Nitrogen use efficiency in *Lolium perenne* L. with low and high fructan content

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Abstract

Perennial ryegrass (*Lolium perenne* L.) is an economically important resource in New Zealand, particularly for its use as a pasture grass. Nitrogen fertiliser is frequently applied after defoliation by grazing and, when assimilation by the plant is limited, causes detrimental impacts on the surrounding environment. The main water-soluble storage carbohydrates in *L. perenne* are fructans, which are primarily located in the sheath tissue of the plant. Fructans are remobilised following defoliation. Carbon plays a crucial role in nitrogen uptake and assimilation, and it has been suggested that nitrogen use efficiency (NUE) in the early stages of plant re-foliation following defoliation (grazing) is greater in plants that have greater concentrations of stored carbohydrate. This study investigated the potential interaction between the fructan concentrations and NUE within three cultivars of ryegrass, two of which, Expo and Aber Magic, were regarded as high sugar grasses. To manipulate the fructan stores further, half the plants were grown in cooler conditions, which was hypothesised to increase fructan content. NUE was determined using ^{15}N supplemented urea.

The cooler environmental treatment led to greater fructan accumulation in the plant base (sheath) material of all three cultivars, with neither 'high sugar' cultivar having significantly more fructan accumulation than the standard New Zealand variety, Nui. Total nitrogen in harvested sheath (g) matched the pattern seen for fructan, again with no significant difference between cultivars. In both sheath and root tissue, there was a trend for increased uptake of the ^{15}N in those plants with the greater fructan levels, which was statistically significant for the 'Nui' plants.

In conclusion, the relationship between increased carbon stores and increased nitrogen uptake was confirmed. There was no evidence that the putatively 'high fructan' cultivars displayed higher fructan contents in this experiment, but a strong effect of the environmental treatments may have masked any differences between the cultivars. Nonetheless, the cool temperature response displayed here does indicate that the strongest impact of higher fructan content on NUE may be expressed in cooler months during the growing season.

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List of Figures and Tables

Abbreviations

°C	degrees Celsius
g	grams
ha^{-1}	hectare
L	Litre
M	Molar (moles per litre)
min	minute(s)
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
RPM	Revolutions per minute
Tg	Teragram
µl	microlitre
µg	microgram

Chapter 1

Introduction

1.1 Background

Perennial ryegrass (*Lolium perenne* L.) is grown as the leading forage grass for agricultural pastures in temperate areas of New Zealand, Australia, South Africa, South America and Japan (Wilkins & Humphreys, 2003). Ryegrass, particularly in New Zealand, is a vital component for industries involved in multiple aspects of agricultural practices (Hampton *et al.* 2012). Its use as a pasture grass and feed for ruminant in meat and dairy industries, as well as seed export, emphasises New Zealand's dependence on ryegrass as a valued species for its economy (MPI Technical Paper).

The benefits of using *L. perenne* are that it is an inexpensive food source, its elevated and prolonged productivity, high digestibility and sufficient seed production (Wilkins, 1991; Humphreys *et al.* 2010). Pasture grasses are able to endure consistent grazing pressure through quick recovery, increased tiller number and resilience (Lestienne *et al.* 2006). The use of improved pasture grasses can be beneficial for productivity and profitability in an agricultural system. Research into *L. perenne* cultivars is furthering this development through the breeding of high sugar grasses with elevated concentrations of water-soluble carbohydrates (WSC) providing greater metabolisable energy (Amiard *et al*. 2003; Turner *et al.* 2006; Edwards *et al.* 2007) . It is also possible to alter carbohydrate content of grasses by manipulating their environment (White, 1973). While high sugar grasses have been developed to provide increased metabolisable energy (Edwards *et al*. 2007), this projects purpose was to determine if the grasses themselves might have better nitrogen-use efficiency (NUE) compared with standard cultivars.

1.2 Nitrogen in the Environment

Nitrogen (N) is responsible for the metabolic functions in cells and the genetic makeup of all living organisms (Galloway & Cowling, 2002). The earth's atmosphere is composed of an N pool consisting of almost 80% diatomic N (N_2), one of the most abundant natural states of N (Vitousek & Howarth, 1991; Galloway & Cowling, 2002; Galloway *et al.*

2004). Due to the strength of the triple bond, many organisms are unable to utilise N_2, leaving the largest source of N unavailable for biological operation (Galloway *et al.* 1995; Vitousek *et al*. 1997; Galloway *et al*. 2004; Erisman *et al.* 2007). For N molecules to be able to bond to other commonly found elements (carbon, hydrogen and/or oxygen), which compose the framework of living organisms, the triple bond must be severed (Cowling & Galloway, 2002). This is completed through N_2-fixation and N-assimilation events (Cowling & Galloway, 2002). Lightning and bacterial biological N fixation (BNF) are two naturally occurring processes that transform forms of unavailable N_2 to available N compounds (Galloway *et al.* 1995; Galloway *et al*. 2004). It is vital for organisms to fix N or find other means of obtaining converted inorganic states to more readily available forms for utilisation (Vitousek & Howarth, 1991; Galloway et al. 2004).

N is a limiting nutrient in a variety of natural and managed ecosystems (Vitousek & Howarth, 1991; Vitousek *et al*. 1997). Many ecosystems globally are limited by biologically available sources (Vitousek & Howarth, 1991; Vitousek *et al.* 2002; Galloway *et al*. 2004). Consequently, agricultural systems in many parts of the world depend on high inputs of N for high productivity (Vitousek & Howarth, 1991; Vitousek *et al.* 2002).

1.3 Haber Bosch Process

Fertiliser production is a leading cause in the increased rate of biologically available fixed N (Galloway & Cowling, 2002). In the early 1900's, Fritz Haber and Carl Bosch discovered how to synthesise the biologically available form of N ammonia, the process more commonly known today as the Haber Bosch process, and by doing so, revolutionised the world today (Erisman *et al.* 2008). The Haber Bosch process has contributed greatly to a substantial growth in the industry of agricultural fertilisers, consequently expanding crop yield and has ultimately fed between 27-48% of the global population since its first establishment (Erisman *et al.* 2008). However, the consequences of such liberal use of the process led to a varying array of unforeseen environmental changes such as acidification in soils and aquatic ecosystems, greenhouse gas imbalances and alteration to ozone layers (Erisman *et al.* 2007; Erisman *et al.* 2008). The use of these fertilisers is not consistent with the NUE of the plants they're being applied to: this is seen in Tilman *et al.* (2002) where cereals dropped from ~80 to ~30% in NUE in the span of 35 years. This highlights that denitrification of N after application and N losses to the environment are frequent

occurrences (Galloway *et al.* 2004; Erisman *et al.* 2008), and that the way in which fertilisers are applied today is unsustainable and uneconomical.

The development of civilisation has caused considerable shifts in many ecosystems, with increased available N affecting the rate of primary production (Vitousek *et al*. 1997). Anthropogenic activity continues to be a leading cause of alteration to naturally present environments, with agricultural expansion of land being most significant (Matson *et al.* 1997). Specifically in a global sense, the extent to which utilisable N sources have increased has likewise led to an increase in N fixation, as currently 100 Tg N per year is used in the form of fertilisers, initiated by the advent of the Haber Bosch process (Galloway *et al.* 1995; Erisman *et al*. 2008). This demand for N-fertiliser has caused considerable anthropogenic-influenced shifts in the natural cycle of N in the environment (Galloway *et al*. 2004). For a world population estimated to peak at 9 billion, horticultural and livestock practices must continue to increase, with practices continually being reviewed and developed in order to support this growth (Godfray *et al*. 2010). Many improvements are due to significant development in technological innovation to efficiently grow current practices by use of irrigation, greater-yielding semi-dwarf cereals (Hedden, 2003), and use of mainly fertilisers and pesticides (Matson *et al.* 1997). The 'Green Revolutions' introduction of dwarfing genes to cereal crops encouraged a significant yield of wheat and rice plants, by displaying stronger stems capable of supporting heavier heads of grain (not lodging) (Hedden, 2003).

To sustain or amplify current agricultural practices, research into intensification of land use must continue. Whilst the global population currently benefits from the advantages of N addition providing increased food supplies, there is apprehension concerning the inefficiency and unsustainability of current practices. It is particularly well documented that intensification of agriculture is responsible for significant fertiliser-driven alterations to the N cycle (Townsend *et al*. 2003; Erisman *et al.* 2007) and the cause of large N losses to the surrounding environment. Such losses in the form of gaseous emission and runoff (Zhu & Chen, 2002), have led to varying impacts on human health (Townsend *et al*. 2003), eutrophication in nearby waterways and reserves, and overall atmospheric addition to changes in global temperature (Matson *et al.* 1997).

The view of agricultural land adaptation has shifted in recent years. The shift is from one focused on producing greater supplies of crops and animal protein for a continually

growing population, to one of recognition of the damage that agricultural and industrial activities are causing to the environment (Vitousek *et al*. 1997).

1.4 Fertiliser N Use in Agriculture

N is used extensively in the form of fertilisers on a global scale in farming and crop production (Chen *et al.* 2008; Mueller *et al*. 2014). Agricultural practices worldwide apply ~85 – 90 metric tonnes of nitrogenous fertilisers annually (Good *et al.* 2004) with the prevalent forms of N application being presented as inorganic ammonium (NH_4^+), nitrate (NO_3^-), and urea (Dechorgnat *et al*. 2010). Only a small portion of this added N is actually assimilated and utilised by the plants (Whitehead & Edwards, 2015). Excess N from the use of these fertilisers can have detrimental effects on the surrounding environment by means of nitrate leaching (into soil and ground water), and as a release of greenhouse gas emissions in the form of nitrous oxide (N_2O) (Di & Cameron, 2002; Good *et al*. 2004; Easton *et al.* 2009; Rasmussen *et al.* 2009; Masclaux-Daubresse *et al.* 2010).

Additionally, N is expelled in the urine of foraging ruminants (Rasmussen *et al.* 2009). A large portion of the N taken up by the animal is excreted in urine due to degradation of proteins in the rumen by microbial activity, reducing the supply for development and growth of the animal (Rasmussen *et al*. 2009). To enable better utilisation of these proteins, researchers have turned to developing increased NUE in the ruminant. One approach is by use of high sugar grasses containing elevated stores of carbohydrates (Miller *et al.* 2001; Edwards *et al.* 2007). Increased metabolizable energy sources, in the form of high WSC grasses, can reduce degradation resulting in a reduction of N excreted, when shoot material is consumed (Miller *et al*. 2001; Edwards *et al*. 2007; Easton *et al*. 2009). This could contribute to reducing the negative effect of ruminant excretion on the surrounding environment (Di & Cameron, 2002; Easton *et al.* 2009).

1.5 Water-Soluble Carbohydrates in *L. perenne*

The main WSC in *L. perenne* are fructans (Turner *et al.* 2006). Fructans are non-structural storage carbohydrates, polymers of fructose, which are synthesised in the vacuole from stored sucrose (Pavis *et al.* 2001; Turner *et al.* 2006). Specific enzymes called fructosyltransferases are responsible for the synthesis of fructans which is regulated by photosynthetic activity (Vijn & Smeekens, 1999). Labelled as reserve carbohydrates, fructans are stored in several locations, readily accessible for remobilisation (Pollock & Cairns, 1991). They are located in the stubble/sheath and roots of a plant, and when the assimilated carbon (C) supply from photosynthesis for growth is lower than required, they are remobilized (Sullivan and Sprague, 1943; Steen and Larsson, 1986; Guerrand *et al*. 1996; Morvan *et al.* 1997; Pavis *et al.* 2001; Chalmers *et al.* 2005). Fructans are also accumulated in the elongation zone of the leaf, which is enclosed by leaf sheaths at the stem base (Schnyder *et al.* 1987). Remobilization of fructans is involved in the rapid regeneration of new plant shoots after defoliation (Liu *et al.* 2015; Guo *et al.* 2017).

1.6 Nitrogen and Carbon Interaction

The relationship between available N and C stores in plants plays a role in NUE (Turner *et al.* 2015; Roche *et al.* 2016; Guo *et al.* 2017). Compared to standard cultivars of ryegrass, it has been suggested that NUE in the early stages of plant re-foliation following defoliation is greater in plants that have greater concentration of stored carbohydrates (Louahlia *et al.* 2008). The remobilization of carbohydrates is critical to the conversion of inorganic nitrate to organic forms, as C provides the backbone for the assimilation of N into organic compounds (Krapp & Traong, 2006). C has been observed to play a crucial role in N uptake and assimilation (Krapp & Traong, 2006; Roche *et al.* 2016; Guo *et al.* 2017).

Roche *et al*. (2016) looked at assimilation of N once taken up by the ryegrass plants. They conducted experiments looking at degradation of fructan stores on addition of nitrate to plants deficient in N. They observed that there was very fast remobilisation of low-molecular weight WSC in the plant when N was added. Further, they observed that the plant tissue (a combination of shoots and sheath) held large fructan stores and suggested that the tissue was responsible for remobilisation of high-molecular weight WSC on

addition of N. They suggested that the fructan pools promoted the uptake and assimilation of nitrate and concluded that C limited N uptake (Roche *et al.* 2016).

Following defoliation, the ability of the plant to perform photosynthesis is prevented for a brief period of time (Guo *et al*. 2017), influencing carbohydrate levels in the plant and ultimately nutrient uptake. Guo *et al.* (2017) looked at the relationship between C and N following defoliation, identifying the interaction between them and the dependence of nitrate uptake on remobilised carbohydrates. They observed that nitrate uptake in response to a shift in the C/N ratio of plants was correlated with a signalling mechanism indicating available C metabolites. Guo *et al.* (2017) found that remobilisation of fructans occurred in an N-dependent manner, and concluded that remobilisation of fructans was stimulated by N addition for growth following defoliation. They also showed that defoliation caused considerable shifts in the C store in the plants which impacted the efficiency of nitrate uptake.

The Roche *et al.* (2016) and Guo *et al.* (2017) papers highlight the important interaction between N and C. The availability of high sugar grasses allows an alternative approach to investigate the relationship between C and NUE in perennial ryegrass.

1.7 Aims and Objectives

My hypotheses for this project are as follows:

(1) That the cultivars with a 'higher sugar' content (i.e. Expo and Aber Magic) will provide greater quantities of stored fructan that will be mobilised following defoliation of leaf material and nitrate addition and;

(2) That due to these greater stores of fructan, that the high sugar cultivars will effectively have a greater NUE than the standard cultivar.

To test the hypotheses, seeds of three cultivars of perennial ryegrass were obtained from PGG Wrightson (Christchurch). These were Nui, regarded as the standard perennial ryegrass cultivar with 'normal' levels of fructan, and Expo and Aber Magic, both of which are regarded as 'high sugar grasses' (Rasmussen *et al.* 2009; Turner *et al.* 2015). All cultivars were diploid. Dr Alan Stewart (PGG Wrightson) indicated that Aber Magic might

be expected to have higher fructan content than the Expo variety, and both somewhat higher than Nui, but cautioned that the levels may, in fact, not be greatly different.

The expression of high sugar phenotypes in *L. perenne* has been documented in research carried out on European soils (Tas *et al.* 2006; Taweel *et al.* 2006). Literature suggests that cooler night temperatures may influence the rate of fructan accumulation in high sugar *L. perenne* cultivars in New Zealand and Australia (Parsons *et al.* 2004; Turner *et al.* 2015). Due to differences in climate between New Zealand and Europe, a period of cooler temperatures may be required for constant expression of high sugars in grass tissues (Parsons *et al.* 2004).

The first objective of the study was to manipulate the cultivars of ryegrass to ensure contrasting fructan levels. To attempt this, it was proposed that the plants should be exposed to two different environmental treatments. The treatments applied were ‘outside’, with a cooler night temperature and ‘glasshouse’, with an overall warmer environment. This would then supply material to test the interaction between stored fructan and NUE post defoliation.

Chapter 2

Materials and Methods

2.1 Plant Material and Experimental Design

Seeds of three cultivars of perennial ryegrass (Nui, Expo and Aber Magic) were obtained from PGG Wrightson (Christchurch). The experiment was run from November 2016 (early summer) until March 2017 (early autumn). This was conducted at the glasshouse facilities of the University of Canterbury (43°31'24.3"S 172°35'14.8"E), both within a glasshouse and outside in the vicinity of the glasshouses.

The experimental set up of the perennial ryegrass seeds in trays was the same as that used by Dawar (2010). Seeds were sown in 30 trays, 10 trays per cultivar. Trays were 420 mm x 300 mm with a depth of ca. 65 mm, and were filled with a mix of 75% unfertilised soil and 25% plasterers sand. The seeds were planted in four rows with ca. 60 mm between rows and ca. 1 cm between each seed (approximately 150 seeds per tray). The trays were arranged in two blocks, each with five trays of the three cultivars. Each tray was moved to a randomised location within the block each week for the first nine weeks of seedling establishment.

Measurement of the length of the longest shoot and tiller number were taken every 3-4 days. The average temperature of the glasshouse was 21.2°C (min: 10.3°C, max: 46.0°C), and vents were opened when the temperature rose above 23°C. Plants were under an automatic watering system schedule, which watered the plants two-three times a week. Shade cloth had previously been set up above the trays. An addition of granular N fertiliser (Yates Thrive All Purpose Soluble Fertiliser) dissolved in water was made up at 10% strength, with one addition at 20% when the tips of the leaves looked yellow. Seedlings were allowed to establish for nine weeks, and following this establishment phase, the addition of N was discontinued. After the nine weeks, half of the trays (block one: five of each cultivar) were moved outdoors. This was to provide a cooler environmental treatment compared to those in the glasshouse. The average temperature in the glasshouse following the establishment phase was 20.8°C (min: 8.9, max: 44.3) and the average temperature outside was 18.8°C (min: 6.4, max: 37.2).

2.2 ^{15}N Application to Defoliated Plants

Three defoliation events occurred following the nine week establishment period. The first defoliation event occurred seven days before the end of the establishment phase. Seven days after defoliation, trays from block one were placed outside. Two further defoliation events occurred at 21 day intervals. Three days following the last defoliation event the plants were supplemented with nitrate (NO_3^-) labelled with ^{15}N to determine assimilation of ^{15}N into the N-pool in the plant. ^{15}N-labelled NO_3^- (0.34 ml 99% ^{15}N to 17.6 g of urea) was applied to plants in solution form at a rate of 25 kg N ha^{-1} to shoots and surface of the soil. This concentration was mixed with ca. 1.5 L of water per block, distributed over plants as 100 ml per tray from a measuring cylinder.

Samples were taken from each tray on the day of the third defoliation, on the day of ^{15}N application (three days following the third defoliation) and the final destructive harvest seven days following nitrate application. Leaf (shoot), sheath and roots were harvested at each harvest event. This was done by harvesting leaf (shoot) material above 4 cm and sheath material in the early morning. Sheath material is defined as tissue above the ground to 40 mm, which includes the elongation zone and mature sheaths of the leaf sections of the plant (Guo *et al.* 2017). The root material was washed thoroughly shortly after to remove all adhering soil particles. At the time of harvest, the plant material was then frozen in liquid N_2.

2.3 Preparation of Plant Material for ^{15}N and Fructan Analysis

Following the harvest of plant material, frozen samples were kept at -20 °C. As fresh weight was not able to be taken at the time of harvest due to time constraints, the material was weighed after being frozen (this was then labelled as fresh/frozen weight). Weights were taken by removing material from their falcon tubes and weighing in a weigh boat immediately after removing from the freezer. Following weight measurements, the lids were removed from the falcon tubes and nappy liners were cut to size, folded over once and secured over the open end of the tube with an elastic band. These samples were then placed in an oven heated to between 50-60 °C to allow the material to dry thoroughly. However, the larger samples should have been placed in paper bags to allow them to dry thoroughly. This resulted in the larger samples (such as the majority of those following nitrate application) showing a white fungus which spread throughout the samples in the

falcon tubes. Once identified as contaminated, these samples were removed from the tubes and then placed in paper bags to further dry them. It was determined that the fungal contamination should not interfere with the ^{15}N analyses, nor the fructan content for the samples harvested prior to N addition as they were smaller quantities and dried well.

Samples were dried to a consistent weight. From the dry samples, smaller portions of 130-150 mg of the weighed material were taken for ^{15}N and fructan analysis. The samples for ^{15}N and fructan analysis were ground in a ball mill (Retsch Mixer Mill 400) until a fine powder was produced. Not all samples were analysed due to time constraints.

Samples of ground material for ^{15}N analysis were accurately reweighed to between 50-60 mg, placed in Eppendorf tubes and sent to the University of Waikato, Hamilton for analysis.

2.4 Fructan Assay

Four out of the five biological replicates per cultivar per environmental treatment prior to the ^{15}N addition, were chosen for fructan analysis. These corresponded with the samples sent for ^{15}N analysis. Fructan analysis was carried out using the Fructan Megazyme Assay Kit (McCleary *et al.* 2000; Revanna *et al.* 2013) to analyse fructan content in the plant material. A complete table of solutions is provided in Table 2.1.

Prior to commencement of the experiment, enzyme solutions A and B used were taken out of the freezer and placed on ice to defrost for 30 min – 1 h with thawing completed by rubbing the tubes between hands for 10 second increments whenever required. The 40 °C water bath was also set up and the AccuBlock™ Digital Dry Bath was heated to 80 °C.

Table 2.1 Solutions and materials provided by the Megazyme Fructan Assay kit and solutions prepared prior to commencement of analysis.

Solutions supplied with the Megazyme kit

Bottle 1: **Sucrase** plus β-amylase, pullulanase and maltase as a freeze-dried powder.

Bottle 2: **Fructanase**. Recombinant *exo*-inulinase and recombinant *endo*-inulinanase as a freeze-dried powder.

Bottle 3: **Fructan Control Flour**. Fructan freeze-dried in the presence of α-cellulose.

Bottle 4: **Sucrose Control Flour**. Sucrose freeze-dried in the presence of α-cellulose.

Bottle 5: **D-Fructose Standard Solution**. (1.5 mg/ml) in 0.2% (w/v) benzoic acid.

Solutions prepared

Buffer 1: **Sodium maleate buffer** (100 mM, pH 6.5) Maleic acid and distilled water (adjusted with 2 M sodium hydroxide solution).

Buffer 2: **Sodium acetate buffer** (100 mM, pH 4.5) Glacial acetic acid and distilled water (adjusted with 1 M sodium hydroxide solution).

Enzyme Solution A: Combination of Bottle 1 and 22 ml of Buffer 1 *(stored at -4°C*)

Enzyme Solution B: Combination of Bottle 2 and 22 ml of Buffer 2 *(stored at -4°C*)

PAHBAH Reducing Sugar Assay Reagent:

- **Solution A**: *p*-hydroxybenzoic acid hydrazide, distilled water and concentrated hydrochloric acid
- **Solution B**: Trisodium citrate dihydrate, distilled water, calcium chloride dihydrate and sodium hydroxide
- **PAHBAH Working Reagent**: 1:9 ratio of PAHBAH Solution A to Solution B

Sodium hydroxide (50 mM): Sodium hydroxide and distilled water

Reagent 3 (Alkaline borohydride): (10 mg/ml sodium borohydride in 50 mM sodium hydroxide)

Reagent 4 (Acetic acid) (200 mM): Glacial acetic acid and distilled water

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The procedure is best described in steps:

Step 1

The method used measured samples at 0-12% fructan content for extraction, with 18.5 mg of sample (also used for sucrose control flour) having 1.85 ml of distilled water added to it in a 2 ml Eppendorf tube. The 12-50% fructan extraction was used for the control fructan flour: 3.7 mg of sample had 1.85 ml of distilled water added to it in a 2 ml Eppendorf tube. The tube was then placed in an AccuBlock^{TM} Digital Dry Bath set at 80 °C. The samples were heated for 20 min, where every 2 min the tubes were briefly placed on a vortex mixer, and every other minute they were tipped or shaken to mix the contents.

Step 2

The samples were allowed to cool to room temperature and then centrifuged in an Eppendorf Centrifuge 5424 for one minute at a time at 8000 RPM (for 2-3 min), checking in between to see if material had pelleted and the solution was clear. This was to pellet cell debris in the sample, leaving the fructan remaining in the supernatant.

Step 3

Aliquots of 0.2 ml of the sample supernatant were pipetted into sterile 15 ml falcon tubes. Tubes were allocated for fructan and sucrose cellulose controls, biological samples, sample blanks and borohydride check (as per guidelines in Megazyme Fructan Assay Procedure). These were labelled as shown in Table 2.2. Four samples were run concurrently in each experimental run, with triplicate technical replicates. Each tray of plants was treated as a biological replicate. Into each falcon tube, 0.2 ml of Enzyme Solution A was pipetted. The tubes were then incubated in a 40 °C water bath for 30 min. Following the incubation period, 0.2 ml of Reagent 3 (alkaline borohydride solution) was added to each falcon tube and placed on a vortex mixer to thoroughly mix the solutions. The tubes were then incubated again in a 40 °C water bath for 30 min. Following the second incubation, 0.5 ml of Reagent 4 (acetic acid) was pipetted into each of the tubes. A vigorous effervescence

was observed when this solution was added. The tubes were then vortexed and termed ‘Solution S’.

Table 2.2 Labelling for samples and controls run in the experiment

Sample labels:
Fructan Cellulose Control (F) and Sample Blank (FSB)
Sucrose Cellulose Control (S) and Sample Blank (SSB)
Borohydride Check (BH)
Samples (e.g. N1 = Nui tray 1) and sample blank (e.g. N1SB)
D-Fructose Standard Solution (DF)
Reagent Blank (RB)

Step 4

Aliquots of 0.2 ml of each Solution S were pipetted into three individual falcon tubes (except for BH). This was completed for all samples and sample blanks, one tube for BH (replacing Solution S with D-fructose Standard) and three for each of the controls and blank controls (fructose and sucrose). Following this, 0.1 ml of Enzyme Solution B was aliquoted into each sample tube and 0.1 ml of Buffer 2 was aliquoted into sample blank, BH check and control blank tubes. These tubes were then incubated a third time in a 40 °C water bath for 30 min. Tubes of D-fructose Standard were also prepared by combining 0.2 ml of D-fructose Standard with 0.9 ml Buffer 2, then placing aliquots of this solution in four separately labelled 15 ml falcon tubes. Aliquots of 0.1 ml of Buffer 2 were also added to D-fructose Standard tubes. The Reagent blank was prepared by pipetting aliquots of 0.3 ml of Buffer 2 into three separate 15 ml falcon tubes.

Step 5

Tubes were separated into two groups. 5.0 ml of PAHBAH working solution was added to the first group and placed in a boiling water bath for exactly 6 min. Following this, the first group was placed in a cool water bath (18-20 °C) for approximately 5 min. The second group was subjected to the same treatment. 0.2 ml of each solution was then pipetted into a 96 well plate. The absorbance of the plate was read immediately after plating at 410 nm using a SpectraMax M5 plate reader.

2.5 Calculation of Fructan and Nitrogen Content

Fructan content was calculated using an equation supplied with the Megazyme Fructan Assay Kit. The values for each part of the equation are listed in Table 2.3:

$$= \Delta_A \times F \times 5 \times V \times (1.1 / 0.2) \times (100/W) \times (1/1000) \times (162/180)$$

$$= \Delta_A \times F \times (V / W) \times 2.48$$

Table 2.3 Components of the equation used to calculate fructan content in plant tissue

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ΔA = sample absorbance - sample blank absorbance (both read against the reagent blank)

F = factor to convert absorbance values to µg of D-fructose = (54.5 µg D-fructose)/(absorbance for 54.5 µg D-fructose)

5 = factor to convert from 0.2 ml as assayed to 1.0 ml

V = volume (ml) of extractant used (i.e. 1.85ml)

(1.1/0.2) = 0.2 ml was taken from 1.1 ml of enzyme digest for analysis

W = weight (mg) of sample extracted

(110/W) = factor to express fructan as a percentage of flour weight

(1/1000) = factor to convert from µg to mg

162/180) = factor to convert from free D-fructose, as determined, to anhydrofructose (and anhydroglucose), as occurs in fructan

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The percentage uptake of N following ^{15}N-labelled urea addition was calculated using equations in Dawar (2010) on ^{15}N recovery in plants, to determine the estimated assimilation of ^{15}N after addition.

Sum of fertiliser derived N in the plant (% N dff):

$$= \left(\frac{\%^{15}N \; excess \; in \; sample}{\%^{15}N \; excess \; in \; fertiliser} \right) \times 100$$

Sum of applied N utilised by the plant (%):

$$= \left(\frac{\%^{15}N \; dff \; \times yield \; of \; N}{rate \; of \; N \; application} \right) \times 100$$

Total N in plant tissue was calculated using the following equation:

$$= \frac{\%N \; \times (g \; tissue \; Dry \; Weight)}{100}$$

2.6 Statistical Analysis

Analysis of variance (ANOVA) was used to test for the effects of treatment, time and cultivar using the R statistical platform (R Development Core Team, 2008) with post hoc comparisons being made using least significance difference tests (Sokal & Rohlf, 1981). Differences were considered significant if probabilities (P) were less than 0.05.

Chapter 3

Results

Plant material was harvested 21 days after the second defoliation event (this was considered the third defoliation event), prior to N addition. Fertiliser plus ^{15}N was added three days after the third defoliation event and the plant material harvested again after seven days. The treatments applied were 'outside', with a cooler night temperature (average: 18.8°C (min: 6.4, max: 37.2)) and 'glasshouse', with an overall warmer environment (average: 20.8°C (min: 8.9, max: 44.3)). Full temperature measurements between the second and third defoliation events were not available due to a recording error, so it is not possible to compare the temperatures over the final stages of the experiment. The main focus was to identify whether the cooler treatment led to an increase in fructan content and whether this is turn increased NUE. A key focus was on the sheath material as this is where perennial ryegrass stores fructan (Guerrand *et al*. 1996, Pavis *et al.* 2001).

3.1 Dry Weight of Plant Tissue

Dry weight of sheath material collected 21 days post-defoliation showed a significant effect of treatment (P <0.001) (Table 3.2A). The treatment effect was shown to be statistically significant for all the cultivars, with the outside treatment showing a greater sheath weight than in the glasshouse (Figure 3.2A). Root material had a similar trend to sheath in regards to treatment (P <0.001) (Table 3.3A), with all cultivars grown outside having greater root weight than those grown in the glasshouse (Figure 3.3A). An effect of cultivar was also significant (P <0.001) (Table 3.3A). Dry weight of shoot material showed a significant effect of treatment (P <0.001) and cultivar (P= 0.015) (Table 3.1A). The treatment effect was shown to be statistically significant for all the cultivars, with plants grown under the glasshouse treatment presenting a greater shoot dry weight than those grown outside (Figure 3.1A).

Table 3.1 Analysis of variance table for dry weight of shoot material in cultivars of ryegrass (A and B correspond to graphs A and B, respectively), see Figure 3.1 for results

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	0.01826	0.00913	5.365	0.0149	*
treatment	1	0.23898	0.23898	140.437	6.17e-10	***
cultivar:treatment	2	0.00266	0.00133	0.781	0.4728	
Residuals	18	0.03063	0.00170			

A

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	0.0170	0.0085	2.818	0.0861	.
treatment	1	0.3470	0.3470	114.794	3.06e-09	***
cultivar:treatment	2	0.0197	0.0099	3.261	0.0619	.
Residuals	18	0.0544	0.0030			

B

Table 3.2 Analysis of variance table for dry weight of sheath material in cultivars of ryegrass (A and B correspond to graphs A and B, respectively), see Figure 3.2 for results

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	0.0006	0.0003	0.002	0.997535	
treatment	1	2.2269	2.2269	19.471	0.000336	***
cultivar:treatment	2	0.0855	0.0428	0.374	0.693272	
Residuals	18	2.0587	0.1144			

A

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	1.787	0.894	7.303	0.00476	**
treatment	1	9.445	9.445	77.198	6.29e-08	***
cultivar:treatment	2	0.793	0.397	3.242	0.06272	.
Residuals	18	2.202	0.122			

B

Table 3.3 Analysis of variance table for dry weight of root material in cultivars of ryegrass (A and B correspond to graphs A and B, respectively), see Figure 3.3 for results

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	0.4869	0.2435	12.172	0.000453	***
treatment	1	0.4052	0.4052	20.260	0.000276	***
cultivar:treatment	2	0.0294	0.0147	0.735	0.493398	
Residuals	18	0.3600	0.0200			

A

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	1.205	0.603	4.868	0.0204	*
treatment	1	5.076	5.076	41.006	4.98e-06	***
cultivar:treatment	2	0.251	0.125	1.012	0.3831	
Residuals	18	2.228	0.124			

B

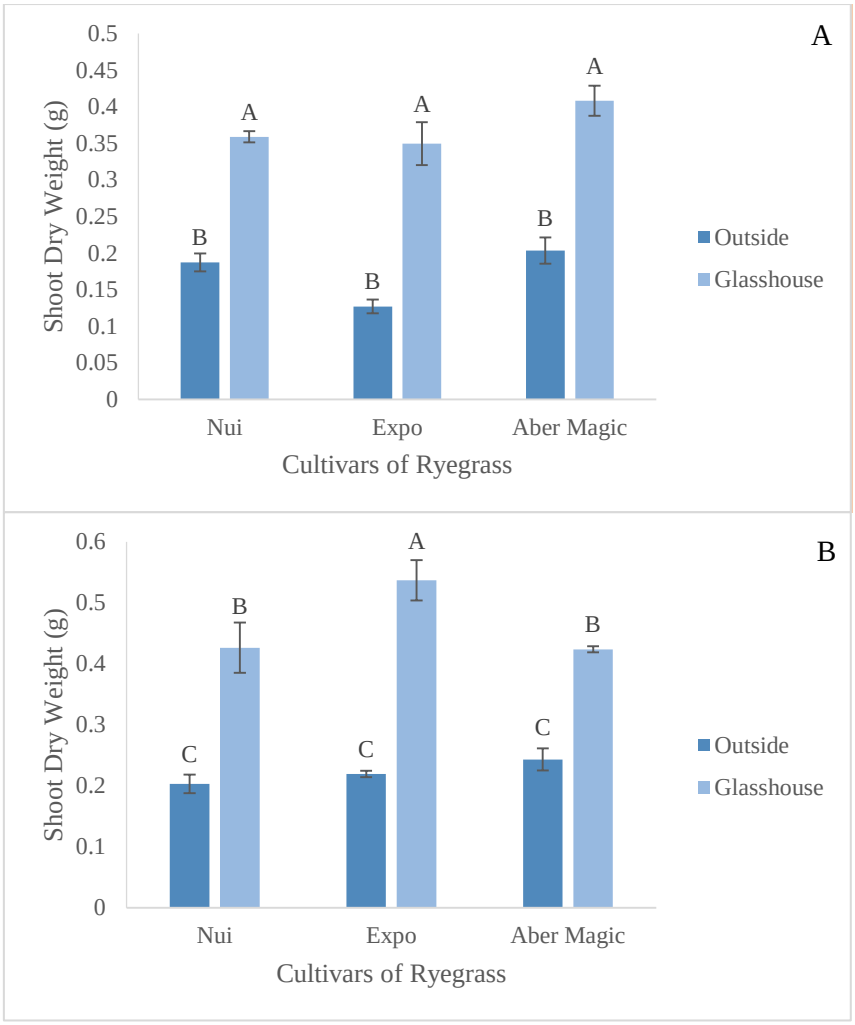

Figure 3.1 Comparison of the effect of the two environmental treatments (outside and glasshouse) on calculated shoot dry weight (g) of three cultivars of *Lolium perenne* L. The dry weight was obtained from plant material harvested 21 days following the second defoliation event (A), and 10 days following the third defoliation event, seven days after N addition (B). Values are means (± standard error of the mean, SEM) where n = 4. Different letters indicate statistically different values at P < 0·05 using least significant difference test of treatment means.

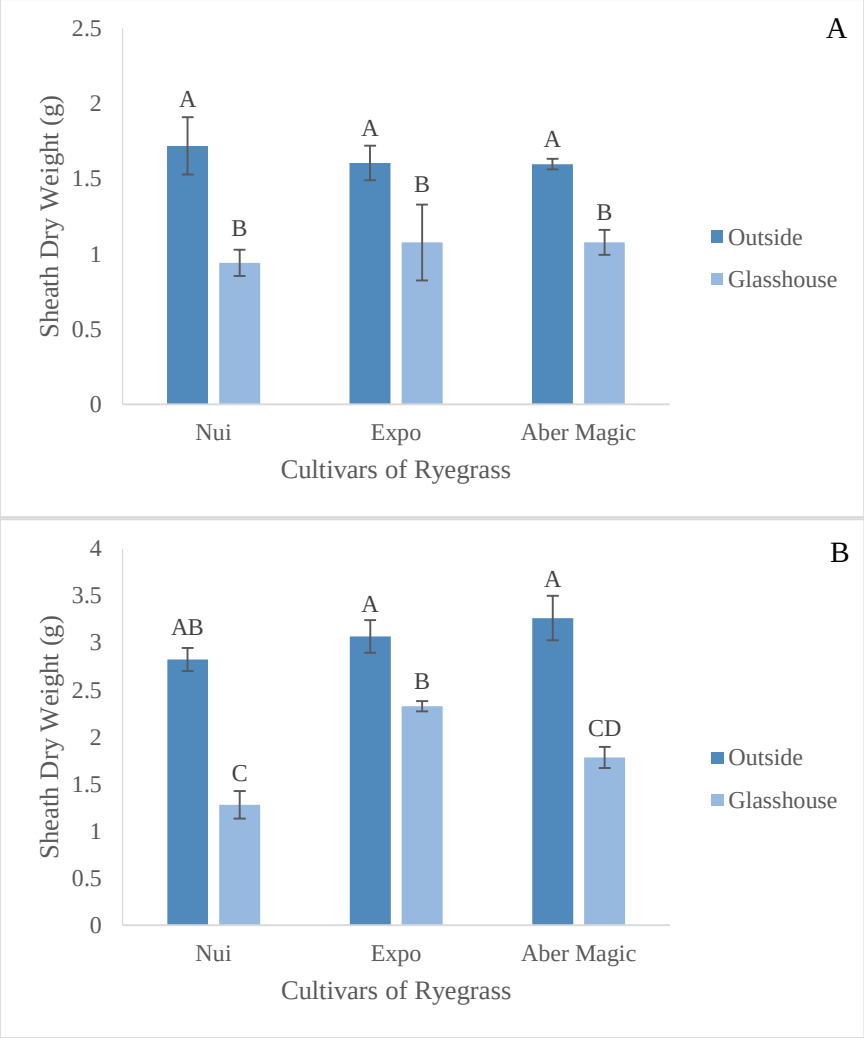

Figure 3.2 Comparison of the effect of the two environmental treatments (outside and glasshouse) on calculated sheath dry weight (g) of three cultivars of *Lolium perenne* L. The dry weight was obtained from plant material harvested 21 days following the second defoliation event (A), and 10 days following the third defoliation event, seven days after N addition (B). Values are means (± standard error of the mean, SEM) where n = 4. Different letters indicate statistically different values at $P < 0{\cdot}05$ using least significant difference test of treatment means.

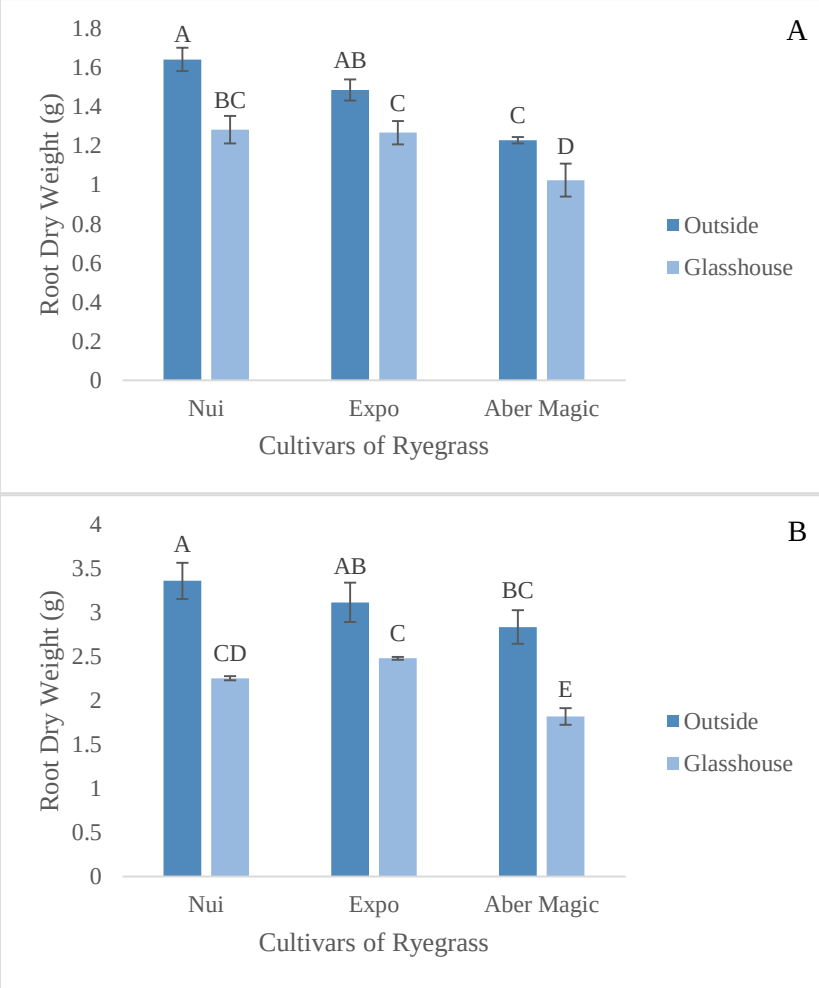

Figure 3.3 Comparison of the effect of the two environmental treatments (outside and glasshouse) on calculated root dry weight (g) of three cultivars of *Lolium perenne* L. The dry weight was obtained from plant material harvested 21 days following the second defoliation event (A), and 10 days following the third defoliation event, seven days after N addition (B). Values are means (± standard error of the mean, SEM) where n = 4. Different letters indicate statistically different values at $P < 0{\cdot}05$ using least significant difference test of treatment means.

3.1 Dry Weight of Plant Tissue - Continued

Plant material was also harvested seven days after N addition. Dry weight of sheath material showed a significant effect of treatment (P <0.001) and cultivar (P= 0.004), showing plants grown in outside conditions had a greater sheath weight than those of the same cultivar grown under glasshouse conditions (Table 3.2B, Figure 3.2B). There was also a statistically significant difference seen between Expo and Aber Magic values when grown in the glasshouse, where Expo presented a greater dry weight than Aber Magic (Figure 3.2B). Root material displayed similar results to sheath, with treatment (P <0.001) and cultivar (P= 0.02) showing a significant effect on plants grown outside (Table 3.3B, Figure 3.3B). Expo and Nui presented a greater root weight than Aber Magic when grown in the glasshouse, and Nui had a significantly greater root weight than Aber Magic when grown outside (Figure 3.3A). Dry weight of shoot material showed a significant effect of treatment (P <0.001) (Table 3.1B). All cultivars grown in the glasshouse had a greater shoot weight than those of the same cultivar grown outside, with Expo showing a greater shoot weight than Nui and Aber Magic (Figure 3.1B).

3.2 Fructan Content

Before N addition, significant effects of treatment (P <0.001) and cultivar (P= 0.012) were identified in fructan content of ryegrass cultivars (Table 3.4). Fructan sheath content in cultivars grown outside was significantly greater than those cultivars grown in the glasshouse (Figure 3.4). This, however, was only shown independently for each cultivar: Nui plants grown outside had greater fructan content than Nui plants grown in the glasshouse (Figure 3.4).This was also seen in Expo and Aber Magic. However, glasshouse grown Expo plants were significantly greater in levels of fructan content than Nui (Figure 3.4).

The Nui cultivar was analysed to determine fructan content in all harvested material prior to N addition. Treatment (P <0.001) and plant material (P <0.001) were each statistically significant, with a significant interaction (P= 0.006) (Table 3.5). Sheath and shoot material had significantly greater fructan content in plants grown outside than those grown in the glasshouse (Figure 3.5). Fructan content in the root material was not significantly different (Figure 3.5). Sheath material grown in the glasshouse also had significantly greater fructan content than shoot and root material (Figure 3.5).

Table 3.4 Analysis of variance table for fructan content in sheath material in cultivars of ryegrass 21 days after second defoliation event, see Figure 3.4 for results

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	25.65	12.83	5.745	0.0118	*
treatment	1	205.41	205.41	92.007	1.69e-08	***
cultivar:treatment	2	3.13	1.56	0.700	0.5096	
Residuals	18	40.19	2.23			

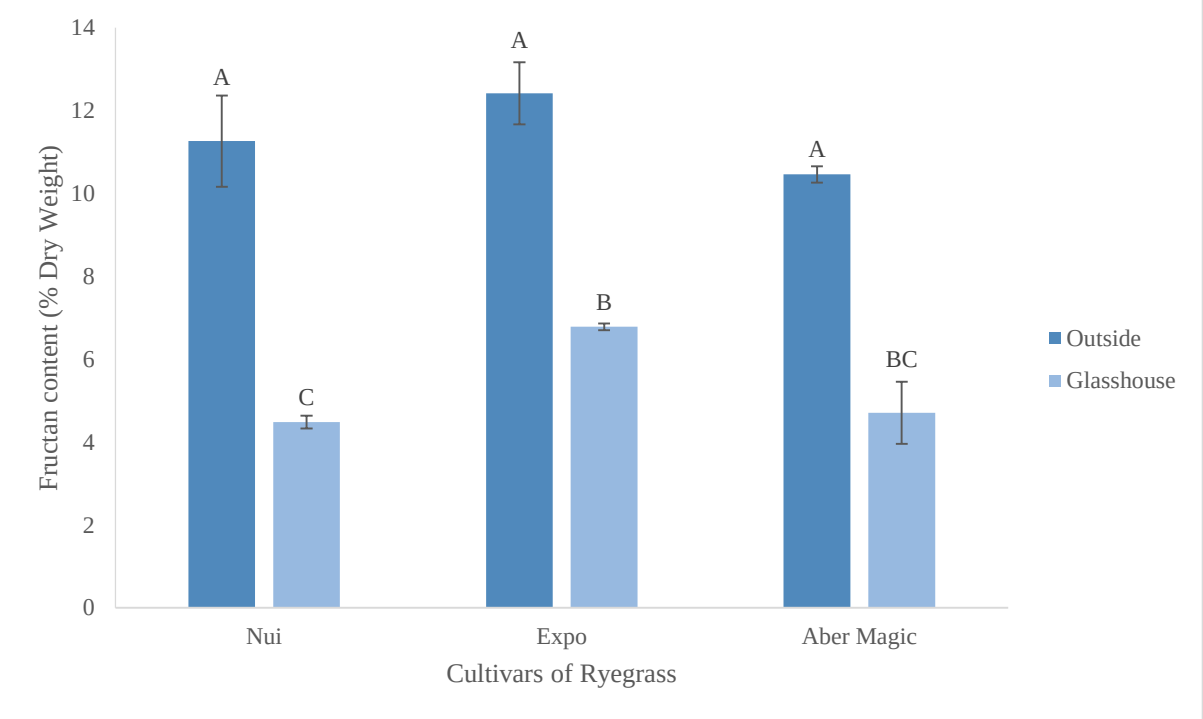

Figure 3.4 Fructan content calculated on a dry weight basis in the sheath tissue in three cultivars of *Lolium perenne* L. under two different treatments (outside and glasshouse), 21 days after the second defoliation event. Values are means (± standard error of the mean, SEM) where n = 4. Different letters indicate statistically different values at P < 0·05 using least significant difference test of treatment means.

Table 3.5 Analysis of variance table for fructan content 21 days after second defoliation event in harvested plant material from cultivar 'Nui', see Figure 3.5 for results

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
treatment	1	102.89	102.89	45.883	2.4e-06	***
material	2	154.26	77.13	34.395	7.1e-07	***
treatment:material	2	30.83	15.41	6.874	0.00606	**
Residuals	18	40.36	2.24			

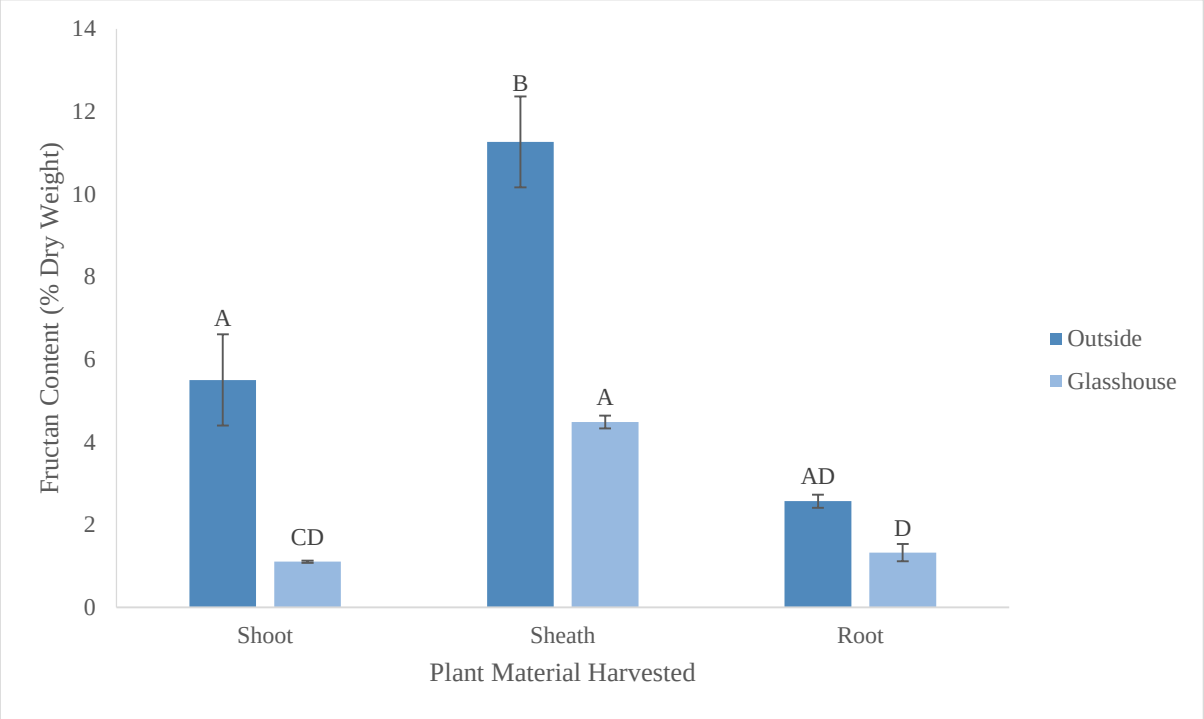

Figure 3.5 Comparison of the effect of two environmental treatments (outside and glasshouse) on calculated fructan content (% Dry Weight) of plant material harvested from *Lolium perenne* L., cultivar Nui. This was 21 days after the second defoliation event. Values are means (± standard error of the mean, SEM) where n = 4. Different letters indicate statistically different values at $P < 0{\cdot}05$ using least significant difference test of treatment means.

3.3 Percentage Uptake of N in Plant Tissue

The data for % uptake of N following application of urea supplemented with ^{15}N was analysed. The effect of treatment on the % uptake of N in sheath material was statistically significant (*P*= 0.004) (Table 6B), where Nui grown outside had a greater uptake of N than the same plants grown in the glasshouse (Figure 6B). However, no effect of cultivar or interaction was seen (*P*= 0.413 and *P*= 0.431, respectively) (Table 6B). Root material displayed similar results to sheath, with treatment (*P* <0.001) being the only factor of significance (Table 6C). Nui and Aber Magic presented greater uptake in outside grown plants than in the glasshouse when compared to their individual cultivar (Figure 6C). The effect of the factor treatment on % uptake of N in shoot material following N addition was statistically significant (*P*= 0.013) (Table 6A), where Expo had greater uptake of N when grown in the glasshouse than outside (Figure 6A).

Table 3.6 Analysis of variance table for % uptake of N in shoot, sheath and root material in cultivars of ryegrass (A, B and C correspond to graphs A, B and C respectively), see Figure 3.6 for results

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	0.1303	0.0652	0.898	0.4250	
treatment	1	0.5503	0.5503	7.580	0.0131	*
cultivar:treatment	2	0.2972	0.1486	2.047	0.1582	
Residuals	18	1.3069	0.0726			

A

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	0.0578	0.0289	0.928	0.41330	
treatment	1	0.3316	0.3316	10.655	0.00431	**
cultivar:treatment	2	0.0549	0.0275	0.882	0.43096	
Residuals	18	0.5601	0.0311			

B

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	0.00176	0.00088	0.498	0.616	
treatment	1	0.04393	0.04393	24.891	9.51e-05	***
cultivar:treatment	2	0.00655	0.00327	1.854	0.185	
Residuals	18	0.03177	0.00176			

C

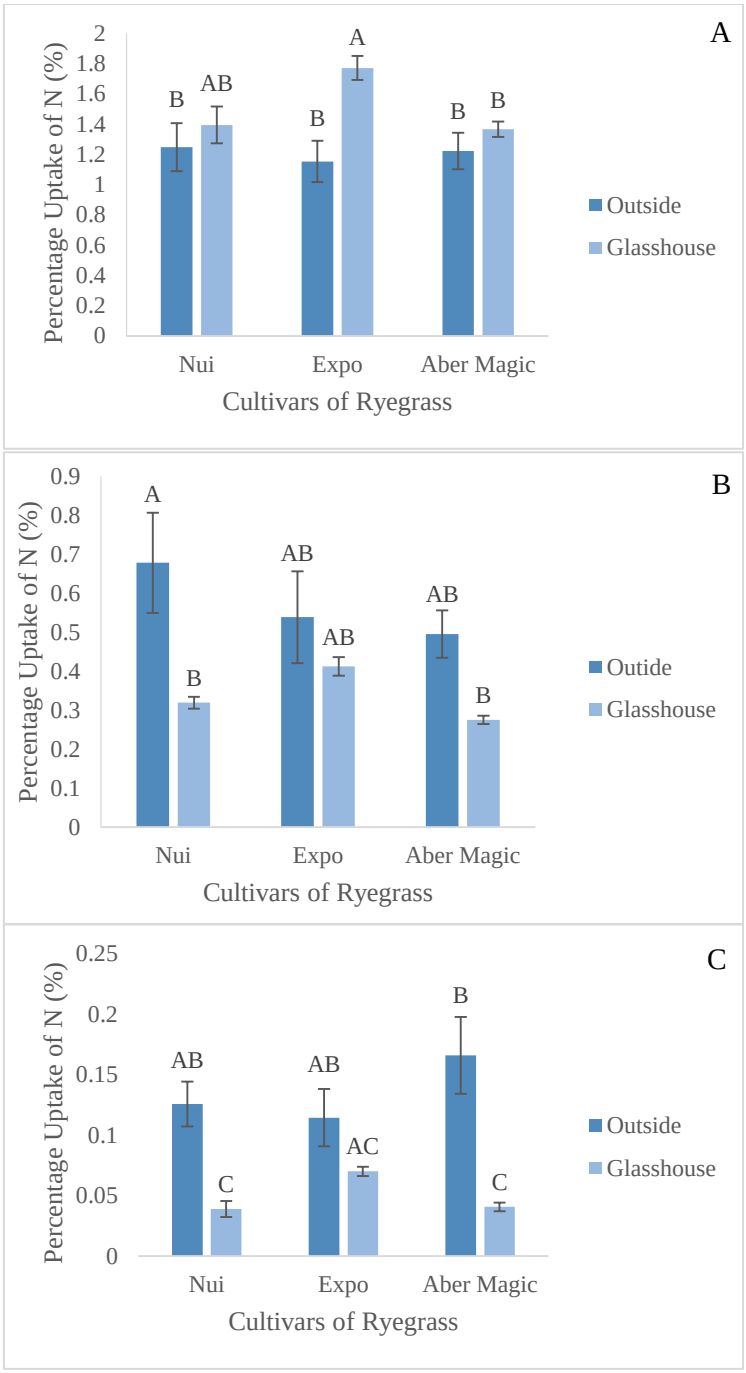

Figure 3.6 Comparison of the effect of two environmental treatments (outside and glasshouse) on calculated % uptake of applied N in shoot, sheath and root material harvested 10 days after the third defoliation event and seven days after N addition, from three cultivars of *Lolium perenne* L. % uptake of N in shoots (A), sheath (B) and root (C) material. Values are means (± standard error of the mean, SEM) where n = 4. Different letters indicate statistically different values at $P < 0{\cdot}05$ using least significant difference test of treatment means.

Table 3.7 Analysis of variance table for the response of total N in shoot tissue following ^{15}N addition, see Figure 3.7 for results

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	0.0000169	0.0000084	1.913	0.1623	
treatment	1	0.0013004	0.0013004	294.728	< 2e-16	***
time	1	0.0013064	0.0013064	296.088	< 2e-16	***
cultivar:treatment	2	0.0000279	0.0000139	3.160	0.0544	.
cultivar:time	2	0.0000323	0.0000162	3.664	0.0356	*
treatment:time	1	0.0003099	0.0003099	70.241	5.56e-10	***
cultivar:treatment:time	2	0.0000319	0.0000159	3.611	0.0372	*
Residuals	36	0.0001588	0.0000044			

Table 3.8 Analysis of variance table for the response of total N in sheath tissue following ^{15}N addition, see Figure 3.8 for results

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	0.000127	0.000063	0.744	0.482605	
treatment	1	0.004618	0.004618	54.249	1.10e-08	***
time	1	0.011491	0.011491	134.984	9.76e-14	***
cultivar:treatment	2	0.000593	0.000296	3.483	0.041421	*
cultivar:time	2	0.000139	0.000070	0.817	0.449701	
treatment:time	1	0.001535	0.001535	18.026	0.000146	***
cultivar:treatment:time	2	0.000086	0.000043	0.505	0.607917	
Residuals	36	0.003065	0.000085			

Table 3.9 Analysis of variance table for the response of total N in root tissue following ^{15}N addition, see Figure 3.9 for results

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
cultivar	2	0.0000865	0.0000432	2.910	0.0674	.
treatment	1	0.0009080	0.0009080	61.123	2.86e-09	***
time	1	0.0030855	0.0030855	207.697	< 2e-16	***
cultivar:treatment	2	0.0000729	0.0000364	2.452	0.1004	
cultivar:time	2	0.0000040	0.0000020	0.134	0.8748	
treatment:time	1	0.0004251	0.0004251	28.615	5.15e-06	***
cultivar:treatment:time	2	0.0000608	0.0000304	2.045	0.1441	
Residuals	36	0.0005348	0.0000149			

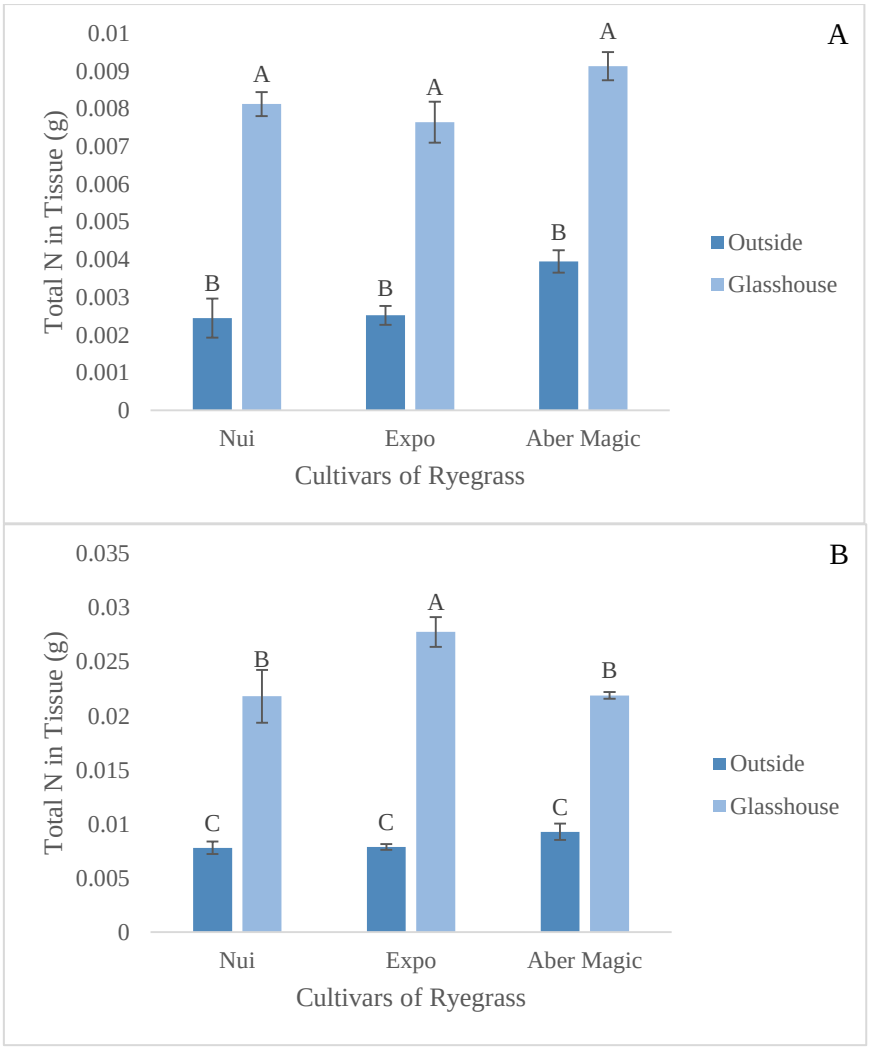

Figure 3.7 Total N from harvested shoot tissue was calculated 21 days following the second defoliation event (A), and 10 days following the third defoliation event, seven days after N addition (B). Values are means (± standard error of the mean, SEM) where n = 4. Different letters indicate statistically different values at $P < 0{\cdot}05$ using least significant difference test of treatment means.

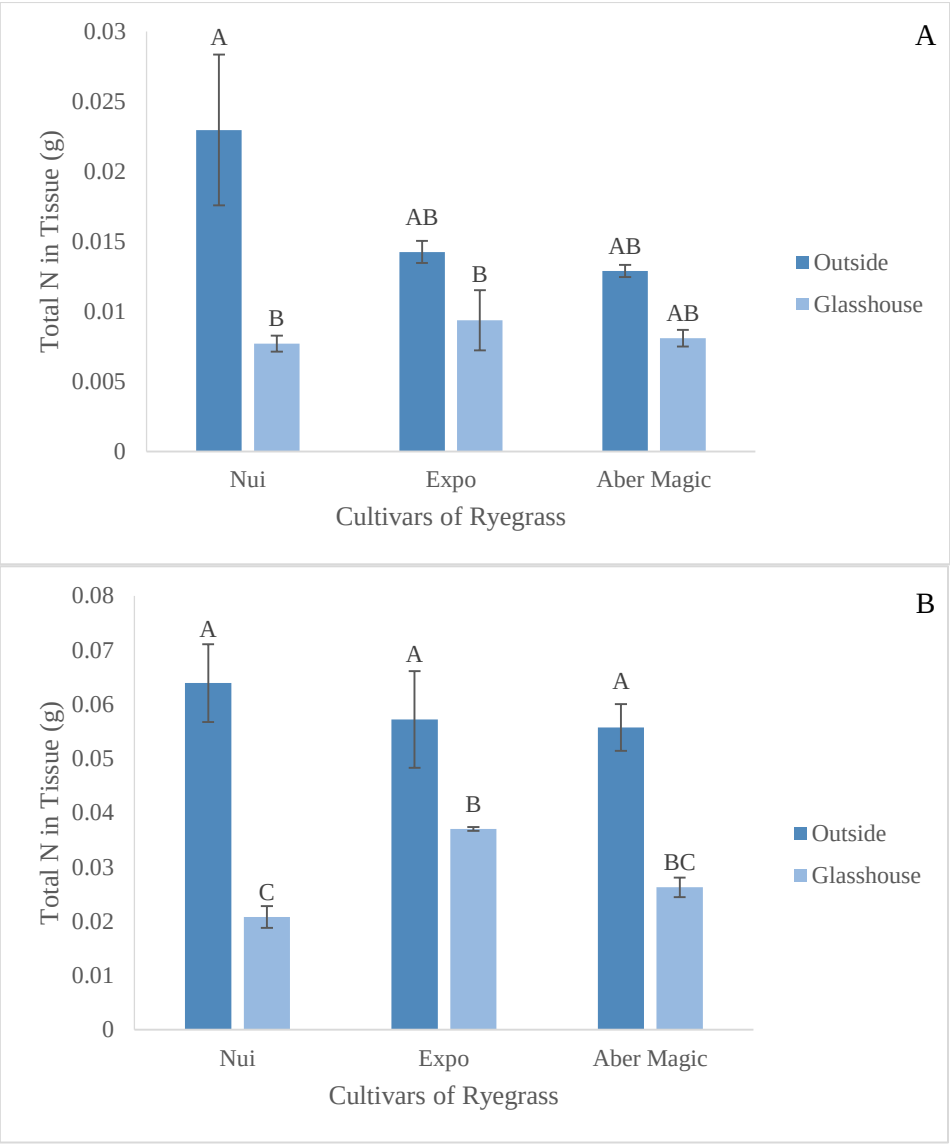

Figure 3.8 Total N from harvested sheath tissue was calculated 21 days following the second defoliation event (A), and 10 days following the third defoliation event, seven days after N addition (B).Values are means (± standard error of the mean, SEM) where n = 4. Different letters indicate statistically different values at P < 0·05 using least significant difference test of treatment means.

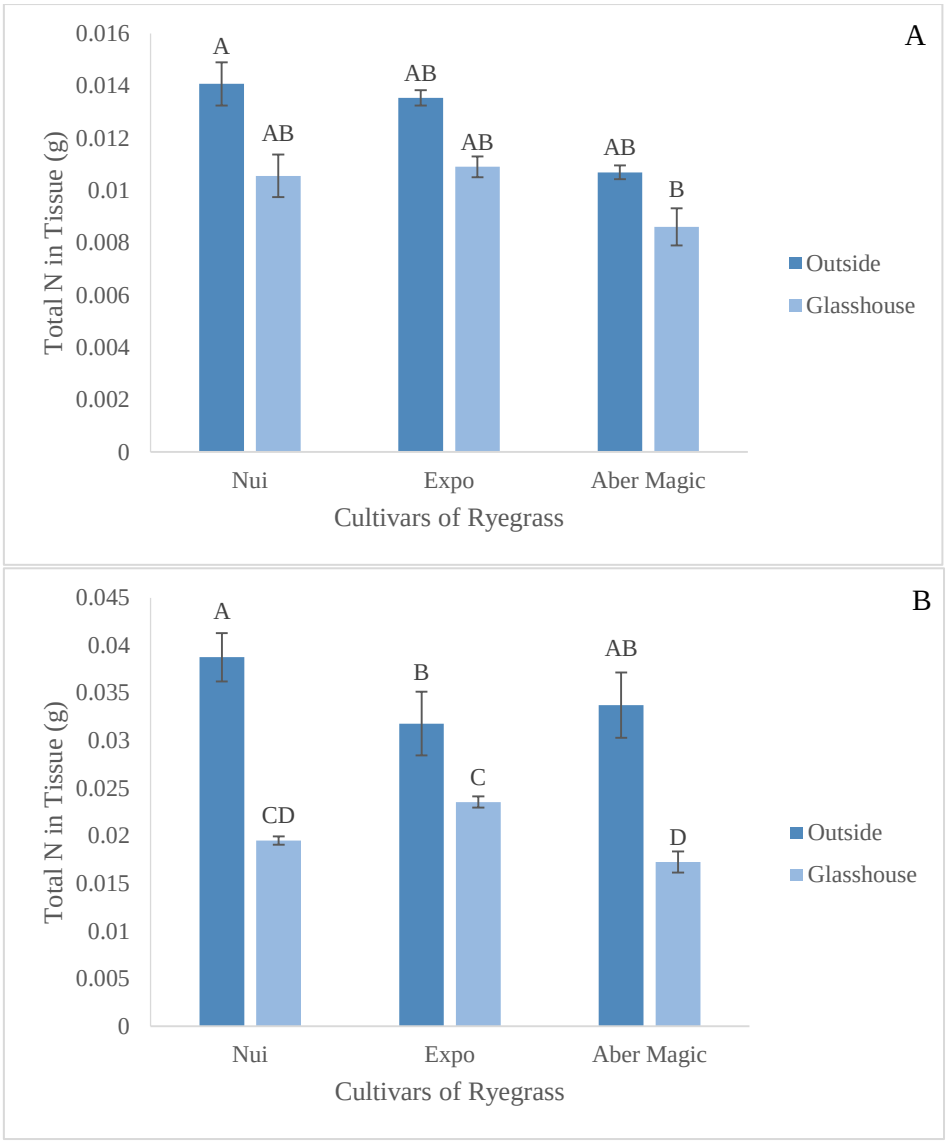

Figure 3.9 Total N from harvested root tissue was calculated 21 days following the second defoliation event (A), and 10 days following the third defoliation event, seven days after N addition (B).Values are means (± standard error of the mean, SEM) where n = 4. Different letters indicate statistically different values at $P < 0{\cdot}05$ using least significant difference test of treatment means.

3.4 Total N in Harvested Tissue

Total N in harvested tissue (i.e. total dry matter yield x N content/Dry Weight) was also measured. Treatment was shown to have a significant effect in sheath material (P <0.001) (Table 8), where plants grown outside showed a greater total N in sheath tissue than those grown in the glasshouse (Figure 8A and 8B). The factor of time was also seen as statistically significant (P <0.001) (Table 8), where it showed greater total N in sheath tissue material following N application (Figure 8B). The interaction between treatment and time was shown to be significant (P <0.001) (Table 8). There was also an interaction of cultivar and treatment on total N in sheath tissue (P= 0.041) (Table 8). Nui was consistently expressing higher total N in sheath material when grown outside (both before and after N addition), than Nui grown in the glasshouse (Figure 8A and 8B). After N addition, Expo and Aber Magic also expressed this difference (Figure 8B).

Total N in root tissue followed a similar trend to sheath material, with significant treatment (P <0.001) and time (P <0.001) effects, complemented by an interaction between the two (P <0.001) (Table 9). No significant differences were found between treatments or cultivars before N addition (Figure 9A). However, after N addition, a clear treatment effect was shown to be statistically significant for all the cultivars, with the outside treatment showing a greater total N in root tissue than in the glasshouse (Figure 9B). Nui had a greater total N than Expo when both were grown outside, and Expo had a greater total N than Aber Magic when both were grown in the glasshouse (Figure 9B). Three interactions were significant for total N in shoot tissue: cultivar and time (P= 0.036), treatment and time (P <0.001), and a significant three-way interaction between cultivar, treatment and time (P= 0.037) (Table 7). Before N addition, plants grown in the glasshouse showed a greater N content in shoot tissue than those grown outside (Figure 7A). This was only shown independently for each cultivar (i.e. Nui grown in the glasshouse was significantly different from Nui grown outside). There was also a greater total N in shoot tissue following N application in glasshouse grown plants compared to outside for all cultivars, and Expo showed greater total N in glasshouse grown plants compared to Nui and Aber Magic (Figure 7B).

Chapter 4

Discussion

Perennial ryegrass is a vital component of the agricultural industries in New Zealand (Hampton *et al.* 2012). Manipulating the ability of ryegrass to more efficiently uptake N from inputs such as fertilisers may reduce the environmental damage associated with fertiliser use. As an interaction between N assimilation and stored fructan has been shown (Guo *et al.* 2017), the key aim of this thesis was firstly to manipulate the fructan content of *L. perenne* cultivars, and secondly, to observe if NUE changed depending on the level of these fructans. The first aspect was to determine whether a cooler environment encouraged this accumulation, and where this accumulation occurred, with specific interest in the sheath material.

This study looked at how cooler night temperatures influenced the rate of fructan accumulation in high sugar *L. perenne* cultivars, as lower temperatures have been shown to encourage the accumulation of stored WSC within the plant (Parsons *et al.* 2004; Turner *et al.* 2015). Although the so-called high sugar cultivars (Expo and Aber Magic) did not have substantially greater fructan accumulation within plant organs when compared to Nui, there was a consistency across all cultivars in which the outside treatment of the experiment (effectively the cooler treatment providing colder night temperatures) provided a greater accumulation of fructans than those grown in the glasshouse.

The expression of a high sugar phenotype in *L. perenne* has been documented in research carried out on European soils (Tas *et al.* 2006; Taweel *et al.* 2006). However, the cultivation of these high sugar grasses in New Zealand and Australia has shown inconsistencies amongst studies (Parsons *et al.* 2004; Turner *et al.* 2014). It has been suggested that a potential genotype x environmental interaction is occurring (Halling *et al.* 2004; Edwards *et al.* 2007). Based on the work conducted by Parsons *et al.* (2004), lower temperatures have been suggested as crucial for expression of the high sugar phenotype in European derived ryegrass cultivars, as the inconsistencies observed when the same cultivars are grown in New Zealand pastures are not replicated in Europe (i.e. European studies are consistent with an increase in WSC content in high sugar cultivars) (Edwards *et al.* 2007; Turner *et al.* 2015). The results in this thesis did show an increase in fructan

accumulation in cultivars Expo and Aber Magic under the lower temperature but did not support the genotype x environmental hypothesis, as similar responses were seen with Nui.

Previous literature has highlighted that the sheath (known as stubble) is responsible for storage of fructans, which are readily accessible for remobilisation (Pollock & Cairns, 1991; Chalmers *et al.* 2005; Guo *et al.* 2017). Research by Guo *et al.* (2017) and Roche *et al*. (2016) identified the shoot and sheath material as being the region for the most active remobilised WSC after N addition. Fructan content was analysed in cultivars of *L. perenne* to test the hypothesis that the cultivars with 'higher sugar' content (i.e. Expo and Aber Magic) would provide greater quantities of stored fructan in the sheath material. Sheath compared with shoot and root material, regardless of treatment, had a significantly greater amount of accumulated fructan content (Figure 4). Figure 4 also identified a clear treatment effect in shoot and sheath material, where outside grown material had greater fructan content than that grown in the glasshouse. This leads to the conclusion that the outside treatment was more effective in increasing WSC content, than growth in the glasshouse, but there was no difference between cultivars.

The N application to plants was urea supplemented with ^{15}N, and the data for this was calculated to generate the % uptake of ^{15}N in the plants. It was hypothesised that greater stores of fructan may support a greater N assimilation, or NUE. The results of % uptake of N in plant material varied depending on the cultivar. Sheath and root material showed a trend of greater % uptake of N in material harvested from outside plants than those in the glasshouse, with Nui being statistically significant. However, this effect was not seen in the shoots, where shoots from cultivar Expo represented the opposite effect and showed greater % uptake of N in the glasshouse grown plants.

The total N in plant tissue combined the growth of tissue and N content in the tissue. The data indicates that total above ground N in sheath tissue is greater in plants grown outside compared to those grown in the glasshouse. These results support the conclusion that the cooler environmental treatment encouraged increased fructan accumulation in the sheath material, and hence allowed a greater assimilation of N by the plant tissue.

Conclusions and Future Research

The experimental treatments of cooler and warmer environments produced results that were specific for those two environments and provided a tool to study the fructan/N interaction. Clearly, 'high sugar' grasses could be beneficial both for ruminant NUE and plant NUE. As this experiment was only conducted during the summer, it is possible that the effect on NUE may have been diminished due to increases in photosynthetic activity. Increases in NUE are more likely to be observed during the spring/autumn period where colder temperatures at night would likely increase accumulation of fructan, potentially leading to a more profound interaction between N and fructan content.

This research focused on the accumulated fructan stores before N addition. Future research could include similar analyses being performed on material harvested after N addition, to observe where fructan stores are remobilised within the plant, and where they accumulate following defoliation. This would be able to link back to uptake of N (%) and total N in tissue data, to compare how N and fructans interact, following defoliation and N addition.

Future work could also include replicated experimentation under field conditions to support the findings in this thesis, by measuring plant growth during a variety of time points throughout a growing season.

Interest in the interaction between C and N in *L. perenne* cultivars provided an opportunity for my thesis to develop on the current literature surrounding this relationship, expanding on from the studies of Guo *et al.* (2017) and Roche *et al.* (2016). The efforts of my practice come as an addition to research in this area, and to focus on how we can increase sustainability in New Zealand's agricultural practices with the continuation of fertiliser application on our pastures.

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