil fertility. As varietal differences in DM and N yield were so minimal in Core Experiment 1, it seems unlikely that even highly significant differences ($P < 0.001$) in morphological variates such as stem leaves affected yield and fertility-building capacity. It is possible that differences in aboveground morphology affected belowground morphology (for example traits like taproot diameter, order of branching, number of nodules etc), which was not measured in Core Experiment 1. However this is unlikely, as Chmelikova *et al.* (2015) documented both above and belowground morphological variation in RC and found no correlation between the two.

Morphological variation was likely most relevant in the stem hairs variate. Astred and Ruby were significantly hairier than all other varieties ($P < 0.001$ – Tables 3.14, 4.7 and 4.8) and were also significantly less susceptible to winter leaching. Given how stem hairs are associated with longer drying times and therefore mineralization rates in forage legumes (Lenssen, *et al.* 2001, Taylor 2008b), increased stem hair density in these varieties may have contributed to their capacity to limit winter leaching, although the lack of removal of aboveground biomass as a

disease control measure was likely more influential. It is also notable how Astred and Ruby were shown to be significantly less susceptible to clover rot than all other varieties. This finding contradicted the conventional viewpoint, that tetraploid red clover is more resistant than diploid (Frame 1976, Ohberg *et al.* 2005, Vleugels, *et al.* 2013c), but may be explained by increased hair density. Stem hairs are generally associated with resistance to herbivory and this is thought to be their evolutionary driving force (Levin 1973), but other research has documented how they may contain bactericidal and fungicidal chemicals and therefore contribute to disease resistance (Wang *et al.* 2001, Prats *et al.* 2007, Lu *et al.* 2013). It remains unclear if this was the case in Core Experiment 1, but the apparent correlation between hair density and clover rot resistance may be a promising area for future research in RC breeding programs.

6.2.2 Belowground Biomass

The contribution of belowground biomass to soil fertility in RC cropping can be split into two components: the senescence and mineralization of the root system following termination and the senescence and mineralization of the root system whilst crop growth is taking place. Mineralization of the entire root system following termination will be more significant than that of fine root hairs and senescing nodules throughout the growing season. This less significant component is broadly known as rhizodeposition (Paterson 2003) and can be subject to leaching throughout the winter period, as well as uptake from the clover crop itself, another form of N recycling. Rhizodeposition is discussed in Section 6.2.3.

6.2.2.1 Management

Cutting is thought to reduce the size of the root system in forage legume leys (Lawson *et al.* 2000, Vinther 2006), as the crop transfers nutrients from root storage to newly growing shoots (Volenec, *et al.* 1996). This implies although cutting is optimal management for aboveground biomass, it may impact negatively on the contribution to soil fertility from belowground biomass. Root biomass was however not documented in Core Experiment 1, due to time constraints, and the impact of cutting management on subsequent crop growth was variable. It is therefore difficult to accurately determine the effect of cutting on the mineralization of the root system and how this relates to subsequent crop production.

It may be possible to make inferences about the effect of cutting on the root system from Core Experiment 2. This experiment initially included the cut as a factor in statistical analyses, but it was omitted from results given how non-significant it was consistently found to be. The lack

of effect of cutting on subsequent clover regrowth and root biomass implies any reduction in root size incurred by cutting was too minor to significantly affect plant growth. Cutting in the first year of growth did not effect aboveground biomass production in the second, and the effect of cutting on soil fertility may have been more influential on the belowground biomass component of fertility-building.

6.2.2.2 Variety Selection

Some research suggests varietal differences can influence the contribution of the belowground biomass to soil fertility in RC cropping. Some authors cite early varieties as putting down significantly more root biomass (Christie *et al.* 1992, Sabljic 2016) and others cite tetraploids as having significantly larger root systems than diploids (Thilakarathna *et al.* 2012a), but this was not observed in this study. Differences in *Rhizobia* relationships among varieties are also unlikely as the Australian variety Astred was shown to successfully nodulate with British *Rhizobia*. The significant differences observed in root biomass in Core Experiment 2 were more likely to be the result of increased disease pressure in Astred and Ruby than genotypic differences (although they were found to be less susceptible to clover rot, this meant the disease treatment of removing infected biomass could not be undertaken and infected biomass was left behind, which may have infected the taproot and reduced its biomass). This implies that although the literature predicts varietal effects on belowground biomass, this study suggests variety is a non-significant factor in contributing belowground N in the form of senescing root systems.

6.2.3 Rhizodeposition

Use of the broad term rhizodeposition to describe contributions from plant roots to soil N (and C) is controversial. Some authors describe rhizodeposition as solely root exudate and mucilage (zu Schweinsberg-Mickan *et al.* 2010), but others include root system turnover (Scandellari *et al.* 2010), which will be more significant and occur over a longer time-frame. Defining the extent of the rhizosphere is also problematic (Hogh-Jensen and Schjoerring 2001), i.e. the distance extending from plant roots to be defined as the rhizosphere is arbitrary and every author may have their own view. Time and equipment to study the rhizosphere was limited to SMN accumulation in this study, and therefore this discussion only refers to this particular form of rhizodeposition.

6.2.3.1 Management

Management clearly affected SMN leaching susceptibility in Core Experiments 1 and 2, but the effect of cutting on rhizodeposition throughout the growing season was more obscure. Here number of cuts was non-significant in both experiments but accumulation was high in Core Experiment 1 (~25 kg ha⁻¹ from the beginning of the trial), whereas accumulation was negligible in Core Experiment 2 (~4 kg ha⁻¹). This appeared to correlate with aboveground N yields, which were low in Core Experiment 1 but high in Core Experiment 2 (i.e. low N demand from the crop facilitated rhizodeposition, whilst high demand took it up and lowered rhizodeposited SMN).

Optimal management for rhizodeposition in the first year of RC cultivation may then synchronize with that of aboveground biomass; i.e. cutting and removing the first, when N yields are low, but cutting and mulching the second, when N yields are high. In the second year of cultivation, in which N yields tended to be lower in cut 2, this management may not be optimal.

Additionally, neither management strategy addresses leaching susceptibility over the winter period. Mulching to build soil fertility is common practice in organic farming, but mulches themselves can be susceptible to nutrient loss (Loges, *et al.* 2010). For example, Neeteson (1989) found leaching from a mulched RC crop to be significantly higher than that of a mineral fertilizer application in similar soil conditions to this study (clay). High rates of leaching in RC mulch was also described by Bergstrom *et al* (2004) but this study took place on a sandy soil, in which increased leaching would be expected. Numerous other authors describe high rates of leaching associated with mulching in RC across varying soil types (Nelson and King 1996, Lahti and Kuikman 2003, Romanovskaja and Tripolskaja 2003).

If removing the second cut can reduce the contribution to soil fertility from aboveground biomass, and mulching it can be prone to significant leaching over the winter period, optimal management for soil N following RC cultivation may then be to cut and remove once and leave the remaining growth to overwinter. This management is that of the 1 cut treatment applied in this study, the treatment which yielded significantly more total mustard DM and more wheat DM at the Second Biomass Assessment. This management may limit winter leaching through increased groundcover, possibly provide a larger root system for mineralization during subsequent crop growth, and return a large amount of N to the system. This N will then be released following soil incorporation prior to subsequent cultivation, but this release will be

slow as leaving it to flower permits lignification, which lengthens the mineralization process in forage legumes (McDonagh *et al.* 1995, Clement *et al.* 1998). This may allow for two release points of N during subsequent cultivation, the rapid mineralization of the low C:N ratio spring grown biomass and slow mineralization of the lignified tissues from the previous years growth. This may act as a buffer against untimely N release and better ensure synchronicity of N mineralization with the N demand of the proceeding crop. The presence of senescing aboveground biomass may of course inhibit spring growth the following year, but this should not cause concern because number of cuts was found to be non-significant in the DM and N yields of the first clover cut in Core Experiment 2.

6.2.3.2 Variety Selection

As with belowground biomass, significant differences were observed for variety in rhizodeposited SMN throughout Core Experiment 1 (Ruby and Astred were significantly higher ($P < 0.001$) following the winter). This was not repeated in Core Experiment 2, however some varieties experienced significant temporal fluctuations ($P < 0.05$) such as Milvus, whilst others such as Maro did not (Figure 4.5). Differences in Core Experiment 1 were more likely caused by disease incidence and management and not genotypic characteristics, as previously described.

Temporal differences in rhizodeposition in Core Experiment 2 indicated the correlation between rhizodeposition and aboveground N yields may have been more complicated than a simple inverse relationship. Little change occurred for all varieties between February and June, but Ruby and Astred (the varieties which yielded the least DM and N at the first cut) appeared to decrease SMN in the February-June growth period. This contradicts most of the literature on the relationship between SMN and RC aboveground DM and N yields, which tends to decrease an inverse relationship. For example Marley *et al.* (2013b) described how increasing DM yields correlate with decreasing SMN in RC cropping. Cupina *et al.* (2017) documented RC DM yields to be higher in soils with lower mineral nitrogen content, and Jia *et al.* (2006) showed SMN levels decreased with increasing N yields in alfalfa. However, what precisely counts as 'high' in the context of these findings is important. Yields of 191 kg ha^{-1} and 236 kg ha^{-1} for Ruby and Astred respectively would be considered high by most accounts (Carlsson and Huss-Danell 2003). This study also documented these varieties to have significantly less nodules than all other varieties, albeit in August and not June. If these varieties had fewer nodules at the time of this assessment they may then have been more dependent on already present SMN for nutrition, and less on biofixation. This may have caused the reduction in SMN

in these varieties, whereas other varieties, which yielded higher DM and N but didn't reduce SMN may have been more reliant on biofixation.

Variety was found to be insignificant for every individual measurement of SMN apart from the assessment following the winter period of Core Experiment 1, in which Astred and Ruby were shown to be higher than other varieties. This was more likely caused by applied disease management, as previously described. This implies that variety, in itself, was not a significant factor in the contribution of rhizodeposited N on subsequent soil fertility throughout the course of this study.

6.3 The Bioassay Phase

6.3.1 Cultivation

Effective termination was achieved in almost all cases in the H treatment, as negligible volunteering occurred in H plots. This was expected to occur, but it remains a result worth reporting, as some authors cite difficulties in effectively terminating RC using herbicides (Ceballos *et al.* 2004, Laird *et al.* 2016). The volunteering issue in the NH treatment clearly indicated the rotavation applied prior to cultivation did not effectively terminate the RC. The decision to include an NH treatment at all may have been unwise, given how RC can be difficult to terminate even with industrial-scale cultivators (Currell 2011, Anderson *et al.* 2016). Other research specifically asserts RC crops require deep moldboard ploughing to ensure effective termination if herbicides are not applied (Duiker and Curran 2007).

Specialist management may be capable of turning this difficulty in RC cropping into a positive. Wyngaarden *et al.* (2015) described how strip-tillage can be performed in such a manner as to cultivate volunteers as relay crops for subsequent cereal crops. Other research from organic systems describe how late autumn cutting can weaken crowns and terminate them over the winter period (Anderson 2017), but this was only found to be effective in the third year of cultivation. Volunteering was documented to be inhibitive of growth in almost all biomass assessments in this trial, indicating herbicide applications are optimal management in terminating RC leys if effective termination cannot be guaranteed with tillage alone. Concerns over effective termination with herbicides proved unfounded as glyphosate application was adequate in the H treatment.

Cultivation clearly impeded growth in mustard cultivation. The H treatment yielded significantly more DM and N ($P < 0.001$) but the ANCOVA found the covariate to be non-significant for cultivation, indicating the reduced yields in the NH treatment may have been caused by the treatment itself and not volunteering. The rapid growth rate of the mustard may have made it a more effective competitor than the wheat and facilitated it to grow without significant impediment from the volunteers. However, as volunteering rapidly proceeded before the second crop could establish, the omission of herbicide completely restricted all subsequent growth. Any further research of this nature should ensure termination is effectively carried out prior to cultivation in order to avoid this problem.

In wheat, cultivation was shown to be highly significant ($P < 0.001$) in both DM and N yields by the Second Biomass Assessment. The H treatment yielded near twice that of the NH treatment at this assessment (Table 5.4), but cultivation was non-significant at the First Biomass Assessment. This implies the inhibitive effect of the volunteering was most significant in the period of growth between these assessments (GS 25-69 (Zadoks 1974)), growth stages known to be particularly vulnerable to weed pressure in wheat (Worthington *et al.* 2013). The highly significant volunteer clover covariate at the Second Biomass Assessment (Table 5.4) supports the hypothesis that volunteering was the cause of the significantly lower yields in the NH treatment in the initial ANOVA. The adjusted means reflect an estimate of what would have happened without the effect of the volunteering.

No significant differences were observed for any treatments in this estimate and no significant interactions were observed. The best estimate for the effect of inclusion or omission of herbicide in this study must then be that it is non-significant, as increased or reduced N availability associated with either treatment would likely not affect plant growth during the grain filling stage. However this is only an estimate, and another trial with effective, mechanized tillage would be required to fully understand the impact of herbicide inclusion or omission on spring wheat following RC cultivation. The H treatment yielded significantly higher ($P < 0.001$) DM and N than the NH in all variates in the Final Biological Assessment, indicating this study found herbicide application to be optimal in subsequent wheat production, but given the results of the previous assessments the limited growth in the NH treatment was more likely caused by volunteering than the treatment itself.

6.3.2 Management

The effect of management on subsequent crop production was obvious in mustard. 1 cut yielded significantly more DM and N ($P < 0.001$) in both the first two mustard crops and total yield. Higher rates of SMN (Table 3.12), less leaching of nutrients like P and K and potentially larger root systems may have played a role in this. Incorporation of aboveground biomass, which was removed in the second cut, may also have contributed to soil fertility, although this contribution was likely reduced by the removal of infected tissues prior to the winter period of Core Experiment 1. Cut was shown to be non-significant in the final mustard cultivation, but this may have been caused by the reduced photoperiod and cool weather rather than soil fertility associated with the management treatment, as autumnal conditions can limit growth in mustard (Angadi *et al.* 2004). A highly significant difference of 20 kg ha⁻¹ ($P < 0.001$) between 1 and 2 cuts for the total N yield clearly shows 1 cut was optimal management for subsequent crop production in mustard.

Cutting management was significant in wheat DM at the Second Biomass Assessment (Table 5.4) and grain protein (Table 5.7). 1 cut yielded more than 2 cut in both variates. The absence of significance for this treatment at the initial biomass assessments, but effect at the second may be explained by delayed mineralization of the root system. Taproots are expected to mineralize at a slower rate than aboveground biomass (Skuodiene *et al.* 2012, Ferreira *et al.* 2015), likely because of their higher C:N ratio and suberin content, although this cannot be asserted from this study as these properties were not investigated. Delayed mineralization of the root system during wheat cultivation may explain the significantly higher yields of DM and N under 1 cut in the Second Biomass Assessment, but lack of effect of cut on these variates in the First Biomass Assessment (Table 5.3), if the root systems of the 1 cut treatment were indeed larger than the 2 cut. The ANCOVA in the Second Biomass Assessment did however correct means to show no significant effect of cut on DM and N yields, which implies the differences observed in the initial ANOVA may have been caused by differing rates of volunteering between cut treatments, and not differing contributions from mineralizing taproots.

Although 1 cut yielded significantly more DM and N at the Second Biomass Assessment, it failed to convert this biomass into significantly higher grain yields at the Final Biological Harvest. This may have been caused by SMN levels in GS 20-59, as increased available SMN in the pre-anthesis stage can negatively impact on efficiency of N partitioning in the grain-filling stage (Ehdaie and Waines 2001). This may have occurred in this study, if indeed SMN levels were higher in the 1 cut treatment at this phase, but this is not possible to SMN as time

constraints prevented SMN from being documented in the bioassay cultivation. The reduced leaching and increased soil-incorporated biomass associated with the 1 cut treatment did not significantly increase yields in wheat cultivation, but in mustard. The total N measurements for both crops under 1 cut (99.6 kg ha⁻¹ for wheat and 107.3 kg ha⁻¹ for mustard – Tables 5.8 and 5.11) would imply the differences observed here are significant, although this is not possible to say for sure as the limitations associated with the NH treatment in mustard required a different number of replications than in wheat. The higher mustard yields may also have been boosted by non-N components of soil fertility, for example an increase in SOM associated with the higher rate of aboveground biomass incorporation.

1 cut appeared to be optimal management in fertility-building with RC, but to base one's conclusions solely on the 'treatment and effect' conceptual framework may be shortsighted. Accurate inferences can be made from this framework, but it ignores the social and economic elements of optimal management; elements which are increasingly being viewed as more worthy of consideration both in agronomic research and its extension (Giller *et al.* 2015, Coe 2016). In this instance 1 cut may be preferable to 2 for subsequent soil fertility, but in mixed farming systems farmers may desire forage for the winter period more than optimal yields of subsequent cereal production. Time constraints in the busy harvest period in arable systems may also pressurize farmers seeking optimal management for their RC leys. Cutting may be required to control diseases, as was the case in this study, and failure to cut in response to disease incidence may reduce root biomass and capacity to produce aboveground biomass prior to cultivation the following spring, which may offset the benefit associated with the management of 1 cut. Declarations of 1 cut as optimal management in RC based on the results of this study can therefore only be made from the agronomic perspective and broader recommendations require a more nuanced approach.

6.3.3 Variety Selection

Significant varietal differences in phenology, yield and morphology were observed in this study (Chapters 3 and 4). The effect of variety on subsequent soil fertility (as assessed using the bioassay crops) was less obvious. Variety was found to be non-significant for almost all recorded variates in wheat cultivation. Where it was found to be significant it may have been trivial, for example grain protein (Table 5.7), where a range of 1.2% from low to high was reported and all varieties were below the protein percentage desired by bakers. Some varieties produced taller wheat than others (Table 5.5), but again differences were significant but small and unlikely to have impacted on yield, given how variety was non-significant for all yield

components. Variety was also non-significant for all variates in the First Biomass Assessment, but when the clover covariate was included Ruby and Astred yielded less DM and N than other varieties. This was more likely to be caused by increased volunteering in other varieties such as Maro and Amos, as these were late varieties with increased growth expected in the first flush. Critical values for variety in wheat cultivation may have been increased by variability associated with volunteering and experimental design (as discussed in Section 5.7.4), but the final results of this study indicate variety selection was non-significant in the fertility-building capacity of RC leys in subsequent cereal production.

High variability in the First Biomass Assessment in the mustard cultivation may also have caused critical values to be high and find no statistical significance between varieties, as Ruby and Astred did appear to yield more DM than other varieties, though non-significantly. Astred and Ruby did however yield more N than other varieties, and this difference was clearly shown in the interaction between variety and cut in N yield (Figure 5.23). Variety was then significant in the second crop, with Ruby and Astred yielding among the highest for DM and N (Table 5.9), albeit with yields reduced when compared the first crop due to weed pressure. The adjusted means in the ANCOVA changed little in statistical difference, indicating weed pressure was uniform throughout the experiment, so inferences about the effect of variety on this mustard crop can be robustly made. Variety was non-significant in the final crop, but this may have been the result of limiting growth conditions, as previously described.

Significant varietal differences were observed for both DM and N yield when all mustard crops were summed (Table 5.11) The total DM yields of Ruby and Astred were matched by that of Claret and Maro, but Ruby yielded more N than all other varieties ($P < 0.05$). Here Ruby contributed more to soil fertility, but this more likely reflects its higher resistance to clover rot and the incorporation of uninfected aboveground biomass (which had been removed to a greater extent in more susceptible varieties). This hypothesis is supported by several observed interactions between variety and cut, for which the difference between 1 and 2 cut in Ruby appeared more significant than that of other varieties (Figures 5.14, 5.15, 5.22, 5.23, 5.26). The higher rate of biomass removal in highly infected varieties reduced the contributions to soil fertility from aboveground biomass regardless of cutting management, but the lower removal rate of more susceptible varieties maintained this effect, contributing more aboveground biomass in the 1 cut treatment, and this may have increased yields the following year. The effect of variety on soil fertility-building capacity was then significant, but the results of this

study indicate this significance resulted from disease occurrence and management, not intrinsic phenological, genotypic or morphological qualities of the varieties themselves.

All RC varieties trialled significantly differed in capacity to yield DM, N and protein. All were also phenologically diverse, as breeders describe, and were morphologically diverse for most traits, with Astred exhibiting the most morphological distinction from other varieties.

6.4 Conclusions

6.4.1 RC Cultivation

- RC varieties differed significantly in morphology, phenology, disease resistance, DM/N yield, and protein percentage
- Cutting management significantly affected DM and N yields of RC varieties trialled
- All varieties effectively outcompeted weeds
- RC varieties differed in nodule number and root biomass in the second year of cultivation, but this was more likely caused by differences in disease susceptible than genotype
- Differences in nodule number and root biomass did not appear to effect DM and N yields
- The CVA described a correlation between stem hair density and growth habit (i.e. plants with hairy stems grew prostrately and hairless stems grew erectly)

6.4.2 Fertility-Building

- One cut was consistently significantly less susceptible to leaching
- Interactions between variety selection and cutting management significantly affected the accumulation of SMN
- Variety was significant in leaching susceptibility, but this was more likely resulting from disease management than genotype
- Rhizodeposition was high when aboveground yields were low, but low when aboveground yields were high

- Disease incidence consumed aboveground biomass to an extent which affected fertility-building capacity in some varieties
- Stem hair density may affected fertility-building capacity, directly through delayed mineralization of aboveground biomass or indirectly by contributing to disease resistance

6.4.3 Bioassay Phase

- The inclusion of a herbicide significantly affected the yields of subsequent crops, but this was more likely caused by the absence of volunteers than the effects of the treatment
- Omission of herbicide failed to terminate the RC crop
- RC volunteering inhibited investigations into the effect of herbicide inclusion or omission on subsequent crop production
- The ANCOVA gave an estimate of the effect of herbicide inclusion or omission was that it would be non-significant
- One cut gave higher DM and N yields in mustard cultivation in the H treatment, but not in wheat.
- Ruby yielded the highest DM and N in mustard in the H treatment, whilst Amos yielded the lowest. Variety was non-significant in wheat DM and N, but this may have be caused by increased variability in data associated with volunteering

6.4.4 Statistical Procedures

- Cananical Variate Analysis is effective at illustrating and describing the relationship existing between variates in groups
- Analysis of Covariance can be used to estimate the effect of nuisance variates such as volunteering on main variates
- High variability caused by volunteering, and experimental design, may have incurred Type 2 Errors in wheat production

6.5 Recommendations for Future Research

As discussed in Section 6.2.1, a superficial correlation between stem hair density and clover rot resistance was observed. The CVA biplots of variance in morphological traits showed a statistically robust correlation between stem hair density and growth habit (Figures 3.9, 3.11, 4.7 and 4.9), although one cut was required to bring about this correlation. These biplots indicate more prostrate varieties were also hairier. Further research in plant breeding on this correlation may be capable of generating new, hairier and more prostrate varieties which may have increased resistance to clover rot, persist longer in the sward and improve fertility-building capacity.

The bulk of the current literature on biofixation in RC focuses on assessing the %Ndfa (% Nitrogen derived from atmosphere) in the aboveground biomass (these studies are summarized in Tables 1.4-1.6). The findings of this study questions the merit of these studies. Assessment of %Ndfa remains of academic interest, but if cuttings are removed and belowground N is ignored, the relevance of this assessment for subsequent soil-fertility is dubious. This study also described an apparent inverse relationship between aboveground N yields and SMN accumulation. Low N yields in the first year gave high SMN accumulation (Tables 3.6 and 3.12), whilst high N yields in the second year gave low SMN accumulation (Tables 4.2 and 4.6). Further research on biofixation should admonish readers about this relationship to avoid miscommunicating the relevance of %Ndfa in fertility-building, and explore new methods of relating %Ndfa to fertility-building capacity. This was attempted by the 'root factor' calculation described by some authors (Unkovich, *et al.* 2010, Peoples, *et al.* 2012), but it remains a largely unstudied area.

The lack of effective termination in the NH treatment clearly impeded many aspects of this study. As discussed in Section 6.3, this challenge in effectively terminating RC leys has been described by many authors, but few studies on how this challenge is to be overcome have been undertaken. Further research on optimal termination strategies that do not require herbicide applications would remove the concern of potential volunteering from RC cultivation and may increase the adoption of RC rotations in cereal production.

This study found 1 cut and remove management preferable to 2 cut and remove. This was explained by a number of effects on all three components of fertility-building with forage legumes (aboveground, belowground and rhizodeposition). Another promising area of research may to repeat the treatments but to also include of treatment of mulching the second cut. This

would provide the soil cover of the 1 cut and remove treatment, but the biomass returned to the soil following cultivation the following season would be in a more advanced state of mineralization (having been killed by cutting the previous autumn). This treatment may either supply additional N to the subsequent crop in the crucial spring growth phase, or may be subject to increased N losses over the winter and reduce available N for subsequent production. Investigations into this treatment in future research may further optimize RC use in fertility-building.

6.6 Recommendations for Farmers

Farmers should choose RC varieties, management strategies and cultivation techniques to suit their individual requirements. Those looking interested in forage production will desire the highest possible yields of DM, N and protein and should select an intermediate variety, such as Maro, and two cuts should be obtained. Cutting should take place at the early bloom stage to avoid a drop-off in forage quality. Those interested in grazing management should select a prostrate variety, such as Astred.

Farmers selecting optimal management for fertility-building should cut only once, as this facilitates the incorporation of more biomass into the soil prior to cultivation the following year. It also leaves biomass to act as a cover crop over the winter period, which can limit soil N losses from leaching and denitrification over the winter period.

Clear recommendations in cultivation strategies are difficult to make. Herbicide application significantly increased subsequent crop yields, but this more likely resulted from the elimination of volunteers, which were so problematic in the non-herbicide treatment. Farmers operating minimal tillage systems should include a herbicide prior to cultivation, as effective termination may not be guaranteed without deep tillage. Those operating deeper tillage could choose either option. Reasonable expectations would be that herbicide inclusion would cause rapid mineralization, whilst omission would cause slow mineralization, but the results of this thesis were compromised by the volunteering issue and cannot confirm this.

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APPENDICES

All non-significant interactions are given here in appendices corresponding to the data cited in the main text (i.e. Appendix 3.12 contains the non-significant interactions omitted in Table 3.12 etc).

Appendix 3.12 Effect of interaction between soil mineral nitrogen accumulation and cut in Core Experiment 1

Variety & Cut	Winter 2015 kg ha ⁻¹	Spring 2016 kg ha ⁻¹	Leached N kg ha ⁻¹
Amos 1	39.7	15.2	22.8
Astred 1	40.8	25.1	12.7
Claret 1	40.1	25.5	15.4
Maro 1	39.3	16.5	21.3
Milvus 1	40.0	21.3	19.0
Ruby 1	40.4	25.9	11.6
Amos 2	39.1	18.4	21.8
Astred 2	37.0	23.9	12.0
Claret 2	38.4	22.5	19.2
Maro 2	41.9	16.3	23.2
Milvus 2	40.9	16.9	23.7
Ruby 2	39.2	21.5	15.6
<i>S.E.D. (273 df)</i>	1.5	1.99	2.09
<i>P</i>	0.061	0.065	0.231

Appendix 4.5 Effect of interaction between variety and cut on *Sclerotinia* disease index

Variety & Cut		Sclerotinia Disease Index
Amos	1	247.5
Astred	1	217.5
Claret	1	220
Maro	1	217.5
Milvus	1	207.5
Ruby	1	265
Amos	2	107.5
Astred	2	140
Claret	2	115
Maro	2	120
Milvus	2	142.5
Ruby	2	140
<i>S.E.D. (33 df)</i>		23.78
<i>P</i>		0.248

Appendix 4.6 Effect of interaction between variety and cut on soil mineral nitrogen accumulation in Core Experiment 2

Variety & Cut		June 2016 kg ha ⁻¹	August 2016 kg ha ⁻¹	February 2017 kg ha ⁻¹	Leached
Amos	1	22.7	20.7	16.3	4.4
Astred	1	18.5	20.4	17.3	3.2
Claret	1	16.3	20.1	20.4	0.2
Maro	1	18.8	23.25	21.4	1.9
Milvus	1	17.2	21.7	23.7	2.0
Ruby	1	17.2	19.9	15.9	4.0
Amos	2	15.7	21.3	19.3	2.0
Astred	2	17.6	20.4	16.1	4.3
Claret	2	22.6	21.5	17.3	4.2
Maro	2	22.7	19.9	16.6	3.3
Milvus	2	17.7	24.3	15.5	8.7
Ruby	2	16.5	23.4	17.5	5.9
<i>S.E.D. (273 df)</i>		1.71	2.34	2.71	2.42
<i>P</i>		0.059	0.334	0.091	0.076

**Appendix 5.2 Effect of interaction between variety, cut and cultivation on establishment
and tiller number following different RC varieties**

Variety	Cut	Cultivation	Establishment plants m ²	Number of Tillers m ²
Amos	1	H	172	245.3
Amos	1	NH	278.6	281.3
Amos	2	H	302.6	244
Amos	2	NH	209.3	237.3
Astred	1	H	226.6	234.6
Astred	1	NH	366	284
Astred	2	H	153.3	284
Astred	2	NH	285.3	270.6
Claret	1	H	262.6	221.3
Claret	1	NH	312	253.3
Claret	2	H	244	238.6
Claret	2	NH	256	280
Maro	1	H	304	260
Maro	1	NH	214.6	269.3
Maro	2	H	238.6	266.6
Maro	2	NH	265.3	278.6
Milvus	1	H	292	302.6
Milvus	1	NH	310.6	278.6
Milvus	2	H	240	293.3
Milvus	2	NH	225.3	280
Ruby	1	H	206.6	212
Ruby	1	NH	218.6	216
Ruby	2	H	226.6	277.3
Ruby	2	NH	265.3	224
S.E.D. (49 df)			49.5	32.07
P			0.254	0.233

**Appendix 5.3 Effect of interaction between variety, cut and cultivation on wheat DM
and N following different RC varieties – First Assessment**

Variety	Cut	Cultivation	ANOVA		ANCOVA	
			Wheat DM t ha ⁻¹	Wheat N kg ha ⁻¹	Wheat DM t ha ⁻¹	Wheat N kg ha ⁻¹
Amos	1	H	0.64	26.9	0.54	23.2
Amos	1	NH	0.50	20.2	0.63	25.2
Amos	2	H	0.58	23.9	0.55	22.9
Amos	2	NH	0.56	22.9	0.60	24.1
Astred	1	H	0.52	23.1	0.44	20.2
Astred	1	NH	0.52	18.6	0.57	20.5
Astred	2	H	0.51	19.8	0.40	15.8
Astred	2	NH	0.45	18.3	0.52	21.2
Claret	1	H	0.66	29.1	0.49	22.2
Claret	1	NH	0.35	15.0	0.66	26.4
Claret	2	H	0.62	28.4	0.45	21.4
Claret	2	NH	0.40	17.4	0.63	25.9
Maro	1	H	0.53	22.0	0.37	18.0
Maro	1	NH	0.41	16.9	0.63	25.1
Maro	2	H	0.58	19.1	0.43	21.2
Maro	2	NH	0.51	22.2	0.66	27.8
Milvus	1	H	0.66	24.5	0.60	18.8
Milvus	1	NH	0.60	24.1	0.70	28.0
Milvus	2	H	0.61	24.0	0.48	18.4
Milvus	2	NH	0.45	18.7	0.61	25.0
Ruby	1	H	0.56	26.4	0.46	18.6
Ruby	1	NH	0.44	18.9	0.42	18.7
Ruby	2	H	0.45	23.6	0.35	15.7
Ruby	2	NH	0.46	18.3	0.45	18.1
S.E.D. (49 df)			0.1	3.8	0.8	4.0
P			0.357	0.666	0.695	0.816

**Appendix 5.4 Effect of interactions between variety, cut and cultivation on number of stems and wheat DM and N following different RC varieties (ANOVA and ANCOVA) –
Second Assessment**

Variety	Cut	Cultivation	ANOVA		ANCOVA		Wheat DM t ha ⁻¹	Wheat N kg ha ⁻¹
			Wheat DM t ha ⁻¹	Stems m ²	Wheat N kg ha ⁻¹	Stems m ²		
Amos	1	H	6.67	524	74.6	479.8	4.69	74.7
Amos	1	NH	3.73	225.3	43.2	262.6	5.42	40.5
Amos	2	H	7.22	386.6	81.7	351.0	5.54	85.6
Amos	2	NH	3.25	329.3	38.4	359.5	4.69	32.4
Astred	1	H	5.98	406.6	70.7	358.3	4.32	74.5
Astred	1	NH	3.96	258.6	48.2	305.7	5.55	44.1
Astred	2	H	6.21	392	73.3	343.6	4.56	77.0
Astred	2	NH	3.52	216	42.0	261.7	5.06	37.3
Claret	1	H	7.04	488	78.5	445.6	5.43	82.5
Claret	1	NH	3.72	238.6	43.8	281.6	5.36	40.1
Claret	2	H	6.58	432	75.2	389.6	4.91	79.2
Claret	2	NH	3.93	285.3	44.4	329.9	5.63	41.4
Maro	1	H	8.06	378.6	96.6	329.6	6.4	100.3
Maro	1	NH	3.65	352	44.3	400.4	5.27	40.5
Maro	2	H	7.70	473.3	83.5	424.3	6.04	89.1
Maro	2	NH	3.12	214.6	36.5	259.5	4.61	31.1
Milvus	1	H	6.92	469.3	85.4	432.0	5.37	87.8
Milvus	1	NH	2.97	282.6	36.5	320.9	4.61	32.5
Milvus	2	H	6.69	444	77.9	406.6	5.11	82.2
Milvus	2	NH	3.64	300	42.3	344.9	5.52	41.3
Ruby	1	H	6.69	416	79.0	380.4	5.11	83.3
Ruby	1	NH	4.01	241.3	48.2	281.4	5.78	45.7
Ruby	2	H	5.12	377.3	62.8	341.7	3.55	67.2
Ruby	2	NH	2.51	261.3	30.5	302.9	4.33	28.6
S.E.D. (49 df)			0.86	77.7	9.50	51.66	0.95	10.65
P			0.375	0.178	0.459	0.186	0.122	0.137

Appendix 5.5 Effect of interactions between variety, cut and cultivation on number of ears and ears, straw and total DM following different RC varieties

Variety	Cut	Cultivation	ANCOVA				
			Plant Height cm	Ears m ⁻²	Ears DM t ha ⁻¹	Straw DM t ha ⁻¹	Total DM t ha ⁻¹
Amos	1	H	80.6	358.7	5.18	3.47	8.65
Amos	1	NH	75.1	136.0	1.84	1.38	3.22
Amos	2	H	80.2	314.7	5.03	3.42	8.45
Amos	2	NH	76.6	240.0	3.23	2.48	5.71
Astred	1	H	79.3	366.7	5.43	4.06	9.50
Astred	1	NH	78.8	213.3	2.51	2.29	4.80
Astred	2	H	80.9	368.0	4.97	3.44	8.41
Astred	2	NH	77.8	220.0	2.56	1.92	4.48
Claret	1	H	80.5	376.0	5.43	3.77	9.21
Claret	1	NH	78.7	204.0	2.85	2.12	4.98
Claret	2	H	80.2	376.0	5.80	4.59	10.39
Claret	2	NH	79.8	192.0	2.73	1.68	4.41
Maro	1	H	77.0	372.0	6.22	4.34	10.56
Maro	1	NH	77.4	169.5	2.08	1.57	3.65
Maro	2	H	82.2	390.7	6.27	4.19	10.45
Maro	2	NH	70.2	130.7	1.69	1.44	3.13
Milvus	1	H	75.3	400.3	6.13	3.51	9.65
Milvus	1	NH	74.9	205.3	2.31	1.89	4.20
Milvus	2	H	76.8	356.0	5.05	3.63	8.68
Milvus	2	NH	77.0	181.3	2.46	1.64	4.11
Ruby	1	H	76.5	393.3	5.86	3.98	9.84
Ruby	1	NH	79.4	257.3	3.79	2.75	6.54
Ruby	2	H	79.3	484.0	6.20	4.07	10.27
Ruby	2	NH	76.4	238.7	3.23	2.26	5.49
	S.E.D. (49 df)		3.05	53.65	0.70	0.49	1.11
	P		0.177	0.073	0.250	0.122	0.179

Appendix 5.6 Effect of interactions between variety, cut and cultivation on grain yield, thousand grain weight (TGW) and harvest index (HI) following different RC varieties

Variety	Cut	Cultivation	Grain Yield t ha ⁻¹	TGW	HI
Amos	1	H	4.21	4.21	44.1
Amos	1	NH	1.33	1.33	36.8
Amos	2	H	5.38	5.38	44.2
Amos	2	NH	2.90	2.90	37.5
Astred	1	H	5.39	5.39	44.6
Astred	1	NH	1.79	1.79	35.6
Astred	2	H	3.97	3.97	43.7
Astred	2	NH	1.73	1.73	39.2
Claret	1	H	5.44	5.44	44.1
Claret	1	NH	2.32	2.32	35.7
Claret	2	H	5.97	5.97	42.7
Claret	2	NH	1.65	1.65	36.8
Maro	1	H	6.36	6.36	43.1
Maro	1	NH	1.39	1.39	37.3
Maro	2	H	5.72	5.72	44.3
Maro	2	NH	1.27	1.27	37.8
Milvus	1	H	6.03	6.03	44.7
Milvus	1	NH	1.38	1.38	38.6
Milvus	2	H	5.08	5.08	42.9
Milvus	2	NH	1.83	1.83	39.1
Ruby	1	H	5.97	5.97	43.1
Ruby	1	NH	3.05	3.05	35.1
Ruby	2	H	5.49	5.49	44.5
Ruby	2	NH	2.35	2.35	35.7
S.E.D. (49 df)			0.950	1.168	2.34
P			0.376	0.301	0.249

Appendix 5.7 Effect of interaction between variety, cut and cultivation on grain yield, thousand grain weight and harvest index (N HI) and grain protein following different RC varieties

Variety	Cut	Cultivation	Grain N kg ha ⁻¹	Total N kg ha ⁻¹	N HI	Grain Protein (%)
Amos	1	H	60.5	79.4	78.8	10.5
Amos	1	NH	23.8	31.7	73.1	12.3
Amos	2	H	70.6	88.2	78.1	11.0
Amos	2	NH	44.7	58.7	74.2	11.1
Astred	1	H	77.5	98.2	79.5	10.7
Astred	1	NH	36.6	49.3	72.9	12.3
Astred	2	H	60.4	78.0	79.1	10.2
Astred	2	NH	33.7	45.6	74.1	11.6
Claret	1	H	87.2	106.8	77.9	11.6
Claret	1	NH	39.7	53.3	75.3	12.1
Claret	2	H	82.8	107.2	78.3	10.8
Claret	2	NH	30.2	41.5	74.3	12.9
Maro	1	H	88.4	110.6	80.1	11.6
Maro	1	NH	27.2	36.6	72.8	12.0
Maro	2	H	88.3	98.9	80.9	10.8
Maro	2	NH	24.0	32.7	71.9	12.1
Milvus	1	H	87.8	101.3	79.8	10.5
Milvus	1	NH	27.5	39.9	71.1	11.2
Milvus	2	H	77.6	95.8	78.6	10.8
Milvus	2	NH	31.5	41.2	72.5	11.6
Ruby	1	H	83.3	108.2	80.5	11.0
Ruby	1	NH	46.7	63.9	75.4	11.0
Ruby	2	H	77.1	108.6	81.2	10.0
Ruby	2	NH	37.4	51.5	74.8	10.7
S.E.D. (49 df)			12.51	14.21	3.75	1.190
P			0.706	0.497	0.455	0.402

**Appendix 5.8 Effect of interaction between variety, cut and cultivation on mustard DM
and N following different RC varieties**

Variety	Cut	Cultivation	ANOVA		ANCOVA	
			Mustard DM t ha ⁻¹	Mustard N kg ha ⁻¹	Mustard DM t ha ⁻¹	Mustard N kg ha ⁻¹
Amos	1	H	1.82	58.55	1.75	56.43
Amos	1	NH	1.06	31.32	1.39	44.02
Amos	2	H	1.74	59.61	1.69	58.00
Amos	2	NH	1.34	41.43	1.68	54.61
Astred	1	H	2.05	68.55	1.92	63.83
Astred	1	NH	1.97	75.81	2.00	77.33
Astred	2	H	2.00	60.77	1.87	56.05
Astred	2	NH	1.64	52.17	1.79	57.79
Claret	1	H	1.87	76.94	1.79	74.04
Claret	1	NH	1.25	42.51	1.60	55.86
Claret	2	H	1.65	57.47	1.57	54.57
Claret	2	NH	1.01	32.35	1.27	42.62
Maro	1	H	1.53	49.32	1.41	44.68
Maro	1	NH	1.56	52.84	1.70	57.83
Maro	2	H	1.79	65.94	1.66	61.31
Maro	2	NH	1.33	42.92	1.40	45.84
Milvus	1	H	1.70	59.86	1.55	53.87
Milvus	1	NH	1.43	45.38	1.36	42.81
Milvus	2	H	1.54	66.95	1.39	60.96
Milvus	2	NH	1.54	58.91	1.50	57.15
Ruby	1	H	2.32	99.51	2.16	93.19
Ruby	1	NH	1.83	75.27	1.73	71.25
Ruby	2	H	1.81	61.04	1.65	54.72
Ruby	2	NH	1.41	50.89	1.33	47.53
S.E.D. (49 df)			0.23	9.22	0.31	11.9
P			0.073	0.194	0.059	0.126

**Appendix 5.9 Effect of interaction between variety, cut and cultivation on mustard DM
and N and weed DM following different RC varieties**

Variety	Cut	ANOVA		ANCOVA	
		Mustard DM t ha ⁻¹	Mustard N kg ha ⁻¹	Mustard DM t ha ⁻¹	Mustard N kg ha ⁻¹
Amos	1	0.41	11.5	0.89	12.0
Astred	1	0.79	24.7	0.70	21.2
Claret	1	1.17	37.5	0.91	27.9
Maro	1	0.95	29.5	0.86	26.1
Milvus	1	0.87	26.3	0.72	20.8
Ruby	1	0.72	22.5	0.31	25.0
Amos	2	0.07	1.8	0.27	9.2
Astred	2	0.24	8.2	0.27	9.3
Claret	2	0.42	12.1	0.60	19.0
Maro	2	0.78	24.9	0.71	22.1
Milvus	2	0.26	7.4	0.50	16.6
Ruby	2	0.61	19.5	0.54	16.9
S.E.D (129 df)		0.24	7.41	0.20	6.06
P		0.341	0.234	0.095	0.884

**Appendix 5.10 Effect of interaction between cut and variety on mustard DM and N
following different RC varieties**

Variety & Cut		Mustard DM t ha ⁻¹	Mustard N kg ha ⁻¹
Amos	1	0.42	11.1
Astred	1	0.62	16.4
Claret	1	0.79	28.5
Maro	1	0.68	16.9
Milvus	1	0.64	19.5
Ruby	1	0.61	15.5
Amos	2	0.50	13.5
Astred	2	0.53	18.9
Claret	2	0.49	19.4
Maro	2	0.53	19.5
Milvus	2	0.54	18.2
Ruby	2	0.77	23.1
<i>S.E.D. (129 df)</i>		0.124	4.59
<i>P</i>		0.133	0.198

**Appendix 5.11 Effect of interaction between cut and variety on total mustard DM and N
in treatment following different RC varieties (Total)**

Variety & Cut		Mustard DM t ha ⁻¹
Amos	1	2.65
Astred	1	3.46
Claret	1	3.83
Maro	1	3.17
Milvus	1	3.21
Ruby	1	3.64
Amos	2	2.32
Astred	2	2.77
Claret	2	2.56
Maro	2	3.10
Milvus	2	2.33
Ruby	2	3.18
<i>S.E.D. (129 df)</i>		0.33
<i>P</i>		0.163