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Understanding the physiological basis of post-flowering nitrogen (N) dynamics in spring barley to improve nitrogen use efficiency (NUE).

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Abstract

Identifying ways to improve nitrogen use efficiency (NUE) in cereal production is a major international research priority. It has been estimated that globally only around 40% of N from fertilizer is recovered in the grain of cereal crops. Rates of post-anthesis N uptake (PANU) by crops are often low and high quantities of N can be found in the soil at harvest, apparently left unused by the crop. The low rate of PANU needs to be overcome to minimise the negative environmental impacts caused by the use of N fertilizers. A better understanding of the physiological control of PANU as an interrelated mechanism of soil N availability, plant N demand, N remobilisation and root growth and activity is needed to further improve NUE. The aim of this project was to investigate the dynamics of N uptake and remobilisation during the post-flowering period in relation to the N status at flowering, the soil N supply and plant N demand during grain filling.

Controlled environment experiments were conducted on spring barley cv Westminster grown in sand-perlite using ^{15}N labelling techniques to discriminate between the fate of N taken up before anthesis and that taken up during grain filling. The results showed that barley roots have the capacity to uptake N from repeat low concentration (1 mM) applications with high efficiency throughout grain filling (>90%). The efficiency of PANU was not affected by the plant N status at flowering. Higher N status plants remobilized a larger quantity of N from vegetative tissue for allocation to the grain, but the remobilisation efficiency was little affected. The temporal dynamics of remobilisation from each organ, however, showed some differences, with a slower onset of remobilisation from leaf sheaths and stems in plants of higher N status compared to plants of low status.

The role of N supply and grain N demand in the control of PANU was tested by varying the concentration of N applied after flowering and by partial de-graining. Regardless of the N supply, de-grained plants accumulated

significantly less N than the control plants between early and late grain filling. However, there was no significant difference between de-grained and control plants in their net N uptake measured by short term (24 hours) ^{15}N labelling. De-graining and increased N supply increased the partitioning of ^{15}N to vegetative tissues, mostly towards the tillers. These results indicate that there is a large sink demand for N post-flowering even after grain numbers are reduced. They also suggest that the observed reduction in the total N accumulation is not the result of reduced PANU, but the consequence of a different mechanism, potentially N losses from the plant to the atmosphere. However, this hypothesis was not supported by measurements of NH_3 volatilization from leaves and ears of de-grained and control plants. Further experiments are needed to determine the cause of the reduced N accumulation in de-grained plants.

A field experiment was conducted in 2019 to compare post-anthesis soil nitrate depletion by spring barley with that of spring oats, a species regarded as having a high N uptake efficiency. Spring oats depleted soil nitrate to a greater extent than spring barley and this effect was consistent across four varieties of each species tested at anthesis, but the soil N dynamics change during the grain filling period. The poorer depletion by barley was associated with its smaller average root length density in the topsoil, but variability in the data prevented the establishment of critical root length densities for nitrate uptake by each species.

These experiments have shown that spring barley has a large demand for N during grain filling and maintains a high physiological capacity for its uptake. The relatively poor depletion of soil nitrate by field grown barley crops during this period may be associated with restricted access of roots to N rather than physiological controls over its uptake. Improvements in root distribution may be a suitable target to increase N uptake efficiency of spring barley.

Lay Summary

The main fertilizer elements in crop production are nitrogen (N), phosphorus (P), and potassium (K) with N being the nutrient that most often limits growth and subsequently productivity. The consumption of N fertilizers in agriculture has been massively increased since the 1980's in an attempt to improve yields. Unfortunately, crops and in particular cereal crops, use nitrogen fertilizers inefficiently. Nitrogen can be lost from the soil-plant system through several pathways causing significant environmental problems. Therefore, an essential goal in modern agriculture is to minimize N fertilizer inputs, which will help to reduce production costs and environmental deterioration, while maintaining high yields and grain quality.

It is essential to increase the nitrogen use efficiency (NUE) of crop species. This can be improved through two complementary ways. Firstly, by improvements in crop management and fertilizer techniques, and secondly through plant breeding and the development of more efficient varieties. The latter requires information on which phenotypic traits control NUE within a breeding population.

The present research was conducted in spring barley due to its high demand for the malting industry and the highly specific parameters on grain quality. Firstly, the physiological capacity of barley roots to capture N was tested using a simple root medium and the isotope ^{15}N. The results showed that barley has the physiological capacity to absorb N right through the end of grain filling and the N uptake is not influenced by the N status of the plant at flowering. I then tested the relationship between N uptake, the supply of N post-flowering and the grain demand for N. The grain N demand was altered by removing 50% of the barley ears (de-graining treatment). The results showed that even though there was a reduction on the net N accumulation between two growth stages during the grain filling period, the uptake of N was not affected. The post-anthesis N supply, however, largely influences the uptake of N. The higher the

N supply, the higher the uptake of nitrogen. Volatilization of ammonia (NH_3) was measured in an attempt to explain the reduced N accumulation previously observed. Ammonia was measured in control and de-grained plants, but no differences were observed between them. Lastly, the ability of barley to deplete N compare to other cereal crops such as oats was assed under field condition during 2019. Differences in the ability to deplete soil N appears to be likely a specific characteristic of the different species at anthesis but the N dynamics change during the grain filling period.

More information is needed to understand the relationship between soil N, roots and soil microorganism. However, given the high capacity of barley roots observed when grown under a simple medium, there seems to be opportunities to improve the root architecture of barley, particularly the proliferation of roots at deeper soil profiles.

Declaration

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and that this work has not been submitted for any other degree or professional qualification.

Diana Carolina Garzon Obando

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Abbreviations

N	Nitrogen
NH_3	Ammonia
NH_4^+	Ammonium
NO_3^-	Nitrate
NO_2^-	Nitrite
KCl	Potassium chloride
M	Molar
mM	Millimolar
cv	Cultivar
NUE	Nitrogen use efficiency
NupE	Nitrogen uptake efficiency
NutE	Nitrogen utilization efficiency
PANU	Post-anthesis N uptake
NR	Nitrogen Remobilisation
NRE	Nitrogen Remobilisation Efficiency
MGW	Mean grain weight
DW	Dry weight
FW	Fresh weight
DAF	Days after flowering
GS	Growth Stage
SMN	Soil Mineral Nitrogen
SOM	Soil organic matter
RLD	Root Length Density

cRLD	Critical Root Length Density
LATS	Low-affinity transporter system
HATS	High-affinity transporter system
GPC	Grain protein content
ADF	Apoplastic dilution factor
EAF	Extracted apoplast fluid
LSD	Least Significant differences
SEM	Standard error of the mean

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Chapter 1: Literature review

1.1 Barley: a crop of interest for research

Barley is ranked fourth among the most cultivated cereals in the world. It is grown on approximately 47.7 million hectares in 106 countries worldwide (FAOSTAT, 2022). It is cultivated both in highly productive agricultural systems and, also in dry and marginal areas (Miralles et al., 2020).

In the UK, barley is the second most important cereal after wheat, grown on nearly 1.2 million ha and producing 8.4 million tonnes in 2020 (FAOSTAT, 2022). Figures 1.1 shows worldwide changes in the area harvested of barley and its production from 2002 to 2020. Figure 1.2 shows the world and UK's yield trends over the same period.

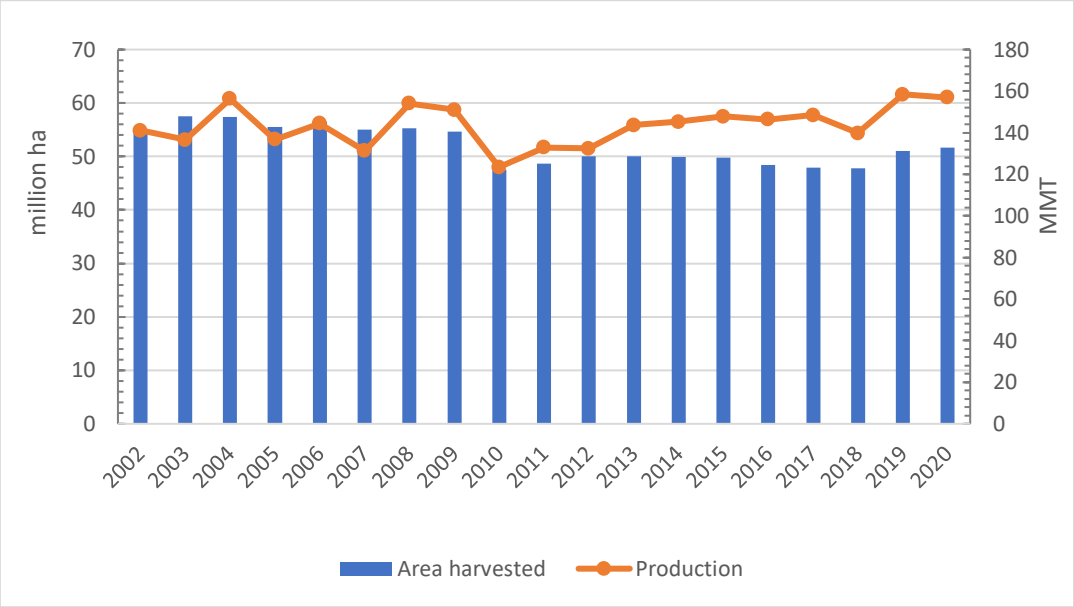


Figure 1.1 World changes in area harvest, expressed in million hectares, and average barley production, expressed in million metric tonnes (MMT) from 2000 to 2020 (FAOSTAT, 2022).

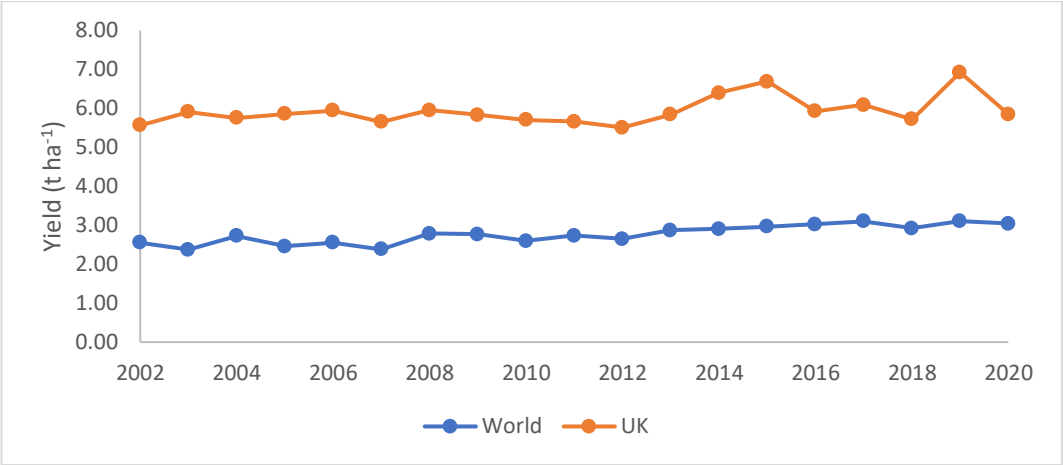


Figure 1.2 Barley yields (t ha^{-1}) worldwide and in the UK from 2002 to 2020 (FAOSTAT, 2022).

Besides its importance as a crop for animal feed and malting for alcohol production, it is an established model plant for agronomic, genetic, and physiological studies (Criado et al., 2015; Raun and Johnson, 1999). Due to its diploid nature, it is a good genetic model for other crops in the Triticeae family (Karunarathne et al., 2020). Though a better understanding of the physiological basis of many agronomic traits is needed. Fortunately, barley germplasm resources are considerable, with much potential for exploitation. Consequently, substantial gains in crucial sustainability characteristics should be achievable in the future, together with increased understanding of the physiological basis of several agronomic traits, particularly water and nutrient use efficiency (Newton et al., 2011).

Barley was domesticated about 10,000 years ago the crop was domesticated there from its wild relative *Hordeum spontaneum* (Badr et al., 2000). Barley was domesticated as a source of human food, but over time, its major usage evolved to the extent that, in highly developed countries, its importance as a food crop is very limited (Swanston et al., 2014). Worldwide, animal feeding is the main use of barley (Baik and Ullrich, 2008). Even in countries like Morocco, with relatively high levels of human food use, around 80% of the barley grown

is used to feed animals (Baik and Ullrich, 2008). However, in some developing countries such as Ethiopia, the total annual barley grain production is almost entirely destined for human consumption, making of barley the major staple food in the highlands (Grando and Gormez, 2005).

In the UK, there are two crops of barley grown, one sown mainly in September-November known as winter barley and the other sown in February-May known as spring barley. Winter barley varieties are generally higher yielding, they require a period of exposure to cool temperatures called vernalization to initiate flowering and must also be cold hardy. Spring barley varieties do not require vernalization but are usually resistant to cold temperatures (Kling and Hayes, 2004).

The growing cycle of the different barley crops depend on which area in the UK is the crop sown. For example, in England, spring barley tends to have a growing cycle of 120-130 days whereas in Scotland it can be as long as 150-160 days (Kling and Hayes, 2004). Figure 1.3 shows the development and growth phases of a winter barley crop sown in October. The timing at which different paraments such as spikes (or ear) number and grain number is also shown (Bingham et al., 2006).

Winter barley is typically utilised in brewing for ales, lagers and roast products, spring barley on the other hand, is widely used for brewing and distilling (Kok et al., 2019).

Globally, around 15-20% of the barley production is used in the malting industry for brewing or whisky making (Miralles et al., 2020; Newton et al., 2011). The dominance of barley as a brewing cereal result from its capacity to provide a suitable substrate and source of enzymes, to produce sugars that are used by yeasts in the fermentation process (Swanston et al., 2014).

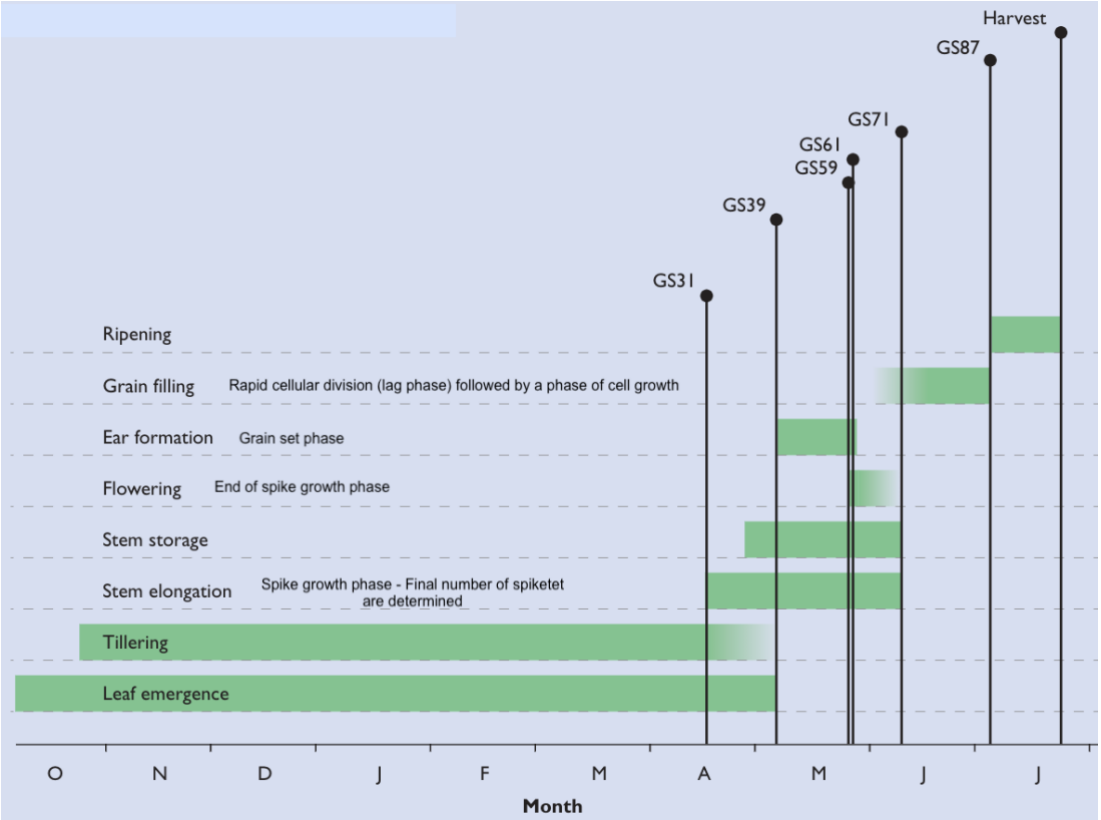


Figure 1.3 Barley growth stages and timing for developmental stages modified from (Bingham et al., 2006).

Malting is the process in which raw grain is made ready to become the main ingredient in the brewing process. This is achieved by employing a controlled germination of grain in moist air (MacLeod and Evans, 2016). The malting process can be divided in three main stages: steeping, germination and kilning. In the steeping stage, the acquiescent grains imbibe water and hydrates the embryo and endosperm. In the germination stage, enzymes are synthesized, activated and mobilized and the embryo begins to develop. In the kilning phase, grain growth is halted using a heat treatment which dries the grains to constant and low moisture for storage (MacLeod and Evans, 2016).

The commercial quality of malting barley is highly specific and parameters such as grain size, germination potential and grain N concentration are carefully considered (Newton et al., 2011). The required grain nitrogen

percentage in barley varies depending on the final use of the malt. In the UK the brewing industry requires N concentrations between 1.6% and 1.75% (MAGB, 2022). For export and lager beers a concentration of 1.7% to 1.85% is required (MAGB, 2022). Barley for distilling malt requires a lower N level, typically about 1.5%. In difficult seasons higher levels, up to 1.65% can be used (Newton et al., 2011). Due to the high demand for barley in the malting industry, achieving high yields is as important as obtaining an optimal commercial quality of the grain (Criado et al., 2018).

1.1.1 Barley diversity: Types and germplasm

Barley is currently classified according to ear structure. Barley ears can produce either one or three grain per node, giving 2-row and 6-row varieties (AHDB, 2018a). Of the 3 spikelets which are inserted at each node of the rachis, only the central one is fertile in the 2-row type (Le Gouis, 1991). The major gene responsible for the row-type differences in barley is the Six-rows spoke 1 (Vrs1) which affects the pistil development of the lateral spikelets (Zwirek et al., 2019). Two-row varieties usually have a higher number of tillers per plant. On the other hand, six-row varieties usually have more seeds per inflorescence. Thus, the compensatory effects of yield components lead to similar levels of yield potential (Kling and Hayes, 2004). It is widely recognised that two-row barley is favoured for malting throughout most of the world, whereas sox-row is large used only for feed. However, in the USA and Mexico, six-row barley is used extensively for this purpose (Kling and Hayes, 2004).

Wild barley is two-row. The six-row trait was selected shortly after domestication (Zwirek et al., 2019). Genetic variation is essential for crop improvement. However, during the domestication process and the transition from wild genotypes to modern cultivars the genetic diversity of crop species, including barley has been drastically reduced, potentially leaving behind useful genes and alleles (Tanksley and McCouch, 1997). Fortunately, an enormous amount of natural barley diversity can still be found, either within gene banks

or in the wild (Ellis et al., 2000). In 2010, FAO estimated that there are over 400,000 barley accessions in the world. These accessins include cultivars (15%), landraces (43%), breeding lines and genetic stocks (28%) and wild Hordeum species (14%). Due to their high adaptability to a range of conditions barley landraces are recognized as an important genetic resource with which to search for tolerance to biotic and abiotic stresses (Monteagudo et al., 2019). Wild barley and landraces which predate modern elite lines offer the breeder the potential to find unexploited genetic diversity (Stevens et al., 2021).

There is ample evidence of intraspecific variation in NUE in barley and thus potential for improving resource use efficiency through breeding (Anbessa et al., 2009; Bingham et al., 2012). Improvements in both N uptake efficiency and N utilization efficiency (main components of NUE) may be required to make substantial gains in yield under conditions of low or moderate nitrogen availability. However, progress requires the development of an understanding of the phenotypic traits that govern NUE, their genetic control and their expression in a range of environments (Newton et al., 2011).

1.2 Global N consumption in relation to cereal crops

Over the past century, crop yield and soil fertility have significantly increased mainly from the greater inputs of fertilizer, pesticides and water alongside the rapid adoption of modern high-yielding crop varieties and other technologies of the ‘Green Revolution’ (Lu and Tian, 2017; Tilman et al., 2002). The rise in fertilizer production and application contributed considerably to improving agricultural productivity and decreasing hunger worldwide (Tilman et al., 2002). Because the nutrient requirements of crop species vary, their contribution to N, P and K consumption differs significantly. For example, cereal crops have a much greater impact on N-fertilizer consumption than legume crops. Modern short-stature varieties of wheat and rice have contributed greatly to the increase in global N fertilizer application because of their positive response to N, high yields, and high harvest index and improved

lodging resistance (Ladha et al., 2005). Short-stature or semi-dwarf varieties refer to plants that grows to a lesser height than usual for its kind (Dalsrmple, 1980).

Although most of the chemical fertilizers were consumed in developed countries over the past 60 years, the use of fertilizer has now increased in developing countries. In 1960, developed countries accounted for 88% of world fertilizer consumption but by 2001, 63% of world fertilizer was consumed by developing countries (Ladha et al., 2005).

World N-fertilizer consumption in agriculture (Fig 1.1) was 9 MMT (million metric tonnes) in 1960. It increased dramatically after the Green Revolution, reaching 82.5 MMT in 2002. By 2019, the total world N-fertilizer reached 107.7 MMT (FAOSTAT, 2022). Almost 50% of the world fertilizers is applied to cereals to maintain yields (Heffer et al., 2017). The global demand for N-fertilizer is dictated by cereal grain production (Tilman et al., 2002).

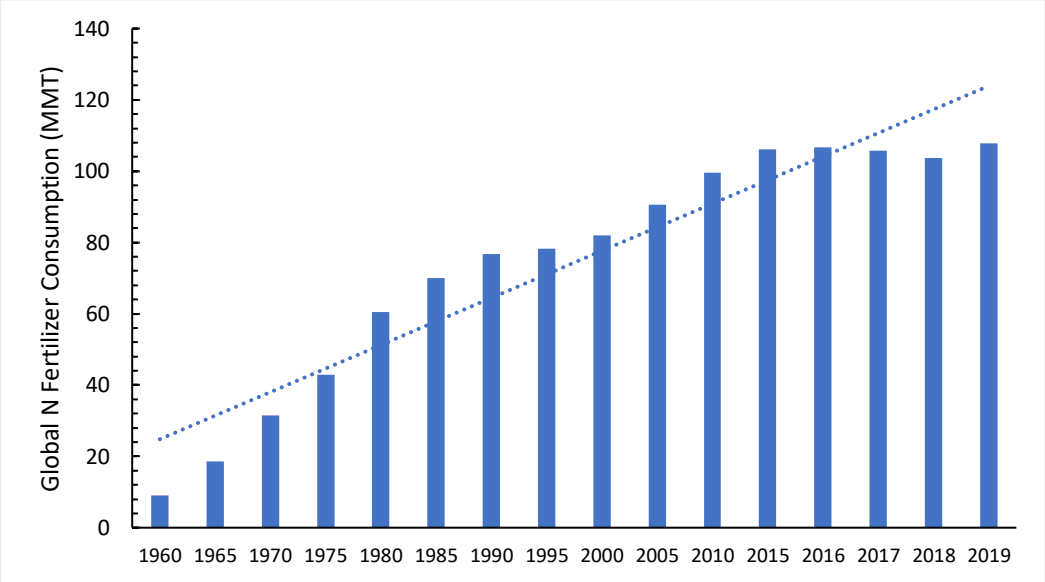


Figure 1.4 Global consumption of agricultural N-fertilizer from 1960 to 2019 in million metric tonnes (MMT) (FAOSTAT, 2022).

In the UK, the consumption of fertilizer nutrients reached 1.48 MMT during the 2018/2019 period, of which 1.04 correspond to nitrogen-based fertilizers. Among cereal crops, winter wheat showed the largest application rates of N-fertilizer whereas spring barley received the lower rates of mineral N (FAOSTAT, 2022).

It has been reported that, on average, globally, cereal crops use nitrogenous fertilizer inefficiently. Only about 40% of the applied N-fertilizer is absorbed by the crop (Sylvester-Bradley and Kindred, 2009). In the UK, this figure is about 60% if the best management guidelines are followed. The remainder nitrogen can be lost through processes such as volatilization, denitrification and leaching from soil–plant systems to water bodies and the atmosphere, generating pollution issues (Ladha et al., 2005). These processes are described in section 1.4.

A major goal of modern agriculture is to minimise fertilizer inputs while maintaining high yields and grain quality (Criado et al., 2015). Reducing the use of N fertilizers is important to minimize the negative environmental impact and to reduce the cost associated with fertilizers production and their utilization. Therefore, it is essential to reduce the amount of fertilizers used in agriculture and breed for crops with better resource use efficiency to restore and maintain ecosystem functioning while meeting the demand of a growing population (Heffer et al., 2017).

1.3 Manufacture of synthetic N fertilizer

Large-scale production of mineral nitrogen fertilizers began in the 1950s. This dramatically contributed to increase crop yields and its use is likely to continue to increase in the future (Ahlgren et al., 2008). Unfortunately, the production of nitrogen fertilizer is highly consuming of energy, representing 1.2% of the global primary energy demand (Ahlgren et al., 2008). Fertilizer prices have

been driven by surging energy costs, supply restrictions, and trade policies (Baffes and Chian, 2021).

The most important factor affecting fertilizer costs has been the sharp increase in the price of natural gas (Hebebrand and Laborde, 2022). Natural gas is used as feedstock and as energy source in the production of ammonia, which is the base material for N fertilizers (Dawson and Hilton, 2011). Ammonia is formed in the Haber–Bosch process which converts hydrogen and nitrogen to ammonia (N_2+3H_2 à $2NH_3$). The process uses very high temperatures (300°C–500°C) and pressure to break apart the N_2 (Nevins et al., 2020). The hydrogen originates from natural gas and the nitrogen from air.

Fertilizer prices have increased enormously over the last year and a half with soaring gas prices. Natural gas prices spiked, especially in Europe due to the conflict in Ukraine. Yara, which is one of the world's largest fertilizer makers, announced in March 2022 that its European production capacity of ammonia and urea was decreasing by 55% due to the surge in natural gas prices.

Moreover, the resent announcement of China to suspend fertilizer exports to ensure local availability has increased the concerns for N-fertilizers supply.

It is therefore not just the cost of fertilizer production that is a problem, it is also the security of supply. Supplies are being shortened because less is being manufactured as a result of the high gas prices.

High fertilizer costs represent an extra pressure on food prices, compromising food security, making access to food more difficult. Moreover, the economic optimum amount of N fertilizer rate is highly dependent on grain prices and more specifically, the ratio of N fertilizer to grain price. With the increasing cost of fertilizer, many farmers, may need to reassess N application rates. While the prices if grain and fertilizers are likely to remain high, not every region will be affected in the same way; poorest countries will suffer the most.

1.4 Dynamics of soil - crop N

1.4.1 Key soil N transformations

Nitrogen and water one of the most limiting elements for plant growth and crop productivity (Tatsumi et al., 2019). Although N is the primary gas (N_2) in the atmosphere (78%), it is inaccessible in this form to plants, but it can be converted to ammonia NH_3 through biotic and abiotic processes. NH_3 is the precursor of ammonium (NH_4^+) and nitrate (NO_3^-) which comprise the main forms of N that are available for plant uptake. This section outlines the key soil N transformations.

N fixation: N can be added to directly to the soil from the atmosphere through either biological or physical/chemical processes. Biological N_2 fixation naturally occurs in legume plants through a mutualistic relationship with a specific group of bacteria, rhizobia (Nevins et al., 2020). Legumes can be grown as a cover crop after harvest but before the next vegetable planting to help supply N to the soil (Nevins et al., 2020). The association of cereals with diverse bacteria, including nitrogen-fixing bacteria called diazotrophs, has now been widely studied. Biological N fixation by diazotrophic bacteria reduced N_2 to NH_3 using nitrogenase enzyme systems (Rosenblueth et al., 2018). Nitrogen-fixation in cereals is not high enough to support the plant's needs, but it has been the aim of different studies to increase N fixation in cereals (Bloch et al., 2020; Wen et al., 2021).

The industrial conversion of N_2 to NH_3 is used worldwide to produce synthetic fertilizers as described in the previous section (see section 1.3).

Mineralization: Nitrogen mineralization is the biological process by which organic N is converted to NH_4^+ (Benbi and Richter, 2002). N mineralization is completed by a large variety of soil microbes that can mineralize decomposed organic material, such as crop residues, soil organic matter, or compost. The amount of N mineralized to NH_4^+ depends on the N concentrations in the

decomposing organic material and the decomposition rate (Nevins et al., 2020). Mineralization rates are maximised in well-drained soils, with adequate moisture and low C:N. When soil microbes have met their N requirements from decomposition, excess N accumulates as NH_4^+ in the soil. (Nevins et al., 2020).

Nitrification: Soil NH_4^+ can be converted to nitrite (NO_2^-) and then NO_3^- through the process of nitrification by soil microbes. Nitrification is regulated by abiotic conditions, such as soil O_2 concentrations and pH, as well as soil NH_4^+ concentration. It is carried out by three microbial groups, (1) autotrophic ammonia oxidizers, (2) autotrophic nitrite oxidizers, and (3) heterotrophic nitrifiers. When conditions are ideal for nitrification, NO and N_2O are produced in low concentrations. However, when nitrification has started but is not completed the concentrations of these gases increase. The main reasons for an incomplete nitrification are low oxygen levels and acidic soils (Nevins et al., 2020). The optimum temperature for nitrification is around 32° C. Below 10° F the rate slows rapidly, but nitrification can continue until 0° C (Prosser, 2005).

There are other transformations processes that, by contrast, limit the amount of N available to the plants in the soil. These includes immobilization and de-nitrification. De-nitrification, leaching and volatilization are the main ways by which N can be lost from the soil. Losses of N via leaching or volatilization can also be included as key components of the soil N cycling transformations.

Immobilization: When ammonia and nitrate are used by microbes to meet their biological N requirements, N gets immobilized and therefore becomes unavailable to the roots of plants. The rate of immobilization and the amount of N immobilized depends on the starting concentrations of NH_4^+ and NO_3^- in the soil, the amount of C added to the soil, the size of the microbial pool, and the specific microbes present in the soil. High C concentrations relative to N causes microbes to acquire the available N from the soil so they can metabolise the added C (Nevins et al., 2020).

Denitrification: Is the conversion of NO_3^- to gaseous N forms such as N_2 and N_2O. The process occurs under low oxygen conditions, and it is facilitated by microbes. *Pseudomonas* are commonly known denitrifying soil bacteria. The presence of these microbes depends on several factors, such as soil organic matter, pH, moisture content, and temperature (Nevins et al., 2020).

Leaching: Nitrate-N leaching is a prominent process of N loss in agricultural ecosystems due to its negative charge being as that of most soil particles (Huang et al., 2017). Nitrate is leached as water drains through the soil profile, moving out of the range of plant rooting systems**.** Leached NO_3^- leads to groundwater contamination or surface water eutrophication as it promotes algae growth (Ladha et al., 2005; Nevins et al., 2020). The rate of leaching in soils is influenced by factors such as soil type, affecting drainage characteristics and rainfall rates (Nevins et al., 2020). High residues of N have been found in barley crops by harvest (Bingham et al., 2012). Leaching of N can be reduced by matching the use of fertilizers with the crop N demand and/or by improving the root system, especially root proliferation at lower soil layers (Nevins et al., 2020).

Volatilization: As already described, N can be lost from soils to the atmosphere through denitrification. It can also be lost as ammonia volatilization which occurs when NH_3 gas is lost to the atmosphere. It has been reported that losses of N through volatilization can occur from the soil as well as from the above-ground tissue (Harper et al., 1987; Schjoerring et al., 1993). NH_3 volatilization is mainly controlled by the amount of organic material added to the soil (e.g urea, manure) which causes a rise in the soil pH. A pH greater than 8.0 creates a soil environment that prevents conversion of NH_3 to NH_4^+ facilitating the volatilization of NH_3 to the atmosphere (Nevins et al., 2020). Ammonia volatilization from plant tissue depends on the ammonia stomatal compensation point which is defined as the NH_3 concentration in the air within the substomatal cavities at which no net NH_3 exchange with the atmosphere

takes place (Farquhar et al., 1980). It is determined by the concentration of NH_4^+ and H^+ in the leaf apoplastic solution (Søren Husted et al., 2000).

Investigating the biological and non-biological processes controlling N transformations in the soil is of major importance to improve our understanding of the soil N dynamics. The implementation of new molecular techniques to determine beneficial microorganisms involved in the biological processes of N transformation, plus the implementation of crop simulation models to predict the effect of biotic and abiotic factors will potentially allow us to increase the environmental quality, hopefully improving crop production, and agriculture sustainability. A detailed section for strategies to improve root-soil microbe interactions is given in section 1.6.

1.4.2 N sources and uptake

The major sources of N in agricultural soils are nitrate (NO_3^-) and ammonium (NH_4^+), NO_3^- being the predominant form of N available for crops. NO_3^- is carried towards the root by bulk flow and diffusion and it is absorbed into the epidermal and cortical symplasm (Glass, 2003; Segonzac et al., 2007). NO_3^- is actively transported across the plasma membrane by both low and high affinity transporter systems. Members of the transporter's systems family are regulated by internal signals including nitrogen compounds and by the demand for nitrogen from the shoot (Crawford and Glass, 1998).

Nitrate uptake is facilitated by specific transporters belonging to high affinity and low affinity transport systems (HATS and LATS, respectively) (Fan et al., 2017; Glass, 2003; Masclaux-Daubresse et al., 2010).

HATS are active when the concentration of NO_3^- in the soil is low <250 µM. LATS, by contrast, predominate at high soil NO_3^- concentration (>250 µM) (Glass, 2003).

Low affinity nitrate transporters (LATS) are coded by the Nitrate transporter 1 (NRT1) gene family, which has been re-named as the NPF family (Léran et al., 2014). In *Arabidopsis thaliana*, 53 genes belong to this family (Masclaux-Daubresse et al., 2010) and 93 members in the rice (*Oryza sativa* L.) genome (Léran et al., 2014). High affinity nitrate transporters (HATS) belong to the NRT2 family with 7 members in *Arabidopsis thaliana* (Miller et al., 2007) and 5 in the rice genome (Feng et al., 2011).

Although the NPF members are believed to function as the main components of the LATS for nitrate when the concentration of N in the soil is high, some NPF proteins have revealed dual affinity for nitrate. This is the case of NPF6.3/NRT1.1 in *Arabidopsis* and NRT1.1B in rice (K. H. Liu et al., 1999; R. Wang et al., 1998).

The specific location of these proteins within the root system is still unclear. 51 of the genes expressed in *Arabidopsis* are likely to exhibit different tissue expression patterns suggesting a specialised and unique function for at least some of them (Masclaux-Daubresse et al., 2010). Both NPF and NRT2 gene families are expressed in the root epidermis and in root hairs (Lea and Azevedo, 2006). However, some experiments have suggested that HATS are expressed in the epidermal, cortical and endodermal cell layers of mature roots and not in young roots (Lea and Azevedo, 2006), whereas LATS are suggested to be expressed in the epidermal cells of young roots (Huang et al., 1996). Therefore, it is likely that young roots may be responsible for most of the absorbed nitrogen via low-affinity transports, as soil N surrounding older regions of the root is more likely to be depleted (Glass, 2003).

Recent studies have shown the HATS respond to plant N demand and contribute the majority of total uptake capacity at high NO_3^- concentrations (>2.5 mM) raising questions regarding the roles and activity of each uptake system (Garnett et al., 2013). The authors showed major changes in the high-affinity nitrate uptake capacity throughout the lifecycle in maize plants. These

variations correlated to changes in the relative growth rates of shoots and roots. In this experiment the reduction in NO_3^- supply led to a dramatic increase in NO_3^- uptake capacity. These led to changes in the transcript levels of the putative high-affinity transporters, suggesting a model with short-term post-translational regulation and longer-term transcriptional regulation of NO_3^- uptake capacity.

Ammonium is the other major form of N taken up by plants. Because ammonium is toxic to plant cells, plants tend not to accumulate high concentrations of ammonium ions (Glass, 2003). Ammonium uptake and assimilation are tightly regulated (Masclaux-Daubresse et al., 2010; Tegeder and Masclaux-Daubresse, 2018). In plants, ammonium transport and homeostasis are controlled by a) saturable high-affinity Ammonium Transporters (AMTs) and b) non-saturable low-affinity uptake systems (aquaporins or cation channels) (Masclaux-Daubresse et al., 2010; Tegeder and Masclaux-Daubresse, 2018). Like the nitrate transporters, some AMT genes are also expressed in root hairs (Loqué et al., 2005). In Arabidopsis, four AMTs function in ammonium acquisition by roots, with AMT1.1, AMT1.3 and AMT1.5 being involved in the direct uptake from soil via the epidermis (Loqué et al., 2005). AMT1.2 is expressed in cortical and endodermal cells and mediates absorption of ammonium from the apoplast. Collectively, AMT1.1, AMT1.2 and AMT1.3 import up to 95% of the NH_4^+ (Masclaux-Daubresse et al., 2010; Tegeder and Masclaux-Daubresse, 2018).

Organic forms of N can also be taken up by roots. Root amino acid uptake systems belong to three families within the Amino-acid-Polyamine-Choline (APC) transporter superfamily: 1) the Amino Acid Permeases (AAPs), 2) Lysine/Histidine-likes Transporters (LHTs), and 3) Proline and Glycine Betaine Transporters (ProTs) (Masclaux-Daubresse et al., 2010; Tegeder and Masclaux-Daubresse, 2018).

1.4.3 Nitrogen assimilation

Once the nitrate is transported into the roots cells, it has four possible fates: (1) it is converted to NO_2^- by nitrate reductase and reduced to NH_4^+ by nitrite reductase and ultimately incorporated into amino acids through the GS/GOGAT pathway (Fig 1.2), (2) efflux back across the plasma membrane, (3) influx and store in the vacuole; or, (4) transport to the xylem for long-distance translocation to the shoot (Crawford and Glass, 1998). The loading of nitrogen from root cortical cells to xylem is driven by transpiration at the leaf surface (Tyree, 2003) and it is thought to be regulated by the concentrations of cycling amino acids (Crawford and Glass, 1998).

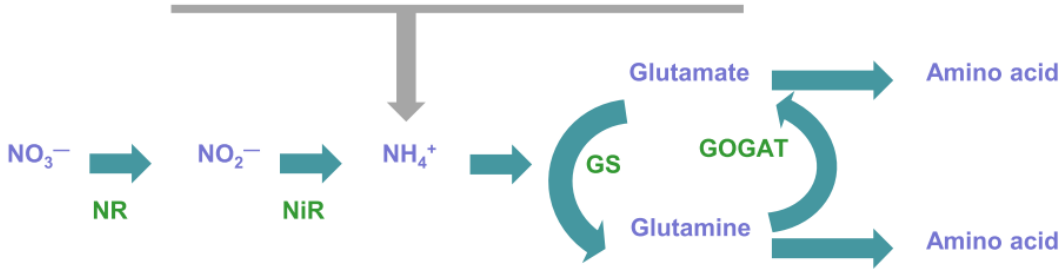


Figure 1.5 Main N assimilation pathways reported in wheat (Le Gouis et al., 2016).

NO_3^- reduction takes place in the cytoplasm of roots and shoots. After reduction to NO_2^- it is translocated to the chloroplast where it is reduced to NH_4^+ (Masclaux-Daubresse et al., 2010). Ammonium is mainly assimilated in the plastids/chloroplast by the glutamine synthetase/ glutamate synthase (GS/GOGAT) pathway. The glutamine synthetase fixes ammonium on a glutamate molecule to form glutamine. This glutamine subsequently reacts with 2- oxoglutarate to form two molecules of glutamate, this step being catalysed by the glutamate synthase.

It has been reported that three different enzymes probably participate in the assimilation of NH_4^+. These are: cytosolic asparagine synthetase (AS), carbamoylphosphate synthase (CPSase) and the mitochondrial NADH-glutamate dehydrogenase (GDH) (Masclaux-Daubresse et al., 2006).

Absorbed NO_3^- and NH_4^+ are assimilated by the crop and used to build the photosynthetic machinery which contains large quantities of photosynthetic proteins (principally Rubisco) and the structural proteins to support plant growth. A third pool of N called reserve N has been suggested to be part of the overall N source in plants (Pask et al., 2012).

Once the N compounds are transported via the xylem to the source organs (leaves, stems and leaf sheaths) they are used for leaf functions, stored in the vacuoles, or transported into the phloem to the sinks. The majority of N is exported into the phloem as amino acids (Masclaux-Daubresse et al., 2010). N released from the phloem is assumed to be via symplasmic movement through plasmodesmata to the neighbouring parenchyma cells (Masclaux-Daubresse et al., 2010).

1.4.4 Nitrogen remobilisation (NR) and senescence dynamics

Nitrogen uptake and assimilation during the grain filling period is usually not enough to meet the high demand of the developing grains (Masclaux-Daubresse et al., 2010). Thus, the remobilisation of N to the grains is critical for grain productivity and yield (Chardon et al., 2012).

There are two approaches to determine N remobilisation in plants; one is the ‘apparent remobilisation’ method and the second one involves the use of the isotope ^{15}N. The former involves the determination of the amount of total N content in the different parts of the plant at different times through the season. The latter uses ^{15}N for long-term labelling, which allows for the determination of N fluxes (Gallais et al., 2006).

Currently, controversy exists regarding how nitrogen remobilisation is regulated during grain filling period (Kong et al., 2016). It has been suggested that the extent of remobilisation is dependent on N availability, environmental conditions and genotype and it is also influenced by the post anthesis N uptake (Bancal, 2009; Borrell et al., 2001; Martre et al., 2003; Moll et al., 1982; Slimane et al., 2013)

The relationship between NR and senescence has been widely studied in cereal crops such as wheat, maize and barley (Chardon et al., 2012; Kong et al., 2016; Masclaux-Daubresse et al., 2010). While various studies had indicated that the initiation of NR in cereal crops occurs immediately after anthesis (Dreccer, 2006; Wang et al., 2013), senescence, which involves the degradation of macromolecules and cell constitutes, begins approximately 8-16 days after anthesis (Kong et al., 2016). N remobilisation during leaf senescence is strongly regulated by chloroplast and vacuole protease activities as well as by the various long-distance transporters (Xu et al., 2012).

High rates of NR have been observed when accelerated senescence takes place (Bogard et al., 2010) leading to a high protein content but may negatively impact grain yield due to the reduced C assimilation. Moreover, (Van Oosterom et al., 2010) demonstrated that a delay in vegetative senescence in sorghum increased the duration of leaf photosynthesis during the grain filling period and resulted in a delay nitrogen remobilisation, negatively impacting the protein deposition in the grains.

GPC is not only influenced by the N remobilisation efficiency, but also by the post-anthesis N uptake capacity of the roots. This has been demonstrated in wheat, barley, sorghum and maize (Borrell et al., 2001; Distelfeld et al., 2014; Gallais et al., 2006; Kichey et al., 2007; Taulemesse et al., 2016).

Although N uptake and remobilisation have proven to be of major importance for the grain protein filling, the genetic and physiological coordination between the two remains poorly understood.

1.5 Nitrogen use efficiency (NUE): Definition and its components

Nitrogen use efficiency has been defined as the yield of grain (grain dry matter yield) per unit of available nitrogen in the soil, which can be from soil, fertilizer or both (Moll et al., 1982). The authors divided NUE into two components: (1) N-uptake efficiency (NupE) and (2) N-utilization efficiency (NutE).

$$\mathrm{NUE} = \frac{\text{Grain yield (kg)}}{\text{N supply (kg: applied N} + \text{SMN)}}$$

Nitrogen uptake efficiency is usually calculated as the total amount of N in the above-ground parts of the plant at harvest divided by the available N in the soil. It considers the capacity of the roots to acquire N. If root data is available, it should be included in the equation. NupE can also be measured as the amount of N taken up in relation to the unit of root dry mass, when root data is available (Le Gouis et al., 2016; Xu et al., 2012).

$$\mathrm{NupE} = \frac{\text{N uptake}}{\text{N supply}}$$

Nitrogen utilization efficiency is calculated as the ratio of grain dry matter yield to above-ground crop N uptake. It considers the efficiency of assimilation or remobilisation of plant-acquired N to be converted to total plant biomass or grain yield. Some authors distinguish between utilization of N derived from fertilizer and that from soil N mineralisation. This can be estimated by calculating the difference in yield and N uptake between crops supplied with fertilizer and those grown without fertilizer (Beatty et al., 2010; Delogu et al., 1998).

$$\text{NutE} = \frac{\text{Grain yield}}{\text{N uptake}}$$

1.6 Strategies to improve NUE

The nitrogen use efficiency of crop production must be improved so that cereal yields can be increased to meet an expanding global demand for grain without corresponding increases in N fertilizer inputs. Improvements in NUE can be sought in two complementary ways, firstly through changes to crop management and fertilizer practice, and secondly, through plant breeding and the development of more efficient varieties. The latter approach requires information on which phenotypic (morphological and physiological) traits govern NUE, the genes responsible for their control and the scale of variation in these traits in the breeding population.

1.6.1 Crop management and fertilizer practices

Nitrogen use efficiency can be improved by adopting fertilizer, soil, water, and crop management practices that will maximize crop N uptake and minimize its losses. Strategies to improve fertilizer N use by crops can focus on two approaches: (i) increasing the use of applied N-fertilizer during the growing season and (ii) decreasing N losses by increasing the recovery of residual fertilizer N by subsequent crops (Ladha et al., 2005).

Strategies to improve NUE through crop management and fertilizer practices include: (1) site-specific N management which aims to optimize the supply of soil nutrients over time and space to match the requirements of crops through four key principles: right product, right rate, right time and right place (Verma et al., 2020). The implementation of site-specific N managements requires knowledge of soil characteristics and the ability to monitor crop nutrient status to adjust N fertilizer inputs accordingly. Estimating soil N enables agricultural advisors and farmers to estimate the amount of N fertilizer that is required as

well as the number of applications and timing. Chlorophyll meters and leaf colour charts are promising tools developed to monitor N status of crops (Haripriya and Byju, 2008); a powerful tool used in precision farming is NDVI (Normalized difference Vegetation Index) which is use for visible spectrum and adopted to analyse remote sensing measurements, allowing to measure plant status and health. Moreover, remote sensed imagery can be used for mapping soil properties, classification of crop species, detection of crop water stress, monitoring of weeds and crop diseases, and mapping of crop yield (Sishodia et al., 2020), (2) integrated N management which refers to a safe way to dispose of crop residues and produce high-quality compost by a balanced and integrated use of organic (crop residues, organic manure) and inorganic fertilizers. This is to maintain soil fertility and provide plants with an optimum level of nutrients required during the entire growing season (Selim, 2020); (3) slow and/or controlled release N fertilizers can improve the use efficiency of applied nutrients by reducing N-losses and enhancing their beneficial use in plants. These products can slow the release rate of nutrients and the rate of nitrification inhibitors that can interfere with nitrogen transformation processes (Wu et al., 2021). In the UK, the nutrient management guide (RB209) provided by AHDB offers best practices guidance for the application of mineral fertilizers, manure and slurries to crops and grassland (AHDB, 2018b).

An accurate measurement of the soil properties pre and post sowing and an accurate monitoring of the weather conditions throughout the growing season could potentially influence the decisions on N fertilizers.

The long-term benefits of these management practices on soil quality will also have an important influence on the NUE of the entire agroecosystem (Foulkes et al., 2009). Crop management practices that increase soil organic matter, which is a key measure of soil quality, will result in a greater indigenous N supply and a reduction in N fertilizer requirements (Cassman et al., 2002).

1.6.2 Improvements through plant breeding

Improving NUE through plant breeding may be achieved through changes in one or more of the plant's morpho-physiological traits, which directly or indirectly contribute to its superior capacity to uptake and/or utilize the soil available N.

To make progress through breeding, the presence of natural or artificially induced genetic variability is an essential requirement. Genetic variability in NUE of spring barley genotypes has been reported (Anbessa et al., 2009; Bingham et al., 2012). In these experiments, differences between old and modern barley cultivars in NUE and their grain yield responses to low and high N environments were revealed. Bingham et al., (2012) in their analysis of the effects of nearly 75 years of breeding on the NUE of spring barley showed that there was significant variation between genotypes in NupE and NutE. The variation in NupE was associated with differences in fertilizer recovery, rather than acquisition of soil mineral N. High NupE seemed to be the result of a larger uptake of N during the post-anthesis period only. These results suggest that N uptake in these varieties may be driven by a large demand associated with a large grain sink. Moreover, plant breeding for low N environments may not be optimised as selection is mainly done at optimal N levels.

In a similar and recent study performed in wheat, the NUE of four ancient and four modern wheat genotypes was evaluated (Lupini et al., 2021). The authors concluded that the ancient varieties exhibited higher PANU values compared to the modern ones. Increasing N uptake post-anthesis, while maintaining low grain N for malting quality is a major challenge. These results indicate that there is a significant margin to improve NUE in barley through breeding. However, efforts are needed (1) to continue to identify new sources of NUE genes from modern germplasm as well as landraces of barley and (2) for a greater understanding of the physiological processes that determine NUE.

Traits to improve N uptake efficiency (NupE)

The primary processes that contribute to the efficient capture of soil N are the development of a root system that can efficiently explore the soil and the expression of transporter systems in those roots, especially HATS (Goulding et al., 2008; Mălinaş et al., 2022). Moreover, soil microorganisms also play an essential role in determining the availability of N for plant roots.

It has been demonstrated that N uptake is the result of a balance between an active influx and a passive efflux (Crawford and Glass, 1998). Physiological methods, especially the use of isotopes of N (^{15}N and ^{13}N) have been widely used to characterize the regulation of these fluxes for nitrate and ammonium in roots of crop species (Glass et al., 2001). These techniques have shown that the influx and even the efflux of NO_3^- and NH_4^+ from plant roots vary depending on the plant species and cultivar, time of the day, internal N concentration of the root tissue and the external N concentration of NO_3^- and NH_4^+ in the soil.

Recent studies have started to characterize and identify nitrate transporter systems in barley, particularly HATS (Guo et al., 2020). The identification of putative candidate genes and genetic variability within barley germplasm is essential for improving NUE and crop production.

Improvements of root-soil microbe's interactions

Plant roots, including those of cereal crops, release a variety of potentially valuable compounds such as organic acids and sugars into the rhizosphere (region of soil directly influenced by roots). These play an essential role in the chemical, physical, and biological interactions between roots and the rhizosphere (Yadav et al., 2015). These interactions may influence the plant growth and development, change nutrient dynamics and also alter the plants susceptibility towards diseases and abiotic stresses (Yadav et al., 2015).

As discussed in section 1.4.1, soil microorganisms in the rhizosphere are key players in the availability of nutrients, especially N, for plant roots (Yadav et al., 2015). N availability for plant roots may be reduced by microbial competition as various soil microbes use ammonium and nitrate as N sources (Harte and Kinzig, 1993; Sun et al., 2021) and/or transform nitrate to gaseous N by denitrification (Hayashi et al., 2015). On the contrary, N availability can also be enhanced by microbial mineralization of organic N, yielding ammonium in the rhizosphere (Benbi and Richter, 2002; Myrold, 2021; Schimel and Bennett, 2004).

It would be of major interest to develop breeding strategies to promote root colonization by plant-beneficial microbial communities, especially those with the potential to enhance N availability in the rhizosphere, enhance the root system and architecture as well as both plant metabolism and microbial phyto-protection.

Improvements in root architecture

In most agricultural soil, the availability of water and N are greater in deeper soil strata over the growing season (Lynch, 2013). Nitrate ions that are not taken up by the crop are potentially leached down the soil profile to underground water. Consequently, one of the most important characteristics to improve is rooting depth. The construction of an ideotype to maximize nutrients and water capture in cereal crops has been widely proposed (Le Gouis et al., 2016; Lynch, 2013). In these ideotypes, the authors indicated that to maximize N capture, a deeper relative distribution of roots could be beneficial, and further improvements in root architecture are needed.

The primary root traits that may contribute to rooting depth in cereal crops include (1) a large diameter primary root with few but long laterals and the ability to grow in cold soil, (2) many seminal roots with shallow growth angles, thin diameters, many lateral and long root hairs, (3) an intermediate number of crown roots with steep growth angles and few but long lateral branches, the

growth angle of axial roots is a primary determinant of root foraging depth, (4) one whorl of brace roots of high occupancy, a growth angle that is slightly shallower than the growth angle for crown roots, with few but long laterals, and (5) low K_m (the concentration at which 50% of V_{max} is reached) and high V_{max} (maximum uptake capacity) (Lynch, 2013).

In relation to N capture, different experiments have stated that a deeper root growth is more important in relation than an increased root density (Haberle et al., 2006; Kristensen and Thorup-Kristensen, 2004; Thorup-Kristensen, 2006). Theoretical calculations predict a critical root length density (cRLD) of about 1 cm cm^{-3} for water and nitrate (King et al., 2003). In wheat, root length density is typically below the critical root density of *ca*. 1 cm cm^{-3} at soil depths below 80 cm (Ford et al., 2006; Gregory and Brown, 1989; King et al., 2003). In 2003, a modelling study concluded that distributing roots deeper in the soil profile and decreasing specific root length (root DM per unit length) would give greater N capture and yield under low N availability (King et al., 2003).

Root architecture and root function are likely to be multigenic and hence much more difficult to select for. Methods for phenotyping cereal roots in the field include the use of rhizotrons and the measurement of root parameters from soil cores following root washing and image analysis (Jia et al., 2019; Smit and Groenwold, 2005). However, due to practical difficulties, maximum rooting depth in the field is rarely measured. Therefore, there is little evidence that root depth has changed systematically by breeding. Breeding for root characteristics has seldom been implemented to date, probably due to the difficulties of scoring root phenotypes directly.

Clearly total nutrient uptake by plants is a function of root biomass, root morphology, root age, root and plant growth rates, root physiological capacity and the root proliferation and the interaction with micro-organisms in regions of abundant nutrients (Glass, 2003)**.** The understanding, identification, and

incorporation of these traits in various crops through breeding approaches is a helpful tool to improve NUE.

Traits to improve N utilization efficiency (NutE)

As explained, the NUtE addresses the yield produced per unit of N acquired by the crop. The harvest index (HI) and the N harvest index (NHI) relate to the biomass and the N allocated, respectively to yield relative to the whole plant (Congreves et al., 2021). These indices are useful for identifying genotypes with enhanced capability of allocation growth or N resources towards the economic portion of plants, therefore, useful indices for plant breeding (Congreves et al., 2021). Moreover, using NHI can indirectly determine the amount of N left in the soil at harvest, which can be at risk of loss through leaching.

NutE may be improved, principally by, (1) modifying specific leaf N and therefore improving photosynthesis per unit of N, (2) delaying canopy senescence and (3) increasing N remobilisation efficiency (Foulkes et al., 2009; Gaju et al., 2011; Garnett et al., 2015; Sandhu et al., 2021).

Improving leaf photosynthesis per unit of N

Strategies to improve the photosynthetic N utilisation efficiency involve increasing the leaf area index and decreasing the specific leaf N (Gastal and Lemaire, 2002). Both conditions are needed for a better radiation use efficiency, which is a measure of the efficiency conversion of intercepted radiation to biomass (Sinclair and Muchow, 1999).

Rubisco, which is a major regulatory enzyme for carbon fixation, is also the most abundant leaf protein (Evans, 1983). However, it is also involved in photorespiratory losses leading to the release of previously fixed CO_2 and NH_3, which can be as high as 20% of the total N fixation in C3 crops such as wheat and barley (Bauwe et al., 2010; Sandhu et al., 2021).

Several components can be targeted to increase photosynthetic activity by decreasing photorespiration through Rubisco, increasing carboxylase activity of Rubisco and by introducing mechanisms to increase carbon concentration in the vicinity of Rubisco which will ultimately increase NutE (Murchie et al., 2009; Reynolds et al., 2000; Zhu et al., 2010).

Delaying senescence

The kinetics of canopy maturation, a highly controlled and regulated process (Hörtensteiner and Feller, 2002), impacts on both final yield and remobilisation efficiency (Hawkesford and Griffiths, 2019).

The senescence of a canopy limits further photosynthesis, reserve N accumulation and ultimately yield. Therefore, delaying leaf senescence enables continued photosynthesis with age which has the potential to increases grain yield and carbon filling into seeds (Masclaux-Daubresse et al., 2010). However, it has also been proved that delayed senescence could lead to a decrease in N remobilisation efficiency (Van Oosterom et al., 2010).

Within this context, delaying leaf senescence should be achieved through the remobilisation of stored N in preference to photosynthetic N from vegetative tissues so the photosynthetic capacity of the canopy is maintained for longer (Barraclough et al., 2014).

Genetic diversity in terms of senescence and ‘stay-green’ phenotypes has been reported in cereal crops like wheat (Bogard et al., 2011; Gaju et al., 2011). However, ensuring active remobilisation of N to the grains during the post-anthesis period is critical to improve NUE. Therefore, several factors including Rubisco degradation, stem nitrogen assimilation, and stay-green phenotypes provide major targets to improve NutE.

Improving remobilisation efficiency

During the grain filling period, the N found in cereal grains such as barley generally comes from two sources: post-anthesis N uptake (PANU) and N remobilisation from vegetative tissues. It has been reported that the remobilisation of pre-absorbed N to the grains accounts for between 60 to 92% of the total grain N (Barmeier et al., 2021; Kichey et al., 2007).

The amount of N remobilised to the grains depends on a number of factors, making it a difficult trait to select for. It has been reported that N remobilisation depends on: the soil N supply (Moll et al. 1982), the extent of PANU and the environmental conditions (Bogard et al., 2010; Borrell et al., 2001; Kichey et al., 2007; Martre et al., 2003). The partitioning and remobilisation of N is also believed to be genetically controlled (Barraclough et al., 2014).

A negative relationship has been observed between grain protein concentration (GPC) and yield, with higher yielding crops generally decreasing grain protein concentration (Bogard et al., 2010). Selecting genotypes that deviate from this negative relationship known as the grain protein deviation (GPD), for example, selecting those genotypes that maintain protein content with high yield, has been suggested as a tool for breeding programs aiming to address this issue (Garnett et al., 2015). Moreover, delaying the last fertilizer application to around heading has been shown to increase GPC without reducing grain yield in wheat (Bogard et al., 2010). GPD is a potential target for breeding as it appears to be relatively robust across different environments and would be valuable in increasing total N uptake by maturity (Bogard et al., 2010) helping to reduce the N left at harvest which has been associated with environmental degradation as previously discussed.

In barley (Jukanti and Fischer, 2008) found a QTL associated with accelerated senescence and high GPC. This and other studies (Distelfeld et al., 2008)

raise the possibility that total N remobilization and/or N remobilization efficiency explain the physiology of GPD.

The use of N fertilizers is an indispensable management practice for crop productivity. However, the environmental damages caused by the inefficient capture of N by crops are well recognised. Reducing the use of N fertilizers, therefore, is of great importance.

NUE is a complex trait dependant on a number of factors. Therefore, there is strong need to target multiple mechanisms/enzymes/factors to enhance NUE. Future research is dependent on in-depth understanding of the regulatory mechanisms of N metabolism and the ability to combine genetic, agronomic and environmental variables to the cropping system. Figure 1.6 shows an schematic representation of key traits and processes influencing NUE.

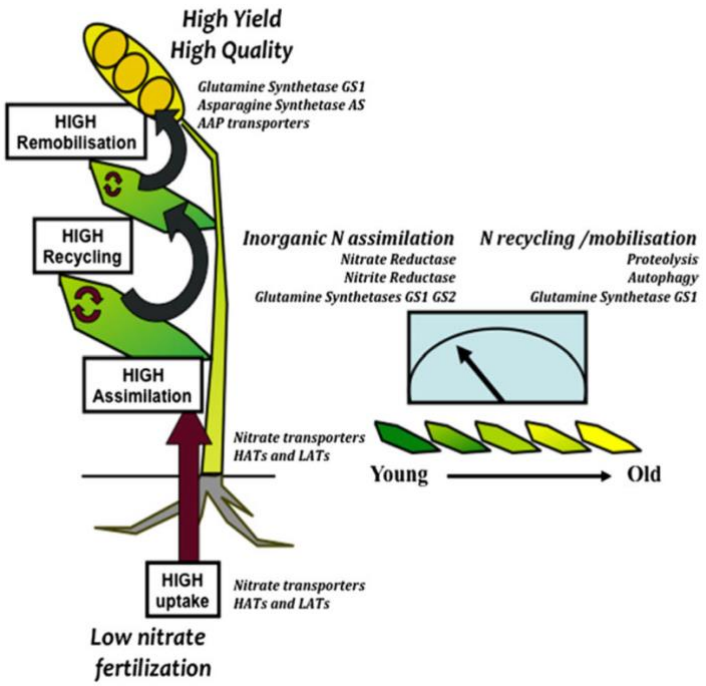


Figure 1.6 Schematic representation of an ideal yielding plant, key traits and processes for NUE are shown (Chardon et al., 2012).

1.7 Aims of this thesis

The overall aim of this project is to investigate the dynamics of N uptake and remobilisation during the post-flowering period in relation to the N status at flowering, the soil N supply and plant N demand during grain filling. The following hypotheses will be addressed:

Chapter 2 investigates the effects of plant N status at anthesis on N remobilisation and PANU of a spring barley cultivar under N nutritional conditions that resemble those of malting barley production systems. The specific hypotheses were to a) the plant N status at anthesis influences the onset and efficiency of N remobilisation from different vegetative tissues; b) plant N status at anthesis affects the temporal dynamics and efficiency of N uptake through the grain filling period; c) losses of N accumulated prior to anthesis occur and the extent of this losses is influenced by the plant N status at anthesis.

Chapter 3 investigates the response of barley to changes in N supply and demand using ^{15}N labelling in a split-root system. The specific hypotheses tested in chapter 3 were: a) post-anthesis N uptake in spring barley is regulated by grain sink demand and soil N supply b) a reduction in the grain sink demand results in greater N recycling from shoot to roots, and c) vegetative (non-grain) tissues remain a strong sink for N taken up after anthesis when grain N demand is reduced by de-graining.

Chapter 4 is a follow-up of chapter 3, where the emission of NH_3 is measured in control (intact) plants and compared to the emissions of NH_3 in de-grained plants. The hypothesis was that de-grained plants emit greater amount of NH_3 than control plants.

Chapter 5 explores the post-anthesis crop and soil N dynamics in barley and oat crops under field conditions. The specific hypotheses were: a) Oats have

the ability to deplete SMN to a greater extent than barley cultivars, and this greater depletion by oats is a characteristic of the species, b) Oats are a more productive cropping system is in relation to its fertilizer N input compared to barley, and c) The greater SMN depletion by oats is the result of a greater RLD.

Chapter 2: Post anthesis N dynamics in relationship to plant N status at anthesis

2.1 Introduction

Nitrogen (N) is the most important nutrient element limiting crop productivity. The use of N fertilizers is essential for producing a high yield of quality grain. However, nitrogen use efficiency (NUE), defined as grain yield per unit of N available from soil and fertilizer is low. The Food and Agriculture Organization (FAOSTAT, 2022) reported that, in 2019, approximately 108 million tonnes of N fertilizers were used in agriculture globally. However, as little as 33% of the applied N is recovered in the grain of cereals globally (Raun and Johnson, 1999).

This incomplete N capture has major negative impacts on the environment through nitrate pollution of water and the emission of gasses such as ammonia and nitrous oxide (Ladha et al., 2005; McIsaac, 2020). Therefore, breeding high yielding genotypes that use N efficiently is an urgent priority to reduce the impact of agriculture on the environment whilst meeting the growing global demand for food.

The N acquired by cereal crops is used in the formation of grain yield and protein over a series of distinct developmental phases (Miralles et al., 2020). During the pre-anthesis vegetative stage, plants rapidly increase their leaf area, shoot and root biomass, thereby increasing their capacity for resource capture. The maximum rate of N accumulation occurs during the period of stem extension and is critical to the establishment of a large canopy size (green area index), number of spikes per m^2 and, number of grains per spike (Luo et al., 2020). Nitrate and ammonium taken up by the root system during this period are assimilated into amino acids and used in the synthesis of photsynthetic proteins, of which rubisco is the most abundant, and the structural proteins in supporting and vascular tissues of the shoot (Pask et al. 2012).

During the reproductive stage, following flowering and grain set, it has been observed that most of the N allocated to the grain is obtained through remobilisation of N from the stems and leaves (Barmeier et al., 2021; Bogard et al., 2010). However, post-anthesis N uptake makes an important contribution, especially in conditions of high soil fertility, and is positively correlated to grain protein concentration (Foulkes et al., 2009; Hawkesford and Griffiths, 2019).

Construction of crop ideotypes can be useful for identifying suites of physiological traits that might be suitable targets for selection in plant breeding programmes (Reynolds and Langridge, 2016). It has been proposed that altering N remobilisation from leaves and delaying leaf senescence may increase N use efficiency (specifically N utilization efficiency; grain yield per unit of N absorbed) by prolonging photosynthetic activity and carbon assimilation during grain filling (Foulkes et al., 2009; Oorbessy Gaju et al., 2014; Le Gouis et al., 2016). This idea is supported by observations of higher yields in varieties with functional stay-green phenotypes (Guiamét and Giannibelli, 1996). However, restricting N remobilisation in this way could compromise grain N concentrations and lead to a reduction in grain quality, depending on the market requirements. Barley requires a low N concentration for distilling and higher N for brewing certain types of beer and for animal feed (Fox et al., 2003). The decrease in grain N concentrations could be minimised if N were preferentially remobilized from non- photosynthetically active or less important sources first such as true stems and leaf sheaths, or if, during early grain growth, grain N was preferentially supplied by post-anthesis N uptake (PANU).

Pask et al. (2012) have argued that true stems of wheat contain large quantities of soluble ‘reserve’ protein that is available for remobilisation in addition to structural protein which is not remobilized. Maximising PANU and reducing residual soil mineral N concentrations would have the additional

benefit of minimising N losses to the environment through post-harvest nitrate leaching.

Optimising the temporal dynamics of N remobilisation whilst maximising PANU is, therefore, a potential route to increasing NUE. Currently, our understanding of the dynamics and control of N remobilisation from different organs and PANU of cereals is incomplete (Le Gouis et al., 2016). Control of N uptake, remobilisation and partitioning has been widely considered in terms of the supply and demand relationships for N (Borrell et al., 2001; Martre et al., 2003). Some studies have shown that sink characteristics may control grain N accumulation (Mattsson et al., 1993; Mi et al., 2000; Wyss et al., 1991), whilst others suggest that N supply from remobilisation and PANU determine grain N content (Bancal, 2009).

PANU itself may be controlled by both N availability in the root medium and plant N demand. During the post-anthesis period the greatest demand for N is from the grain, although vegetative organs may also represent a significant sink for N. PANU was smaller in a small-eared than a large-eared genotype of wheat and less responsive to grain removal at anthesis (Mi et al., 2000), consistent with control by grain N demand. On the other hand, it is widely recognised that increases in N supply at anthesis, through late fertilizer application, can increase PANU (Efretuei et al., 2016; Hocking and Steer, 1995; McKenzie et al., 2006; Woolfolk et al., 2002). Relatively little is known about the temporal changes in N uptake through the grain filling period. Some authors have suggested that plants maintain a high capacity of N uptake through the grain filling period (Egle et al., 2008; Mattsson et al., 1993). Others have observed complex dynamics with a high uptake rate during the early grain filling period followed by a later decline (Taulemesse et al., 2015). Such a decline could conceivably result from a reduced demand for N towards the end of grain filling or a decrease in capacity for uptake. Root senescence during grain filling can lead to a decrease in root length density between anthesis and

harvest (P. J. Gregory et al., 1978a) and modelling studies have simulated reductions in N uptake associated with decreased dry matter allocation to roots during mid to late grain filling (Bertheloot et al., 2011a). Moreover, there appears to be a negative relationship between PANU and N remobilisation from vegetative tissue (Triboi and Triboi-Blondel, 2002). High N uptake may lead to a delay in leaf senescence and associated decline in N content of vegetative tissue.

However, it is unclear whether the higher uptake rate is a cause or an effect of the delayed leaf senescence. Stay green varieties have been found to have higher rates of PANU and longer maintenance of leaf greenness (Borrell et al., 2001; Gooding et al., 2005). Stay-green genotypes are characterized by delayed senescense, these plants can maintain their leaves photosynthetically active and subsequently improve the grain filling process (Borrell et al., 2001). However, stay-green plants may also remobilise less N from vegetative tissues. Delay senescence could be an advantage in feed varieties and potentially in malting varieties too due to the favourable longer period of active photosynthesis during the grain filling and higher grain yield, whereas, for bread-making varieties, the lower grain protein concentration associated with delay senescence is a disadvantage (Bertheloot et al., 2011; Gaju et al., 2011; Gaju et al., 2014).

An additional, but often overlooked, component of the N dynamics are losses from the plant during the grain filling period. Some studies have reported a net reduction in above ground N content between anthesis and harvest (McTaggart and Smith, 1995). Such losses might occur via root exudation, incomplete remobilisation from leaf tissue prior to shedding, or volatilization of ammonia from senescing tissue (McTaggart and Smith, 1995; Schjørring et al., 1989a).

It is apparent from the above that post-anthesis N dynamics are a complex interplay between PANU, N remobilisation from vegetative tissue and

partitioning to the grain, the regulation of which may involve inter-organ signalling of plant N status (Bertheloot et al., 2011; Gaju et al., 2011; Gaju et al., 2014; Liu et al., 2018). We might expect, therefore, these dynamics to depend not only on N availability in the root zone during grain filling, but also on plant N status at anthesis, both of which are likely to vary according to local site and crop management factors.

The aim of the experiment reported here was to determine the effects of plant N status at anthesis on N remobilisation and PANU of a spring barley cultivar under N nutritional conditions that resemble those of malting barley production systems. The specific objectives were to 1) determine the effects of plant N status at anthesis on the onset and efficiency of N remobilisation from different vegetative tissues; 2) determine the effects of plant N status at anthesis on the temporal dynamics and efficiency of N uptake through the grain filling period; 3) quantify post-anthesis losses of N accumulated prior to anthesis and to determine whether these are influenced by plant N status at anthesis. To achieve these objectives plants were grown in sand-perlite with N supplied as ^{15}N prior to anthesis. At anthesis, ^{15}N was flushed from the root medium and N supplied as ^{14}N between anthesis and harvest. The experimental system allowed remobilisation of N deposited before flowering to be distinguished from that captured and partitioned during grain filling.

2.2 Materials and methods

2.2.1 Plant material, establishment and growth conditions

PVC cylinders (40 cm depth, 7 cm diam) were sealed at one end with a polyester mesh (100 µm aperture, Cadisch Precision Meshes Ltd, Hertfordshire-UK.) and filled with 1.5 kg of sand:perlite mixture (2:1 v/v). The mesh allowed excess water to drain but minimized the emergence of roots from the cylinder. Each cylinder was placed in an individual plastic dish (9 cm depth, 9 cm diam) to avoid potential nutrient solution cross contamination.

Cylinders were watered with deionized water prior transplanting, covered to prevent evaporation of water from the surface and allowed to drain for 48 h to reach field capacity. The total weight of the cylinder moistened to field capacity was recorded. Caryopses of a two-row spring barley variety (*Hordeum vulgare*, cv Westminster) were germinated in the dark at 20 °C for 5 days on filter paper moistened with deionized water. Westminster was first on the recommended list in 2005. It is a two-row feed variety from Nickerson UK. It was first sold as a malting variety, but in 2011 its status was changed to a feed variety. It was selected for this project to maintain continuity with previous research on NUE at SRUC.

When the primary roots had reached a length of approximately 5cm one seedling was transplanted to each cylinder. Plants were placed in a growth cabinet (Modular climate chamber, SNIJDERSLabs, The Netherlands) with a 20 ºC daytime temperature (16 h) and 15 ºC night temperature. Relative air humidity was set at 70%. Light was supplied by white light emitting diodes (LED) giving a photon irradiance at plant height of 356 ± 18 µmol m^{-2} sec^{-1} of photosynthetically active radiation (PAR).

2.2.2 Experimental design and treatments

The experiment consisted of three nitrogen supply regimes applied up to flowering and five destructive sampling dates from flowering to grain maturity. The experiment was laid out within the growth cabinet in a randomised block design (Photo 2.1). One plant for each nitrogen treatment x sampling date combination was randomly allocated to a position within each of five replicate blocks.

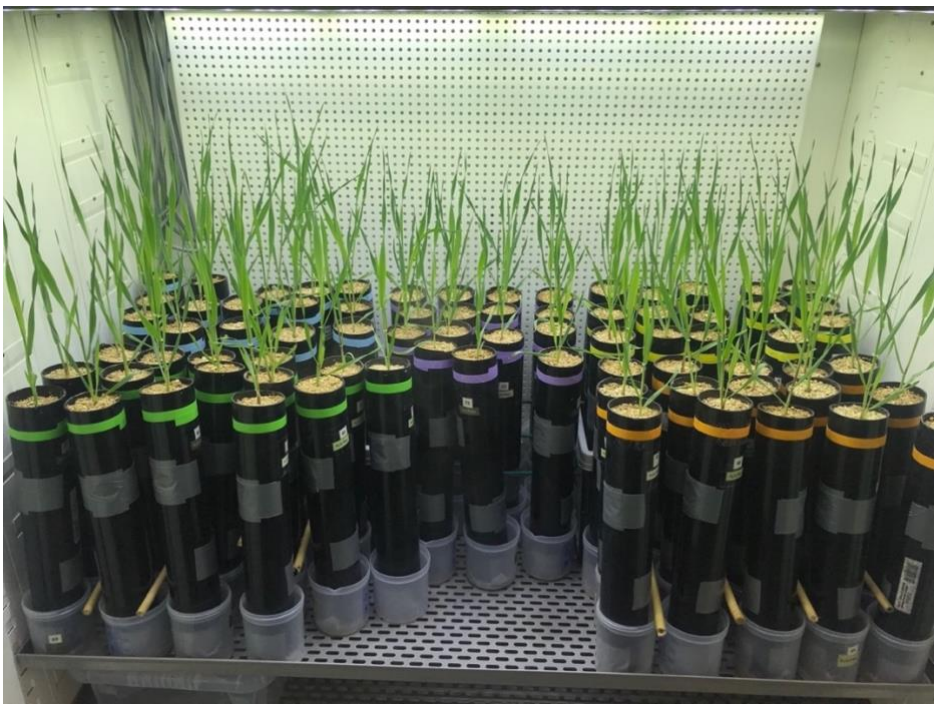


Photo 2.1.1 Photo of the experiment laid out in the growth cabinet. Plants are at GS 39. Colour bands at the top of each cylinder were used to differentiated between blocks.

From transplantation of seedlings to flowering each plant received 75 ml weekly of a complete nutrient solution containing one of three different NH_4NO_3 concentrations 2 (N1), 4 (N2) and 6 (N3) mM N (Table 2.1). The N was enriched by ^{15}N at both the NH_4 and NO_3 to 10% atom. This regime supplied plants with a dose of 4.2, 8.4 and 12.6 mg N every seven days for treatments N1, N2 and N3, respectively. At flowering, also referred here as anthesis, when the main shoot ear emerged (Zadoks GS59; (Tottman, 1987), the unabsorbed nutrient solution remaining in the substrate (sand:perlite) was flushed out.

Three litres of $MgSO_4.7H_2O$ (2 mM) were used to flush out the root medium to remove any residual ^{15}N that was not captured by the plant followed by an additional litre of deionised water.

Table 2.1.1 Different nitrogen nutrient solutions used during the experiment.

		N1	N2	N3
Macro-elements (mM)	KH_2PO_4	1	1	1
	K_2SO_4	1.5	1.5	1.5
	$MgSO_4\ 7H_2O$	2	2	2
	$CaCl_2\ 2H_2O$	3.5	3.5	3.5
	$^{15}NH_4{}^{15}NO_3$	**2**	**4**	**6**
Micro-elements (µM)	H_3BO_3	30	30	30
	$CuSO_4.4H_2O$	0.3	0.3	0.3
	$MnSO_4.4H_2O$	5	5	5
	$ZnSO_4\ H_2O$	0.7	0.7	0.7
	Na_2MoO_4	0.1	0.1	0.1
	EDTA Na-Fe	50	50	50

From flowering until the end of grain filling the plants received 75 ml weekly of the same background nutrient solution composition but using non-^{15}N enriched $^{14}NH_4{}^{14}NO_3$ at a N concentration of 1 mM.

During the entire growth period deionized water was applied regularly to maintain the substrate moisture content near field capacity. Every two days each cylinder was weighed to determine the amount of water lost. Deionised water was applied to refill the cylinders to their corresponding field capacities. As each cylinder was placed in an individual container, any solution that drained out through the mesh was added back to the cylinder via the substrate surface.

2.2.3 Sampling and biomass measurements

There were five destructive harvest times. The first one took place once the main shoot flowered (Zadoks GS 59; (Tottman, 1987), this was one day after the ^{15}N labelled solution was flushed out. Flowering occurred synchronously

across the different N treatments. The remaining sampling dates were 7 days after flowering (DAF), 17 DAF, 27 DAF and 34 DAF corresponding to GS59+128 ºC days, GS59+311ºC days, GS59+475 ºC days and GS59+604 ºC days when grain physiological maturity and the end of grain filling was reached (base temperature at 0 ºC). The N solution was applied seven days before every sampling date.

At each harvest date, designated cylinders were removed from the growth cabinet and the number of fully developed tillers was recorded. Shoots were cut from the roots at the stem base and separated into leaves (green and senescent), leaf sheaths, stems, and ear. At the last harvest time, GS59+604 ºC days, the ears were divided into grains and chaff from main shoot and tillers. All grain weights are described as 100% dry weight (DW). Each plant component was weighed and then the samples were oven-dried at 80 °C for 48 hours for dry weight determination to the nearest 0.01 g.

The sand:perlite mixture was released from the cylinder and placed in a tray, where the roots were carefully removed. Any substrate adhering to the roots was carefully removed using a gentle flow of water onto a tray. This caused the roots to separate from the mixture allowing them to be collected. This process reduced the risk of root tissue losses. Root tissue, including fine roots were carefully collected. The roots were gently blotted dry and weighed fresh, chopped into approximately 2 cm lengths and a representative subsample of approximately 20 % fresh weight (FW) was taken, weighed fresh and stored frozen at -20 ºC to await root length determination. The remaining root tissue was weighed fresh and dried at 80 °C for 48 h.

The mixture left after removing the roots was retained for N determination (see below). The sand:perlite mixture, hereafter referred as soil medium, was stored in a cold room at 4 °C for 24 h.

Additionally, at each harvest time, before the destructive sampling SPAD readings were recorded on the flag leaf, leaf 1 (leaf below the flag leaf), leaf 2

and leaf 3 of the main shoot of each plant sampled. Measurements were taken using a SPAD 502DL Plus Chlorophyll Meter, Spectrum Technologies, Inc. Aurora, USA.

At GS59+128DD (7DAF), leaf area was determined on the main shoot of each plant prior to drying shoot tissue. The projected area of leaf laminae was measured using a LI-3100 area meter, LI-COR, Lincoln, NE, USA.

2.2.4 Measurements of root length

Approximately 20 % of roots (FW) was taken for root length determination. The samples were stored at -20 ºC before the analysis. The analysis was performed once the experiment finished. The washed roots were allowed to thaw at room temperature within plastic bags before being placed in a clear rectangular tray with 50ml of water to facilitate the spread of the roots in the tray. The samples were placed in a scanner (Epson®, Expression 12000XL, UK) and the analysis of root length was performed on scanned images using WinRhizo Pro 2017 (Regent Instruments Inc. Canada) root image analysis system.

2.2.5 Sand:perlite and plant tissue analyses

Total N content in the sand:perlite was measured to establish whether there was sufficient N remaining in the root medium for determination of ^{15}N released from the roots post-flowering. On each sampling date, after removing the roots, the soil medium was homogenised thoroughly by hand. To avoid cross-contamination of samples each plant and hence, each root sampling was performed individually cleaning all the materials used between each plant sampled. An extra 30 g approximately was taken for moisture content determination.

For determination of NH_4^+ and NO_3^- approximately 10 g of the soil medium was weighed to the nearest 0.01 g and shaken with 50 ml of 2 M KCl for one hour. The extracts were then centrifuged at 4700 rpm for 20 min and the supernatant

collected for colorimetric analysis using an automated wet chemistry analyser (SAN^{++}, Skalar). For moisture content determination, approximately 30 g FW were taken and oven-dried at 104°C for 24h before weighing.

Total N and C of the soil medium was determined by drying a sample at 60 ºC for 72 h. Once dried it was ground to a fine powder using a ball mill (SPEX 8000 Mixer/Mill) and total N and C concentrations were determined by an automated Dumas combustion method using a Flash 2000 elemental analyser (Thermo Scientific UK). Approximately 15-20mg of sample was place in tin capsules for the determination of total N and C.

After the determination of dry weight, plant tissue was ground to a fine powder using a ball mill (SPEX 8000 Mixer/Mill) and total N concentration measured by an automated Dumas combustion method using a Flash 2000 elemental analyser as described above. Abundance of ^{15}N in sand:perlite and plant tissue samples was measured using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (IRSM). Analyses were conducted by the UC Davis Stable Isotope Facility (UC Davis, California, USA).

2.2.6 Nitrogen calculations and data analysis

The following assumptions are made in this experiment: a) there is no discrimination between ^{14}N and ^{15}N for N distribution within the plant and, b) both isotopes are remobilized in the same proportion c) the nitrogen taken up post-anthesis is uniformly mixed with the ^{15}N already in the plant.

Total N and ^{15}N abundance

At each sampling date, the following equation was used to determine total nitrogen content in each plant component represented by "*c*"

$$Total\ N\ content_{(c)} = (\%\ N_f * DW_f)/100 \qquad Equation\ 2.1.1$$

To calculate the ^{15}N atom enrichment derived from pre-anthesis fertilizer in a particular plant fraction ($\%^{15}N_f$), the measured ^{15}N atom % was adjusted for natural ^{15}N abundance of 0.36933 atom %. This value was based on previous experiments conducted on spring barley at SRUC as well as a literature review. The ^{15}N accumulated in any plant fraction from fertilizer was then given as:

$$Total\ ^{15}N\ content_{(c)} = (\%\ ^{15}N_c * Total\ N\ content_{(c)}) /100 \qquad Equation\ 2.1.2$$

The total N content at the whole plant level at each harvest point was determined by summing the total N content found in each plant component (leaves + sheaths + stems + roots + ears). The same operation was used to calculate the total accumulated ^{15}N at the whole plant level.

Nitrogen Remobilisation

^{15}N Remobilisation from each fraction

One of the advantages of having a system where any remaining N in the root medium at anthesis was flushed out, is that remobilisation of ^{15}N from different plant component can be measured from anthesis to the end of grain filling, regardless of the new nitrogen taken up from flowering. At this stage we assumed that, before each harvest time, all the nitrogen absorbed after anthesis was uniformly mixed with the already existing ^{15}N in the plant.

The ^{15}N remobilisation (NR; mg^{15} N plant^{-1}) was estimated as the amount of ^{15}N in the plant component at anthesis (GS59, T1) which was not recovered in the plant component at the next harvest time or at grain maturity, 128 ºC days, 311 ºC days, 475 ºC days or 604 ºC days equivalent to 7, 17, 27 and 34 days after flowering (T2, T3, T4, T5 respectively).

$$^{15}NR_c = Total\ ^{15}N\ content_{(f,T1)} - Total\ ^{15}N\ content_{(f,T2,\ T3,T4,T5)} \qquad Equation\ 2.1.3$$

The ^{15}N remobilisation efficiency was calculated as:

NRE= Equation 2.3 ÷ $^{15}N_{T1}$ * 100 *Equation 2.1.4*

Total N remobilisation

To calculate the total amount of N (mg N plant^{-1}) deposited in a particular plant component that was subsequently remobilised, the % atom enrichment of the tissue has to be considered. The total N remobilised from each plant organ was calculated as:

Total N Rem = Total N content $_{(fcT1)}$ – (Total ^{15}N content $_{(c,\underline{T5})}$ ÷ % $^{15}N_{(x,T5)}$)

Equation 2.1.5

The N remobilisation was also calculated per every time interval, T1-T2, T2-T3, T3-T4 and T4-T5.

Nitrogen uptake

The post anthesis N uptake (PANU; mg N plant^{-1}) was calculated as:

PANU = Total $N_{(T5)}$ – Total $N_{(T1)}$ *Equation 2.1.6*

The N taken up by a tissue "$_{(f)}$" at any time interval "$_{T}$" was calculated as:

$Nup_{c,T}$ = (Equation 2.6) – change in N content $_{(c,\ T1\text{-}T2)}$

Equation 2.1.7

Calculation was done at every time interval.

To calculate the N uptake efficiency the total amount of N taken up by a plant was divided by the N supplied post anthesis.

NupE = (Equation 2.7 $_{(plant)}$ ÷ post anthesis N supply) * 100 *Equation 2.1.8*

2.2.7 Determination of N losses

Loss of ^{15}N absorbed pre-anthesis during the grain filling period was calculated as the difference between the total ^{15}N plant^{-1} between anthesis (T1) and grain maturity (T5). There was insufficient N in the soil medium at anthesis and the final harvest to the determine ^{15}N and estimate the net loss of ^{15}N to the root medium during grain filling.

2.2.8 Nitrogen Nutrition Index (NNI)

Nitrogen nitration index is the most widely recognised tool for the accurate diagnose of in-season crop N status (Lemaire et al., 2008).

The NNI is usually determined through a critical nitrogen dilution curve. The critical nitrogen concentration is the minimal concentration of total N in shoots that produced the maximum above-ground dry matter, at a given time and field condition (Justes et al., 1994).

To calculate the nitrogen nutrition index of the plants at anthesis, in this experiment, a value of 1.74 % (N %) was used. This is the crop N concentration at GS59 for crops grown with optimum fertilizer rate for yield under field conditions. However, given the environmental conditions of the columns used in this experiment, which are considerable different than the field conditions in which the calibration curve is established, a “relative NNI” was calculated assuming N3 plants represent non-liming N conditions. The same procedure used by Justes et al. (1994) was used. By plotting the plant N% vs the accumulated above-ground biomass of N3 plants a unique critical nitrogen dilution curve was obtained. The obtained equation Nc = 3.377 x DW $^{-0.823}$ led to the determination of the relative NNI as follow:

$$NNI = \frac{Plant\ N\%}{Nc}$$

2.2.9 Statistical Analysis

Analysis of variance (ANOVA) of the data were carried out to determine the statistical significance of differences of mean values between treatments using GenStat (19th Edition VSN International Ltd.). Two-way ANOVA (in randomised blocks) was used to determine statistical significance of differences between N treatments across time. For individual analyses at specific harvest time e.g. yield components at maturity a one-way ANOVA and multiple comparisons analysis (LSD α=0.05) were performed. Residuals were checked and shown to have a normal distribution and homogeneous variance and thus transformation was not required. Bi-plot procedures to test associations between traits were carried out in RStudio version 2.4.5.

2.3 Results

2.3.1 Effects of pre-anthesis N supply on biomass accumulation, root length, leaf area.

Biomass accumulation

The three levels of $^{15}NH_4{}^{15}NO_3$ (2, 4 and 6 mM, respectively referred to as N1, N2 and N3) that were used pre-anthesis led to highly contrasting differences in plant biomass at anthesis (Fig. 2.2). Thereafter, even though the N supply post-anthesis was identical, the overall dry matter increased at each harvest time in each N treatment. By the end of grain filling plants accumulated an average (± standard error of mean) of 3.22 ± 0.33 g plant^{-1}, 5.73 ± 0.17 g plant^{-1} and 7.13 ± 0.50 g plant^{-1} with N1, N2 and N3 treatment, respectively. There was an almost 2.9x increase in biomass from anthesis to the end of grain filling in each N treatment (Fig. 2.2, Table S2.1). The N treatment as well as the time of harvest and the interaction between these two were statistically significant ($p<0.001$; Table 2.2). Plants in all N treatments showed a rapid increase in total biomass during the first half of grain filling and then a decrease in the rate of growth thereafter. This was most pronounced at the highest N supply.

The different N treatments supplied pre-anthesis resulted in a significant difference in the DW of each tissue fraction between anthesis and the end of grain filling ($p<0.001$, Table 2.2). The biomass of each fraction was greater with a greater N supply (Fig. 2.2, Table S2.1).

In general, the DW of all fractions increased between flowering and mid grain filling. For leaves the biomass then declined with the most pronounced changes occurring in the N2 and N3 treatments (NxT interaction P=0.009). For leaf sheaths, stem and roots, after the initial increase, the DW remained relatively stable until the end of grain filling. Ear DW increased throughout the grain filling period at each N supply.

Table 2.1.2 Analysis of variance of dry weights of whole plants and different plant tissue fractions as affected by N supply pre anthesis.

Effect	Leaves	Sheaths	Stems	Roots	Ears	Total
N supplied (N)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Harvest (T)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
(NxT)	0.009	0.113	<0.001	0.105	<0.001	<0.001
N LSD (5%)	0.009	0.035	0.086	0.061	0.096	0.216
T LSD (5%)	0.0125	0.046	0.111	0.078	0.124	0.279
NxT LSD (5%)	0.0217	n.s	0.192	n.s	0.216	0.483

Data are the means of five replicate plants. LSD = Least significant differences; n.s not significant.

Root Length

The pre-anthesis N supply significantly influenced the root length of plants ($p<0.001$). The time after flowering also showed a significant influence

(p<0.001). However, there was no interaction between the different N treatments and the time of harvest (p=0.140).

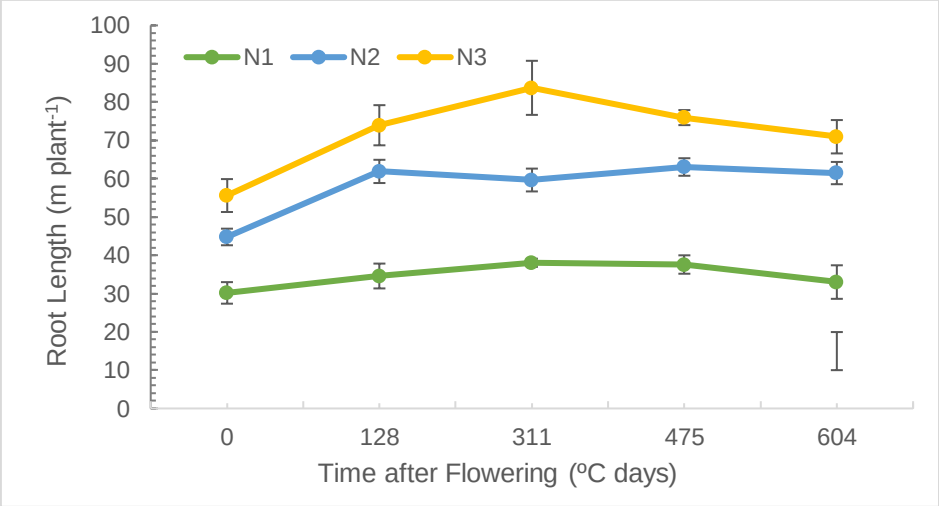


Figure 2.1.1 Changes in root length through the grain filling period for plants supplied with 2 mM (N1), 4 mM (N2) and 6 mM N (N3) before flowering. Data are the means of five replicate plants ± SEM. LSD of the interaction NxT is shown.

Thus, root length was increased with increasing N supply and these differences were observed throughout the grain filling period (Fig.2.1). In general plant root length increased from flowering to around mid-grain fill thereafter there was a small decline. These changes were most pronounced for the N1 and N3 treatments.

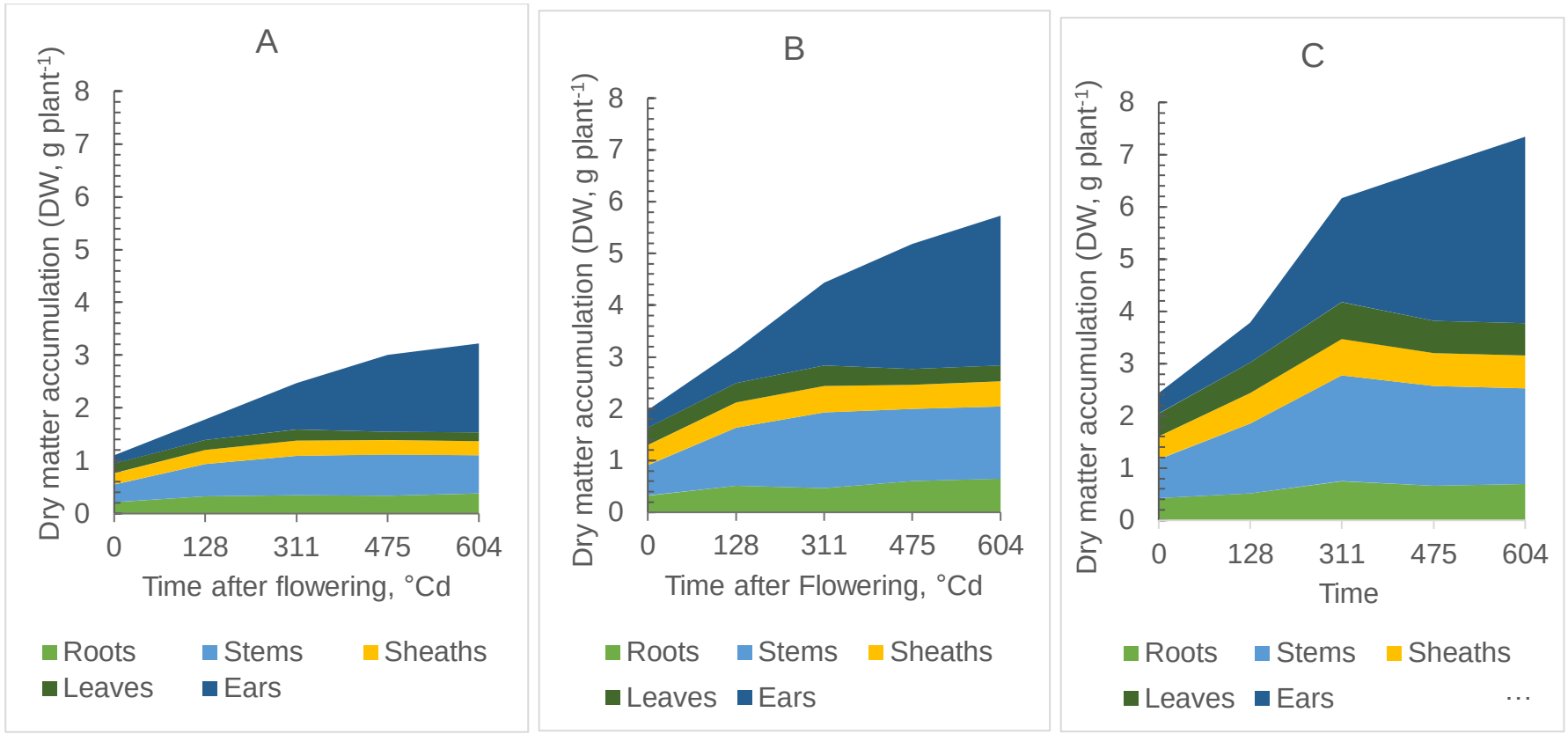


Figure 2.1.2 Total dry weights (DW) and changes in DW of leaves, leaf sheaths, stems, roots, and ears of N1 (A), N2 (B) and N3 (C) from anthesis to the end of grain filling. Data are the means of five plant replicates.

Leaf Area

On average the total leaf area of the main shoot of N1 plants was 31.71 cm^2, this is about 40% less compared with the plants treated with high N treatment (52.75 cm^2). There were no significant differences between N2 and N3 plants (Fig. 2.3).

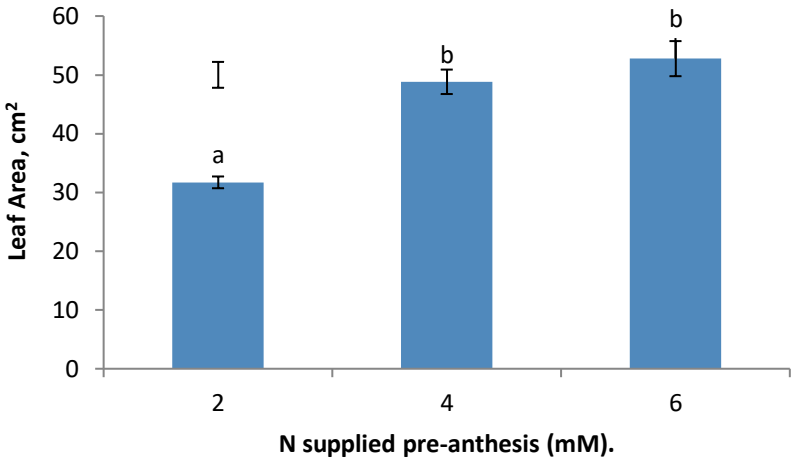


Figure 2.1.3 Effect of 2, 4 and 6mM of N pre-anthesis on total leaf area ($p<0.001$) determined on the main shoot at GS59+128DD. Values are mean of five plants per treatment ± SEM. Bar on the top left side is LSD (α=0.05).

2.3.2 Effects of pre-anthesis N supply on yield, yield components, NNI and plant N concentration

Increasing the pre-anthesis N supply significantly increased the grain yield (g DW $plant^{-1}$) as well as the number of grains per plant (p <0.001, Table 2. 3). At the end of grain filling plants grown under the N2 and N3 treatments, also produced a greater number of developed ears than those given the lowest (N1) supply (p=0.051, Table 2.3).

The number of grains on the main shoot was significantly affected by the N treatment applied pre-anthesis (p=0.002, Table S2.2). Main shoots had an average of 15.6, 17.6 and 18.8 grains in N1, N2 and N3 plants, respectively (Table 2.4). The grain number of the main shoot was significantly greater

under the N2 and N3 treatments compared to the N1 treatment, indicating that the significant differences observed in the overall number of grains were not just associated with the greater tillering at high N supply. Overall, an average of 11.8, 14.2 and 16.3 grains were recorded per ear in N1, N2 and N3 treatments, respectively ($p < 0.001$, Table 2.3).

Table 2.1.3 Effect of three nitrogen levels supplied pre-anthesis on grain yield per plant and its components.

N supply treatment	Grain weight, g DW plant^{-1}	Grain number plant^{-1}	Grain number ear^{-1}	Ear number plant^{-1}	Mean grain weight, mg DW
N1	1.38 a	30.2 a	11.8 a	2.6 a	46.26 a
N2	2.43 b	48.2 b	14.2 b	3.4 a	50.86 a
N3	2.86 c	58.2 c	16.3 c	3.6 a	49.27 a
p value	<0.001	<0.001	<0.001	0.051	0.311
LSD (α=0.05)	0.199	6.8	1.638	n.s	n.s

Data are the means of five plants. Within a column, means followed by a different letter are statistically significant (LSD= Least significant differences).

The concentration of N in the grains was only influenced by the pre-anthesis N supply in the grains of main shoots ($p=0.002$, Table 2.4). There were no significant differences in the N concentration of the grains of tillers. Plants treated with the highest N supply (N3) pre-anthesis had an N concentration of 1.4 % in the main shoot grains whereas those given the N2 treatment reached 1.2 % and those given the N1 treatment only reached 1.1 % (Table 2.4).

Table 2.1.4 Number of grains and N concentration in grains of main shoots and tillers of plants treated with 2, 4 and 6mM of N pre-anthesis.

N supply	Main shoot		Tiller	
	Number	N%	Number	N%
N1	15.6 a	1.14 a	14.6 a	0.94 a
N2	17.6 b	1.22 a	30.6 b	1.00 a
N3	18.8 b	1.43 b	39.4 c	0.98 a
p value	0.001	0.002	<0.001	0.419
l.s.d (α=0.05)	1.44	0.135	6.68	0.0998

Data are the means of five plants. Within a column, means followed by a different letter are statistically significant. LSD=Least significant differences; n.s =not significant.

The whole plant N concentration observed at anthesis was significantly affected (p = 0.018) by the pre-anthesis N treatment (Table 2.5). However, by the end of grain filling no significant differences were observed between treatments in the N concentration.

According to the estimates of nitrogen nutrition index (NNI), when using 1.74% as the critical value for N, corresponding to field conditions, only plants treated with the lowest pre-anthesis N supply (N1) appeared to be deficient in N at anthesis with an NNI of 0.88 (Table 2.5) and those given the N2 and N3 treatments had NNIs of 1.0 and 1.13 respectively. By contrast, by using N3 as a non-liming N supply, the relative NNI indicates that N1 and N2 were N deficient (Table 2.5).

Table 2.1.5 Effect of pre-anthesis N supply on whole plant N concentration at anthesis and maturity and the NNI at anthesis calculated using the field N% and the critical curve relative to N3 plants (relative NNI).

N supply	Plant N Concentration (N % DW)		NNI	Relative NNI
	Anthesis	Maturity		
N1	1.41 a	0.72 a	0.88 a	0.43 a
N2	1.63 ab	0.71 a	1.00 ab	0.75 b
N3	1.85 b	0.75 a	1.13 b	1.00 c
p value	0.018	0.208	0.007	<0.001
LSD (α=0.05)	0.27	n.s	0.248	0.046

Data are the means of five plants. Within a column, means followed by a different letter are statistically significant. LSD=Least significant differences; n.s =not significant.

2.3.3 Effects of pre-anthesis N supply on the accumulation of N at tissue level

At anthesis, the different treatments of pre-anthesis N increased the amount on N accumulated by each plant component ($p<0.001$, Table 2.6). Fig. 2.4 shows that at anthesis the highest amount of N was found in the leaf's fractions. The N content of each plant organ was reduced as the grain filling progress ($p<0.001$), apart from the ears fraction which showed a significantly increased. Only in the roots, the interaction between N treatment and harvest time was not significant ($p=0.753$). The N content in this organ was maintained relatively stable throughout the grain filling period. The pattern of the N changes over time differed between plant component and pre-anthesis N supply treatments. The N content of leaves was reduced from anthesis to the end of grain filling. Leaves of plants treated with N1, N2 and N3 had an initial

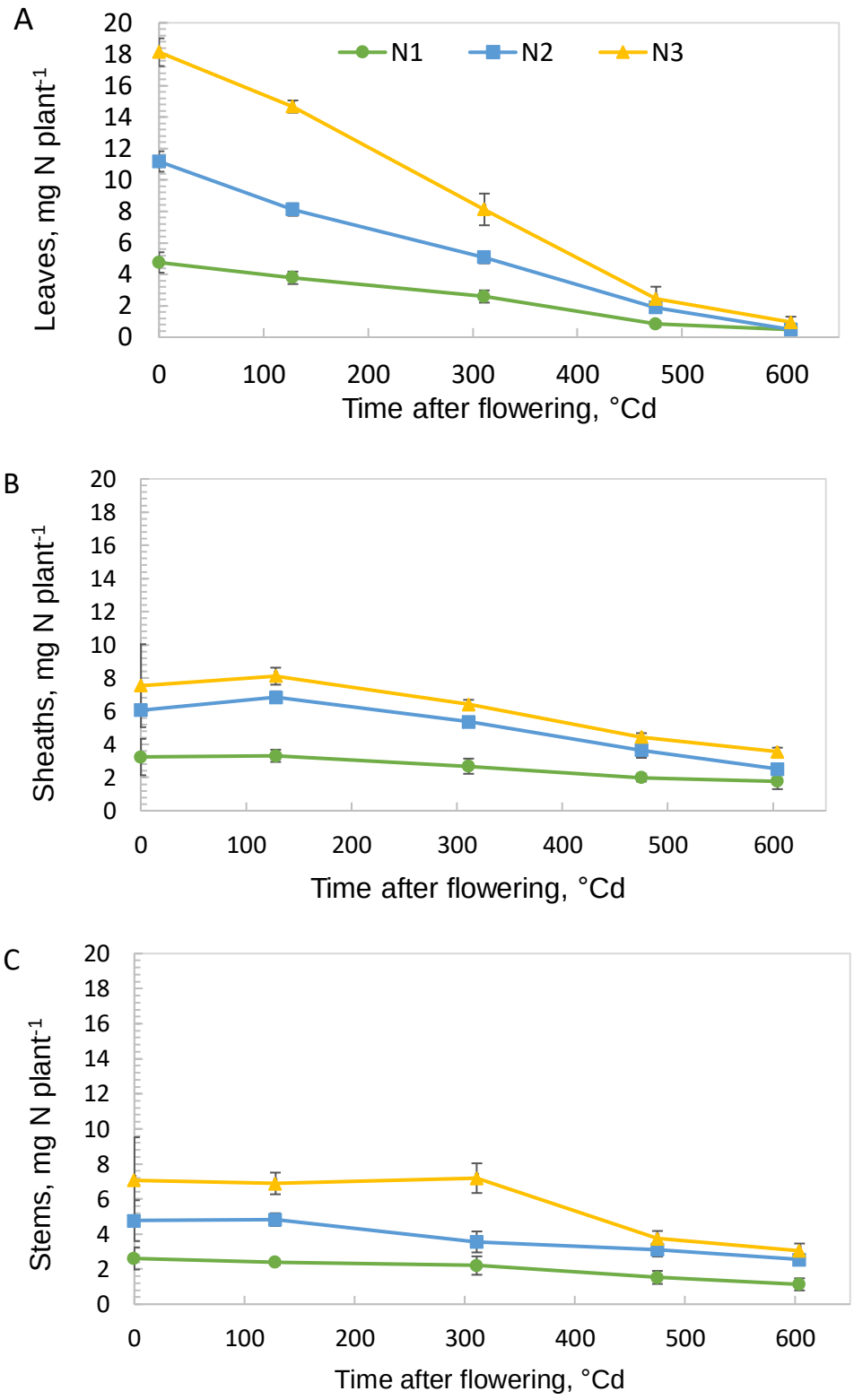
N content of 4.7, 11.1 and 18.1 mg N plant^{-1}, respectively. By the end of grain filling leaves had 0.48, 0.49 and 0.96 mg N plant^{-1} for N1, N2 and N3 respectively. The rate of reduction as shown by the slope in Fig.2.4 A, suggests that it was greater at the highest N treatment.

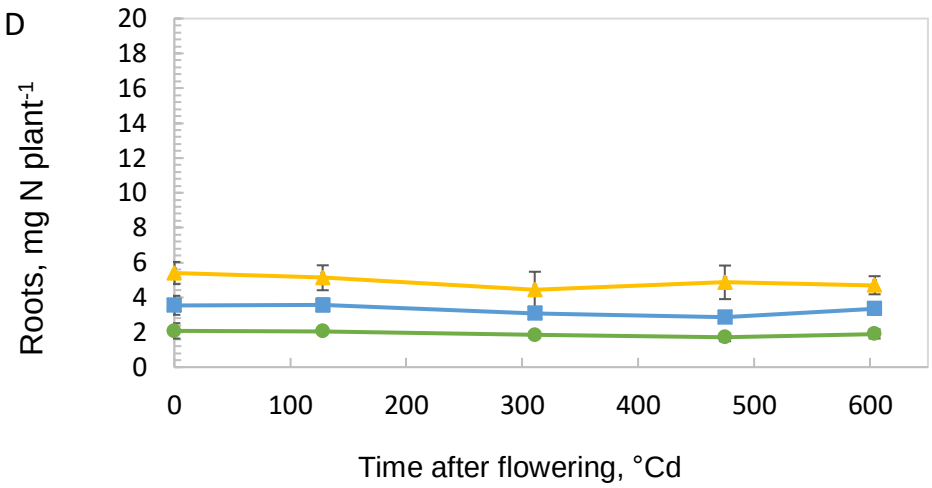
On the other hand, the decline in N content of sheaths and stems was less pronounced and in fact, there appears to be a delay on the decrease of N content in these organs. For sheaths (Fig 2.4 B), from T1 to T2 there was a slight increase on the N content from 3.2 to 3.3 mg N plant^{-1} in N1 plants, 6.0 to 6.8 mg N plant^{-1} in N2 plants and 7.5 to 8.1 mg N plant^{-1} in N3. Thereafter, the N content in sheaths declined, reaching 1.8, 2.5 and 3.6 mg N for N1, N2 and N3 treatments, respectively. Stems (Fig 2.4 C) were able to maintain their N content relatively stable until mid-grain filling, thereafter there was a slight N decline with N3 plants showing the greatest reduction.

Table 2.1.6 Analysis of variance of nitrogen (N) content of plant tissue fractions as affected by N supply pre-anthesis during the grain filling period.

Effect	Leaves	Sheaths	Stems	Roots	Ears
N supplied pre-anthesis (N)	<0.001	<0.001	<0.001	<0.001	<0.001
Harvest Time (T)	<0.001	<0.001	<0.001	0.011	<0.001
(N x T)	<0.001	0.001	<0.001	0.753	<0.001
N LSD	0.331	0.442	0.466	0.290	0.997
T LSD	0.428	0.570	0.602	0.374	1.288
NxT LSD	0.741	0.988	1.043	n.s	2.230

LSD=Least significant differences; n.s =not significant.


A
Leaves, mg N plant-1
N1
N2
N3
Time after flowering, °Cd
B
Sheaths, mg N plant-1
Time after flowering, °Cd
C
Stems, mg N plant-1
Time after flowering, °Cd



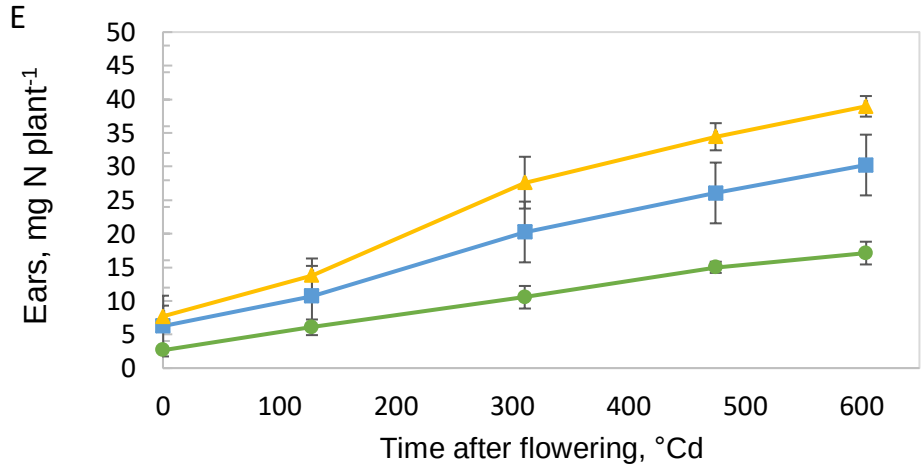


Figure 2.1.4 Change in N content of each plant organ (A) leaves, (B) sheaths, (C) stems, (D) roots and I ears from anthesis to the end of grain filling. Data are means of five plants ± SEM

At the end of the grain filling period, grains were separated from chaff and also, from main shoots and tillers. The N supplied pre-anthesis increased the N accumulated in each of these fractions with N3 treatment showing the greatest N accumulation ($p<0.001$, Table 2.7)

Table 2.1.7 Effects of pre-anthesis N supply on the total N content (mg) of the different ear components.

N supply	N accumulated (mg N plant^{-1})			
	Grain main shoot	**Grain tillers**	**Chaff main shoot**	**Chaff tillers**
N1	17.11 a	4.58 a	2.30 a	1.10 a
N2	30.22 b	13.24 b	3.42 b	2.43 b
N3	38.96 c	16.68 c	5.50 c	4.04 c
p value	<0.001	<0.001	<0.001	<0.001
LSD (α=0.05)	1.756	1.961	0.439	0.5018

Data are the means of five plants. Statistically significantly groups are shown. LSD=Least significant differences; n.s =not significant.

Table 2.8 shows the amount of N left in each vegetative organ including senesce leaves at the end of grain filling alongside its N concentration (%). The amount of N left in completely senesced leaves at maturity was bigger that the amount found in those posing some visible chlorophyll. The amount of N found in every plant fraction at the end of grain filling, increased significantly with the increase on pre-anthesis N supply. Plants treated with N3 showed the greatest amount of N left. However, only the N concentration in senesce leaves was affected by the different N supplies.

Table 2.1.8 Amount of total N remaining (mg N plant^{-1}) and N concentration in each vegetative organ at the end of grain filling.

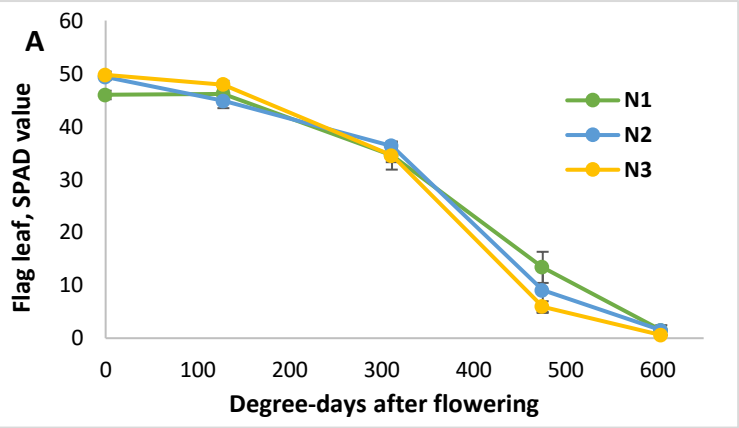
N supply	Leaves		Senesce leaves		Sheaths		Stems		Roots	
	mg N	N %	mg N	N %	mg N	N %	mg N	N %	mg N	N %
N1	0.48 a	0.9 a	0.60 a	0.50 a	1.77 a	0.65 a	1.13 a	0.15 a	1.90 a	0.52 a
N2	0.49 ab	0.83 a	1.62 b	0.63 b	2.52 b	0.52 a	2.56 b	0.18 b	3.36 b	0.52 a
N3	0.96 b	0.94 a	2.16 c	0.71 b	3.56 c	0.57 a	3.04 b	0.17 ab	4.70 c	0.68 a
p value	0.025	0.363	<0.001	0.036	<0.001	0.210	<0.001	0.107	<0.001	0.415
LSD. (α=0.05)	0.363	n.s	0.403	0.558	0.493	n.s	0.538	n.s	0.581	n.s

Data are the means of five replicate plants. Statistically significantly groups are shown. LSD=Least significant differences; n.s =not significant.

2.3.4 Post-anthesis leaf N dynamics through SPAD reading

Fig 2.5 shows the changes on SPAD measurements from anthesis to the end of grain filling in the different leaves of the main shoot. The analysis of variance (Table 2.9) indicated that the pre-anthesis N supply only influenced the changes in SPAD readings in the oldest leaf (Leaf 3, $p = 0.023$) of the main shoot. The time of harvest significantly reduced the SPAD readings of all the leaves analysed ($p < 0.001$). There was a significant interaction between the pre-anthesis nitrogen treatments and the harvest time on leaves 1, 2 and 3 ($p<0.001$, 0.008 and 0.049, respectively, Table 2.9), with N3 plants initially showing higher SPAD values and the lowest from mid-grain filling.

At anthesis, N1 plants showed the lowest SPAD value in the flag leaf (45.9) but also in leaf 1 (41.8), leaf 2 (39.4) and leaf 3 (34.6). N2 and N3 treated plants showed almost identical values of SPAD at anthesis. Interesting, leaves of N1 plants were able to maintain their chlorophyll content better than leaves of N2 and N3 especially in the flag leaf, leaf 1 and leaf 2. For example, at 475 °C days after flowering, N1 plants had a SPAD value of 10.1 in the leaf below the flag leaf (leaf 1) whereas N2 showed a SPAD value of 3.2 and N3 plants showed an average of 0.14 in the leaf 1 (Fig 2.5 B).



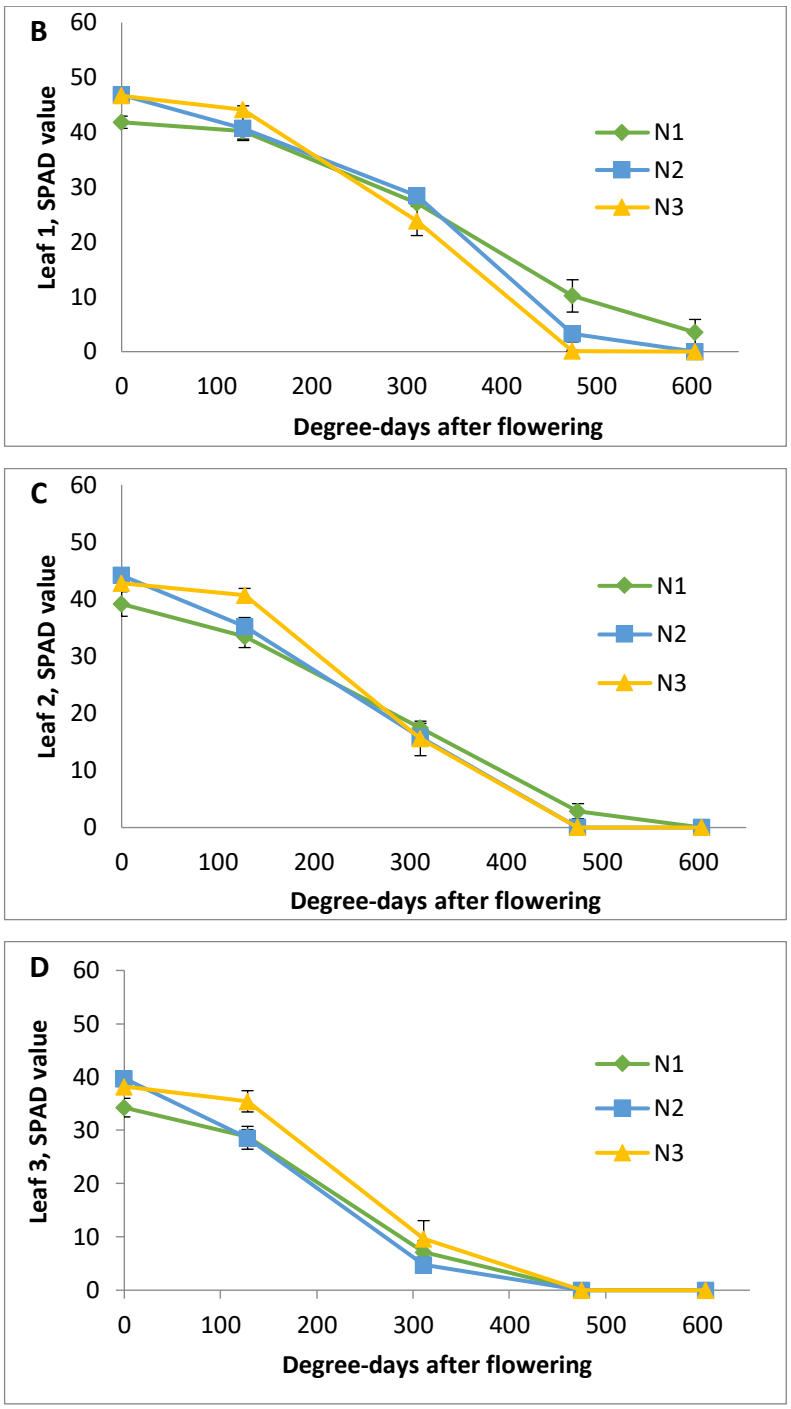


Figure 2.1.5 SPAD readings of (A) flag leaf, (B) leaf 1-below flag leaf, (C) leaf 2 and (D) leaf 3 of the main shoot, from anthesis to the end of grain filling. Data are means of five replicate plants ± SEM.

Table 2.1.9 Analysis of variance of SPAD readings taken on different leaves post-anthesis.

Effect	Flag leaf	Leaf 1	Leaf 2	Leaf 3
N supplied pre-anthesis (N)	0.844	0.243	0.488	0.023
Harvest time (T)	<0.001	<0.001	<0.001	<0.001
N x T	0.147	<0.001	0.008	0.049
LSD 5% (T)	3.020	2.480	2.323	2.500
LSD 5% (N x T)	n.s	4.296	4.023	4.330

LSD=Least significant differences; n.s = not significant

A link between biomass accumulation, N content and SPAD measurements is difficult to establish considering that both biomass and N accumulation account for all the leaves in the plant whereas the SPAD measurements were only taken in the leaves of main shoots. However, the SPAD measurements were significantly reduced ($p<0.001$, Table 2.9) from anthesis to the end of grain filling in all the measured leaves. Flag leaf and leaf 1 exhibit some chlorophyll content measured trough the SPAD towards the end of grain filling with N1 plants showing the higher SPAD values at these harvest times (Fig 2.5 A and B).

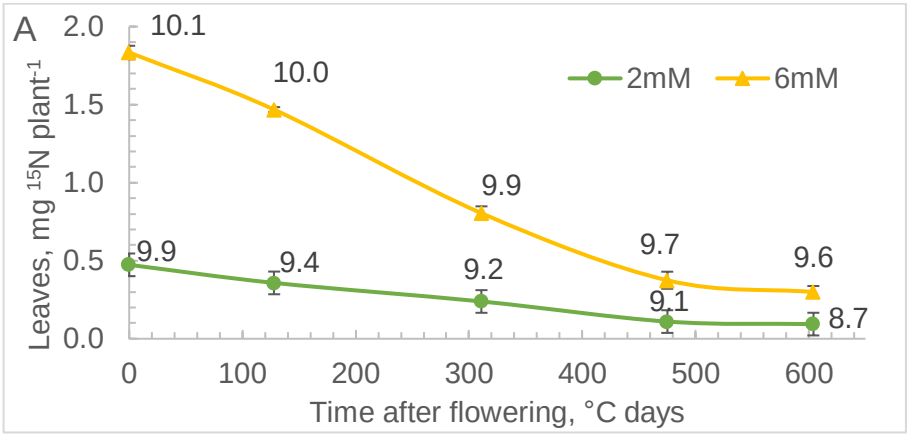
2.3.5 Effects of pre-anthesis N supply on N remobilization

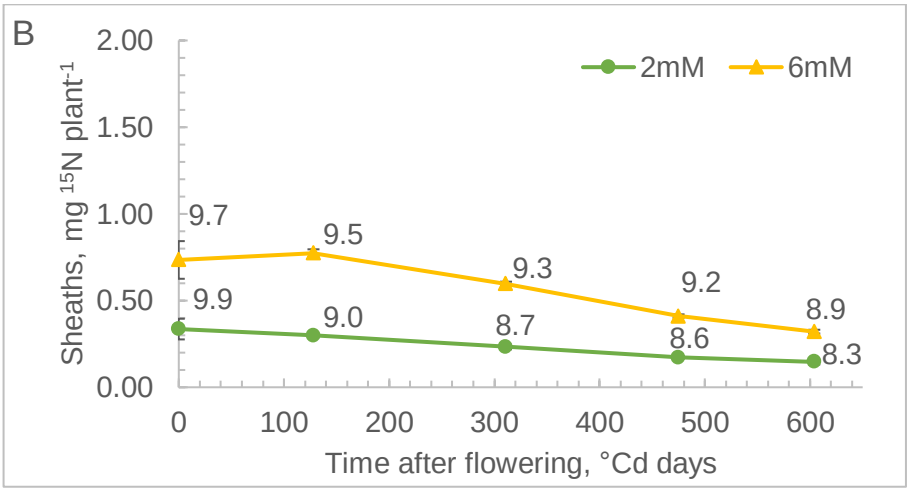
Onset of the remobilization of ^{15}N from different vegetative organs

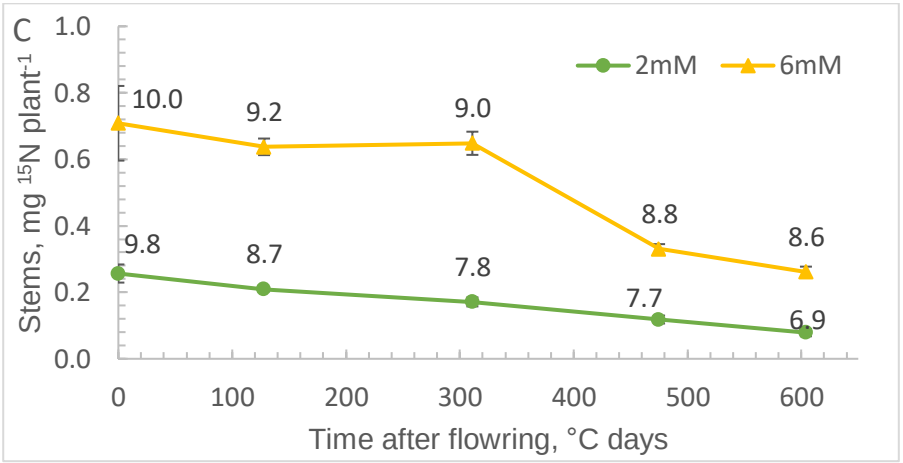
The ^{15}N content of each fraction was only analysed in plants treated with 2 and 6mM N supply pre-anthesis. The two extremes were chosen for the analysis given the cost involved and due to the responses of total N content observed between the different N treatments.

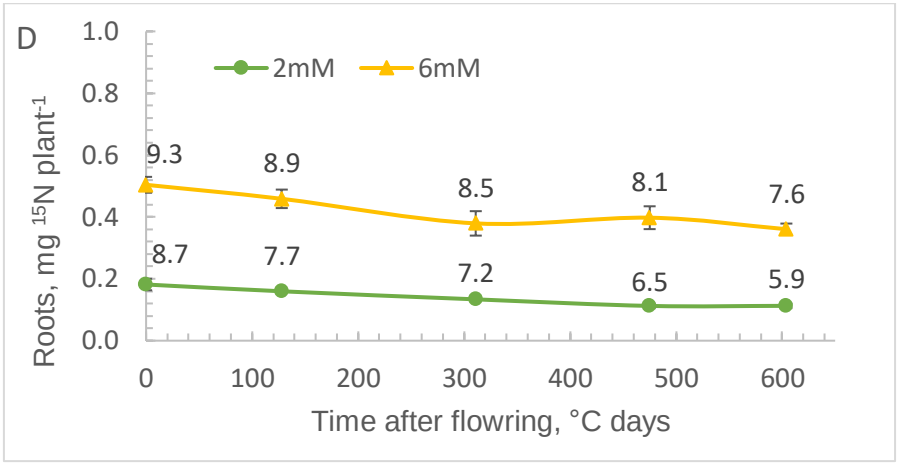
The onset of the ^{15}N remobilisation differed between the different organs. Fig. 2.6 shows the changes in the amount of ^{15}N (mg ^{15}N plant^{-1}) in each plant

fraction from anthesis to the end of grain filling. In the graph, the % atom enrichment at each harvest time is also shown.









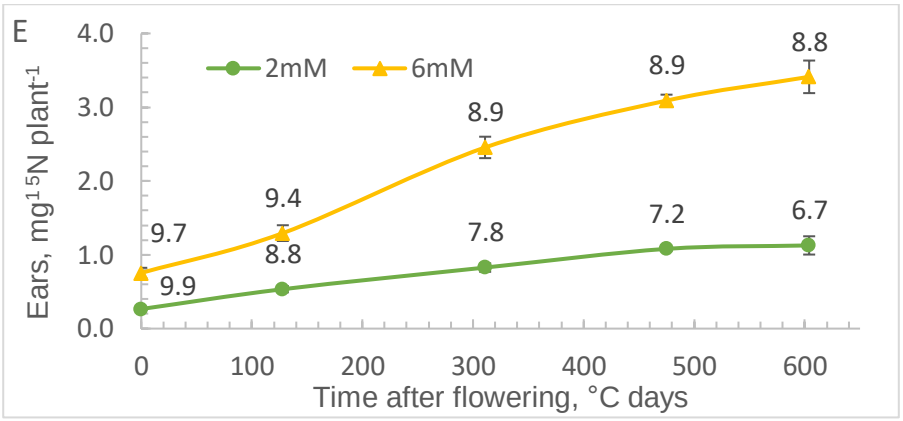


Figure 2.1.6 Change in the amount of ^{15}N content of each organ plant (A) leaves, (B) sheaths, (C) stems, (D) roots and I ears during the grain filling period. Data labels are the % atom enrichment. Data are means of five plants ± SEM.

Analysis of variance of the changes in the amount (mg) and atom enrichment of each plant fraction is shown in tables 2.10 and 2.11 respectively.

Table 2.1.10 Analysis of variance of the changes in the amount of ^{15}N content of each plant component treated with three N supplies pre-anthesis during the grain filling period.

Effect	**Leaves**		**Sheaths**		**Stems**		**Roots**		**Ears**	
	p value	LSD	*p* value	LSD	*p* value	LSD	*p* value	LSD	*p* value	LSD
N treatment	<0.001	0.031	<0.001	0.051	<0.001	0.051	<0.001	0.030	<0.001	0.095
Harvest time	<0.001	0.048	<0.001	0.080	<0.001	0.081	<0.001	0.047	<0.001	0.151
N x T	<0.001	0.068	0.003	0.113	<0.001	0.114	0.403	n.s	<0.001	0.213

LSD=Least significant differences; n.s =not significant

Table 2.1.11 Analysis of variance of the changes in the ^{15}N atom enrichment of each plant component treated with three N supplies pre-anthesis during the grain filling period.

Effect	**Leaves**		**Sheaths**		**Stems**		**Roots**		**Ears**	
	p value	LSD	*p* value	LSD	*p* value	LSD	*p* value	LSD	*p* value	LSD
N treatment	<0.001	0.073	<0.001	0.187	<0.001	0.254	<0.001	0.147	<0.001	0.127
Harvest time	<0.001	0.110	<0.001	0.119	<0.001	0.161	<0.001	0.093	<0.001	0.080
N x T	<0.001	0.162	<0.001	0.265	<0.001	0.360	<0.001	0.207	<0.001	0.180

LSD=Least significant differences; n.s =not significant

Based on the remobilisation dynamics observed it appears that the remobilisation of pre-absorbed N from the leaves fraction occurs right from anthesis and possibly earlier. There is a steady decline in the ^{15}N content of stems and sheaths, these organs were able to maintain their ^{15}N content until 311 ºC days after flowering when it started to decline. The ^{15}N content of roots slightly decline, though the atom enrichment in this plant component was greatly reduced by the end of grain filling. Thus, compared to leaves and roots, there seems to be a slight delay on the start of the remobilisation of ^{15}N from sheaths and stems, especially at high N supply.

The onset of ^{15}N remobilisation from roots seems to occur before anthesis, especially in plants treated with 2 mM of N where the atom enrichment measured at flowering was already reduced.

The analysis of variance showed that the N supply pre-anthesis significantly affected the amount of ^{15}N found in each plant fraction at each harvest time. The interaction between N supply and harvest time was significantly different for each plant component apart from roots. The atom enrichment was also influenced by the N supplied pre-anthesis and it was significantly different from anthesis to the end of grain filling. There also was a significant interaction between N supply and harvest time with regards of atom % enrichment.

Nitrogen remobilisation efficiency of vegetative organs

The overall nitrogen remobilisation efficiency (NRE, %), calculated from the ^{15}N data, from each vegetative organ (leaves, sheaths, stems and roots) is shown in table 2.11.

Table 2.1.12 Overall nitrogen remobilisation efficiency (NRE %) from each vegetative organ.

N supply	Leaves	Sheaths	Stems	Roots	Total
N1	79.8	51.0	68.7	35.1	64.3
N3	83.7	50.2	60.9	27.0	66.7
p value	0.070	0.639	0.215	0.227	0.491
LSD	n.s	n.s	n.s	n.s	n.s

Data are the mean of five replicate plants; LSD=Least significant differences; n.s =not significant.

The N treatment did not influence the NRE of any organ. However, there were appreciable differences between the plant organs with leaves being the most efficient with an NRE of around 80 %, followed by stems, sheaths and finally roots which were the least efficient.

2.3.6 Effects of plant N status at anthesis on the temporal dynamics and efficiency of N uptake through the grain filling period.

The pre-anthesis N treatments significantly influenced the total N content of the plants from anthesis to the end of grain filling ($p<0.001$). However, the interaction between N treatment and harvest time was not significant ($p=0.222$).

Fig. 2.7 shows that at anthesis plants had accumulated significantly different amounts of N at every N supply. At anthesis, plants had accumulated on average a total of 15.3, 31.80 and 45.83 mg N plant^{-1} for N1, N2 and N3 treatments, respectively. Thereafter, and regardless of the N treatment, plants continued to uptake N until the end of grain filling. By the end of grain filling plants treated with low N (N1) reached 23mgN plant^{-1} that is, on average, 7.6 mg more N than its initial N content. Plants treated with medium N (N2) accumulated 40.7 mg N plant^{-1}, 8.9 mg more than its initial N content and N3 plants accumulated 53.3 mg N, 7.5 mg more than its initial N content.

Simple regression by groups used to compare the N accumulation over time under each N treatment showed that the intercept of each N treatment was significantly different ($p<0.001$), but the slopes showed no significant difference ($p=0.738$).

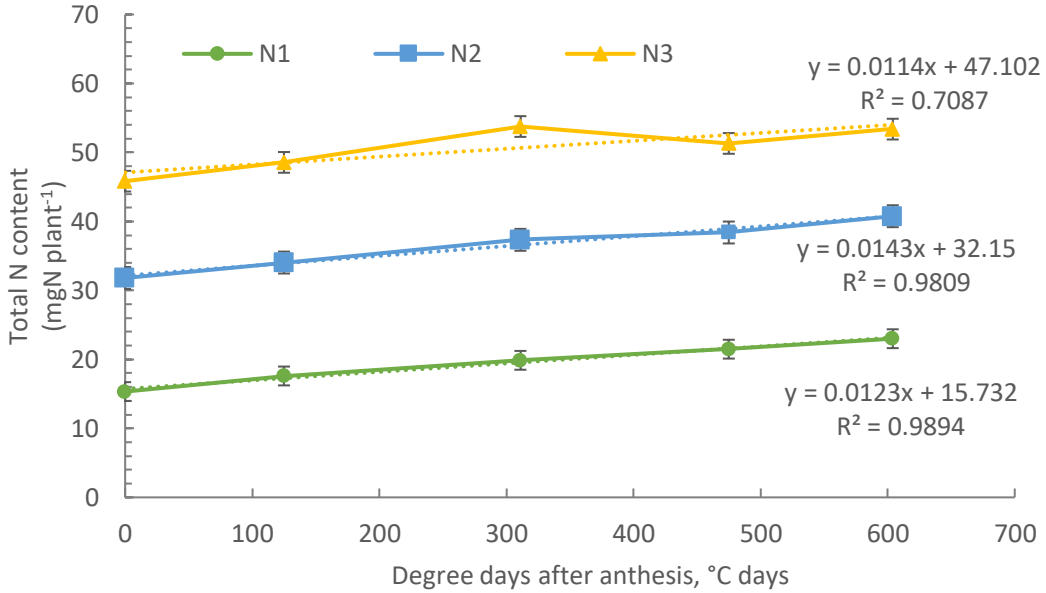


Figure 2.1.7 Total N content (mg N plant^{-1}) at each harvest time. Data are means of five replicate plants at each harvest time ± SEM.

Between 311 and 475 °C days after flowering there appeared to be a small reduction in the N content of plants in the N3 treatment. This is likely to arise from sampling variation, with plants at 311 °C days having an unusually large N content. The difference in total N between 311 and 475 °C days was 5.2 mg of N, however, this is not possible given that the post anthesis N supply was 2.1 mg in between harvest times and no N residual was detected in the root-medium at sampling (section 2.3.6). Therefore, this phenomenon must be carefully considered.

A high NupE was observed through the grain filling period regardless of the pre-anthesis N supply ($p=0.361$, Table 2.13).

Table 2.1.13 Post-anthesis N uptake and uptake efficiency of plants treated with three N supplies pre-anthesis.

N supply	mg N plant^{-1}	NupE (%)
N1	7.7 a	91.2 a
N2	8.9 a	106.1 a
N3	7.5 a	89.8 a
p-value	0.361	0.361
LSD	n.s	n.s

Data are the mean of five plants. LSD= Least significant differences; ns = not significant.

2.3.7 Summary of ear N accumulation and the relationship between key traits measured

Ear N accumulation through the grain filling period

Using ^{15}N labelling, it was possible to accurately quantify the fate of the pre-anthesis N absorbed by each plant component and, therefore, its remobilisation. The amount of N imported by the different organs during the post-anthesis period was also accounted for (Fig. 2.8)

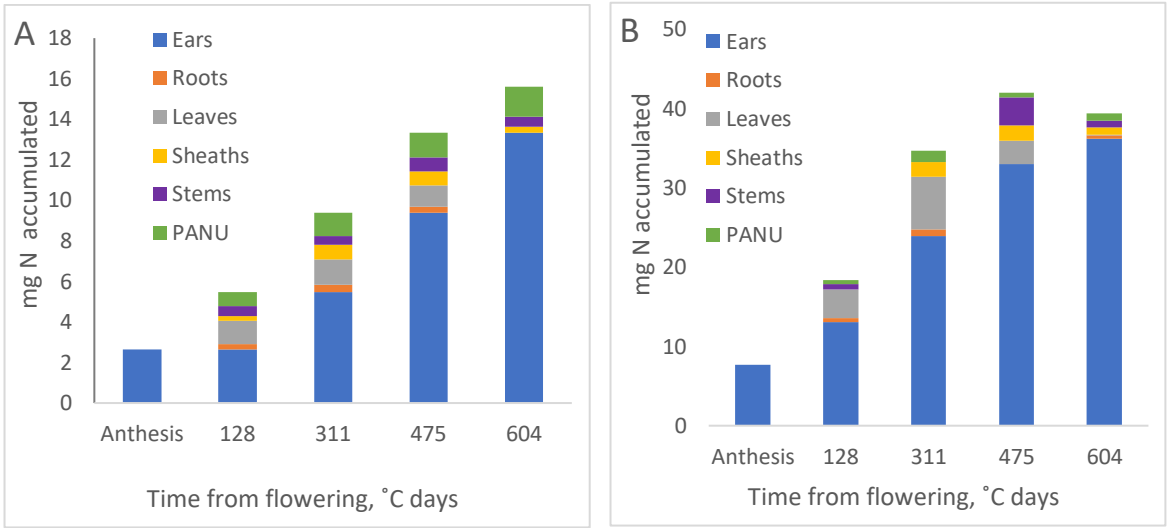


Figure 2.1.8 Contribution from N remobilization from each organ and PANU through the grain filling period of plants treated with low N (A) and high N (B).

Relationship between different key traits measured

Fig 2.9 shows a multi-variate analysis for the key traits measured in this experiment per N level.

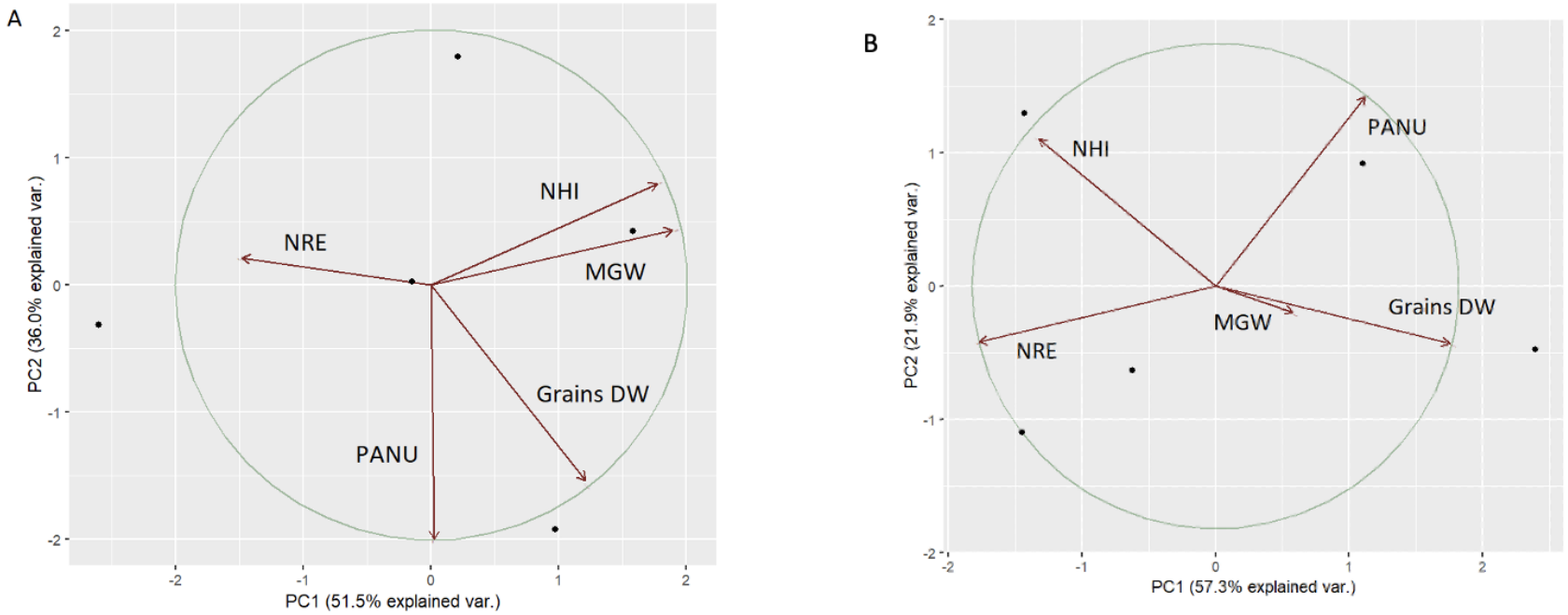


Figure 2.1.9 Bi-plot for post-anthesis N uptake (PANU), N remobilization efficiency (NRE), grain dry weight (Grain DW), nitrogen harvest index (NHI) and mean grain weight in low N (A) and high N (B) treatments.

PANU was negatively associated with NRE under both low and high N supply (R^2 = 0.56 and 0.58, respectively). Under low N supply pre-anthesis PANU and NRE were negatively associated with NHI (R^2 = 0.33 and 0.16, respectively). NRE was also negatively associated with Grain DW (R^2 = 0.63), whereas PANU showed a strong association with Grains DW (R^2 = 0.82). Under high N supply pre-anthesis, on the other hand, PANU and NRE showed a positive relationship with NHI (R^2 = 0.26 and 0.58, respectively). The negative association between NRE and grain DW was stronger that the observed under low N supply conditions (R^2 = 0.94) and the positive relationship between PANU and Grain DW was weaker (R^2 = 0.27).

2.3.8 Overview of N remobilization and uptake

An overview of the partitioning of the N (taken up and remobilized) through the different plant organs from anthesis to the end of grain filling in plants treated with low N and high is shown in the flow chart (Fig. 2.10).

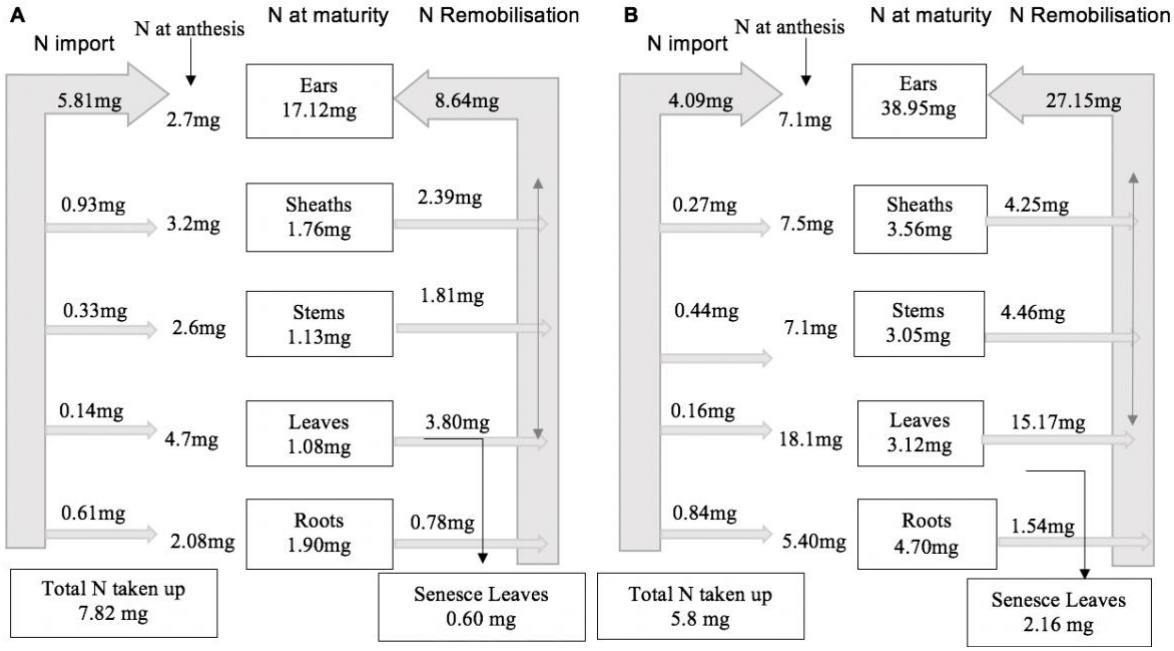


Figure 2.1.10 Dynamics of N taken up and remobilised from anthesis to grain maturity under low (A, 2mM) and high (B, 6mM). Values are the mean of five individual plants.

2.3.9 Quantification of ^{15}N losses during the grain filling period.

At each sampling date, results showed no detectable N (0.00%) in each replicate of the rooting medium sampled, regardless of the pre-anthesis N treatment applied. The same results (0.00 mg) were observed when NH_4 and NO_3 were determined using KCl extractions at each harvest point. As 2.1mg of N was applied each week during the grain filling period, but none was detected at sampling seven days later, the results indicate that in this system barley plants were capable of taking up N efficiently almost until maturity. Given that the amount of N in the root medium was beyond the limits of detection, analysis of ^{15}N loss to the root was not possible.

The amount of ^{15}N in plant tissue remained stable throughout the grain filling period (p= 0.156, Fig. 2.11). This suggests that there were no losses of previously accumulated N. The amount of ^{15}N found in senesced leaves was included in the overall ^{15}N budget.

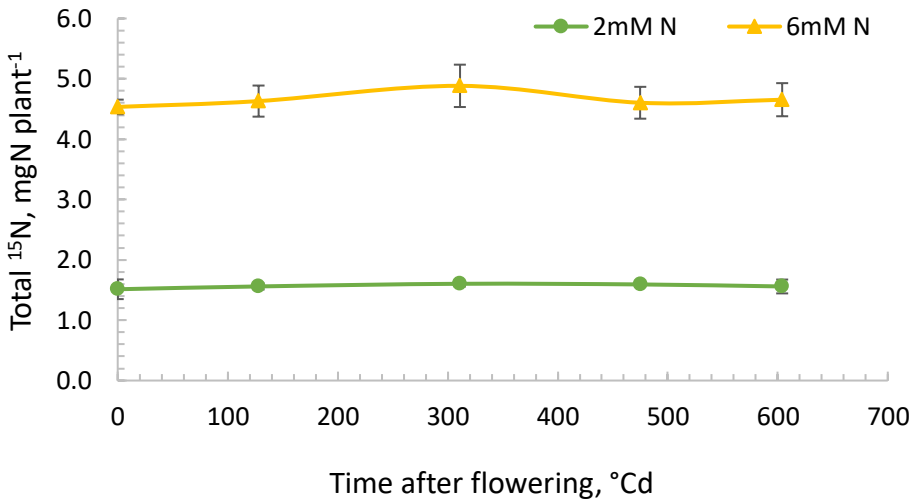


Figure 2.1.11 Changes in the amount of pre-absorbed ^{15}N. Data are means of five replicate plants at each harvest time ± SEM.

Table 2.1.14 Analysis of variance of the changes in the amount of ^{15}N content in whole plants during the grain filling period at three N supplies pre-anthesis.

Effects	p value	LSD
N supply (N)	<0.001	0.178
Harvest time (T)	0.156	n.s
Interaction N x T	0.566	n.s

LSD= Least significant differences; ns = not significant.

2.4 Discussion

To improve our understanding on the N dynamics in barley plants during the grain filling period with relation to their N status at anthesis an experiment was conducted where plants were grown under different N supply conditions during the vegetative period. The ^{15}N labelling technique allowed the fate of the N absorbed pre-anthesis to be tracked during grain filling and to measure accurately whether losses of the previously assimilated N occurred during various stages of the grain filling period. Under field conditions, during the developmental growth cycle of barley crops, the highest rate of N uptake is reported to occur during stem elongation, at this stage it is likely that the N concentrations in the soil are being rapidly depleted (Jones et al., 2015; Mălinaş et al., 2022). Typically, at anthesis under field conditions, the concentrations of soil mineral nitrogen (SMN) are 1 – 2 mM, and during the grain filling period there is little change. The shoot N concentration (N%) at anthesis, of plants grown under optimum nitrogen supply is reported to be between 1.68 and 1.79 % (data from Chapter 5). Hence, the different N concentration pre-anthesis used in this experiment to create a range of shoot

N % at anthesis and the 1 mM of N used during the grain filling period intended to recreate the conditions typical of field crops.

The low N availability of plants treated with 2 mM of N during the vegetative period was a limiting factor for growth and, ultimately, yield. These plants not only produce on average one tiller less than plants treated with high N, but also the leaf area (LA) was significantly lower (Fig 2.3). The number of grains in plants treated with low N was reduced by half compared with plants treated with high N.

The calculated NNI (Nitrogen Nutrition Index) suggests that plants supplied with N2 had the same plant N nutrition status as plants grown under field conditions, with optimum N supply and that those under the N3 treatment has a supra optimal tissue N concentration. However, there was an increase in plant biomass and grain yield when N was increased from N2 to N3 indicating that N2 plants grown under control environmental conditions were not optimal for growth. This must be due to the different environmental conditions at which the columns were grown compared to field conditions. Therefore, a relative NNI was calculated assuming that N3 plants represent non-limiting N conditions (Table 2.5).

Nevertheless, the evidence suggests that plants treated with N3 were not far from optimal at the start of anthesis as LA was not significantly increased at N3 compared to N2. The NNI is a good indicator of the contrasting N status of the plants at anthesis.

Onset, and efficiency of ^{15}N remobilisation

Even though there was no significant effect of plant N status at anthesis on the overall nitrogen remobilisation efficiency (NRE) during grain filling at the whole plant level, there were differences in the NRE of the different plant components, with leaves being the most efficient organ remobilizing N. This observation has been widely reported in cereal crops, especially in wheat and

maize. Gaju et al., (2014) studied the partitioning and remobilisation of N in relation to leaf senescence, grain yield and grain nitrogen concentration in wheat cultivars, they found that regardless of the N status of the plant more N was remobilized in the post-anthesis period from the leaf lamina than the stem. Overall, there was a larger residual N left at the end of grain filling in leaf sheaths and stems than in leaf lamina, but at a lower tissue N%. Pask et al., (2012) estimated the amount of structural (SN), reserve (RN) and photosynthetic (PN) N allocated to different organs of winter wheat. At anthesis, they found that most of the PN is allocated in the leaf lamina and less to the true stem. Most of the SN, on the other hand is allocated to the true stem and less to leaves and ears. Similarly, the highest RN was allocated to the true stem. Our results support the idea that organs with higher structural or reserve nitrogen have an overall lower NRE (e.g. stems and sheaths compared to leaves, Table 2.10), hence the high amount of N left in these organs. At the higher N supply (N3) a greater quantity of N was remobilized from each organ than at the lower supply (N1) and there was a larger residual N in these tissues at the end of grain filling. However, this also reflects the greater initial N content and organ DW at the higher N supplies as there was relatively little difference in residual N concentration in the tissue at grain maturity. SPAD readings taken on the leaves of main shoots also followed this pattern (Fig 2.5), with the highest values observed at anthesis in N3 plants but relatively little differences at grain maturity among the N treatments.

There were also, differences observed in the onset of ^{15}N remobilised from the different vegetative organs during the grain filling period. Regardless of the N supplied pre-anthesis, the remobilisation of N declined towards the grain filling.

At 2 mM of pre-anthesis, leaves showed a steady reduction of total N and ^{15}N, but this reduction was more pronounce at 6mM of N supply. On the other hand, there appears to be a slight delay on the onset of N remobilisation of leaf sheaths and to a lesser extent, stems. In fact, leaf sheaths showed a slight

increase on the total N during the first week after flowering even though ^{15}N was remobilised. Suggesting that leaf sheaths can maintain their N content for longer. Something similar was observed in the stems fraction.

However, we need to consider that at 311 ºC days after anthesis the stems of plants treated with 6mM seem to be slightly bigger than those harvested after that. Therefore, this interpretation is heavily dependent on that one data point and needs to be carefully considered. Still, it is evident that both stems and sheaths have a good capacity to retain N (post-anthesis). There are indications that onset from sheaths and leaves is affected by plant N status at anthesis. Onset appears to occur earlier from these organs in lowest n treatment (N1) compared to N2 and N3.

In the leaves fraction of plants treated with high N supply (6 mM), while the total N is reduced, the changed in the isotopic ratio ^{14}N:^{15}N from anthesis to mid-grain filling was little affected (Fig. 2.6 A). The fact that the isotopic ratio was only reduced from 10.1 % to 9. 9% from anthesis to mid-grain filling would suggest that there was a limited amount of N being imported to the leaves during this period. Given that the leaves represent the primary photosynthetic machinery of the plant, an early N remobilisation from this organ could restrict the photosynthetic activity and limit assimilate production for grain filling.

Therefore, ideally the remobilisation of N should start from non-photosynthetically active tissues such as stems followed by sheaths and leaves. Modifying NRE to delay senescence could be advantageous in feed or malting cultivars to favour a longer period of active photosynthesis during grain filling and higher grain yield (Gaju et al., 2014). By prolonging photosynthesis, a larger amount of carbohydrate should be provided to the above-ground growth, but also more carbohydrates should also be provided to roots supporting a prolong N uptake, which would ultimately benefit to reduce the high residues of N left in the soil at harvest (Dreccer, 2006).

^{15}N remobilisation in roots of plants treated with 2 mM and 6 mM is present right from anthesis (Fig. 2.6 D), even earlier. However, at both N supplies, they can maintain their total N content relatively stable (Fig. 2.4 D) through the grain filling period. This mechanism is not observed in any other plant fraction, regardless of the N supply. (Mattsson et al., 1993) studied the translocation and remobilisation of N in barley during vegetative and reproductive growth.

They observed that the N pool of the root system was continuously exchanging and that the net losses of N were small. The dilution on the isotopic ratio of roots observed in this experiment suggests that some of the ^{15}N accumulated had already been remobilised somewhere else within the plant but N in the form of ^{14}N must have been absorbed, given that roots are able to maintain their N content relatively stable through the grain filling period.

The root length (RL) showed an increase early during the grain filling period followed by a small reduction. This decline in RL could suggest that the capacity of these roots to uptake N was declining. However, there is no evidence to support this statement. On the contrary, under these conditions, the roots showed an intrinsic capacity to uptake N right to the end of grain filling, and regardless of the N status of the plants at anthesis.

Temporal dynamics of N uptake and N uptake efficiency

Under our controlled environmental conditions in sand:perite, plants exhibited a high nitrogen uptake until the end of grain filling regardless of the N supplied pre-anthesis. Similar observations have been reported in barley (Egle et al., 2008; Mattsson et al., 1992) and wheat (Oscarson, 2000; Taulemesse et al., 2015). However, it has also been observed that in cereal crops like wheat grown under N limiting conditions the N uptake gradually decreases during the post-anthesis period (Oscarson et al., 1995). Other evidence suggests that the PANU can 75issue75e with an increase on the N supply (Taulemesse et al., 2016).

In the present study the contribution that PANU made to the N content of the plants and specially to the grains was considerable. More than 80% of the total N taken up post-flowering was absorbed by the ears in plants treated with 2mM of N pre-anthesis. This contribution of PANU to the grains has been widely observed but to a lesser extent. It has been reported that in spring barley, the post-anthesis N uptake accounts for 3 to 50% of the total grain N (De Ruiter and Brooking, 1994, Barmeier et al., 2021) and similar values have been reported for wheat (Andersson, 2005).

In this experiment the dynamics of the N uptake remained unchanged through the grain filling period regardless of the N supplied pre-anthesis. However, at 311 ºC days after anthesis there seemed to be an increase on the uptake of N in plants treated with 6mM of N pre-anthesis, followed by a slight decline (Fig. 2.7). This increase was higher than the N supplied post flowering during that period which will make this phenomenon impossible. Given the natural variation within plants it is possible that this set of plants was slightly bigger or simply a sample error could have occurred. Nevertheless, the PANU calculated as the differences in total N content from maturity to anthesis accurately accounted for almost all the N supplied during this time (~90 %). Given that the N % in the grains and particularly in the grain of tillers was low, suggesting plenty of sink capacity, a higher N supplied, or a range of N supplies post-flowering could have help to identify the relationship between PANU and the N availability in the root medium.

Moreover, given that the columns used in this experiment have a depth of 40 cm, root length density (RLD) was unlikely to limit N uptake. Contrary to field conditions were roots below 80 cm are likely to be below the critical density for N uptake. RLD and the relationship with N depletion is discussed with detail in chapter 5 of this thesis.

Losses of N absorbed pre-anthesis

Having a system where ^{15}N is supplied regularly until anthesis, and the regular sampling of plants allowed accurate measurements of losses of previously assimilated N and to determine when these losses may occur during the grain filling period. The results of this experiment show that the amount of ^{15}N at every sampling time remains constant through the grain filling period in both low and high N supply (Fig 2.11). Nitrogen losses from entire plants have been study in cereal crops. For example, (Rroço and Mengel, 2000) investigated the release of N from roots as well as the loss of N from above-parts of wheat spring wheat from tillering to maturity using ^{15}N. They concluded that the released rates of labelled N into the soil and the volatile N losses were low from tillering to ear emergence and increased progressively towards grain filling. Losses of N to the soil accounted for 13% in year 1 and 11% in year 2. Whereas unaccounted losses represented a 4.1% in both years. Similarly, other experiments were a ^{15}N labelling technique was also used have not been able to account for all the ^{15}N supplied. (Mattsson et al., 1993) found about 80% recovery of the ^{15}N supplied and concluded that there were indications that a fraction of the remobilized nitrogen was actually lost from the plants.

In this study, at each harvest time there was no detectable N in the root medium either, confirming both that there are no N losses to the root medium and that the roots maintained their capacity to absorb N to the end of grain filling. Given the high NRE (64 and 67 % for N1 and N3, respectively), the high demand for N from grains that was evident given the low concentration of N achieved at maturity especially from tiller (~1.00 %) and the low N supply post-anthesis it is not surprising that losses of N did not occur.

The high post-anthesis N uptake observed in this experiment plus the fact that there was no N left in the root medium at harvest contradicts the well-known reality that occurs under field conditions, where high amounts of nitrogen are left untouched at harvest (L-Baeckström et al., 2006). Therefore, it is possible

that losses of N are more likely to occur in systems where the N supply is increased or when the N demand within the plant is reduced.

2.5 Conclusion

Under our artificial root medium, barley roots showed an intrinsic capacity to uptake N with the same efficiency throughout the grain filling period. The remobilisation efficiency of N absorbed pre-anthesis was not affected by the N status of the plant but there were highlighted differences between the NRE within the different vegetative organs. Leaves showed the highest NRE, followed by leaf stems and sheaths. The roots fraction showed the lowest NRE. Recoveries of post-flowering N fertilizer were high, on average, 90% of the post-flowering fertilizer N was recovered and uptake was not affected by the plant N status at the start of grain filling. ^{15}N data also showed that there were no detectable N losses through the grain filling period.

NUE could be improved by delaying in the remobilisation of N, especially in upper leaves. This would contribute to increasing grain yield through improved photosynthesis during the grain-filling period. Moreover, the relatively poor depletion of soil nitrate by field grown barley crops may be associated with restricted access of roots to N rather than physiological controls over its uptake. Improvements in root distribution may be a suitable target to increase N uptake efficiency of spring barley during the grain filling period.

The results observed in this chapter lead to questions about the control of the N uptake post-anthesis in relation to N supply post-anthesis as well as the plant N demand. The next experiment was set up to investigate these relationships by using contrasting N supplies post-anthesis and reducing the grain N demand.

Chapter 3: Relationship between N supply and grain N demand in the control of the post-anthesis N uptake

3.1 Introduction

At maturity of spring barley crops, large quantities of soil mineral N have been found to remain seemingly unused (Bingham et al., 2012). Maximizing post-anthesis N uptake therefore becomes an important target to minimise the NO_3^- remaining in the soil at harvest, which is at risk of leaching. Leaching of nitrate from soil represents a threat to the environment given that it can lead to water pollution and eutrophication (Ladha et al., 2005; Wang et al., 2015). Even though soil mineral N concentrations can be high under field conditions, the N taken up by cereals during the grain filling period is generally small. It has been reported that post-anthesis N uptake contributes between 3 and 33% to the total grain N among barley species (Barmeier et al., 2021). During the developmental growth cycle of cereal crops, the highest rate of N uptake is observed during stem elongation (Jones et al., 2015; Mălinaş et al., 2022).

There are several possible mechanisms that could explain a low PANU and high residual soil mineral N at harvest. First, ageing and senescence of the root system could lead to a decline in the physiological capacity of the root system for N uptake during grain filling. Second, there may be a low demand for N uptake by the plant if grain N requirements are met by remobilisation from vegetative tissue. Third, N uptake may be limited by soil N availability at the root surface, even when concentrations in the bulk soil appear high, if movement of N to the root is restricted by poor root distribution or soil conditions.

It has been reported that after anthesis root growth slows down and the root system begins to senesce (Gregory et al. 1978). This could potentially explain the low rates of PANU observed under field conditions. However, the relationship between N uptake and a well-developed root system cannot be considered separately from the availability of N in the soil and the demand for

N in the plant. These appear to be interrelated mechanisms involved in the control of PANU.

A decline in the post anthesis N uptake has been often reported even under controlled environment conditions (Delogu et al., 1998; Garnett et al., 2013; Oscarson et al., 1995; Taulemesse et al., 2015). Taulemesse et al. (2015) reported a strong initial NO_3^- uptake at anthesis in winter wheat plants grown in semi-hydroponic systems, followed by a rapid decline after anthesis. This response was observed irrespective of the plant N status at anthesis. Similar results have been reported for spring wheat grown hydroponically under N-limiting conditions where the net N uptake rate gradually decreased throughout the pos–anthesis period (Oscarson et al., 1995). The authors measured the kinetics of net NO_3^- uptake on several occasions during the vegetative and reproductive stages. While V_{max} decreased with age, K_m values did not differ with age or among different cultivars. Garnett et al., (2013) studied the response of maize NO_3^- transporter systems to N supply as well as N demand across the lifecycle. Nitrate uptake capacity and transcript levels of high- and low- affinity NO_3^- transporters were determined across the lifecycle of a dwarf maize variety (Gaspe Flint) grown under reduced and adequate nitrate conditions. The NO_3^- uptake capacity was extremely variable throughout the lifecycle of Gaspe Flint with a general trend to decrease as plants approached maturity. This was correlated with plant nitrogen demand regardless of the external N supply.

Even though there is ample evidence that suggests a decline in PANU in cereal crops, such changes were not found in the previous chapter of this thesis. The root system of spring barley maintained a high uptake capacity throughout grain filling when plant N status was moderate to low and presumably the demand for N remained high. Thus, a decline in the physiological capacity of roots with plant age is unlikely to explain observations of low PANU in spring barley, although this may be dependent on the root length density (RLD). Mattsson et al., (1993) also concluded that it is unlikely that post-anthesis performance of the root system limits the productivity of barley. Instead, a

major cause for the decline in the ability of plants to acquire NO_3^- was suggested to be linked to the sink strength of the ears (Mattsson et al., 1993).

In cereal crops, during the grain filling period the largest sink for nitrogen comes from the grains. The idea that N uptake is regulated by the plant's demand for N has been widely supported (Cooper and Clarkson, 1989; Garnett et al., 2013; Mi et al., 2000). Mi et al., (2000) demonstrate that the PANU in wheat is related to grain sink size. Their results showed that a cultivar with large ears and number of grains had a high potential for N uptake after anthesis and additional N application at anthesis could increase its PANU and grain N content. In contrast, a cultivar with small ears, showed low N uptake after anthesis, and additional N application at anthesis had little effect on PANU. In addition, when one-third of the ear of the cultivar with large grains was removed, the post-anthesis N uptake was greatly reduced, suggesting a feedback regulation of sink size on the post-anthesis N uptake. Garnett et al. (2013) supported the hypothesis that growth rate determines the rate of N uptake. The authors showed that NO_3^- uptake capacity increased when peaks in shoot growth were observed suggesting an increase in the plant N demand, and conversely NO_3^- uptake capacity decreased rapidly when the shoot growth reduced.

When the plant growth demand is met by providing a sufficient supply of N, a rapid cycling of soluble nitrogen has been reported to occur in the whole plant (Cooper and Clarkson 1989). This nitrogen cycling pattern could exceed the larger stable pools of nitrogen in shoot and root tissues, possibly acting as the signal that coordinates growth potential and N demand by the shoot with feedback regulation of NO_3^- uptake by the root system (Cooper et al., 1986; Rufty and Sinclair, 2020). Cooper and Clarkson (1989) proposed that a feedback control system synchronizes the N uptake with growth rate. When growth conditions are unfavourable for shoot development, the feedback system may down-regulate NO_3^- uptake and, when the tissue N concentration increases as a consequence of an increase In N supply, there could also be a feedback downregulation on N uptake.

The relative contribution of N supply and grain sink demand to the control of PANU in spring barley has been studied under field conditions. Experiments to explore the response of PANU to fertilizer N supplied at anthesis and reductions in grain sink demand by de-graining the ear were conducted at SRUC between 2011 and 2014 (publication in preparation). In these experiments, the PANU appeared to be controlled simultaneously by both N demand from grain and soil N availability. Applications of additional N fertilizer at anthesis increased PANU but the partial de-graining treatment reduced PANU compared to intact plants. However, the mechanisms that underly the dual control are not known. In these experiments PANU was estimated from the difference in above ground crop N content at anthesis and harvest. Measurements of the effects of treatments to vary N supply and grain N demand on N uptake and recycling of N between shoot and root are needed to probe the mechanisms of control.

Finding the balance between appropriate N fertilizer applications that meet the plant N demand to obtain adequate levels of grain protein content and grain yield is a major goal for modern agriculture. It has been suggested that applications of N fertilizer at around anthesis in the field are a potential tool to increase PANU where high grain N concentrations are required (Bingham et al., 2012; Plett et al., 2018). However, if considerable amounts of soil mineral N are found at harvest, a better solution to improve PANU may be to first establish the reasons for such small PANU and then breed varieties that are more efficient at taking up N from the soil.

The aim of the current chapter was to investigate the response of barley to changes in N supply and demand using ^{15}N labelling in a split-root system under controlled environmental conditions. This would provide more precise control over the N supply and demand relationships during grain filling than could be achieved in the field, and therefore a greater insight into the mechanisms of their control. By reducing the grain sink capacity of the plants, a reduction on the biomass and therefore, on the uptake capacity was expected. Different levels of N supply post-anthesis were used to establish the

relationship between N demand and supply. The specific hypotheses tested in this experiment were: a) post-anthesis N uptake in spring barley is regulated by grain sink demand and soil N supply b) a reduction in the grain sink demand results in greater N recycling from shoot to roots, and c) vegetative (non-grain) tissues remain a strong sink for N taken up after anthesis when grain N demand is reduced by de-graining.

3.2 Materials and methods

3.2.1 Plant material and split root system

A special box was designed and constructed to grow plants with a split root system (Fig. 3.1). The design consisted of two individual PVC trunks (5x5x30 cm each) glued together. An open-ended Eppendorf tube (the bottom of the tube was removed) was secured in a recess cut into the partitioning wall between the two compartments. Here is where the seedlings were placed, and the roots divided equally between each compartment.

Figure 3.1 Box design to create a split root system.

Each box was filled with 1.2 kg of sand:perlite (2:1 v/v, 600 g in each root compartment). A mix of washed and graded coarse granular sand (RHS sand, UK) and 2.0 – 5.0 mm standard perlite (Sinclair, UK) were used. The lower end of each compartment of the box was covered with a polyester mesh (100 µm aperture, Cadisch Precision Meshes Ltd, Hertfordshire, UK) to allow excess nutrient solution to be drained while containing the substrate and preventing

outgrowth of roots. Each root compartment was placed in its own individual plastic container to avoid cross contamination of nutrient solution between the two root compartments. After packing, the sand-perlite was watered with deionized water prior to transplanting and covered to prevent evaporation of water from the surface and allowed to drain for 48 h to reach field capacity.

Barley seeds (*Hordeum vulgare* cv Westminster) were germinated in the dark on damp filter paper for 7 days at 20 ºC. Seedlings were selected for uniformity and transferred to the Eppendorf tube within the root box, where the seminal roots were divided equally between two compartments and directed into holes created in the sand-perlite by a dissecting needle.

Plants in their root boxes were placed in a growth cabinet (Modular climate chamber, SNIJDERSLabs, The Netherlands) with a 20 ºC day temperature (16 h) and 15 ºC night temperature. Light was supplied by white light emitting diodes (LED) giving a photon irradiance at initial plant height of 356 ± 18 µmol m^{-2} sec^{-1} of photosynthetically active radiation (PAR) and relative air humidity was set at 70%. As the plants grew the tray containing root boxes was lowered so that the irradiance at the top of the plants was maintained close to the initial value.

3.2.2 Experimental design and treatments

The experiment was set up in a factorial design with post anthesis N supply and de-graining as the two factors. There were three levels of N supply and two levels of de-graining (de-grained where 50 % of the ear was removed and an intact control). There were two destructive samples taken during the grain filling period, one at early milk and the second at early dough (Zadoks GS 73 and GS 83, respectively; Tottman, 1987). The experiment was laid out within the growth cabinet in a randomised block design. One control and one de-grained plant for each nitrogen treatment x sampling date combination was randomly allocated to a position within each of five replicate blocks.

3.2.3 Nitrogen nutrition regime and nitrogen treatments

From transplantation to anthesis each plant received 80 ml weekly of the same nitrogen solution containing 168 mg N/L (6 mM) and a balanced supply of other macro- and micro-nutrients (Table 3.1). Each root compartment was given 40 ml of the solution. From de-graining onwards, each root compartment received 40 ml weekly of one of the three N supplies (1, 3 or 6 mM). N treatments are referred as N1, N2 and N3 in this chapter. Table 3.1 shows the full composition of the solutions used pre- and post- anthesis.

Table 3.1 Nutrient solutions used during the investigation of source-sink regulation of N uptake.

	Concentration (mM)			
Macroelements	**Pre-anthesis**	**Post – anthesis**		
KH_2PO_4	1	1	1	1
K_2SO_4	1.5	1.5	1.5	1.5
$MgSO_4$ $7H_2O$	2	2	2	2
$CaCl_2$ $2H_2O$	3.5	3.5	3.5	3.5
NH_4NO_3	6	1	3	6
Total N (mg/L)	168	28	84	168
Weekly N supply (mg)	13.4	2.2	6.7	13.4

Microelements: H_3BO_3 50µM, $CuSO_4.4H_2O$ 0.4 µM, $MnSO_4.4H_2O$ 5 µM, $ZnSO_4.H_2O$ 0.7µM, Na_2MoO_4 0.10 µM and EDTA Na-Fe 50 µM.

To avoid the nutrient solution running through the container, the columns were weighed every two days leaving always only 80g between their field capacity weight and the current weight. Deionised water was used in between N solution application to maintain columns well-watered. By flowering, plants were taking up between 90 and 120 ml every two days.

3.2.4 Sink adjustment (de-graining treatment)

Anthesis (also referred to as flowering, as anthesis coincides with ear emergence in barley) was recorded when 50 % of the main shoots had fully emerged ears (Zadoks GS 59). Six days (109 ºC days) after flowering the de-graining treatment was imposed. All the visible ears of each corresponding de-grained plant were 50 % de-grained. The total number of grains per ear was counted to remove exactly half of the grains. The top half of each ear was carefully cut using a small stainless steel surgical scissor. The scissors were cleaned carefully before and after each cut. The removed grains were retained, dried at 80 ºC for 48 h, weighed to the nearest 0.01 g and stored to await N analysis. 24 hours after de-graining, plants were supplied with one of three different nitrogen solutions (Table 3.1). This was repeated every 7 days thereafter.

3.2.5 Measurements of ^{15}N uptake

^{15}N influx was measured at two times during the grain filling period. One at GS 73, 14 days after flowering (256 ºC days) and the second one at GS 83, 28 days after flowering (512 ºC days). In each case plants were given 80ml of their corresponding N solution. 40 ml of $^{14}NH_4{}^{14}NO_3$ was supplied to one of the root compartments whilst the other root compartment received 40 ml of the same nutrient solution in the form of $^{15}NH_4{}^{15}NO_3$ (10 atm % excess). To avoid cross contamination of N from the surface of the sand:perlite, the N applications were performed using a 200 mm stainless steel needle. The N solution was injected by placing the needle in the middle of each root compartment at 20 cm depth. Plants were destructively sampled 24 hours after ^{15}N application.

For each plant, the number of shoots (main shoot and tillers) and the number of grains per shoot were recorded. Plants were then separated into main shoot and tiller leaves, leaf sheaths, stems, grains and chaff. Grains were separated from the chaff by hand. The roots of each compartment were analysed as a separate sample.

Roots were separated from the mixture of sand:perlite in each root compartment. After cutting the shoots at the base of the plant, each root compartment was separated and the sand:perlite mix placed into a tray. The roots were then carefully picked from the mix and gently washed under a slow water flow over a tray to allow the collection of any separated root pieces. This process minimised the risk of root material losses. Roots samples were then gently blotted dry to remove surface water.

The samples of individual tissue fractions were placed in paper bags and oven-dried at 80 °C for 48 hours for dry weight (DW) determination. After weighing to the nearest 0.01 g, plant tissue was ground to fine powder using a ball mill.

3.2.6 Analysis of total N and ^{15}N content

The total N concentration in weighed samples of dried and milled tissue was determined by an automated Dumas combustion method using a Flash 2000 elemental analyser (Thermo Scientific UK). Further samples were weighed to the nearest 0.001 mg and sealed in tin capsules (8 x 5 mm), before shipping to the UC Davis Stable Isotope Facility (Davis, CA, USA) for ^{15}N determination. Samples were analysed using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (IRMS) (Sercon Ltd., Cheshire, UK).

3.2.7 Calculations

At each sampling date, the following equations were used to determine total N content in each plant fraction represented by "*f* ":

$$\text{Total N content}_{(f)} = (\%N_f * DW_f)/100 \qquad \text{Equation 3.1}$$

Abundance of ^{15}N, which is the ratio of the amount of ^{15}N to the total amount of N (^{14}N + ^{15}N) was calculated as:

$$\%^{15}N_f = {}^{15}N_f \text{ (atm-\%)} - \text{natural abundance } {}^{15}N \text{ (atm-\%)} \qquad \text{Equation 3.2}$$

A value of 0.36933 atm % has been used for natural abundance. This value was based on previous experiments conducted on spring barley at SRUC as well as a literature review. Once the ^{15}N excess (or enrichment) was calculated, the total amount of ^{15}N was then calculated for each plant tissue.

Total ^{15}N content (*f*) = (Equation 3.2 * Equation 3.1) /100 Equation 3.3

The total N content of the plant at each sample time was determined by summing the total N content found in each plant fraction (leaves + sheaths + stems + roots + chaff + grains). The same operation was used to calculate the total ^{15}N accumulated by the whole plant.

The growth rate was expressed in g DW day^{-1}. It was calculated as:

$$(DW_{GS83} - DW_{GS73}) \div 14 \qquad \text{Equation 3.4}$$

There were 14 days between GS73 and GS83.

The N accumulation between GS73 and GS83 was calculated by pairing the plants by their N content. This was needed to ensure a more precise comparison between plants. At each harvest time, the five replicate plants (blocks) per treatment were arranged from the lowest N content to the highest N content.

3.2.8 Statistical Analysis

All statistical analyses were carried out using analysis of variance for randomised block designs in GenStat 19th Edition (VSN International Ltd). De-graining, N supply treatments and time of destructive harvest were included as fixed factors. Fishers least significant differences (LSDs) were calculated in post-hoc analysis to establish significance of differences between the pairs of treatment means. Homogeneity of variance and normal distribution of residuals was checked for each analysis.

3.3 Results

3.3.1 Effects of post anthesis N supply and de-graining on biomass accumulation and growth rate

Total plant dry weight (DW) was significantly reduced by the de-graining treatment (p=0.017), mostly at GS83 (de-graining x time *p*=0.058; Fig 3.2 and Table 3.2). At GS 83, intact plants treated with 1mM of N post-anthesis (treatment N1) had a total DW of 7.2 g plant^{-1} whereas de-grained plants had a total DW of 6.8 g plant^{-1}, N2 treated plants showed a DW of 7.2 g plant^{-1} for control plants and 6.7 g plant^{-1} for de-grained plants, intact plants treated with 6mM showed a total DW of 7.4 g plant^{-1} and control plants showed a DW of 6.5 g plant^{-1}. The amount of N supplied after anthesis did not influence the total DW of the plants. Plants harvested at GS 83 had a significantly higher biomass (p<0.001) than those at GS 73.

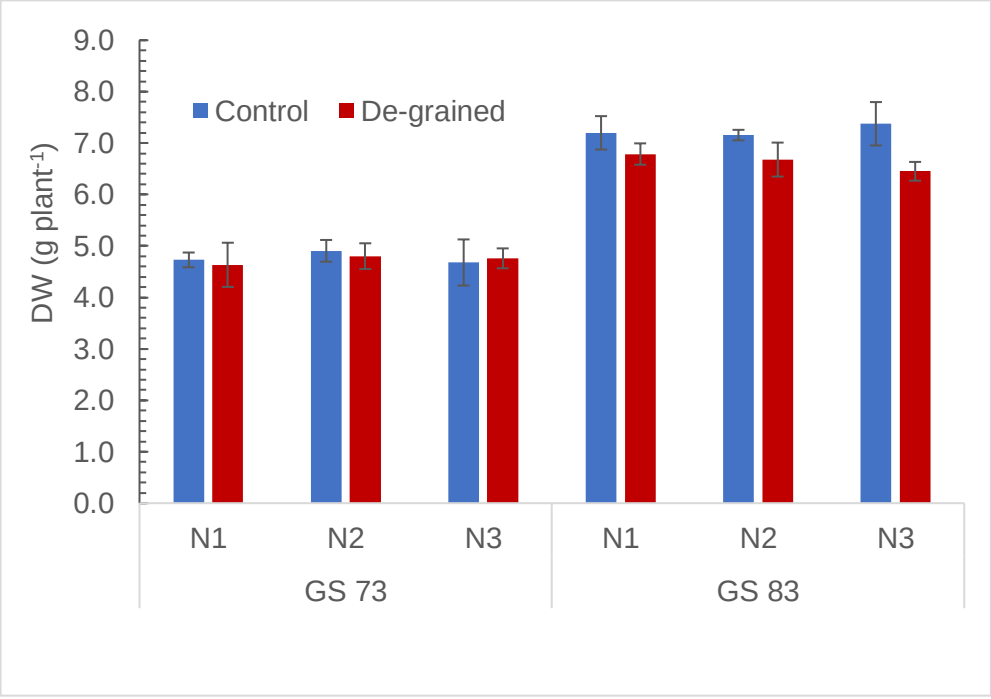


Figure 3.2 Differences in biomass of control and de-grained plants treated with three different N concentration N1, N2 and N3 (1, 3 and 6mM of NH_4NO_3, respectively) post-anthesis and harvested at two growth stages. Data are the means of 5 replicate plants ± SEM.

The de-graining treatment significantly reduced the plant growth rate (g day^{-1}, p=0.03) by 22 % compared to intact (control) plants (Table 3.3). The effects of post-anthesis N supply and the interaction between N supply and de-graining treatment were not statistically significant (p=0.885 and p=0.404, respectively).

Table 3.2 ANOVA results for effects of N supply, de-graining and harvest time on the biomass accumulation of spring barley.

Effect	p value	LSD
De-graining treatment (D)	0.017	0.286
N treatment (N)	0.922	n.s
Harvest date (T)	<0.001	0.286
N x T	0.919	n.s
T x D	0.058	n.s
N x D	0.938	n.s
N x T x D	0.525	n.s

LSD is least significant difference at *p*=0.05; ns not significant.

Table 3.3 Plant growth rate as affected by de-graining and post anthesis N supply treatments.

N treatment	Growth rate (g DW day^{-1})		
	Control	De-grained	Mean N
N1	0.18	0.15	0.16
N2	0.16	0.14	0.15
N3	0.19	0.12	0.16
Overall mean	0.18	0.14	
***p*-value De-graining**	0.03		
***p*-value N treatment**	0.885		
***p*-value D x T**	0.404		

Data are the mean of five replicate plants.

3.3.2 Effect of post-anthesis N supply and de-graining on number of shoots and number of grains

The reduction of 50 % grain sink was successfully achieved through the de-graining treatment. Table 3.4 shows the number of grains on the main shoots as well as tillers after the de-graining treatment was performed.

As intended, the de-graining treatment reduced by half, the number of grains on both main shoots and tillers ($p<0.001$, Table 3.4). However, the total number of shoots with developed ears in control and de-grained plants was not influenced by the de-graining treatment. Only the harvest time influenced the number of shoots, with plants harvested at GS 83 showing a slightly higher shoot number per plant, on average one more than at GS 73. The different post-anthesis N supplies showed no influences on shoot or grain numbers.

3.3.3 Effects of post-anthesis N supply and de-graining on total N content and net N accumulation.

The total N content (Fig. 3.3) was significantly reduced by the de-graining, but increased with the N supply treatments, and with the time of harvest (Table 3.5). At GS 73, control plants treated with 6 mM of N had accumulated 70.0 mg N plant^{-1} whereas de-grained plants accumulated an average of 67.1 mg N plant^{-1}. At GS83, intact plants treated with 6mM of N post-anthesis accumulated a total of 100.7 mg N plant^{-1}, whereas de-grained plants accumulated a total of 90.9 mg N plant^{-1}.

Table 3.4 Shoot number, number of grains on main shoots and tillers of control and de-grained plants treated with three different N supplies post-anthesis.

		Shoots plant^{-1}		Grains main shoot^{-1}		Grains tillers^{-1}	
Harvest	**N supply**	**Control**	**De-grained**	**Control**	**De-grained**	**Control**	**De-grained**
GS 73	N1	3.0	3.2	20.6	11.2	30.8	15.8
	N2	3.0	3	21.2	10.8	33.0	16.2
	N3	3.2	3.4	21.6	10.6	38.2	21.0
GS 83	N1	3.4	3.2	20.2	11.3	38.0	23.0
	N2	3.4	4.0	21.0	11.2	37.2	18.2
	N3	4.2	4.0	20.8	10.4	36.6	12.2
***p*-value**	Degraining (D)	0.7		<0.001		<0.001	
	Harvest time (T)	0.02		0.6		0.5	
	N supply (N)	0.2		0.8		1.0	
LSD	D	n.s		0.643		4.85	
	T	0.479		n.s		n.s	
	N	n.s		n.s		n.s	

Data are the means of five replicate plants. P-value and LSD of the main effects are shows. None of the interactions show significant differences. LSD is least significant difference at p=0.05; ns not significant.

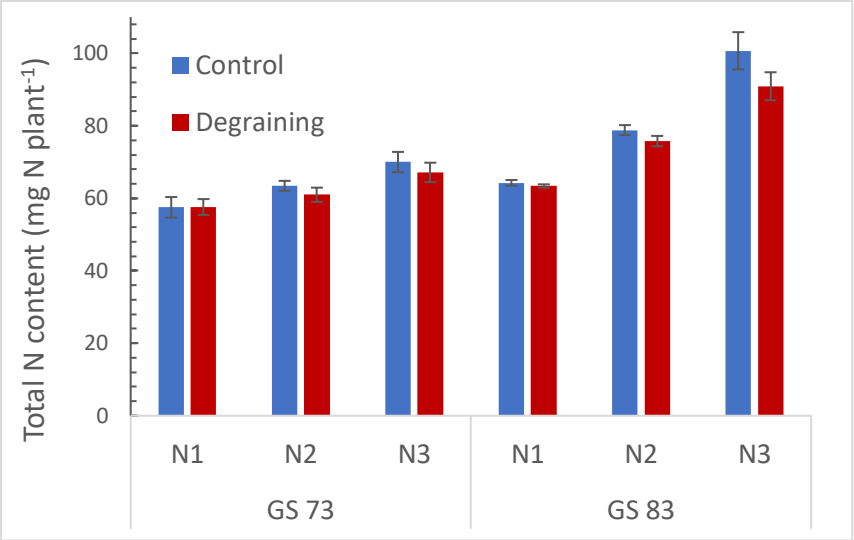


Figure 3.3 Total N content in control and de-grained plants for N1, N2 and N3 post-anthesis. Data are the mean of five replicate plants ± SEM.

With regards to the net accumulation of N (Fig. 3.4), de-grained plants accumulated significantly less N than intact plants between GS 73 and GS 83 ($p=0.039$, Table 3.6). Unsurprisingly, increasing post-anthesis N supply significantly increased the accumulation of N ($p<0.001$), but there was no significant interaction between de-graining and N supply. On average there was a 16 % reduction in the accumulation of N between control and de-grained plants.

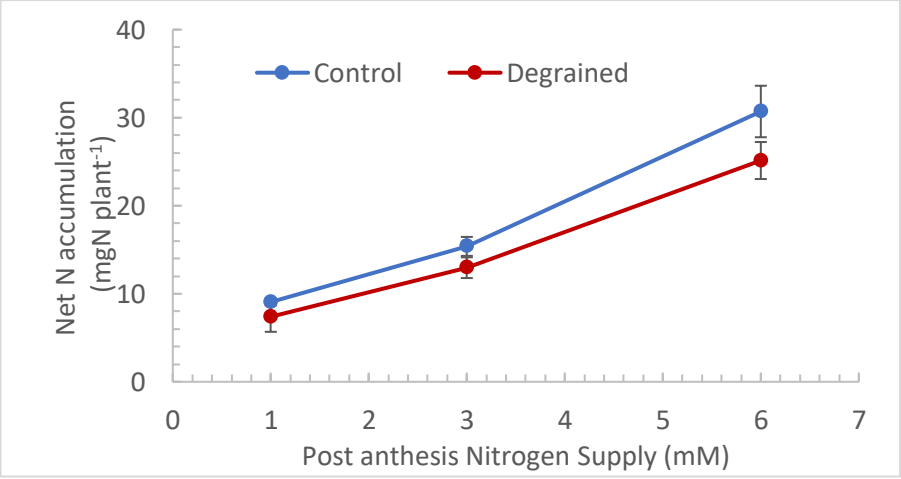


Figure 3.4 Net N accumulation by control and de-grained plants between GS 73 and GS 83 as affected by different N supply. Data are the mean of five replicate plants ± SEM.

Table 3.5 Anova results for effects of different N supplies, de-graining treatment and harvest time on the total N content of spring barley.

Effect	*p*-values	LSD
De-graining (D)	0.038	2.967
N supply (N)	<0.001	3.633
Harvest time (T)	<0.001	2.967
D x N	0.264	n.s
D x T	0.344	n.s
N x T	<0.001	5.138
D x N x T	0.618	n.s

LSD is least significant difference at *p*=0.05; ns not significant.

Table 3.6 Anova of the effects of de-graining treatment and post-anthesis N supply on the net N accumulation between GS 73 and G S83.

Effect	*p*-values	LSD
De-graining (D)	0.039	3.721
N supply (N)	<0.001	3.038
D x N	0.523	n.s

LSD is least significant difference at *p*=0.05; ns not significant.

3.3.4 Effects of post-anthesis N supply and de-graining on net ^{15}N uptake after 24 h.

Fig. 3.5 shows the net uptake of ^{15}N measured over a 24-hour labelling period at GS 73 and GS 83. There was no significant difference between control and de-grained plants (p=0.913, Table 3.7) and no interaction between de-graining and either N supply or growth stage. At GS 83, intact plants treated with 1mM of $^{15}NH_4{}^{15}NO_3$ post-anthesis, had taken up a total of 75.2 µg^{15}N plant^{-1} and, de-grained plants had taken up 74.8 µ^{15}N plant^{-1}. Comparably, the amount of

^{15}N taken up by control and de-grained plants treated with 3 and 6 mM of $^{15}NH_4{}^{15}NO_3$ N post-anthesis was almost identical at each growth stage.

The N supply post-anthesis positively influenced the uptake of ^{15}N over 24h ($p<0.001$) and ^{15}N uptake was greater at GS 83 compared to GS 73 (harvest time $p=0.033$; Table 3.7). Moreover, there was a weak interaction between N supply and harvest time ($p=0.059$) indicating that the greater ^{15}N uptake at the later growth stage occurred mostly at the highest N supply (6 mM). No interaction between de-graining and harvest time was observed.

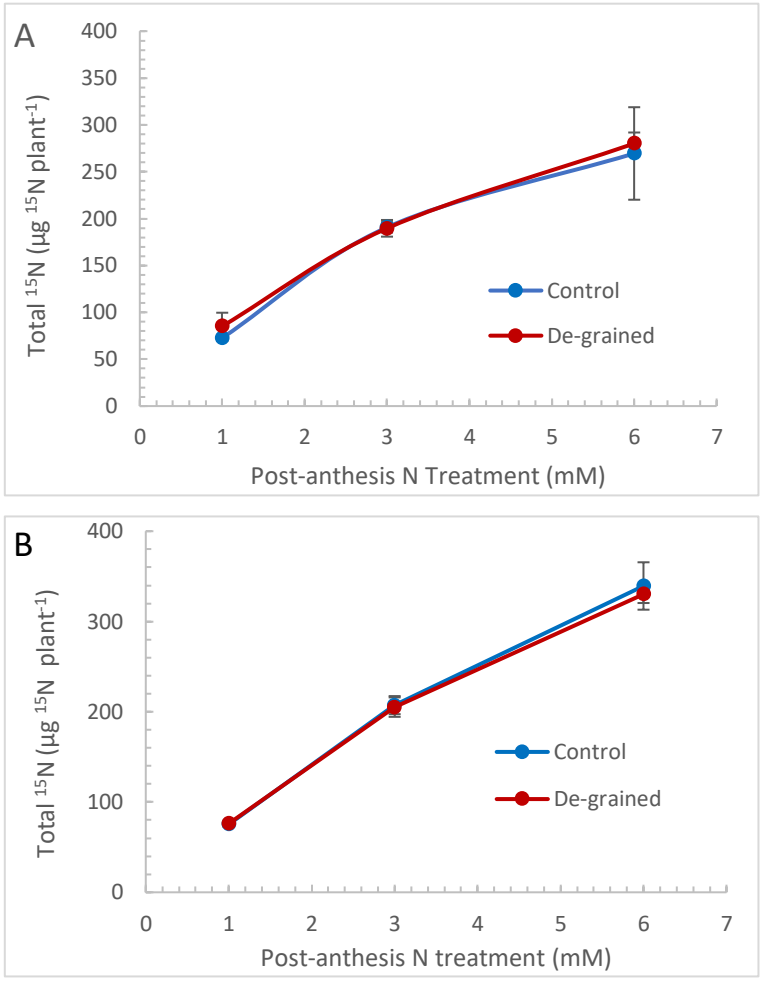


Figure 3.5 Net ^{15}N uptake of control and de-grained plants supplied with 3 different N concentrations (1, 3 and 6mM) over a 24h labelling period (A: GS 73, B: GS 83). Data are the mean of five replicate plants ± SEM.

Table 3.7 Analysis of effects of post anthesis N supply, de-graining treatment and growth stage (harvest time) on the net uptake of ^{15}N measured over 24h.

Effect	*p*-value	LSD
De-graining Treatment (D)	0.913	n.s
N supply (N)	<.001	26.93
Harvest time (T)	0.033	21.99
D x N	0.959	n.s
D x T	0.641	n.s
N x T	0.059	n.s
D x N x T	0.93	n.s

LSD is least significant difference at p=0.05; ns not significant.

The rate of the uptake of ^{15}N by the roots (Fig. 3.6) was calculated as mg^{15} N g DW (roots)$^{-1}$ h^{-1}. When expressed per unit root weight, the rate of ^{15}N uptake showed a similar response as the ^{15}N uptake per plant described above. However, it was not affected by the time of harvest (p=0.258).

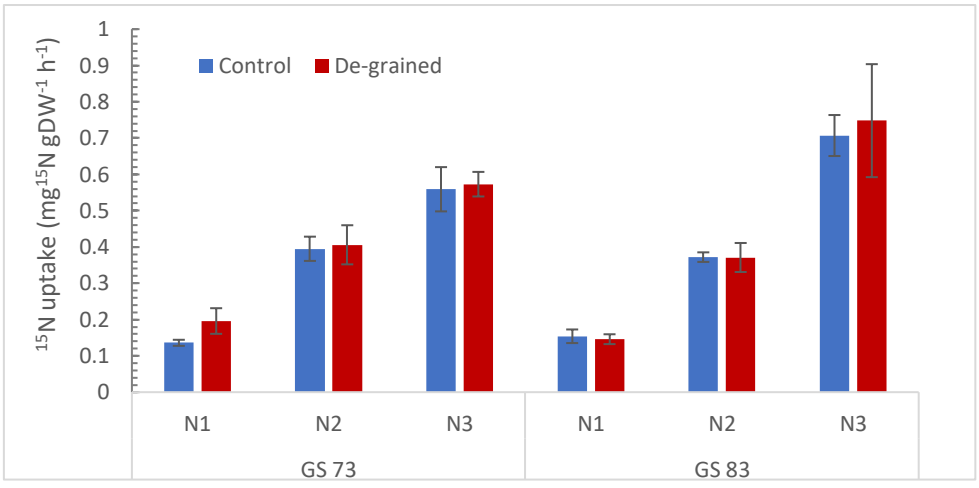


Figure 3.6 Rate of ^{15}N taken up by the root compartment that received ^{15}N. Data are the mean of five replicate plants ± SEM.

Table 3.8 Analysis of variance of the rate of ^{15}N uptake by the roots (mg^{15}N g DW roots^{-1} h^{-1}).

Effect	*p*-values	LSD
De-graining (D)	0.565	n.s
N supply (N)	<0.001	0.084
Harvest Time (T)	0.258	n.s
T x N	0.048	0.119

Only significant interactions are shown. LSD is least significant difference at p=0.05; ns not significant.

3.3.5 Effects of post-anthesis N supply and de-graining on the recycling of N from shoots to roots

Measuring the ^{15}N content of root tissue in the compartment that only received ^{14}N showed that effectively there was a larger recycling of ^{15}N from shoot to roots over a 24 h after labelling in de-grained plants and this was observed at both growth stages (Fig. 3.7).

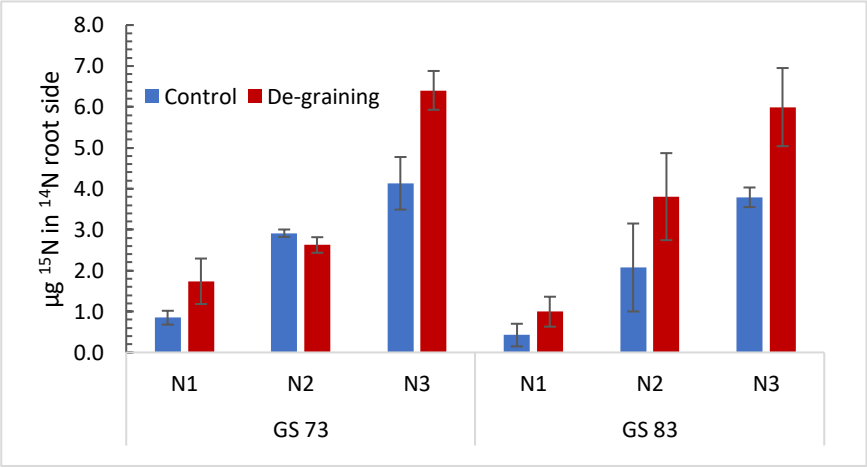


Figure 3.7 Total ^{15}N content (µg^{15}N) recovered in root tissue in the compartment that only received ^{14}N as affected by post-anthesis N supply and de-graining treatments. Data are the mean of five replicate plants ± SEM.

The statistical analysis showed that de-graining treatment and N supply increased the amount of N recycled to the roots ($p<0.001$, Table 3.9). At GS 73, N1 control plants had accumulated 0.85 µg of ^{15}N in the ^{14}N root side whereas, N1 de-grained plants had recycled almost double that amount (1.73 µg of ^{15}N). Plants treated with the N3 treatment recycled considerably more ^{15}N with 4.13 µg of ^{15}N in control plants and 6.40 µg of ^{15}N in de-grained plants. At GS 83, the amount of ^{15}N recycled to the roots was not different to that at GS 73 when averaged across the de-graining and N supply treatments (harvest time $p=0.475$).

Table 3.9 Analysis of variance of the amount of ^{15}N recycled from shoots to roots.

Effect	*p*-values	LSD
De-graining (D)	<0.001	0.712
N supply (N)	<0.001	0.872
Harvest Time (T)	0.475	n.s

Interactions were not significant ($P>0.05$). LSD is least significant difference at $p=0.05$; ns not significant.

The root biomass was not affected by any of the treatments imposed (data not shown), therefore it is unlikely that the greater amount of ^{15}N found in the ^{14}N root compartment is a consequence of a larger root system.

3.3.6 Effects of post-anthesis N supply and de-graining on the partitioning of newly absorbed ^{15}N among different plant organs

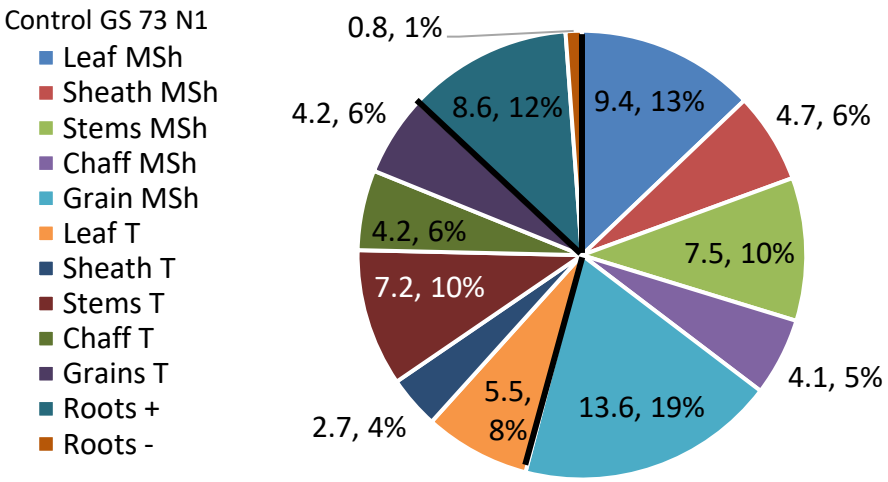
Non-grain tissues remained a strong sink for the uptake of N after anthesis (Fig. 3.8). The N supply post-anthesis significantly increased ($p<0.001$) the amount of ^{15}N allocated to each plant component at each harvest time. This was true for main shoot organs and tillers.

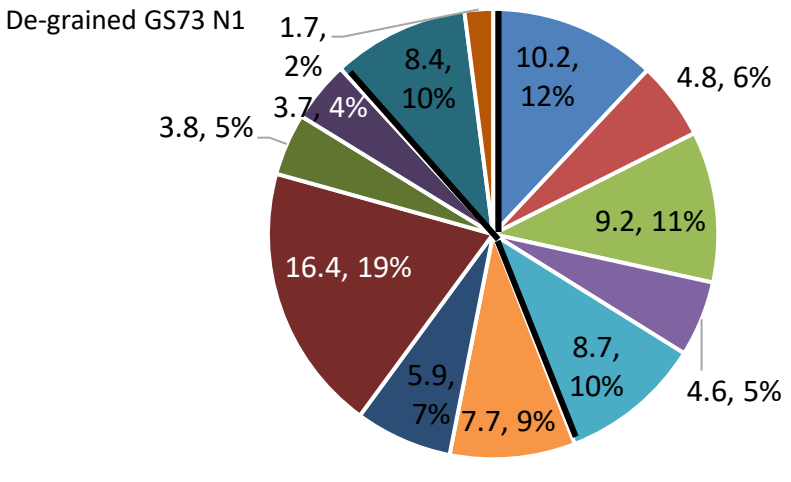
The de-graining treatment on the other hand, only influenced the amount of N allocated in some organs of main shoots and tillers (see Table 3.10 and 3.11). For example, at GS 73, only grains of main shoots and sheaths and stems of tillers were affected. While the amount of ^{15}N in the grains of the main shoots was significantly reduced by the de-grained treatment, the proportion of ^{15}N in the sheaths and stems was significantly increased.

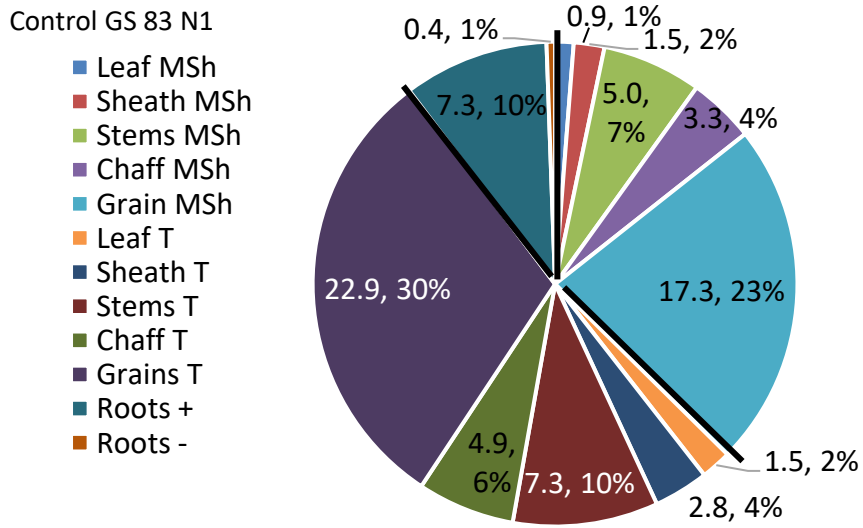
The interaction between de-graining treatment and N supply post-anthesis was only significant in grains of main shoots and leaves and sheaths of tillers.

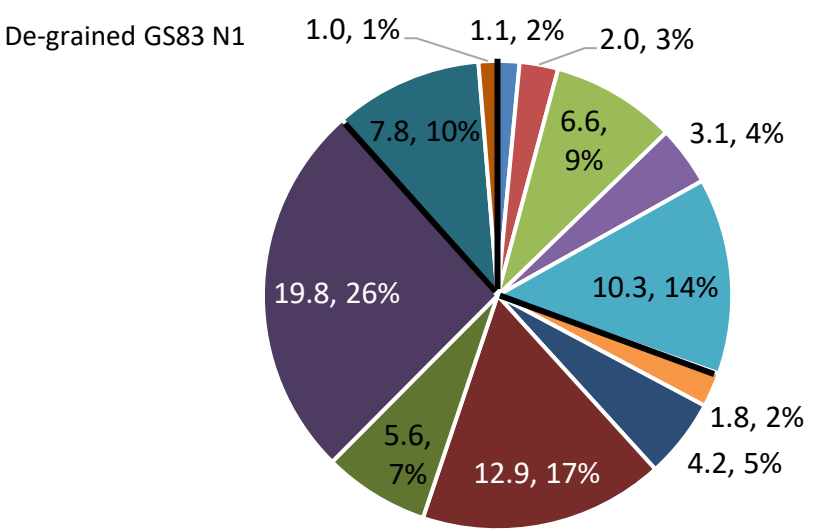
In control plants at GS 73, leaves of main shoots accumulated 9.4 µg ^{15}N in the N1 treatment, 23.3 µg ^{15}N in N2 treatment and 38.0 µg ^{15}N in N3. Although the amount of N in the organs of main shoots was reduced at GS83 compared to GS 73, it increased as the N supply increased. Leaves from main shoots of control plants accumulated 0.9 µg^{15}N in the N1 treatment, 6.5 µg^{15}N in N2, and 30.0 µg^{15}N in N3. This trend was observed among all the plant orgnas of main shoots and tillers.

At GS 83, stems, chaff and grains of main shoots were significantly influenced by the de-graining treatment. While chaff and grains accumulated less N in de-grained plants (given the reduced number of grains) stems showed a higher ^{15}N content in de-grained plants. De-grained plants treated with N2 accumulated 20.4 µg^{15}N whereas in controls only 11.95 µg^{15}N was found. Apart from the chaff, which showed no differences in the amount of ^{15}N in tillers, every plant component of the tillers accumulated significantly more ^{15}N in de-grained plants compared to control at GS83. Stems for example, accumulated 7.3 µg^{15}N in control plants of N1 and 12.9 µg^{15}N in de-grained plants.

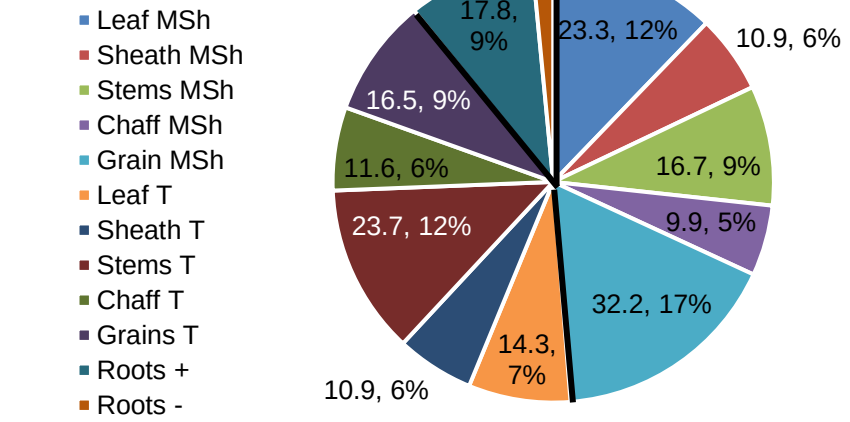

Control GS 73 N1
Leaf MSh
Sheath MSh
Stems MSh
Chaff MSh
Grain MSh
Leaf T
Sheath T
Stems T
Chaff T
Grains T
Roots +
Roots -
0.8, 1%
9.4, 13%
4.7, 6%
7.5, 10%
4.1, 5%
13.6, 19%
5.5, 8%
2.7, 4%
7.2, 10%
4.2, 6%
4.2, 6%
8.6, 12%


De-grained GS73 N1
1.7, 2%
8.4, 10%
10.2, 12%
4.8, 6%
3.7, 4%
3.8, 5%
9.2, 11%
16.4, 19%
8.7, 10%
4.6, 5%
5.9, 7%
7.7, 9%

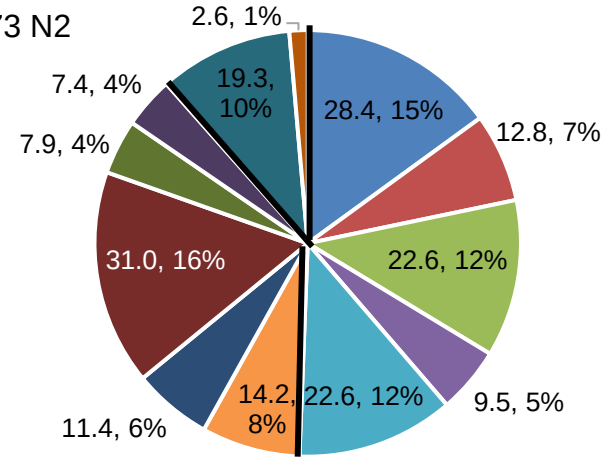

Control GS 83 N1
Leaf MSh
Sheath MSh
Stems MSh
Chaff MSh
Grain MSh
Leaf T
Sheath T
Stems T
Chaff T
Grains T
Roots +
Roots -
0.4, 1%
0.9, 1%
1.5, 2%
5.0, 7%
3.3, 4%
17.3, 23%
1.5, 2%
2.8, 4%
7.3, 10%
4.9, 6%
22.9, 30%
7.3, 10%


De-grained GS83 N1
1.0, 1%
1.1, 2%
2.0, 3%
7.8, 10%
6.6, 9%
3.1, 4%
10.3, 14%
1.8, 2%
4.2, 5%
12.9, 17%
5.6, 7%
19.8, 26%

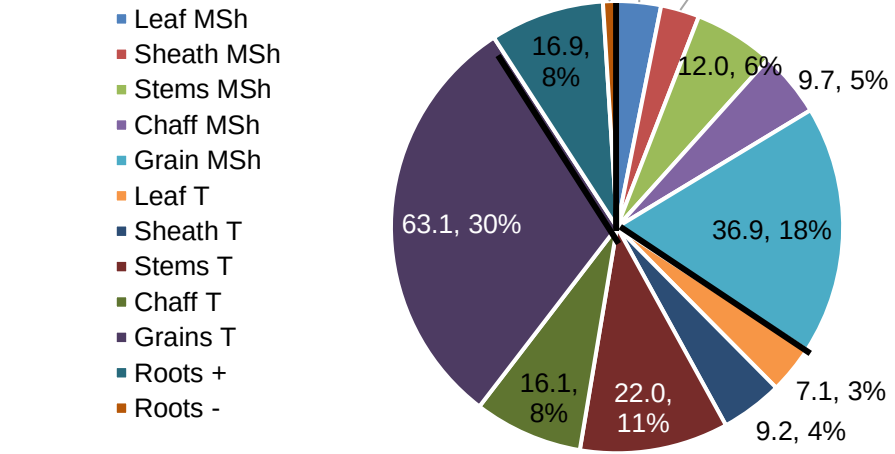
Control GS 73 N2



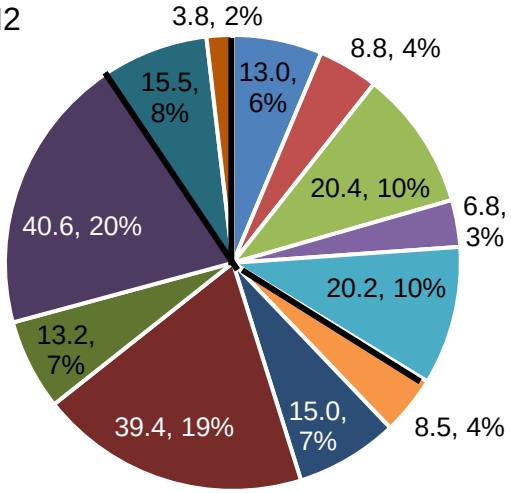
De-grained GS73 N2



Control GS 83 N2



De-grained GS83 N2



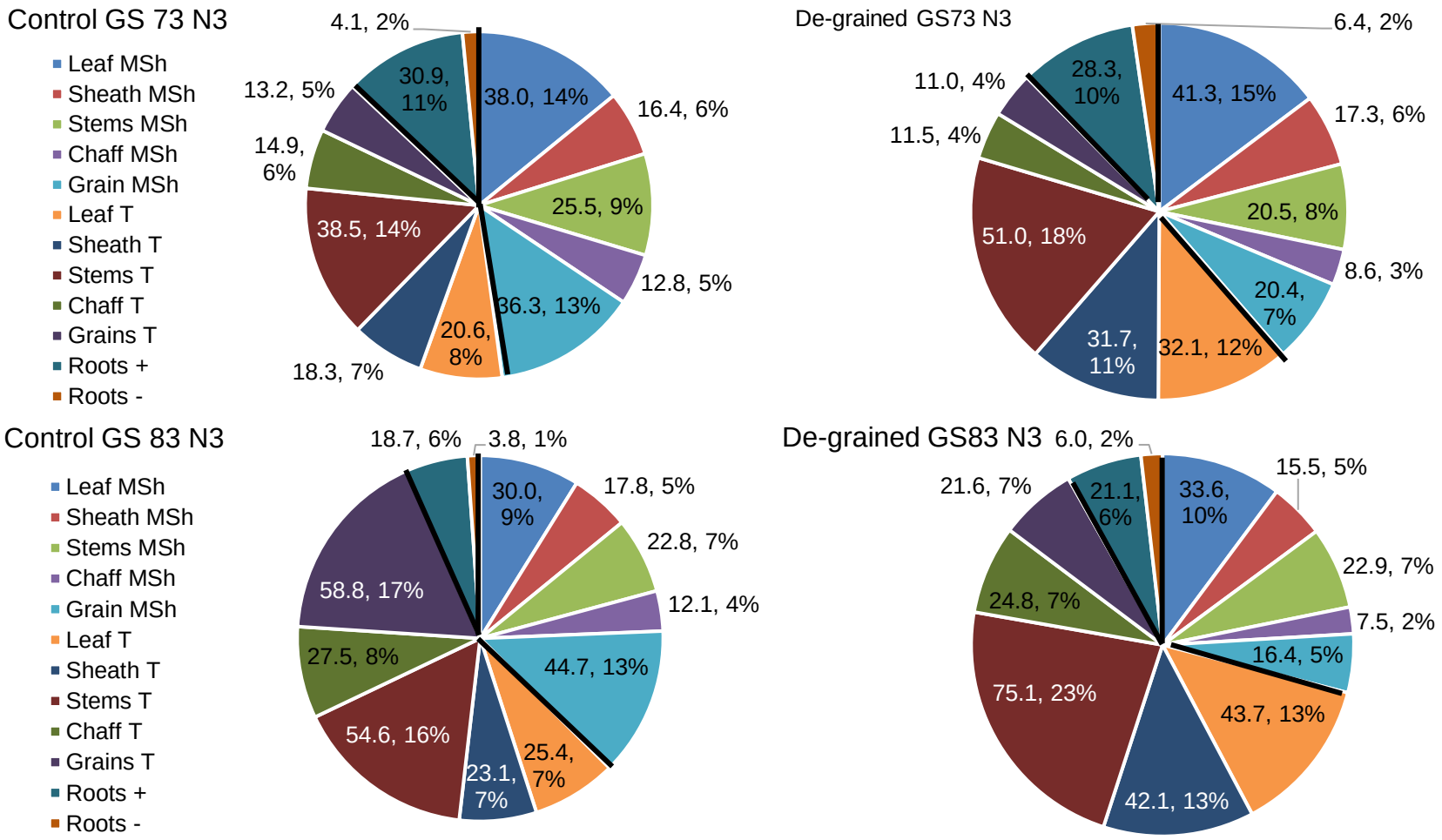


Figure 3.8 ^{15}N distribution after 24h labelling. Data are µg^{15}N in a particular organ, and its corresponding percentage (%) of the total ^{15}N uptake. Black lines divide main shoots from tillers and roots. Data are the mean of five replicate plants.

Table 3.10 Analysis of variance of effects of de-graining treatment and different post-anthesis N supply on the ^{15}N content of different plant organs in main shoots of barley.

	Main Shoot				
GS 73	**Leaf**	**Sheath**	**Stem**	**Chaff**	**Grain**
De-grain treatment (D)	0.232	0.455	0.705	0.271	0.002
LSD (D)	n.s	n.s	n.s	n.s	6.21
N supply (N)	<0.001	<0.001	<0.001	<0.001	<0.001
LSD (N)	6.110	3.516	5.740	2.893	7.600
D x N	0.781	0.840	0.156	0.195	0.395
GS 83	**Leaf**	**Sheath**	**Stem**	**Chaff**	**Grain**
De-grain treatment (D)	0.161	0.654	0.023	0.003	<0.001
LSD (D)	n.s	n.s	1.478	1.553	6.00
N supply (N)	<0.001	<0.001	<0.001	<0.001	<0.001
LSD (N)	6.99	2.875	3.789	1.902	7.35
D x N	0.782	0.173	0.086	0.067	0.022
LSD (D x N)	n.s	n,s	n.s	n.s	10.390

LSD is least significant difference at p=0.05; ns not significant.

Table 3.11 Analysis of variance of effects of de-graining treatment and different post-anthesis N supply on the ^{15}N content of different plant organs in tillers of barley.

	Tillers				
GS 73	**Leaf**	**Sheath**	**Stem**	**Chaff**	**Grain**
De-grain treatment (D)	0.093	0.043	0.019	0.097	0.139
LSD (D)	n.s	6.36	9.64	n.s	n.s
N supply (N)	<0.001	<0.001	<0.001	<0.001	0.029
LSD (N)	6.690	7.790	9.640	3.721	6.570
D x N	0.189	0.303	0.861	0.537	0.408
GS 83	**Leaf**	**Sheath**	**Stem**	**Chaff**	**Grain**
De-grain treatment (D)	<0.001	0.002	0.004	0.416	<0.001
LSD (D)	2.952	4.950	8.69	n.s	11.50
N supply (N)	<0.001	<0.001	<0.001	<0.001	<0.001
LSD (N)	3.616	6.060	10.64	6.430	14.08
D x N	<0.001	0.013	0.225	0.912	0.075
LSD (D x N)	9.880	4.066	n.s	n.s	n.s

LSD is least significant difference at p=0.05; ns not significant.

A reduced amount of N was found in the grains of de-grained plants as expected, given the reduced number of grains. However, in main shoots the grain nitrogen concentration was significantly increased with the de-graining and the post-anthesis N supply treatments. This was more pronounced at GS83 (Fig 3.9), where the grains of de-grained plants seem to have reached saturation with the N2 and N3 treatments.

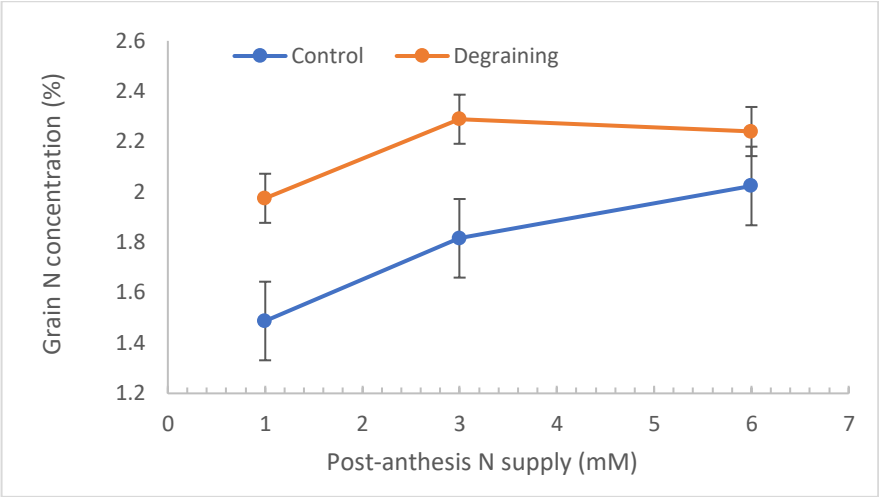


Figure 3.9 Grain N concentration of main shoots at GS 83 in control and de-grained plants at three different post-anthesis N supplies (1, 3 and 6mM of NH_4NO_3). Data are the mean of five replicate plants ± SEM.

Table 3.12 Analysis of variance of effects of post-anthesis N supply and de-graining on grain N concentration of main shoots and tillers of spring barley.

Effect	**Main shoots**		**Tillers**	
	p-values	LSD	*p*-values	LSD
De-graining (D)	<0.001	0.109	0.075	n.s
N supply (N)	<0.001	0.133	0.002	0.145
Harvest Time (T)	<0.001	0.109	0.206	n.s
D x T	0.004	0.154	0.008	0.168

Only significant interactions are shown. LSD is least significant difference at p=0.05; ns not significant.

3.3.7 Determination of ear N accumulation

Fig 3.10 shows the ear N accumulation in control plants vs ear N accumulation in the de-graining treatment. A 1:1 line was added for comparison. There is a greater deviation from the 1:1 line with the highest N supply. Control plants grown with N1 post-anthesis accumulated an average of 19.7 mg N in the ears, like de-grained plants which accumulated 19.6 mg N in the ears. The correlation was reduced as the N supply post-anthesis was increased (Fig 3.10).

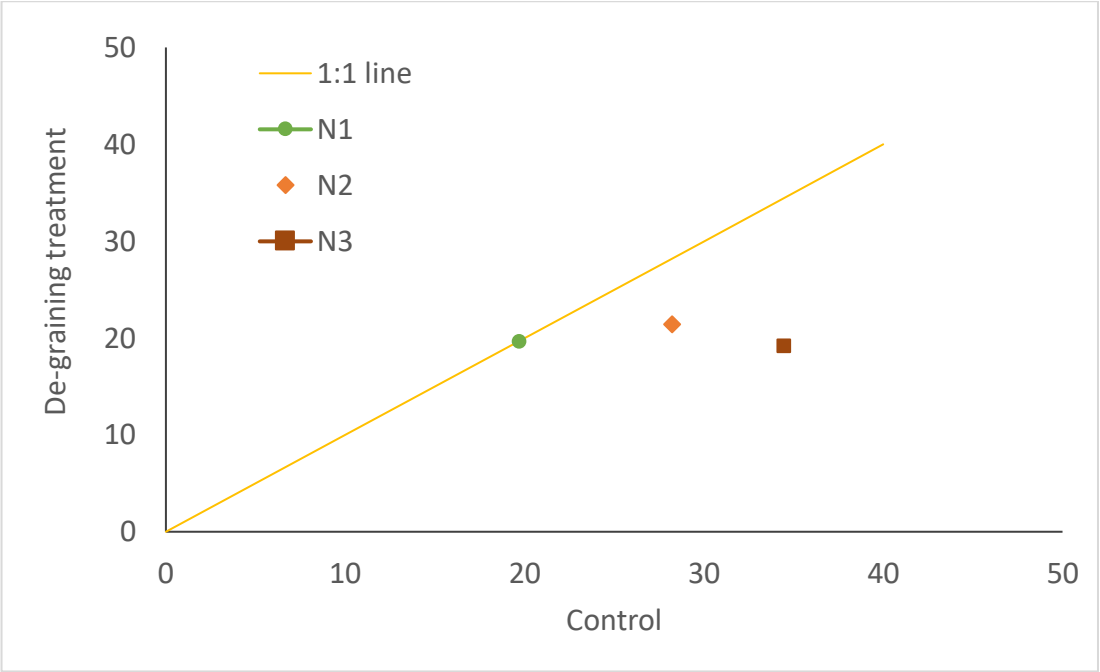


Figure 3.10 Ear N accumulation in control plants vs ear N accumulation in de-grained plants. A 1:1 line is shown. N accumulation is calculated as the differences in N from GS 73 to GS 83. Data shown is the mean of five replicate plants.

3.4 Discussion

The absorption of N by plants is believed to be faster than is required for their current growth (under optimal conditions), which results in N accumulation in the tissue and the formation of N reserves (Liu et al., 2018). Depending on the physiological status of the plant nitrate can be stored or reduced and assimilated in both, roots and leaves (Masclaux-Daubresse et al., 2010). Stored N is a pool for maintaining leaf expansion and synthesizing photosynthetic proteins (Liu et al., 2018). The stored N is believed to act as a buffer pool for asynchrony between N supply and N demand for growth.

Previous work with field-grown spring barley has suggested that PANU is controlled by both N supply and grain sink demand (Bingham, unpublished). In the current study there is clear evidence that PANU responded positively to an increase in N supply. This was observed when measuring both the net N accumulation and the net ^{15}N uptake. Net N accumulation was measured as

the increase in N content in the plants over a 14-days interval, while ^{15}N uptake was measured over a 24-hour period. By contrast, reducing the grain sink demand by partial de-graining significantly reduced the amount of net N accumulated between GS 73 and GS 83, but net ^{15}N uptake was not affected by the de-graining treatment.

The N demand of a crop depends on the increase in dry matter (Schenk, 1996). The de-graining treatment significantly reduced the DW of these plants (Fig. 3.2) and a greater recycling of N from the shoots to the roots was observed (Fig. 3.7). Given that (1) a reduction on the crop N demand seems to have been achieved due to the reduced DW observed, and (2) a greater amount of N was found in the roots of de-grained plants, a reduction in the ^{15}N uptake was expected but was not observed.

One possible explanation for the lack of response of ^{15}N uptake to de-graining is that there was a large unsatisfied sink demand elsewhere in the plant, and that the N taken up by the roots was allocated to organs other than grains. In fact, when looking at the partitioning of the newly absorbed N within the plant from the ^{15}N data (Fig. 3.8), it is evident that at GS73 in de-grained plants, the newly absorbed ^{15}N is preferably allocated to the leaves and stems of main shoots and leaves, sheaths and stems of tillers whereas in control plants a large amount of ^{15}N is directly translocated to the grains.

Given that the amount of N recycled from shoots to roots was increased by de-graining but had no effect on ^{15}N uptake it would appear that the N recycling measured here does not provide an indication of the N status and demand of the plant and therefore did not act as a signal for the regulation of N uptake.

To date, it has been established that there are at least two signal systems that may exert a regulatory control on the uptake of nitrogen. These are sugars and reduced N compounds (Kant, 2018; Li et al., 2022; Miller et al., 2007). The former signalling molecules are related to photosynthetic processes and constitute a positive feed-forward signal, whereas reduced N compounds such as amino acids are related to general growth and constitute negative feedback

(Li et al., 2022). Moreover, Cooper and Clarkson (1989) suggested that a greater pool of amino acids cycling between the roots and shoots, and an increased concentration of certain amino acids in the phloem sap causes an inhibition of NO_3^- uptake. Therefore, it is clear that such signaling substances must have the potential to decrease or increase nitrate uptake either at the transcitional or post-transcriptional level (Li et al., 2022).

In this experiment, even though a larger amount of ^{15}N was found in the receiver roots of split-root plants following de-graining, this total ^{15}N content was measured in the bulk root tissue and provides no information about the form this ^{15}N is in or its location within the roots. Therefore, establishing whether this ^{15}N should have acted as a signal is not possible. More information is needed on the type of compounds of the ^{15}N labelled that is going back down to the root system and also their location within the root tissue.

As already stated, although the de-graining treatment showed no effect on the net ^{15}N uptake, between GS 73 and GS 83, a significant reduction in net N accumulation was observed. Why was the net N accumulation reduced when the net uptake at each individual GS was unaffected? How do we reconcile these apparently contradicting findings?

The net N accumulation was measured by difference over a harvest interval and involved pairing of plants for statistical analysis, whereas ^{15}N uptake was measured on individual plants. It is possible that the pairing process introduced errors into the analysis. However, the manipulation of grain sink demand by ear trimming has been widely utilised in cereal crops research to investigate the relationship between source and sink in yield formation (Madani et al., 2010), photosynthesis and N remobilisation (Zhang et al., 2014), grain weight responses (Rharrabti et al., 2010) and overall N dynamics through the grain filling period (Wu et al., 2022).

A reduction in PANU following de-graining treatments has been previously observed under field conditions when PANU was measured by net N

accumulation over a harvest interval (Bingham, publication in preparation). The consistency in response found in measurements of net accumulation in different studies suggests that the results in this experiment are unlikely to be a consequence of an artefact-introduced by pairing of plants. However, some precaution is needed when extrapolating these results to the field scale, given that under the control condition used for this experiment the rooting depth was restricted to 30 cm.

The ^{15}N represents a measure of what is getting up into the plant over 24-h, but the net N accumulation is showing a reduction in de-grained plants. That would suggest that there may be a net loss of N over the harvest interval that is not being measured within the 24-h ^{15}N labelling. Therefore, possible losses might be coming from (1) senescence and decay of unlabelled roots, (2) efflux of unlabelled N that ultimately becomes unavailable for re-uptake by the plant due to microbial activity or (3) volatilization of previously absorbed N and thus unlabelled.

It is important to note the ^{15}N uptake measured over 24-h is a net process and involves both gross influx and efflux. Thus, if increased efflux of N from roots after de-graining does contribute to the difference in response of net accumulation and ^{15}N uptake, it must involve previously absorbed unlabelled N and not ^{15}N. The role of N efflux may be an inevitable consequence of plant N nutrition due to the accumulation of potentially toxic ions, such as NH_4^+ in the cytosol (Britto et al., 2001). The net effect of increasing efflux is to reduce the efficiency of the net N uptake (Glass, 2003). However, the data from this experiment shows that the differences in the net N accumulation are most likely to occur from the above-ground tissue rather than the roots (data not shown), therefore the volatilization of N from the shoots is the most likely explanation for the reduced net N accumulation found in de-grained plants. Shedding of leaves does not contribute to the reduced N accumulated as all leaves, both living and senescent, were recovered and their N content determined.

Evidence suggests that losses through NH_3 volatilization are likely to occur especially from ear emergence towards maturity (Harper et al., 1987). McTaggart and Smith (1995) stated that the extent of losses through volatilization seems to be determined by the concentration of N in the different plant tissues and imply it may be greater when the grain sink is reduced. In this experiment, the de-grained treatment only increased the N concentration of grains in the main shoots and stems in tillers (Table S3.1, Table S3.2). Increased volatilization of N from these organs may therefore occur.

The evidence presented in this chapter suggests that the traditional view, that crop N uptake and crop growth are regulated by either soil N supply or by crop demand needs to be reconsidered and replaced by a more dynamic and integrated approach in which soil N availability, N uptake, assimilation and distribution and plant growth and development are considered as an interrelated mechanism (Plett et al., 2018).

3.5 Conclusion

The nitrogen uptake capacity of barley is tightly correlated with the N supply and to a lesser extent the plant N demand. Plants responded positively to the increase in N supply at early milk and early dough, though the net N uptake doesn't seem to increase linearly. There was no evidence of a decline in the net uptake of ^{15}N between GS 73 and GS 83 and in fact, there was an increase in the ^{15}N uptake at early dough compared to the early milk stage. The high capacity of barley roots for post-anthesis N uptake from a simple medium was once again confirmed.

The reduced N accumulation observed in de-grained plants compared to control plants when the net ^{15}N influx was not affected suggests that there is a loss of N from de-grained plants that is independent of current N uptake by roots. This is more likely to be the result of NH_3 emissions from above-ground tissue and will be tested in the next chapter.

Vegetative tissues remain strong sinks for the allocation of newly absorbed N

in de-grained plants. A larger pool of N in de-grained plants was demonstrated to travel from shoots to roots. However, more information is needed with regards the form of this N pool and the allocation within the tissue to explain its lack of effect on net ^{15}N uptake. Finding key molecules that provide a signal for the regulation of N uptake remains an important subject among the scientific community.

Chapter 4: Does de-graining increases NH_3 emissions from plants?

4.1 Introduction

Experimental reductions in the demand for N by the grains has often been achieved through de-graining treatments where part of the ear is removed. In barley plants, a reduction in the grain N demand results in a reduction in the net N accumulation. Such a net N reduction has been observed under field conditions as well as under controlled environment conditions (Bingham, publication in preparation, Chapter 3, (Bancal, 2009). However, this reduction does not seem to be the result of a decline in the N uptake capacity of the roots, as the last experiment (Chapter 3) showed no difference between control and de-grained plants in the net uptake of ^{15}N measured after 24 hours and this was observed regardless of the concentration of N supplied post-anthesis.

Evidence from field experiments suggests that even in the absence of de-graining treatments there can be a net reduction in above ground N content of some crops during grain filling (McTaggart and Smith, 1995; Rroço and Mengel, 2000; Schjørring et al., 1989b). It has been suggested that the extent of N loss is determined by the concentration of N in the plant tissues (McTaggart and Smith, 1995). McTaggart and Smith (1995) observed that when the N uptake was low, there was no evidence of significant loss of N. It was also implied that under these conditions the developing grains were adequate sinks to accommodate the translocation of N from other plant tissues.

To find an explanation for the overall reduced N content of de-grained plants, the hypothesis is that in plants with 50% of the ear removed, there must be a net loss of nitrogen. Losses of N have been suggested to occur as a result of root exudation (Cooper et al., 1986), ammonia volatilization particularly from senescing tissue (Mattsson and Schjoerring, 1996; Schjørring et al., 1989b) or simply by the shedding of dead leaves during the grain filling period (Mattsson et al., 1993).

N lost by the shedding of dead leaves cannot account for the reduction in N content observed with de-graining in chapter 3 as all plant tissue, including senescent leaves, was collected and analysed in chapter 3 experiment.

Efflux of N back across the plasma membrane could be a possible explanation for the reduced N content of de-grained plants. However, if efflux occurs this N must become unavailable for the roots, probably due to microbial activity and given that the net ^{15}N uptake was unaffected, any efflux of N must come from unlabelled N. Root exudates including sugars and N compounds such as amino acids are believed to be passively lost from the root and used by rhizosphere microbes (Canarini et al., 2019).

Volatilization of NH_3 from plant surfaces has been widely studied, especially during the 1980's and 90's. However, while some authors have concluded that NH_3 emissions are of minor significance from the plant N economy point of view (Mattsson and Schjoerring, 1996), others have reported that significant amounts of N can be lost through volatilization of ammonia (Harper et al., 1987). Schjoerring et al., (2000) stated that ammonia emission may lead to a significant loss of up to 5 % of the shoot N content. Therefore, seen in the context of atmospheric NH_3 pollution even small quantities still merit attention (Mattsson and Schjoerring, 1996, Schjoerring et al., 2000).

Harper et al., (1987) examined the cycling of N in a wheat crop, and the effect of early N fertilizer application on N absorption from the soil and N released to the atmosphere. In this experiment, N was lost as volatile NH_3 from the soil and from the above-ground plants after fertilization and during the senescence period. Around 9.8 % of the N applied was lost from plants between anthesis and maturity. NH_3 losses after anthesis were believed to be controlled by plant senescence and remobilisation of N to the grains.

By contrast, the amount of nitrogen lost as NH_3 from the above-ground parts of spring barley crops has been estimated to be between 0.5 and 1.5 kg NH_3-N ha^{-1} (Schjoerring et al., 1993; Sutton et al., 1995). In this experiment, three levels of NH_4NO_3 were applied at sowing (medium 40 kg ha^{-1}, high 120 kg ha^{-}

^{1} and excessive 160hg ha^{-1}). There was a higher NH_3 loss from plants in the high N treatments. In this experiment, contrary to the Harper (1987) study, the highest values for NH_3 emissions were measured around anthesis. The NH_3 emissions declined as the plants reached full maturity. It is possible that the high N remobilisation efficiency observed in all treatments may have helped to minimize the volatilization of ammonia in this experiment.

Farquhar et al., (1980) established the concept that plants have a compensation point (X_{NH_3}) for exchange of ammonia with the atmosphere. The NH_3 compensation point is defined as the NH_3 concentration in the air within the substomatal cavities at which no net NH_3 exchange with the atmosphere takes place (Farquhar et al., 1980). Plant stomata are a channel of gas exchange, including NH_3, between a leaf and the atmosphere (X. Chen et al., 2012). When the atmospheric NH_3 concentration is higher than the compensation point (X_{NH_3}), NH_3 may be absorbed by the leaves, while at ambient NH_3 concentrations below the X_{NH_3} plants may emit NH_3 to the atmosphere. The magnitude and direction of the NH_3 fluxes are dependent on multiple factors such as environmental conditions, crop growth characteristics and timing of fertilizer application (Schjoerring et al., 2000; Wang et al., 2013).

The compensation point value is the NH_3 concentration in the intercellular air spaces of plant tissues at equilibrium with ammonium in the apoplast (Husted and Schjoerring 1995; Loubet et al., 2002). It is determined by the concentration of NH_4^+ and H^+ in the leaf apoplastic solution as these two parameters control the concentration of dissolved NH_3 in equilibrium with gaseous NH_3 within the substomatal cavities (Husted et al., 2000). The exchange of NH_3 between plants and the atmosphere is therefore strongly affected by apoplastic pH and ammonium concentrations (Husted et al., 1996; Husted et al., 2000) which in turn may vary over the season and with crop management such N fertilizer application (Loubet at al. 2002).

It is plausible that by reducing the grain N demand, de-graining could increase soluble N pools modifying the ammonium concentration in the apoplast and

possibly its pH. Therefore, the hypothesis tested was that de-grained plants increase their apoplastic pH and NH_4^+ concentration, particularly in senescing leaves and therefore release more NH_3 to the atmosphere. Increased NH_3 volatilization might then explain the decrease in net N accumulation observed in plants following de-graining without a corresponding decrease in N uptake by root systems.

The aim of this study was to measure ammonia emissions from leaves and ears of intact and de-grained plants grown under control environmental conditions at high N supply. A system was developed where the ear, and two leaves of the main shoot where enclosed in individual chambers, and the emissions of NH_3 were collected in HCl. Moreover, a vacuum infiltration technique was used to extract apoplastic solution with very little cytoplasmic contamination for measurement of its NH_4^+ concentration and pH.

4.2 Materials and Methods

4.2.1 Plant growth and maintenance

Caryopses of a two-row spring barley variety (*Hordeum vulgare*, cv Westminster) were germinated in the dark at 20 °C for 5 days on filter paper moistened with deionized water. When the primary roots had reached a length of approximately 5 cm, one seedling was transplanted to a PVC cylinder filled with 1.5 kg of sand:perlite mixture (2:1 v/v) as described in Chapter 2. Plants were placed in a growth cabinet (Modular climate chamber, SNIJDERS Labs, The Netherlands) with a 20 ºC daytime temperature (16 h) and 15 ºC night temperature. Relative air humidity was set at 70 %. Light was supplied by white light emitting diodes (LED) giving a photon irradiance at plant height of 356 ± 18 µmol m^{-2} sec^{-1} of photosynthetically active radiation (PAR).

Each plant was supplied with 80 mL of a full nutrient solution (Table 4.1). Nutrient solution was supplied every 7 days. Deionized water was supplied at additional times to maintain the substrate moisture content near field capacity, as described in chapter 2.

Table 4.1 Nutrient solution composition supplied to barley plants every seven days during growth.

Macro-nutrients (mM)	K_2SO_4	1.5
	$MgSO_4\ 7H_2O$	2
	$CaCl_2\ 2H_2O$	3.5
	NH_4NO_3	6
Micro-nutrients (µM)	H_3BO_3	30
	$CuSO_4.4H_2O$	0.3
	$MnSO_4.4H_2O$	5
	$ZnSO_4\ H_2O$	0.7
	Na_2MoO_4	0.1
	EDTA Na-Fe	50

Anthesis (also referred to as flowering) was recorded when 50 % of the main shoots had fully emerged ears. Six days (109 ºC days) after flowering the de-graining treatment was imposed. All the visible ears of each designated plant had 50 % of their grains removed, as described in chapter 3. Control pants were left intact.

Measurements of NH_3 emissions and apoplastic NH_4^+ and pH were made in separate experiments.

4.2.2 Ammonia emissions experiment

Six control and six de-grained plants were randomly allocated within the growing cabinet and used for the ammonia emissions experiment. At GS 79, which was 14 days after de-graining, an older visibly senescing leaf (leaf 4), a young leaf (leaf 2) (flag leaf being leaf 1) and the ear of main shoot were used to measure the emission of NH_3. Given the complexity of the design, measurements were conducted over 3 consecutive days (two control and two de-grained plants were measured each day). The equipment for collection of

ammonia emissions is illustrated in Fig. 4.1. The design is an adaptation from (Pearson et al., 1998), the authors used this set up to measure ammonia emissions from leaves of wild plants and *Hordeum vulgare* treated with methionine sulphoximine.

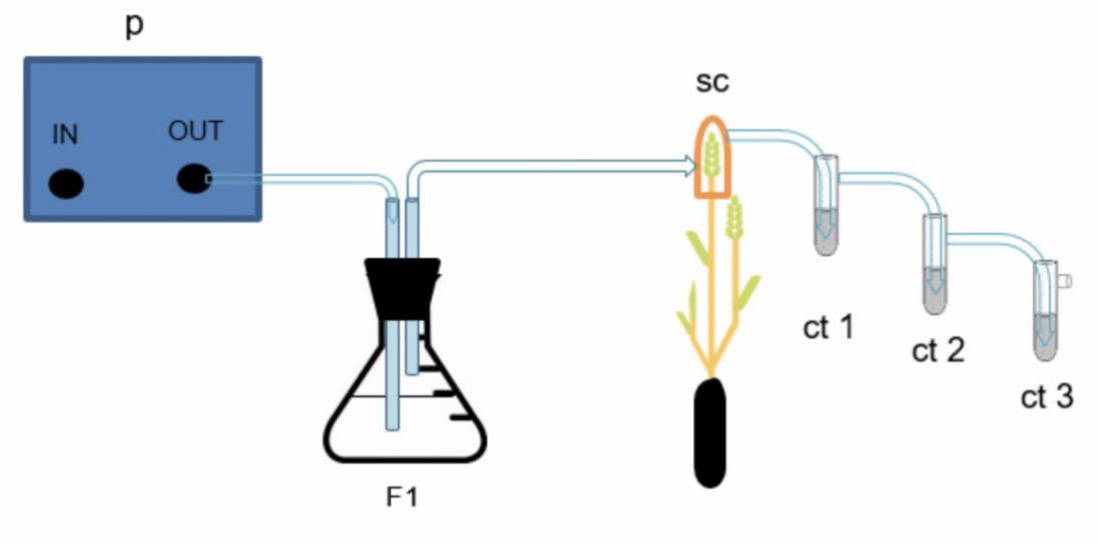


Figure 4.1 System design to collect gaseous NH_3 emissions from ears and leaves of spring barley plants (Pearson et al., 1998).

The system consisted of an air pump (p) connected to a glass conical flask (F1) filled with 1000 mL of 0.1 M HCl. The flask was sealed airtight with a rubber bung fitted with a glass inlet and outlet tube. The pump and HCl trap supplied ammonia-free air at a rate of 400 ml min^{-1} to a sampling chamber (sc) containing the leaf or ear to be measured. PTFE tubing was used to connect the outlet from the sampling chamber to three collection glass tubes (ct) in series. Each ct was filled with 3 ml of 0.1 M HCl to trap NH_3 as NH_4^+ when air from the sample chamber was bubbled through. The upper end of the sampling chamber was sealed with a rubber bung fitted with an outlet and the lower end where the plant tissue was contained was cushioned with cotton wool and sealed using several layers of Parafilm (Bemis Company, Inc). This allowed an airtight seal to be achieved while avoiding damage to the plant tissue. The system operated under positive pressure using ammonia-free air at the sample chamber inlet thus avoiding ambient air being drawn into the chamber and collection tubes modified from (Pearson et al., 1998).

The system was run for 24 hours with separate gas samples collected over the dark and light periods. Sample collection commenced at the start of the dark period immediately after plants were given their weekly dose of nutrient

solution. Nutrient solution was supplied, and the ammonia collection started immediately. Once the sampling after the 8 h dark period (night) took place, the collection tubes were replaced with fresh tubes and sampling resumed for a further 16 H of light (day). The solutions in ct1, 2 and 3 were collected individually and immediately frozen to -20 ºC. The NH_3 (in the form of NH_4^+ in solution) was analysed the following day using a San^{++} continuous flow analyser (Skalar Analytical B.V., The Netherlands). The NH_3 collected from ct1, ct2 and ct3 was summed to calculate the total NH_3 emissions over the dark and light periods. After sampling, the projected area of leaf lamina and ears was measured using a LI-3100 area meter (LI-COR, Lincoln, NE, USA). The ammonia emissions are expressed in nmol m^{-2} s^{-1}. In order to determine the percentage that ammonia volatilization makes to the overall N budget; several assumptions were made. First, the leaf area of the plants was assumed to be 450 cm^2, this was used for control and de-grained plants. No distinction between control and de-grained plants was made given that the de-grained treatment was made after anthesis by which the leaf number and tiller number are established. The nmol m^{-2} s^{-1}were converted to mg NH_3 $plant^{-1}$ day^{-1}. The total N content of the dried plants was determined by an automated Dumas combustion method using a Flash 2000 elemental analyser (Thermo Scientific UK) as described in chapter 2 and 3.

Before the main experiment was conducted a series of preliminary experiments were carried out to determine the effectiveness of the system for the collection of NH_3. This was tested in two ways: Firstly, checks were made without plants in the sampling chamber, using known amounts of NH_3 generated from stock solutions of NH_4Cl to quantify capture and test for carry over of NH_3 between sampling times. Secondly, a selected group of plants was treated with 0.5 mM of MSO (Metsulphoximine, DL-methionine-DL-sulphoximine) to inhibit glutamine synthetase (GS) activity 24 h before the collection of NH_3. This was to test whether it was possible to detect NH_3 under conditions known to promote its emission from plant tissue (Mattsson and Schjoerring, 1996).

4.2.3 Determination of apoplastic pH and NH_4^+ concentration

A second set of six control and six de-grained plants were used for the determination of apoplastic pH and NH_4^+ concentration. The measurements were taken using the same leaves (leaf 4 and leaf 2) as in the ammonia emissions experiments.

The first measurement took place at 14 days after the de-graining treatment was imposed and one hour after the plants were supply with N solution. A second measurement was taken 7 days after the N was applied to the root medium.

4.2.4 Extraction of apoplastic fluid

A vacuum infiltration technique was used for the extraction of apoplastic fluid from leaves of spring barley.

Firstly, leaves (4 and 2, also referred as old and young leaves, respectively) were cut at the base of the stem, washed with deionized water and dried with laboratory tissue. The fresh weight of each leaf was recorded. Each leaf was cut into six different segments of approximately 5 cm and separated into two equal subsamples. One for the infiltration with indigo carmine and the other for infiltration with deionized water.

Each subsample was placed in a 60 ml syringe which was filled with 40 mL of indigo carmine (50 µM). The infiltration was achieved by placing a finger over the end of the syringe and moving the plunger up, right to the end, to create a vacuum. After holding the vacuum for 10 seconds, the plunger was returned to its original position, releasing the vacuum. This process was repeated 6 times to achieve infiltration. After the infiltration, leaf pieces were blotted dry and placed on a rectangular piece of Parafilm. The leaf pieces were positioned individually, alongside each other. The Parafilm was then rolled in parallel to the edges of the leaves using a 1ml pipette tip. this samples were placed In the barrel of a 10 mL syringe with the plunger removed. The 10 mL syringe

was then placed in a 50 mL centrifuge tube. The assembly was centrifuged at 2000 g for 5 min at 4 ºC. The same process was repeated but using the second leaf subsample with deionized water instead of indigo carmine.

Prior to the main experiment, a series of preliminary experiments were carried out to optimise the amount of apoplastic fluid extracted whilst minimising membrane damage and cytoplasmic contamination of extracts (see below). This involved varying the vacuum suction created during infiltration, the duration of infiltration and the time and speed of centrifugation.

4.2.5 Calculation of the apoplastic dilution factor

An apoplast dilution factor was calculated following the method of (O'Leary et al., 2014). The optical density of indigo carmine solution and the deionized water used for the infiltrations was measured spectrophotometrically at 610nm (FLUOstar® Omega, BMG Labtech Ltd, UK). The corrected absorbance of the solution prior to the infiltration, hereafter named infiltrate was then calculated:

$$OD_{610,\ infiltrate} = OD_{610,\ indigo\ carmine} - OD_{610,\ deionized\ water} \qquad \textit{Equation 4.1}$$

After infiltration, the absorbance of the extracted apoplast fluid (EAF) was also measured at 610nm. To calculate the corrected absorbance of the fluid the same equation was used:

$$OD_{610,\ EAF} = OD_{610,\ EAF,\ indigo\ carmine} - OD_{610,\ EAF,\ deionized\ water} \qquad \textit{Equation 4.2}$$

Using the corrected absorbance of the indigo carmine infiltrate and the corrected absorbance of the apoplast fluid extracted in indigo carmine solution, the apoplast dilution factor (ADF) can be determined:

$$ADF = \textit{Equation 4.1} \div (\textit{Equation 4.1} - \textit{Equation 4.2}) \qquad \textit{Equation 4.3}$$

In this experiment, an ADF for young and older leaves for control and de-grained plants was determined.

4.2.6 Leaf apoplastic [NH_4^+] and [H^+] determination

The concentration of H^+ was determined through the pH measurements. The pH of extracts collected during the centrifugation was measured directly with a micro pH electrode (PerpHecT ROSS Micro combination pH electrode, Thermo Scientific, UK). pH was converted to H^+ using:

$$[H^+] = 1 \times 10^{-pH} \qquad \textit{Equation 4.4}$$

The NH_4^+ concentration of the apoplast extractions was measured directly using a San^{++} continuous flow analyser (Skalar Analytical B.V., The Netherlands). Samples were diluted with 0.1 M HCl to achieve the minimum analysable volume of 1.5 ml.

The NH_4^+ and H^+ concentrations calculated were multiplied by the apoplastic dilution factor to estimate the concentration in the apoplast. This accounts for the dilution of the apoplastic fluid by the infiltration solution.

4.2.7 Cytoplasmic contamination

From each plant used for the determination of pH and [NH_4^+], a leaf (leaf 3) was taken for the determination of MDH (malate dehydrogenase) activity following the method of (Søren Husted and Schjoerring, 1995). Comparison of MDH activity of bulk leaf homogenates and apoplastic extracts was performed. Leaf homogenates were prepared by milling 0.1g of the fresh leaf in 500µl of extraction buffer (TES 0.1 M, EDTA 0.2 mM and DTT 2 mM, pH 7.5) using a TissueLyser LT (Qiagen, USA).

MDH activity of bulk and apoplast extracts was assessed by adding 10µl of extracts to a solution containing 230 µl of 10 mM K/Na phosphate, 30 µl of 2 mM NADH and 30 µl of 20 mM OAA (oxaloacetate) and measuring the change in the absorbance at 340 nm at 25 ºC in a spectrophotometer (FLUOstar® Omega, BMG Labtech Ltd, UK).

4.2.8 Calculation of NH_3 compensation points

The X_{NH_3} was calculated from the apoplastic concentrations of NH_4^+ and H^+ according to the following equation (Loubet et al., 2002):

$$X_{NH3} = M_{NH_3} \times K_H \times K_D \times \frac{[NH_4^+]}{[H^+]} \times 10^9 \qquad \textit{Equation 4.5}$$

where, X_{NH3} is the stomatal compensation point (in µg NH_3 m^{-3} or nmol mol^{-1}) MNH_3 is the molecular mass of NH_3 (in g mol^{-1}), K_H is the dimensionless Henry association constant and K_D is the acidity dissociation constant of the NH_3/NH_4 acid-base couple (in mol l^{-1}). The product of K_H and K_D is 2.25 x 10^{-13} at 20 °C.

4.2.9 Statistical analyses

A two-way ANOVA was carried out to establish the statistical significance of differences between treatment means (de-graining and plant fraction) and their interactions using GenStat (19th Edition VSN international Ltd).

For the emission of ammonia experiment, the ANOVA design consisted of de-graining x plant tissue treatments at each time of collection. There were six control and six de-grained plants randomly allocated within the chamber cabinet with two replicates for each of the three sampling days. The plant tissue factor had three levels: old leaf, young leaf and ear.

For the $[NH_4^+]$, $[H^+]$ and X_{NH3} the ANOVA design also consisted of de-graining x plant tissue treatments. Here there were six control and de-grained plants and two ages of leaves per plant (old and young) and the measurements took place one hour and again 7 days after N application to the root medium.

The time of measurement was not included as a factor in the statistical analysis. Separate analyses were performed for individual measurement times, this is dark and light periods for the ammonia emission experiment and one hour and seven days after N supply. LSD α=0.05, were used to compare pairs of mean values. Residuals were checked and shown to have a normal

distribution and homogeneous variance and thus transformation was not required.

4.3 Results

4.3.1 Ammonia emissions form leaves and ears of control and de-grained plants

Preliminary experiments showed that the system described in Fig 4.1 was effective and reliable for the collection of NH_3. Without plant tissue in the sampling chamber there was no detectable NH_3 collected, whereas when a leaf was placed in the chamber NH_3 was measured. Leaves of plants treated with MSO emitted a significantly greater amount of NH_3 compared to untreated plants ($p=0.034$). On average, the rate of NH_3 emitted from ears and leaves of MSO treated plants was 5.7 nmol m^{-2} tissue surface s^{-1}, whereas for control plants the rate of NH_3 emissions was 1.5 nmol m^{-2} tissue surface s^{-1}.

Using the sampling system described it was, therefore, possible to measure the emissions of NH_3 from intact and de-grained plants (Fig. 4.2). Regardless of the time of collection, the de-graining treatment did not influence the amount of NH_3 emitted from plants (Table 4.2). At GS79, when averaged over the different tissue fractions, the rate of NH_3 emission from intact plants during the night was 1.65 m^{-2} tissue surface s^{-1} and during the day was 1.16 m^{-2} tissue surface s^{-1} compared to 1.61 and 1.08 m^{-2} tissue surface s^{-1} respectively for de-grained plants. Overall, the time of collection showed a significant increment in the rate of NH_3 emissions during the dark period ($p=0.002$, ANOVA not shown).

During the 8 h of night, there was no significant difference in rate of emission between the different tissue fractions analysed when averaged over the de-graining treatments ($p=0.212$, Table 4.2), nor was there a significant de-graining x tissue fraction interaction.

On the other hand, at the end of the 16 h of daylight, the average rate of NH_3 emission during the 16 h day was significantly different among the different

plant fractions (p=0.038, Table 4.2), but there was no de-graining x tissue fraction interaction (p=0.401). Thus, during the day, the rate of NH_3 emission was significantly greater from old leaves than young for both, control and de-grained plants. The rate of NH_3 emission from the de-grained ears was significantly higher than that from young leaves but not significantly different from old leaves (Fig. 4.2).

Table 4.2 Analysis of variance (ANOVA) of the effect of de-graining and tissue fraction on the rate of emissions of NH_3 from spring barley collected during the day and the night.

Effect	Night		Day	
	***p*-value**	**LSD**	***p*-value**	**LSD**
De-grained Treatment (D)	0.895	n.s	0.539	n.s
Tissue fraction (F)	0.212	n.s	0.038	0.320
D x F	0.167	n.s	0.401	n.s

n.s = not significant; LSD (5%) least significant differences

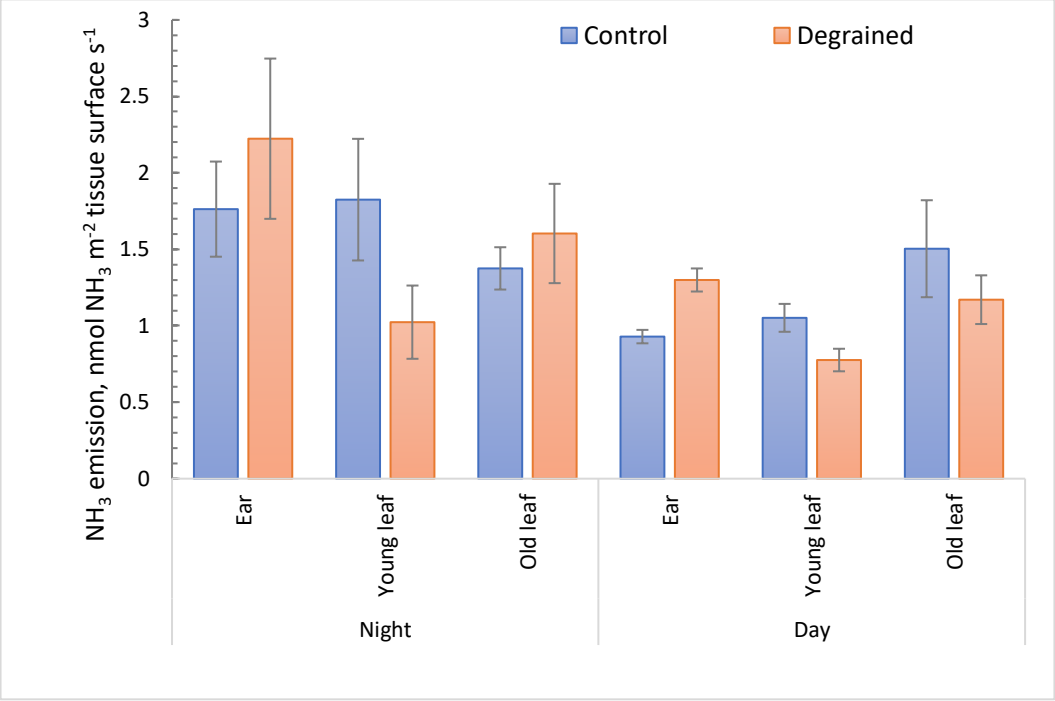


Figure 4.2 Rate of volatilization of NH_3 from ears and leaves of intact (control) and de-grained plants. Collection performed during 16h of light and 8h of dark. Data are the mean of six replicate plants ± SEM.

On average, control plants emitted 0.26 mg NH_3 $plant^{-1}$ day^{-1} and de-grained plants an average of 0.25 mg NH_3 $plant^{-1}$ day^{-1}. The total N content in control plants was 115.3 mg N and in de-grained plants was 110.5 mg N. Therefore, control and de-grained plants volatilised around 0.23% of their total N content.

4.3.2 Leaf apoplast [NH_4^+]

The de-graining treatment did not affect the concentration of NH_4^+ in the apoplast at either time of measurement. Table 4.3 shows no significant effect of de-graining or leaf age and no interaction between de-graining and leaf fraction (age) on apoplastic [NH_4^+] whether measured one hour or seven days after supply N to the root medium.

Fig. 4.3 shows the apoplastic [NH_4^+] in leaves of intact and de-grained spring barley plants measured one hour and seven days after N supply. Concentrations ranged from 146 to 245 µM.

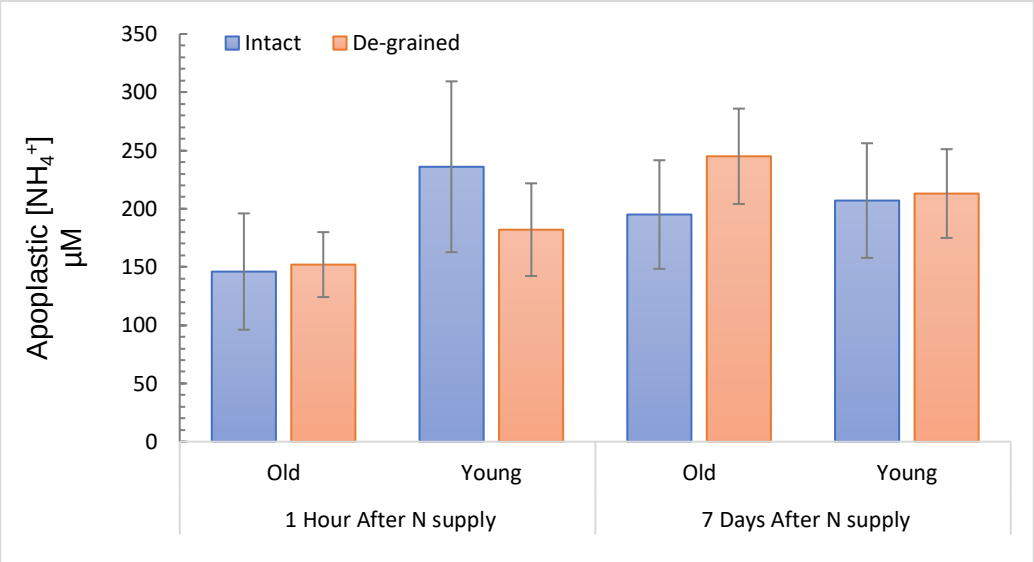


Figure 4.3 Apoplastic NH_4^+ concentration in a young and old leaf of main shoots of spring barley. Measurements taken one hour and seven days after N supply. Data are the means of six control and de-grained plants ± SEM.

Table 4.3 Analysis of variance (ANOVA) of the effect of de-grained, treatment and the different leaf on the apoplastic [NH_4^+] after one hour and 7 days of N supply.

Effect	*p* value	
	1h	*7 days*
De-graining treatment (D)	0.622	0.528
Leaf (F)	0.227	0.818
D x F	0.538	0.642

Overall, the MDH activity of the extracts was very low compared to that of bulk tissue. MDH activity in the apoplastic extracts was, on average, less than 0.9 % of that in the bulk extracts indicating that contamination by cytoplasmic contents was negligible. There were a couple of samples were the MDH activity was as high as 3.5 % in which case contamination may have led to a slight overestimation in the apoplastic NH_4^+ concentration.

4.3.3 Leaf apoplastic pH

Fig. 4.4 shows the apoplastic pH of young and old leaves of intact (control) and de-grained plants one hour and seven days after N application. The de-graining treatment reduced the leaf apoplastic pH of de-grained plants seven days after N application (p= 0.006, Table 4.4) but not one hour after N supply. The reduction in pH seven days after N application was more pronounced in old leaves, although did not lead to a significant de-graining x leaf fraction interaction (p=0.134). Statistically, there were no significant differences between the different leaves analysed, regardless of the time at which the pH was measured.

After 1 hour of N supply, the apoplastic pH of old leaves of intact (control) and de-grained plants and the young leaf of control plants was 6.4 while the young leaf of de-grained plants showed a pH of 6.6.

Seven days after N supply the apoplastic pH of de-grained old leaves was 6.0, the lowest recorded during this experiment, whereas controls showed a pH of 6.6. With regards of younger leaves, control plants had a pH of 6.6 whereas the leaf pH of de-grained plants was 6.5.

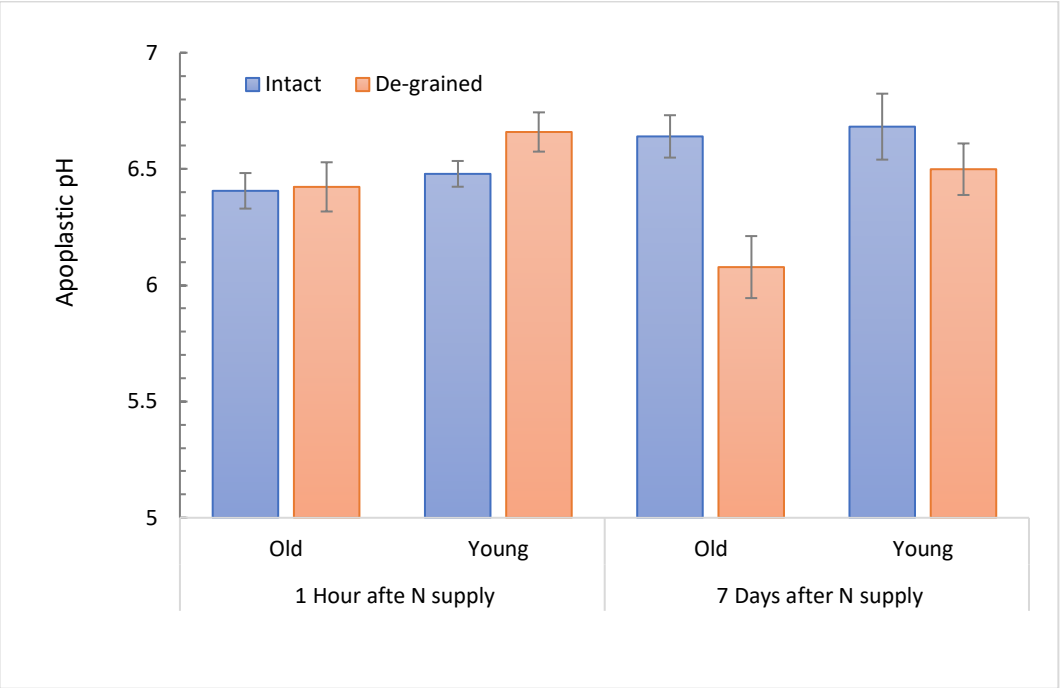


Figure 4.4 Apoplastic pH in old and young leaves of intact (control) and de-grained spring barley plants. Measurements taken one hour and seven days after N supply. Data are the means of six control and de-grained plants ± SEM.

Table 4.4 Analysis of variance (ANOVA) of the effect of de-graining and tissue fraction on the leaf apoplastic pH of spring barley leaves measured one hour and seven days after N supply.

Effect	***p* value**		***LSD***	
	1h	*7days*	*1h*	*7 days*
De-graining treatment (D)	0.21	0.006	n.s	0.252
Leaf (F)	0.055	0.069	n.s	n.s
D x F	0.295	0.134	n.s	n.s

n.s = not significant; LSD (5%) least significant differences

4.3.4 Stomatal compensation point (X_{NH3})

The leaf apoplastic [NH_4^+] and, the [H^+] obtained from the measured pH, were used to calculate the stomatal compensation point (XNH_3). Values of XNH_3 are shown in Fig. 4.5. The XNH_3 was calculated for old and young leaves, one hour after the N nutrient solution was applied and also after seven days of N supply. The de-graining treatment did not affect the calculated XNH_3 at any time (Table 4.5), nor was there a significant effect of leaf or a de-graining x leaf interaction. Values of the compensation point ranged from 1.6 to 4.2 µg NH_3 m^{-3} across the different treatment combinations.

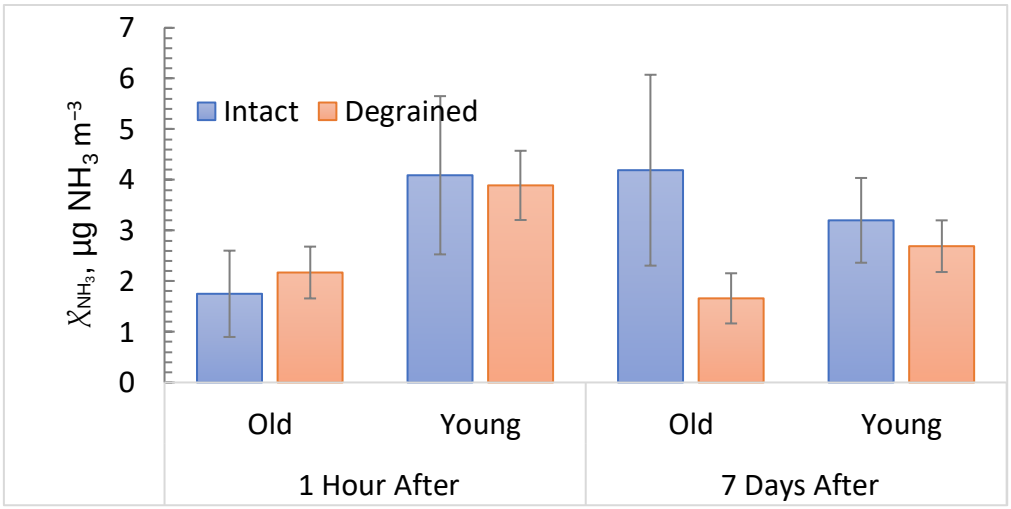


Figure 4.5 Stomatal compensation point (XNH_3) of young and old leaves of intact and de-grained barley plants one hour and seven days after N supply. Data are the means of six replicates plants ±SEM.

Table 4.5 Analysis of variance (ANOVA) of the effect of de-graining and leaf (young and old) on the stomatal compensation point XNH_3 of spring barley leaves measured one hour and seven days after N supply.

Effect	*p* value	
	1h	7 days
De-grained treatment (D)	0.806	0.176
Leaf (F)	0.136	0.912
D x F	0.812	0.396

4.4 Discussion

The lower net N accumulation between GS 73 and GS 83 observed in de-grained plants relative to intact controls (Chapter 3) could be explained if a net loss of previously assimilated N is determined. This chapter investigates whether the volatilization of NH_3 is a possible explanation for the reduced net N accumulation observed in de-grained plants.

During the post-anthesis stage, some N may be taken out of the xylem and transferred directly to the phloem for immediate supply to rapidly growing sinks (Tegeder and Masclaux-Daubresse, 2018), most likely to the developing grains. The transfer between xylem and phloem requires N retrieval from the transpiration stream into the xylem parenchyma cells via proton symport (Van Bel, 1990) and symplasmic N movement towards the phloem, followed by an apoplastic loading step into the phloem (Tegeder and Masclaux-Daubresse, 2018).

It is, therefore, plausible to hypothesise that a reduction in grain N demand imposed by partial degraining would increase the amount of soluble N in the form of NH_4^+ in the leaf apoplast. An increase in apoplastic NH_4^+ concentration is a parameter known to influence the compensation point for exchange of ammonia between plants and the atmosphere (Husted and Schjoerring 1995; Loubet et al., 2002).

The emission of NH_3 may also be associated with protein breakdown processes as the plant starts to senesce, should some of the liberated NH_4^+ leak from the cytosol into the leaf apoplast (Schjoerring et al., 1993).

In the current work the potential for emissions of NH_3 from intact and de-grained barley plants was assessed by two methods; by direct measurement of the NH_3 released and captured in an acid trap (HCl), and indirectly by measuring the leaf apoplastic [NH_4^+] and pH to determine the stomatal compensation point.

The direct determination of the rate of NH_3 emissions showed that significant amounts of NH_3 were emitted from leaves and ears of barley plants, regardless of whether or not they were de-grained. The emissions of NH_3 showed no significant differences between intact-control and de-grained plants regardless of the tissue measured. The percentage that NH_3 volatilization makes to the overall N budget is relatively small. Only 0.23% of the total N was measured as volatilised NH_3. In order to explain the reduced accumulation of total N observed in de-grained plants compared to control plants of chapter 3 the amount of N loss as ammonia in de-grained plants should have been at least 0.56 mg NH_3, instead only 0.25 mg NH_3 was measured. To date, NH_3 emissions from plants where the grain sink has been reduced (de-graining treatment) has not been reported. However, ammonia emissions from barley plants grown under field conditions have been widely reported as discussed below.

Mattsson and Schjoerring (1996) demonstrated that ammonia emissions from intact young barley plants can occur. The authors monitored the emissions of NH_3 from entire barley plants using an automated NH_3 monitor with regular measurements for 24 hours during the light and dark period. The rate of NH_3 emissions ranged from 0.5 nmol m^{-2} s^{-1} during the dark period to 1 nmol m^{-2} s^{-1} during the light period. The results from the experiment presented here showed a rate of NH_3 emissions from intact plants during the night of 1.6 m^{-2} tissue surface s^{-1} and during the day of 1.1 m^{-2} tissue surface s^{-1}. Thus, rates were in the range reported elsewhere. Slightly higher rates would be expected from the current experiment compared to those from some other studies because NH_3-free air was passed over the plant tissue surface which would have increased the diffusion gradient for emission.

Mattsson and Schjoerring (1996) also stated that plants supplied with NH_4^+ could potentially release greater amounts of NH_3. than those supplied with NO_3^-. Moreover, it is well established that when plants are treated with NH_4NO_3, as in the current work, NH_4^+ is absorbed more readily than NO_3^- (Cooper et al., 1986). The absorption of NH_4^+ by the roots may lead to a higher

concentration of NH_4^+ in the apoplast and also an acidification of the apoplast solution. NO_3 applications will result in an increase of the leaf apoplast pH (Hoffmann et al., 1992).

Leaf apoplast [NH_4^+] of barley plants has been reported to range between 52 µM in plants grown for two weeks and 360 µM in plants grown until just prior to anthesis (Mattsson et al., 1997; Wang et al., 2013). As highlighted above, the apoplastic [NH_4^+] is highly dependent on the N supply to the root medium and the capacity of these roots to uptake the available N (Mattsson et al., 1997; Nielsen and Schjoerring, 1998; Settelmacher, 2019). The results from this current experiment fit accurately with those already published with a range of apoplastic [NH_4^+] between 146 and 245µM. However, there were no significant differences between control and de-grained plants.

As discussed, an increase in the concentration of apoplastic NH_4^+ was expected following de-graining due the reduced grain sink demand and the higher recycling on N observed from shoots to roots in Chapter 3. However, the comparable [NH_4^+] in both de-grained and control plant tissues, including leaves at advanced stages of senescence, suggest that any increase in soluble N pool may be retained effectively within the cytosol or loaded rapidly into the phloem thus preventing a large increase in apoplastic concentration. Moreover, this pool of mobile N that is recycled from shoot to roots is more likely to be in the form of organic compounds such as amino acids (Masclaux-Daubresse et al., 2010). Determining the form of the circulation N pools in control and de-grained plants is therefore essential to understand how N compounds may or may not act as signalling compounds to regulate the uptake of N and their relationship with plant N demand.

There were no differences between control and de-grained plants in the apoplastic pH measured one hour after N application. This was true for both leaves analysed. The apoplastic pH of old and young leaves of control plants increased 0.2 units reaching 6.6 when measured seven days after N application. With regards de-grained plants, the apoplastic pH was significantly

reduced in old leaves (pH 6.0) at seven days after N supply compared to the pH measured one hour after N supply. Wang et al., 2013 reported pH values of barley leaves of 6.5 under favourable conditions. After seven days of N supply, old leaves of de-grained plants showed the highest [NH_4^+] in the apoplast (although not significantly different) and the lower pH, consistent with the association between high concentrations of NH_4^+ and the acidification of apoplastic pH. These leaves showed the lowest compensation point (XNH_3).

The stomatal compensation point is influenced by the equilibrium between [NH_4^+] and [NH_3] in the apoplast and will increase with an increase in the both apoplastic [NH_4^+] and [H^+] (S. Husted et al., 1996; Søren Husted et al., 2000). The NH_4^+ concentration and the pH values, as well as stomatal compensation points measured in this experiment were comparable to published values, supporting the validity of the experimental approaches used in this chapter.

In barley, NH_3 compensation points between 0.9 and 8 $\mu gNH_3\ m^{-3}$ have been measured (Husted et al., 1996; Mattsson et al., 1997). Plants with stomatal compensation points close to zero tend to absorb NH_3 from the atmosphere, whilst those with higher compensation points may emit NH_3. The compensation points calculated on the basis of apoplastic [NH_4^+] and [H^+] measured in this experiment did not differ between de-grained and control, correlating with the direct measurements of NH_3 emissions and with published values, suggesting that volatilization of NH_3 from barley leaves is likely to occur regardless of whether the grain sink demand for N was altered.

Given that the ammonia compensation point is a function of the ratio between [NH_4^+] and [H^+], the reduced apoplastic pH observed in old and young leaves of de-grained plants seven days after N supply is a characteristic toward lower compensation points (higher H^+ concentration) which support the trend to lower NH_3 emissions observed in this experiment during the day. There is no evidence of an increase in NH_3 volatilization from de-grained plants.

Although it is possible that some NH_3 volatilization might contribute to the reduced N accumulation observed in de-grained plants a different mechanism

must also be responsible for this phenomenon. Losses of N have also been suggested to occur as a result of root exudation (Cooper et al., 1986), or simply by the shedding of dead leaves during the grain filling period (Mattsson et al., 1993). The later, as already stated is the least possible explanation as in these experiments the N was measured in shedding tissues. Further experiments are needed to determine the reduced accumulation of N observed in de-grained plants, with a focus on root exudation.

4.5 Conclusion

The apoplastic [NH_4^+] and pH measurements, are valuable tools for the determination of the likelihood of ammonia emissions from plants. There were no significant differences between control and de-grained plants in their apoplastic NH_4^+ concentration. Seven days after N application the leaves of de-grained plants showed a significantly lower apoplastic pH, a characteristic trend towards lower compensation points which support the lower NH_3 emissions measured in this experiment. Importantly there was no evidence of an increase in the NH_3 emissions from de-grained plants.

Losses on N from plants via volatilization of NH_3 occurred in both control and de-grained plants, therefore the reduced net N accumulation observed in de-grained plants cannot be explained by an increase in NH_3 emissions.

Chapter 5: Investigating the post-anthesis N uptake and soil N depletion of spring barley and spring oats under field conditions

5.1 Introduction

Global fertilizer consumption for agricultural use has increased dramatically over the last 40 years, reached 188.5 million tonnes (Mt) during 2019, of which 108 Mt correspond to N-fertilizer (FAOSTAT, 2022). The use of nitrogen (N) fertilizers plays an essential role in crop productivity and, it has been estimated that over 70% of the world N-fertilizer is applied to cereal crops to maintain high yields (Heffer et al., 2017). Unfortunately, cereal crops use nitrogenous fertilizer inefficiently (Raun and Johnson, 1999) resulting in high production costs and considerable impacts on the environment.

Nitrogen use efficiency (NUE) has been defined as the yield of grain per unit of available nitrogen in the soil (Moll et al., 1982). This NUE can be divided into two processes: uptake efficiency and utilization efficiency. The former refers to the capacity of the plant roots to remove N from the soil and, the later to the ability of the plant to use the absorbed N in grain yield formation (Lea and Azevedo, 2006).

During the growth of cereal crops, the highest rate of N uptake is observed during stem elongation (Jones et al., 2015; Miralles et al., 2020). After anthesis, N uptake declines, the canopy starts to senesce and remobilisation and translocation of N from vegetative organs to the grains occurs (Barraclough et al., 2014; Hawkesford and Griffiths, 2019). In spring barley crops, at maturity, large quantities of mineral N have been found to remain apparently unused in the soil (Bingham et al., 2012). The physiological mechanisms behind this phenomenon remain unclear.

It is unlikely that the physiological capacity of spring barley roots is the reason for the apparent unused N left in the soil at harvest. Previous results reported in this thesis (Chapter 2) alongside published work (Mattsson et al., 1993) have demonstrated that roots of spring barley crops have a high physiological

capacity to acquire N from the root medium throughout the growing season and late into the grain filling period. Although, it is soil N uptake at depth where root density is low, that is important in relation to improving N uptake efficiency. Moreover, de-graining experiments have demonstrated that reducing the grain sink demand does not influence the N uptake capacity of the root system (Chapter 3). However, these experiments were conducted under controlled environmental conditions and using only one barley variety.

Nitrogen fertilizer requirement and NUE greatly varies among cereal crops and are highly dependent on environmental conditions such as type of soil and irrigation (AHDB, 2018b). Among spring cereal cultivars, nitrogen for spring sown wheat varies between 40 and 210 kg N ha^{-1} depending on the soil type and SNS index (Soil Nitrogen Supply = amount of nitrogen already in the soil that is available for uptake by the crop). Spring barley and, in particular, malting spring barley have lower N requirements and high NUE have been observed (Raun and Johnson, 1999; Sandhu et al., 2021; Sylvester-Bradley and Kindred, 2009). N for spring sown barley grown for feed varies from 40 kg N ha^{-1} to 160 kg N ha^{-1} whereas malting barley has an N requirement of between 30 and 130 kg N ha^{-1}depending on the soil type and SNS index. Less extensively grown cereal crops such as oats and rye have the lowest N requirements ranging from 20 to120 kg N ha^{-1} (AHDB, 2018b).

Previous SRUC research has shown that oats deplete soil nitrate to lower concentrations than barley (unpublished work). This was observed when comparing spring barley cultivar Westminster against spring oat cultivar Firth. No differences have been observed in their relative depletion of soil ammonium concentrations. Currently the mechanisms that underlie these responses are unknown.

The root length density (total length of roots per unit of soil volume; RLD) is an essential trait to estimate the soil volume explored by a root system. The crop's water and nutrient absorbing ability per unit of soil volume is usually calculated using relationships with RLD (Tinker and Nye, 2000). The concept of a critical

root length density (cRLD) at which water capture became near complete was introduced by Gregory and Brown (1989) and was estimated that be around 1 cm cm^{-3}. RLD of wheat and barley crops measured in the upper 20cm of soil profile usually exceeds cRDL and below 30-40cm depth RLD is typically lower than 1cm cm^{-3} (Bingham and Wu, 2011; Carvalho et al., 2014; White et al., 2015). As such mean root length densities of 0.5-10 cm cm^{-3} of cereals in the upper layer of most soils are considered sufficient to capture most of the available water during the yield-formation stage (King et al., 2003). RLDs higher than 1 cm cm^{-3} are likely to only contribute to a small increase in the total amount of water absorbed by the crop during this period (King et al., 2003).

Sensitivity analyses derived from pot-experiments have shown that with regards to the capture of mobile nutrients such as NO_3^-, the length of roots was of minor importance, but it became the most sensitive parameter when modelling uptake of immobile nutrients (Barber, 1995). However, this model requires a realistic term for the relative distribution of roots down to the soil profile and therefore, cannot be translated to field work.

King et al., (2003) developed a quantitative model using wheat root systems that links the size and distribution of the root system to the capture of water and nitrogen during the grain filling period. By analogy with water, King et al. argued that mobile ions such as nitrate would behave in a similar way to water with respect to the cRLD for uptake. The authors concluded that a winter wheat crop could capture about 55% of the available water and nitrate to a root depth of 2m by anthesis. However, this model assumes a maximum possible rooting depth and an even distribution of available resources with depth.

Measures of SMN in spring cereal crops, have shown different N concentration at 0-30cm depth compared to 30-60cm depth at anthesis, with the upper layer containing higher NO_3^- and NH_4^+ concentrations (Bock, 1986; Mengel et al., 2006).

The aim of the experiments reported in this Chapter was to investigate the post-anthesis crop and soil N dynamics in barley and oat crops. The specific objectives of these experiments conducted under field conditions were; a) to quantify differences between oats and barley cultivars in their ability to deplete SMN and to determine whether previous reports of a greater depletion by oats is a general characteristic of the species, b) to compare how productive each cropping system is in relation to its fertilizer N input, and c) to quantify the relationship between RLD and NO_3^- concentration in the soil to determine whether the greater depletion by oats is the result of a greater RLD. Moreover, the PANU and NRE of Westminster grown under field conditions can be compared to the PANU and NRE of those grown under controlled-environmental conditions.

5.2 Materials and Methods

5.2.1 Site conditions and crop management

Two field experiments were conducted in 2019 at an SRUC site near Edinburgh, UK. Experiments were located at Boghall Farm, Midlothian (latitude 55.878 ^{o}N, longitude 3.198 ^{o}W) on a sandy clay loam soil of the Macmerry series with an organic matter content of 7.34 % and topsoil pH of 6.4. The previous crop was spring barley.

Seedbed preparation followed ploughing and plots (10x2 m) were sown with spring barley (*Hordeum vulgare)* and spring oat (*Avena sativa*) cultivars (dependent on the experiment detailed below) at a rate of 360 viable seeds m^{-2} on the 26th of March. N, P and K fertilizer was applied as a top dressing to the soil in a rate of 120 kg N ha^{-1}, 60 kg P_2O_5 ha^{-1} and 60 kg K_2O ha^{-1}. N fertilizer was applied as ammonium nitrate granules at the 2 - 3 leaf stage of the crop (Zadoks GS 12–13,Tottman, 1987). P and K fertilizer was applied to the seed bed.

Manganese and sulphur and a rigorous crop protection programme were applied according to standard farm practice to avoid micronutrient deficiencies and to control weeds, pests, and diseases.

5.2.2 Variety Experiment

Experimental design

Four cultivars of spring barley (Westminster, Laureate, Concerto and RGT Planet) and four cultivars of spring oats (Firth, Yukon, WPB Elyann, Aspen) were grown under the conditions detailed above. The experiment was conducted in a split-plot design with four replicate blocks; species were randomised within main plots and cultivars in sub-plots. Table 5.1 shows the cultivars used in this experiment with their corresponding year of released and breeder.

Table 5.1 Year of release for cultivars used in this experiment and breeder company according to the AHDB recommended List.

Variety	Species	Year of released	Breeder
Westminster	Barley	2005	Nickson
Laureate	Barley	2016	Syngenta Participations AG
Concerto	Barley	2009	Limagrain UK
RGT Planet	Barley	2015	RAGT Seeds
Firth	Oats	2000	KWS UK
Yukon	Oats	2017	Nordsaat, Germany
WPB Elyann	Oats	2017	Wiersum BV, Netherlands
Aspen	Oats	2015	Bauer, Germany

Crop measurements

Barley cultivars were sampled at anthesis and physiological maturity whereas oats were sampled at anthesis, at barley maturity and when oats reached physiological maturity. For barley cultivars the anthesis date of sampling was the 3rd of July 2019 and for oats it was the 8th of July 2019. Barley maturity was reached on the 20th of August 2019 and oat maturity on the 6th of September 2019.

A 0.5 m length of four adjacent rows of plants were taken from each of two locations at diagonally opposite ends of the plots. The outer two rows of plots and plants within 0.5 m of the plot ends were not sampled to avoid edge effects. Shoots were cut at ground level and carefully removed from the plot retaining any senesced and senescing leaf tissue. The sampled plants were combined to give one bulked sample per plot and were placed ear first into polythene bags to prevent moisture loss and transferred to the laboratory for assessment.

Samples were stored in their bags at 4 ^{o}C in the dark and processed within 48 h. Shoots were divided at random into five subsamples. One representative subsample was weighed fresh, the number of ear-bearing and non-ear-bearing shoots recorded and then separated into ears, leaf laminae and stem, dried for 48 h at 80 ^{o}C in an oven and each fraction weighed to the nearest 0.01 g.

At maturity, grains were threshed from the dried ears using a laboratory thresher (Wintersteiger LD 180, Austria), and weighed. The chaff was recovered and treated as a separate plant fraction. The dry weight of the chaff was calculated as the ear weight minus the grain weight. After milling the plant fractions into fine powder, the tissue N concentration was determined as described in chapters 2 and 3.

Soil measurements

After plant sampling, soils were sampled for determination of mineral N (ammonium and nitrate) concentrations. Soil samples were taken from the two harvested areas in each plot where the plants had been sampled and combined into one for processing. Soil cores were taken to a depth of 60 cm using a 2.5 cm diameter auger (a single core per sample location), separated into two depth intervals, 0-30 and 30-60 cm, placed in polythene bags and analysed immediately on return to the laboratory.

For the soil mineral N (SMN) analysis, the soil sample was well mixed by hand and 10 g of soil was extracted in 50 ml of 2 M KCl for 1 h. Any visible roots were removed this soil sample. The extracts were then centrifuged at 4700rpm for 20 min and the supernatant collected for colorimetric analysis using an automated wet chemistry analyser (SAN^{++}, Skalar Analytical B.V., The Netherlands). For moisture content determination, 30 g of fresh soil was taken and oven-dried at 104°C until constant weight. After harvest soil dry bulk density was determined at soil depths of 10-15, 25-30, 45-50 and 70-75 cm (Rowell, 1994). At each depth, two samples were taken from each of two locations per site.

Calculations

N contents in above ground biomass fractions were calculated from the dry weight of the fractions expressed per m^2 of ground area sampled and the tissue N concentration. The following calculations were then made:

$$HI = \frac{G_w}{G_w + S_w}$$

Where HI is the harvest index, G_w is the grain yield and S_w is the straw plus chaff weight.

$$NHI = \frac{N_g}{N_{\text{off}}}$$

NHI is the nitrogen harvest index, N_g is grain N and N_{off} is the N in the above ground biomass.

$$NutE_g = \frac{G_W}{N_{off}}$$

$NutE_g$ is the N utilization efficiency (expressed on a grain basis).

$$NutE_b = \frac{B}{N_{Off}}$$

$NutE_b$ is the N utilization efficiency on a total biomass basis, *B* is the total above ground biomass at harvest calculates as $\frac{G_W}{HI}$

The fertilizer N use efficiency term used in this experiment was the partial factor productivity (PFP) calculated as total biomass per unit of N fertilizer supplied (Dobermann, 2007).

PANU was calculated as the differences between total aboveground N at maturity and total aboveground N at anthesis.

Nitrogen Remobilization (NR) is the amount of N in the crop component at anthesis which is not recovered in the crop at harvest. NRE = N content in each crop component at anthesis – N content of each crop component at harvest.

Nitrogen remobilization efficiency (NRE) is the proportion of N in the crop component at anthesis which is not present in the crop component at harvest. NRE = N content at anthesis – N content at harvest / N content at anthesis.

Grain number m^{-2} was as calculated as Gw divided by MGW (mean grain weight). Yield and MGW are expressed on a 100% dry matter basis. MGW as calculated from the number of grains in a 10g grain sample.

To calculate SMN per ha^{-1} concentrations of nitrate and ammonium in KCl extracts were converted to concentrations per unit soil dry weight after

correcting for dilution by the water content of fresh soil. Values were then used to calculate the total quantity of nitrate and ammonium per unit ground area from the depth and dry bulk density of the 0-30 and 30-60 cm soil layers. The SMN was given as the sum of the nitrate and ammonium contents.

5.2.3 Root Length Density (RLD) experiment

Crop maintenance, experimental design

One spring barley cultivar (Westminster) and one spring oat cultivar (Firth) were grown under the conditions detailed above in a completely randomised block design with crop species randomised with each of three replicate blocks.

To create a range of RLD in the topsoil and subsoil, plant density was varied by thinning plants after establishment as follows. When plants were at the 1-2 leaf stage and before significant root growth had occurred an area 1.0 x 0.5 m was marked out within a central part of each plot and seedlings removed by hand. Care was taken to remove as much of the root system as possible (Fig 5.1). Weed growth within the plant free area was prevented by use of a robust herbicide programme and regular hand weeding of any small seedlings as required.

Sampling of soil for measurement of RLD and soil SMN concentration took place three weeks after anthesis. Within each plot, samples were taken from each of three positions relative to the crop (Fig 5.1). Position 1 was near the middle of the plant-free area, this was 50 cm from the top and 20 cm from the left of the crop rows, as illustrated in Fig 5.1. The second position was at the boundary between the plant-free area and a plant row, with the centre of the soil corer 5 cm from the plant row. The third sampling (position 3) was taken from a well plant-populated area. The soil corer was carefully positioned midway between two adjacent rows of plants. The row spacing was 11.5 cm.

Within each position samples at two depths were taken, 10-25 cm and 40-55 cm using a soil corer (8 cm diam, 15 cm depth). The first 10 cm and the middle

layer (25 cm to 40 cm) were discarded. Samples were placed in polythene bags and taken to the laboratory for analysis. A total of 36 samples were taken, 2 species x 3 replicates (blocks) x 3 positions x 2 depths.

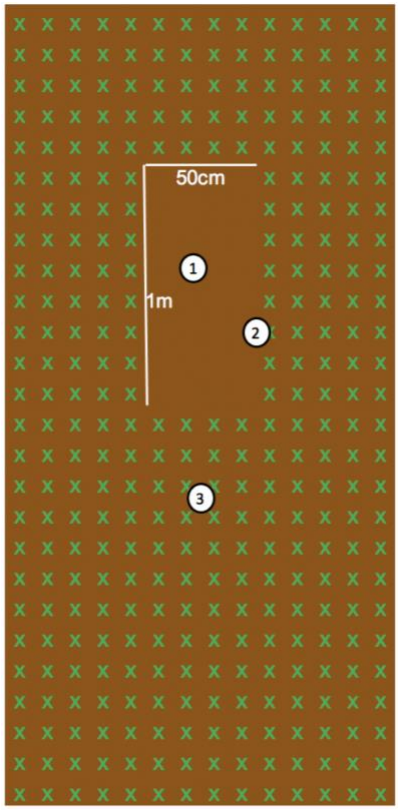


Figure 5.1 Graphic illustration of the positioning of the core for soil sampling.

SMN analysis, root washing and scanning

Soil samples were immediately taken to the laboratory for SMN determination by using the approach described above. Samples were then kept in their polyethene bags in a cold room at 4^{o}C and washed in plot order.

Root washing started immediately after SMN analysis. Roots were gently, but thoroughly washed from the soil in reverse osmosis water using a Delta-T root washer (Delta-T Devices Ltd., Cambridge, England). The Delta-T root washer allows four soil samples to be washed at the same time in four separate buckets. The flow of water in each bucket can be individually regulated.

Washed roots were carefully clean, blotted dry and frozen to -20 ^{o}C in 50 % ethanol solution. Root length was determined by placing the clean roots in a clear rectangular tray with 50ml of water to facilitate the spread of the roots in the tray. The samples were placed in a scanner (Epson®, Expression 12000XL, UK). Images were taken at 600 dpi and saved as TIFF format for root length determination. The analysis of root length was performed on scanned images using the root image analysis system WinRhizo Pro 2017 (Regent Instruments Inc. Canada).

5.2.4 Meteorological data

Mean daily temperature and monthly total rainfall data from June to August 2019 were compared to the 1991 - 2020 long term averages (Met Office, 2022). While in June the mean temperature differed little from the long-term mean (± 0.5 °C), in July the mean daily temperature was 1.5 and 2.5 °C greater than the long term mean and in August it was 0.5 and 1.5 °C greater. With regards rainfall June, July and August 2019 were wetter months compared to the average for 1991 – 2020. In June and July there was an increase of about 25 % whilst in August it was around 75 % greater than the long-term average.

5.2.5 Statistical analysis

All statistical analyses were carried out using Genstat 19th Edition (VSN International Ltd). For the variety experiment, a general analysis of variance with varieties nested within species was performed to determine the significance differences in N offtake, SMN and total N in the crop-soil system. Time of harvest was added as a factor to establish differences among sampling times. For the RLD experiment, the effect of soil depth, position within the plot and species was analysed individually for RLD and NO_3^-N concentrations, and then to establish the direct relationship between the two variables an analysis of variance with RLD as covariate was carried out. A multi-variate analysis (biplot) to test associations between traits was carried out in RStudio version 2.4.5

LSD (5%) were used to compare pairs of mean values. Residuals were checked and shown to have a normal distribution and homogeneous variance and thus transformation was not required.

5.3 Results

5.3.1 Variety experiment

5.3.1.1 Yield and yield component

Yield and its components, grain number m^{-2} and mean grain weight differed significantly between species and varieties (Table 5.2). The highest yield within spring barley varieties was 7.57 t ha^{-1} from Laureate whereas for oats the highest yield was observed with WPB Elyann (10.77 t ha^{-1}). The same was observed for the yield component grain no m^{-2} (Table 5.2). There were significant differences between species in the straw biomass ($p<0.001$), but within each species no significant differences were observed between varieties (p=0.608, Table 5.2). On average, straw biomass at maturity of spring barley was 5.94 t ha^{-1} whilst for spring oats it was significantly higher, on average 11.64 t ha^{-1}.

5.3.1.2 Crop growth characteristics at maturity

There were no significant differences between species or their varieties in the harvest index (HI) or grain N concentration at maturity (Table 5.3). Spring barley had an average HI of 0.53 and a grain N % of 1.88, compared to 0.49 and 1.82 respectively for spring oats.

The total above-ground N offtake (Total N_{off}), and the straw (chaff included) N offtake (Straw N_{off}) at maturity, were significantly different between species ($p<0.001$), but not between their varieties (Table 5.3). N offtake within spring barley ranged from 179 kg N ha^{-1} to 213 kg N ha^{-1}, and for spring oats it ranged from 285 kg N ha^{-1} to 308 kg N ha^{-1}. With regards straw N offtake, the average for spring barley was 71 kg N ha^{-1} and the average in spring oats was 115 kg N $ha^{-1.}$

Significant differences between species and their varieties were observed for the straw N concentration, the grain N offtake and the nitrogen harvest index (NHI). Straw N concentration was significantly higher in spring barley than for oats (p=0.01). Between the different varieties within each species (p=0.004), Westminster for spring barley showed the highest straw N% (1.37%) and for oats, Firth showed the highest straw N% (1.10%). These varieties also had the lowest grain N offtakes for their species and also the lowest NHI.

Table 5.2 Mean data for yield and yield components of four varieties of spring barley and spring oats. Data calculated from manually sampled areas. Weights are expressed on a 100% dry matter basis.

Species	Variety	Yield (t ha^{-1})	DM maturity t/ha	MGW (mg grain^{-1})	Grain no. m^{-2}	Straw (t ha^{-1})
Barley	Concerto	6.12	11.37	40.02	15247	5.25
	Laureate	7.57	13.75	42.67	17785	6.18
	RGT Planet	7.68	14.25	43.80	17527	6.57
	Westminster	5.61	11.36	44.21	13189	5.76
Oats	Aspen	10.41	22.07	38.99	26909	11.66
	WPB Elyann	10.77	22.67	38.32	28094	11.90
	Firth	7.72	18.88	30.88	25022	11.17
	Yukon	9.70	21.54	40.38	24100	11.85
***p*-value**	Species (S)	0.002	<0.001	0.01	0.002	<0.001
***p*-value**	S x Variety	<0.001	0.007	0.001	0.032	0.608
LSD	S x Variety	1.163	2.354	3.624	3067.4	n.s

n.s =not significant; LSD (5%) = least significant difference; MGW= mean grain weight. Varieties are nested within species; therefore, individual variety effect is not given.

Table 5.3 Mean data for crop growth characteristics at maturity of four spring barley and spring oats varieties. Data calculated from manually sampled areas.

Species	Varieties	HI	Grain N%	Straw N%	Total N_{off} kg N ha^{-1}	Grain N_{off} kg N ha^{-1}	Straw N_{off} kg N ha^{-1}	NHI
Barley		0.53	1.88	1.20	198.1	126.8	71.3	0.64
	Concerto	0.54	1.94	1.15	179.1	119.0	60.1	0.66
	Laureate	0.56	1.86	1.17	212.6	140.7	71.9	0.66
	RGT Planet	0.54	1.82	1.13	213.8	139.8	74.0	0.65
	Westminster	0.49	1.91	1.37	186.9	107.6	79.3	0.58
Oats		0.49	1.82	0.98	288.9	174.3	114.6	0.60
	Aspen	0.49	1.78	0.86	285.4	184.6	100.8	0.65
	WPB Elyann	0.55	1.69	0.93	292.2	181.9	110.3	0.62
	Firth	0.46	1.91	1.10	269.2	146.4	122.8	0.54
	Yukon	0.46	1.90	1.05	308.3	184.1	124.2	0.60
p-value	Species (S)	0.213	0.380	0.01	<0.001	<0.001	<0.001	0.045

p-value	S x Variety	0.061	0.120	0.004	0.358	0.018	0.278	<0.001
LSD (5%)	S x Variety	n.s	n.s	0.139	n.s	25.98	n.s	0.044

n.s =not significant; LSD = least significant difference; HI = harvest index, NHI= N harvest index; N_{off}= N offtake. Varieties are nested within species; therefore, individual variety effect is not given.

A higher N offtake from grains of spring oats compared to spring barley was observed at maturity ($p<0.001$), ranging from 146 kg N ha^{-1} for Firth cultivar to 184.6 kg N ha^{-1} in the Aspen cultivar. The N offtake of spring barley grains ranged from 107.6 kg N ha^{-1} for Westminster to 140.7 kg N ha^{-1} for Laureate.

With regards NHI, significant differences were observed between species ($p=0.045$) and between their varieties ($p<0.001$). Overall, barley showed a slightly higher NHI, on average 0.64, whereas for oats the average NHI was 0.60.

5.3.1.3 Total N offtake (N_{off}) and SMN depletion

Nitrogen offtake

Fig. 5.2 shows the mean data of N_{off} and SMN at anthesis, at barley maturity (where oats were also harvested) and at oats maturity. Total N offtake (N_{off}) at anthesis showed no significant differences between species or their varieties (Table 5.4). On average, the N_{off} of spring barley at anthesis, ranged from 179.12 kg N ha^{-1} for the Concerto cultivar to 205.23 kg N ha^{-1} for the Laureate cultivar. In spring oats, the N_{off} ranged from 185.32 kg N ha^{-1} for the Firth cultivar to 194.34 kg N ha^{-1} for the Aspen cultivar.

At barley maturity, there was a slight (~ 4 to 17 %) increase in the N_{off} in all the spring barley varieties except for Concerto, compared to the total N_{off} at anthesis. Spring oats were also sampled on the same calendar day as barley maturity and showed a larger increase in N_{off} compared to both its N_{off} at anthesis and the N_{off} of spring barley at maturity. However, this was also variety dependent. Analysis of variance showed that there was a significant interaction between species x varieties x time of harvest ($p = 0.029$, Table 5.5) when the analysis was confined to the period anthesis to barley maturity. There was no significant increase in N_{off} of WPB Elyann and Firth between anthesis and barley maturity, but there was a ~ 50 % increase in Aspen and Yukon.

Comparing N_{off} at barley and oat maturity showed significant differences between species ($p<0.001$) but no significant differences between their

cultivars (p=0.173). At maturity, the total N offtake of spring barley cultivars ranged from 179.1 kg N ha^{-1} for Concerto to 213.7 kg N ha^{-1} for RGT Planet. Spring oats on the other hand, had significantly higher N offtake at maturity, with Firth showing an average of 269.2 kg N ha^{-1} and Yukon an average of 308.2 kg N ha^{-1}.

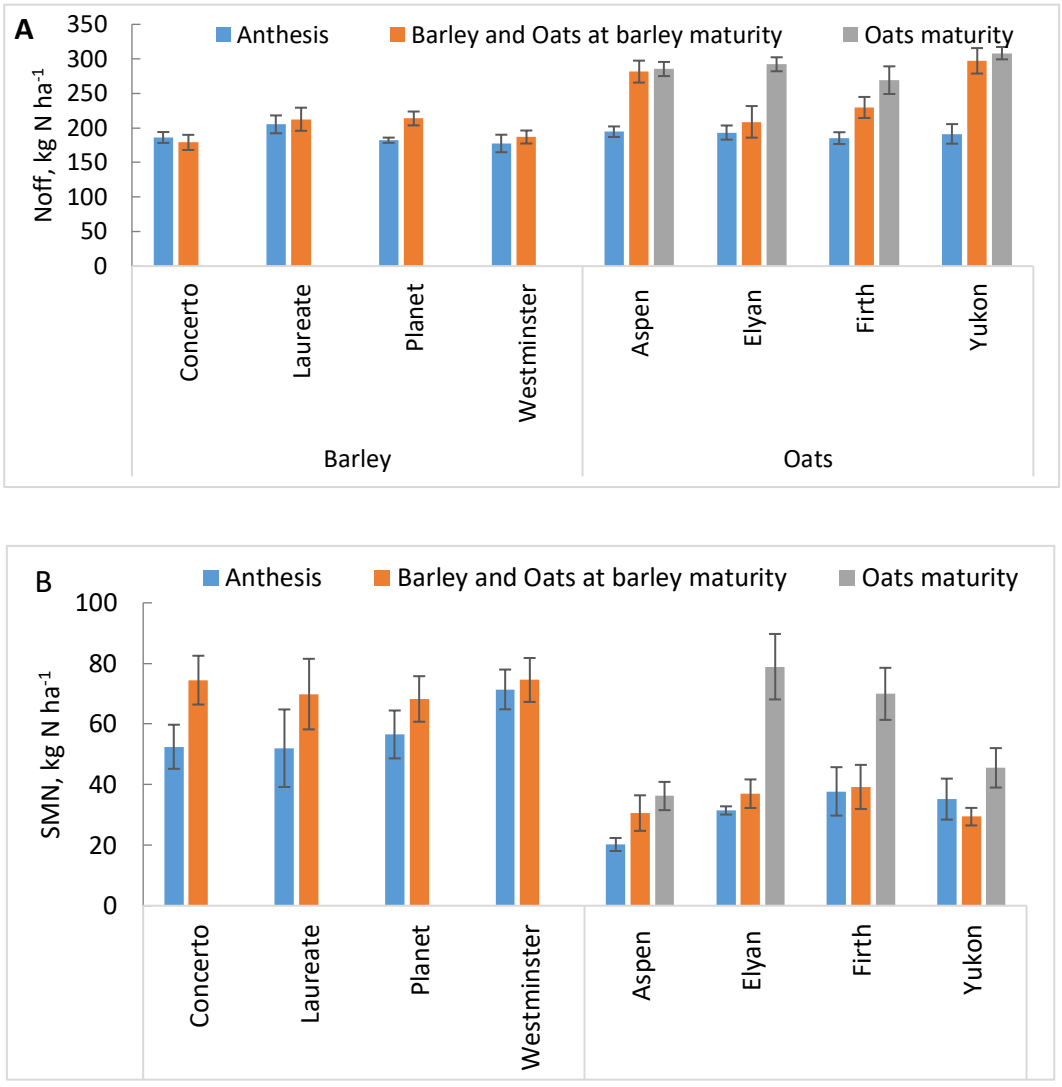


Figure 5.2 Mean data of total nitrogen offtake ($N_{off,}$ A) and soil mineral nitrogen (SMN, B) measured at anthesis and maturity in spring barley, and anthesis, on the same calendar day as barley maturity and physiological maturity of spring oats. Data are mean values of four replicates ± SEM.

Table 5.4 Species differences in total N offtake and soil mineral nitrogen (SMN) at anthesis, on the calendar date when barley was at physiological maturity, and when both barley and oats were at physiological maturity. Values are means of four replicates for each of four cultivars.

		Total N_{off} kg N ha^{-1}			SMN kg N ha^{-1}			Total crop available N (N_{off} + SMN) kg N ha^{-1}		
Species		**Anthesis**	**Barley maturity**	**Maturity**	**Anthesis**	**Barley maturity**	**Maturity**	**Anthesis**	**Barley maturity**	**Maturity**
Barley		187.8	198.1	198.1	58.1	71.8	71.8	245.9	270.1	270.1
Oats		191.2	254.3	288.7	31.2	34.1	57.7	222.4	288.4	346.4
p	Species	0.578	<0.001	<0.001	<0.001	<0.001	0.013	0.006	0.198	<0.001
p	S x V	0.368	0.006	0.173	0.235	0.921	0.01	0.841	0.09	0.345
LSD (5%)	S x V	n.s	45.89	n.s	n.s	n.s	22.52	n.s	n.s	n.s

n.s = not significant; LSD = least significant difference. Varieties are nested within species (S x V); therefore, individual variety effect is not given. At barley maturity oats were also harvested.

Table 5.5 ANOVA of effect of species, varieties and time of harvest from anthesis to calendar date of barley maturity in total N offtake, soil mineral nitrogen (SMN) and Total N_{off} + SMN.

Means		Total N_{off}	SMN	SMN + N_{off}
Species (S)	Barley	193.1	65.0	258
	Oats	222.8	32.6	255.4
Time (T)	Anthesis	189.5	44.6	234.1
	Barley Maturity	226.3	52.9	279.3
S x T	Barley x Anthesis	187.8	58.1	245.9
	Barley x B Maturity	198.3	71.8	270.1
	Oats x Anthesis	191.2	31.2	222.3
	Oats x B Maturity	254.3	34.1	288.4
S x V	Concerto	182.7	63.5	246.2
	Laureate	208.9	60.9	269.9
	RGT Planet	198.4	62.4	260.9
	Westminster	182.2	73.0	255.2
	Aspen	238.1	25.4	263.5
	WPB Elyann	201.1	34.2	235.3
	Firth	207.5	38.5	246.0
	Yukon	244.3	32.2	276.7

Effect	p	LSD	p	LSD	p	LSD
S	<0.001		<0.001		0.754	n.s
T	<0.001		0.024		<0.001	
S x V	0.007	27.36	0.335	n.s	0.194	n.s
S x T	<0.001	19.35	0.136	n.s	0.017	24.1
S x V x T	0.029	38.7	0.756	n.s	0.196	n.s

n.s = not significant; LSD (5%) = least significant difference. Varieties are nested within species (S x V); therefore, individual variety effect is not given

Table 5.6 ANOVA of effect of species, varieties and time of harvest from anthesis to crop maturity (barley and oat at maturity) in total N offtake, soil mineral nitrogen (SMN) and Total N_{off} + SMN.

Means		Total N_{off}	SMN	SMN + N_{off}
Species (S)	Barley	193.1	65.0	258
	Oats	240	44.4	284.4
Time (T)	Anthesis	189.5	44.6	234.1
	Maturity	243.5	64.7	308.3
S x T	Barley x Anthesis	187.8	58.1	245.9
	Barley x Maturity	198.3	71.8	270.1
	Oats x Anthesis	191.2	31.2	222.3
	Oats x Maturity	288.8	57.7	346.4
S x V	Concerto	182.7	63.5	246.2

Laureate	208.9	60.9	269.9
RGT Planet	198.4	62.4	260.9
Westminster	182.2	73.0	255.2
Aspen	239.9	28.2	268.2
WPB Elyann	242.8	55.2	298.0
Firth	227.3	53.8	281.1
Yukon	249.9	40.4	290.1

Effect	p	LSD	p	LSD	p	LSD
Species	<0.001		<0.001		<0.001	
Time	<0.001		<0.001		<0.001	
S x V	0.064	n.s	0.003	14.14	0.248	n.s
S x T	<0.001	15.52	0.074	n.s	<0.001	19.75
S x V x T	0.444	n.s	0.128	n.s	0.695	n.s

n.s = not significant; LSD = least significant difference at 5%. Varieties are nested within species (S x V); therefore, individual variety effect is not given

The analysis of variance carried out on anthesis and crop maturity (barley and oats at their physiological maturity) data, showed that while the species and the time of harvest as individual factors were significant, as was the species x time of harvest interaction ($p<0.001$), there was no interaction between species x variety x time of harvest ($p=0.444$, Table 5.6). Thus, all oat varieties accumulated a significantly greater amount of N in above ground tissue between anthesis and maturity compared to barley.

SMN depletion

At anthesis, while the N_{off} was fairly similar among the different species and their cultivars, the SMN was significantly higher ($p=0.01$) in spring barley compared to the SMN measured in spring oats (Fig. 5.2b; Table 5.3). SMN in spring barley plots at anthesis, was on average of 58.1 kg N ha^{-1} whereas in

spring oats plots it was 31.2 kg N ha^{-1}. There were no significant differences between the varieties within each species in the SMN at anthesis (p=0.235, Table 5.2).

The SMN of spring oat plots measured at the time of barley physiological maturity was significantly lower than that of barley plots (p<0.001) and differences between their cultivars were not found (Fig. 5.2b; Table 5.3).

With regards SMN at crop maturity, statistically significant differences were observed between species (p=0.013). The SMN of spring barley was, statistically, significantly higher than the SMN of spring oat plots, but the species differences were variety dependant (p=0.01). On average, spring barley plots showed a SMN between 68 and 74 kg N ha^{-1} across varieties, whereas for spring oats, cultivars such as Aspen and Yukon showed significantly lower SMN (36.2 kg N ha^{-1} and 45.5 kg N ha^{-1}, respectively) than WPB Elyann and Firth (80 and 70 kg N ha^{-1}, respectively).

A statistical analysis (ANOVA) was performed, including the time of harvest as a factor, to determine if the SMN changed significantly between the different harvest times. Tables 5.4 and 5.5 show the effect of species, varieties and time of harvest, and their interactions on the SMN. Between anthesis and the date of barley maturity, and also between anthesis and crop maturity of each species, there was a significant overall increase in SMN when averaged across species. A weak (p=0.074) species x time interaction in the anthesis to crop maturity analysis (Table 5.5) indicates that the increase in SMN between anthesis and crop maturity tended to be larger in oats than barley when averaged over varieties.

Total crop available N (soil mineral nitrogen plus total N offtake)

The total crop available N in the plant-soil system can be calculated by adding up the total soil mineral nitrogen and the total N offtake of the crop. Fig. 5.3 shows the total N in the system at the different harvest times for barley and oats.

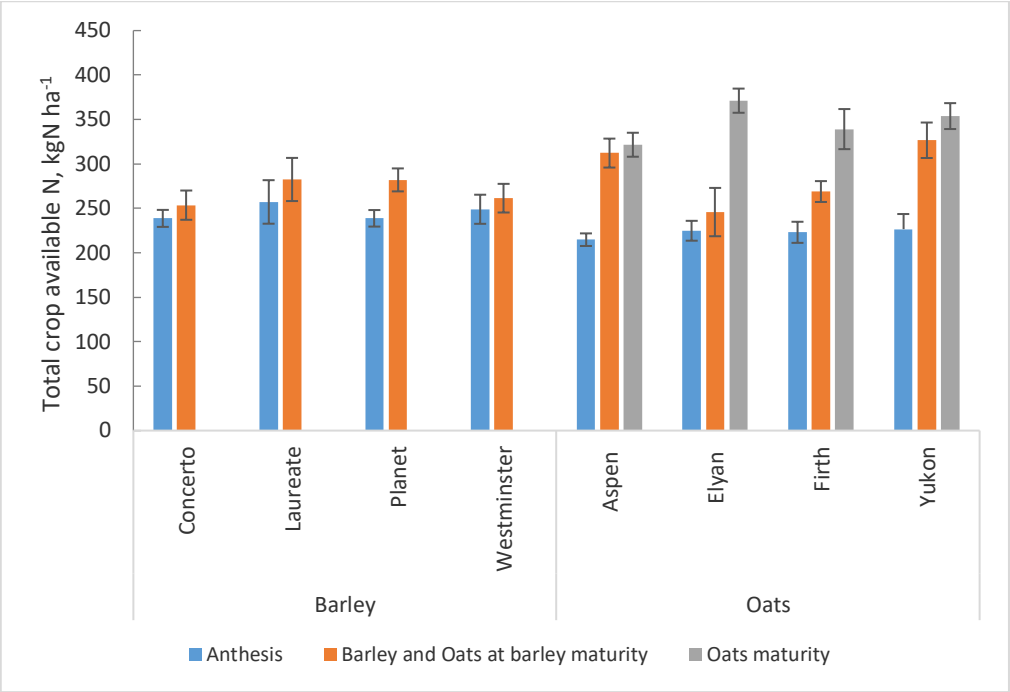


Figure 5.3 Total N in the plant-soil system (SMN + N_{off}) of spring barley and spring oats cultivars, at anthesis, at barley maturity and oats maturity.

At anthesis, barley showed a significantly higher crop available N than oats ($p=0.006$, Table 5.4). There were no significant differences between their varieties ($p=0.841$). On average, barley crops showed a total of 245.9 kg N ha^{-1} while oats showed a total of 222.3 kg N ha^{-1} in the crop-soil system. The total amount in the crop-soil system showed that when barley has reached maturity, but oats were yet to mature, no significant differences were observed between species or varieties ($p=0.198$ and $p=0.09$ respectively, Table 5.4).

When comparing the total N in the crop-soil system between species at their physiological maturity, oats showed a significant higher total N ($p<0.001$), even though the SMN was considerably lower in oats at maturity specially in Aspen and Yukon cultivars. The higher N in the crop-soil system observed in the oats plots was consistent across varieties, with an average of 346.4 kg N ha^{-1} compared to the 270.1 kg N ha^{-1} measured in the barley plot.

Between anthesis and the date at which barley reached maturity, no significant differences were found between species or their varieties (Table 5.5). There

was a strong effect of time and species x time ($p<0.001$ and $p=0.017$, respectively) with both crops showing significantly higher available N at barley maturity and barley showing higher N in the soil-system at anthesis than oats at anthesis.

On the other hand, between anthesis and crop maturity, a strong effect was found among species, time and species x time ($p<0.001$) on the total available N in the crop-soil system. The varieties within the species showed no differences nor the species x variety x time interaction.

5.3.1.4 Post-anthesis N uptake and Nitrogen remobilization efficiency from vegetative tissue

The PANU of the oats was, in general, higher than the PANU of the barley cultivars ($p < 0.001$, Table 5.7, Fig 5.4). On average, oats had taken up a total of 97.6 kg N ha^{-1} whereas barley cultivars had taken up an average of 10.3 kg N ha^{-1} with concerto showing no PANU but instead a net loss of N (- 7.13 Kg N ha^{-1}). There were no significant differences within the different cultivars.

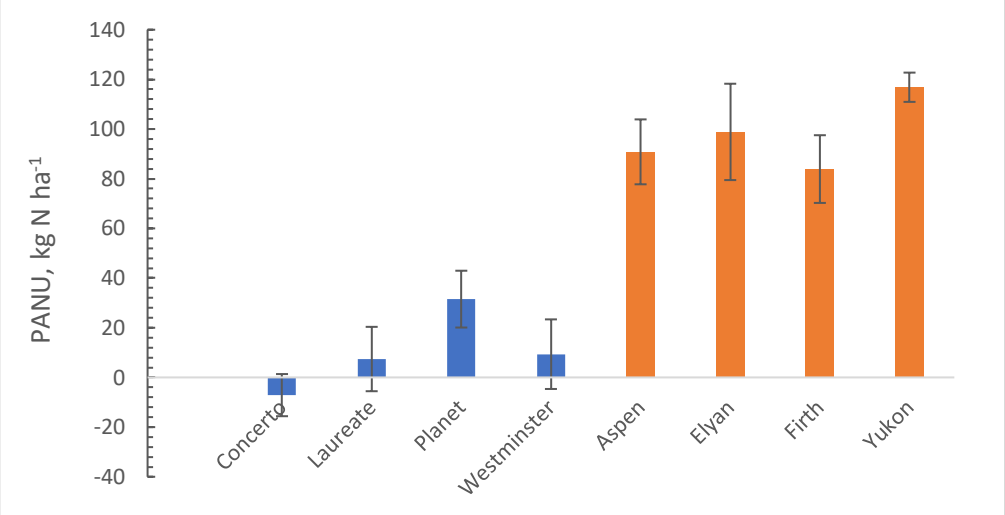


Figure 5.4 Post-anthesis N uptake of barley and oats cultivars. Data are mean values of four replicates ± SEM.

Although the PANU was higher in oats, the nitrogen remobilization efficiency from the vegetative tissues as well as the total NRE was lower in this species

(Table 5.7). Therefore, a strong negative relationship between PANU and total NRE was observed in both crop species (R^2 = 0.64 and 0.70 for barley and oats respectively).

On average, the remobilization efficiency of barley was 60.6%, whereas for oats it was almost half. 32.5%. The most efficient variety in barley was concerto, and in oats was Aspen with 66.1% and 42.7%, respectively.

Table 5.7 Effect of post-anthesis N uptake (PANU) and Nitrogen remobilization efficiency (NRE) form vegetative tissue (leaves and stems) and total NRE in barley and oats cultivars.

Species	Variety	PANU	NRE Leaves	NRE Stems	Total NRE
Barley		10.30	75.47	48.21	60.61
	Concerto	-7.13	80.73	54.54	66.13
	Laureate	7.40	76.91	56.08	65.49
	RGT Planet	31.52	71.98	47.59	57.53
	Westminster	9.36	72.27	34.63	53.30
Oats		97.60	52.79	6.35	32.52
	Aspen	90.82	60.12	15.60	41.75
	WPB Elyann	98.85	59.17	7.09	36.08
	Firth	83.89	52.56	-0.59	29.45
	Yukon	116.84	39.30	3.31	22.78
p-value	Species	<0.001	<0.001	<0.001	<0.001
	Species x Variety	0.288	0.001	0.278	0.043
LSD	Species	1.926	4.63	10.7	6.55
	Species x Variety	n.s	9.27	n.s	13.09

n.s = not significant; LSD = least significant difference at 5%. Varieties are nested within species (S x V); therefore, individual variety effect is not given

5.3.1.5 Nitrogen utilization efficiency (NutE)

The N utilization efficiency (NutE) was calculated on a grain weight basis as well as on total crop biomass at maturity basis. Table 5.6 shows that differences between species in $NutE_g$ and $NutE_b$ were not statistically significant. The NutE expressed on a grain weight basis (g) was significantly different between the varieties within each species ($p<0.001$). RGT Planet showed the highest $NutE_g$ (35.9 kg grain kg N_{off}^{-1}) amongst the barley varieties whereas Westminster showed the lowest (30.1 kg grain kg N_{off}^{-1}). Within oats the highest $NutE_g$ was observed for the cultivar WPB Elyann (36.6 kg grain kg N_{off}^{-1}) and Firth showed the lowest $NutE_g$ (28.76 kg grain kg N_{off}^{-1}).

Table 5.8 Differences between four spring barley and four spring oat cultivars in nitrogen utilization efficiency calculated on a grain weight ($NutE_g$) and crop biomass basis ($NutE_b$).

Species	Variety	**$NutE_g$** kg grain kg N_{off}^{-1}	**$NutE_b$** kg biomass kg N_{off}^{-1}
Barley		34.00	64.00
	Concerto	34.06	63.63
	Laureate	35.83	64.58
	RGT Planet	35.96	66.70
	Westminster	30.11	61.01
Oats		33.48	68.82
	Aspen	36.66	74.42
	WPB Elyann	36.91	68.37
	Firth	28.76	62.99
	Yukon	31.58	69.49
p-value	Species (S)	0.513	0.053

p-value	S x V	<0.001	0.354
LSD (5%)	S x V	2.958	n.s

n.s =not significant; LSD=least significant of difference. Varieties are nested within species (SxV); therefore, individual variety effect is not given.

5.3.1.6 Partial factor productivity (PFP)

A common index of NUE is the partial factor productivity (PFP), which describes the crop biomass produced per kg of N fertilizer applied (Dobermann, 2007). The PFP was significantly higher for oats than barley ($p<0.001$, Table 5.9) when calculated at both anthesis and crop maturity. There were also significant differences among the different varieties within the species at crop maturity ($p=0.012$) but not anthesis.

Table 5.9 Partial Factor Productivity (PFP) expressed as kg of above ground crop biomass per kg of fertilizer N applied, for spring barley and spring oat cultivars. Data are the mean of four replicates.

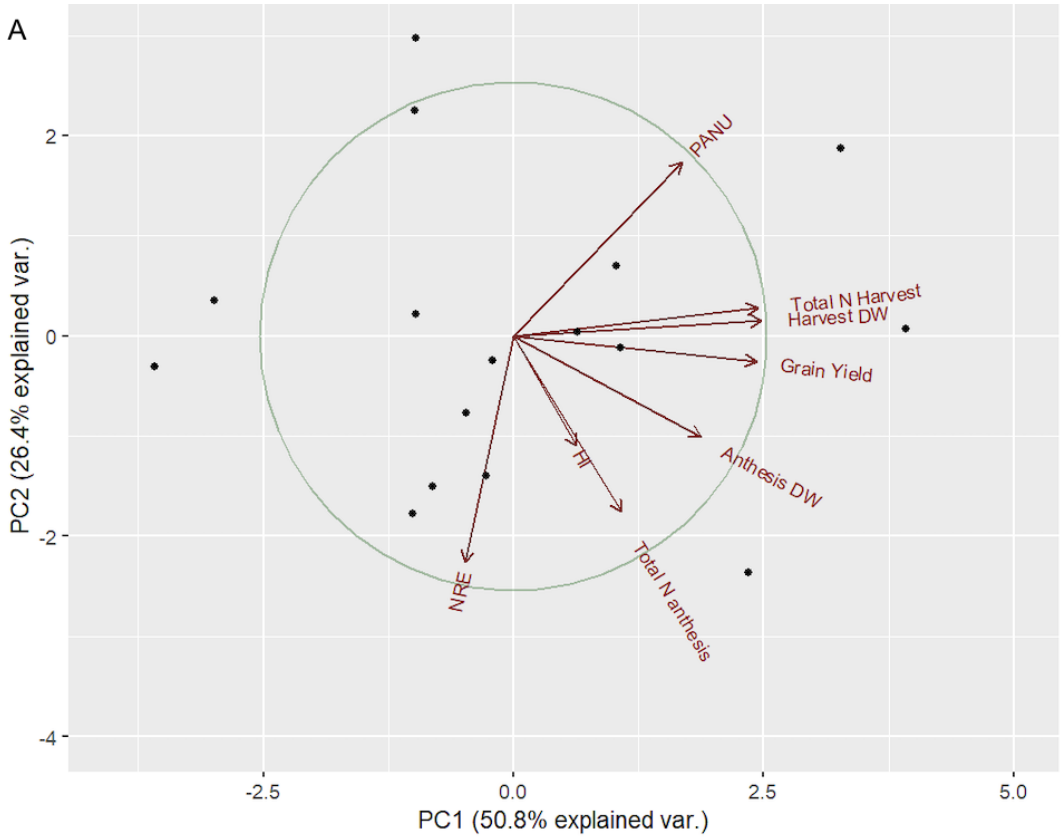
	PFP at anthesis	**PFP at maturity**
Barley	70.90	105.70
Concerto	70.96	94.78
Laureate	74.81	114.54
RGT Planet	74.30	118.74
Westminster	63.53	94.72
Oats	87.80	177.43
Aspen	91.72	183.89
WPB Elyann	86.87	188.94
Firth	83.75	157.38
Yukon	87.87	179.5

p-value		
Species (S)	<0.001	<0.001
S x V	0.265	0.012
LSD (5%) S x V	n.s	20.43

n.s =not significant; LSD=least significant of difference. Varieties are nested within species (SxV); therefore, individual variety effect is not given.

5.3.1.7 Multi-variate analysis of key traits measured

A multi-variate analysis including PANU, NRE, grain yield, harvest index (HI), DW at anthesis, DW at harvest, N content at anthesis and N content at harvest was carried out to establish the relative contribution or trade-off among traits for each group species. Fig. 5.5 shows the biplot of this multi-variate analysis.



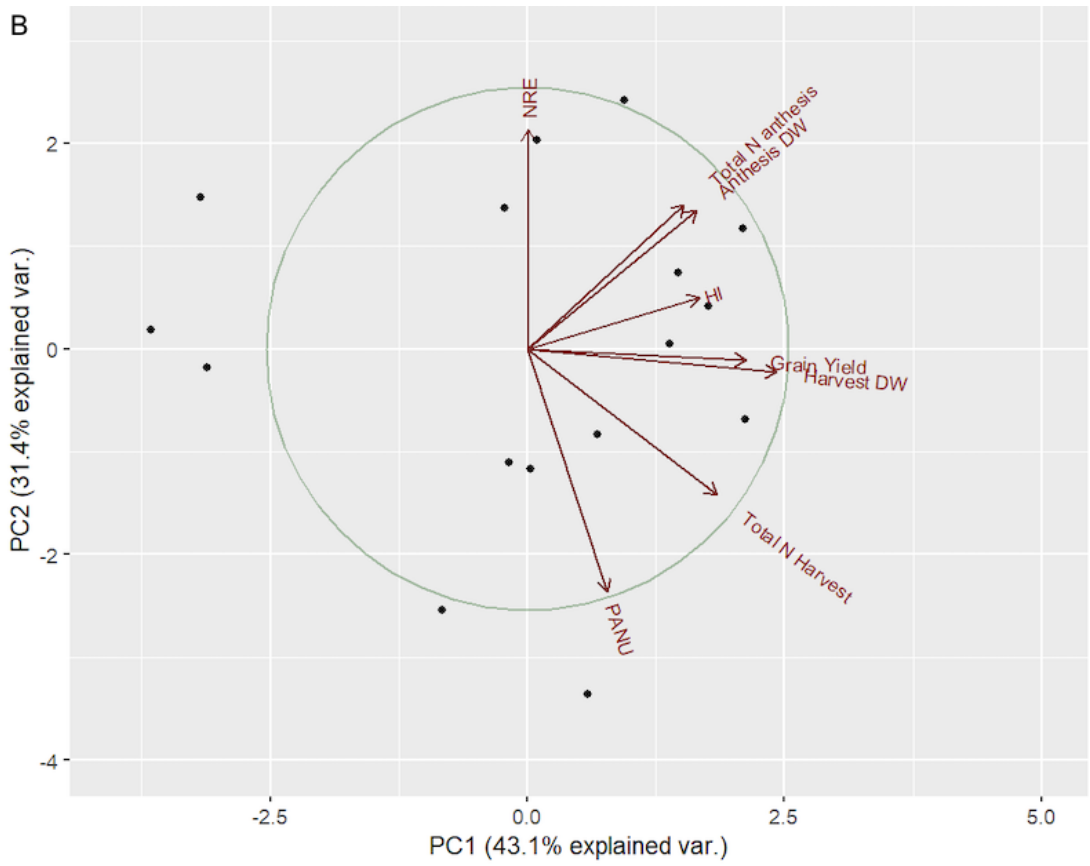


Figure 5.5 Biplot for grain yield, N remobilization efficiency (NRE), post-anthesis N uptake (PANU), Dry weight (DW) at anthesis, DW at harvest, total N content at anthesis, total N content at harvest and Harvest index (HI) for barley (A) and oats (B).

In barley, PANU was positively and strongly associated among cultivars with grain yield. DW and total N at harvest (R^2 = 0.63, 0.68 and 0.68 respectively). PANU was negatively associated with NRE and total N content at anthesis (R^2 = 0.64 and 0.35). NRE, showed a positive association with HI (R^2 = 0.49) but almost no association with grain yield (R^2 = 0.01). Whereas grain yield was strongly associated with DW and total N at harvest (R^2 = 0.93 and 0.88). It was also positively associated among cultivars with DW and total N content at anthesis and HI (R^2 = 0.66, 0.35 and 0.47, respectively).

In oats, PANU was positively associated among cultivars with grain yield, DW at harvest and total N at harvest (R^2 = 0.30, 0.35 and 0.76, respectively). PANU negatively associated among the cultivars with NRE, DW at anthesis and total

N content at anthesis (R^2 = 0.70, 0.28 and 0.36 respectively). NRE, on the other hand, showed weak positive associations with grain yield (R^2 = 0.17), DW and Total N at anthesis (R^2 = 0.28), and HI (R^2 = 0.20). NRE was negatively associated among cultivars with total N content at harvest (R^2 = 0.51). Grain yield and DW at harvest showed a strong positive association among oats cultivars (R^2 = 0.93). Grain yield and HI showed an association of 0.55. The DW and the total N content at anthesis as well as at harvest were strongly associated among the cultivars (R^2 = 0.82 and R^2 = 0.65, respectively.) Harvest index was positively correlated with all the traits, but the strongest correlations were observed with grain yield (R^2 = 0.55) and DW at harvest (R^2 = 0.61).

For oats and barley, there was almost no association between PANU and HI (R^2 = 0.09 and 0.01, respectively).

5.3.2 Root length density experiment

5.3.2.1 Root length density measurements

There were significant main effects of species, position of sampling and soil depth on the root length density (RLD) (Table 5.10). When averaged over the other factors RLD was greater in oats than barley. It was also greater in the topsoil compared to subsoil and declined progressively when the plant density was reduced from sampling position 3 to 1. There was a strong (p=0.019) species x position interaction. Thus, the RLD for oats (averaged over soil depth) declined from 3.15 cm cm^{-3} in the well-populated parts of the plot to 0.84 cm cm^{-3} in the weeded area 20 cm from the nearest crop plant, compared to a decline from 1.31 to 0.63 cm cm^{-3} for barley.

Table 5.10 Root length density (RLD, cm cm^{-3}) of barley (cv Westminster) and oat (cv Firth) plots sampled at three different positions within the plot and at two soil depths three weeks after anthesis. Data are the means of three replicates.

		Depth		
Species	**Position**	**10-25 cm**	**40-55 cm**	**Mean**
Barley	1. No plants	0.96	0.31	0.63
	2. Boundary	2.25	0.88	1.56
	3. Well-populated	1.52	1.10	1.31
Oats	1. No plants	1.39	0.29	0.84
	2. Boundary	2.31	0.41	1.36
	3. Well-populated	3.88	2.41	3.15
Mean species	Barley	1.58	0.76	1.17
	Oats	2.53	1.04	1.78
Mean position	1. No plants			0.74
	2. Boundary			1.46
	3. Well-populated			2.23
Mean depth		2.05	0.90	
Effect		**p**	**LSD**	
Species		0.042	0.59	
Position		0.001	0.722	
Depth		<0.001	0.59	
Species x Position		0.019	1.021	
Species x Depth		0.253	ns	

Position x Depth	0.492	ns
Species x Position x Depth	0.896	ns

n.s = not significant; LSD (5%) = Least significant difference.

As expected, the area where plants were pulled showed the lowest RLD. However, even in this position oats showed a greater RLD, especially in the topsoil. For Westminster, in the topsoil (10-25cm) the RLD increased form 0.96 cm cm^{-3} in the area where no plants were allowed to grow, to 1.52 cm cm^{-3} in the well populated position. In the Firth variety on the other hand, in the topsoil the RLD increased from 1.39 cm cm^{-3} in the position 1 to 3.88 cm cm^{-3} in position 3.

5.3.2.2 Soil NO_3^--N concentration

There were significant effects of crop species ($p=0.008$) and sampling position ($p<0.001$) on the soil NO_3^--N concentration, but no overall effect of soil depth (Table 5.11). There were also no interactions between species, sampling position and depth on soil nitrate NO_3^--N concentration. Thus, concentrations were 75% greater in barley plots compared to oats and three to four times greater in areas where crop plants had been removed compared to the well-populated and boundary areas. (Table 5.11).

On average, in the topsoil, the nitrate N concentration in the barley plots ranged from 16.1 mg NO_3^--N kg dry $soil^{-1}$ in position 1 (no plants) to 5.05 mg NO_3^--N kg dry $soil^{-1}$ in position 3 (well-populated). In the oat plots, the NO_3^- N concentration ranged from 5.62 mg NO_3^--N kg dry $soil^{-1}$ in position 1 to 2.67 mg NO_3^--N kg dry $soil^{-1}$ in position 3.

Table 5.11 Soil NO_3^- N concentration in mg NO_3^--N kg dry $soil^{-1}$ of barley (cv Westminster) and oat (cv Firth) plots taken at three different positions within the plot and two soil depths three weeks after anthesis. Data are the means of three replicates.

		Depth		
Species	**Position**	**10-25 cm**	**40-55 cm**	**Mean**
Barley	1. No plants	16.1	14.21	15.15
	2. Boundary	6.81	5.69	6.25
	3. Well-populated	5.05	2.90	3.97
Oats	1. No plants	5.62	12.12	8.87
	2. Boundary	2.95	4.11	3.53
	3. Well-populated	2.67	1.57	2.12
Mean species	Barley	9.32	7.60	8.46
	Oats	3.75	5.93	4.84
Mean position	1. No plants			12.01
	2. Boundary			4.89
	3. Well-populated			3.05
Mean depth		6.53	6.77	
Effect		**p**	**LSD**	
Species		0.008	2.557	
Position		<0.001	3.132	
Depth		0.853	ns	
Species x Position		0.318	ns	
Species x Depth		0.127	ns	
Position x Depth		0.441	ns	
Species x Position x Depth		0.442	ns	

n.s = not significant; LSD (5%) = Least significant difference.

5.3.2.3 Relationship between RLD and NO_3^--N concentration in the soil

When RLD was added as a co-variate in the analysis of variance of soil NO_3^- N concentration a weak species effect was still seen (p=0.059, Table 5.12). The overall mean calculated and adjusted for the covariate showed a higher soil [NO_3^-] in barley plots (8.3 mg NO_3^- kg dry $soil^{-1}$) compared to oat plots (4.96 mg NO_3^- kg dry $soil^{-1}$).

Table 5.12 Anova table of effect of species, position and soil depth within positions on the soil [NO_3^- -N] concentration of spring barley and spring oat plots when RLD was used as a co-variate.

Effect	p-value	LSD (5%)
Species	0.059	n.s
Position	<0.001	3.809
Depth	0.939	n.s
Species x Position	0.281	n.s
Species x Depth	0.162	n.s
Position x Depth	0.447	n.s
Species x Position x Depth	0.442	n.s
Covariate: RLD	0.678	n.s

n.s = not significant; LSD (5%) = Least significant difference.

Fig 5.6 shows individual measurements of RLD for Westminster (A) and Firth (B) cultivars and their corresponding soil nitrate N concentrations taken from three different positions within the plots.

In oats, with the exception of one outlier, at the expected cRLDs (1 to 2 cm cm^{-3}) there was a lower nitrate N concentration compared to the barley plots. As the RLD was reduced from a maximum of 4.58 cm cm^{-3}, the soil NO_3^--N concentration was maintained between around 0.34 and 3.59 mg NO_3^--N kg DW^{-1} soil until RLD fell below 0.5 cm cm^{-3}, after which the NO_3^--N concentrations rose sharply.

In barley, this relationship was much more variable. Above an RLD of 0.5 cm cm^{-3} there was a range of high soil nitrate-N concentrations (5 to 17 mg N kg DW^{-1} soil) and only a few individual plants showing lower nitrate-N concentrations. Over the values of RLD regarded as critical for water and nitrate uptake (1-2 cm cm^{-3}) the soil nitrate-N concentration in barley ranged from 2.0 to 17.6 mg NO_3^--N kg DW^{-1} soil, whilst for oats it was 0.34 to 2.55 mg N kg DW^{-1}.

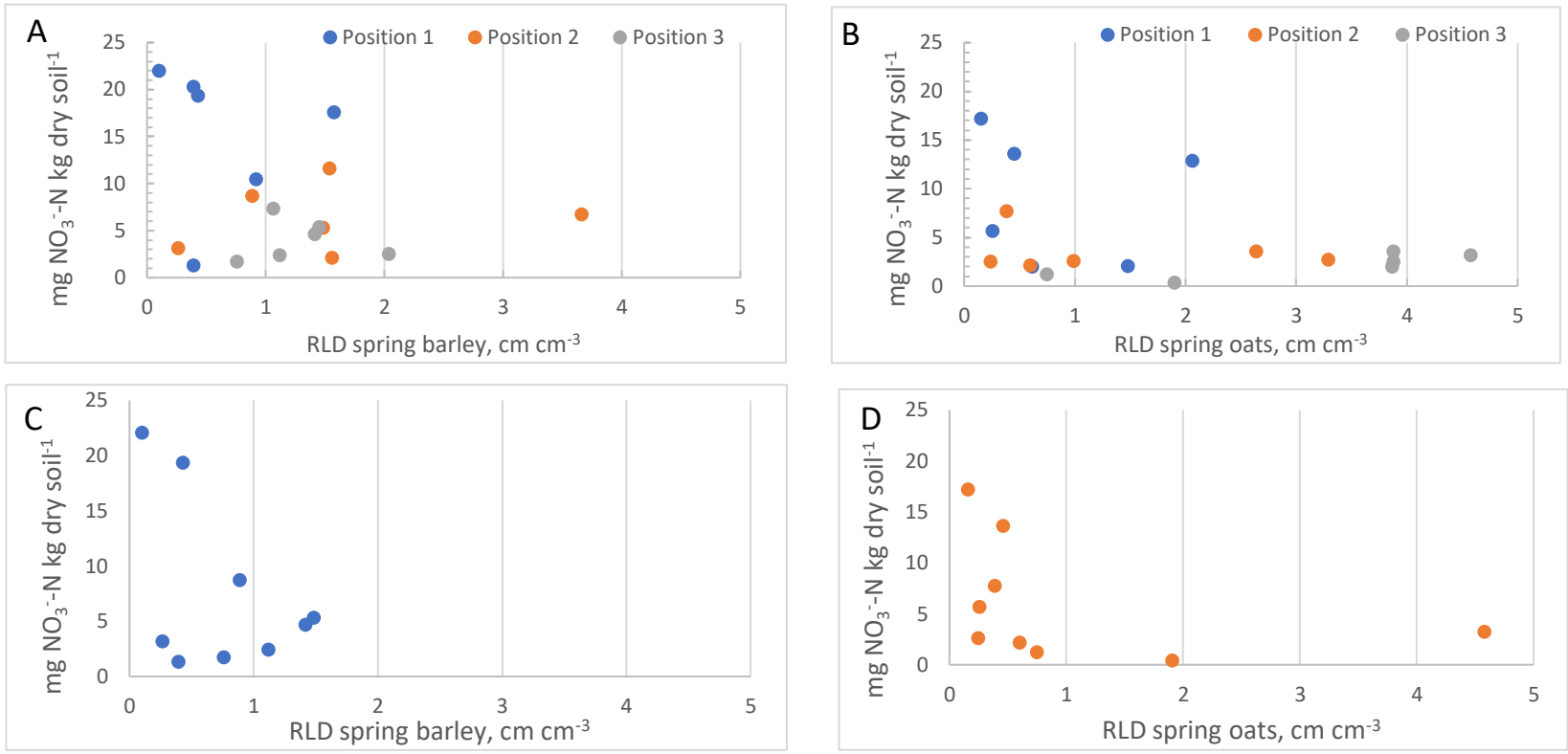


Figure 5.6 Relationship between RLD and soil NO_3^- N concentration in spring barley (A,C) and spring oat plots (B,D). A and B are individual data points collected at each position. C and D are individual data points for RLD and soil NO_3^- N concentration in the lower soil horizon (depth 40-55 cm)

5.4 Discussion

Are differences in soil N depletion between barley and oat consistent across varieties?

The relative ability of spring barley and spring oats to deplete the soil N pool has been studied previously using a single cultivar of each (Bingham, unpublished work). The spring barley cultivar Westminster was shown to deplete SMN to a lesser extent that the oat cultivar Firth and leave a larger residual soil nitrate concentration at harvest. The experiment in this chapter was set up to establish whether the differences previously observed between the cultivars Westminster and Firth are a characteristic of the species and thus consistent across varieties, and to determine whether the greater residual SMN under barley is a function of a smaller root length density.

Differences between barley and oats with regards SMN have to be considered independently at each sampling time due to the differences in the rate of crop development and changing soil N dynamics observed during the post-anthesis period. Anthesis dates per individual variety were not recorded as they were not more than two days apart.

At anthesis, the four barley varieties used in this experiment, showed consistently higher SMN contents than the oat varieties, suggesting that the poorer apparent SMN depletion observed in barley is a characteristic of the species at this particular stage of crop development. On average, oats showed 47% less SMN than barley at anthesis.

At barley maturity the differences in SMN content between barley and oats were maintained, although between anthesis and this harvest time there was an increase in SMN especially in the barley plots.

By contrast, the differences in SMN observed when both crops reached their respective physiological maturity were variety dependent with oat varieties WPB Elyann and Firth showing SMN contents comparable to those measured in the barley plots at maturity. These two varieties showed a significant

increase in the residual SMN left at physiological maturity compared to that measured at anthesis or at barley maturity. The possible reasons for an increase SMN during the post-anthesis stage will be discussed later.

Is the greater residual soil nitrate concentration in barley the result of a smaller RLD?

Differences in the ability of various plant species to deplete the inorganic N pool of the soil have been found to be associated with differences in root growth, particularly in rooting depth and penetration (Kage, 1997; Shahzad et al., 2018; Thorup-Kristensen, 2006).

The ability of oats to take-off rapidly, scavenge nutrients and compete with weeds better than any other spring oats has been recognised (van Heyzen, 2021). It has also been suggested that oats have an excellent fibrous root structure and will put more emphasis than barley into rooting before shooting (van Heyzen, 2021). With this context, the hypothesis of a larger RLD being responsible for the lower SMN residue in oats was tested.

RLD is considered to be a precise predictor of potential water capture (Carvalho et al., 2014) and subsequently nitrate capture due to its high mobility and negative charge (Glass, 2003). For 90% capture of available water the critical root length density (cRLD) of barley cv Rum has been estimated to be 0.96 cm cm^{-3} (Carvalho et al., 2014). (Wiesler and Horst, 1994) found a correlation between RLD of maize and subsoil nitrate concentrations where values of RLD were low. No correlation was found in the topsoil where RLD was typically greater than 3.8 cm cm^{-3}. Kage (1997) reported that the lower RLD of faba beans could account for the greater residual SMN found with this species compared to oats

In the current experiment the relationship between RLD and soil nitrate concentration was determined by using plant thinning and depth of soil sampling to vary the RLD. Root length density and soil nitrate were measured three weeks after anthesis, which is the period where the SMN was

significantly and consistently different between barley and oat varieties. It is important to mention that the differences observed in the SMN contents of barley and oat plots in the variety experiment were largely the result of differences in the soil NO_3^--N concentrations rather than the NH_4^+-N concentrations (Table S5.1). Therefore, only soil NO_3^--N concentrations were considered for this experiment.

Overall, roots of Firth had a higher RLD than Westminster, especially in the topsoil. Furthermore, the average diameter of Firth roots was significantly greater than Westminster (Table S5.2). The smaller RLD of barley is, therefore, consistent with the greater residual SMN observed in this crop compared to oats. However, when compared at the same RLD of ~1cm cm^{-3}, regarded as being around the cRLD for water and nitrate uptake by cereal crops (King et al. 2003), NO_3^--N concentrations were generally lower in oats (between 0.75 and 2.5 mg NO_3^--N kg DW^{-1} soil) compared to barley (1.1 to 10.4 mg NO_3^--N kg DW^{-1} soil). These results suggest that the smaller RLD of barley may contribute to, but not account entirely for, the greater SMN found with this crop during grain filling. This conclusion is supported by the analysis of crop species effects on soil nitrate concentrations using RLD as a covariate. A weak effect of species remained when RLD was used as a co-variate, suggesting that the differences in soil nitrate concentration in oat and barley plots cannot be explained entirely by differences in RLD. Certainly, other root characteristics such as the amount and role of root cortical aerenchyma (RCA) contribute to differences in the ability of the root systems to acquire N (Saengwilai et al., 2014). For example, in maize, under low N conditions it was revealed that the formation of RCA increased, and the rooting depth was also increased by 31%. The ability of this root systems to explore deeper layers of soil may have contributed to the increase leaf N content observed and ultimately the 58% increase in grain yield (Saengwilai et al., 2014).

Is the greater residual SMN in barley associated with a lower crop N uptake?

If the greater SMN content of barley plots at anthesis was simply the result of a smaller N uptake and hence depletion of the available SMN, a smaller crop N offtake would be expected compared to oats. Like the SMN analysis across varieties, the N offtake needs to be considered per individual sampling time. The results show that at anthesis, even though there was a greater SMN content in the barley plots, the N offtake by barley and oats was comparable. There were no significant differences between species or their varieties in the N uptake at anthesis. Thus, a smaller N uptake and depletion of soil N may not be the cause of the larger SMN observed in barley at anthesis.

There was also evidence that the crop and soil N dynamics changed over the grain filling period and that the changes differed in scale between barley and oats. Between anthesis and crop maturity there was a tendency for SMN to increase in both barley and oats. In barley there was little increase in N offtake over this period, but in oats N offtake increased substantially. At maturity, oats had a significantly greater N offtake than barley and the SMN was significantly lower in two of the oat varieties, suggesting that oats had access to a larger soil N pool than barley or perhaps, the grain sink strength was greater in oats.

Do differences in soil N transformations contribute to apparent differences in residual soil N between barley and oats?

Given that SMN differences measured at anthesis cannot be entirely explained through differences in RLD or N offtake and, given that there is an increase in the amount of SMN between anthesis and maturity the results suggest that soil N transformations such as net mineralization contribute to the differences in residual SMN observed between barley and oats.

Nitrogen mineralization is the process of conversion of organic N forms to NH_3 or NH_4 and to NO_3^- by microbes (Benbi and Richter, 2002). It plays an essential role in plant N availability and critically affects ecosystem productivity (Herman et al., 2006; Pinton et al., 2000). The above average temperature and rainfall

conditions observed during July and August of 2019 would be expected to be favourable for mineralization processes.

The SMN content of soil may be affected simultaneously by inputs via mineralization or fertilizer additions and removal via crop N uptake, immobilization, leaching and gaseous emissions. The sum of SMN and crop N offtake provides an index of the crop-available N in the crop-soil system at any given time. During the post-anthesis period, there was an increase in the total crop available N of both crops, although it was most pronounced in oats. This suggests that there was significant net mineralization of N between anthesis and crop maturity, but that the extent differed between crop species. The timing also appeared to differ between varieties of oats. Thus, net mineralization seemed to occur later during grain filling (from the date of barley maturity to physiological maturity of oat) for WPB Elyann and Firth compared to Aspen and Yukon. These results suggest that net mineralization is influenced by crop genotype presumably via differences in root-soil-microbe interactions. As mineralization may occur simultaneously with N uptake, the SMN content will depend on the balance of the two processes. Where mineralisation exceeds uptake an increase in SMN will result. Thus, differences in the residual SMN found between barley and oats at crop maturity may be strongly influenced by effects of crop species on soil N transformations.

N mineralization is completed by a variety of soil microbes that can mineralize decomposed organic material, such as crop residues, soil organic matter, or compost (Nevins et al., 2020). The amount of N mineralized depends on the N concentrations in the organic material and the decomposition rate (Nevins et al., 2020). Nitrogen mineralization can also be influenced by the root system itself. Roots exudates may supply microbes with carbon. Microbes are believed to be commonly C limited (Paul and Clark, 1996) but are less commonly N limited than plants because they have the enzymatic capacity to access and utilize macromolecules of organic N making them available to the roots (Paul and Clark, 1996).

The interactions between plant roots and microbes in the rhizosphere play an important role in the nutrient availability (Pinton et al., 2000). Roots exude large and complex amounts of organic compounds such as sugars, amino acids and organic acids into the nearby soil (Canarini et al., 2019). The majority of the root exudates are believed to alter the structure and activity of soil microbial communities (Shi et al., 2011).

The release of root exudates is therefore a major factor influencing soil N availability. The location from which these organic compounds are released within the roots is an important consideration. For example, if the supply of C is high near young roots and declines in older root sections, then C-limited microbes in a mature rhizosphere would mineralize NH_4 during catabolism of N-rich cell components (Myrold, 2021). Root-C enhancement of microbial numbers and activity may attract bacterivores, which on consumption of low C:N microbial biomass, release N as NH_4 into the rhizosphere. Protozoa and other soil fauna release an estimated 30 % of consumed bacterial N into the rhizosphere (Griffiths et al., 1992) where it then becomes available for plant uptake.

With this context, the higher SMN content observed in the barley plots of this experiment compared to oat plots might also be partially attributed to differences in the interactions between roots-soil-microbes. Comparing, qualitatively and quantitatively, the compounds released by the root system of both species could provide an insight into the differences observed between the species. Similarly, understanding the relationship and interactions between plant roots and microbes, for example, evaluating the competition for soluble N between roots and microbes in the different crop species would be helpful to determine the possible differences observed in N depletion.

The challenges associated with root sampling under field conditions has limited the studies of crop root growth and function, parameters essential for understanding soil N dynamics, N losses and alternatives for reducing leaching.

5.5 Conclusion

SMN contents are the net outcome of several process that may occur simultaneously in the soil. These are removal of N from the SMN pool by crop uptake, nitrate leaching or transformational losses such as immobilisation or denitrification and subsequent gaseous emissions. These losses may be opposed by inputs to the SMN pool by mineralisation of soil organic matter or losses from the plant through root senescence, exudation or shedding of leaf litter.

During this one experimental year, not only did the ability of the species to deplete SMN by anthesis appear to differ but there were also differences between the species, and even among varieties, in their subsequent nitrogen mineralization dynamics. Overall, the greater residual SMN found in barley at anthesis appeared to be a species characteristic. There was evidence of significant net mineralization of N during grain filling. This was more extensive in oat than barley and the timing of which differed between oat varieties. The differences in RLD between species may partially contribute to differences in depletion of soil nitrate, but the complexity of the root-soil-microbe dynamics and its effects on net mineralization needs to be considered too.

Chapter 6: General discussion and implications for NUE

Improving crop productivity and product quality together while minimizing N inputs is, globally, an essential goal for modern agriculture. In such context, the concept of an ideal plant (ideotype) for improved NUE can be created.

The ideotype approach for biological models was first proposed by (Donald, 1968). He defined crop ideotype as a plant model which is expected to yield a greater quantity or quality of grain, oil or other useful product when developed as a cultivar within a defined environment (Donald, 1968). More recently (Martre et al., 2015) defined ideotype as the combination of morphological and/or physiological traits, or their genetic bases, to optimize crop performance.

Ideally, a barley crop should form a good photosynthetic canopy with a low tissue N requirement and/or by capturing more N from the soil and with minimal N fertilizer applications. Specifically an ideal barley crop should: 1) remove nutrients from the soil with high efficiency and therefore, show high uptake efficiency through the growing season, including during the post-anthesis period to minimise SMN residues at harvest, 2) assimilate, recycle and remobilise N with high efficiency while maintaining maximum photosynthetic capacity before and during the grain filling period, to support whole plant growth, yield, and to ensure grain quality (Chardon et al., 2012; Foulkes et al., 2009).

The implications of the key findings from this research on the main components of NUE (N uptake and N utilization) are discussed below.

6.1 Improving N uptake efficiency

Table 6.1 Summary of some of the opportunities to define an ideotype for increasing PANU in a barley crop, based on this thesis findings.

Traits	Improving PANU
Root traits	Deep, extensive root system that can explore deeper layers of soil and utilizes more water and solubilized mineral nutrients. However, risk of soil mining through the years.
Shoot traits	Enough (few) leaves in an increasing angle top to bottom to maximised light intercept. Short and strong stems to avoid lodging. Large and erect ear, enough capacity for photosynthates.
Fertilizer practices	Minimum input of N fertilizers. Late application of N fertilizers (around anthesis). However, risk of developing infertile tillers.
Use of sensors and monitoring devices	Assessment of soil conditions before and during the growing season, monitoring the crop from emergence. These tools allow to make better decisions through the entire season.

The current research has shown an apparent inability of barley roots to deplete N to low concentrations in soil, but a high physiological capacity for N uptake from a simple medium. This project also demonstrated that the physiological capacity of barley roots appeared not to be downregulated by age or by the grain sink demand and there was evidence that there is a capacity for N utilization in non-grain tissue when the supply is high. So, understanding why

barley is not able to deplete the soil mineral N and why there is a high residue of N left in the soil at harvest is essential to make further improvements in NUE.

In the field, at 30 cm depth, nitrate concentrations at anthesis and during grain filling in barley were between 1.6 and 2.6 mM, and ammonium concentrations ranged from 0.4 to 1.2 mM, yet PANU was relatively low. In sand-perlite, by contrast, barley was able to deplete nitrate and ammonium to non-detectable levels from supply concentrations as high as 6 mM throughout the grain filling period.

It has been suggested that improvements in N uptake by cereal crops might be achieved through the manipulation of root N transporter systems. Recent studies have shown that overexpressing nitrate transporter systems (e.g. *OsNRT2*.*3b*, *OsNRT2*.1, *HvNRT2*.1) improve yield and ultimately NUE in cereals crops such as rice and barley (Chen et al., 2020; Feng et al., 2017; Guo et al., 2020; Luo et al., 2018). However, these experiments are typically conducted under controlled environment conditions, and in the majority of cases, plants are grown hydroponically. While, these are valuable and informative experiments, whether manipulating transporter numbers will be effective in the soil under field conditions is questionable.

If the bulk soil NO_3^- concentrations of 2.6 mM measured in the field in the current study occur in the root surface the HATS should be saturated, and the LATS should be actively operating, but the low post-anthesis N uptake observed raise questions about the efficiency with which the roots are able to access that bulk soil N. Therefore, manipulating transporter activity and numbers may not be the most effective mechanism to improve NUE if the major limitation is the getting the supply of N from the bulk soil to the root surface. (Kage, 1997) demonstrated that the nitrate uptake of faba beans is limited by the transport of nitrate from the bulk soil to the root surface. In this case it was associated with an inadequate root length density.

The apparent unused N left in the soil at harvest is at risk of leaching through the soil profile during the winter season and therefore potentially causing water pollution. Where RLD is potentially limiting one of the primary root traits to improve N capture and to minimise the risk of nitrate leaching is to increase rooting depth and rooting density (Foulkes et al., 2009). Given that losses of N may occur when N is leached beyond the root zone of the crop, maximum N uptake and depletion of soil nitrate before harvest is essential (Thorup-Kristensen, 2006). The SMN remaining at harvest will not be protected from leaching even if the crops have deeper roots.

Improvements in root architecture to ensure maximum N capture before harvest could focus on a deeper relative proliferation of roots, especially in deeper sub soil layers of the soil profile (Foulkes et al., 2009). In fact, at greater depths a root length density below a critical threshold of 1 cm cm^{-3} would potentially increase the risk of nitrate leaching (Ford et al., 2006; King et al., 2003). During the 2019 field experiment at 40-55cm depth in the soil profile, barley crops (cv. Westminster) showed a RLD of 1.1 cm cm^{-3} whereas oats (cv. Firth) showed a RLD of 2.4 cm cm^{-3}. This RLD is likely to decrease in the deeper layers of the soil profile, where the SMN also increase as demonstrated in Chapter 5 of this thesis. Therefore, N is at higher risk of leaching if unabsorbed by the crop and if the soil water content exceeds its field capacity. A maximum possible rooting depth of 2 m has been reported for winter wheat grown in the UK (P. J. Gregory et al., 1978). At such depths the RLD of a barley crop is likely to be well below the cRLD of 1cm cm^{-3}. Crop management practices such as the use of a cover crop during winter can be adopted to minimise leaching of N remaining at harvest (Ladha et al., 2005).

Differences between species and varieties need to be further explored to determine genetic variability in the root systems and therefore potential for improvement of N capture, especially at deeper soil layers. Although increasing RLD seems to be a promising target, it is unlikely to be the whole answer to maximise N uptake and minimise residual N because soil transformation processes such as mineralization and nitrification also need to

be considered. Even at RLDs in the topsoil close to or above the generally accepted cRLD there was a large SMN content during early grain filling suggesting that improvements in RLD in this soil layer may not lead to significant improvements in depletion of SMN.

The data from the field experiment showed high levels of mineralization followed by nitrification taking place during the post-anthesis period for barley and oats. The higher level of N depletion observed at anthesis by oats was in some varieties counteracted by these transformation processes later during grain filling. Therefore, it is important to determine the major factors contributing to post-anthesis soil N mineralization. Weather predictors and temperature in particular may be useful tools in such a scenario. Moreover, understanding the interaction between roots and microbes in the soil could potentially help to identify key targets to prevent high levels of mineralization late in the growing season. For example, it has been suggested that root exudates influence the rate of mineralization and nitrification processes (Canarini et al., 2019; Y. Liu et al., 2022; Paterson et al., 2006), because they provide an easily accessible energy source for microorganisms that result in greater soil N mineralization. Senescing roots might exude organic nitrogen compounds, such as amino acids (Canarini et al., 2019) that are then converted into inorganic nitrogen or it could be that the root sugar exudates lead to mineralisation of soil organic matter (SOM) that is already present (Liu et al., 2022). Knowing the source of this mineralized N will help us understand the relationship between roots and soil microbes and to target process such as root senesce in an effort to optimise the acquisition and availability of the soil resources.

Improving root systems to maximise depletion of SMN pre-anthesis and preventing or slowing mineralization of N post-anthesis might be desirable in terms of reducing the requirement for fertilizer N application and to minimise the risk of nitrate leaching after harvest, but is it sustainable in the long term? The results of the field experiment showed that in 2019 grain N offtake exceeded fertilizer N inputs (Table 5.1) indicating that there was a large uptake

of soil N. If this were to occur in every growing season, soil N will in effect be mined by cropping and will need to be replaced, challenging the current desire to use less N fertilizer. Therefore, finding ways to obtain N from sources other than fertilizers (e.g. N-fixation) is key to finding the balance between crop N uptake and soil fertility. This is essential to reduce the negative nitrogen balance represented by the unsustainable nitrogen mining of agricultural soils that has already been reported (Lassaletta et al., 2014).

6.2 Improving N utilization efficiency

The efficient remobilization of N from the vegetative organs to the grains during the post-anthesis stage and the maintenance of a sufficiently large photosynthetic capacity to ensure complete grain filling are widely considered to be key mechanisms to achieve a high N utilization efficiency (Chardon et al., 2012; Foulkes et al., 2009).

Experiments conducted in the first chapter of this thesis showed that the remobilization of N from vegetative organs to the grains was highly efficient and that the N concentration of non-photosynthetic organs such as stems was already very low by the end of grain filling. As such, opportunities to further improve the N remobilization efficiency may be limited. However, the biggest limitation of this work was that the controlled environment experiments were performed on one cultivar only. Exploring genetic variability in terms of differences in the N content of non-photosynthetic plant tissues at anthesis and differences in the post-anthesis N remobilization efficiency from these organs may potentially identify opportunities to breed for varieties capable of translocating larger amounts of N to the grains without compromising the plants photosynthetic capacity (Bertheloot et al., 2008).

Le Gouis et al., (2016) stated in their review that a high N remobilization efficiency from the stem would potentially favour high N utilization efficiency through delaying leaf lamina senescence in wheat crops. However, it has also been shown that delayed senescence could lead to a decrease in N remobilisation efficiency (Van Oosterom et al., 2010) and a dilution of the GPC

by prolonging carbon assimilation and supply of carbohydrates to the grain (Distelfeld et al., 2014; Gaju et al., 2016; Hawkesford and Griffiths, 2019).

Within the plant tissues, N can be found in the form of photosynthetic N, structural N and reserve N (Lemaire and Gastal, 1997; Pask et al., 2012). Therefore, in order to achieve high remobilization efficiency while maintaining a high photosynthetic capacity the remobilisation of N should come predominantly from reserve and structural N. (Gallais et al., 2006), indicated that N remobilization can occur in the same leaf simultaneously with different activity levels depending on the senescence level. The same leaf can thus begin to remobilize N and still maintain its photosynthetic capacity.

Given the specific requirements of barley grains for the malting industry (up to 1.8% grain N), more N remobilized and translocated to the grains may not necessarily mean better grain quality. Instead, breeding for varieties that utilise less N fertilizer to form a good canopy and synchronise the remobilization of N (i.e. the onset and the rate) with the whole-plant senescence might be a better approach to reduce the utilization of synthetic N fertilizers. Therefore, pre- and post-anthesis N dynamics needs to be considered as a whole; pre-anthesis N uptake to ensure yield and post-anthesis N uptake to ensure grain quality and minimised SMN residue. NUE is a complex trait and the main components, N uptake and N utilization are influenced by several factors. There are no definite answers when breeding for NUE as this depends on environmental conditions, genotypes, developmental stage, soil N and water availability. Despite the wide literature that already exists on NUE and its improvement, there is, still, a significant challenge ahead in understanding where the inefficiencies come from and in finding the most accurate and appropriate phenotyping and genetic techniques to increase NUE.

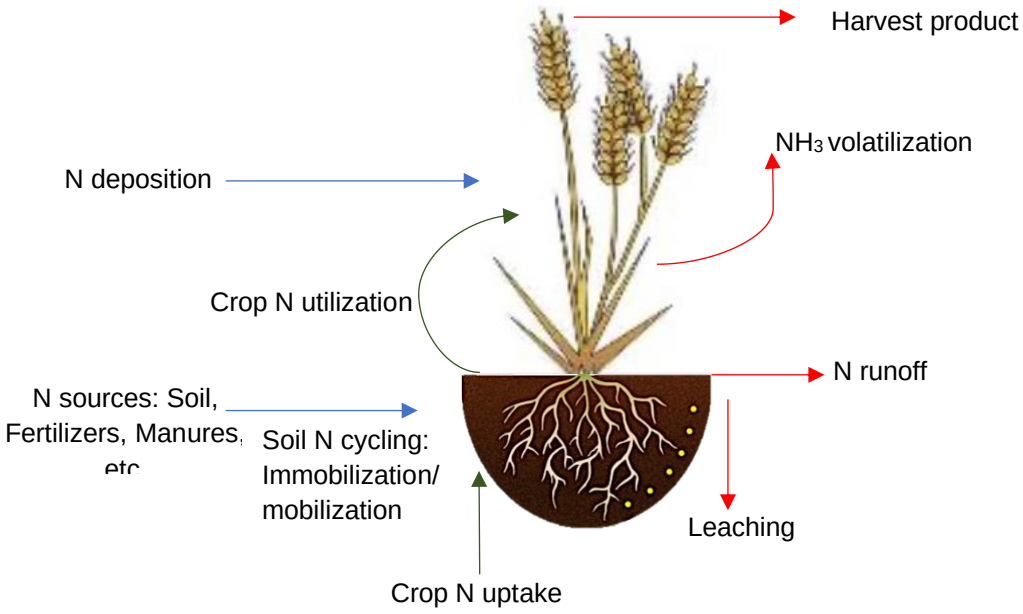


Figure 6.1 Conceptual framework for the overall N budget.

6.3 Recommendations for future research

6.3.1 Regulation of N uptake and net N accumulation during grain-filling

The question about the cause of the reduced net N accumulation from GS 73 to GS 83 observed in de-grained plants (Chapter 3) remains unanswered, and further research is required to address it. This might involve experiments to determine whether the reduction in N accumulation is the result of losses of N assimilated before de-graining. This could be done by using an approach similar to that used in Chapter 2. Labelling plants to constant enrichment using ^{15}N before de-graining and then switching them to a supply of ^{14}N from de-graining onwards would help distinguish between effects of de-graining on concurrent net N accumulation and those on N retention.

The relationship between N-source and N-sink needs to be further explored with relation to the regulation of N uptake. Looking at the expression of a range of different transporters when the grain sink demand has been reduced may provide an insight on whether transporters are affected at all, or if any are being

up or downregulated, or whether some transporter(s) dominates the uptake of nitrate and ammonium over others under these conditions. The relationship between N transporter systems and N demand can then be established.

Moreover, looking at what may be happening to the relative influx and efflux components of net N uptake would be another way to explore and try to find an explanation for the unexpected fact that the ^{15}N uptake doesn't seem to be affected by the increase in amount of N that is recycled back into the root system from the shoot. Exploring what compounds are being translocated from the shoots to the roots will help us to understand what constitutes a signal for the regulation of N uptake and to make comparisons with already existing literature.

Most experiments on the regulation of N uptake have been conducted on relatively young plants. During the grain filling period the root system of cereals is senescing, along with the canopy, and there is a net loss of root tissue (Gregory, 1994). This is seldom considered in research on the regulation of N uptake. The expression of transporters and role of shoot-root signalling of plant N status should be investigated in root tissue of defined age and progress of senescence in order to provide a more complete understanding of the physiological capacity of root systems for N uptake during grain filling.

6.3.2 Identification of the source of the post-anthesis N mineralization

Further research is needed to understand the mechanisms responsible for net N mineralization during grain filling. This includes the study of root aging and exudation before and after crop harvest. If the death and decay of root tissue is a major contributor of soil organic matter (SOM) and therefore a source of energy for soil microorganisms, delaying the senescence of roots becomes an important characteristic to tackle. Slowing down the root senescence process may help to prevent the rise of soil N when there is no crop to take it up. Similarly, establishing when during the growing season and what compounds are exuded from the root system will help us to establish the relationship between the time and rate of N mineralization. By quantifying how different

root exudates affect the contribution of N mineralization will enhance our understanding of the C for N exchange in the plant rhizosphere.

6.3.3 Exploiting soil microbial communities

Because roots and their exudates affect microbial activity and soil C and N mineralization, microbial communities and their enzyme activity profiles vary within species (Zhu et al., 2014). Some species may be more effective in terms of priming N release from SOM than others (Rosolem et al., 2017), and similarly some species may be better at depleting SMN due to the microbial communities within their rhizosphere. Therefore, exploring differences in microbial communities present in the rhizosphere of different species through the growing season may provide a better understanding of the relationship between soil N transformation processes and specific microbial activity.

Another area of interest and potential research is to explore possible sources to fix N_2 other than through legumes. It is well established that the mutualistic association between legumes and some bacteria can fix significant quantities of N and provide effective yields without additional N fertilisers (Rosolem et al., 2017). Finding free-living microorganisms that can fix N_2 and that are independent of a symbiotic relationship has become of particular interest for cereal crops. Diazotrophic bacteria have shown the potential for such purpose (Ladha et al., 2016).

High throughput gene-editing techniques provide the tools and the engineering framework to effectively develop microbes with the potential to function in N-rich soils conditions which would normally supress N fixation (Bloch et al., 2020). However, whilst this would supply high levels of biologically fixed N_2 in a soil N background that might be sufficient to meet the demand of high yielding crops, it will be necessary to ensure that problems of gaseous emissions and nitrate leaching associated with high rates of fertilizer application are not simply replaced by similar problems from high rates of N_2 fixation.

Phenotyping methods combined with molecular information are essential tools to develop new genotypes to improve NUE, yield and grain quality of cereal crops. Exploring the genetic diversity of cereal genotypes becomes critical for the manipulation of candidate genes aiming to improve or to provide a better understanding of the mechanism controlling NUE. Accurate phenotyping is needed to help plants to adapt to source-limiting environments and to be able to phenotype large population. Remote sensing has an enormous potential to determine NUE phenotypes. Through remote sensing vegetation spectral data can be obtained. Other devices such as SPAD, NDVI, ASD, UAV-RSP with multispectral sensors have become an easy operational base for high-throughput field phenotyping in large-scale field studies(Liang et al., 2021). Therefore, successful application of phenotyping tools to advance crop breeding through the monitoring and identification of essential trails requires: 1) high resolution of imaging and environmental sensors, 2) quilaty data that facilitates computer vision, machine learning and geographic information system (GIS), 3) capacity infrastructure for data management and analysis and 4) automated environmental data collection. Accelerated breeding for agriculturally relevant crop traits is key to the development of improved varieties (Shakoor et al., 2017).

Worldwide, improving NUE has become a top research priority. Given the complexity of this trait, breeding for better NUE can be achieved by selecting from a wide diversity of physiological traits and therefore, its improvement could be the result of the modification of several crop and potentially microbial components.

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Appendix

Table S2.1 Dry weights of each plant tissue and whole plants treated with three levels of N pre-anthesis from anthesis to the end of grain filling.

N supply	Harvest	DW, g plant^{-1}					
		Leaves	Sheaths	Stems	Roots	Ears	Total
N1	Flowering	0.18	0.21	0.33	0.22	0.16	1.1
	128 DAF	0.19	0.27	0.61	0.32	0.38	1.78
	311 DAF	0.21	0.29	0.75	0.35	0.88	2.47
	475 DAF	0.16	0.27	0.78	0.34	1.46	3.01
	604 DAF	0.17	0.27	0.72	0.38	1.68	3.22
N2	Flowering	0.33	0.39	0.59	0.32	0.34	1.97
	128 DAF	0.37	0.49	1.12	0.52	0.65	3.14
	311 DAF	0.39	0.51	1.47	0.47	1.61	4.44
	475 DAF	0.32	0.47	1.39	0.6	2.41	5.2
	604 DAF	0.31	0.48	1.39	0.65	2.89	5.73
N3	Flowering	0.49	0.44	0.75	0.43	0.39	2.48
	128 DAF	0.52	0.59	1.33	0.52	0.76	3.72
	311 DAF	0.54	0.7	2.03	0.75	1.99	6
	475 DAF	0.41	0.62	1.91	0.66	2.94	6.54
	604 DAF	0.41	0.62	1.84	0.7	3.57	7.13
p-value	N treatment (N)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Harvest time (T)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	NxT	0.009	0.113	<0.001	0.105	<0.001	<0.001
LSD 5%	N	0.009	0.035	0.086	0.061	0.096	0.216
	T	0.0125	0.046	0.111	0.078	0.124	0.279
	N x T	0.217	n.s	0.192	n.s	0.216	0.483

Data are the mean of five replicate plants; n.s = not significant; LSD (5%) = Least significant difference.

Table S2.2 Number of grains and N concentration in grains of main shoots and tillers of plants treated with 2, 4 and 6mM of N pre-anthesis.

N supply	Main shoot		Tiller	
	Number	N%	Number	N%
N1	15.6 a	1.14 a	14.6 a	0.94 a
N2	17.6 b	1.22 a	30.6 b	1.00 a
N3	18.8 b	1.43 b	39.4 c	0.98 a
p value	0.001	0.002	<0.001	0.419
LSD (α=0.05)	1.44	0.135	6.68	0.0998

Data are the means of five replicate plants. LSD (5%) = Least significant difference.

Table S3.1 N concentration (N%) in the different main shoot and tiller fractions of spring barley treated with 1, 3 and 6mM of NH_4NO_3 and harvested at GS 73 and GS 83.

			GS 73			GS83		
Plant tissue		Treatment	N1	N2	N3	N1	N2	N3
Main shoot organs	Leaf	Control	2.53	2.67	3.22	1.16	1.40	1.98
	Sheath	Control	1.25	1.13	1.39	0.61	0.73	1.09
	Stem	Control	0.38	0.39	0.50	0.26	0.26	0.31
	Grain	Control	1.58	1.70	1.84	1.49	1.82	2.02
	Chaff	Control	1.48	1.60	1.73	0.88	1.06	1.22
	Leaf	Degraining	2.58	2.83	3.08	1.04	1.44	2.18
	Sheath	Degraining	1.13	1.16	1.30	0.55	0.69	1.07
	Stem	Degraining	0.40	0.43	0.39	0.25	0.32	0.35
	Grain	Degraining	1.71	1.78	1.83	2.00	2.29	2.24
	Chaff	Degraining	1.53	1.63	1.68	0.90	1.00	1.28
Tiller fractions	Leaf	Control	3.03	3.14	3.53	1.48	1.95	2.63
	Sheath	Control	1.13	1.48	1.78	0.82	1.05	1.47
	Stem	Control	0.38	0.39	0.50	0.26	0.26	0.31
	Grain	Control	1.54	1.74	1.70	1.27	1.55	1.89
	Chaff	Control	1.86	2.01	2.33	1.31	1.52	1.91

Leaf	Degraining	2.84	3.29	3.30	1.38	2.03	2.93
Sheath	Degraining	1.48	1.53	1.79	0.76	1.18	1.88
Stem	Degraining	0.40	0.43	0.39	0.25	0.32	0.35
Grain	Degraining	1.53	1.56	1.72	1.74	1.94	1.85
Chaff	Degraining	1.89	1.94	2.09	1.37	1.59	2.10

Table S3.2 Analysis of variance of effects of post-anthesis N supply and de-graining on N concentration of main shoots and tillers fractions of spring barley.

	Main shoot					Tillers				
Effect	Leaf	Sheath	Stem	Chaff	Grain	Leaf	Sheath	Stem	Chaff	Grain
De-graining treatment	n.s	n.s	n.s	n.s	<0.001	n.s	n.s	<0.001	n.s	n.s
N supply	<0.001	<0.001	0.011	<.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
Harvest	<0.001	<0.001	<0.001	<.001	<0.001	<0.001	<0.001	<0.001	<0.001	n.s
D x N	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
D x H	n.s	n.s	n.s	n.s	0.004	n.s	n.s	0.004	0.04	0.008
N x H	n.s	n.s	n.s	0.028	n.s	0.006	n.s	n.s	n.s	n.s
D x N x H	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

n.s = not significant

Table S5.1 Nitrate and ammonium concentration in the top (0-30cm) and subsoil (30-60cm) of four barley and four oats at anthesis, barley maturity and oats maturity. Data are the means of four replicates.

		mg N kg dry soil^{-1}											
		Anthesis				**Barley Maturity**				**Crop Maturity**			
		0-30 cm		30-60 cm		0-30 cm		30-60 cm		0-30 cm		30-60cm	
		NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+
Barley Total		6.46	2.85	2.80	1.94	6.94	1.24	7.57	1.33	6.94	1.24	7.57	1.33
Barley Variety	Concerto	6.45	1.95	2.87	1.56	6.83	1.11	8.45	1.30	6.83	1.11	8.45	1.30
	Laureate	5.42	2.60	2.21	2.28	6.89	1.25	7.16	1.35	6.89	1.25	7.16	1.35
	RGT Planet	5.86	4.21	2.28	1.33	6.43	1.48	6.94	1.41	6.43	1.48	6.94	1.41
	Westminster	8.10	2.64	3.85	2.57	7.60	1.13	7.74	1.28	7.60	1.13	7.74	1.28
Oats Total		2.48	2.01	2.01	0.97	2.85	1.71	2.31	1.27	6.52	2.43	2.94	1.99
Oats Variety	Aspen	1.82	1.36	0.90	0.79	2.32	1.83	2.15	1.01	2.71	1.90	2.02	2.01
	WPB Elyann	2.80	2.09	1.71	0.97	3.57	1.35	2.57	1.36	9.96	2.36	4.69	1.98
	Firth	3.14	2.45	2.29	1.18	3.14	2.08	2.96	1.20	8.75	2.37	3.82	1.91
	Yukon	2.15	2.13	3.16	0.96	2.37	1.59	1.56	1.52	4.66	3.07	1.21	2.06

***p*-value**	Species	<.001	0.048	0.178	<.001	<.001	0.066	<.001	0.68	0.651	<.001	<.001	<.001
	S x V	0.098	0.156	0.356	0.138	0.927	0.772	0.673	0.702	0.013	0.143	0.043	0.992
LSD (5%)	S x V	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	3.779	n.s	2.21	n.s

n.s = not significant; LSD=Least significant differences; Varieties are nested within species; therefore, individual variety effect is not given.

Table S5.2 Average diameter of barley and oats roots collected from three different positions and two different depths.

	Species	**Diameter (mm)**
	Barley	0.26
	Oats	0.32
***p*-value**	Species	<0.001
	Position	0.116
	Species x Position	0.994
	Position x Depth	0.176
	Species x Position x Depth	0.137