Understanding Nitrogen Uptake, Partitioning and Remobilization to Improve Grain Protein Content in Wheat

By

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Abstract

In cereals production, nitrogen (N) fertiliser management is necessary to maximise yield and grain protein content (GPC). Unfortunately, uptake of N fertilisers is less than 50% in wheat, necessitating the focus on improving N uptake/utilization and better N fertiliser management. Another major problem is the negative correlation between grain yield and GPC. The physiological traits correlating to this negative relationship are not yet understood hence, this makes it difficult for breeders to select for high yield and high GPC wheat simultaneously. Therefore, there is a need to identify the physiological traits that are responsible for the negative correlation.

The first aim of this research was to understand the effects of foliar N application applied post-anthesis to improve GPC as an N management practice. N applied in split dosage, on the soil at sowing, followed by foliar application after anthesis was aimed at improving grain quality. Two Australian varieties of bread wheat, Gregory (low GPC) and Spitfire (high GPC) were used in field-like and controlled conditions. Foliar N was applied at different growth stages. There was a significant increase in Gregory GPC following foliar N application at heading and seven days-post-anthesis (7 DPA). These findings indicate the effectiveness of foliar N application at specific growth stages to increase GPC in low GPC wheat.

The second aim was to investigate the leaf surface structures that could be correlated to efficient foliar N uptake including, wax shape and the wax chemical compositions in four bread wheat cultivars Spitfire, Gregory Kukri and RAC875 at stem elongation and 7 DPA. Trichome density, primary alcohols and alkanes were correlated to foliar N uptake. Foliar N uptake was also characterised by identifying the time taken for foliar N entry, the forms of N (nitrate, ammonium or urea) preferentially taken and the N transporters involved. Here, ^{15}N isotope labelling was used in Gregory and Spitfire

using single and combined N forms, and accumulation into the grain and other plant-parts assessed. There was maximum foliar N accumulation in shoots of Gregory 2 h post-treatment. For the N form preferentially taken up, urea and urea ammonium nitrate (UAN) had high accumulation in the grain tissues. The N transporters genes investigated for their involvement in foliar N uptake included *TaNRT1.1, TaNRT2.1, TaAMT1.1, TaAMT2.1* and *TaDUR3.* Their expression pattern was either upregulated or downregulated after foliar N treatment.

The third aim was to identify feedback effects of foliar N on root N uptake and accumulation, and on root-expressed nitrate transporter gene expression. In this experiment, labelled nitrate ($^{15}NO_3^-$) was used in a flux experiment to quantify root N accumulation after a foliar UAN pre-treatment. Root N accumulation was lower in foliar-treated plants, suggesting negative feedback regulation on root uptake. For the root nitrate transporters, the expression of *TaNRT1.1* and *TaNRT2.1* was downregulated by foliar treatment in the early time points and upregulated at later time points.

The fourth aim was to decipher the negative correlation between grain yield and grain protein content by identifying the physiological traits associated with the two important agronomic traits. 15 genotypes from a genetic diversity panel contrasting for GPC/GPD were grown in field condition, and in a controlled semi-hydroponics set up with different N treatments. Several physiological traits associated with grain yield and grain N concentration under various N treatments were identified.

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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To accomplish great things, we must not only act, but also dream; not only plan, but also believe.

~ Jacques Anatole Thibault (1844-1924)

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Above all I would like to thank God for bringing me this far, giving me the courage, good health, and sound mind to complete my PhD.

List of abbreviations

Z31	Zadok Stage 31
°C	Degree Celsius
µM	Micromole
AMTs	Ammonium Transporters
AN	Ammonium Nitrate
ATP	Adenosine Triphosphate
cDNA	complementary DNA
cHATs	constitutively active High-Affinity Transporters
DAP	di-Ammonium Phosphate
DNA	Deoxyribonucleic Acid
FAO	Food and Agricultural Organization of the United Nation
FAOSTAT	Food and Agricultural Organization of the United Nation Statistics
g	gram(s)
GOGAT	Glutamine 2-Oxoglutarate Amino Transferase
GPC	Grain Protein Content
GPD	Grain Protein Deviation
GS	Glutamine synthetase
ha	hectare
HATs	High-affinity Transporters
hg	hectogram
iHATs	Induced High-affinity Transporters
kD	kiloDalton
Kg	Kilogram (s)
LAI	Leaf Area Index

LATs	Low-affinity Transporters
m	meter
Mt	Million tonnes
N	Nitrogen
NH_3	Ammonia
NH_4^+	Ammonium
NiR	Nitrite Reductase
nm	nanomolar
NNP	Nitrate-Nitrite Porters
NPFs	Nitrate Transporter 1/ Peptide Transporter Family
NR	Nitrate Reductase
NRTs	Nitrate Transporters
PANU	Post-Anthesis N Uptake
PTR	Peptide Transporters
SOA	Sulfate of Ammonia
t	tonnes
UAN	Urea Ammonium Nitrate
UAS	Urea Ammonium Sulfate
DPA	Days Post Anthesis
Tg	teragran
NO_3^-	Nitrate
iw1	inhibitor wax 1
Urea	CH_4N_2O
LN	Low N
HN	High N

HI	Harvest Index
NHI	Nitrogen Harvest Index
w/w	weight by weight
w/v	weight by volume
P	Phosphorus
µl	Microlitre
h	hour
min	minute
TKW	Thousand Kernel Weight
DW	Dry Weight
FW	Fresh Weight
mM	Millimolar
GY	Grain Yield
PCA	Principal Component Analysis

Chapter 1

Literature review

1.1 N importance in agriculture

Nitrogen (N) is an indispensable nutrient for plants, even though it is of limited availability (Bernard and Habash 2009). It is necessary for growth, development, and reproduction, and is a constituent of important cellular molecules including amino acids, chlorophyll, nucleic acids, and plant hormones (Bernard and Habash 2009, Guo *et al.* 2014). N is available from the soil in organic forms, for example, amino acids and other degraded N compounds obtained from plant and animal residues, and inorganic forms, such as ammonium and nitrate, mostly derived from synthetic fertilisers.

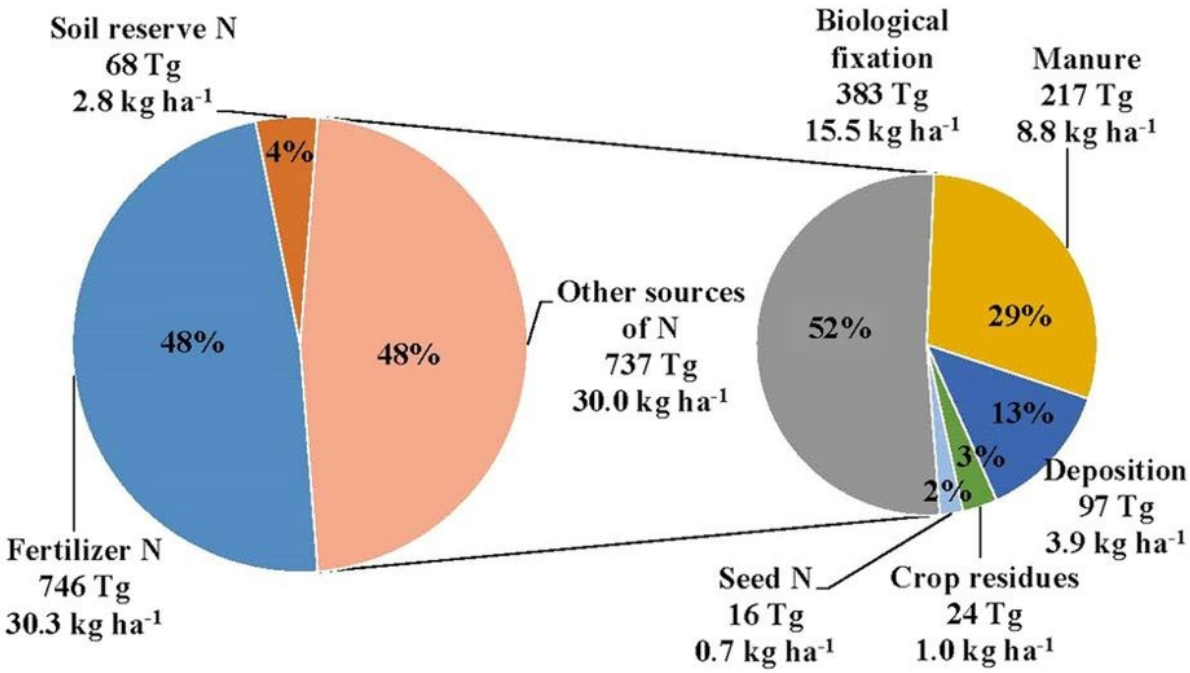

Figure 1: A 50-year (1961 to 2010) world assessment of the contributions of different N sources to three important cereal crops, rice, wheat and maize. Units for each source of N are shown in Tg, % and kg ha^{-1}. The estimated N contributions include 48% from fertilisers, 4% soil reserve N and 48% from other sources (left pie chart). Contributions from the 'other sources' include 52% from biological fixation, 29% from manure, 13% from deposition and 5% from crop residue and seed N (Ladha et al. 2016).

A global assessment of the contribution of N sources to cereal crops rice, wheat and maize was made over a period of 50 years, using data collected from yield, N content of harvested grain and straw, crop production area, fertiliser N inputs, and changes in soil N reserves (Fig. 1). This estimated that 48% of cereal crop N is from synthetic

fertiliser and 4% from reserved soil N (Ladha *et al.* 2016). The remaining 48% is from other sources of N including biological fixation, manure, atmospheric deposition, crop residues and seed N (Ladha *et al.* 2016).

The N taken up by plants from fertilisers or N fixation is removed as grain or remains in plant residue left after harvest. This residue is converted to organic N followed by mineralisation to i) plant-available N ii) inorganic N and iii) soluble organic N that can be taken up by plants, immobilised by microbes or lost in the environment (Fig. 2). The extra N not taken up by crops is lost in the environment resulting in nitrate leaching, volatilization, and denitrification (Sylvester-Bradley and Kindred 2009).

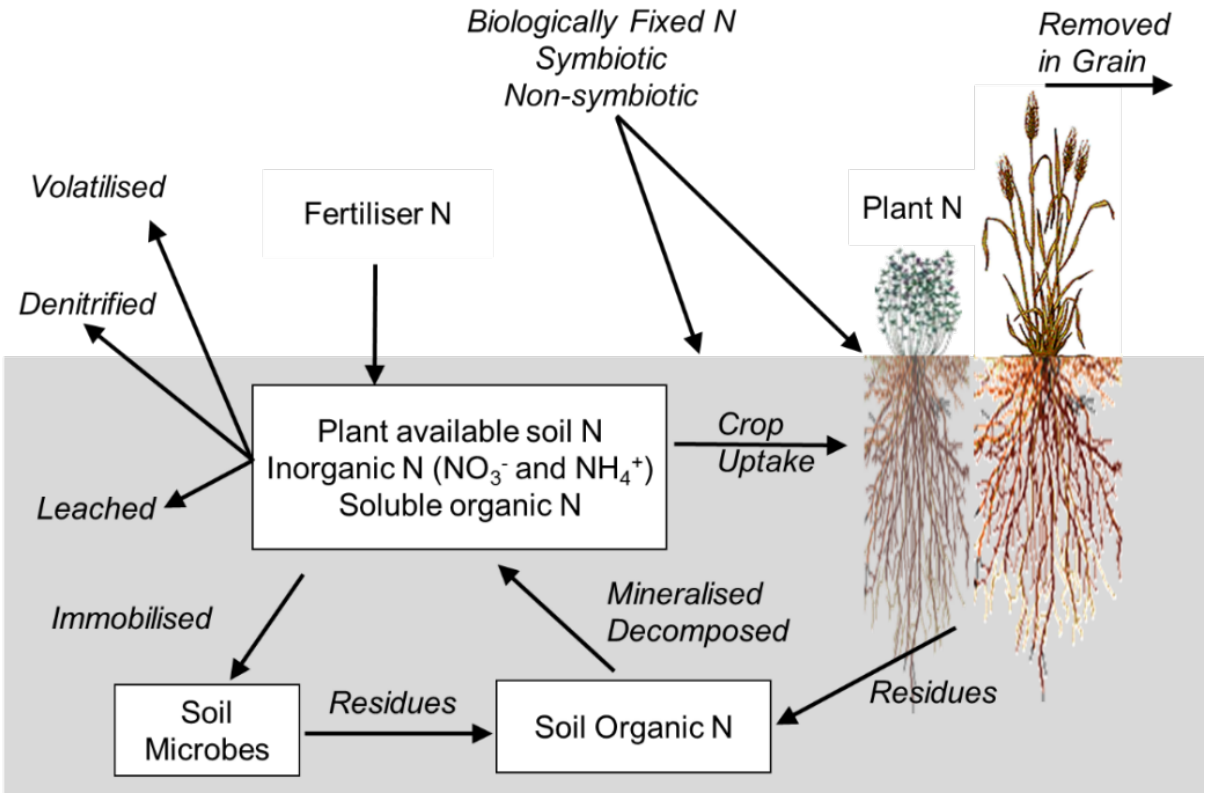

Figure 2: N cycle showing different N sources, N fate upon getting to the soil and losses of excess N from the soil. The sources of N include fertilisers and biologically fixed N. Once the N from these sources is in the soil, it becomes available to the plant as inorganic N (NO_3^- and NH_4^+) or soluble organic N. Once taken up by the plant it is stored as plant N and at maturity removed as grain or in other plant material or is left behind in plant residues. Plant residues are decomposed to soil organic N and inorganic N that is available in the next season for growing plants. Excess fertiliser N is lost through leaching, denitrification, and volatilization (Jeff et al. 2018).

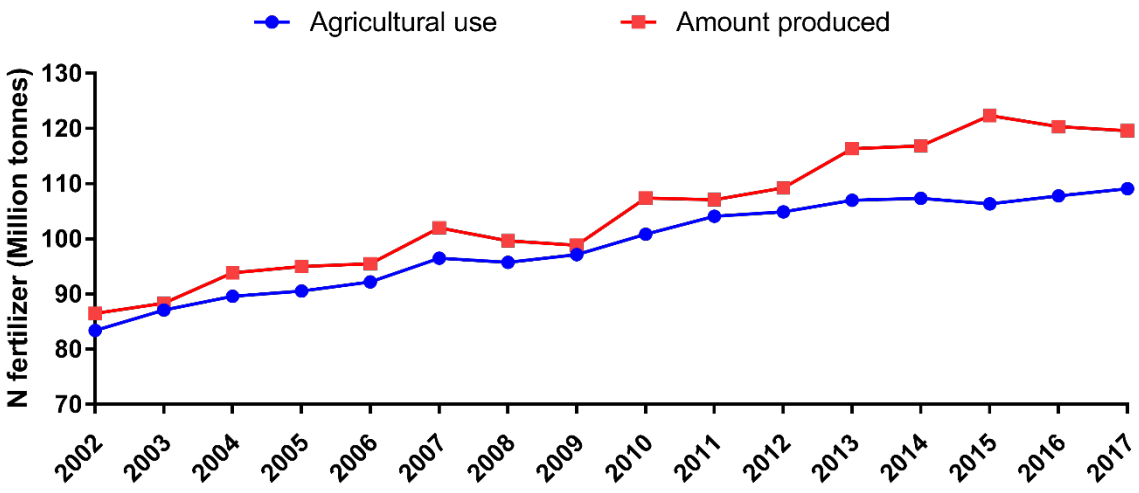

Figure 3: Global synthetic N fertiliser production and agricultural use in 15 years (2002 -2017), showing a gradual increase in both fertiliser produced, and amount used in agriculture. The amount produced in this period was always higher than that what was used in agriculture. Data sourced from (www.fao.org) (FAO 2019b)

According to FAO data, the amount of synthetic N fertilisers produced globally between 2002 and 2017 has increased from 86 million tonnes (Mt) to 120 Mt (Fig. 3) (FAO 2019b). The agricultural use of the produced N fertilisers has also increased in this 15-year timeline, from 83 Mt to 110 Mt (Fig. 3) (FAO 2019b).

Of the total synthetic N fertiliser produced, over 50% is estimated to be used for the growth of cereal crops including rice, wheat, and maize, at 16%, 18% and 16%, respectively (Ladha *et al.* 2016). These three important cereal crops contribute a total of over 200 Mt of cereal proteins each year, which is consumed by humans and animals (Shewry 2007). The utilization of N-containing fertilisers in cereal crops has not only increased yield but also the quality of grains, translating to increased premiums paid to farmers. Nonetheless, cereal crops take up only 40%-50% of N applied as fertiliser; the rest being wasted (Sylvester-Bradley and Kindred 2009).

Mueller and colleagues built a model called the ‘trade-off frontier’, which estimates the minimum N required to produce a range of production levels for wheat, maize, and rice (Fig. 4). Data was collected and mapped for the major cereal crops between 1997 and 2003, that included N application (kg/ha), excess N rate (kg/ha) and yield (t/ha), and

this was used to model an N use trade-off frontier (Mueller *et al.* 2014). The maps show the high N application (Fig. 4a) and the excess N not used (Fig. 4b) in specific regions, including Australia, China, the USA, and parts of Western Europe, of between 150 – 200 kg/ha. However, these regions also have the highest yields for cereal crops (Fig. 4c). The trade-off is, therefore, excess N application for higher yields at the cost of excess unused N that is detrimental to the environment.

With a rapidly increasing world population, food production including the three important cereal crops, rice wheat and maize must also increase exponentially. This translates to additional fertiliser usage that needs improved management practices to improve the uptake and use while also minimising wastage (Dobermann and Cassman 2005, Lassaletta *et al.* 2014).

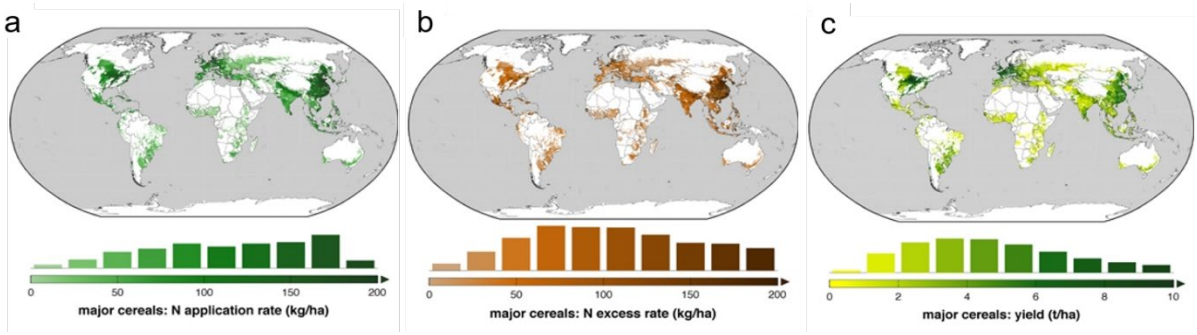

Figure 4: A mapped 'tradeoff frontier' for major cereal production. a, N application rates (kg/ha), b, N excess rates (kg/ha), c, yield (kg/ha), for the major cereal crops wheat, rice, and maize. The data used to make the maps was were collected between 1997 and 2003 and show higher cereal yield, N fertiliser application and N excess in major cereal production regions in Australia, China, the USA and parts of Western Europe. Low cereal yield, N fertiliser application, and N excess are shown in Africa and some parts of South America (Mueller et al. 2014).

1.2 Wheat production statistics

Wheat is an important cereal in the world as a food and source of protein, with many uses, including bread-making, confectionery, pasta, and noodles, and as feed for livestock. According to data from FAOSTAT, average wheat yields internationally have increased steadily for the past 50 years from 1.3 t /ha in 1967 to 3.5 t/ ha in 2017, an

almost 3-fold increase (Fig. 5) (FAO 2019b). The increase is as a result of advanced technology, termed ‘the green revolution’, with the production of rust-resistant, semi-dwarf wheat and improved N utilization (Enghiad *et al.* 2017, FAO 2003).

Australia is one of the top five wheat exporters in the world, with 7.5% global wheat trade in 2018 generating 3.1 billion US dollars (www.worldstopexports.com/wheat-exports-country). Spring wheat is the main wheat type grown in Australia, but recently growing of winter wheat has also been on the rise (Brennan and Quade 2004). The Australian wheat belt is found in five states; Western Australia, South Australia, New South Wales, Victoria, and Queensland (Wang *et al.* 2018).

Data from FAO 2019 from 50 years of wheat production in Australia, from 1967-2017, shows a slow but gradual increase in yield from 0.83 t/ha in 1967 to 2.6 t/ha in 2017, a three-fold increase (Fig. 6). The production quantity follows a similar trend. However, the area of wheat harvested has not increased, indicating that the yield increase in Australia is a result of other agricultural strategies including development and use of adapted cultivars, and proper management strategies including fertiliser use and water conservation. (Godden *et al.* 1998). Grain production in Australia is largely rain-fed and potential productivity is primarily dependant on water and N availability (Jeff *et al.* 2018). The annual N fertiliser used in Australian cereal production is over 1 Mt, with a recovery rate of less than 50% of applied N ending up in the grain (ABARES 2013, Raun and Johnson 1999). It is, therefore, necessary to improve the uptake and utilization of fertiliser N in cereals including wheat, to maintain high yields, improve grain quality, and reduce wastage (that incur losses to farmers as well as causing environmental degradation).

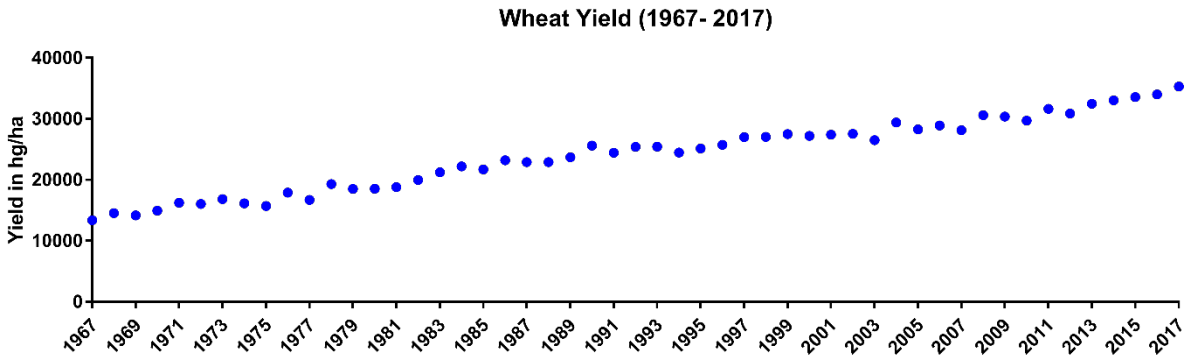

Figure 5: Global average wheat yields (hg/ha) for a period of 50 years (1967-2017), showing a gradual increase over this period (FAO 2019a).

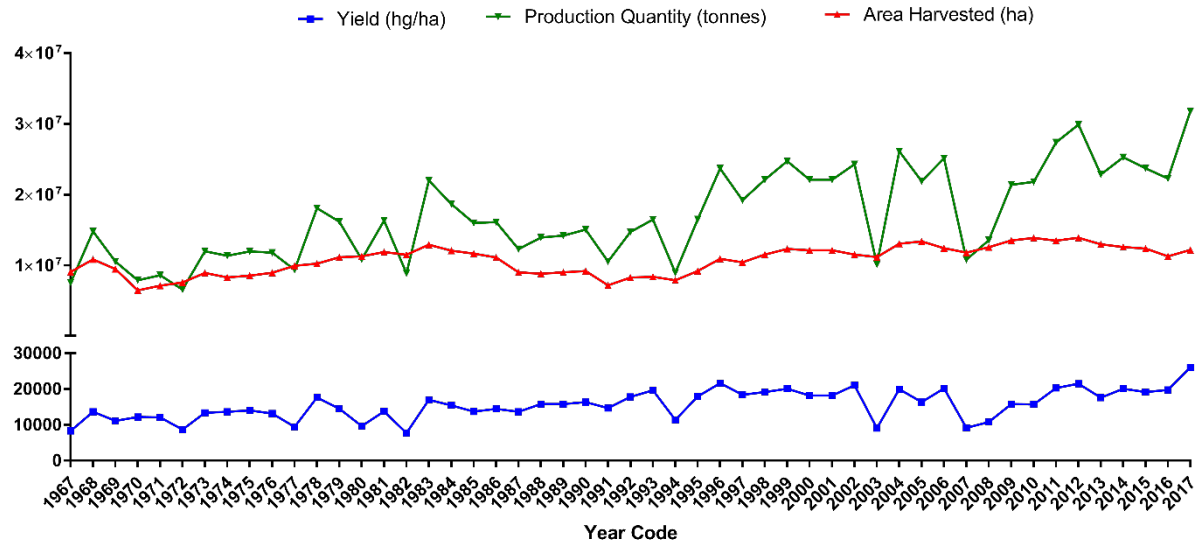

Figure 6: Australian wheat statistics; yield, production quantity, and area harvested for the past 50 years (1967 - 2017). The annual yield (hectograms/hectare; blue line) and production quantity (tonnes; green line) follow a similar trend, with an overall increase across time and with sharp decreases and increases in some years. The area of harvest (ha; red line) has remained relatively constant for the past 50 years. Data sourced from FAOSTAT (www.fao.org) (FAO 2019b)

1.3 Yield and grain quality responses to N

Crop yield response to N input is nonlinear, and this can be used to improve yield without applying excess N that is harmful to the environment (Sutton *et al.* 2013). The yield response to low N application rates is steep compared to a shallow to flat response in high rates of N application (Dobermann and Cassman 2005) (Mueller *et al.* 2014). When N is applied in low amounts, the yield increase potential is largely due to high uptake rates since N is the only limiting factor to growth and yield potential. However, upon an increased N supply, there is a minimal yield increase potential due

to low uptake rates, and more limiting factors other than N (Cassman *et al.* 2003). The nitrogen use efficiency (NUE) in field conditions is determined by genetic and environmental factors (Guttieri *et al.* 2017). NUE is defined as the yields attained per unit of available nitrogen, (grain yield/soil N supply) or described as NUE = NupE x NutE (Le Gouis *et al.* 2000, Melino *et al.* 2015, Moll *et al.* 1982).Nitrogen uptake efficiency (NupE) = Nt/ Ns, described by (ability to remove N from the applied soil fertilizers, (total above-ground N/soil N supply)) and nitrogen utilization efficiency (NutE) = Gw/Nt (ability to use available N to produce grain, (grain yield/total above-ground N)). Both NupE and NutE as described from the equation are important in determining NUE. For example, spring and winter wheat having enhanced NupE, when grown in zero N input field sites were found to have improved NUE, giving an indication of the complexity of NUE in current agricultural setting (Le Gouis *et al.* 2000, Ortiz-MonasterioR *et al.* 1997). Field-grown winter wheat investigated for N accumulation at vegetative growth stage had reserve N accounting for 44% of above ground N, which was located mainly in the stem (Pask *et al.* 2012). The reserve N remobilization at the post-anthesis stage from the field-grown wheat was 48%. This was low compared to chaff, leaf sheath and leaf laminae at 56%, 61% and 76% respectively (Pask *et al.* 2012).To further explain, in well fertilised crop, the reserve N remained in the stem at harvest, which reduced the NutE (Pask *et al.* 2012). Apart from yield, the other important agronomic trait in cereal production is grain quality measured as Grain Protein Concentration (GPC). GPC determines the classification and end-use of wheat produced. The sources of plant N that contribute to GPC come from the remobilization of vegetative tissues N to the grain as well as post-anthesis N uptake. The GPC level varies in wheat as a result of genetic variability in N utilization, environmental influences, and management practices. When wheat varieties are grown in different

environments with varying N application rates, GPC often varies. For example, wheat grown under high N and high rainfall has more yield and lower GPC than crops grown under low N and with a dry finish to the season. Such an environment accelerates senescence with more N remobilised from vegetative tissues to the grains (Shewry 2007).

Both grain yield and GPC contribute to the production value of a wheat crop (Cormier *et al.* 2016, Oury *et al.* 2010). A negative relationship between grain yield and GPC makes the simultaneous genetic improvement of yield and grain quality difficult (Shewry 2007). The cause of this negative relationship at a physiological level is hypothesised to be the competition for energy between N fixation and carbon assimilation, or dilution of the plant N by carbon compounds (Acreche and Slafer 2009, Munier *et al.* 2005). Previous studies have proposed the use of the deviation from the regression line between grain yield and GPC, termed as grain protein deviation (GPD), to identify genotypes with higher GPC than expected from their grain yield (Monaghan *et al.* 2001)

1.4 Grain Protein Deviation (GPD)

GPD is the deviation from expected GPC for a given yield. Monaghan and colleagues found that using GPD, cultivars with a higher GPC than was predicted from grain yield alone were identified (Monaghan *et al.* 2001). Therefore, GPD may be used to study the variability of physiological processes controlling the inter-relationship between carbon and N metabolism translating to yield and GPC (Bogard *et al.* 2010). Carbon and N metabolism is differentially affected by post-anthesis N uptake and pre-anthesis N uptake, with underlying physiological traits possibly explaining the genotypic variation observed for GPD. Examples of these traits may be the regulation of N uptake

depending on plant N status, or maintenance of root activity during the grain-filling period (Bogard *et al.* 2010).

Previous research findings indicate that genotypes with positive GPD tend to have higher levels of N uptake after flowering, and greater N remobilization (Barbottin *et al.* 2005). GPD is strongly determined by post-anthesis N uptake and is independent of the anthesis date or total N at anthesis. However, there are genetic variations in GPD which are related to post-anthesis N uptake in association with anthesis date (Bogard *et al.* 2010, Monaghan *et al.* 2001).

Identifying genotypes with a positive GPD in different N environments is an important step towards uncoupling the negative relationship between yield and GPC (Monaghan *et al.* 2001, Oury *et al.* 2003). Focusing on post-anthesis N uptake of different wheat varieties may help understand the cause of this negative correlation. Furthermore, N management strategies that match N supply to growth stage demands, including split applications, may be employed to achieve both greater yields and high GPC.

1.5 Nitrogen dynamics in plants

There are three essential stages related to N nutrition in plants: uptake, assimilation, and remobilization. The three stages are important in determining N effectiveness towards yield and grain quality. Many studies have focused on the various aspects affecting uptake, assimilation, and remobilization. In the following discussion, each of these stages will be discussed with a primary focus on wheat.

1.5.1 Uptake of the different N forms

For N uptake, the roots are particularly important as a direct point of contact between plants and soil N which can either be organic (urea and amino acids) or inorganic

(nitrate, ammonium) (Crawford and Glass 1998). Also, wheat root architecture and the physiological state of the N transport system involving transporters are important (Griffiths and York 2020, Melino *et al.* 2015). The rate of N uptake is highly dependent on plant growth stage and plant demand, with uptake increasing in a sigmoidal fashion leading up to anthesis. In wheat, the greatest N demand occurs during stem elongation (Hooper 2013).

To facilitate N uptake, at a molecular level, N is moved from the soil solution to plant cells via a transporter system. Transporters are specific to particular nutrients in their common chemical forms, which is important for preferential uptake of ions that are in demand while blocking those not needed or toxic (Griffiths and York 2020). While most studies on N transporters are pioneered in Arabidopsis, there are also studies in other crops including wheat (Melino *et al.* 2015, Ping *et al.* 2011, Yin *et al.* 2007).

Several types of transporters, named according to the N substrate they transport (nitrate, ammonium, urea), have been characterised (Mérigout *et al.* 2008). These transporters are nitrate transporters (NRTs), ammonium transporters (AMTs) and urea transporters (DUR3) (Crawford and Glass 1998, Wang *et al.* 2008, Williams and Miller 2001).

The following is a description of the current knowledge of N uptake of different N forms as well as the transport proteins involved. Much of the information centers on root N uptake and root-expressed transporters, cloned and characterised in the model plant Arabidopsis. There is still not much information on leaf N uptake or the involvement of N transporters in leaf tissue N uptake. However, where possible, known studies on leaf N uptake and the transporters involved will be discussed. Also, information is included about N uptake and transporters involved in wheat.

1.5.1.1 Nitrate uptake

Nitrate is a highly soluble ion and is the main form of N delivered to the root surface through mass flow from the soil solution, driven by the transpiration stream. It is also the main form of N taken up by plants (Marschner 2011). The initial N absorption pathway through roots is primarily the entry of nitrate into the cell and this is mediated by nitrate transporters (Ping *et al.* 2011). Recent research on the kinetic parameters for nitrate uptake in wheat showed a higher maximal uptake rate (I_{max}) compared to barley (Griffiths and York 2020), suggesting that nitrate transporters in wheat are particularly efficient.

1.5.1.1.1 Nitrate Transporters

Nitrate transporters are divided into two classes of membrane proteins that mediate active transport of nitrate, possibly via symport with protons, across the plasma membrane (Crawford 1995). These are the peptide transporters (PTRs)/NRT1 and nitrate-nitrite porters (NNPs)/NRT2 (Crawford 1995, Forde 2000). Recently, the PTR/NRT1 transporters were re-named NPFs (Nitrate Transporter 1/ Peptide Transporter Family); I will herein use the term NPF to describe this class. NPFs and NRTs each consist of multiple genes with different regulatory roles and kinetics and are differentially regulated (Forde 2000, Léran *et al.* 2014, Williams and Miller 2001). They are both categorised in the Major Facilitating Superfamily with two sets of six transmembrane domains linked by a longer cytoplasmic loop (Pao *et al.* 1998, Williams and Miller 2001). NPFs are Low-affinity Transporters (LATs) and NNPs/NRT2s are High-affinity Transporters (HATs), further divided into Induced High-affinity Transporters (iHATs) and constitutively active High-Affinity Transporters (cHATs), whereby iHATs are induced by nitrate, while cHATs are constitutively expressed in the

absence of nitrate (Aslam *et al.* 1992, Crawford and Glass 1998, Williams and Miller 2001). LATs are active when N concentration is high, in the millimolar range, while HATs are active under low N, with K_m values in the micromolar range (Miller *et al.* 2007). The identified plant nitrate transporters are induced by nitrate and play a distinct role in nitrate uptake (Crawford and Glass 1998, Tsay *et al.* 2007).

NPFs are involved in transporting many types of N-containing substrates, including amino acids and peptides, in addition to nitrate (Tsay *et al.* 2007). Arabidopsis has fifty-three *NPF* genes, some of which have been functionally characterised, whereas rice has eighty *NPF* genes with only just a few characterised members. These high numbers suggest an essential role of this family of transporters in higher plants. Perhaps, besides nitrate/peptide transport, they also transport other substrates or perform additional functions (Buchner and Hawkesford 2014, Plett *et al.* 2010, Tsay *et al.* 2007). In Arabidopsis, the NRT1.1 dual affinity transporter was found to be strongly expressed in guard cells and involved in the opening of the stomata (Guo *et al.* 2003). In wheat, sixteen *NPF* genes have been identified that are homologous to characterised LATs genes, and these are mainly expressed in roots (Buchner and Hawkesford 2014). Bread wheat is a hexaploid that has a very large genome, and there are likely many more NPF members that are yet to be identified. Recent advances in wheat genome sequencing and associated resources will facilitate the identification of more NPFs in wheat.

The functionality of the second class of nitrate transporters, NRT2s, has been found to require a second protein, NAR2/NRT3, forming a two-component nitrate uptake system (Okamoto *et al.* 2006, Orsel *et al.* 2006). Both NAR2/NRT3 and NRT2s are regulated by different N forms, that is, they are induced by nitrate and repressed by N

metabolites (Feng *et al.* 2011). The Arabidopsis genome has seven *NRT2* genes that have been characterised, and these are mostly expressed in roots except *AtNRT2.4*, *AtNRT2.5,* and *AtNRT2.7*, which are expressed in leaf tissue (Okamoto *et al.* 2003, Orsel *et al.* 2006, Plett *et al.* 2010). A previous study has suggested that *AtNRT2.4* has a role in root nitrate uptake as well as loading of nitrate into phloem in shoots (He *et al.* 2016). *AtNRT2.5* is associated with the negative regulation of programmed cell death and N remobilization during senescence (Lezhneva *et al.* 2014). *AtNRT2.7* is expressed in shoot and leaf and is associated with photosynthesis/ light-harvesting (He *et al.* 2016). In barley, four *NRT2* genes have been cloned (Trueman *et al.* 1996, Vidmar *et al.* 2000). In bread wheat, five *NRT2* genes have been identified with *NRT2.1* gene being the first to be characterised (Guo *et al.* 2014, Yin *et al.* 2007). Wang and colleagues studied expression patterns of *NRT2.1, NRT2.2* and *NRT2.3* in wheat seedlings (Wang *et al.* 2011). The three nitrate transporters were expressed in roots and regulated by nitrate levels.

1.5.1.2 Ammonium uptake

The uptake of ammonium occurs on a much lower scale than nitrate since it is immobile, has less solubility, and its concentration is 10 to 1,000 times lower than that of nitrate in unfertilised soils. Soil solution ammonium concentrations in agricultural soils typically vary between 20 and 200 µM (Barber 1995, Hawkesford *et al.* 2012, Miller and Cramer 2005). In rice, normally grown in paddy fields, ammonium is the preferred source of N (Williams and Miller 2001). Ammonia (NH_3) is protonated readily to form ammonium (NH_4^+) in low pH soil conditions (Gaur *et al.* 2012, Yamaya and Oaks 2004). In hydroponically grown wheat, low concentrations of ammonium were measured in the xylem sap at 0.4 mM when 4 mM ammonium was supplied (Cramer

and Lewis 1993). Ammonium uptake systems in plants are categorised into low- and high-affinity transport systems (Li *et al.* 2009)

1.5.1.2.1 Ammonium transporters (AMT) family

Channels regulate low-affinity ammonium uptake while proton-coupled transporters regulate high-affinity uptake (Davenport and Tester 2000, Williams and Miller 2001). Expression of ammonium transporters is regulated by N status of the soil, glutamine concentration and ammonium accumulation (Rawat *et al.* 1999). Additionally, AMTs have a diurnal pattern of expression which reflects a diurnal pattern of ammonium uptake (Gazzarrini *et al.* 1999). The first discovery of AMTs was in Arabidopsis (Ninnemann *et al.* 1994). In Arabidopsis, eight ammonium transporters were identified through screening of cDNA libraries with *AtAMT1.1* as a classical example (Gazzarrini *et al.* 1999, Sohlenkamp *et al.* 2000). *AtAMT1.1* is a high-affinity ammonium transporter encoding a 53 kD protein, that is expressed in roots and leaves (Ninnemann *et al.* 1994, Williams and Miller 2001). There are twelve AMTs identified in rice, categorised into three subfamilies: *OsAMT1, OsAMT2* and *OsAMT3* (Williams and Miller 2001). Previous research on the expression pattern and localization of nine AMTs in rice identified three copies each of *OsAMT1*, *OsAMT2,* and *OsAMT3* at two different stages (young seedling and tillering stage). From the results, *OsAMT1.1*, *3.2* and *3.3* were expressed in roots and older leaves, while *OsAMT2.1*, *OsAMT 2.2*, *OsAMT 2.3* and *OsAMT 3.1* were expressed in the stem and new leaves (Li *et al.* 2012). In wheat, only two ammonium transporters (*TaAMT1.1*; AY525637) and *TaAMT1.2*; AY525638) have been functionally characterised by heterogeneous expression in yeast or Xenopus oocytes (Jahn *et al.* 2004, Sogaard *et al.* 2009). *TaAMT1.1* and *TaAMT2.1* showed significant expression changes in wheat roots, sampled at different growth stages, and provided with both nitrate and ammonium

under N-limited and N-adequate conditions. The expression generally matched the pattern of in-season N uptake of wheat plants (Liu *et al.* 2015b).

1.5.1.3 Urea uptake

Uptake of urea is generally low in plants as a result of very low concentrations (< 70 μM) in agricultural soils (Wang *et al.* 2008). The low soil concentrations are due to the ubiquitous existence of a nickel-activated urea-hydrolyzing enzyme, urease. The enzyme converts urea to ammonium which can be subsequently taken up and assimilated by plants (Wang *et al.* 2012, Watson *et al.* 1994). However, despite minimal uptake, it is speculated that plants through an evolutionary adaptation, might have developed strategies for utilizing urea through high-affinity uptake systems (Kojima *et al.* 2006). Physiological evidence for the root uptake of urea before degradation has been demonstrated in several independent experiments (Gerendás *et al.* 1998, Hine and Sprent 1988, Merigout *et al.* 2008). Zanin and colleagues showed the capability of maize plants to take up urea via an inducible and high-affinity transport system (Zanin *et al.* 2014).

1.5.1.3 Urea transporters

Previously, urea uptake in plants was found to be either via the high-affinity urea symporter DUR3, or through aquaporins (Wang *et al.* 2008). In Arabidopsis, the high-affinity transporter *AtDUR3* has been cloned and functionally characterised (Liu *et al.* 2003a). *AtDUR3*, a singleton gene in Arabidopsis, encodes an integral membrane protein (Liu *et al.* 2003a). *AtDUR3* is expressed at low levels in shoots (Merigout *et al.* 2008). *DUR3* homologs in rice and maize have been cloned and functionally characterised (Wang *et al.* 2008, Wang *et al.* 2012, Zanin *et al.* 2014). There is little information, on the wheat *DUR3* orthologue. Overall, the uptake pathway for urea is

not well understood at a molecular level, despite physiological evidence of direct uptake by both root and leaf cells (Liu *et al.* 2003b).

There is a knowledge gap on leaf N uptake and the N transporters involved, across all plants including cereal crops. For nitrate and ammonium uptake in wheat, the focus has been on roots. N uptake systems that may facilitate nitrate and ammonium uptake applied as foliar have not been studied in wheat. Investigating the uptake of N through leaves and identification of the N transporters that facilitate this uptake in wheat may reveal their role in foliar N uptake.

1.5.2 N Assimilation within plant organs

Once inside the plant, nitrogenous solutes transportation occurs over long distances through the xylem in vascular plants. Subsequently, N can either be assimilated, stored, or transported to sink tissues via the phloem, depending on plant N demand (Fig. 7) (Garnett *et al.* 2013, Xu *et al.* 2012). The majority of nitrate is assimilated once within the roots (Garnett *et al.* 2013, Miller and Smith 1996). The ability to break down nitrate in the roots is dependent on the ratio of N exudates in the xylem and concentration of the enzyme nitrate reductase (Pate 1980). Alternatively, nitrate is transported to the shoot where it is first converted to nitrite via nitrate reductase (NR) in the cytoplasm, and then nitrite is converted to ammonium via nitrite reductase (NiR) in the chloroplast (Garnett *et al.* 2013). Ammonium directly derived from the soil or formed from nitrate reduction or photorespiration is converted to glutamine and glutamate by glutamine synthetase and glutamate synthase (GS/GOGAT) enzymes in the cytoplasm and plastid (Suzuki and Knaff 2005). The enzyme glutamate synthetase (GS) catalyzes the ATP-dependent fixation of an ammonium molecule to the carboxyl group of glutamate to form glutamine (Masclaux-Daubresse *et al.* 2010) (Bernard and

Habash 2009). Subsequently, 2 oxoglutarate molecules react with glutamine to form glutamate and the process is catalysed by glutamine 2-oxoglutarate amino transferase (GOGAT). Overall, the GS-GOGAT process produces glutamate that is converted to other amino acids via two types of enzymes: transferases and transaminases (Bernard and Habash 2009). The amino acids formed are precursors for organic molecules, including proteins, nucleic acids, and secondary metabolites, and are transported via the phloem (Bernard and Habash 2009).

The assimilation of N following root N uptake has been studied and well understood. However, there is little know knowledge on the assimilation process of foliar absorbed N. It is vital to understand the assimilation of leaf absorbed N, as well as phloem translocation which can then be correlated to foliar N uptake efficiency. The reduction rate of foliar-applied nitrate concerning absorption may also need to be quantified. Also, there is insufficient knowledge on the activity of nitrate reductase in the mature leaves at anthesis.

1.5.3 N partitioning and remobilization

Large amounts of N are remobilised from vegetative tissues to support the development of young leaves before anthesis, and subsequently, post-anthesis grain filling in cereal crops. Vegetative plant N helps to build yield potential and provides the N reserves for remobilization to the developing grain (Hooper 2013).

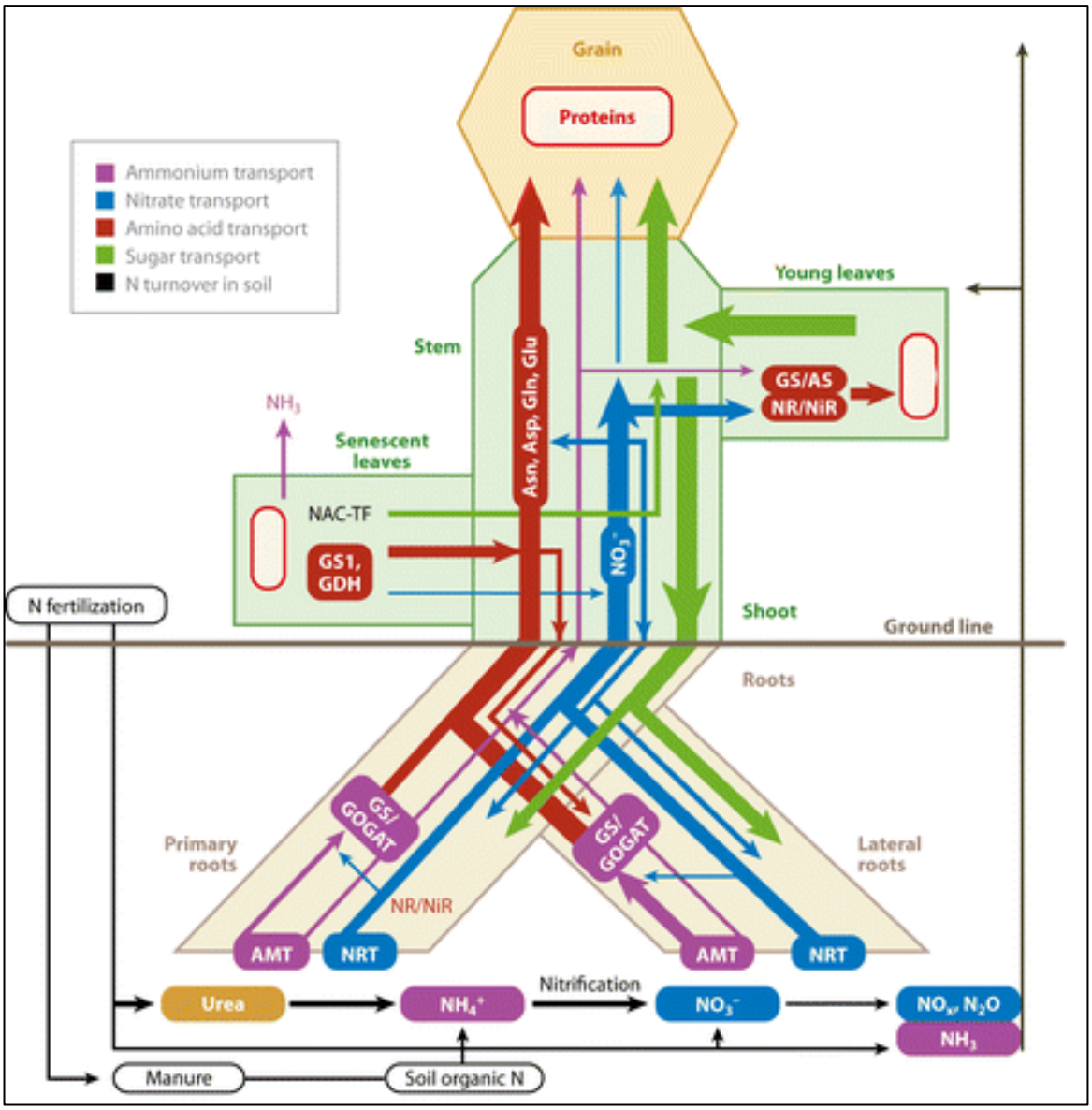

Figure 7: A Scheme of routes of N uptake, transportation, and assimilation in plants. N taken up from the rhizosphere by roots is mainly in the form of ammonium and nitrate. The thicknesses of the arrows represent the relative amounts of nitrogen and sugar in the plant in the different tissues including, root, shoot, stem, young leaves, senescing leaves, and grain. AMT, ammonium transporter; AS, asparagine synthetase; Asn, asparagine; Asp, aspartate; GDH, glutamate dehydrogenase; Gln, glutamine; Glu, glutamate; GOGAT, glutamine-2-oxoglutarate aminotransferase; GS, glutamine synthetase; NAC-TF, certain transcription factors belonging to the NAC family; NiR, nitrite reductase; NR, nitrate reductase; NRT, nitrate transporter (Xu et al. 2012).

The remobilization of stored N is associated with the source/sink-dependent growth patterns of cereal crops (Buchner and Hawkesford 2014). Factors influencing N remobilization to the grain include soil N availability at different stages of plant development, the timing of N fertiliser application, and environmental conditions such

as light and various biotic as well as abiotic stresses (Hirel *et al.* 2007). Remobilization from vegetative tissues involves catabolism of much of the total N from senescing leaves, whereby proteins are broken down into amino acids and moved via the phloem to the developing grains. Remobilised N makes up 95% of seed protein (Taylor *et al.* 2010, Xu *et al.* 2012). During senescence, N is remobilised influencing grain yield and quality (Buchner and Hawkesford 2014). There is genetic variation in senescence which is also influenced by environmental factors (Barbottin *et al.* 2005). Previous research led to the identification of the *Gpc-1 (NAM-1)* gene in wheat, associated with accelerated senescence and increased GPC (Distelfeld *et al.* 2014, Uauy *et al.* 2006). Rice, wheat, maize, oilseed, and barley have approximately 90%, 90%, 50% 95% and 90% of total N respectively remobilised from vegetative tissues for grain filling (Fig. 8 (Buchner and Hawkesford 2014, Gregersen *et al.* 2008). The remainder of the grain N is from post-flowering N absorption. Maize has lower remobilization at 50%, while the rest of the grain N (35-55%) originates from post-silking N uptake (Gallais and Coque 2005).

1.5.3.1 Importance of remobilization and post-anthesis N uptake for grain quality

Post-anthesis grain filling is a critical stage of development, which involves remobilization of previously stored tissue N and post-anthesis N uptake from the soil (Habash *et al.* 2007). It is still unclear whether the remobilization of N stored before anthesis, or uptake after anthesis, are determinants of grain yield and grain N (Martre *et al.* 2003). In wheat, grain N comes from the two main sources: from vegetative tissues to the grains via remobilization/translocation, and post-anthesis N uptake (PANU) directly to the grains. Previous research defined remobilization as (Total above-ground N at anthesis – vegetative N at maturity) (Kong *et al.* 2016, Wu *et al.* 2018). Gallagher and colleagues concluded that an increase in grain N is likely due to

high remobilization and efficient translocation of N from shoot to the grain after anthesis (Gallagher *et al.* 1983). However, the relative contribution of remobilization on grain N is dependent on genotype, water, and N availability (Barbottin *et al.* 2005, Cox *et al.* 1985, Kichey *et al.* 2007). According to previous research PANU is defined as (Total above-ground N at maturity – Total above-ground N at anthesis (Taulemesse *et al.* 2016, Wu *et al.* 2018) and contributes between 5 to 50% of wheat grain N (De Ruiter and Brooking 1994, Gooding and Davies 1992, Van Sanford and MacKown 1986).

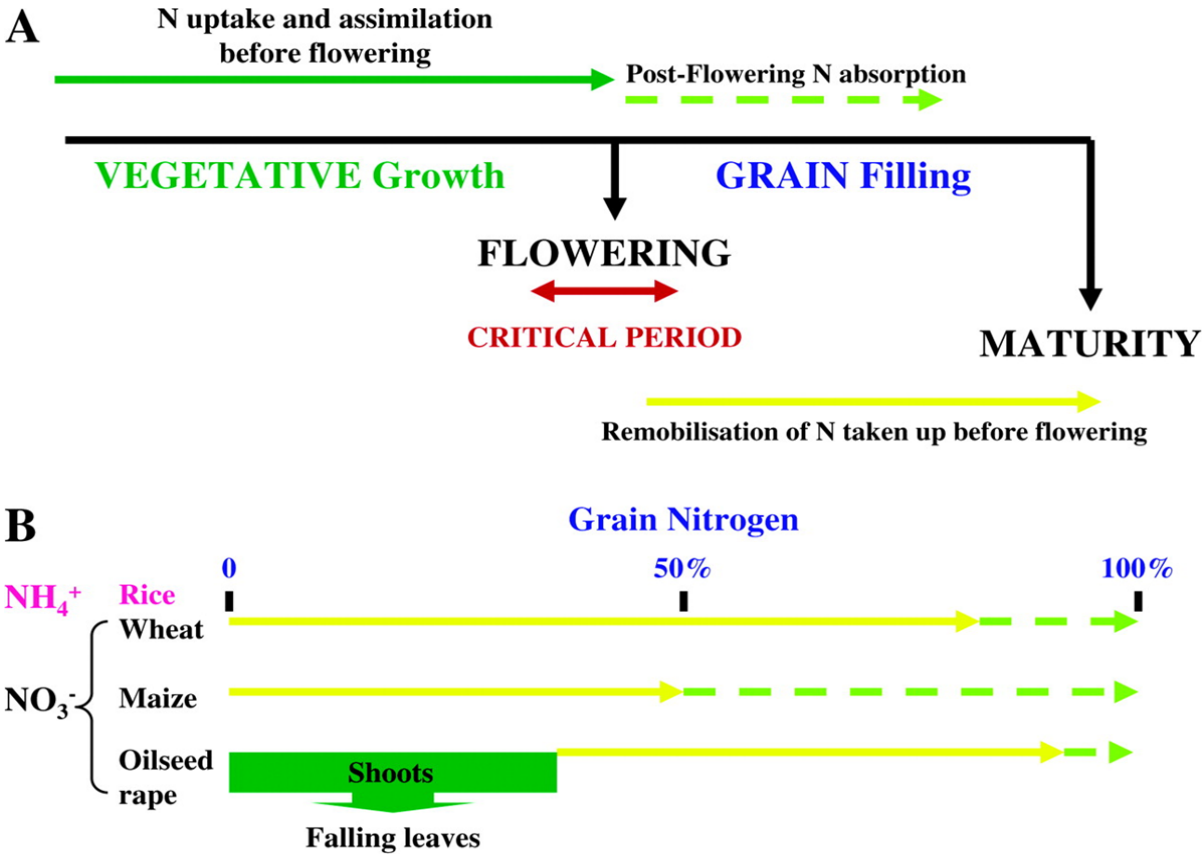

Figure 8. N management (uptake, assimilation, and remobilization) in rice, wheat, maize, and oilseed rape. (A) Vegetative growth, during which N taken up by the roots as nitrate or ammonium is assimilated to build up plant structural components and grain filling stage where post-flowering N absorption and remobilization of N taken up before flowering occurs. (B) N remobilization and post-flowering N uptake (percentage) towards grain filling in rice, wheat, maize, and oilseed rape. Wheat, maize, and oilseed rape preferentially use nitrate, while rice uses ammonium as an N source. In Oilseed rape, a large amount of the N taken up during the vegetative growth phase is lost due to the falling of the leaves (Hirel et al. 2007)

1.6 N management strategies to improve uptake and utilization

To improve grain yield and increase GPC, N management strategies, for example, a split N application has been proposed, whereby N is supplied according to plant demand at different growth stages. Previous research showed the application of N fertiliser at heading stage increases GPC without affecting grain yield (Subedi *et al.* 2007). Unfortunately, the success of this practice is dependent on good climatic conditions, especially water availability during the post-anthesis stage, as well as soil N uptake, which is often low at this stage (Bogard *et al.* 2010). The application of foliar N at later growth stages may offer efficient uptake through shoots, replacing the inefficient/almost-dormant root uptake in dry conditions. Root N uptake activity is slower towards plant maturity due to reduced water availability at the end of the growing season in Mediterranean environments (Bogard *et al.* 2010, Ellen and Spiertz 1980). Therefore, a foliar N application may be an alternative to get the N into the plants at the later growth stages, complementing soil N application.

1.7 Foliar N application

Foliar spraying of fertilisers has traditionally been used to correct nutrient deficiency of micronutrients that have limited phloem mobility, for example, calcium, zinc, boron, and iron (Fernández and Eichert 2009, Kannan 2010). In other cases, foliar fertilization is used as a supplement to soil fertilization or in conditions where soil nutrient availability is limited, to preserve both crop yield and quality with minimal environmental impact (Fageria *et al.* 2009).

Previous experiments on foliar N application have demonstrated an increase in grain N content (Reeves 1954, Smith *et al.* 1991, Strong 1982). Foliar spray applied in wheat at growth stage Z31 (stem elongation stage) and/or at the post-anthesis stage

increased yield potential and boosted grain protein levels (Wylie *et al.* 2003). Furthermore, there was an increase of 1 - 2% grain protein upon late application of foliar N compared to granular urea in dry years in field conditions (Wylie *et al.* 2003). Bly and Woodard and colleagues concluded that GPC was highest when foliar N was applied at the anthesis growth stage of hard red winter wheat and hard red spring wheat (Bly and Woodard 2003). Since there are few studies on foliar N application and foliar absorption in wheat, distinguishing root and foliar N uptake may not be straight forward in field conditions. However, experiment carried out in a controlled, whereby the soil is covered to ascertain no root N uptake of the liquid N solution occurs may ascertain foliar N absorption. This would quantify foliar N absorption efficiency and effects on improving GPC. The success rate of foliar application of nutrients depends on many different factors, which will be summarised in the next section.

1.7.1 Factors affecting foliar N uptake

Adoption of foliar N application is limited by the understanding of uptake efficiency, the varying performance of different foliar N forms and physiological and molecular factors that allow maximum foliar N penetration (Fernández *et al.* 2013, Gooding and Davies 1992). Many factors may influence the performance of foliar fertilisers, including physicochemical properties of the fertiliser, environmental conditions during foliar applications and the physiological characteristics of the plant sprayed (Fernández and Brown 2013).

1.7.1.1 Leaf morphology and physiology affecting foliar N uptake

Foliar N fertiliser uptake by plants is via leaf tissue as the initial point of contact. Unlike root N uptake (supplied with soil-derived N fertilisers) that has been extensively studied in plants, little is known regarding leaf N uptake, especially in cereal crops. Foliar-

applied nutrients are speculated to penetrate the leaf surface through the cuticle or stomata, with both ways considered as vital (Eichert and Fernández 2012, Eichert and Goldbach 2008). Various leaf surface properties will influence leaf spreading, wetting and retention rate of an applied solution, as well as diffusion of the liquid nutrient to the apoplast and symplast of cells for uptake (Peirce *et al.* 2014a).

Proposed possible uptake pathways for nutrients include through the cuticle directly, or via cuticular cracks, stomatal pores, trichomes, trichome basal cracks, or other modified epidermal cells (Fernández and Brown 2013). These pathways are much debated. The leaf cuticle is an extracellular layer that covers epidermal cells and acts as the boundary layer between plant tissues and the environment, protecting the plant against biotic and abiotic stress and minimizing water loss (Kerstiens 1996). It is made up of cutin, waxes, polysaccharides, and phenolics and is an important determiner of foliar nutrient uptake efficiency (Domínguez *et al.* 2011, Eichert and Fernández 2012). It is hypothesised that foliar-applied liquids would need to pass through, or bypass, the different layers, starting with cuticular wax, cutin and cell wall and finally plasma membrane (Franke 1967). The cutin is a polyester matrix embedded with polymerised long fatty acid and soluble waxes made from hydrophobic compounds. This chemical composition gives the cuticle a hydrophobic property (Pollard *et al.* 2008). Hydrophobicity makes it easier for lipophilic molecules/non-ionic molecules to penetrate, for example, urea. Cuticular hydrophobicity is thought to make ionised nutrient diffusion difficult (Fernández *et al.* 2013). However, there is evidence of entry of ionic molecules like ammonium and nitrate through aqueous pores, which arise from hydration of membrane-bound polar functional groups (Liu *et al.* 2015a, Schönherr 2000)

The waxes are divided into epicuticular and intracuticuar waxes, whereby the epicuticular portion is made of aldehydes, alkanes, primary alcohols, secondary alcohols, and ketones while the intracuticular layer is made of terpenoids and sterols (Wang *et al.* 2014). The compounds determine the crystal shapes of waxes (Bi *et al.* 2016). The epicuticular waxes have different crystal types including plates, platelets, ribbons, rods, threads, and crust (Barthlott *et al.* 1998, Samuels *et al.* 2008)

Other leaf surface properties that may influence the penetration of solutes include trichome density and stomata density (Eichert and Fernández 2012, Fernández and Brown 2013). The stomata (specialised cells on the epidermal layer through which water and gas pass), in the cuticle layer, are thought to be possible entry points for foliar liquids. Studies in different plant species have shown an increase in uptake of foliar solution from the abaxial side of the leaf where the stomata density was high (Eichert and Goldbach 2008). The correlation between stomata density and foliar uptake rates was explained to be a result of the high permeability of the peristomatal cuticle covering the guard cells (Eichert and Fernández 2012). There is also evidence of direct entry through stomatal pores from previous research (Eichert and Goldbach 2008, Eichert *et al.* 2008). The role of trichomes (an outgrowth of the epidermis, sometimes described as hair-like) in the uptake of foliar-applied fertiliser is yet to be fully understood (Eichert *et al.* 1998, Fernández *et al.* 2013). However, studies have provided evidence suggesting foliar entry of solutions through the basal cells of trichomes where aqueous pores preferentially occur (Schönherr 2006).

Leaves from a single plant may vary in surface properties, and on a leaf, variation may occur between the adaxial and abaxial sides (Kerstiens 1996). It is therefore vital to conduct studies on individual leaves, with a focus on both adaxial and abaxial sides to

characterize the leaf surface when correlating foliar uptake efficiency to leaf surface morphology (Peirce *et al.* 2014b). Leaf area index (LAI; leaf area/ground area, m^2 / m^2) determines spray interception and is another of the factors influencing the success of foliar absorption of N, with wheat LAI of 2 - 4 considered adequate (Thorne and Watson 1955). Other possible factors that could influence foliar uptake of nutrients include leaf shape and age, plant architecture and growth stage (Puente and Baur 2011, Troughton and Hall 1967).

1.7.1.2 Physiochemical properties of foliar sprays

Physiochemical properties of foliar liquids include molecule size, solubility, density, pH, surface tension, ionic charge, and retention on the plant (Eichert and Fernández 2012). These properties contribute to the uptake efficiency of the nutrient by plant leaf tissues (Fernández *et al.* 2013). Smaller-sized molecules can move through the leaf layers faster since the cuticle is highly size-selective (Buchholz *et al.* 1998, Mercer 2007). The diameter of hydrated ions is below 1 nm, making their movement easier (Eichert and Fernández 2012). Furthermore, the solubility and pH determine the formation of both ionic and non-ionic molecules that traverse the leaf surface at different rates through the hydrophobic cuticle. As mentioned above, non-ionic, and lipophilic molecules penetrate the hydrophobic cuticle easily compared to ionic and hydrophilic molecules (Eichert and Fernández 2012). The alternative entry pathway for ionic molecules is through aqueous pores whereby cations and anions must penetrate in equivalent amounts (Schönherr 2006). Also, ion uptake is against a concentration gradient both in leaves and roots (Fageria *et al.* 2009). The surface tension of a droplet determines the area of spread, which affects the efficiency of uptake. A high surface tension means the droplet has a low spread and poor uptake efficiency. To manipulate

this, adjuvant (any material added to a foliar solution to enhance uptake of the active ingredient) is added to foliar solutions to lower the surface tension, leading to a greater leaf–droplet contact area and increased retention time to improve leaf surface entry (Mercer 2007, Peirce *et al.* 2016, Troughton and Hall 1967). Adjuvants include surfactants, oils, pH buffers, or humectants (Somervaille *et al.* 2012). Epicuticular wax and contact angles on wheat were found to influence retention, leaf wetting and spreading of chemical sprays (Fernández and Brown 2013). Despite knowledge of physicochemical properties and leaf morphological features, there is an information gap relating to the correlation of these properties to foliar uptake of N. This makes research on leaf physiology and fertiliser properties vital to determine N penetration and uptake (Peirce *et al.* 2014a).

1.7.1.3 Environmental and time factors

The effectiveness of foliar sprays is influenced by environmental factors. These factors include humidity, temperature, rain, and wind strength, which affect foliar formulation properties as well as plant responses (Fernández *et al.* 2013). High humidity can cause fertiliser surface runoff, while low humidity may cause rapid drying and crystallization of liquid fertilisers on leaf surface leading to an increased leaf burning effect (Gamble and Emino 1987). High temperatures above 20°C are not ideal for foliar sprays as the applied liquid quickly dries off causing leaf burn, while very low temperature/frost may cause the liquid to freeze and reduce uptake efficiency. Excess wind disrupts the uniform distribution of foliar droplets and coagulates fine mist into larger droplets that may be more likely to also cause leaf burns. It is, therefore, necessary to apply foliar fertilisers during suitable weather conditions for maximum uptake and minimal leaf burn effects.

The timing of foliar N application is crucial for the effectiveness of grain yield and grain protein content (Gooding and Davies 1992). Grain yield does not seem to be affected by N applied after heading. However, grain N is significantly increased by foliar N application at anthesis and a few weeks after anthesis (Dampney and Salmon 1990) (Finney *et al.* 1957). Previous experiments carried out on the optimum timing for foliar urea applications to increase grain N have given relatively similar results with anthesis as the optimal time (Finney *et al.* 1957) or up to fourteen days after anthesis (Dampney and Salmon 1990, Gooding and Davies 1992) (Fig. 10). Reduced grain N responses from applications much later than anthesis may be due to lower green leaf area for the interception of the spray and/or less translocation to the grain (Gooding and Davies 1992).

1.7.1.4 Types of foliar N sprays

Urea is commonly used as a foliar fertiliser due to low cost, and possibly due to its non-ionic form, which easily traverses the hydrophobic cuticle layer of the leaf. However, urea has been reported to cause foliar injury through rapid hydrolysis by urease resulting in the accumulation of hydrolytic products, ammonium, and nitrate (Hinsvark *et al.* 1953, Wittwer *et al.* 1963). Urea is also highly volatile as it is broken down to ammonium that is converted to ammonia (Fig. 9) (Foy *et al.* 1953, Gooding and Davies 1992, Mérigout *et al.* 2008, Wylie *et al.* 2003). Its low salt index prevents desiccation of leaf cells through osmosis, although this is not true in all cases (Gray 1977). Fertilisers such as Urea Ammonium Sulfate (UAS), and Sulfate of Ammonia (SOA) are less volatile compared to urea (Fig. 9) but cause leaf burns that damage plants (Thorne and Watson 1955). Ammonium Nitrate (AN) has the lowest volatility but is the most potent concerning leaf damage (leaf spotting) (Doyle 2013).

Urea Ammonium Nitrate (UAN), on the other hand, is commonly used today as it has high N, low volatility rates compared to urea and a high solubility rate (Woolfolk *et al.* 2002). UAN contains all three N forms, urea, ammonium, and nitrate. In Australia, commercially available UAN formulation includes EasyN® that has 42.5% N w/v (21.5% urea, 10.5% ammonium and 10.5 % nitrate) (Incitec Pivot Fertilisers) and N42® containing 42% N w/v (21. 5% urea, 10.25% ammonium and 10.25 % nitrate) (Yara Australia Pty. Ltd.). Additional chemical properties of UAN, include a salting-out temperature at zero degrees, a pH of 6 - 7 and 10% higher viscosity than water. The density of UAN is 1.32 kg/L and the commercial product contains a corrosive inhibitor to protect storage tanks, (https://www.incitecpivotfertilisers.com.a).

Overall, the detrimental effects of foliar fertiliser, include leaf scorching, burning, or tipping which may have direct effects on yield. In wheat, the leaves appear discoloured at the tips while in some cases no leaf discoloration occurs after foliar application of urea (Dampney and Salmon 1990). To minimize leaf scorching, the following guidelines are recommended when applying foliar N fertilisers: foliar to be applied when there is no frost, early in the morning with no dew, or early evening using stream jets or coarse nozzles (for example, Turbo T with a droplet size of approximately 250 microns); avoid applying foliar N on water-stressed crops or wet foliage; avoid windy weather and temperatures above 20°C (Doyle 2013, Southern Farming Systems 2014). Overall, there is little information on which of the three N forms, urea, ammonium, and nitrate, are easily taken up as foliar and which commercial fertiliser is likely to do well as a foliar formulation for wheat.

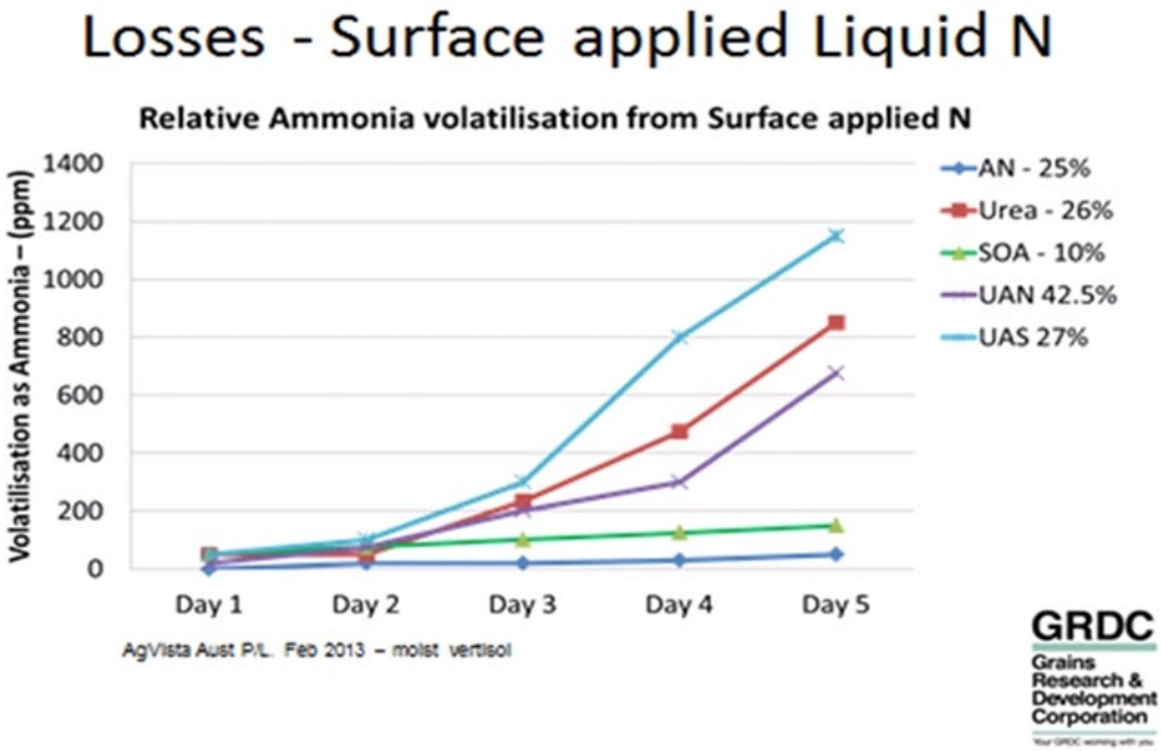

Figure 9: A plot showing the volatility of different liquid fertilisers, Ammonium Nitrate (AN), urea, Sulfate of Ammonia (SOA), Urea Ammonium Nitrate (UAN) and Urea Ammonium Sulfate (UAS), across a period of 5 days. N concentrations (%) in the different liquid fertilisers are shown in the legend. The ammonia volatilization from all fertilisers is low for the first two days, but between days three and five differences in volatility become clear. UAS has the highest volatility, followed by urea and UAN, while SOA and AN have the lowest volatility (Doyle 2013).

1.8 The use of foliar N application in wheat and knowledge gaps

It is necessary to determine the optimal time for foliar application of N to achieve an increase in GPC in specific genotypes, as this knowledge could be vital to wheat growers (Bly and Woodard 2003). The most appropriate type of liquid fertiliser also needs to be determined, as some cause serious leaf damage and increase susceptibility to diseases, thus decreasing total yields. For example, Wylie and colleagues reported a case where foliar urea and UAN increased susceptibility to yellow spot disease and attracted armyworms (Wylie *et al.* 2003). Various forms of foliar liquid fertilisers are available to growers, although most are expensive in

comparison to granular fertilisers, and a cost-benefit analysis is necessary for decision making by farmers.

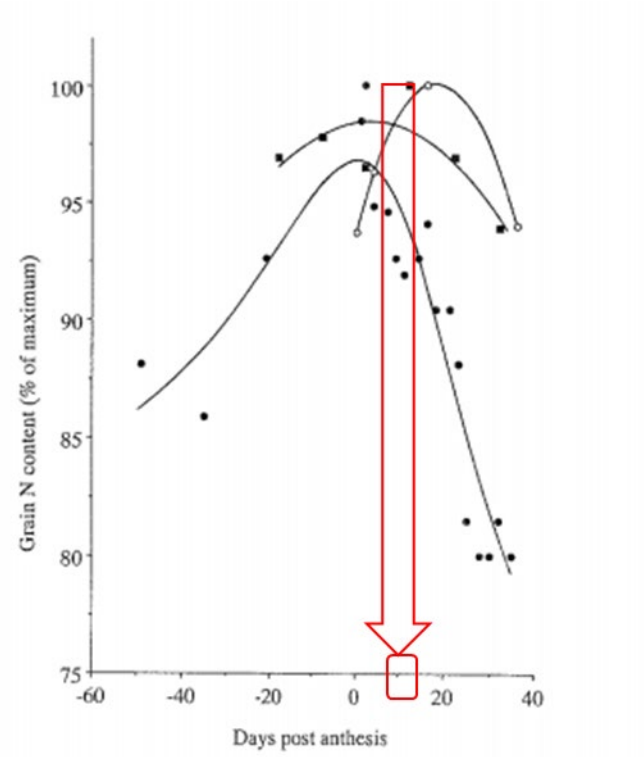

Figure 10: The effects of foliar urea N application in winter wheat (grown in USA and UK) at different timings on grain N content. Three separate regression curves are shown: quadratic divided by quadratic (●, r=0.92) for 33kg/ha urea (Finney et al. 1957), quadratic (■ , r=0.51) for 40kg/ha urea (Dampney and Salmon 1990) and quadratic (○, r=1) for 40kg/ha urea (Astbury and Kettlewell 1990). The red arrow shows the ideal theoretical timing for foliar N application, at between 7 and 14 days post-anthesis, for maximum grain N. Adapted from (Gooding and Davies 1992).

Understanding the effectiveness of penetration of different N forms through leaves, and the effects of leaf morphological traits on foliar uptake efficiency, are essential for maximizing the benefits of N applied as foliar. Moreover, identification of the specific N transporters that are involved in the uptake and transport of foliar-applied N could facilitate their manipulation for improving leaf-applied uptake. To date, there has been very little research focusing on uptake of foliar-applied N, either in wheat or plants more generally.

An initial step is the identification of a proper wheat growth stage to apply foliar N, with a primary focus of matching N supply with plant demand. This would aid in the optimization of foliar fertiliser application, leading to consistently high yields and boosting GPC while reducing the amount of fertiliser used limiting environmental

pollution. Moreover, evaluation of post-anthesis foliar N uptake is necessary to dissect the yield-protein trade-off and select for GPC without an associated penalty on yield. It should be noted that foliar absorption by wheat grown in hot and dry climates such as Australia, the target environment for my research, may be have a greater role to play. Foliar N applications may be less available for absorption by the roots, as soil during late stages of growth is very dry and root activity very low

A focus on the morphological aspects of wheat leaves that may correlate to N uptake via foliar application is also vital. These include leaf wax quantity and chemical composition, shapes of the waxes, trichomes, and stomata density in wheat varieties contrasting for foliar N uptake. At a molecular level, identifying leaf-expressed transporters that are associated with foliar N uptake is necessary.

Since there is little information relating to the specific forms of N that may be preferentially taken up through the leaf, research on this is also necessary for the proper formulation and effective application of foliar N fertilisers with minimal N losses. Furthermore, it is also necessary to investigate if the N taken up through foliar affects root N uptake and root transporters activity.

Also, for a better understanding of N use in wheat, investigating the N uptake, partitioning and remobilization to the grain tissue are necessary especially in wheat varieties contrasting for GPC/GPD. This will lead to a better understanding of the underlying physiological traits and genetic variability in the negative relationship between grain yield and GPC.

1.9 Thesis scope and outline

Previous research focused on soil N application. The objective of this study was to characterize the physiological and molecular aspects of foliar N fertiliser application

and its role in increasing GPC. Another objective was to understand N utilization, in wheat varieties from a diversity panel contrasting in GPC/GPD under different N treatments.

The specific aims of this project were:

I. To determine the most suitable reproductive stage to apply foliar N fertiliser for a stable yield and an increase in grain N in high-yielding wheat genotypes contrasting for GPC (Chapter 2).

II. To investigate wheat foliar N uptake at a physiological and molecular level, by focusing on the leaf surface properties that may be involved in foliar N uptake, the penetration/uptake rate of different forms of N using ^{15}N-labeled N nutrients, and N transporters (nitrate transporters, ammonium transporters and/or urea transporters found through *in silico* analysis to be expressed in leaf tissues) involved in foliar N uptake. (Chapter 3 and 4).

III. To identify the effects of foliar N on root N uptake. (Chapter 4).

IV. To understand N utilization in wheat varieties contrasting for GPC/GPD selected from a diversity panel (Chapter 5).

References

ABARES (2013). Agricultural commodity statistics 2013.

Acreche, M. M. and G. A. Slafer (2009). "Variation of grain nitrogen content in relation with grain yield in old and modern Spanish wheats grown under a wide range of agronomic conditions in a Mediterranean region." The Journal of Agricultural Science 147(06): 657-667.

Aslam, M., R. L. Travis and R. C. Huffaker (1992). "Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley (Hordeum vulgare L.) seedlings." Plant Physiol 99(3): 1124-1133.

Astbury, J. and P. Kettlewell (1990). "Optimising the management of nitrogen-containing fluid fertilizers for breadmaking quality of intensively-managed wheat in the United Kingdom: field experiments in 1989." Advances in Fluid Fertilizer Agronomic and Application Management Technology: 201-217.

Barber, S. A. (1995). Soil nutrient bioavailability: a mechanistic approach, John Wiley & Sons.

Barbottin, A., C. Lecomte, C. Bouchard and M.-H. Jeuffroy (2005). "Nitrogen remobilization during grain filling in wheat." Crop science 45(3): 1141-1150.

Barthlott, W., C. Neinhuis, D. Cutler, F. Ditsch, I. Meusel, I. Theisen and H. Wilhelmi (1998). "Classification and terminology of plant epicuticular waxes." Botanical journal of the Linnean society 126(3): 237-260.

Bernard, S. M. and D. Z. Habash (2009). "The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling." New Phytol 182(3): 608-620.

Bi, H., S. Luang, Y. Li, N. Bazanova, S. Morran, Z. Song, M. A. Perera, M. Hrmova, N. Borisjuk and S. Lopato (2016). "Identification and characterization of wheat drought-responsive MYB transcription factors involved in the regulation of cuticle biosynthesis." J Exp Bot 67(18): 5363-5380.

Bly, A. G. and H. J. Woodard (2003). "Foliar Nitrogen Application Timing Influence on Grain Yield and Protein Concentration of Hard Red Winter and Spring Wheat." Agronomy Journal 95: 335-338.

Bogard, M., V. Allard, M. Brancourt-Hulmel, E. Heumez, J. M. Machet, M. H. Jeuffroy, P. Gate, P. Martre and J. Le Gouis (2010). "Deviation from the grain protein concentration-grain yield negative relationship is highly correlated to post-anthesis N uptake in winter wheat." J Exp Bot 61(15): 4303-4312.

Brennan, J. P. and K. J. Quade (2004). Analysis of the Impact of CIMMYT Research on the Australian Wheat Industry.

Buchholz, A., P. Baur and J. Schönherr (1998). "Differences among plant species in cuticular permeabilities and solute mobilities are not caused by differential size selectivities." Planta 206(2): 322-328.

Buchner, P. and M. J. Hawkesford (2014). "Complex phylogeny and gene expression patterns of members of the NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family (NPF) in wheat." Journal of experimental botany: eru231.

Cassman, K. G., A. Dobermann, D. T. Walters and H. Yang (2003). "Meeting cereal demand while protecting natural resources and improving environmental quality." Annual Review of Environment and Resources 28(1): 315-358.

Cormier, F., J. Foulkes, B. Hirel, D. Gouache, Y. Moënne-Loccoz, J. Le Gouis and F. Ordon (2016). "Breeding for increased nitrogen-use efficiency: a review for wheat (T. aestivumL.)." Plant Breeding 135(3): 255-278.

Cox, M. C., C. O. Qualset and D. W. Rains (1985). "Genetic variation for nitrogen assimilation and translocation in wheat. I. Dry matter and nitrogen accumulation." Crop Sci 25(3): 430-435.

Cramer, M. and O. Lewis (1993). "The influence of nitrate and ammonium nutrition on the growth of wheat (Triticum aestivum) and maize (Zea mays) plants." Annals of Botany 72(4): 359-365.

Crawford, N. M. (1995). "Nitrate: nutrient and signal for plant growth." Plant Cell 7(7): 859-868.

Crawford, N. M. and A. D. Glass (1998). "Molecular and physiological aspects of nitrate uptake in plants." Trends in plant science 3(10): 389-395.

Dampney, P. and S. Salmon (1990). "The effect of rate and timing of late nitrogen application to breadmaking wheats as ammonium nitrate or foliar urea-N, and the effect of foliar sulphur application. I. Effect on yield, grain quality and recovery of nitrogen in grain." Aspects of Applied Biology(25): 229-241.

Davenport, R. J. and M. Tester (2000). "A weakly voltage-dependent, nonselective cation channel mediates toxic sodium influx in wheat." Plant Physiol 122(3): 823-834.

De Ruiter, J. and I. Brooking (1994). "Nitrogen and dry matter partitioning of barley grown in a dryland environment." New Zealand journal of crop and horticultural science 22(1): 45-55.

Distelfeld, A., R. Avni and A. M. Fischer (2014). "Senescence, nutrient remobilization, and yield in wheat and barley." J Exp Bot 65(14): 3783-3798.

Dobermann, A. and K. G. Cassman (2005). "Cereal area and nitrogen use efficiency are drivers of future nitrogen fertilizer consumption." Sci China C Life Sci 48 Suppl 2(2): 745-758.

Domínguez, E., J. A. Heredia-Guerrero and A. Heredia (2011). "The biophysical design of plant cuticles: an overview." New phytologist 189(4): 938-949.

Doyle, S. (2013). "Liquid Nitrogen: pros and cons of different formulations." Grain Research and Development Corporation.

Eichert, T. and V. Fernández (2012). "Uptake and release of elements by leaves and other aerial plant parts." Marschner's Mineral Nutrition of Higher Plants 3: 71-84.

Eichert, T. and H. E. Goldbach (2008). "Equivalent pore radii of hydrophilic foliar uptake routes in stomatous and astomatous leaf surfaces–further evidence for a stomatal pathway." Physiologia Plantarum 132(4): 491-502.

Eichert, T., H. E. Goldbach and J. Burkhardt (1998). "Evidence for the uptake of large anions through stomatal pores." Botanica Acta 111(6): 461-466.

Eichert, T., A. Kurtz, U. Steiner and H. E. Goldbach (2008). "Size exclusion limits and lateral heterogeneity of the stomatal foliar uptake pathway for aqueous solutes and water-suspended nanoparticles." Physiol Plant 134(1): 151-160.

Ellen, J. and J. Spiertz (1980). "Effects of rate and timing of nitrogen dressings on grain yield formation of winter wheat (T. aestivum L.)." Fertilizer research 1(3): 177-190.

Enghiad, A., D. Ufer, A. M. Countryman and D. D. Thilmany (2017). "An overview of global wheat market fundamentals in an era of climate concerns." International Journal of Agronomy 2017.

Fageria, N., M. B. Filho, A. Moreira and C. Guimaraes (2009). "Foliar fertilization of crop plants." Journal of plant nutrition 32(6): 1044-1064.

FAO (2003). "Crop breeding: the green revolution and the preceding millennia," United Nations, Food and Agriculture Organization.

FAO (2019a). FAO cereal supply and demand. World Food Situation. Rome, Italy., United Nations, Food and Agriculture Organization.

FAO (2019b). Global fertilizer production and agricultural use 2002 - 2017. Rome, Food and Agriculture Of The United Nation.

Feng, H., M. Yan, X. Fan, B. Li, Q. Shen, A. J. Miller and G. Xu (2011). "Spatial expression and regulation of rice high-affinity nitrate transporters by nitrogen and carbon status." J Exp Bot 62(7): 2319-2332.

Fernández, V. and P. H. Brown (2013). "From plant surface to plant metabolism: the uncertain fate of foliar-applied nutrients." Frontiers in plant science 4: 289.

Fernández, V. and T. Eichert (2009). "Uptake of hydrophilic solutes through plant leaves: current state of knowledge and perspectives of foliar fertilization." Critical Reviews in Plant Sciences 28(1-2): 36-68.

Fernández, V., T. Sotiropoulos and P. H. Brown (2013). "Foliar fertilisation: Principles and practices." International Fertiliser Industry Association (IFA): Paris, France: 140.

Finney, K., J. Meyer, F. Smith and H. Fryer (1957). "Effect of foliar spraying of Pawnee wheat with urea solutions on yield, protein content and protein quality." Agronomy journal 49: 341-347.

Forde, B. G. (2000). "Nitrate transporters in plants: structure, function and regulation." Biochim Biophys Acta 1465(1-2): 219-235.

Foy, C., G. Montenegro and S. Barber (1953). "Foliar feeding of corn with urea nitrogen." Soil Science Society of America Journal 17(4): 387-390.

Franke, W. (1967). "Mechanisms of foliar penetration of solutions." Annual Review of Plant Physiology 18(1): 281-300.

Gallagher, L. W., K. M. Soliman, D. Rains, C. Qualset and R. Huffaker (1983). "Nitrogen Assimilation in Common Wheats Differing in Potential Nitrate Reductase Activity and Tissue Nitrate Concentrations 1." Crop science 23(5): 913-919.

Gallais, A. and M. Coque (2005). "Genetic variation and selection for nitrogen use efficiency in maize: a synthesis." Maydica 50(3/4): 531.

Gamble, P. and E. Emino (1987). "Morphological and anatomical characterization of leaf burn in corn induced from foliar-applied nitrogen." Agronomy Journal 79(1): 92-96.

Garnett, T., V. Conn, D. Plett, S. Conn, J. Zanghellini, N. Mackenzie, A. Enju, K. Francis, L. Holtham, U. Roessner, B. Boughton, A. Bacic, N. Shirley, A. Rafalski, K. Dhugga, M. Tester and B. N. Kaiser (2013). "The response of the maize nitrate transport system to nitrogen demand and supply across the lifecycle." New Phytol 198(1): 82-94.

Gaur, V. S., U. Singh, A. K. Gupta and A. Kumar (2012). "Influence of different nitrogen inputs on the members of ammonium transporter and glutamine synthetase genes in two rice genotypes having differential responsiveness to nitrogen." Molecular biology reports 39(8): 8035-8044.

Gazzarrini, S., L. Lejay, A. Gojon, O. Ninnemann, W. B. Frommer and N. von Wirén (1999). "Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into Arabidopsis roots." The Plant Cell 11(5): 937-947.

Gerendás, J., Z. Zhu and B. Sattelmacher (1998). "Influence of N and Ni supply on nitrogen metabolism and urease activity in rice (Oryza sativa L.)." Journal of Experimental Botany 49(326): 1545-1554.

Godden, D., R. Batterham and R. Drynan (1998). "Climate change and Australian wheat yield." Nature 391(6666): 447-448.

Gooding, M. J. and W. P. Davies (1992). "Foliar urea fertilization of cereals: A review." Fertilizer Research 32(2): 209-222.

Gray, R. C. (1977). Foliar fertilisation with primary nutrients during the reproductive stage of plant growth.

Gregersen, P. L., P. B. Holm and K. Krupinska (2008). "Leaf senescence and nutrient remobilisation in barley and wheat." Plant Biol (Stuttg) 10 Suppl 1: 37-49.

Griffiths, M. and L. M. York (2020). "Targeting root ion uptake kinetics to increase plant productivity and nutrient use efficiency." Plant Physiology 182(4): 1854-1868.

Guo, F. Q., J. Young and N. M. Crawford (2003). "The nitrate transporter AtNRT1.1 (CHL1) functions in stomatal opening and contributes to drought susceptibility in Arabidopsis." Plant Cell 15(1): 107-117.

Guo, T., H. Xuan, Y. Yang, L. Wang, L. Wei, Y. Wang and G. Kang (2014). "Transcription Analysis of Genes Encoding the Wheat Root Transporter NRT1 and NRT2 Families During Nitrogen Starvation." Journal of Plant Growth Regulation 33(4): 837-848.

Guttieri, M. J., K. Frels, T. Regassa, B. M. Waters and P. S. Baenziger (2017). "Variation for nitrogen use efficiency traits in current and historical great plains hard winter wheat." Euphytica 213(4): 87.

Habash, D. Z., S. Bernard, J. Schondelmaier, J. Weyen and S. A. Quarrie (2007). "The genetics of nitrogen use in hexaploid wheat: N utilisation, development and yield." Theoretical and Applied Genetics 114(3): 403-419.

Hawkesford, M., W. Horst, T. Kichey, H. Lambers, J. Schjoerring, I. S. Møller and P. White (2012). Functions of macronutrients. Marschner's mineral nutrition of higher plants, Elsevier: 135-189.

He, F., A. A. Karve, S. Maslov and B. A. Babst (2016). "Large-Scale Public Transcriptomic Data Mining Reveals a Tight Connection between the Transport of Nitrogen and Other Transport Processes in Arabidopsis." Front Plant Sci 7(1207): 1207.

Hine, J. C. and J. I. Sprent (1988). "Growth ofPhaseolus vulgarison Various Nitrogen Sources: The Importance of Urease." Journal of Experimental Botany 39(11): 1505-1512.

Hinsvark, O., S. Wittwer and H. Tukey (1953). "The Metabolism of Foliar-Applied Urea. I. Relative Rates of C14O2 Production by Certain Vegetable Plants Treated with Labeled Urea." Plant physiology 28(1): 70.

Hirel, B., J. Le Gouis, B. Ney and A. Gallais (2007). "The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches." J Exp Bot 58(9): 2369-2387.

Hooper, G. M. a. P. (2013). "Nitrogen decision-Guidelines and rules of thumb." Grain Research and Development Corporation.

Jahn, T. P., A. L. Moller, T. Zeuthen, L. M. Holm, D. A. Klaerke, B. Mohsin, W. Kuhlbrandt and J. K. Schjoerring (2004). "Aquaporin homologues in plants and mammals transport ammonia." FEBS Lett 574(1-3): 31-36.

Jeff, B., M. Lynne, F. Mark, W. Nina and M. Marta (2018). Nitrogen Dynamics In Modern Cropping Systems. GRDC. Australia.

Kannan, S. (2010). Foliar fertilization for sustainable crop production. Genetic engineering, biofertilisation, soil quality and organic farming, Springer: 371-402.

Kerstiens, G. (1996). "Cuticular water permeability and its physiological significance." Journal of Experimental Botany 47(12): 1813-1832.

Kichey, T., B. Hirel, E. Heumez, F. Dubois and J. Le Gouis (2007). "Wheat genetic variability for post-anthesis nitrogen absorption and remobilisation revealed by 15N labelling and correlations with agronomic traits and nitrogen physiological markers." Field Crops Research 102: 22-32.

Kojima, S., A. Bohner and N. von Wiren (2006). "Molecular mechanisms of urea transport in plants." J Membr Biol 212(2): 83-91.

Kong, L., Y. Xie, L. Hu, B. Feng and S. Li (2016). "Remobilization of vegetative nitrogen to developing grain in wheat (Triticum aestivum L.)." Field Crops Research 196: 134-144.

Ladha, J. K., A. Tirol-Padre, C. K. Reddy, K. G. Cassman, S. Verma, D. S. Powlson, C. van Kessel, B. R. D. de, D. Chakraborty and H. Pathak (2016). "Global nitrogen budgets in cereals: A 50-year assessment for maize, rice, and wheat production systems." Sci Rep 6(1): 19355.

Lassaletta, L., G. Billen, B. Grizzetti, J. Anglade and J. Garnier (2014). "50 year trends in nitrogen use efficiency of world cropping systems: the relationship between yield and nitrogen input to cropland." Environmental Research Letters 9(10): 105011.

Le Gouis, J., D. Béghin, E. Heumez and P. Pluchard (2000). "Genetic differences for nitrogen uptake and nitrogen utilisation efficiencies in winter wheat." European Journal of Agronomy 12(3): 163-173.

Léran, S., K. Varala, J.-C. Boyer, M. Chiurazzi, N. Crawford, F. Daniel-Vedele, L. David, R. Dickstein, E. Fernandez and B. Forde (2014). "A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family members in plants." Trends in plant science 19(1): 5-9.

Lezhneva, L., T. Kiba, A. B. Feria-Bourrellier, F. Lafouge, S. Boutet-Mercey, P. Zoufan, H. Sakakibara, F. Daniel-Vedele and A. Krapp (2014). "The Arabidopsis nitrate transporter NRT2.5 plays a role in nitrate acquisition and remobilization in nitrogen-starved plants." Plant J 80(2): 230-241.

Li, B.-z., M. Merrick, S.-m. Li, H.-y. Li, S.-w. Zhu, W.-m. Shi and Y.-h. Su (2009). "Molecular Basis and Regulation of Ammonium Transporter in Rice." Rice Science 16(4): 314-322.

Li, S.-M., B.-Z. Li and W.-M. Shi (2012). "Expression Patterns of Nine Ammonium Transporters in Rice in Response to N Status." Pedosphere 22(6): 860-869.

Liu, D., L. Yang, Q. Zheng, Y. Wang, M. Wang, X. Zhuang, Q. Wu, C. Liu, S. Liu and Y. Liu (2015a). "Analysis of cuticular wax constituents and genes that contribute to the formation of 'glossy Newhall', a spontaneous bud mutant from the wild-type 'Newhall'navel orange." Plant molecular biology 88(6): 573-590.

Liu, J., J. Fu, H. Tian and Y. Gao (2015b). "In-season expression of nitrate and ammonium transporter genes in roots of winter wheat (Triticum aestivum L.) genotypes with different nitrogen-uptake efficiencies." Crop and Pasture Science 66(7): 671-678.

Liu, L. H., U. Ludewig, W. B. Frommer and N. von Wiren (2003a). "AtDUR3 encodes a new type of high-affinity urea/H+ symporter in Arabidopsis." Plant Cell 15(3): 790-800.

Liu, L. H., U. Ludewig, B. Gassert, W. B. Frommer and N. von Wiren (2003b). "Urea transport by nitrogen-regulated tonoplast intrinsic proteins in Arabidopsis." Plant Physiol 133(3): 1220-1228.

Marschner, H. (2011). Marschner's mineral nutrition of higher plants, Academic press.

Martre, P., J. R. Porter, P. D. Jamieson and E. Triboi (2003). "Modeling grain nitrogen accumulation and protein composition to understand the sink/source regulations of nitrogen remobilization for wheat." Plant Physiol 133(4): 1959-1967.

Masclaux-Daubresse, C., F. Daniel-Vedele, J. Dechorgnat, F. Chardon, L. Gaufichon and A. Suzuki (2010). "Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture." Ann Bot 105(7): 1141-1157.

Melino, V. J., G. Fiene, A. Enju, J. Cai, P. Buchner and S. Heuer (2015). "Genetic diversity for root plasticity and nitrogen uptake in wheat seedlings." Functional Plant Biology 42(10): 942-956.

Mercer, G. (2007). "A simple diffusion model of the effect of droplet size and spread area on foliar uptake of hydrophilic compounds." Pesticide biochemistry and physiology 88(2): 128-133.

Mérigout, P., V. Gaudon, I. Quilleré, X. Briand and F. Daniel-Vedele (2008). "Urea use efficiency of hydroponically grown maize and wheat." Journal of plant nutrition 31(3): 427-443.

Merigout, P., M. Lelandais, F. Bitton, J. P. Renou, X. Briand, C. Meyer and F. Daniel-Vedele (2008). "Physiological and transcriptomic aspects of urea uptake and assimilation in Arabidopsis plants." Plant Physiol 147(3): 1225-1238.

Miller, A. J. and M. D. Cramer (2005). Root nitrogen acquisition and assimilation. Root physiology: From gene to function, Springer: 1-36.

Miller, A. J., X. Fan, M. Orsel, S. J. Smith and D. M. Wells (2007). "Nitrate transport and signalling." J Exp Bot 58(9): 2297-2306.

Miller, A. J. and S. J. Smith (1996). "Nitrate transport and compartmentation in cereal root cells." Journal of Experimental Botany 47(7): 843-854.

Moll, R., E. Kamprath and W. Jackson (1982). "Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization 1." Agronomy journal 74(3): 562-564.

Monaghan, J. M., J. W. Snape, A. J. S. Chojecki and P. S. Kettlewell (2001). "The use of grain protein deviation for identifying wheat cultivars with high grain protein concentration and yield." Euphytica 122(2): 309-317.

Mueller, N. D., P. C. West, J. S. Gerber, G. K. MacDonald, S. Polasky and J. A. Foley (2014). "A tradeoff frontier for global nitrogen use and cereal production." Environmental Research Letters 9(5): 054002.

Munier, J., N. G and C. Salon (2005). "Are the carbon costs of seed production related to the quantitative and qualitative performance? An appraisal for legumes and other crops." Plant, Cell & Environment 28(11): 1388-1395.

Ninnemann, O., J. C. Jauniaux and W. B. Frommer (1994). "Identification of a high affinity NH4+ transporter from plants." EMBO J 13(15): 3464-3471.

Okamoto, M., A. Kumar, W. Li, Y. Wang, M. Y. Siddiqi, N. M. Crawford and A. D. Glass (2006). "High-affinity nitrate transport in roots of Arabidopsis depends on expression of the NAR2-like gene AtNRT3.1." Plant Physiol 140(3): 1036-1046.

Okamoto, M., J. J. Vidmar and A. D. Glass (2003). "Regulation of NRT1 and NRT2 gene families of Arabidopsis thaliana: responses to nitrate provision." Plant Cell Physiol 44(3): 304-317.

Orsel, M., F. Chopin, O. Leleu, S. J. Smith, A. Krapp, F. Daniel-Vedele and A. J. Miller (2006). "Characterization of a two-component high-affinity nitrate uptake system in Arabidopsis. Physiology and protein-protein interaction." Plant Physiol 142(3): 1304-1317.

Ortiz-MonasterioR, J. I., K. D. Sayre, S. Rajaram and M. McMahon (1997). "Genetic Progress in Wheat Yield and Nitrogen Use Efficiency under Four Nitrogen Rates." Crop Science 37(3): cropsci1997.0011183X003700030033x.

Oury, F.-X., P. Berard, M. Brancourt-Hulmel, E. Heumez, P. Pluchard, M. Rousset, G. Doussinault, B. Rolland, M. Trottet and A. Giraud (2003). "Yield and grain protein concentration in bread wheat: a review and a study of multi-annual data from a French breeding program [Triticum aestivum L.]." Journal of Genetics and Breeding (Italy).

Oury, F.-X., H. Chiron, A. Faye, O. Gardet, A. Giraud, E. Heumez, B. Rolland, M. Rousset, M. Trottet and G. Charmet (2010). "The prediction of bread wheat quality: joint use of the phenotypic information brought by technological tests and the genetic information brought by HMW and LMW glutenin subunits." Euphytica 171(1): 87-109.

Pao, S. S., I. T. Paulsen and M. H. Saier, Jr. (1998). "Major facilitator superfamily." Microbiol Mol Biol Rev 62(1): 1-34.

Pask, A. J. D., R. Sylvester-Bradley, P. D. Jamieson and M. J. Foulkes (2012). "Quantifying how winter wheat crops accumulate and use nitrogen reserves during growth." Field Crops Research 126: 104-118.

Pate, J. (1980). "Transport and partitioning of nitrogenous solutes." Annual Review of Plant Physiology 31(1): 313-340.

Peirce, C., T. McBeath, V. Fernández and M. McLaughlin (2014a). "Wheat leaf properties affecting the absorption and subsequent translocation of foliar-applied phosphoric acid fertiliser." Plant and soil 384(1-2): 37-51.

Peirce, C. A., C. Priest, T. M. McBeath and M. J. McLaughlin (2016). "Uptake of phosphorus from surfactant solutions by wheat leaves: spreading kinetics, wetted area, and drying time." Soft Matter 12(1): 209-218.

Peirce, C. A. E., T. M. McBeath, V. Fernández and M. J. McLaughlin (2014b). "Wheat leaf properties affecting the absorption and subsequent translocation of foliar-applied phosphoric acid fertiliser." Plant and Soil 384(1-2): 37-51.

Ping, W., Z.-L. Wang, R.-G. Cai, L. Yong, X.-G. Chen and Y.-P. Yin (2011). "Physiological and molecular response of wheat roots to nitrate supply in seedling stage." Agricultural Sciences in China 10(5): 695-704.

Plett, D., J. Toubia, T. Garnett, M. Tester, B. N. Kaiser and U. Baumann (2010). "Dichotomy in the NRT gene families of dicots and grass species." PLoS One 5(12): e15289.

Pollard, M., F. Beisson, Y. Li and J. B. Ohlrogge (2008). "Building lipid barriers: biosynthesis of cutin and suberin." Trends Plant Sci 13(5): 236-246.

Puente, D. W. and P. Baur (2011). "Wettability of soybean (Glycine max L.) leaves by foliar sprays with respect to developmental changes." Pest Manag Sci 67(7): 798-806.

Raun, W. R. and G. V. Johnson (1999). "Improving nitrogen use efficiency for cereal production." Agronomy journal 91(3): 357-363.

Rawat, S. R., S. N. Silim, H. J. Kronzucker, M. Y. Siddiqi and A. D. Glass (1999). "AtAMT1 gene expression and NH4+ uptake in roots of Arabidopsis thaliana: evidence for regulation by root glutamine levels." Plant J 19(2): 143-152.

Reeves, J. (1954). "Some effects of spraying wheat with urea." J Aust I Agr Sci 20: 41-45.

Samuels, L., L. Kunst and R. Jetter (2008). "Sealing plant surfaces: cuticular wax formation by epidermal cells." Annu Rev Plant Biol 59: 683-707.

Schönherr, J. (2000). "Calcium chloride penetrates plant cuticles via aqueous pores." Planta 212(1): 112-118.

Schönherr, J. (2006). "Characterization of aqueous pores in plant cuticles and permeation of ionic solutes." Journal of Experimental Botany 57(11): 2471-2491.

Shewry, P. R. (2007). "Improving the protein content and composition of cereal grain." Journal of Cereal Science 46(3): 239-250.

Smith, C., J. Freney, R. Sherlock and I. Galbally (1991). "The fate of urea nitrogen applied in a foliar spray to wheat at heading." Fertilizer research 28(2): 129-138.

Sogaard, R., M. Alsterfjord, N. Macaulay and T. Zeuthen (2009). "Ammonium ion transport by the AMT/Rh homolog TaAMT1;1 is stimulated by acidic pH." Pflugers Arch 458(4): 733-743.

Sohlenkamp, C., M. Shelden, S. Howitt and M. Udvardi (2000). "Characterization of Arabidopsis AtAMT2, a novel ammonium transporter in plants." FEBS Lett 467(2-3): 273-278.

Somervaille, A., G. Betts, B. Gordon, V. Green, M. Burgis and R. Henderson (2012). "Adjuvants—Oils, surfactants and other additives for farm chemicals, revised 2012 edition." Grains Research & Development Corp, Canberra, ACT, Australia.

Southern Farming Systems (2014). Evaluation of Late Nitrogen Applications to Achieve Yield Potential and increased protein content in wheat. Kingston, Grain Research and Development Corporation.

Strong, W. (1982). "Effect of late application of nitrogen on the yield and protein content of wheat." Australian Journal of Experimental Agriculture 22(115): 54-61.

Subedi, K. D., B. L. Ma and A. G. Xue (2007). "Planting Date and Nitrogen Effects on Grain Yield and Protein Content of Spring Wheat." Crop Science 47(1): 36-44.

Sutton, M. A., A. Bleeker, C. Howard, J. Erisman, Y. Abrol, M. Bekunda, A. Datta, E. Davidson, W. de Vries and O. Oenema (2013). Our nutrient world. The challenge to produce more food & energy with less pollution, Centre for Ecology & Hydrology.

Suzuki, A. and D. B. Knaff (2005). "Glutamate synthase: structural, mechanistic and regulatory properties, and role in the amino acid metabolism." Photosynth Res 83(2): 191-217.

Sylvester-Bradley, R. and D. R. Kindred (2009). "Analysing nitrogen responses of cereals to prioritize routes to the improvement of nitrogen use efficiency." Journal of Experimental Botany 60(7): 1939-1951.

Taulemesse, F., J. Le Gouis, D. Gouache, Y. Gibon and V. Allard (2016). "Bread Wheat (Triticum aestivum L.) Grain Protein Concentration Is Related to Early Post-Flowering Nitrate Uptake under Putative Control of Plant Satiety Level." PLoS One 11(2): e0149668.

Taylor, L., A. Nunes-Nesi, K. Parsley, A. Leiss, G. Leach, S. Coates, A. Wingler, A. R. Fernie and J. M. Hibberd (2010). "Cytosolic pyruvate,orthophosphate dikinase functions in nitrogen remobilization during leaf senescence and limits individual seed growth and nitrogen content." Plant J 62(4): 641-652.

Thorne, G. N. and D. Watson (1955). "The effect on yield and leaf area of wheat of applying nitrogen as a top-dressing in April or in sprays at ear emergence." The Journal of Agricultural Science 46(04): 449-456.

Troughton, J. H. and D. M. Hall (1967). "Extracuticular Wax and Contact Angle Measurements on Wheat (Triticum Vulgare L.)." Australian Journal of Biological Sciences 20(3): 509-526.

Trueman, L. J., A. Richardson and B. G. Forde (1996). "Molecular cloning of higher plant homologues of the high-affinity nitrate transporters of Chlamydomonas reinhardtii and Aspergillus nidulans." Gene 175(1-2): 223-231.

Tsay, Y. F., C. C. Chiu, C. B. Tsai, C. H. Ho and P. K. Hsu (2007). "Nitrate transporters and peptide transporters." FEBS Lett 581(12): 2290-2300.

Uauy, C., A. Distelfeld, T. Fahima, A. Blechl and J. Dubcovsky (2006). "A NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat." Science 314(5803): 1298-1301.

Van Sanford, D. and C. MacKown (1986). "Variation in nitrogen use efficiency among soft red winter wheat genotypes." Theoretical and Applied Genetics 72(2): 158-163.

Vidmar, J. J., D. Zhuo, M. Y. Siddiqi and A. D. Glass (2000). "Isolation and characterization of HvNRT2. 3and HvNRT2. 4, cDNAs encoding high-affinity nitrate transporters from roots of barley." Plant Physiology 122(3): 783-792.

Wang, B., L. Liu, G. J. O'Leary, S. Asseng, I. Macadam, R. Lines-Kelly, X. Yang, A. Clark, J. Crean, T. Sides, H. Xing, C. Mi and Q. Yu (2018). "Australian wheat production expected to decrease by the late 21st century." Glob Chang Biol 24(6): 2403-2415.

Wang, J., H. Hao, R. Liu, Q. Ma, J. Xu, F. Chen, Y. Cheng and X. Deng (2014). "Comparative analysis of surface wax in mature fruits between Satsuma mandarin (Citrus unshiu) and 'Newhall'navel orange (Citrus sinensis) from the perspective of crystal morphology, chemical composition and key gene expression." Food chemistry 153: 177-185.

Wang, P., Z.-l. Wang, R.-g. Cai, Y. Li, X.-g. Chen and Y.-p. Yin (2011). "Physiological and Molecular Response of Wheat Roots to Nitrate Supply in Seedling Stage." Agricultural Sciences in China 10(5): 695-704.

Wang, W. H., B. Köhler, F.-Q. Cao and L.-H. Liu (2008). "Molecular and physiological aspects of urea transport in higher plants." Plant Science 175(4): 467-477.

Wang, W. H., B. Kohler, F. Q. Cao, G. W. Liu, Y. Y. Gong, S. Sheng, Q. C. Song, X. Y. Cheng, T. Garnett, M. Okamoto, R. Qin, B. Mueller-Roeber, M. Tester and L. H. Liu (2012). "Rice DUR3 mediates high-affinity urea transport and plays an effective role in improvement of urea acquisition and utilization when expressed in Arabidopsis." New Phytol 193(2): 432-444.

Watson, C., M. H, P. Poland, D. Kilpatrick, M. Allen, M. Garrett and C. Christianson (1994). "Soil properties and the ability of the urease inhibitor N-(n-butyl) thiophosphoric triamide (nBTPT) to reduce ammonia volatilization from surface-applied urea." Soil Biology and Biochemistry 26(9): 1165-1171.

Williams, L. and A. Miller (2001). "Transporters Responsible for the Uptake and Partitioning of Nitrogenous Solutes." Annu Rev Plant Physiol Plant Mol Biol 52(1): 659-688.

Wittwer, S., M. Bukovac and H. Tukey (1963). "Advances in foliar feeding of plant nutrients." Fertilizer technology and usage: 429-455.

Woolfolk, C. W., W. R. Raun, G. V. Johnson, W. E. Thomason, R. W. Mullen, K. J. Wynn and K. W. Freeman (2002). "Influence of Late-Season Foliar Nitrogen Applications on Yield and Grain Nitrogen in Winter Wheat." Agronomy Journal 94(3): 429-434.

Wu, H., J. Xiang, Y. Zhang, Y. Zhang, S. Peng, H. Chen and D. Zhu (2018). "Effects of Post-Anthesis Nitrogen Uptake and Translocation on Photosynthetic Production and Rice Yield." Sci Rep 8(1): 12891.

Wylie, P., A. Mayfield, F. Harrop and A. Roe (2003). The potential of fluid fertilisers for broadacre cropping in Australia HOR 00002 Australia, Grains Research and Development Corporation.

Xu, G., X. Fan and A. J. Miller (2012). "Plant Nitrogen Assimilation and Use Efficiency." Annual Review of Plant Biology 63(1): 153-182.

Yamaya, T. and A. Oaks (2004). Metabolic Regulation of Ammonium uptake and Assimilation. Nitrogen Acquisition and Assimilation in Higher Plants. S. Amâncio and I. Stulen. Dordrecht, Springer Netherlands: 35-63.

Yin, L.-P., P. Li, B. Wen, D. Taylor and J. O. Berry (2007). "Characterization and expression of a high-affinity nitrate system transporter gene (TaNRT2.1) from wheat roots, and its evolutionary relationship to other NTR2 genes." Plant Science 172(3): 621-631.

Zanin, L., N. Tomasi, C. Wirdnam, S. Meier, N. Y. Komarova, T. Mimmo, S. Cesco, D. Rentsch and R. Pinton (2014). "Isolation and functional characterization of a high affinity urea transporter from roots of Zea mays." BMC Plant Biol 14(1): 222.

Chapter 2

The effectiveness of foliar-applied nitrogen in improving wheat grain quality

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
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Abstract

In wheat production, nitrogen fertiliser management is necessary to maximize yield and optimize grain protein content (GPC). Since the uptake and utilisation of N fertiliser are low in wheat, an N management strategy involving application in split dosage with soil N application at sowing followed by a foliar N application during the reproductive stages may improve grain yield and grain quality. We investigated the effects of post-anthesis foliar N application on grain yield and GPC in wheat. Two Australian high-yielding varieties of bread wheat (*Triticum aestivum*), Gregory and Spitfire contrasting for GPC were grown in field and glasshouse conditions. N fertiliser was applied as a basal granular form (urea; 46% N w/w) at sowing, and subsequently as foliar N in liquid form (UAN 42.5% N w/v). In field conditions, this experiment was conducted in two consecutive years (2016 and 2017) and foliar N was applied at 7 or 14 days after anthesis (DPA). In the glasshouse, foliar N was applied at each of five growth stages (GS49_awn emergence, GS59_heading, GS65_anthesis, 7 DPA and 14 DPA). Plants were harvested at maturity and processed for yield components, and GPC. Foliar N application did not affect yield components for either cultivar in field conditions. Compared to controls, GPC and grain N yield per m^2 were significantly increased for Gregory following foliar N treatment at 7 DPA. Spitfire, on the other hand, showed no GPC increase after foliar N treatment but maintained high GPC across all treatments. Similar trends were observed in glasshouse conditions. Furthermore, grain N yield per spike in Gregory increased following foliar N application at GS59_heading and 7 DPA, compared to control. These findings demonstrate the potential for foliar application of N fertiliser post-anthesis as an agronomic management practice to improve GPC and grain N yield in low-medium GPC wheat varieties without yield penalties.

Keywords: Nitrogen, Foliar fertilisation, Grain N, Post-anthesis, GPC, Gregory, Spitfire

Introduction

Nitrogen (N) is an important macronutrient required by plants for growth, development, and reproduction (Worland *et al.* 2017). It is freely available in the atmosphere, but only a limited amount is available to non-N fixing plants, necessitating the use of organic and synthetic forms of N, including manure and chemical fertiliser. Utilisation of N-containing fertilisers in cereal crops has not only increased yield but also the quality of grains through higher grain protein content (GPC), translating to improved premiums paid to farmers in some countries and diversified uses in the food industry (Bly and Woodard 2003, Woolfolk *et al.* 2002). Overall, grain yield is a major priority in the cereal industry, while GPC is also gaining a forefront due to its importance.

The downside of using these fertilisers is the fact that they are made from energy-consuming processes that rely on fossil fuels, causing harm to the environment (Garnett *et al.* 2015, Robertson and Vitousek 2009). Also, most crops have poor N uptake and utilisation efficiency (Sylvester-Bradley and Kindred 2009), leading to overuse of N fertilisers that also causes environmental pollution. In Australian agriculture, the annual N fertiliser used is over 1 million tons (ABARES 2013). Unfortunately, in wheat, the recovery rate of N is low with less than 40% applied N ending up in the grain (Raun and Johnson 1999). The low recovery efficiency of N is explained by losses through denitrification, volatilisation, leaching, especially from fertiliser sources, and N immobilisation in organic matter by soil microbes (Fageria 2014). Therefore, the poor uptake and utilisation of N fertiliser by crops necessitate the development of proper application and management strategies and breeding of crops with improved N efficiency (Robertson and Vitousek 2009).

Wheat is an important crop in the world, providing human food and livestock feed (Ladha *et al.* 2016). With a vastly growing human population and increasing demands for food production, growers must produce more wheat. This means either increasing land acreage to grow the crop or using more fertiliser to increase yield and grain quality. Unfortunately, wheat takes up only 40-50% of applied N from fertilisers, while the remainder is unutilized within the growing season (Sylvester-Bradley and Kindred 2009). A global assessment of total N input into wheat fields from 1960-2010 was estimated at 1.147 X 10^9 tonness, while N harvested from wheat as grain and straw was 6.16 X 10^8 tonnes (Ladha *et al.* 2016).

Several strategies are available to improve N uptake and utilisation. For example, applying N at several wheat growth stages instead of as a single sowing dose. The split application helps to match N demand with N supply (Garnett *et al.* 2015). N applied early in the season is invested towards yield and when applied later it is more effective at modifying GPC (Brown and Petrie 2006, Ottman *et al.* 2000). Cereal crops normally take up most N before the flowering stage, with reports of N accumulation after anthesis depending on N availability and moisture levels (Dhugga and Waines 1989). In wheat, the total N uptake at flowering could be 90 -100% of the total amount (Hooper 2013). However, plants can take up more N during the grain filling stage in high N conditions and depending on sink N uptake capacity, soil moisture levels and root activity (Bogard *et al.* 2010, Heitholt *et al.* 1990). Research conducted by Cassman and colleagues showed that N applied at sowing and later in the season was important to maximize yield and GPC, respectively, in wheat (Cassman *et al.* 1992, Gooding and Davies 1992).

Application of N fertiliser as split dosage may include a soil N application at sowing and a complimentary foliar N application at later growth stages. Foliar N application

later in the growing season, may have crucial benefits because N applied has more management flexibility as farmers can adjust the N rates according to the plant growth needs (Woolfolk *et al.* 2002). Other advantages of an additional late foliar N application include reduced N losses through leaching and denitrification. Also, foliar N application is potentially more effective than soil-based methods of application late in the season when soil moisture and root activities are low (Bogard *et al.* 2010).

Previous research showed foliar fertiliser applications had variable effects on GPC which could be related to incorrect application times, unsuitable fertiliser formulations, insufficient knowledge of soil-available nutrients, and environmental conditions (Oosterhuis 2009). However, other research findings showed an increase in GPC when foliar N was applied late in the growing season, without an associated penalty on yield (Brown and Petrie 2006, Cormier *et al.* 2016, Ottman *et al.* 2000). Furthermore, applying liquid N as a spray solution on wheat leaves, especially the flag leaf, may result in grain getting faster N access (Wuest and Cassman 1992). However, genetic aspects of the response to foliar N application in wheat have not been considered. This study aimed to investigate the effect of foliar N on yield, and GPC when applied at different growth stages in the reproductive phase of two bread wheat varieties contrasting for GPC.

Material and methods

Plant material and experimental conditions

Spring bread wheat (*Triticum aestivum* L.) varieties Gregory (Pelsart/2*Batavia), a medium GPC variety bred by Queensland Department of Primary Industries, and Spitfire (Drysdale/Kukri), a high GPC variety bred by LongReach Plant Breeders, were

grown in field and glasshouse conditions. Both varieties have a high yield potential. Spitfire is an early-mid maturing variety while Gregory has a medium to late maturity.

Field experiments

Gregory and Spitfire were grown in rain-fed, field-like conditions at Waite Campus (34.97° S 138.64° E), in two consecutive years (main growing seasons in 2016 and 2017). Before starting the experiments, soil samples were collected from 0-10 cm topsoil and 10-30cm sub-soil and sent for soil analysis (CSBP, Kwinana WA). The results from the soil analysis showed N was 8 mg/kg of soil for both topsoil and subsoil.

The experiment was a randomized complete block design with two varieties (Spitfire and Gregory) × three treatments (control, foliar treatment 7 days post-anthesis (DPA) and foliar 14 DPA) × four replicates. The field, with an area of 42.7m^2, was divided into four main blocks of 10.6 m^2 with six plots in each. In both years, basal N was applied as granular urea (46% N) at sowing at a rate of 50 kg N/ha. Phosphorus was applied as Superphosphate fertiliser during sowing at a rate of 10 kg P/ha. For each plot (1.7 m^2), seeds were sown in five rows plus a border row, with 5 cm spacings along the rows and 20 cm between rows. Regular agronomic management including weeding and pest control was done up until harvest. The first harvest was done for each plot upon reaching anthesis (determined when 50% of heads in the plot were flowering). In 2017, three plants per plot were harvested at anthesis and pooled for analysis of total N at anthesis (details below). Foliar N was applied at 7 DPA or 14 DPA, using liquid fertiliser UAN (a mixture of urea (21.5% N), ammonium (10.5% N) and nitrate (10.5% N), 42.5% total N w/v) applied with a handheld foliar sprayer at a rate of 40 kg N/ha (12.5ml per plot). Foliar treatment was always done in the evenings when temperatures were below 25 °C, to reduce any burning effect from fertiliser precipitation, and in windless conditions to avoid the fine mist dripping or blowing off the leaves. For control

plots, no treatment was applied in 2016, while in 2017 water with an adjuvant (Spreadwet 1000, active constituent: 1000 g/l Alkoxylated Alcohols, SST Australia, 0.5% v/v) was applied. Harvesting of two 50cm rows per plot was done at maturity. The harvested material was oven-dried at 65 °C for three days, and the plants were then processed for yield components including biomass, grain yield, thousand kernel weight (TKW) and harvest index (HI), and N analysis.

Glasshouse experiment

Gregory and Spitfire were grown in glasshouse conditions in the Plant Phenomics Facility (The Plant Accelerator), on Waite Campus, the University of Adelaide. The glasshouse day/night temperature was 22/15°C and the day/night relative humidity was 54/64%. The experimental design was a randomized complete block with two varieties × two basal N levels (low N (LN) and high N (HN)) × seven treatments × four replicates. The seven treatments included six N application (five foliar N treatments and one soil N treatment). The foliar N applications were done at GS49 (awn emergence), GS59 (heading), GS65 (anthesis), 7 DPA, and 14 DPA. The soil N application using granular fertiliser was done 7 DPA. The last treatment was a control (Spreadwet adjuvant) also applied at 7 DPA. Plants were grown in pots containing 1.2 kg of clay mix soil which was a mixture of cocopeat (a multipurpose growing medium made from coconut husks), sand and clay-loam mix at a ratio 1:1:1 containing a single plant per pot. At sowing, urea (46% N (w/w)) was added in two treatments, a low N of 50 mg N/ kg of soil (approximately 33 kg/ha) and a high N of 100mg N/ kg of soil (approximately 66 kg/ha). Foliar UAN (42.5 % N (w/v)) was applied at a rate of 40 kg N/ha (80 µl on the main tiller (40 µl flag leaf, 20 µl flag leaf -1 and 20 µl flag leaf -2), with half of the volume applied on each of the adaxial and abaxial side of each leaf. Exposed soil was covered with plastic sheeting, to ensure no foliar fertiliser dropped

onto the soil (Fig. 1A). All applications were done mid-morning between 10 am - 12 pm. A 10 µl pipette set at 2 µl was used to apply the droplets evenly on the leaf surfaces (Fig. 1B). The droplets stayed on the leaf surface for several days, giving a clear indication of the oily and sticky nature of the commercial liquid fertiliser. Some burns formed from the droplets after several days (Fig 1C), although these burns disappeared as the plant matured and did not affect plant growth.

Plants were harvested at maturity, oven-dried at 65 °C for three days and processed for yield components and N analysis. For yield components, the main tiller was separated from the other tillers. The main tiller was weighed and then separated into head, leaves, and stem, which were also weighed separately. The head was processed for spike weight, grain number, and total grain weight. Other tillers were retained and processed to determine the total biomass and Harvest Index (HI) per plant.

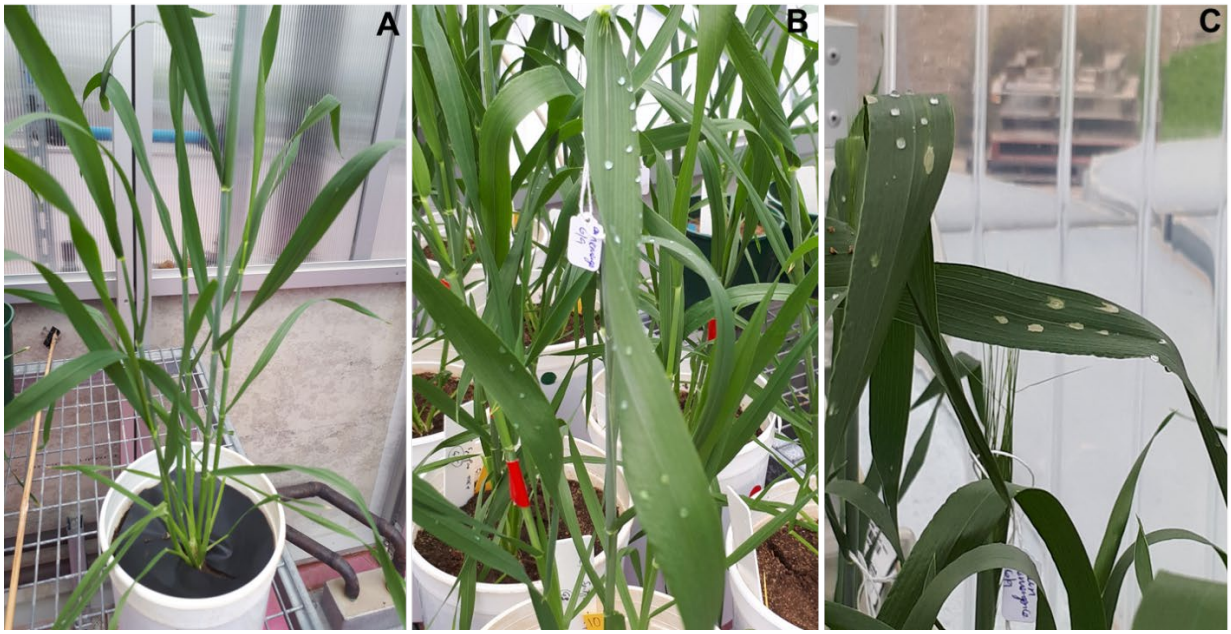

Figure 1: Foliar application in Spitfire and Gregory: A, Exposed soil was covered to prevent foliar fertiliser from dropping to the soil. B, The tiny droplets of liquid UAN applied to the leaves of treated plants. C, Temporary leaf burn on treated tissue.

N analysis

N analysis was done for plants harvested in both field and glasshouse conditions. Plant materials were subsampled and ground using a GenoGrinder® (Spex Sample Prep, USA) at a speed of 1200 rpm. Approximately 100 mg ground material was weighed and capsulated in N-free weighing paper for analysis using a combustion nitrogen/protein analyser (Rapid N exceed®, Elementar Analysensysteme GmbHanalyser, Germany). Aspartic acid (250mg) and blank paper were used as calibration standards. The theoretical N% of aspartic acid (10.52) was divided by the machine's N% output to determine the N factor. The N% measured for each sample was corrected using this calculated N factor. Total N content in the different plant tissues was calculated as plant part N% × DW (g). GPC was calculated as N% multiplied by 5.7.

Statistical analysis

Statistical analysis and plotting of graphical representations were performed using R version 3.4.2 with *agricolae* and *ggplot2* packages (R Core Development Team 2010) and GraphPad Prism version 7 (GraphPad Software). To assess the differences in yield components and accumulated GPC a two-way Analysis of Variance (ANOVA) between foliar-treated plants and controls was done. The statistical differences were assessed using Fisher's least significant difference posthoc test. For analyses involving more than two comparisons, one-way Analysis of Variance (ANOVA) was performed, followed by Tukey's multiple comparison test.

Results

Field foliar N effects on grain yield and grain N

Rainfall and temperature in 2016 and 2017

The field site had differences in the amount of rainfall between 2016 and 2017, especially during the growing season of June – December (Supplemental Fig. 1). In 2016, the average monthly pre-season rainfall between January – June was 56.6mm, while for 2017 the average monthly rainfall was 35.2mm. Pre-season rainfall in 2016 was particularly high in the two months (May – June) before sowing, with an average monthly rainfall of 92.4mm, compared to 2017 at 30.9mm per month. The average monthly rainfall for the growing season in 2016 and 2017 was 93.5mm and 54.7mm, respectively, with in-season rainfall 41% lower in 2017.

Temperature for the two years did not vary significantly (Supplemental Figure 2). The average monthly temperature during the growing season in 2016 and 2017 was 20.1°C and 21.2°C respectively. Between September – December, the period of grain-filling, the average temperature in 2017 was 1.95 °C higher than in 2016.

Effects of foliar N on yield components in field-grown plants

Gregory biomass, grain yield, thousand kernel weight (TKW) and harvest index (HI) were high after foliar N treatment at 7 DPA compared to 14 DPA treatment and control. For Spitfire, biomass and TKW were also highest after foliar treatment at 7 DPA, while grain yield and HI were highest for 14 DPA foliar-treated plants. In 2017, TKW and HI were the highest in the 7 DPA treatment in Gregory while biomass and grain yield were highest in the 14 DPA foliar-treated plants. Biomass, grain yield and TKW were highest in the 7DPA treatment for Spitfire, while HI was the highest in the 14 DPA treatment.

Overall, the biomass and grain yield were much lower in 2017 compared to 2016 (Table 1).

Foliar N effects on GPC and grain N yield in field-grown Spitfire and Gregory

Before foliar treatment was applied post-anthesis in 2017, several plants per plot were harvested at anthesis for both Gregory and Spitfire to quantify pre-anthesis N uptake (Fig. 2). Spitfire had significantly greater pre-anthesis tissue N at 405 mg N/g plant DW, compared to Gregory at 211 mg N/g plant DW.

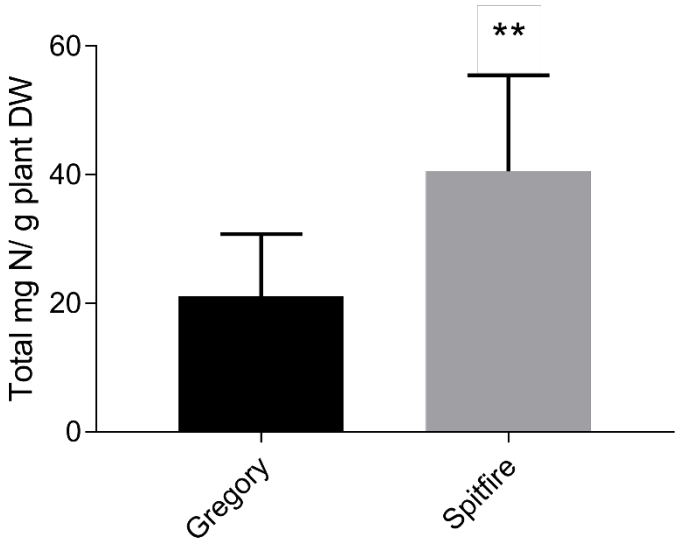

Figure 2: Total plant N in mg/g DW (Dry Weight) at anthesis in Gregory and Spitfire. Error bars indicate the standard error of twelve replicates, and asterisks (**) indicate significant differences (P<0.001) between cultivars.

In both 2016 and 2017, GPC for Gregory was significantly higher after foliar N treatment at 7 DPA, at 13.5% and 11.7% protein, in each year respectively, compared to 10.4% and 8.6% for the control treatment (Fig. 3A and C). Spitfire did not show increased GPC with foliar N application but maintained a high GPC across all treatments for both years (Fig. 3B and D). The average GPC for Spitfire in 2016 and 2017 was 13.4% and 10.5% respectively.

Table 1: Effects of foliar N on biomass, grain yield, TKW (Thousand Kernel Weight) and HI (Harvest Index) in Gregory and Spitfire grown in field-like conditions at Waite Campus in 2016 and 2017. Statistical differences between treatment and controls are ranked using letters. The same letter in each column means the values are not statistically different.

Gregory	Biomass (g m^{-2})		Grain yield (g m^{-2})		TKW (g)		HI	
Planting year	2016	2017	2016	2017	2016	2017	2016	2017
Control	2035±207.98a	1549±157.64a	927.50±119.51a	634.89±58.11a	41.16±1.13a	38.86±0.94a	0.45±0.02a	0.41±0.01a
Foliar (7DPA)	2505±162.04a	1423±222.02a	1176..97±106.41a	599.11±84.69a	42.95±1.01a	43.25±1.17a	0.46±0.01a	0.42±0.01a
Foliar (14DPA)	2292±218.24a	1709±85.96a	961.05±137.80a	706.66±161.76a	37.98±1.49a	39.18±2.90a	0.41±0.02a	0.41±0.00a
Spitfire	Biomass (g m^{-2})		Grain yield (g m^{-2})		TKW (g)		HI	
Planting year	2016	2017	2016	2017	2016	2017	2016	2017
Control	2127±138.67a	1663±154.90a	975.10±103.32a	757.91±66.73a	39.59±2.67a	40.97±0.71a	0.46±0.02a	0.46±0.00a
Foliar (7DPA)	2190±190.14a	1710±11.47a	1024.27±110.53a	715.10±51.15a	43.97±2.02a	41.59±1.98a	0.45±0.02a	0.42±0.00a
Foliar (14DPA)	2047±195.63a	1352±59.97a	980.45±82.08a	611.09±13.47a	40.77±2.58a	40.30±0.47a	0.48±0.01a	0.45±0.01a

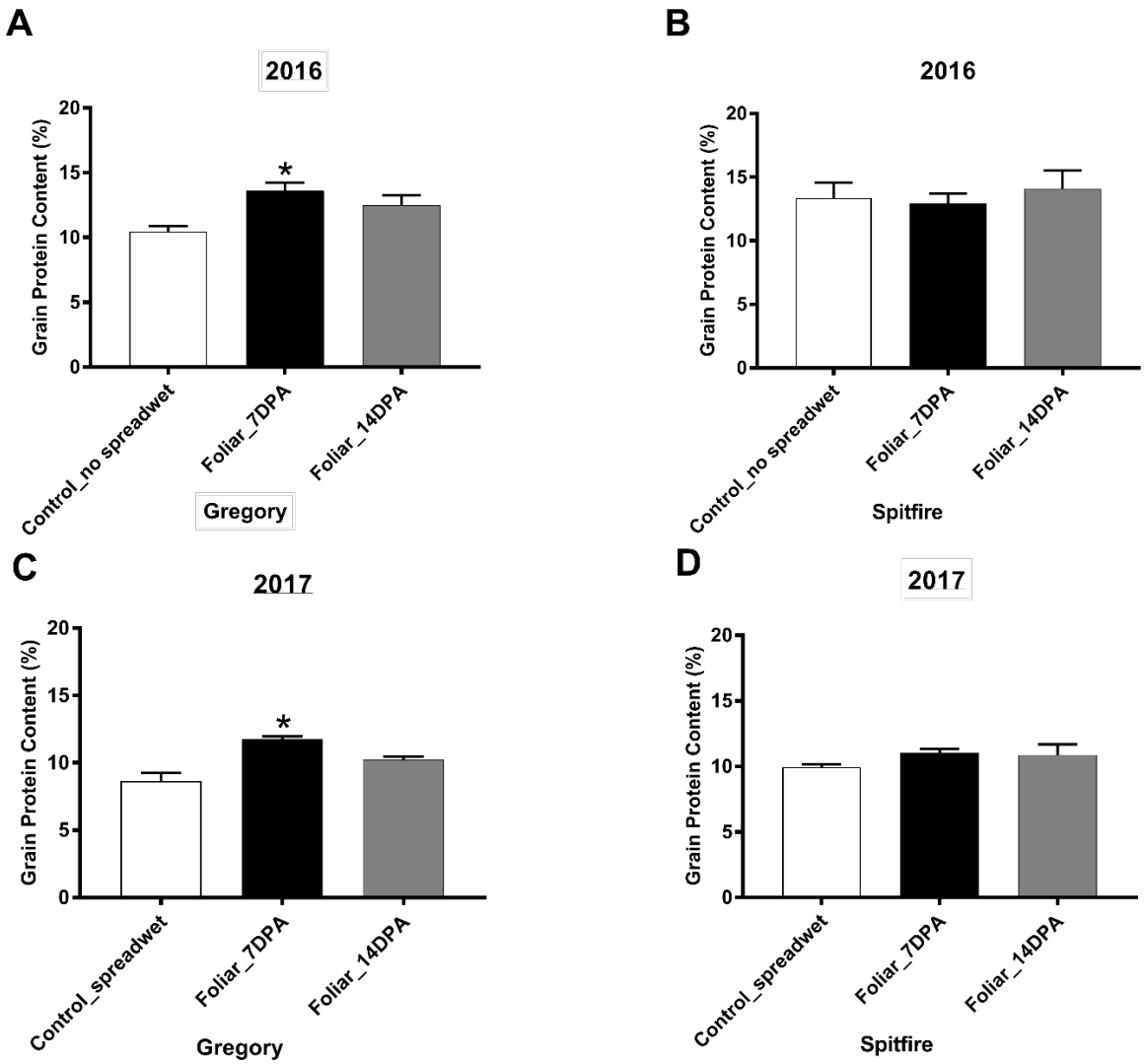

Figure 3: Gregory and Spitfire GPC (Grain Protein Content) calculated from (grain N % × 5.7, after three treatments: Control (no spreadwet (2016) and spreadwet (2017)), and foliar UAN (urea, ammonium, nitrate) applied at 7 or 14 days post-anthesis (DPA). A and C, cv. Gregory (2016 and 2017); B and D, cv. Spitfire (2016 and 2017). Error bars indicate the standard error of four replicates, and asterisks (*) indicate treatments that were significantly different ($P<0.01$) from control for each panel.

In 2016, Gregory had significantly higher grain N yield per m^2 after foliar treatment at 7 DPA, at 915 g N/m^2) compared to the 14 DPA and control treatments at 792 g N/m^2 and 649 g N/m^2, respectively (Fig. 4). In 2017, both foliar treatments resulted in a higher grain N yields per m^2 for Gregory compared to control, with 341 g N/m^2 and 329

g N/m^2 for 7 DPA and 14 DPA, respectively, and 280 g N/m^2 for the control treatment. Overall, grain N yield per m^2 was approximately 60% lower in 2017 for Gregory.

In 2016, Spitfire had the highest grain N yield per m^2 in the 14 DPA foliar-treated plants at 919 g N/m^2, compared to 765 g N/m^2 and 795 g N/m^2 for the 7DPA and control treatments respectively (Fig. 4). For 2017, the highest grain N yield per m^2 was in the 7 DPA foliar-treated plants at 485 g/m^2, compared to 379 g N/m^2 and 436 g N/m^2 for the 14 DPA and control treatments, respectively. This variety maintained a high grain N yield across treatments. Similar to Gregory, the grain N yield per m^2 was 36% - 48% lower in 2017 compared to 2016.

Effects of foliar N applied at different growth stages on grain weight and grain N in glasshouse conditions

To further investigate the effects of foliar N application in different growth stages, foliar N was applied to the flag leaf on the main tiller of individual plants of Gregory and Spitfire at GS49 (awn emergence), GS59 (heading), GS65 (anthesis), 7 DPA, and 14 DPA, grown in a controlled environment in the glasshouse. Also, a granular application treatment at 7 DPA was included to compare with the foliar treatment. Spreadwet was used as a control for this experiment. Two basal N treatments, a low N and high N were applied at sowing. In Gregory, grain weight in the foliar-treated plants across all growth stages was significantly higher than for the Spreadwet control in the two basal N treatments (Table 2). Foliar N treatment at the heading stage had the highest grain weight in the two basal N treatments (3.00 g and 3.43 g), while Spreadwet had the lowest grain weight (2.24 g). Grain weight between low and high basal N applications showed significant N responsiveness ($p= 0.03$), with average grain weights of 2.55g and 2.86g for each treatment, respectively (Table 2, Supplementary Table 1). Gregory grain number was significantly affected ($p= 0.005$) by the interaction between basal N

treatment and growth stage (Supplementary Tables 1). Effects of treatments on other yield components including biomass and spike weight are summarised in Table 2.

Grain weight in Spitfire was significantly affected by growth stage treatment (p=0.02) (Table 2, Supplementary Table 1). Foliar N treatment at 7DPA had the lowest grain weight (1.89g). Biomass was significantly affected by basal N treatment (p=0.000), with average biomass at low basal N and high basal N recorded as 24.91g and 26.77g, respectively (Table 2, Supplementary Table 1). Yield components in Gregory and Spitfire were not significantly different after granular N treatment when compared with foliar treatments. The effects of growth stages, basal N, and the interaction between the two treatments on the yield components are summarized in Supplementary Table 2.

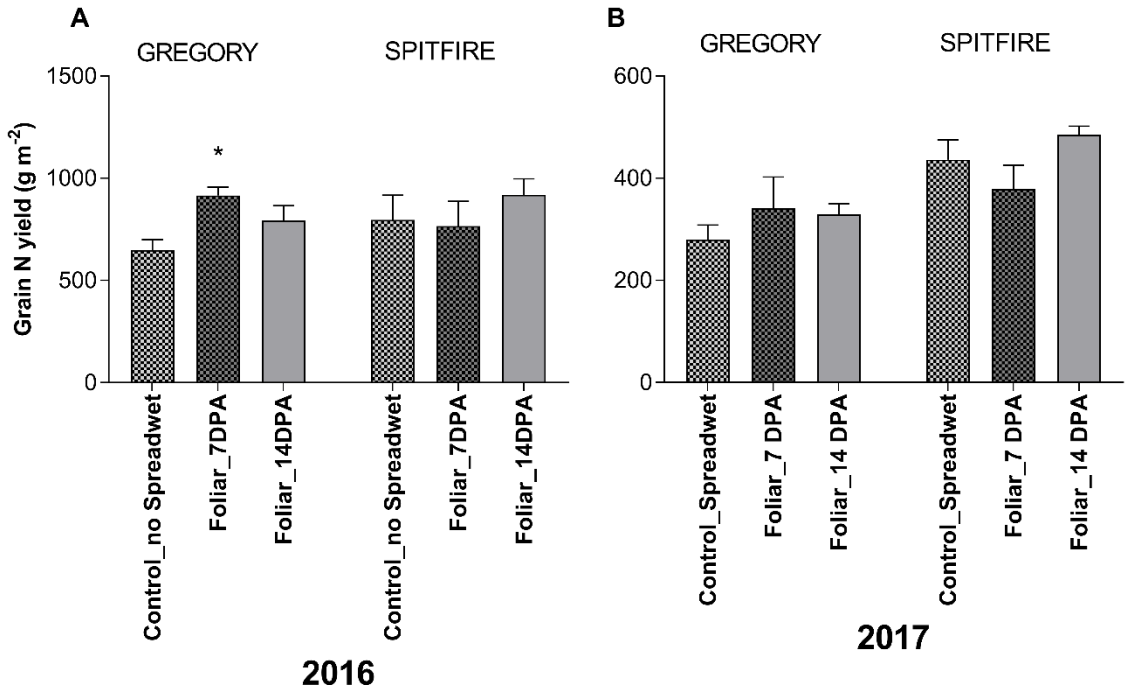

Figure 4: Grain N yields (g N/ m2) for cultivars Gregory and Spitfire in (A) 2016 and (B) 2017 grown in field-like conditions and treated with foliar UAN (urea, ammonium, nitrate) applied at 7 or 14 days post-anthesis (DPA). Error bars indicate the standard error of four replicates, and asterisks (*) indicate treatments that were significantly different (P<0.05) from Control for each cultivar.

Table 2: Effect of foliar N applied at different growth stages on yield components (biomass, spike weight, grain weight, and grain number (no) in bread wheat cultivars Gregory and Spitfire grown in low N (50 mg/N) and high N (100 mg/N) per kg of soil-applied at sowing. N=4, different letters indicate significant differences. Two-way Anova (growth stages and basal N treatment) with Tukey's Post Hoc testing was used to test for statistical significance (P<0.05).

Variety		Growth stage	Biomass (g)	Spike weight (g)	Grain Weight (g)	Grain no	Biomass (g)	Spike weight (g)	Grain Weight (g)	Grain no
	Basal N treatment		LN				HN			
Gregory	LN	GS49_awn emergence	31.82±2.16a	3.46±0.25a	2.51±0.15a	53.25±4.19a	36.57±2.12a	4.08±0.20a	3.39±0.22a	61.25±5.00a
		GS59_heading	31.98±1.76a	3.72±0.29a	3.00±0.11a	50.00±2.34a	36.38±1.77a	4.00±0.07a	3.43±0.02a	68.00±1.00a
		Anthesis	32.91±1.09a	3.73a±0.19	2.61±0.12a	55.50±3.42a	35.88±1.47a	3.40±0.35a	2.72±0.47a	45.60±5.71a
		7 DPA	29.66±1.01a	3.06±0.22a	2.45±0.29a	45.25±3.49a	36.21±1.50a	4.13±0.18a	3.12±0.32a	61.75±3.66a
		14DPA	33.91±1.09a	3.48±0.22a	2.73±0.10a	53.75±3.61a	36.25±2.45a	3.68±0.57a	2.76±0.42a	49.25±8.44a
		Control								
		Granular	32.69±2.50a	3.39±0.11a	2.28±0.07a	50.15±1.84a	36.03±1.19a	3.23±0.27a	2.41±0.18a	44.80±5.32a
		Spreadwet	31.77±1.06a	3.16±0.33a	**2.24±0.20b**	57.5±1.19a	31.92a±2.75a	3.01±0.50a	**2.22±0.36b**	43.33±8.45a
		Average	**32.11**	**3.43**	**2.55**	**52.20**	**35.61**	**3.65**	**2.86**	**53.43**
Spitfire	LN	GS49_awn emergence	24.91±1.27a	2.78±0.22a	2.14±0.08a	45.20±1.15a	31.53±1.06a	3.18±0.40a	2.48±0.12a	50.00±4.06a
		GS59_heading	23.56±2.25a	3.13±0.19a	2.19±0.11a	47.50±2.62a	31.07±1.93a	2.98±0.24a	2.08±0.15a	44.50±0.95a
		Anthesis	27.44±0.89a	3.11±0.15a	2.22±0.12a	48.00±0.81a	31.42±1.55a	2.90±0.13a	2.05±0.15a	47.50±0.64a
		7 DPA	25.99±1.46a	2.98±0.08a	2.09±0.04a	46.00±1.29a	32.98±1.77a	2.61±0.16a	1.89±0.19b	38.5a±4.69
		14DPA	25.36±1.11a	2.76±0.23a	1.96±0.173a	42.00±2.27a	30.55±2.53a	3.12±0.07a	2.28±0.08a	46.25±1.54a
		Control								
		Granular	26.38±0.17a	3.22±0.01a	2.31±0.04a	47.00±1.58a	32.99±1.17a	3.38±0.09a	2.50±0.04a	49.50±0.95a
		Spreadwet	26.09±1.66a	3.11±0.03a	2.19±0.06a	45.75±1.54a	32.60±1.26a	3.20±0.10a	2.32±0.09a	47.50±0.95a
		Average	**25.67**	**3.01**	**2.16**	**45.92**	**31.87**	**3.05**	**2.22**	**46.25**

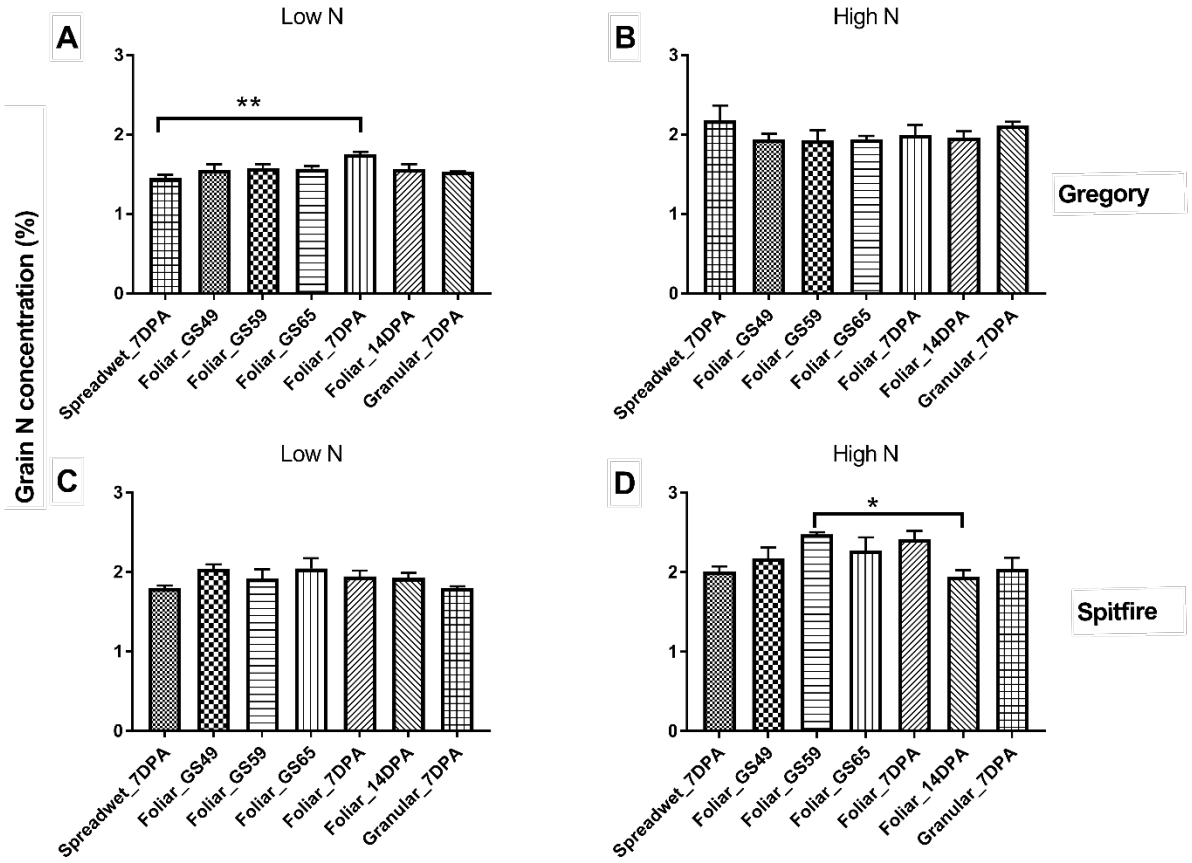

Figure 5: Effects on grain N concentration (%) of foliar N applied to flag leaf of the main tiller of wheat plants at different growth stages: GS49_awn emergence, GS59_heading, GS65_anthesis, 7DPA and 14DPA, compared to a granular fertiliser treatment at 7 DPA and a control (foliar treatment with Spreadwet only at 7DPA) in Gregory and Spitfire, with two basal N treatments (low N (50 mg/N) and high N (100 mg/N) per kg of soil). (A), Gregory at low basal N; (B), Gregory at high basal N; (C), Spitfire at low basal N; (D), Spitfire at high basal Nt. Error bars indicate the standard error of four replicates, and asterisks (*) indicate treatments that were significantly different ($P<0.05$) within each panel.

Foliar N applied at different growth stages affected grain N yield

Grain N concentration (N%) in Gregory under low N basal treatment (Fig. 5A) was significantly higher for the foliar_7 DPA treatment compared to the Spreadwet control, at 1.75% N, translating to a GPC (N% × 5.7) of 10.2%. All other treatments had a grain N concentration that ranged between 1.53 - 1.57% N, (GPC 8.7 - 8.9%). These values were not significantly higher than the Spreadwet control (1.45% N, GPC 8.2%) (Fig. 5A). In the high basal N treatment, grain N concentration ranged between 1.92 - 2.11% N (GPC 10.9 - 12.0%) (Fig. 5B) and was not significantly different from the Spreadwet control at 2.17% N (GPC 12.4%).

In Spitfire, under high basal N treatment (Fig. 5D), when foliar N was applied at heading (foliar_GS59), grain N concentration was significantly higher (p=0.04) at 2.42% N (GPC 14.1%) compared to 14 DPA foliar N treatment at 1.94% N (GPC 11%). The grain N concentration ranged between 1.80 - 2.04% N (GPC range of 10.2 - 11.6%) and 2.17 - 2.41 % N (GPC 12.3 - 13.7%), under low basal treatment and high basal treatments (Fig. 5C and 5D).

The grain N yield per spike (N concentration (%) × spike grain weight) of Gregory grown at low basal N was significantly higher in two growth stage treated with foliar N compared to Spreadwet control: GS59_heading (p = 0.005) and 7 DPA (p= 0.02) (Fig. 6A). For all other foliar treatments, the grain yield/ spike was higher, though not significant, compared to Spreadwet control. The grain N yield per spike for Gregory grown with a high basal N treatment and Spitfire grown in both low and high basal N treatment, was not significantly different in any of the foliar treatments compared to the Spreawet control (Fig. 6B – 6D).

Discussion

Effects of foliar N on grain yield and grain N in field conditions

N fertiliser management has been a focus of efforts to improve uptake and utilisation efficiency for high yield and GPC in wheat. The timing of N application, as well as consideration of N rate, is vital to match the supply of N with plant demand across different growth stages, with both affecting yield and GPC (Midwood 2014). Split application of N fertiliser at sowing and later growth stages, including early post-anthesis stages, is essential especially in utilisation of post-anthesis applied N to improve GPC in wheat (Boman *et al.* 1995, Midwood 2014, Wuest and Cassman 1992).

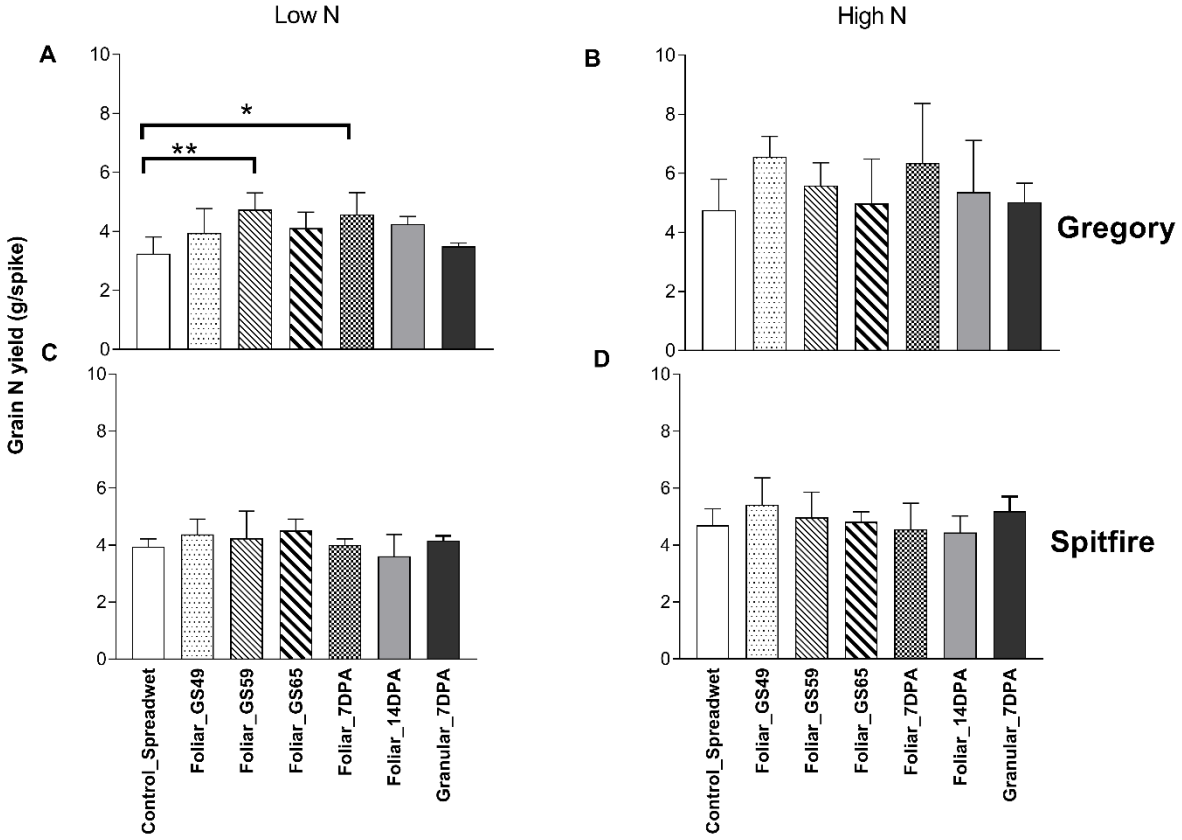

Figure 6: Grain N yield per spike after foliar N treatment of the flag leaf of the main tiller of wheat plants at different growth stages: GS49_awn emergence, GS59_heading, GS65_anthesis, 7DPA and 14DPA, and a granular fertiliser N treatment at 7 DPA compared to a control (foliar treatment with Spreadwet only at 7DPA), in Gregory and Spitfire with two basal N treatments (low N (50 mg/N) and high N (100 mg/N) per kg of soil). (A), Gregory at low basal N; (B), Gregory at high basal N; (C), Spitfire at low basal N; (D), Spitfire at high basal N. Error bars indicate the standard error of four replicates, and asterisks (*) indicate treatments that were significantly different ($P<0.05$) from control (Spreadwet).

Foliar N applied at 7 DPA and 14 DPA in cv. Gregory (a high yielding and medium GPC variety) in field-like conditions did not result in a significant increase in yield components in either of the two trial years. However, these important components including grain yield, biomass, and TKW tended to be higher in foliar-treated plants compared to controls for both Gregory and Spitfire (Table 1). In controlled conditions, grain weight was significantly high in foliar-treated plants compared to controls in Gregory (Table 2). These results may be explained by a potentially reasonable contribution of foliar N applied post-anthesis to yield, through a biomass increase translating to more grains (Table 1). These observations are similar to those of

previous studies where foliar N applied after anthesis was found to increase grain yield, especially in conditions where N was limiting (Abad *et al.* 2004, Gooding and Davies 1992, Strong 1982). However, in the current study, in field conditions, the lack of a significant increase in grain yield in the two varieties may be due to pre-anthesis N being heavily invested in yield compared to N taken up after anthesis. Earlier findings show that primarily the N applied at sowing or early in the season is invested towards yield through biomass accumulation and later N remobilisation for grain number and grain weight (Woolfolk *et al.* 2002). Research by Kelley showed that N applied to wheat at sowing or before heading had a major effect on yield and minimal effect on GPC (Kelley 1995). By comparison, Boman and colleagues showed no increase in yield for maize (*Zea mays*) after foliar application of N at either 7 days before anthesis or 7 DPA (Boman *et al.* 1995). However, the N applied after anthesis had a significant effect on GPC (Boman *et al.* 1995). It is therefore reasonable to conclude that the effect of foliar N on yield may strongly depend on N limitation experienced by the crop, hence the varying results seen from this and other research studies.

Foliar N applied at 7 DPA in Gregory had a significant effect on GPC and grain N yield when determined per unit crop area, and with a similar trend observed across seasons (Fig. 3 and 4). According to Goodings and Davies's review on foliar application, studies of GPC responses to foliar N are more consistently and frequently reported than those of effects of foliar N on yield (Gooding and Davies 1992). Furthermore, our results are in line with previous findings showing that N taken up after flowering increases GPC (Bly and Woodard 2003, Simmons *et al.* 1995, Woolfolk *et al.* 2002). The developing grains in Gregory may have more capacity to accumulate N due to a higher grain number and grain weight compared to Spitfire (Table 2) and therefore a larger N sink. Bly and Woodard concluded that the application of foliar N post-anthesis resulting in

an increased GPC could limit price reductions due to quality and return premiums to growers in favorable years (Bly and Woodard 2003).

Spitfire, a high yielding and high GPC variety showed no increase in GPC after foliar N treatment in the two consecutive years of field experiments. An explanation might be that this variety takes up sufficient N before anthesis, as suggested by our observation that tissue N at anthesis was substantially higher in Spitfire compared to Gregory (Fig. 2), and the accumulated N could be then efficiently remobilized to the grain. Spitfire may also have a less plastic response to N supply than Gregory at later stages of development.

Effects of foliar N applied at different reproductive growth stages on grain yield and grain N under controlled conditions

In controlled conditions, besides investigating the effects of foliar N at different growth stages during the reproductive phase on grain N concentration, we also sought to understand the effect of basal N rate on subsequent foliar N uptake.

Gregory grown under low basal N rate showed a significant increase in grain N concentration for foliar N applied at 7 DPA, while grain N yield per spike was significantly increased for treatments at two reproductive stages: GS59_heading and 7 DPA (Fig. 5 and 6). An increase in grain N concentration and grain N yield per spike at 7 DPA was consistent with the field results. Previous research has also reported a positive effect of foliar N applied post-anthesis on grain N especially when N is limiting (Gooding and Davies 1992). Midwood found an increase in grain N when foliar N was applied at wheat GS55_heading, and also at GS70 (early post-anthesis) similar to this study (Midwood 2014). These findings are indicative that a considerably broad window of growth stages, between awn emergence and heading, may be suitable for applying

foliar N to boost grain N in some wheat varieties, thus giving growers some flexibility in the timing of foliar application.

When grown with high basal N, no significant increase in grain N concentration or grain N yield per spike was observed between control and foliar-treated Gregory or Spitfire (Fig. 5B, 6B and 6D). Foliar treatment overall was effective in increasing grain N content when plants had a low basal N treatment compared to a high basal N treatment. The high basal N treatment may not have provided an N limiting condition, confirming from previous research that the positive effect of foliar N applied post-anthesis on grain N, happened when N was limiting (Gooding and Davies 1992). Comparison of the two basal N rates on foliar N uptake and effect on grain N concentration gives an overall indication of the importance of basal N rate in the uptake of N later in the growing season (Abad *et al.* 2004, Gooding and Davies 1992).

Spitfire did not show a significant difference in grain N concentration or grain N yield per spike between control and foliar-treated plants. The results were similar to those of the field experiment. The variety still maintained a high grain N concentration both for control and foliar-treated plants as well as between the two basal N rates. In addition, grain N concentration was higher with high basal N compared to the low basal N treatment. Spitfire may be responsive to basal N application, but not responsive to foliar N. This scenario matches the field results and could be due to efficient uptake of N before anthesis combined with efficient remobilisation, minimizing the need for extra N from foliar N uptake. As mentioned above, this is suggestive of a less plastic response in Spitfire.

The difference in foliar N uptake capacity between the two Australian elite cultivars Gregory and Spitfire is interesting since it may explain the variability in foliar N uptake and utilisation seen in previous research under field conditions, with some varieties

potentially having different foliar N uptake capacities than other varieties. Furthermore, the variability between genotypes could be due to pre-anthesis N uptake capacity, efficiency in N remobilisation and post-anthesis N uptake from soil determined by sink N uptake capacity (Dhugga and Waines 1989). These factors could, therefore, be used to determine how effective a split N application will be, as well as the optimum basal N supply and what rates to apply at targeted growth stages for maximum yield and GPC. Compared to granular N application in the soil, foliar N application has an advantage only under low basal N conditions in the medium GPC variety Gregory, for which it significantly increases GPC.

Conclusion

The results presented here suggest that foliar application of N fertiliser post-anthesis has potential as an agronomic management practice to improve GPC in wheat. This method of N top-up may be an advantage to wheat varieties that have a high yield and low-medium GPC, particularly where low basal application rates of N fertiliser are used. Furthermore, important factors influencing the effect of foliar-applied N fertilisers, including pre-anthesis uptake capacity, remobilisation efficiency, and sink N uptake post-anthesis should be considered when applying foliar N. The study findings could be used to explain further the variability of foliar N performance from previous research and could be used in the future to manage foliar N fertilisation to achieve an increase in GPC without a penalty on yield.

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References

Abad, A., J. Lloveras and A. Michelena (2004). "Nitrogen fertilization and foliar urea effects on durum wheat yield and quality and on residual soil nitrate in irrigated Mediterranean conditions." Field Crops Research 87(2–3): 257-269.

ABARES (2013). Agricultural commodity statistics 2013.

Bly, A. G. and H. J. Woodard (2003). "Foliar Nitrogen Application Timing Influence on Grain Yield and Protein Concentration of Hard Red Winter and Spring Wheat." Agronomy Journal 95: 335-338.

Bogard, M., V. Allard, M. Brancourt-Hulmel, E. Heumez, J. M. Machet, M. H. Jeuffroy, P. Gate, P. Martre and J. Le Gouis (2010). "Deviation from the grain protein concentration-grain yield negative relationship is highly correlated to post-anthesis N uptake in winter wheat." J Exp Bot 61(15): 4303-4312.

Boman, R., R. Westerman, W. Raun and M. Jojola (1995). "Time of nitrogen application: Effects on winter wheat and residual soil nitrate." Soil Science Society of America Journal 59(5): 1364-1369.

Brown, B. D. and S. Petrie (2006). "Irrigated hard winter wheat response to fall, spring, and late season applied nitrogen." Field Crops Research 96(2-3): 260-268.

Bureau Of Meteorology, B. (2019a). Rainfall Data 2017 and 2018. 47086292. South Australia, Australian Government.

Bureau Of Meteorology, B. (2019b). Temperature Data 2017 and 2018. 47086221. South Australia, Australian Government. IDCJAC0010

Cassman, K. G., D. C. Bryant, A. E. Fulton and L. F. Jackson (1992). "Nitrogen Supply Effects on Partitioning of Dry Matter and Nitrogen to Grain of Irrigated Wheat." Crop Science 32(5): 1251-1258.

Cormier, F., J. Foulkes, B. Hirel, D. Gouache, Y. Moënne-Loccoz and J. Le Gouis (2016). "Breeding for increased nitrogen-use efficiency: a review for wheat (T. aestivum L.)." Plant Breeding 135(3): 255-278.

Dhugga, K. S. and J. G. Waines (1989). "Analysis of Nitrogen Accumulation and Use in Bread and Durum Wheat." Crop Science 29(5): 1232-1239.

Fageria, N. K. (2014). "Nitrogen Harvest Index and Its Association with Crop Yields." Journal of Plant Nutrition 37(6): 795-810.

Garnett, T., D. Plett, S. Heuer and M. Okamoto (2015). "Genetic approaches to enhancing nitrogen-use efficiency (NUE) in cereals: challenges and future directions." Functional plant biology 42(10): 921-941.

Gooding, M. J. and W. P. Davies (1992). "Foliar urea fertilization of cereals: A review." Fertilizer Research 32(2): 209-222.

GraphPad Software GraphPad Prism version 7.00 for Windows. La Jolla California, USA, GraphPad Software.

Heitholt, J. J., L. I. Croy, N. O. Maness and H. T. Nguyen (1990). "Nitrogen partitioning in genotypes of winter wheat differing in grain N concentration." Field Crops Research 23(2): 133-144.

Hooper, G. M. a. P. (2013). Nitrogen Decision - Guidelines And Rules Of Thumb. Australia, GRDC.

Kelley, K. (1995). "Rate and time of nitrogen application for wheat following different crops." Journal of production agriculture 8(3): 339-345.

Ladha, J. K., A. Tirol-Padre, C. K. Reddy, K. G. Cassman, S. Verma, D. S. Powlson, C. van Kessel, B. R. D. de, D. Chakraborty and H. Pathak (2016). "Global nitrogen budgets in cereals: A 50-year assessment for maize, rice, and wheat production systems." Sci Rep 6(1): 19355.

Midwood, J. (2014). Evaluation of Late Nitrogen Applications to Achieve Yield Potential and increased protein content in wheat. grdc, Southern Farming Systems

23 High Street Inverleigh VIC 3321.

Oosterhuis, D. (2009). Foliar fertilization: mechanisms and magnitude of nutrient uptake. Proceedings of the fluid forum.

Ottman, M. J., T. A. Doerge and E. C. Martin (2000). "Durum Grain Quality as Affected by Nitrogen Fertilization near Anthesis and Irrigation During Grain Fill." Agronomy Journal 92(5): 1035-1041.

R Core Development Team (2010). R: a language and environment for statistical computing. Vienna, Austria, Foundation for Statistical Computing.

Raun, W. R. and G. V. Johnson (1999). "Improving nitrogen use efficiency for cereal production." Agronomy journal 91(3): 357-363.

Robertson, G. P. and P. M. Vitousek (2009). Nitrogen in agriculture: Balancing the cost of an essential resource. Annual Review of Environment and Resources. 34: 97-125.

Simmons, S., E. Oelke and P. Anderson (1995). Growth and development guide for spring wheat. University of Minnesota, WWW-F0-02547.

Strong, W. (1982). "Effect of late application of nitrogen on the yield and protein content of wheat." Australian Journal of Experimental Agriculture 22(115): 54-61.

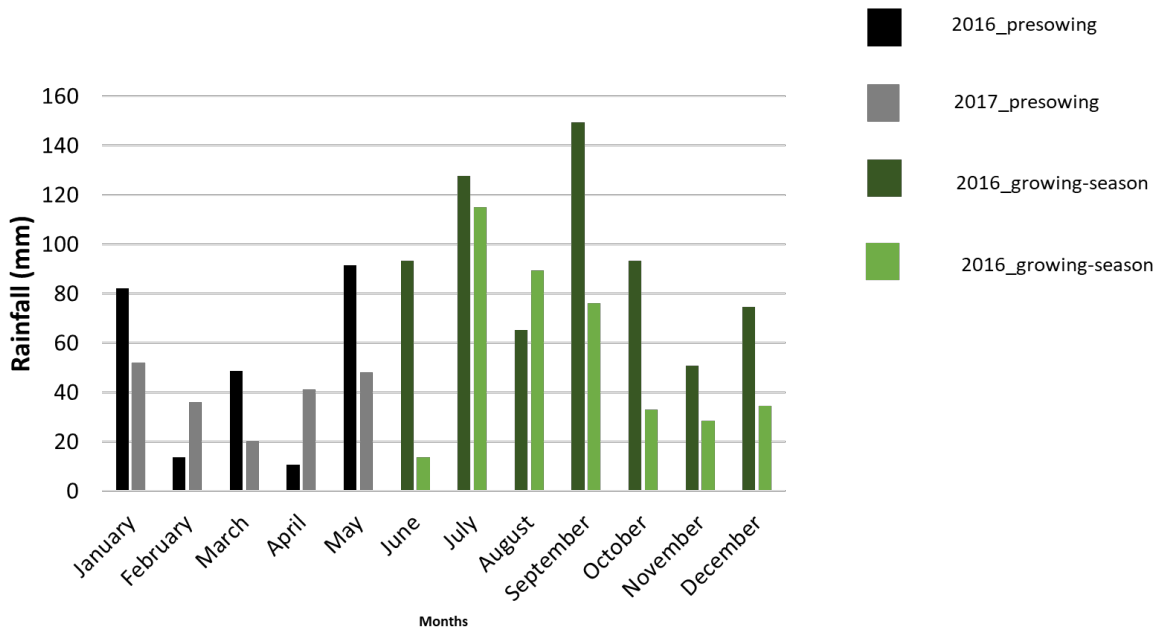
Sylvester-Bradley, R. and D. R. Kindred (2009). "Analysing nitrogen responses of cereals to prioritize routes to the improvement of nitrogen use efficiency." Journal of Experimental Botany 60(7): 1939-1951.

Woolfolk, C. W., W. R. Raun, G. V. Johnson, W. E. Thomason, R. W. Mullen, K. J. Wynn and K. W. Freeman (2002). "Influence of Late-Season Foliar Nitrogen Applications on Yield and Grain Nitrogen in Winter Wheat." Agronomy Journal 94(3): 429-434.

Worland, B., N. Robinson, D. Jordan, S. Schmidt and I. Godwin (2017). "Post-anthesis nitrate uptake is critical to yield and grain protein content in Sorghum bicolor." J Plant Physiol 216: 118-124.

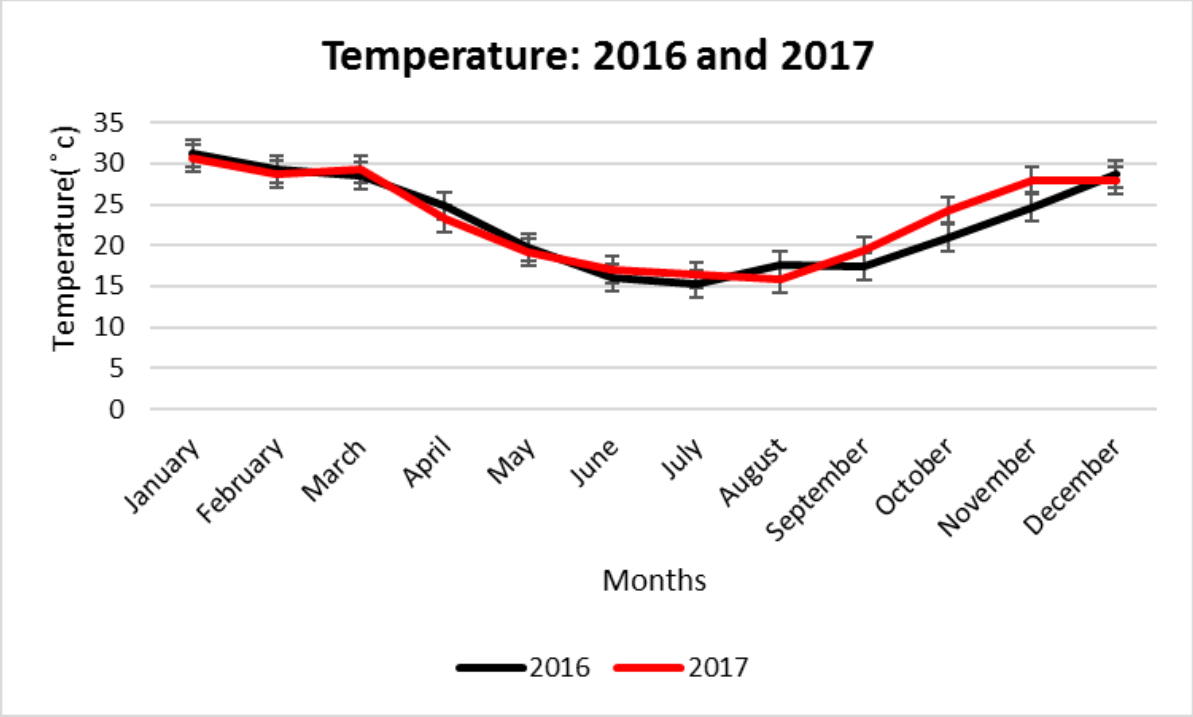
Wuest, S. and K. Cassman (1992). "Fertilizer-nitrogen use efficiency of irrigated wheat: I. Uptake efficiency of preplant versus late-season application." Agronomy journal 84(4): 682-688.

Supplemental Figures and Tables



Supplementary Figure 1: Average rainfall during 2016 and 2017 at Waite campus (34.97° S 138.64° E) (Bureau Of Meteorology 2019a) (Bureau Of Meteorology 2019a). The black and grey bars (January – May) represent the

wheat pre-sowing season. The dark green and light green bars (June - December) represent the wheat growing season.



Supplementary Figure 2: Average monthly daily temperature in 2016 and 2017 at Waite campus (34.97° S 138.64° E) (Bureau Of Meteorology 2019b).

Supplementary Table 1: Tukey's Multiple Comparison Test.Gregory grain number after foliar N treatment at different growth stages under two basal N treatment.

Tukey's multiple comparisons test	P-value	Tukey's multiple comparisons test	P-value	Tukey's multiple comparisons test	P-value	Tukey's multiple comparisons test	P-value
GS49_awn emergence_LN vs. GS59_heading_LN	****	GS59_heading_LN vs. Spreadwet_HN	****	14DPA_LN vs. Spreadwet_LN	****	Spreadwet_LN vs. Spreadwet_HN	****
GS49_awn emergence_LN vs. Anthesis_LN	****	Anthesis_LN vs. 7 DPA_LN	****	14DPA_LN vs. GS49_awn emergence_HN	****	GS49_awn emergence_HN vs. GS59_heading_HN	****
GS49_awn emergence_LN vs. 7 DPA_LN	****	Anthesis_LN vs. 14DPA_LN	**	14DPA_LN vs. GS59_heading_HN	****	GS49_awn emergence_HN vs. Anthesis_HN	****
GS49_awn emergence_LN vs. Granular_LN	****	Anthesis_LN vs. Granular_LN	****	14DPA_LN vs. Anthesis_HN	****	GS49_awn emergence_HN vs. 14DPA_HN	****
GS49_awn emergence_LN vs. Spreadwet_LN	****	Anthesis_LN vs. Spreadwet_LN	***	14DPA_LN vs. 7 DPA_HN	****	GS49_awn emergence_HN vs. Granular_HN	****
GS49_awn emergence_LN vs. GS49_awn emergence_HN	****	Anthesis_LN vs. GS49_awn emergence_HN	****	14DPA_LN vs. 14DPA_HN	****	GS49_awn emergence_HN vs. Spreadwet_HN	****
GS49_awn emergence_LN vs. GS59_heading_HN	****	Anthesis_LN vs. GS59_heading_HN	****	14DPA_LN vs. Granular_HN	****	GS59_heading_HN vs. Anthesis_HN	****
GS49_awn emergence_LN vs. Anthesis_HN	****	Anthesis_LN vs. Anthesis_HN	****	14DPA_LN vs. Spreadwet_HN	****	GS59_heading_HN vs. 7 DPA_HN	****
GS49_awn emergence_LN vs. 7 DPA_HN	****	Anthesis_LN vs. 7 DPA_HN	****	Granular_LN vs. Spreadwet_LN	****	GS59_heading_HN vs. 14DPA_HN	****
GS49_awn emergence_LN vs. 14DPA_HN	****	Anthesis_LN vs. 14DPA_HN	****	Granular_LN vs. GS49_awn emergence_HN	****	GS59_heading_HN vs. Granular_HN	****
GS49_awn emergence_LN vs. Granular_HN	****	Anthesis_LN vs. Granular_HN	****	Granular_LN vs. GS59_heading_HN	****	GS59_heading_HN vs. Spreadwet_HN	****
GS49_awn emergence_LN vs. Spreadwet_HN	****	Anthesis_LN vs. Spreadwet_HN	****	Granular_LN vs. Anthesis_HN	****	Anthesis_HN vs. 7 DPA_HN	****
GS59_heading_LN vs. Anthesis_LN	****	7 DPA_LN vs. 14DPA_LN	****	Granular_LN vs. 7 DPA_HN	****	Anthesis_HN vs. 14DPA_HN	****
GS59_heading_LN vs. 7 DPA_LN	****	7 DPA_LN vs. Granular_LN	****	Granular_LN vs. Granular_HN	****	Anthesis_HN vs. Granular_HN	ns
GS59_heading_LN vs. 14DPA_LN	****	7 DPA_LN vs. Spreadwet_LN	****	Granular_LN vs. Spreadwet_HN	****	Anthesis_HN vs. Spreadwet_HN	****
GS59_heading_LN vs. Spreadwet_LN	****	7 DPA_LN vs. GS49_awn emergence_HN	****	Spreadwet_LN vs. GS49_awn emergence_HN	****	7 DPA_HN vs. 14DPA_HN	****
GS59_heading_LN vs. GS49_awn emergence_HN	****	7 DPA_LN vs. GS59_heading_HN	****	Spreadwet_LN vs. GS59_heading_HN	****	7 DPA_HN vs. Granular_HN	****
GS59_heading_LN vs. GS59_heading_HN	****	7 DPA_LN vs. 7 DPA_HN	****	Spreadwet_LN vs. Anthesis_HN	****	7 DPA_HN vs. Spreadwet_HN	****
GS59_heading_LN vs. Anthesis_HN	****	7 DPA_LN vs. 14DPA_HN	****	Spreadwet_LN vs. 7 DPA_HN	****	14DPA_HN vs. Granular_HN	****
GS59_heading_LN vs. 7 DPA_HN	****	7 DPA_LN vs. Spreadwet_HN	***	Spreadwet_LN vs. 14DPA_HN	****	14DPA_HN vs. Spreadwet_HN	****
GS59_heading_LN vs. Granular_HN	****	14DPA_LN vs. Granular_LN	****	Spreadwet_LN vs. Granular_HN	****	Granular_HN vs. Spreadwet_HN	*

Supplementary Table 2: Interactions of different Treatments (growth stages, basal N treatment and growth stages × basal N treatment) after foliar N applied at different growth stages) in Gregory and Spitfire under a low basal N and high basal N applied at sowing. N=4. Two-way Anova with Tukey's Post Hoc test was used to test for statistical significance

Genotype	**Treatment**	**Biomass (gm)**	**Spike weight (gm)**	**Grain Weight (gm)**	**Grain no**
Gregory	Growth stage	ns	ns	**p≤ 0.02**	ns
	Basal treatment	**p≤ 0.0004**	ns	**p≤ 0.03**	ns
	Growth stage× Basal treatment	ns	ns	ns	**p≤ 0.005**
Spitfire	**Growth stage**	ns	ns	**p≤ 0.02**	ns
	Basal treatment	**p≤ 0.00000**	ns	ns	ns
	Growth stage× Basal treatment	ns	ns	ns	ns

Chapter 3

Morphological and chemical characterisation of wheat leaves, and association with foliar nitrogen uptake

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Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Improving Nitrogen (N) fertilisation management is necessary to maximise plant N use and reduce wastage. This can be achieved through split N dosage as a management strategy that aims to match the nutrient supply more closely with its demand by the plant. With fertilizer applications at sowing and at later growth stages the plant is supplied with N to support vegetative growth and grain filling. However, the N assimilation from the soil may be hindered at later growth stages as a consequence of lower root activity. An alternative way to supply the plant with N is via foliar application. Due to a limited understanding of the physiological and molecular characteristics associated with effective N uptake and utilization, foliar N application is not a common practice. In this study, we sought to identify leaf morphological traits associated with foliar N uptake in four bread wheat genotypes (Spitfire, Gregory, Kukri, and RAC875). The four genotypes were foliar sprayed with commercial N fertiliser at two growth stages, (stem elongation and 7 days post-anthesis (DPA). Upon treatment 7 DPA, Gregory and Kukri had an increased grain N concentration, while Spitfire and RAC875 showed no response.

Leaf wax shapes and chemical composition analysis revealed differences in the flag leaf of the four genotypes harvested at 7 DPA. The gene expression analysis of selected N transporters in leaves of cv. Gregory revealed that *TaNRT1.1, TaAMT1.1, TaAMT2.1*, and *TaDUR3* genes were either downregulated or upregulated following foliar N treatment under both low and high basal soil N supply. Overall, the results suggest that genotypic differences in wax shape, chemical composition and transporter gene expression at 7 DPA, a stage considered optimal for foliar application, could be important for defining wheat varieties efficient in uptake and utilization of foliar-applied nutrients.

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Introduction

Nitrogen (N) fertiliser use has contributed to an increased world food production. However, N uptake in most crops is poor, which results in wastage (Dobermann and Cassman 2005). The management of N fertiliser application is important to ensure efficient uptake and utilization to secure crop production. One N management strategy that has been effectively utilised in cereal crops is the application of N in split dosage: at sowing and again after anthesis. N applied at sowing contributes to yield through accumulated biomass, while N applied post-anthesis is distributed to the grain, contributing to grain quality (Gooding and Davies 1992, Wylie *et al.* 2003). Spreading the fertiliser supply across the crop life cycle may also contribute to reducing N losses. However, plants at later growth stages sometimes have poor root activity due to water unavailability in the soil (Bogard *et al.* 2010). Moreover, nitrate in the soil is water mobile, affecting the availability of N in dry soils. Furthermore, N is likely less available late in the season since plants have already accessed much of it. One way to solve this problem is by providing N through foliar application.

Foliar application of micronutrients, for example, zinc and iron, is commonly practiced (De Valença *et al.* 2017, Eichert and Fernández 2012, Westfall and Bauder 2011). However, foliar N application is uncommon among cereal growers in Australia, although it is a strategy employed in America and Canada to improve the grain yield and quality (http://www.gov.mb.ca/agriculture/crops/soil-fertility/foliar-nitrogen-wheat-protein-enhancement.html). Unlike root N uptake that has been extensively studied in plants, little is known regarding leaf N uptake. Adoption of foliar application is limited specifically by a poor understanding of uptake efficiency, the varying performance of different foliar N forms, and a knowledge gap in the physiological and molecular factors that allow maximum foliar N penetration (Fernández *et al.* 2013, Gooding and Davies

1992). There is minimal research on pathways of foliar N uptake in wheat and no viable link has been demonstrated between leaf morphology/physiology and uptake efficiency of foliar-applied N fertilisers (Fernández *et al.* 2013). Various factors including physiochemical properties of the fertiliser, environmental conditions during foliar application, and the physiological characteristics of the plant, may influence the performance of foliar fertilisers (Fernández and Brown 2013).

Leaf surface properties determine leaf wetting and retention rate as well as diffusion of the liquid nutrient to the apoplast and symplast (Peirce *et al.* 2014b). The leaf surface properties that may influence the penetration of solutes include cuticle composition, epicuticular waxes, trichomes, and stomatal density (Eichert and Fernández 2012, Fernández and Brown 2013). Specifically, cuticular cracks, stomatal pores, trichome basal cracks and other modified epidermal cells have all been proposed as possible entry points for N uptake (Fernández and Brown 2013). The cuticle is a protective extracellular layer that covers epidermal cells of leaves, young shoots and other aerial organs and acts as the point of contact with the environment. It is made up of cutin, waxes, polysaccharides, and phenolics (Domínguez *et al.* 2011). The cuticle determines leaf glaucousness (blue-white colouring on the surface of plants) that has been linked to drought tolerance (Bi *et al.* 2016). Biosynthesis of the main components of leaf cuticle begins in the chloroplast with the synthesis of C16-C18 fatty acids and conversion into acyl-CoAs. Acyl-CoAs are converted to Very Long Chain Fatty Acyl-CoAs (VLCFA) in the endoplasmic reticulum and form precursors for alcohol formation (primary alcohols and alkyl), alkane formation (aldehydes, alkanes secondary alcohols and ketones) and diketone formation (Bi *et al.* 2017). These chemical compounds determine the wax crystal types including plates, platelets, ribbons, rods, threads and crust (Barthlott *et al.* 1998, Samuels *et al.* 2008). Previous studied on herbicide

penetration through the leaf concluded that the presence of specific wax crystal types rather than the amount is vital to regulating successful herbicide penetration (Raj and Allan 1991).

It is hypothesised that foliar liquids pass through the different layers of a leaf surface, starting with cuticular wax, cuticle, cell wall and finally plasma membrane (Franke 1967). Since the leaf wax quantity and composition vary between wheat genotypes, they may influence the entry and uptake of foliar nutrients. Pathways for entry of foliar N and other nutrients are much debated (Peirce et al. 2014a), necessitating species-specific research on leaf physiology and surface properties to determine nutrient penetration and uptake. For example, cuticular hydrophobicity will allow easier entry of non-ionic molecules such as urea through the lipophilic pathway and make ionised nutrient diffusion difficult (Eichert and Fernández 2012, Fernández *et al.* 2013). Conversely, entry of ionised molecules like nitrate and ammonium is through aqueous pores that are membrane-bound and formed from hydration of polar functional groups that rely on cuticle water content and humidity (Eichert and Fernández 2012, Schönherr 2000).

At a molecular level, several known N transporters have been studied in roots of plants and confirmed to facilitate the uptake of various forms of N; nitrate, ammonium and urea. However, similar studies have not been extensively undertaken in leaf and/or shoot. In the root tissue, nitrate is a common form of N taken up by plants, and thus many studies have focused on characterizing nitrate transporters. Nitrate transporters are divided into two classes of membrane proteins that mediate active transport of nitrate, across the plasma membrane, via symport with protons. They are the peptide transporters (PTRs)/NRT1/NPF and the nitrate-nitrite porters (NNPs)/NRT2 (Crawford 1995, Forde 2000). Both are categorised in the Major Facilitating Superfamily with two

sets of six transmembrane domains linked by a longer cytoplasmic loop (Pao *et al.* 1998, Williams and Miller 2001). (PTRs)/NRT1/NPF are Low-Affinity Transporters (LATS) and NNPS/NRT2S are High-Affinity Transporters (HATS), further divided into induced High-Affinity Transporters (iHATS) and constitutively active High-Affinity Transporters (cHATS). Whereas iHATS are induced by nitrate, cHATS are constitutively induced in the absence of nitrate (Aslam *et al.* 1992, Crawford and Glass 1998, Williams and Miller 2001). LATS are also induced by nitrate and active when N concentration is high, showing a linear relationship with external N concentration, while HATS are active under low N, with K_m values in the micromolar range (Miller *et al.* 2007). NRT1.1 is a dual affinity transporter that was found to be expressed and localised in leaf guard cells and involved in stomatal opening in *Arabidopsis thaliana* (Guo *et al.* 2003a). Also, Arabidopsis, NRT2.4, NRT2.6, NRT2.7, and NRT2.5 transporters were also found to be expressed in shoots (He *et al.* 2016, Kiba *et al.* 2012, Orsel *et al.* 2002). NRTs have also been identified in wheat and their expression investigated in the roots and correlated to nitrate uptake (Buchner and Hawkesford 2014a, Melino *et al.* 2015). In Arabidopsis, the *NRT1.1* dual affinity transporter gene was found to be strongly expressed in guard cells and involved in the opening of the stomata (Guo *et al.* 2003b). In wheat, sixteen *NPF* genes have been identified that are homologous to characterised LATS genes, and these are mainly expressed in roots (Buchner and Hawkesford 2014b). Research done previously has shown expression of NRT1.1 in leaves of wheat seedlings (Wang *et al.* 2019).

The other N form taken up and utilised by plant roots is positively charged ammonium. It is transported by a group of transporters known as AMTs. These transporters are further divided into high-affinity and low-affinity ammonium transporters. Rice, normally grown in flooded paddy fields, has ammonium as the preferred source of N because

in this environment with low pH soil conditions, ammonia (NH_3) is protonated readily to form ammonium (NH_4^+) (Gaur *et al.* 2012, Williams and Miller 2001, Yamaya and Oaks 2004). There are 12 AMT transporters identified in rice, categorised under three subfamilies, OsAMT1, OsAMT2 and OsAMT3 (Williams and Miller 2001). Li *et al.,* 2012, investigated the expression pattern and localization of nine AMT genes in rice, three *OsAMT1*, three *OsAMT2,* and three *OsAMT3* at two different stages (young seedling and tillering stage). From the results, *OsAMT1.1*, *3.2* and *3.3* were expressed in roots and older leaves, while *OsAMT2.1*, *OsAMT 2.2*, *OsAMT 2.3* and *OsAMT 3.1* were expressed in the stem and new leaves (Li *et al.* 2012). Two ammonium transporters (TaAMT1.1; AY525637) and TaAMT1.2; AY525638) have recently been identified in wheat (Søgaard *et al.* 2009). However, further studies are required to elucidate whether other orthologues are involved in both root and shoot N uptake.

The quantity of urea uptake is lower than nitrate and ammonium, but physiological evidence for root uptake of urea has been demonstrated in previous research (Mérigout *et al.* 2008) (Wang *et al.* 2012a). For example, rice grown in urea was found to contain large amounts of urea in the shoot, suggesting transportation of urea from root to aerial parts as a molecule (Gerendás *et al.* 1998). The uptake of urea in rice occurs either via the high-affinity urea symporter DUR3, or aquaporins (Wang *et al.* 2008). DUR3 is a high-affinity transporter and is a singleton in rice, with no other known members (Kojima *et al.* 2007, Wang *et al.* 2012b, Zanin *et al.* 2014). In Arabidopss, *AtDUR3* is expressed in roots and shoots under all nitrogen treatments (N presence and N starvation) (Liu *et al.* 2003). The gene was found to be highly expressed in old leaf tissue compared to young developing leaf (Liu *et al.* 2003). Urea, being an uncharged molecule, could be a form of N more easily taken up into the plant through the leaf suggesting the possible role of DUR3 in facilitating foliar N uptake. Urea uptake

by roots seems to be controlled by N availability, while leaf uptake is a diffusion-driven process (Liu *et al.* 2003). Previous experiments demonstrated urea uptake in leaves to follow the transpiration rate of the leaves, indicating a water mass flow-driven process, with the leaf uptake proportional to applied urea (Klein and Weinbaum 1985, Palta *et al.* 1991). There is no published information about the function of DUR3 or related genes in wheat to this date.

We sought to investigate leaf surface morphology through wax imaging and quantification, and gene expression analysis of selected N transporters in the flag leaf. This will improve our knowledge of the leaf morphological characteristics and identify the N transporters associated with uptake of foliar N in wheat. The measurements were taken in four elite Australian bread wheat genotypes, Spitfire and Gregory, Kukri, and the advanced breeding line RAC875. Spitfire and Gregory are elite Australian cultivars that showed a contrasting response to foliar N uptake from a prior study (Chapter 2). Kukri and RAC875 are contrasting for leaf glaucousness, wax quantities and drought tolerance (Bi *et al.* 2016). This study aimed to better understand factors that may affect foliar N uptake of nutrients in bread wheat.

Materials and method

Plant material and growth conditions

Four bread wheat (*Triticum aestivum* L.) genotypes, Gregory, Spitfire, RAC875 and Kukri, were grown in greenhouse conditions with natural lighting at the Australian Plant Phenomic Facility (The Plant Accelerator), Waite Campus in 2017. The controlled day/nighttime temperatures and humidity were 22/15 °C and 54/64 % respectively. The plants were grown in pots with 1.5 kg soil containing a mix of soil with loam, clay, and cocopeat (a multi-purpose growing medium made out of coconut husk) in 1:1:1 ratio. 50 mg N per kg of soil was added from granular urea into the minus N soil.

Foliar treatment at two wheat growth stages

The experiment was arranged in a randomised complete block design with four genotypes, two treatment stages (stem elongation and 7 days post-anthesis (DPA)) and four replications per treatment. The treatment was a foliar N application using commercial urea ammonium nitrate (UAN) (42.5% N w/v) in a volume of 80 µl, applied on the three topmost fully-expanded leaves at stem elongation, or on the flag leaf and the two leaves below the flag leaf, at 7 DPA. The 80 µl, was divided into 40 µl (20 µl adaxial and 20 µl abaxial) for the youngest fully expanded leaf/ flag leaf and 20 µl (10 µl adaxial and 10 µl abaxial) for each of the other two leaves. The application was made using a micro-pipette set to release 2 µl droplets that were evenly distributed on the surface of the leaf. The plants were harvested at maturity, oven-dried at 72 °C for three days and their dry weights recorded. Grain weight and grain N concentration were measured.

N analysis

Grain was ground using a Geno Grinder® (Spex Sample Prep, USA) at a speed of 1200 rpm. Approximately 100 mg ground material was subsampled, weighed and capsulated in N-free weighing paper for analysis of total N using a combustion nitrogen/protein analyser (Rapid N exceed®, Elementar Analysensysteme GmbHanalyser, Germany). Aspartic acid (250mg) and blank paper were used as calibration standards. The theoretical N% of aspartic acid (10.52) was divided by the machine's N% output to determine the N factor. The N% measured for each sample was corrected using this calculated N factor. Total grain N content was calculated as grain N% × grain wt (g), and grain protein content (GPC) was calculated as N% multiplied by the bread wheat conversion factor 5.7.

Wax quantification and chemical analysis

Plants were grown in conditions described above and harvested at stem elongation and 7 DPA. At stem elongation, the second fully expanded leaf was harvested, and at 7 DPA the flag leaf was harvested. The leaves were weighed and snap-frozen in liquid nitrogen. The frozen leaf samples were warmed to ambient temperature for 2 min for wax extraction. For an internal standard, hexadecane (C16 alkane), was dissolved in hexane and applied to the surfaces of leaves in amounts of 1 µg per 0.3g of a leaf sample. After 3 - 5min of applying the internal standard, the leaf samples were immersed in 4 ml chloroform for 1 min followed by drying under a stream of nitrogen for wax extraction.

GC-MS analysis of the extracted wax was conducted in the South Australian Health and Medical Research Institution (SAHMRI). Extracted wax was dissolved in 200 µl of acetonitrile, spiked with 1 µg triacontane (dissolved in chloroform), and derivatised with

50 µl N, O-bis(trimethylsilyl)trifluoroacetamide with 1% (v/v) trimethylchlorosilane at 80 °C for 60 min. A stream of nitrogen was used to air-dry the samples and the residue was reconstituted in 100 µl of chloroform and subjected to GC-MS analysis according to the procedure described by (Cha *et al.* 2009).

Scanning electron microscopy

For this experiment, the design was a randomised complete block design with four genotypes, two replicates per genotype, and six square pieces per leaf taken at two growth stages. The second fully expanded leaf was harvested, during stem elongation and the flag leaf was harvested at 7 DPA. Leaf samples of 1 cm by 1 cm were excised from the central portion of the leaf while avoiding the major vein. Leaf samples were snap-frozen in liquid nitrogen, and stored at -80 °C, followed by freeze-drying. To prevent charging under the electron beam, samples were mounted on a carbon stub, and coated with platinum before observation by Scanning Electron Microscopy (SEM), The epicuticular wax structure was examined at the Adelaide Microscopy Unit (https://www.adelaide.edu.au/microscopy/) using a Hitachi FlexSEM 1000 SEM with a scanning electron detector and a pre-centred tungsten filament. Image analyses were performed using ImageJ software (http://imagej.nih.gov/ij/. Analyses were made for three SEM images to determine trichome density (TD, numbers/µm2), trichome to trichome distance (TTD, µm), stomata length (SL, µm), stomata width (SW, µm) and stomata density (SD, numbers/µm2). For trichome length, stomata width and length, fifteen trichomes per image were measured.

In silico analysis of gene expression for N transporter genes with a putative role in leaf N uptake

N transporter gene identities from Arabidopsis and rice were obtained from literature (Guo *et al.* 2003a, Li *et al.* 2009, Zanin *et al.* 2014), followed by searches on the NCBI database for wheat orthologs (https://www.ncbi.nlm.nih.gov/). Extracted FASTA cDNA sequences were further blasted against the IWGSC RefSeq v1.0 Assembly (http://crobiad.agwine.adelaide.edu.au/afw/blast). The results included predicted chromosomal locations of three wheat homeologs for each transporter, where these were identifiable. Full-length cDNA sequences were extracted using the FETCH-seq tool (http://crobiad.agwine.adelaide.edu.au/afw/fetch).

In silico analysis of expression for five N transporter genes and their homeologues was done using the web application POPSEQ Ordered *Triticum aestivum* Gene Expression (POTAGE) (http://crobiad.agwine.adelaide.edu.au/potage) (Suchecki *et al.* 2017). The expression data in POTAGE is collated from bread wheat Chinese Spring RNA-seq data consisting of five different tissue types (root, leaf, stem, spike, and grain) at three growth stages, each with two replicates. Transporter genes showing expression in leaf tissue in POTAGE were selected for further investigation of their role in foliar N uptake.

Real-time quantitative PCR (RT-QPCR)

Plants were grown in greenhouse conditions in pots containing 1.5 kg soil, as described above. Urea (46% N w/w) was added to the minus N soil at 50 mg N and 100 mg N per kg of soil. The two N treatments were classified as Low N (LN) and High N (HN) basal N, respectively, with a single plant per pot. The experiment was a randomised complete block design with five replicates, two basal N treatments (LN and HN), and four foliar N treatments. Foliar N was applied to the flag leaf at 7 DPA. The four foliar treatments were (i) SpreadWet only (active constituent: 1000 g/l Alkoxylated Alcohols,

SST Australia, 0.5% v/v)) (control), (ii) urea (0.5% SpreadWet containing 3 M N), (iii) ammonium sulfate (0.5% SpreadWet containing 3 M N), (ii) potassium nitrate (0.5% SpreadWet containing 3 M N). Foliar treatments were applied in a total volume of 80 µl (40 µl on the adaxial side and 40 µl on the abaxial side) using a micropipette set to 2 µl dispensing volumes. Flag leaves were harvested at 30 min and 2 h after application of foliar N. The harvested leaf material was snap-frozen and stored at -80 °C before RNA extraction.

Primers were designed using Geneious Prime version 2019.2.1 software. They were designed in consensus regions of the gene homeologs and checked using IDT OligoAnalyzer (https://sg.idtdna.com/calc/analyzer) to confirm their melting temperature and GC content, and check for hairpins, self-dimers, and heterodimers. Primers were tested for amplification of the gene of interest only with a further confirmation done by gel electrophoresis and Sanger sequencing of amplified fragments.

RNA was extracted from frozen leaf tissue using a Direct-zol™ RNA Miniprep Kit (Zymo Research). A DNase I column digestion treatment was included to remove any putative genomic DNA contamination in the RNA sample, and a final elution was done using DNase/RNase-Free water. Concentrations of RNA were measured by UV spectroscopy (NanoDrop, ND-1000 spectrophotometer). Approximately 1 µg RNA was then used for the synthesis of cDNA using SuperScript III™ reverse transcriptase (ThermoFisher SCIENTIFIC). The cDNA was stored at −20 °C until qPCR analysis. For qPCR, 10 µl master mix was prepared, containing 5 µl Precision Fast 2x qPCR Master Mix (Low Rox) with SYBR Green, 0.5 µl forward primer and 0.5 µl reverse primer, 2 µl water and 2 µl (approximately 2.5 ng/µL) cDNA. Real-time PCR was carried out using a QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific).

The thermal cycling consisted of an initial enzyme activation (hot start) at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 20 s and a final extension at 95 °C for 15 s. Each reaction per treatment was performed with four biological replicates, each having three technical replicates. The average threshold cycle (CT) value was calculated for each sample. Two reference genes were used, (glyceraldehyde-3-phosphate dehydrogenase *TaGAPDH*) and (Elongation Factor α *TaEFα*) were used (Supplementary Table 3). CT values obtained for each sample were normalised with the two reference genes. The control treatment samples were used as a calibrator of gene expression. The qPCR data were extracted and analysed in LinRegPCR, a program that analyses quantitative PCR (qPCR) data based on the PCR efficiency of amplification curves (Ramakers *et al.* 2003). Non-baseline corrected data was used, whereby the software did a baseline correction on each sample and determined a window-of-linearity followed by a linear regression analysis to determine the PCR efficiency from the slope of the regression line (Ramakers *et al.* 2003). Finally, the mean PCR efficiency per amplicon was calculated (Peirson *et al.* 2003).

The formula used to calculate the relative expression ratio of the gene of interest is

$E^{-\Delta\Delta CT}$, with E= real-time PCR efficiency of target gene transcript, ΔCT = $CT_{\text{target gene}}$ minus $CT_{\text{reference genes}}$, and $E^{-\Delta\Delta CT}$, where ΔΔCT = [($CT_{\text{target, treated sample}} - CT_{\text{reference gene, treated}}$)-(CT $CT_{\text{target, untreated sample}} - CT_{\text{reference gene, untreated}}$)]. The standard error was computed from the average of the $E^{-\Delta\Delta CT}$ values for each treatment (Schmittgen and Livak, 2008).

Statistical analysis

One-way ANOVA to analyse wax composition differences between the genotypes and test the hypothesis of no differences between means. The means of treatment effects were analysed using Asreml function in R Studio and GraphPad Prism^{TM}. When differences were identified from the ANOVA analysis, multiple comparisons with Tukey's significant differences test was used at $p \leq 0.05$. Gene expression analysis was done using GraphPad Prism^{TM} using one-way ANOVA, multiple comparisons comparing means of treatment with control and a Tukey's significant differences test was used at $p \leq 0.05$.

Results

Foliar N effects on grain yield and grain N

Four bread wheat genotypes, Spitfire, Gregory, Kukri and RAC875, were grown in controlled conditions and treated with foliar N at two growth stages, stem elongation and 7 DPA. For the control treatment, SpreadWet was applied. Multiple comparison test was done to identify significant differences for grain weight and grain N concentration between treatments in the two growth stages for the four genotypes (Supplementary Table. 1). After foliar N treatment at stem elongation, grain weight was significantly higher ($P<0.05$) in Spitfire compared with control, but there were no significant differences for the other three genotypes (Fig. 1A). There were no differences in grain weight when foliar N was applied at 7 DPA in all genotypes (Fig. 1B). At 7 DPA, foliar-N-treatment Gregory and Kukri had significantly higher grain N concentration compared to the controls (Fig. 1D).

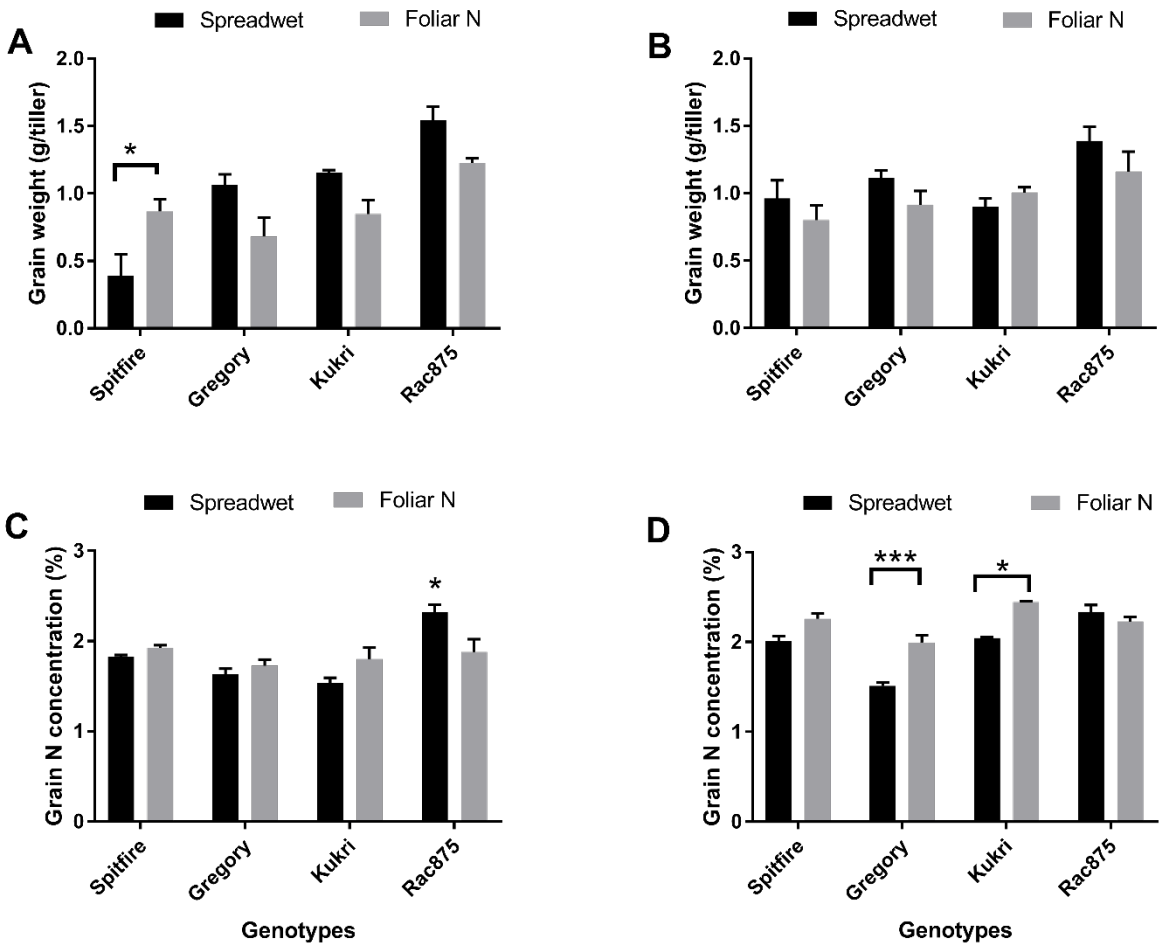

Figure 1: Effects of foliar N application at stem elongation (A and C) and 7 days post-anthesis (B and D), on grain weight (A and B) and grain N concentration (C and D), in four bread wheat genotypes reportedly contrasting for grain protein concentration and leaf surface characteristics. Control plants were treated with SpreadWet only. Error bars indicate the SEM of four replicates, and asterisks (*) indicate treatments that were significantly different from control, (* $p<0.01$, ** $P<0.001$ and ***$P<0.0001$).

Leaf surface wax quantity and chemical composition

To investigate the relationship between foliar nitrogen uptake and cuticular composition, we analysed wax quantity and chemical composition of the second fully-expanded leaf (stem elongation) and flag leaf (7 DPA) in four bread wheat genotypes: Spitfire, Gregory, Kukri, and RAC875. In all genotypes, the leaf surface wax quantities were lower at stem elongation compared to 7 DPA (Fig. 2). At stem elongation, the total wax content for Gregory was significantly higher than all other genotypes (Fig.

2A), while at 7 DPA, Spitfire had significantly higher (p≤0.001) total leaf wax content than Gregory (Fig. 2B). Between the two stages, Spitfire had the highest increase in total wax with a 12-fold increase (p≤0.0001), followed by Kukri with an 8.6-fold increase (p≤0.01) and RAC875 with a 4.6-fold increase (p≤0.03). Gregory, with a 2.6-fold increase, was not significantly changed. At each growth stage, Kukri and RAC875 had similar levels of leaf surface wax.

There were no differences in the wax chemical composition of leaf surface wax at stem elongation; the distribution of alcohols, fatty acids, aldehydes, alkanes, and diketones in the four genotypes was similar in all the four genotypes (Fig. 3). The major wax chemical component was primary alcohol Isotridecyl alcohol with an 82 – 89% prevalence, followed by alkanes (hexacosane and heptacosane) with 5 – 8%, and aldehydes (pentacosanal) with 4 – 9% (Supplementary Fig. 1). Isotridecyl alcohol decreased in the 7 DPA stage.

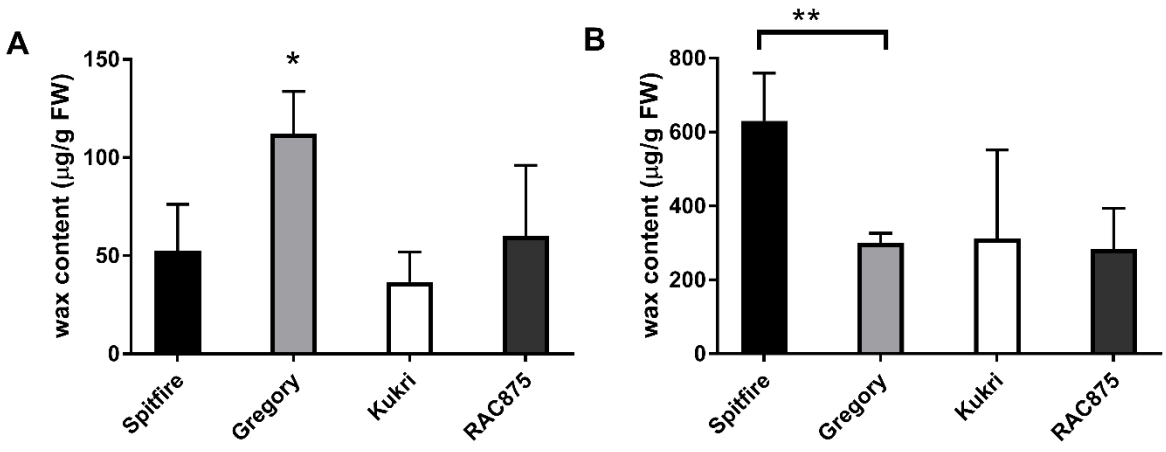

Figure 2: Total leaf surface wax content in leaves of four bread wheat genotypes (Spitfire, Gregory, Kukri, and RAC875) sampled at two growth stages. A. stem elongation (SE) and B. 7 days post-anthesis (7DPA). Error bars indicate the SEM of five replicates, and asterisks (*) indicate significant differences between genotypes; (*P<0.01, **P<0.001, ***P<0.0001).

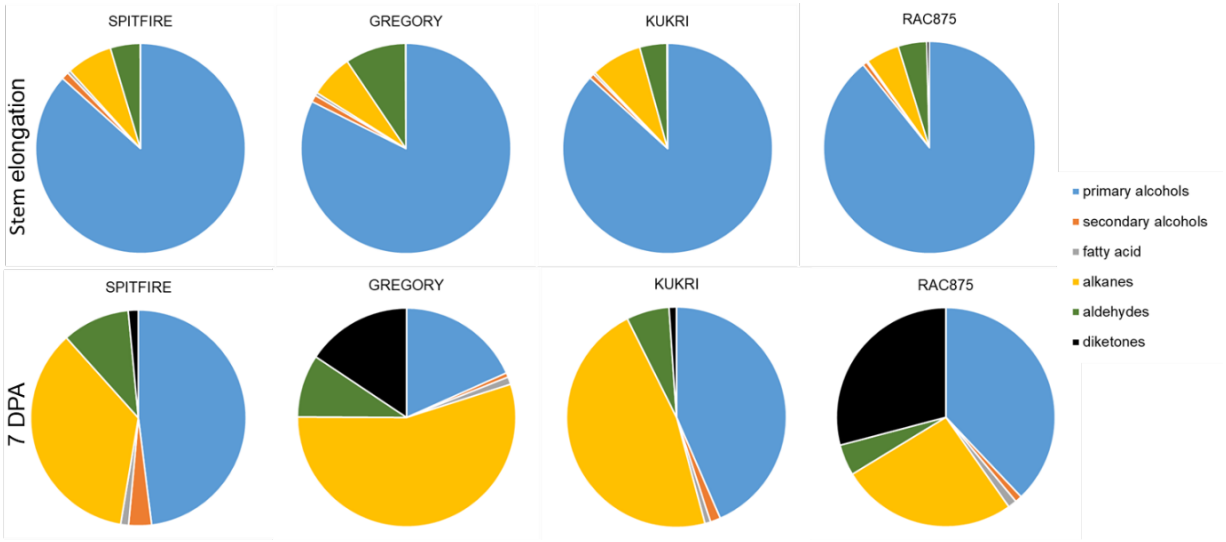

Figure 3: The wax chemical composition of the flag leaf collected at 7 DPA, in four bread wheat genotypes, Spitfire, Gregory, Kukri, and RAC875. The chemical components are primary and secondary alcohols, fatty acids, aldehydes, alkanes, and diketones.

Overall, the abundance of flag leaf wax chemical components at 7 DPA showed differences compared to the stem elongation stage (Fig. 3; Supplementary Table 2). Even though at 7DPA primary alcohols, alkanes and aldehydes remained the primary components, all genotypes showed a decrease in primary alcohols and an increase in alkanes. Interestingly, the major changes were observed in Gregory, which presented both the largest decrease in primary alcohols (reaching 18%) and the largest increase in alkanes (55%). All genotypes had consistently low proportions of secondary alcohols and fatty acids for both stages. Overall, Spitfire and Kukri had a similar surface wax composition, except for a slightly higher alkane proportion in Kukri compared to Spitfire at 47% and 36% respectively. Gregory and RAC875 had a significant increase in the proportions of diketones at (16%) and (29%) respectively. The individual chemical components within each compound class (alcohols, fatty acids, aldehydes, alkanes, and diketones) at 7 DPA are in reported in Supplementary Fig. 2.

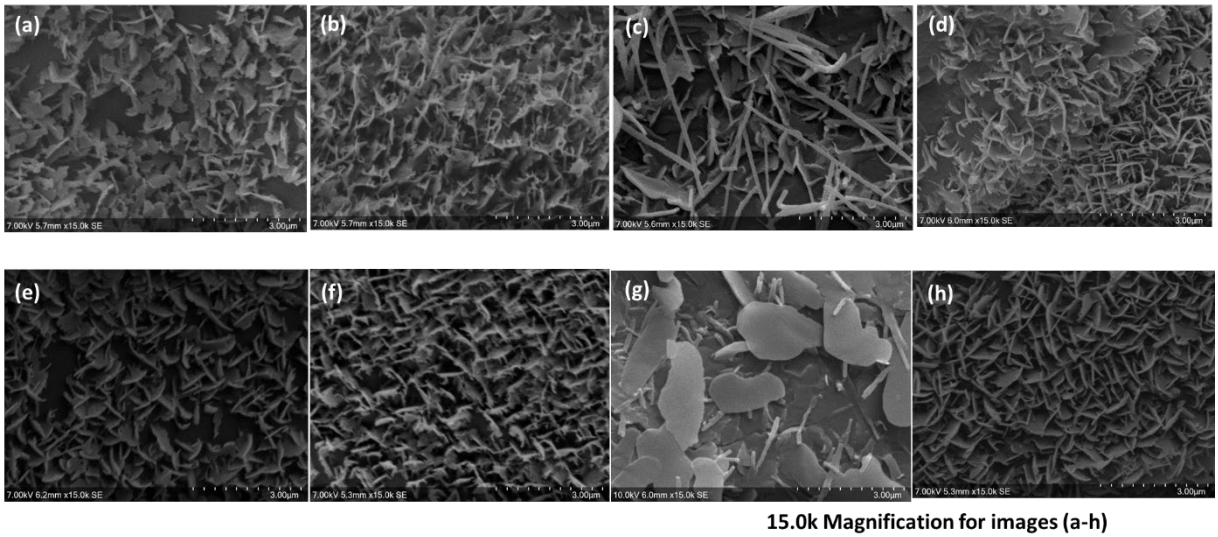

Figure 4: Scanning Electron Microscopy of Gregory and Spitfire leaf at two growth stages, stem elongation (SE) and 7 days post-anthesis (7 DPA). Gregory: (a) SE, adaxial, (b) SE, abaxial, showing plate-like wax shapes, (c) 7 DPA, adaxial, showing numerous long tubular and platelet wax shapes and (d) 7 DPA, abaxial, showing dense plate-like wax shapes. Spitfire: (e) SE, adaxial, (f) SE, abaxial, showing plate-like wax shapes, (g) 7 DPA, adaxial, showing few short tubular and platelet wax shapes and (h) 7 DPA, abaxial, showing dense plate-like wax shapes.

Gregory and Spitfire wax shape at stem elongation and 7 DPA

The wax shapes observed on both the adaxial and abaxial sides of the leaf were similar in Gregory (Fig. 4 a, b) and Spitfire (Fig. 4 e, f) at stem elongation. However, at 7DPA, the flag leaf wax shapes were distinctly different in Gregory (Fig. 4 c, d) and Spitfire (Fig. 4 g, h), especially on the adaxial side of the flag leaf. Gregory had some platelet structures and numerous long tubular shaped-waxed structures. Spitfire, on the other hand, had large platelet structures and fewer short tubular-shaped waxes.

Kukri and RAC875 trichomes and wax shapes at 7DPA

To further explain the differences observed between Spitfire and Gregory, wax shapes were also investigated in Kukri and RAC875. From the SEM images, differences in trichome and wax shape at 7 DPA were investigated under different magnification (Supplementary Fig. 3 a, b, c, d). Kukri had differences in shapes on the adaxial and abaxial sides of the flag leaf. The images in Supplementary Fig. 3 a and b, show a smooth adaxial side and abaxial side with visible short, straight-tips and thick-bottomed

trichomes. Wax shapes on the adaxial and abaxial side of the leaf looked similar and had platelet dense structures (Supplementary Fig. 3 c, d).

The differences in trichome and wax shape 7 DPA were investigated in RAC875, under different magnification (Supplementary Fig. 4 a, b, c, d). The adaxial side had a rough hairy-like surface (Supplementary Fig 4 a, b). The abaxial side had visible sharp, bent tips and thick-bottomed trichomes. Wax shapes on the adaxial and abaxial side of the leaf were different with platelet dense shapes (Supplementary Fig. 4c) on the adaxial side and a mix of platelet and tubular-shaped wax on the abaxial side of the leaf (Supplementary Fig 4d).

Trichome density and trichome shape in Spitfire and Gregory at 7DPA

Scanning Electron Microscopy (SEM) was used to identify the leaf surface structures in four bread wheat genotypes that may be associated with foliar N uptake, with a focus on wax shapes. Additional analysis was done on the trichome shape and density on the leaf surface of Spitfire and Gregory. There were differences in trichome shape and density between Gregory and Spitfire on the flag leaf collected at 7 DPA (Fig. 5; Table1).

Gregory had many visible trichomes on the adaxial side of the leaf (Fig. 5a) with a thin long shape (Fig. 5c). Spitfire, on the other hand, had fewer trichomes on the adaxial side of the leaf compared to Gregory (Fig. 5b). The trichomes shape in Spitfire were short and thick (Fig. 5d). The trichome length and trichome density were significantly greater in Gregory compared to Spitfire (Table 1), while the trichome-to-trichome distance was smaller. However, there were no differences in the flag leaf stomata length, width, and density between the two genotypes.

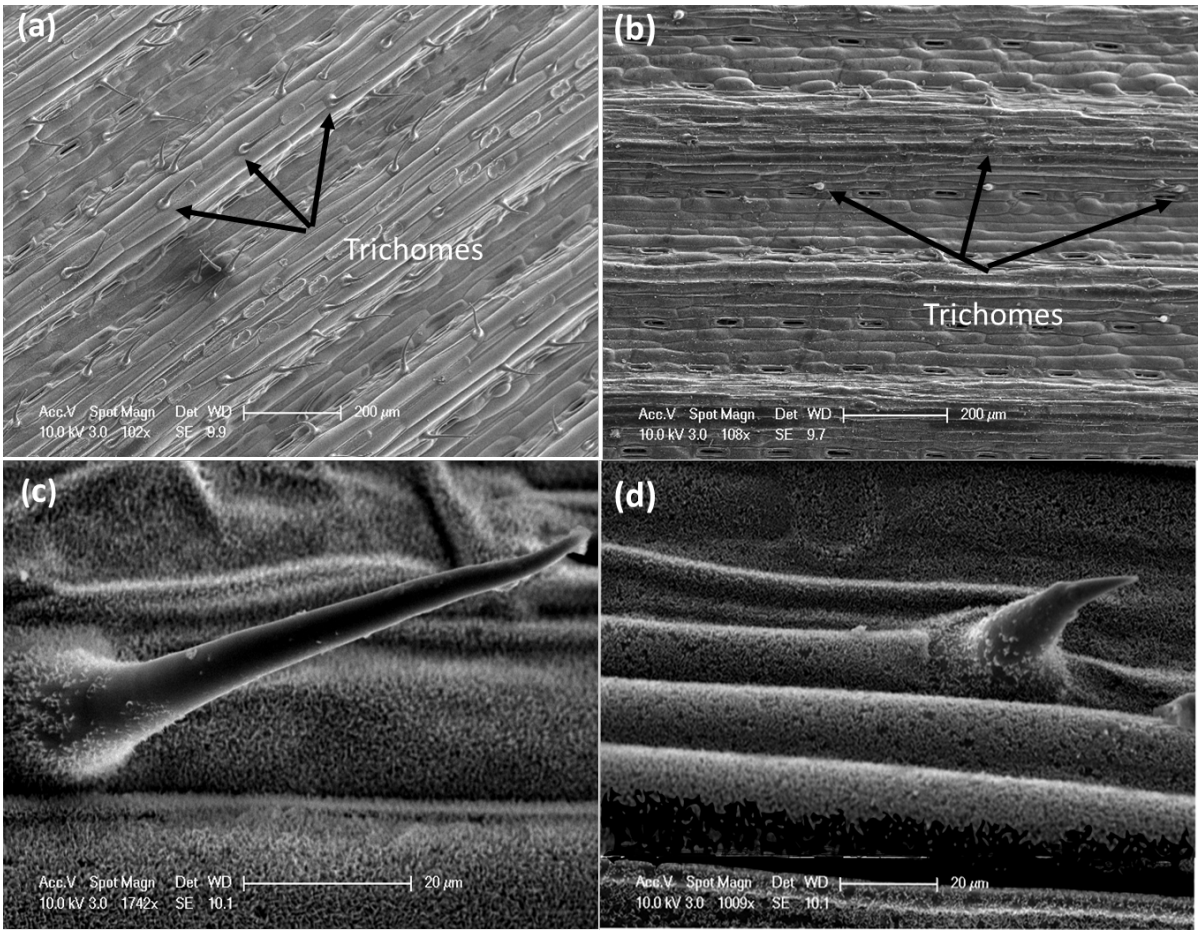

Figure 5: Scanning Electron Microscopy of Gregory and Spitfire flag leaf at 7DPA. a) and c), Gregory adaxial leaf surface with trichomes and typical trichome shape, which is long, thin, and sharp. b) and d) Spitfire adaxial leaf surface with trichomes and typical trichome shape, which is short and thick.

Table 1. Gregory and Spitfire flag leaf trichome length (TL, µm), trichome to trichome distance (TTD, µm), trichome density (TD, n/µm2), stomata length (SL, µm), stomata width (SW, µm) and stomatal density (SD, number/µm2). The data were obtained from three SEM images of the adaxial surface analysed using Image J software. The values are an average (+/SE), and letters indicate significant differences, whereby the same letter means no differences and different letters means significantly different.

Genotype	***Trichome length (µm)***	***Trichome to trichome distance (µm)***	***Trichome density ($n/\mu m^2$)***	***Stomata length (µm)***	***Stomata width (µm)***	***Stomatal density ($n/\mu m^2$)***
Gregory	95.5±3.56a	127.6±11.22a	60.6±4.33a	67.3±1.32a	19.6±0.86a	44.8±4.02a
Spitfire	34.2±2.28b	219.1±21.56b	23.2±2.37b	63.8±1.20a	20.6±0.82a	44.0±3.73a

Gene expression of leaf-expressed N transporters

Nitrogen uptake and transport occur via several proteins. An initial literature review was done to identify N transporters expressed in leaf tissue. Previous studies in *Arabidopsis thaliana* and rice (*Oryza sativa*) have identified various N transporters expressed in leaf tissue, including *NRT1.1, NRT2.4, NRT2.5, AMT1.1, AMT2.1, AMT2.2*, and *DUR3* (Supplementary Table 3). Nucleic sequences of these transporters were used to identify putative wheat orthologs for further *in silico* analysis (see Materials and Methods). From the POTAGE analysis, the N transporter genes expressed in the shoot were *TaNRT1.1*, *TaAMT 1.1*, *TaAMT2.1*, and *TaDUR3,* based on FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values (Supplementary Fig 5). On the other hand, *TaNRT2.4, TaNRT2.5, and TaAMT2.2* had high root expression but low (< 1 FPKM) to no leaf tissue expression (Supplementary Fig 6) and were therefore not considered for further analysis.

Gene expression analysis of the selected genes (*TaAMT 1.1*, *TaAMT2.1*, *TaNRT1.1*, and *TaDUR3*) was performed on Gregory due to its foliar N responsiveness, which resulted in a positive N uptake and increased GPC as demonstrated in Chapter 2. Analyses were conducted on plants grown with both high and low level of basal soil N supply, foliar treated and harvested at 30 min and 2 h post-treatment.

The transcriptional activation of *TaNRT1.1* varied significantly between LN and HN conditions in response to foliar N applications. Under LN conditions, *TaNRT1.1* was significantly downregulated ($p \leq 0.001$) at 30 minutes upon ammonium application but remained unchanged for other N treatments compared to control (SpreadWet surfactant only) (Fig. 6A). In contrast, *TaNRT1.1* expression significantly increased after ammonium ($p \leq 0.001$), decreased following nitrate ($p \leq 0.001$) and remained

unchanged after urea treatment at 2h (Fig. 6A). *TaNRT1.1* was generally downregulated (p≤0.0001) under HN in both time points for all N forms, except upon urea treatment, where expression at 30 minutes was unchanged (Fig. 6B).

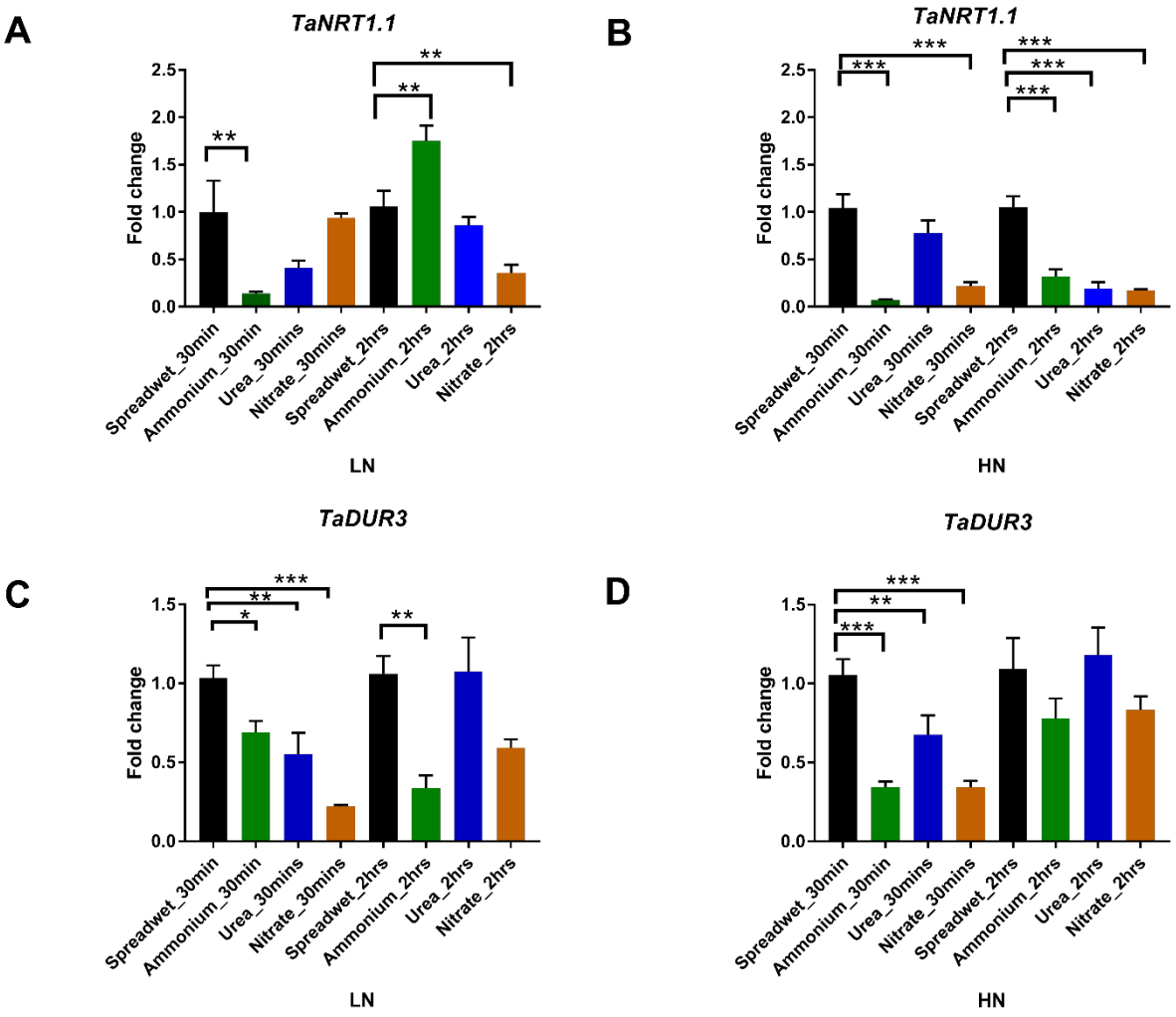

Figure 6: Gene expression (fold change) in the flag leaf of Gregory grown with an initial low N (LN) and high N (HN) basal treatment, followed by foliar treatment with different N forms (ammonium, urea or nitrate) at 7DPA and harvested at two time-points following application (30 min and 2 h). a) TaNRT1.1_LN and b, TaNRT1.1_HN c) TaDUR3_LN and d, TaDUR3_HN. Error bars indicate the SEM of four replicates, and within each time point, asterisks (*) indicates treatments that were significantly different from the control (* P<0.01; ** P<0.001; *** P<0.0001).

The singleton urea transporter, *TaDUR3*, had a largely similar expression except for ammonium treatment at the two time-points under both LN and HN basal treatment conditions. Specifically, at the 30 min post-treatment, *TaDUR3* was significantly

downregulated compared to control after ammonium, urea, and nitrate treatments under both LN and HN conditions (Fig. 6 C, D). *TaDUR3* expression had little change in both LN and HN at 2 h post-treatment for all N foliar applications when compared to control. However, the gene was significantly downregulated ($p \leq 0.001$) following ammonium application in LN (Fig. 6 C, D). There was, however, an upregulation of *TaDUR3* with urea treatment after 2 h compared to 30 min post-treatment.

Under LN basal treatment, *TaAMT1.1* was significantly upregulated as a result of ammonium ($p \leq 0.0001$), and nitrate ($p \leq 0.0001$) treatments at 30 min post-treatment (Fig. 7A). However, at 2 h post-treatment, *TaAMT1.1* was significantly downregulated following nitrate treatment ($p \leq 0.01$) (Fig. 7A). Urea application seemed to not affect *TaAMT1.1* expression under LN. For the HN basal treatment, *TaAMT1.1* was significantly downregulated after ammonium ($p \leq 0.0001$), urea ($p \leq 0.0001$), and nitrate ($p \leq 0.0001$) treatments in the 30 min post-treatment (Fig. 7B). At the 2 h post-treatment, *TaAMT1.1* was significantly downregulated ($p \leq 0.001$) in response to ammonium treatment but remained unaffected in response to the other foliar N forms (Fig. 7B).

The expression of the second ammonium transporter, *TaAMT2.1,* under LN basal treatment was significantly downregulated after nitrate ($p \leq 0.01$) treatment at the 30 min post-treatment and ammonium treatment ($p \leq 0.01$) at the 2 h post-treatment. Under HN basal treatment, *TaAMT2.1* was significantly downregulated after ammonium ($p \leq 0.0001$) and nitrate ($p \leq 0.0001$) treatment at the 30 min post-treatment. At 2 h post-treatment, *TaAMT2.1* was significantly downregulated in response to all the three foliar N treatments.

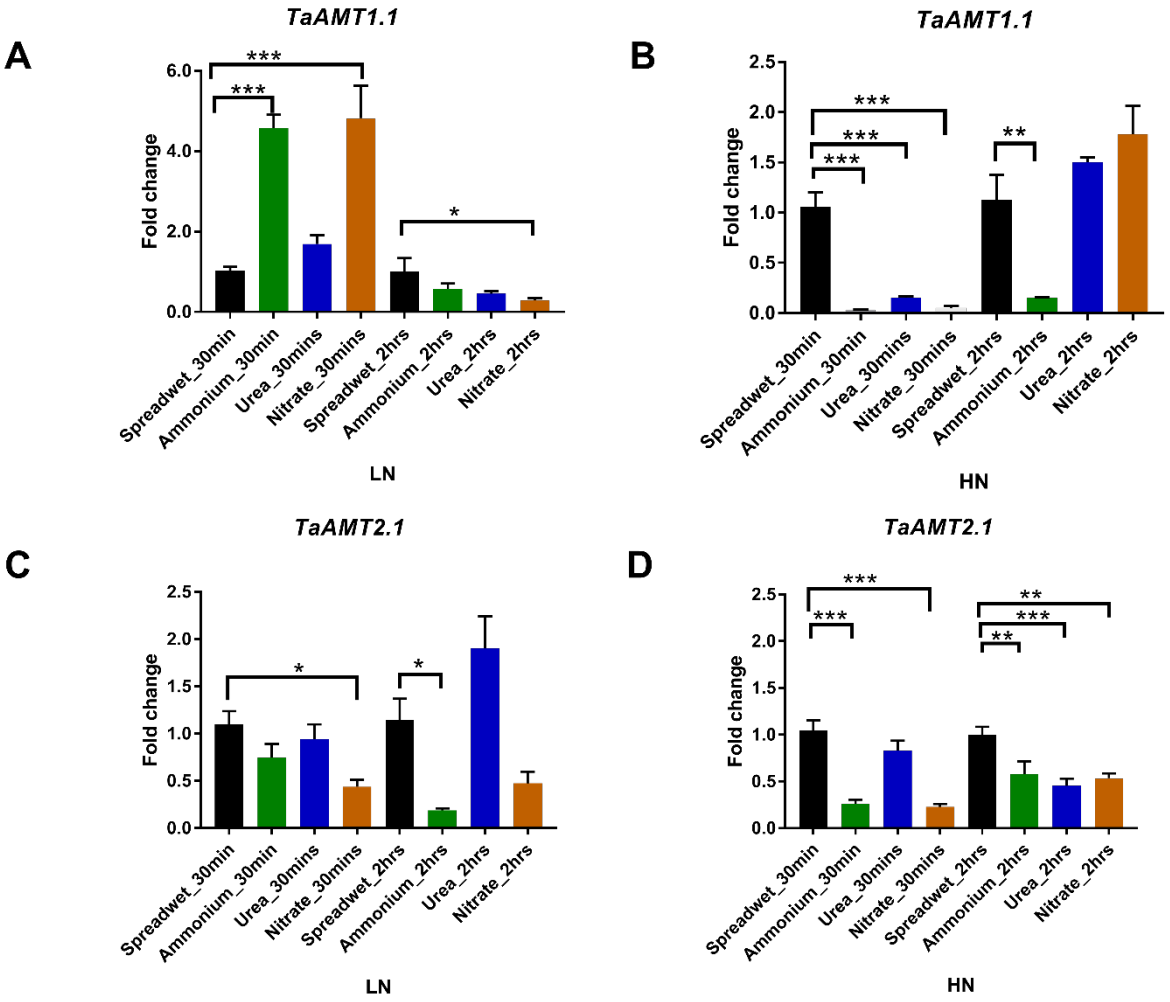

Figure 7: Gene expression (fold change) in Gregory with an initial low N (LN) and high N (HN) basal treatment, followed by foliar treatment with the different N forms (ammonium, urea and nitrate) on the flag leaf at 7DPA and harvested at 2-time points (30 min and 2 h). a) TaAMT1.1_LN and b, TaAMT1.1_HN c) TaAMT2.1_LN and d), TaAMT2.1_HN. Error bars indicate the SEM of four replicates, and asterisks (*) indicates treatments that were significantly different from the control, (*P<0.01, **P<0.001, ***P<0.0001).

Discussion

Leaf surface morphological features have been speculated to influence foliar uptake of solutions in plants (Eichert and Fernández 2012). Studies on the leaf surfaces to identify the specific features that strongly correlate to foliar uptake of applied spray solution are required to validate these claims. In this study we investigated the foliar N uptake in four bread wheat genotypes (Gregory, Spitfire, Kukri and RAC875), followed

by a leaf morphological study to try and identified the specific traits that are correlated to the effective entry of sprayed solution through leaf tissue. We also investigated the expression response of genes coding for leaf-expressed N transporters specific to the three N forms (ammonium, urea, and nitrate). Foliar N applied at stem elongation did not have a significant effect on grain weight in all the genotypes except Spitfire (Fig. 1). To explain the results, Spitfire could be effectively taking up more soil and foliar N at this stage translating to an increased grain weight. At the 7 DPA stage, foliar N applied also had no significant effect on grain weight. Previous research also found similar results, whereby foliar N applied after anthesis had minimal to no effect on grain yield (Chapter 2) (Dampney and Salmon 1990, Dick *et al.* 2016, Strong 1982). The outcome is as expected since N uptake after anthesis is more likely to be invested towards an N increment in the preformed grains. Therefore, the timing of the foliar N application may be crucial in determining N utilization for grain yield and grain N concentration.

We observed that grain N concentration was significantly increased in Gregory and Kukri after foliar N treatment at 7 DPA. This agrees with previous research where grain N concentration increased after foliar treatment late in the season in bread wheat and durum wheat (Chapter 2) (Blandino *et al.* 2015, Dick *et al.* 2016). The N applied as foliar and taken up through the leaf tissue may be effectively moving to the grains resulting in an increased grain N concentration. Furthermore, these results stipulate a genetic variability in foliar N uptake as seen in the different responses to leaf applied N on grain N concentration in the four wheat genotypes, which could be associated with differing leaf morphological features.

The wax quantity significantly increased between stem elongation and 7 DPA stages in all genotypes except Gregory which had a marginal increase (Fig. 2). Wang and

colleagues had similar findings from their research on wheat wax quantity, whereby there was an increase in wax levels between 50 and 200 days during initial leaf development, followed by either a decrease or increase between 200 and 230 days in different genotypes (Wang *et al.* 2015). The reason behind the developmental changes in wheat leaf wax quantity was correlated to leaf expansion and changes in wax chemical composition, involving variation in alcohols, alkanes and diketones (Wang *et al.* 2015). Since the flag leaf was harvested at 7 DPA, we could not report of a decrease or increase in wax quantity towards maturity as observed from previous research (Tulloch 1973, Wang *et al.* 2015). In the current study, there was no significant developmental difference in Kukri and RAC875 total wax quantity like in previous research (Bi *et al.* 2016).

The wheat leaf cuticular layer is made up of primary and secondary alcohols, ketones, alkanes, fatty acids, aldehydes, and esters (Javelle *et al.* 2011, Kunst and Samuels 2009, Wang *et al.* 2015). Wax composition during early leaf development (stem elongation) was mainly composed of alcohols (82 -89%), alkanes (5 - 7%) and aldehydes (4 – 9%) (Fig. 3). Similarly, Wang and colleagues also found alcohols (90%), alkanes (2.8 – 4.4%) and aldehydes (1.0 –3.3%) during the early leaf development stages in wheat (Wang *et al.* 2015).

At 7 DPA, there was a decrease in alcohol levels, and a simultaneous increase in alkanes, and diketones. The latter two were hardly detected in the stem elongation stage (Fig. 3). Previous research on wheat leaf at later growth stages had similar observations on the wax chemical composition, whereby, the alcohol levels decreased and subsequently the alkanes and diketones increased (Tulloch 1973, Wang *et al.* 2015). The developmental variation in alcohols and diketones in wheat was previously

hypothesised to stem from the biosynthetic relationship between the two chemical components (Tulloch 1973).

Alkanes proportions were high in Gregory and Kukri flag leaf at 7 DPA (Fig. 3, Supplementary Table 2). The two genotypes responded to foliar N uptake with a positive effect on grain N concentration. A gene known as 'inhibitor of wax 1', *iw1*, inhibits the formation of diketones in the flag leaf cuticle and causes a non-glaucousness phenotype in wheat. This gene is also proposed to be responsible for an increase in alkanes. (Adamski *et al.* 2013). In the current study, the low diketones and high alkanes in Kukri may explain the importance of *iw1* or the non-glaucousness phenotype in influencing effective foliar N uptake. Gregory in contrast, apart from maintaining high alkanes, had an elevated quantity of diketones which strongly supported the presence of tubular wax shapes (Fig. 4c). Furthermore, Gregory had low total primary alcohols (Supplementary Table 2) which could be further correlated to effective foliar N uptake. The common phenotype between the two genotypes that take up foliar N effectively is an elevated quantity of alkanes, suggesting there could be a correlation between alkanes and foliar N uptake, which could be further investigated. The low primary alcohol levels may also be correlated to foliar uptake and could also be a further point of future study.

There were developmental differences in wax shape in the four genotypes, whereby at stem elongation, platelet wax shapes were prevalent in Gregory (Fig. 4 a, b) and Spitfire (Fig. 4 e, f), and Kukri (Supplementary Fig. 3) as well as Rac875 (Supplementary Fig. 4). Similar observations were made previously on 8-week old wheat plants (Koch and Ensikat 2008). At 7 DPA there was a mix of platelet and tubular wax shapes as seen in Gregory (Fig. 4 c, d) and RAC875 (Supplementary Fig. 4). Similarly, Wang and colleagues reported on wax shapes in wheat leaf surfaces having

developmental differences. In their study, 50 and 100-days old wheat had leaves covered with platelets on the adaxial and abaxial leaf side in four wheat varieties, while 200-days old plants had platelets on the adaxial and tubular wax shapes on the abaxial leaf side in two wheat varieties (Koch *et al.* 2006, Wang *et al.* 2015). Additional research suggested that platelet wax shapes are deposited on the adaxial side, while tubular-shaped wax was found on the abaxial side of the wheat flag leaf (Adamski *et al.* 2013, Wang *et al.* 2014, Zhang *et al.* 2013). This is partly in agreement with the results in the current study, whereby Kukri and RAC875 had platelet wax shapes on the adaxial side, while tubular-shaped wax was found on the abaxial side of the wheat flag leaf (Supplementary Fig.3, 4). In contrast, Gregory and Spitfire had platelet wax shapes on the abaxial side and tubular-shaped wax on the adaxial side of the wheat flag leaf at 7 DPA stage (Fig. 4 c, d) and (Fig. 4 g, h).

The tubular shapes have been suggested to result from a high content of diketones while platelet shapes are positively correlated with alcohols (Adamski *et al.* 2013, Koch and Ensikat 2008). Results from this study showed that all the genotypes with platelet shapes on both sides of the leaf at stem elongation had alcohol as the major wax component. Gregory and RAC875 had an abundant quantity of diketones, consistent with a higher incidence of tubular wax shapes. Overall, the results from the current study and previous research showed that changes in the wax chemical composition corresponded to changes in wax shapes (Wang *et al.* 2015).

The trichome shape and density were distinctly different between Gregory and Spitfire, which speculatively could contribute to the differences in foliar N uptake seen resulting in differences in GPC in Chapter 2. In the current study, Gregory had thrice as many trichomes per unit leaf surface area in the flag leaf compared to Spitfire (Fig. 5; Table 1). Previous work reported the occurrence of numerous aqueous pores at the base of

trichomes and proposed that these may be points of entry for hydrophilic substances into leaf tissue (Schönherr 2006). The current study did not investigate the presence of aqueous pores at the base of trichomes, and as such, we can only speculate on their presence in the numerous trichomes in Gregory, and perhaps an active involvement in the effective entry of foliar N.

Gene expression studies were carried out to measure the expression responses to foliar N treatment in genes encoding leaf specific N transporters under two basal N treatments (LN and HN) in Gregory. Gregory was used because of its foliar N responsiveness resulting in a positive uptake of foliar N and increased GPC from previous experiments (Chapter 2). *TaNRT1.1*, a putative dual affinity transporter, has been extensively researched in wheat roots (Guo *et al.* 2014), but there is no information about leaf tissue expression. However, available POTAGE data shows that this gene has previously been detected *in-silico* as being expressed in the leaf (Supplementary Fig 5). The expression of *TaNRT1.1* in the current study was basal N dependant and regulated by foliar treatment with the different N forms, (nitrate, urea, and ammonium) at different time points (Fig. 6A).

TaDUR3, a singleton urea transporter, was downregulated in both LN and HN basal treatment after foliar treatments with the three N forms at the 30 min and 2h time points (Fig. 6 c, d). These results concur with previous observations in maize root tissue, which identified the downregulation of *ZmDUR3* in urea-supplied plants compared to controls (no urea treatment) being a high affinity and saturable transport system (Zanin *et al.* 2014).

The genes encoding two AMT transporters TaAMT1.1 and TaAMT2.1 were either downregulated or upregulated by the different foliar N treatments under LN and HN

basal N treatment (Fig. 7). Previous research in rice reported the expression of *OsAMT1.1* and *OsAMT2.1*, orthologs of wheat *TaAMT1.1* and *TaAMT2.1,* in root and leaf tissue, with rapid upregulated in N starvation, while *OsAMT1.1* in the roots was downregulated upon N re-supply with nitrate (Su-Mei *et al.* 2012). In the current study, *TaAMT1.1* had relatively high expression under LN basal treatment compared to control with a 4.5-fold change at 30 min after ammonium and nitrate treatment. The two N forms may be triggering upregulation of *TaAMT1.1* for more foliar N uptake early into the treatment (30 min) in LN basal treatment, and later (2 h) downregulation of the gene which could be as a result of excess N already taken up as foliar. The expression of *AMTs* is regulated by N availability, N-type, or plant N levels (Su-Mei *et al.* 2012). Overall, the *AMTs* and *NRTs* belong to a broad family of genes with diverse functions (Williams and Miller 2001). The relatively weak relationship between foliar N uptake and gene expression responses could, therefore, be explained by potential functional redundancies.

In conclusion, these results provide new insight into the interplay of various physiological and molecular traits that may determine the effectiveness of foliar N uptake in different wheat genotypes. Leaf surface structures including wax quantity, shapes and composition at different growth stages could facilitate the adherence of N fertiliser solutions to the leaf surface and the entry rate. These morphological features differ between wheat genotypes and could result in differing success rates of foliar N uptake. At a molecular level, N transporter genes are regulated differently, and this regulation depends on the amount applied as basal N, the form of N (urea, ammonium, and nitrate) applied as foliar and plant N status. Future research on the characterisation of the nitrogen status of the leaf or plant (%N of leaves before application) by Nitrogen

Nutrition Index would potentially help determine if the response to foliar N application is related to the N status (Justes *et al.* 1994).

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References

Adamski, N. M., M. S. Bush, J. Simmonds, A. S. Turner, S. G. Mugford, A. Jones, K. Findlay, N. Pedentchouk, P. von Wettstein-Knowles and C. Uauy (2013). "The I nhibitor of wax 1 locus (I w1) prevents formation of β- and OH-β-diketones in wheat cuticular waxes and maps to a sub-c M interval on chromosome arm 2 BS." The Plant Journal 74(6): 989-1002.

Aslam, M., R. L. Travis and R. C. Huffaker (1992). "Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley (Hordeum vulgare L.) seedlings." Plant Physiology 99(3): 1124-1133.

Barthlott, W., C. Neinhuis, D. Cutler, F. Ditsch, I. Meusel, I. Theisen and H. Wilhelmi (1998). "Classification and terminology of plant epicuticular waxes." Botanical journal of the Linnean society 126(3): 237-260.

Bi, H., N. Kovalchuk, P. Langridge, P. J. Tricker, S. Lopato and N. Borisjuk (2017). "The impact of drought on wheat leaf cuticle properties." BMC Plant Biology 17(1): 85.

Bi, H., S. Luang, Y. Li, N. Bazanova, S. Morran, Z. Song, M. A. Perera, M. Hrmova, N. Borisjuk and S. Lopato (2016). "Identification and characterization of wheat drought-responsive MYB transcription factors involved in the regulation of cuticle biosynthesis." Journal of experimental botany 67(18): 5363-5380.

Blandino, M., P. Vaccino and A. Reyneri (2015). "Late-Season Nitrogen Increases Improver Common and Durum Wheat Quality." Agronomy Journal 107(2): 680-690.

Bogard, M., V. Allard, M. Brancourt-Hulmel, E. Heumez, J. M. Machet, M. H. Jeuffroy, P. Gate, P. Martre and J. Le Gouis (2010). "Deviation from the grain protein concentration-grain yield negative relationship is highly correlated to post-anthesis N uptake in winter wheat." J Exp Bot 61(15): 4303-4312.

Buchner, P. and M. J. Hawkesford (2014). "Complex phylogeny and gene expression patterns of members of the NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family (NPF) in wheat." Journal of Experimental Botany 65(19): 5697-5710.

Cha, S., Z. Song, B. J. Nikolau and E. S. Yeung (2009). "Direct profiling and imaging of epicuticular waxes on Arabidopsis thaliana by laser desorption/ionization mass spectrometry using silver colloid as a matrix." Analytical chemistry 81(8): 2991-3000.

Crawford, N. M. (1995). "Nitrate: nutrient and signal for plant growth." The plant cell 7(7): 859.

Crawford, N. M. and A. D. Glass (1998). "Molecular and physiological aspects of nitrate uptake in plants." Trends in plant science 3(10): 389-395.

Dampney, P. and S. Salmon (1990). "The effect of rate and timing of late nitrogen application to breadmaking wheats as ammonium nitrate or foliar urea-N, and the effect of foliar sulphur application. I. Effect on yield, grain quality and recovery of nitrogen in grain." Aspects of Applied Biology(25): 229-241.

De Valença, A., A. Bake, I. Brouwer and K. Giller (2017). "Agronomic biofortification of crops to fight hidden hunger in sub-Saharan Africa." Global food security 12: 8-14.

Dick, C. D., N. M. Thompson, F. M. Epplin and D. B. Arnall (2016). "Managing Late-Season Foliar Nitrogen Fertilization to Increase Grain Protein for Winter Wheat." Agronomy Journal 108(6): 2329-2338.

Dobermann, A. and K. G. Cassman (2005). "Cereal area and nitrogen use efficiency are drivers of future nitrogen fertilizer consumption." Science in China Series C: Life Sciences 48(2): 745-758.

Domínguez, E., J. A. Heredia-Guerrero and A. Heredia (2011). "The biophysical design of plant cuticles: an overview." New phytologist 189(4): 938-949.

Eichert, T. and V. Fernández (2012). "Uptake and release of elements by leaves and other aerial plant parts." Marschner's Mineral Nutrition of Higher Plants 3: 71-84.

Fernández, V. and P. H. Brown (2013). "From plant surface to plant metabolism: the uncertain fate of foliar-applied nutrients." Frontiers in plant science 4: 289.

Fernández, V., T. Sotiropoulos and P. H. Brown (2013). "Foliar fertilisation: Principles and practices." International Fertiliser Industry Association (IFA): Paris, France: 140.

Forde, B. G. (2000). "Nitrate transporters in plants: structure, function and regulation." Biochimica et Biophysica Acta (BBA)-Biomembranes 1465(1): 219-235.

Franke, W. (1967). "Mechanisms of foliar penetration of solutions." Annual Review of Plant Physiology 18(1): 281-300.

Gaur, V. S., U. Singh, A. K. Gupta and A. Kumar (2012). "Influence of different nitrogen inputs on the members of ammonium transporter and glutamine synthetase genes in two rice genotypes having differential responsiveness to nitrogen." Molecular biology reports 39(8): 8035-8044.

Gerendás, J., Z. Zhu and B. Sattelmacher (1998). "Influence of N and Ni supply on nitrogen metabolism and urease activity in rice (Oryza sativa L.)." Journal of Experimental Botany 49(326): 1545-1554.

Gooding, M. J. and W. P. Davies (1992). "Foliar urea fertilization of cereals: A review." Fertilizer Research 32(2): 209-222.

Guo, F.-Q., J. Young and N. M. Crawford (2003). "The Nitrate Transporter AtNRT1.1 (CHL1) Functions in Stomatal Opening and Contributes to Drought Susceptibility in Arabidopsis." The Plant Cell 15(1): 107-117.

Guo, T., H. Xuan, Y. Yang, L. Wang, L. Wei, Y. Wang and G. Kang (2014). "Transcription Analysis of Genes Encoding the Wheat Root Transporter NRT1 and NRT2 Families During Nitrogen Starvation." Journal of Plant Growth Regulation 33(4): 837-848.

He, F., A. A. Karve, S. Maslov and B. A. Babst (2016). "Large-Scale Public Transcriptomic Data Mining Reveals a Tight Connection between the Transport of Nitrogen and Other Transport Processes in Arabidopsis." Frontiers in Plant Science 7(1207).

Javelle, M., V. Vernoud, P. M. Rogowsky and G. C. Ingram (2011). "Epidermis: the formation and functions of a fundamental plant tissue." New Phytologist 189(1): 17-39.

Kiba, T., A.-B. Feria-Bourrellier, F. Lafouge, L. Lezhneva, S. Boutet-Mercey, M. Orsel, V. Bréhaut, A. Miller, F. Daniel-Vedele, H. Sakakibara and A. Krapp (2012). "The Arabidopsis Nitrate Transporter NRT2.4 Plays a Double Role in Roots and Shoots of Nitrogen-Starved Plants." The Plant Cell 24(1): 245-258.

Klein, I. and S. A. Weinbaum (1985). "Foliar application of urea to almond and olive: leaf retention and kinetics of uptake." Journal of plant nutrition 8(2): 117-129.

Koch, K., W. Barthlott, S. Koch, A. Hommes, K. Wandelt, W. Mamdouh, S. De-Feyter and P. Broekmann (2006). "Structural analysis of wheat wax (Triticum aestivum, cv.'Naturastar'L.): from the molecular level to three dimensional crystals." Planta 223(2): 258-270.

Koch, K. and H.-J. Ensikat (2008). "The hydrophobic coatings of plant surfaces: Epicuticular wax crystals and their morphologies, crystallinity and molecular self-assembly." Micron 39(7): 759-772.

Kojima, S., A. Bohner, B. Gassert, L. Yuan and N. von Wiren (2007). "AtDUR3 represents the major transporter for high-affinity urea transport across the plasma membrane of nitrogen-deficient Arabidopsis roots." Plant J 52(1): 30-40.

Kunst, L. and L. Samuels (2009). "Plant cuticles shine: advances in wax biosynthesis and export." Current opinion in plant biology 12(6): 721-727.

Lezhneva, L., T. Kiba, A. B. Feria-Bourrellier, F. Lafouge, S. Boutet-Mercey, P. Zoufan, H. Sakakibara, F. Daniel-Vedele and A. Krapp (2014). "The Arabidopsis nitrate transporter NRT2. 5 plays a role in nitrate acquisition and remobilization in nitrogen-starved plants." The Plant Journal 80(2): 230-241.

Li, B.-Z., M. M, S.-m. Li, H.-y. Li, S.-w. Zhu, W.-m. Shi and Y.-h. Su (2009). "Molecular Basis and Regulation of Ammonium Transporter in Rice." Rice Science 16(4): 314-322.

Li, S.-M., B.-Z. Li and W.-M. Shi (2012). "Expression Patterns of Nine Ammonium Transporters in Rice in Response to N Status." Pedosphere 22(6): 860-869.

Liu, L.-H., U. Ludewig, W. B. Frommer and N. von Wirén (2003). "AtDUR3 encodes a new type of high-affinity urea/H+ symporter in Arabidopsis." The Plant Cell 15(3): 790-800.

Melino, V. J., G. Fiene, A. Enju, J. Cai, P. Buchner and S. Heuer (2015). "Genetic diversity for root plasticity and nitrogen uptake in wheat seedlings." Functional Plant Biology 42(10): 942-956.

Merigout, P., M. Lelandais, F. Bitton, J. P. Renou, X. Briand, C. Meyer and F. Daniel-Vedele (2008). "Physiological and transcriptomic aspects of urea uptake and assimilation in Arabidopsis plants." Plant Physiol 147(3): 1225-1238.

Miller, A. J., X. Fan, M. Orsel, S. J. Smith and D. M. Wells (2007). "Nitrate transport and signalling." Journal of experimental Botany 58(9): 2297-2306.

Orsel, M., A. Krapp and F. Daniel-Vedele (2002). "Analysis of the NRT2 Nitrate Transporter Family in Arabidopsis. Structure and Gene Expression." Plant Physiology 129(2): 886-896.

Palta, J., I. Fillery, E. Mathews and N. Turner (1991). "Leaf feeding of [15N] urea for labelling wheat with nitrogen." Functional Plant Biology 18(6): 627-636.

Pao, S. S., I. T. Paulsen and M. H. Saier (1998). "Major facilitator superfamily." Microbiology and Molecular Biology Reviews 62(1): 1-34.

Peirce, C., T. McBeath, V. Fernández and M. McLaughlin (2014). "Wheat leaf properties affecting the absorption and subsequent translocation of foliar-applied phosphoric acid fertiliser." Plant and soil 384(1-2): 37-51.

Peirson, S. N., J. N. Butler and R. G. Foster (2003). "Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis." Nucleic acids research 31(14): e73-e73.

Raj, G. and J. Allan, Cessna (1991). Environmental Chemistry of Herbicides. Unites States.

Ramakers, C., J. M. Ruijter, R. H. L. Deprez and A. F. M. Moorman (2003). "Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data." Neuroscience Letters 339(1): 62-66.

Samuels, L., L. Kunst and R. Jetter (2008). "Sealing plant surfaces: cuticular wax formation by epidermal cells." Annu Rev Plant Biol 59: 683-707.

Schönherr, J. (2000). "Calcium chloride penetrates plant cuticles via aqueous pores." Planta 212(1): 112-118.

Schönherr, J. (2006). "Characterization of aqueous pores in plant cuticles and permeation of ionic solutes." Journal of Experimental Botany 57(11): 2471-2491.

Søgaard, R., M. Alsterfjord, N. MacAulay and T. Zeuthen (2009). "Ammonium ion transport by the AMT/Rh homolog TaAMT1;1 is stimulated by acidic pH." Pflügers Archiv - European Journal of Physiology 458(4): 733-743.

Strong, W. (1982). "Effect of late application of nitrogen on the yield and protein content of wheat." Australian Journal of Experimental Agriculture 22(115): 54-61.

Su-Mei, L., L. Bao-Zhen and S. Wei-Ming (2012). "Expression patterns of nine ammonium transporters in rice in response to N status." Pedosphere 22(6): 860-869.

Suchecki, R., N. S. Watson-Haigh and U. Baumann (2017). "POTAGE: A Visualisation Tool for Speeding up Gene Discovery in Wheat." Scientific Reports 7(1): 14315.

Tulloch, A. P. (1973). "Composition of leaf surface waxes of Triticum species: Variation with age and tissue." Phytochemistry 12(9): 2225-2232.

Wang, W.-H., B. Köhler, F.-Q. Cao and L.-H. Liu (2008). "Molecular and physiological aspects of urea transport in higher plants." Plant Science 175(4): 467-477.

Wang, W. H., B. Kohler, F. Q. Cao, G. W. Liu, Y. Y. Gong, S. Sheng, Q. C. Song, X. Y. Cheng, T. Garnett, M. Okamoto, R. Qin, B. Mueller-Roeber, M. Tester and L. H. Liu (2012). "Rice DUR3 mediates high-affinity urea transport and plays an effective role in improvement of urea acquisition and utilization when expressed in Arabidopsis." New Phytol 193(2): 432-444.

Wang, Y., J. Wang, G. Chai, C. Li, Y. Hu, X. Chen and Z. Wang (2015). "Developmental Changes in Composition and Morphology of Cuticular Waxes on Leaves and Spikes of Glossy and Glaucous Wheat (Triticum aestivum L.)." PLOS ONE 10(10): e0141239.

Wang, Y., M. Wang, Y. Sun, Y. Wang, T. Li, G. Chai, W. Jiang, L. Shan, C. Li and E. Xiao (2014). "FAR5, a fatty acyl-coenzyme A reductase, is involved in primary alcohol biosynthesis of the leaf blade cuticular wax in wheat (Triticum aestivum L.)." Journal of Experimental Botany 66(5): 1165-1178.

Westfall, D. and T. Bauder (2011). "Zinc and iron deficiencies." Fact sheet (Colorado State University Extension) Crop series; no 0545.

Williams, L. and A. Miller (2001). "Transporters Responsible for the Uptake and Partitioning of Nitrogenous Solutes." Annu Rev Plant Physiol Plant Mol Biol 52(1): 659-688.

Wylie, P., A. Mayfield, F. Harrop and A. Roe (2003). The potential of fluid fertilisers for broadacre cropping in Australia HOR 00002 Australia, Grains Research and Development Corporation.

Yamaya, T. and A. Oaks (2004). Metabolic Regulation of Ammonium uptake and Assimilation. Nitrogen Acquisition and Assimilation in Higher Plants. S. Amâncio and I. Stulen. Dordrecht, Springer Netherlands: 35-63.

Zanin, L., N. Tomasi, C. Wirdnam, S. Meier, N. Y. Komarova, T. Mimmo, S. Cesco, D. Rentsch and R. Pinton (2014). "Isolation and functional characterization of a high affinity urea transporter from roots of Zea mays." BMC Plant Biol 14(1): 222.

Zhang, Z., W. Wang and W. Li (2013). "Genetic interactions underlying the biosynthesis and inhibition of β-diketones in wheat and their impact on glaucousness and cuticle permeability." PLoS One 8(1): e54129.

Supplementary Material

Supplementary Table 1 Effects of foliar N on grain weight and grain N concentration after application on two growth stages; stem elongation (SE) and 7 DPA. A Tukey's multiple comparisons test was carried out to identify significant differences between the treatments in four genotypes Spitfire, Gregory, Kukri and RAC875.

Grain weight Stem Elongation		
Tukey's multiple comparisons test	Significance	P-Value
Rac875:SpreadWet vs. Kukri:Foliar	**	0.0011
Rac875:SpreadWet vs. Gregory:SpreadWet	*	0.0409
Rac875:SpreadWet vs. Gregory:Foliar	****	<0.0001
Rac875:SpreadWet vs. Spitfire:SpreadWet	****	<0.0001
Rac875:SpreadWet vs. Spitfire:Foliar	**	0.0015
Rac875:Foliar vs. Gregory:Foliar	*	0.015
Rac875:Foliar vs. Spitfire:SpreadWet	***	0.0001
Kukri:SpreadWet vs. Gregory:Foliar	*	0.0459
Kukri:SpreadWet vs. Spitfire:SpreadWet	***	0.0003
Gregory:SpreadWet vs. Spitfire:SpreadWet	**	0.0016
Spitfire:SpreadWet vs. Spitfire:Foliar	*	0.0439
Grain N Concentration_Stem Elongation		
Rac875:SpreadWet vs. Rac875:Foliar	*	0.0261
Rac875:SpreadWet vs. Kukri:SpreadWet	****	<0.0001
Rac875:SpreadWet vs. Kukri:Foliar	**	0.0049
Rac875:SpreadWet vs. Gregory:SpreadWet	***	0.0001
Rac875:SpreadWet vs. Gregory:Foliar_se	***	0.0009
Rac875:SpreadWet vs. Spitfire:SpreadWet_se	**	0.0082
Grain weight_7 DPA		
Rac875:SpreadWet vs. Spitfire:Foliar	**	0.0068
Grain N concentration_7 DPA		
Rac875:Foliar vs. Gregory:SpreadWet	****	<0.0001
Kukri:SpreadWet vs. Kukri:Foliar	*	0.0202
Kukri:SpreadWet vs. Gregory:SpreadWet	***	0.0003
Kukri:Foliar vs. Gregory:SpreadWet	****	<0.0001
Kukri:Foliar vs. Gregory:Foliar	**	0.0032
Kukri:Foliar vs. Spitfire:SpreadWet	**	0.0044
Gregory:SpreadWet vs. Gregory:Foliar	***	0.0004
Gregory:SpreadWet vs. Spitfire:SpreadWet	***	0.0003
Gregory:SpreadWet vs. Spitfire:Foliar	****	<0.0001

Supplementary Table 2: Leaf surface wax chemical composition at two growth stages, stem elongation (SE) and 7 days post-anthesis (7 DPA), for four bread wheat genotypes. The main chemical components were alcohols, fatty acids, aldehydes, alkanes, and diketones, all in µg per gram fresh weight. Errors indicate the SEM of five replicates.

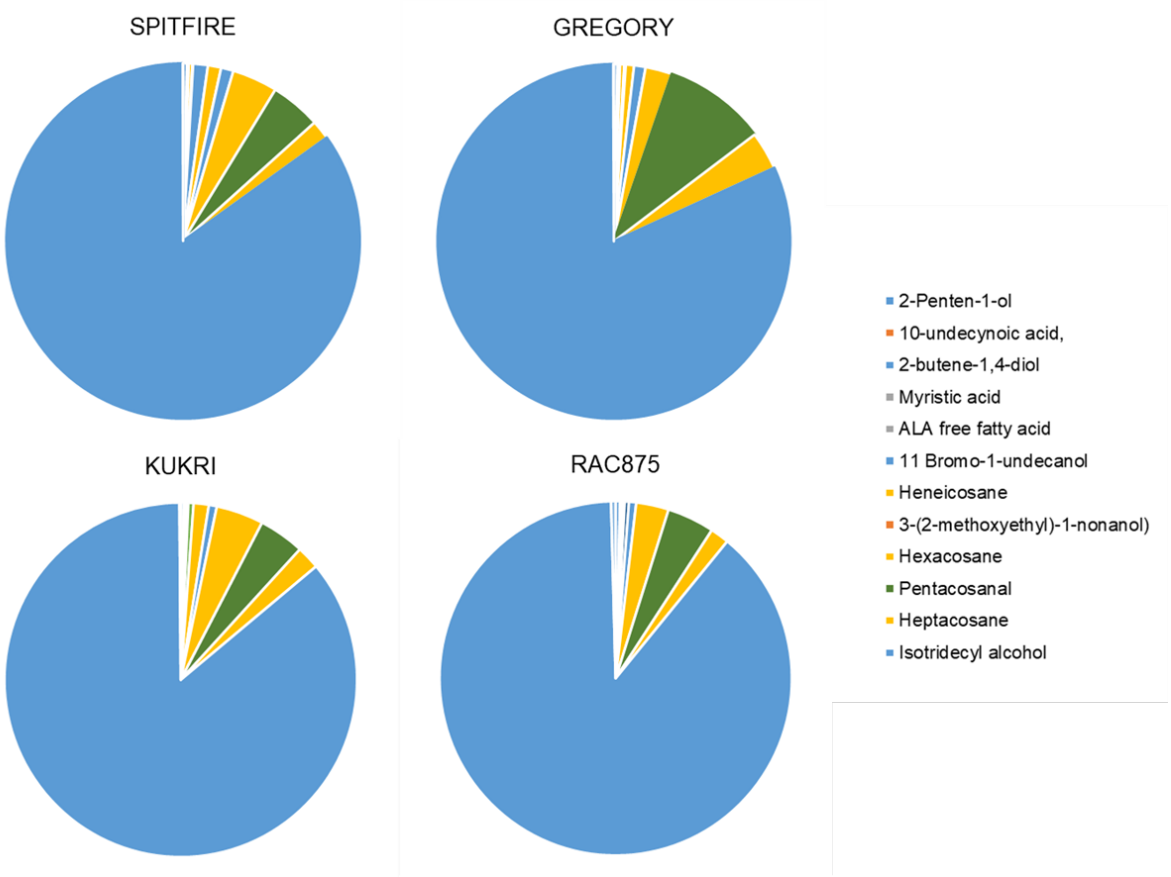
Genotype growth stage	*Primary alcohols*	*Secondary alcohols*	*Fatty acids*	*Alkanes*	*Aldehydes*	*Diketones*
Spitfire_SE	3179±467.8	41±7.1	17±3.5	260±36.7	169±25.0	1±0.3
Spitfire_7DPA	1946±130.9	138±11.7	48±5.0	1446±194.2	411±30.1	61±19.5
Gregory_SE	4703±867.2	60±13.9	26±5.8	379±103.2	536±99.8	3±1.0
Gregory_7DPA	911±255.3	32±14.0	55±11.8	2736±1479	461±100.2	778±382.0
Kukri_SE	2272±715.8	19±1.1	9±0.9	205±26.9	109±5.0	2±1.2
Kukri_7DPA	1533±320.8	53±12.7	29±10.8	1650±186.9	222±41.2	38±9.9
RAC875_SE	3664±515.2	27±3.9	10±1.2	209±24.3	178±42.6	17±5.8
RAC875_7DPA	1434±217.9	38±9.7	49±10.6	986±213.7	173±36.2	1100±148.4

Supplementary Table 3: A list of genes that were found from literature to have an expression in leaf tissues in Arabidopsis and rice.

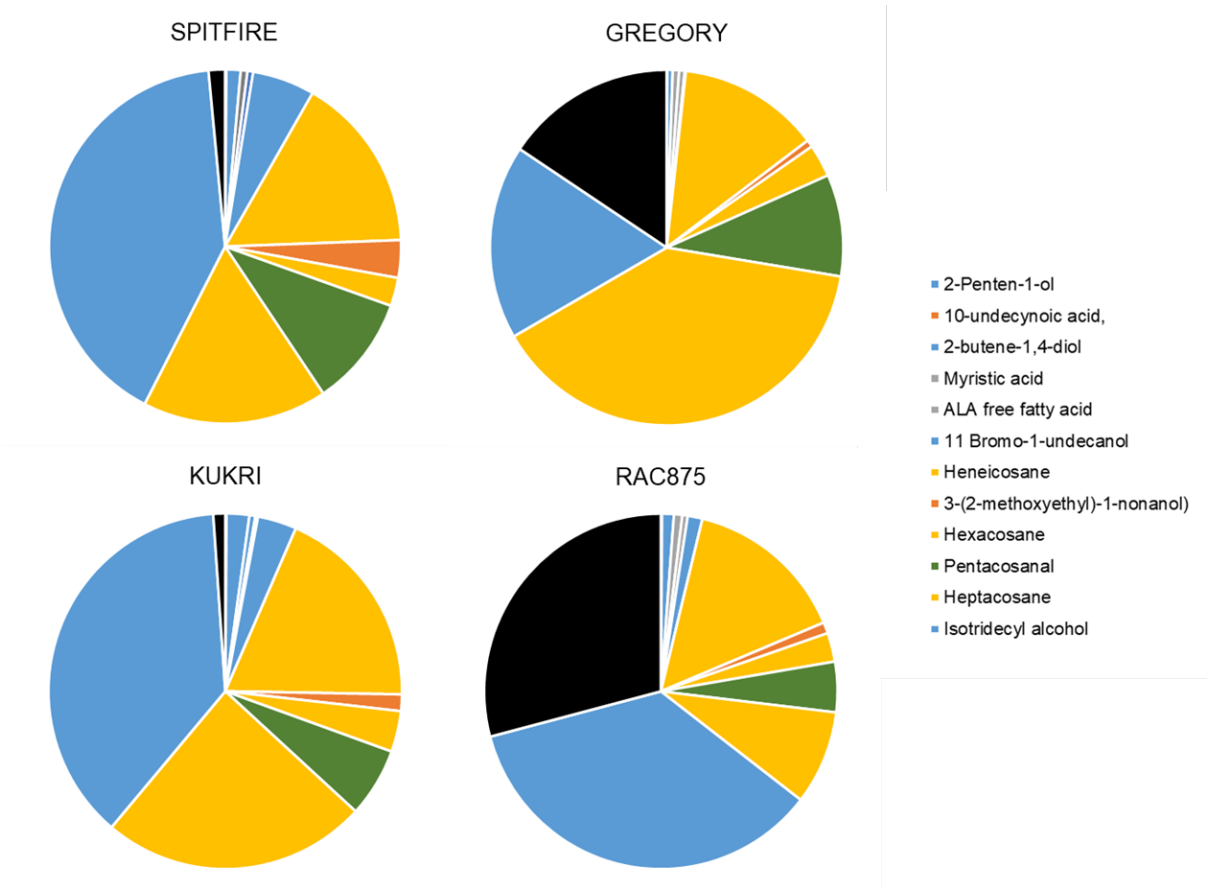
Gene	Tissue expression POTAGE (Wheat)	Tissue expression LITERATURE	Plant	Literature
TaNRT1.1	Root and leaf	Roots, leaf, and guard cell	Arabidopsis	(Guo *et al.* 2003)
TaNRT2.4	Grain	Roots and phloem parenchyma (leaves	Arabidopsis	(Kiba *et al.* 2012)
TaNRT2.5	Root, leaf, and grain	Roots and mature leaves	Arabidopsis	(Lezhneva *et al.* 2014)
TaAMT1.1	Root and leaf	Roots and shoots	Rice	(Li *et al.* 2009)
TaAMT2.1	Root and leaf	Roots and shoots	Rice	
TaAMT2.2	Root, leaf, and spike	Root and leaf	Rice	(Su-Mei *et al.* 2012)
TaDUR3	Roots and leaf	Root and leaf	Maize	*(Zanin et al. 2014)*

Supplementary Table 4: Primer list with primers used for the qPCR experiment. Primers not designed were (TaEFα and TaGADPH) sourced from common laboratory stock and (Melino et al. 2015)

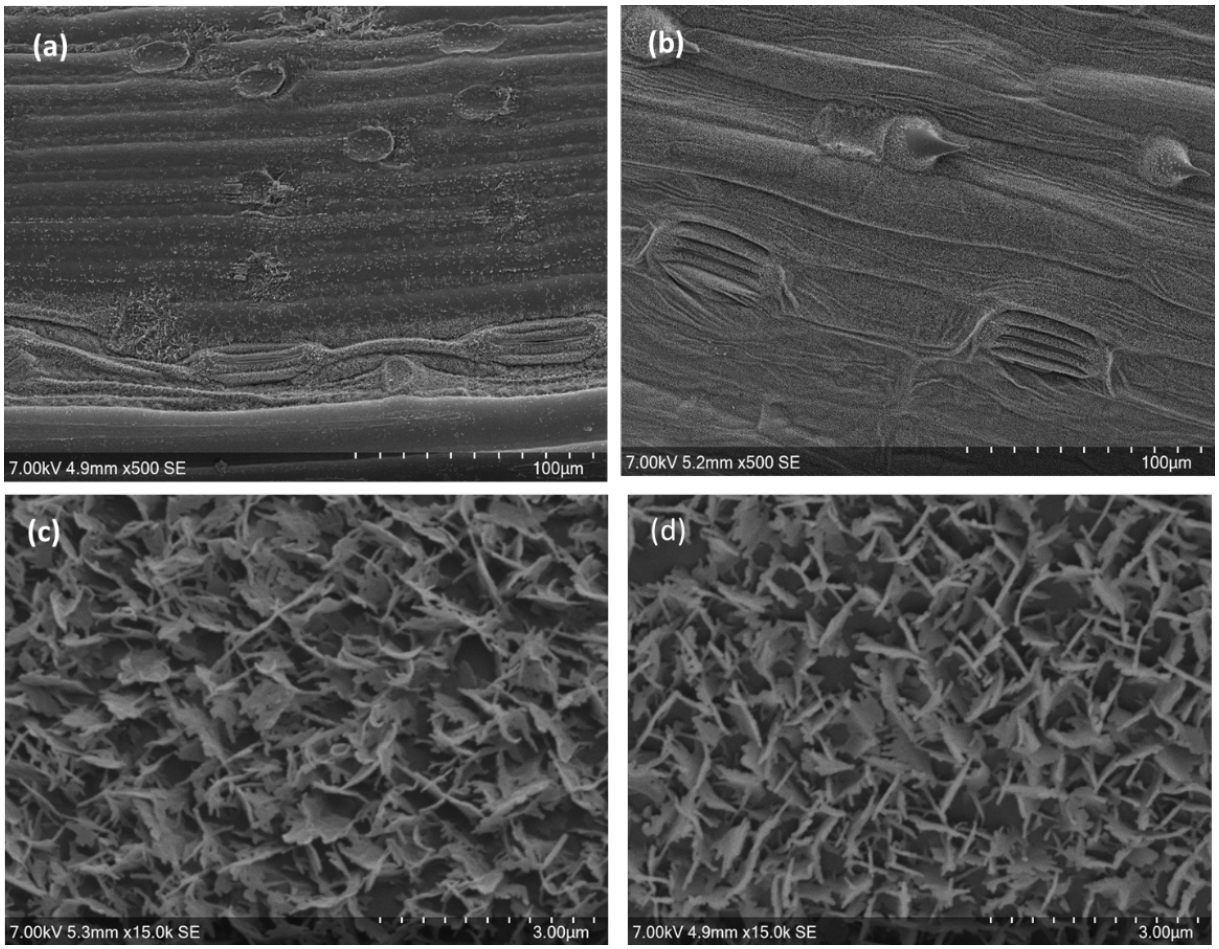
Genes	Forward and reverse primers
TaNRT1.1	Forward: CACGGGAGCAACGACGGCTG Reverse: ATGCGTTTCTCCTTGTACACGTAG
TaDUR3	Forward: ACCTCCAGCAGCAGCCTCGGC Reverse: GAGGCCGCCGGAGCTGAGCAT
TaAMT2.1	Forward: AACTGCAGAGTGTGAGAGCC Reverse: TGCAGCATGTTCGTGCCGGT
TaAMT1.1	Forward: CATCATGCTCACCAACGTGC Reverse: TTGAGGCCGAAGAAGTGCTT
TaEFα	Forward: CAGATTGGCAACGGCTACG Reverse: CGGACAGCAAAACGACCAAG
TaGADPH	Forward: TTTTCACCGACAAGGACA Reverse: AAGAGGAGCAAGGCAGTT



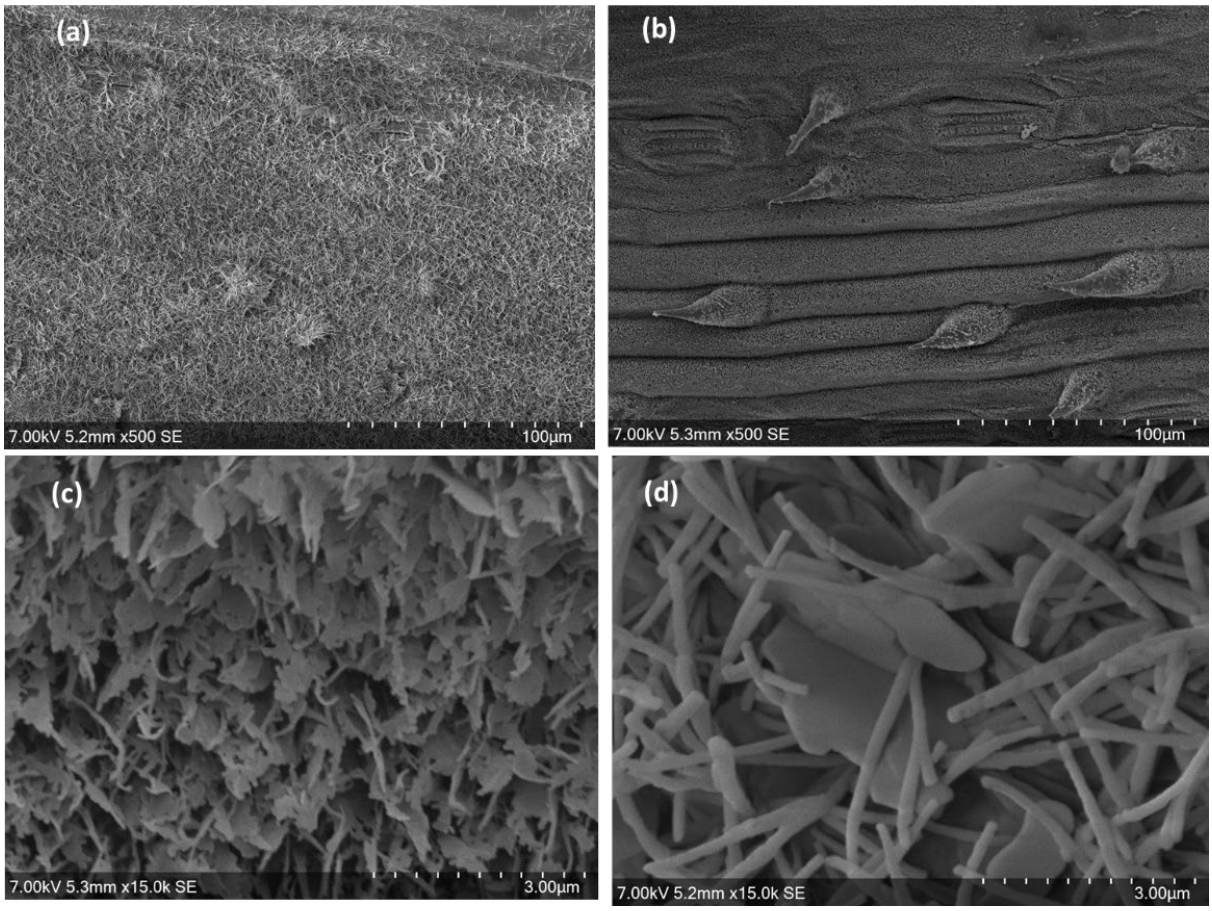
Supplementary Figure 1: The wax chemical composition of leaf sample collected stem elongation, in four genotypes, Spitfire, Gregory, Kukri and RAC875. The chemical components are primary alcohols (2-Penten-1-ol, 2-butene-1,4-diol, 11 Bromo-1-undecanol, and Isotridecyl alcohol), secondary alcohols (3-(2-methoxyethyl)-1-nonanol)), fatty acids (10-Undecynoic acid, Myristic acid, and ALA free fatty acid), aldehydes (Pentacosanal), alkanes (Heneicosane, Hexacosane and Heptacosane), and diketones (Tricosane-2,4-dione



Supplementary Figure 2: The wax chemical composition of the flag leaf collected at 7 DPA, in four genotypes, Spitfire, Gregory, Kukri and RAC875. The chemical components are primary alcohols (2-Penten-1-ol, 2-butene-1,4-diol, 11 Bromo-1-undecanol, and Isotridecyl alcohol), secondary alcohols (3-(2-methoxyethyl)-1-nonanol)), fatty acids (10-Undecynoic acid, Myristic acid, and ALA free fatty acid), aldehydes (Pentacosanal), alkanes (Heneicosane, Hexacosane and Heptacosane), and diketones (Tricosane-2,4-dione).

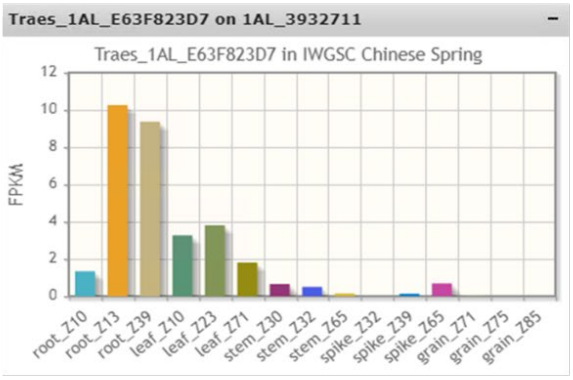


Supplementary Figure 3: Kukri leaf morphological structures on the adaxial and abaxial sides of the flag leaf at 7 DPA (a) smooth shiny adaxial leaf surface, (b), abaxial side with short thick trichomes. (c) and (d) platelet wax shapes on the adaxial and abaxial leaf side. ×500 magnification for image (a) and (b) and ×15.0k magnification for image (c) and (d).

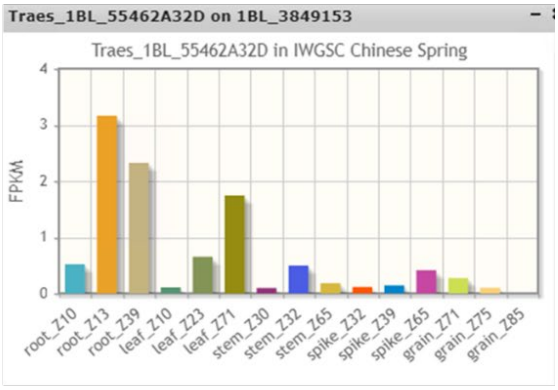


Supplementary Figure 4: RAC875 leaf morphological structures on the adaxial and abaxial sides at 7DPA of the flag leaf (a) rough and hairy-like adaxial leaf surface with no visible trichomes, (b)abaxial side with short, bent tips, and thick-bottomed trichomes. (c), dense platelet wax shapes on the adaxial leaf side and (d), platelet wax shapes and numerous long tubular shapes on the abaxial leaf side. ×500 magnification for image a-b and ×15.0k magnification for image c-d.

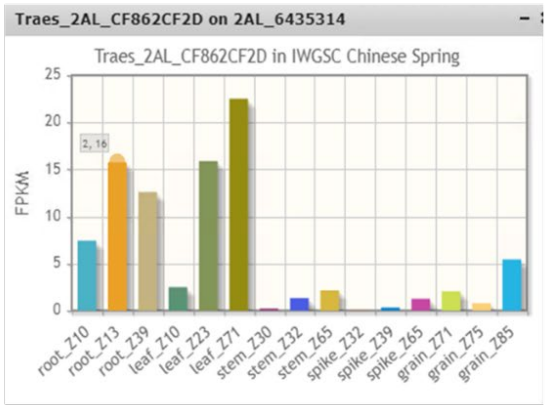
TaNRT1.1



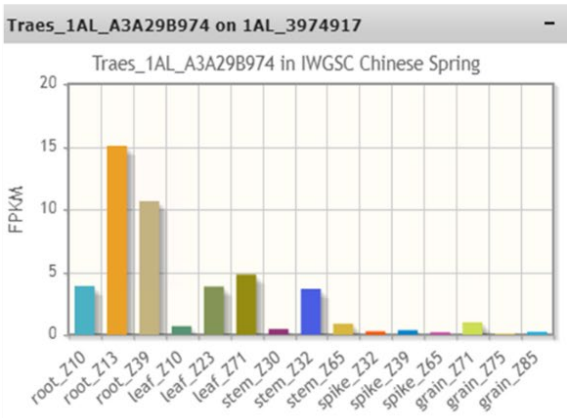
TaDUR3



TaAMT1.1

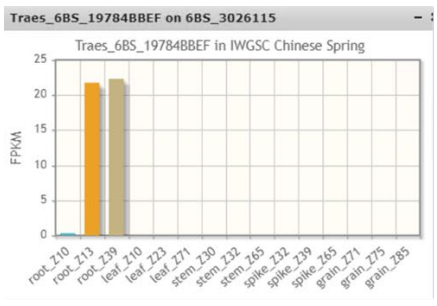


TaAMT2.1

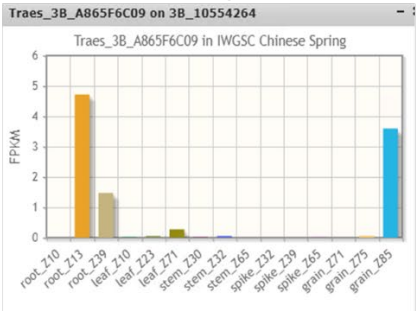


Supplementary Figure 5: POTAGE output showing gene expression of four selected N transporter genes in roots, leaf, stem, and grain. Only these genes expressed in the leaves were selected for qPCR analysis.

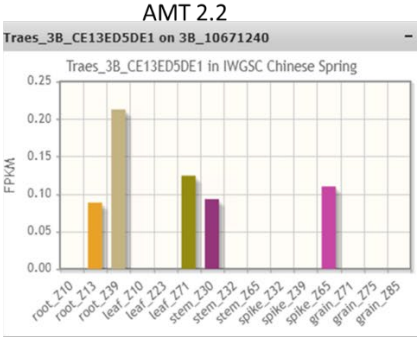
NRT 2.4



NRT 2.5



AMT 2.2



Supplementary Figure 6: POTAGE output showing gene expression of three selected N transporter genes in roots, leaf, stem, and grain. These genes were initially selected from literature but had a low expression or were not expressed in the leaves hence they were not selected for qPCR analysis.

Chapter 4

Foliar uptake of different forms of applied nitrogen, and feedback effects on root nitrogen uptake in wheat

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Statement of Authorship

Statement of Authorship

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Contribution to the Paper	Carried out the study. This included conducting the experiments, data collection, data analysis and manuscript preparation		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	25/05/20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Commercial nitrogen (N) fertilisers generally contain one or a combination of the three N forms: nitrate, ammonium, and urea. Nitrate and ammonium are the main forms of N taken up by plant roots, while the preferred form taken-up for foliar-applied fertilisers remains unknown. This study aimed to investigate the time taken for foliar N to accumulate in wheat plants and identify the N forms preferentially taken up by leaf tissues. Wheat seedlings were treated with foliar application of 15N-labeled urea, ammonium, and nitrate, and harvested at different time points. The plant 15N accumulation and % of applied foliar N translocated into the seedlings was quantified. 15N stable isotope was applied in single or combined forms to the flag leaf of wheat plants 7 days post-anthesis (7 DPA). Plant tissue accumulation of the foliar N was quantified in the grain and other plant parts. 15N accumulation was highest in the wheat seedlings at 2 h post-treatment, with a 17.7 – 26.9% foliar N translocation. In terms of preference in the uptake of different N forms, Urea and urea ammonium nitrate (UAN) had high foliar 15N accumulation in the grain. The proportion of foliar N applied that ended up in the grain in all the treatments ranged from 3 – 35% across all the experiments. The effects of foliar N on root nitrate uptake and accumulation were also investigated, by performing 15N flux and gene expression experiments in combination, following foliar N application. There was a regulatory effect of the foliar N pre-treatment on root nitrate influx, as the pre-treated plants had lower root nitrate accumulation Also, foliar pre-treatment altered the gene expression of two root nitrate transporter genes, TaNRT1.1 and TaNRT2.2. This study demonstrated effective entry of N through wheat leaves and showed a regulatory effect of leaf N application on root nitrate uptake.

Introduction

The use of nitrogen (N) fertilisers is crucial to the success of the agricultural industry. N fertilisers supply plants with nutrients that are necessary for growth and translate to high yields and high-quality produce. It is predicted that the demand for N fertilisers will reach 155 MT globally by 2023 (IFA 2019). In 2017, N fertilisers commonly used in agriculture were urea (79 MT), di-ammonium phosphate (DAP) (17 Mt), and urea ammonium nitrate (UAN) solutions (15 MT) (FAO 2017). In Australia, the most common N fertilisers used are urea and ammonium phosphate (ABS 2018). These fertilisers are normally applied to the soil in granular form and are taken up by the plant through the roots as different species of N (nitrate, ammonium, or urea). Nitrate and ammonium are the predominant N forms in the soil that are taken up by plants (Jackson and Volk 1992). Urea is normally only available in very low concentrations in soil (Wang *et al.* 2012, Zanin *et al.* 2014). Low soil concentrations of urea are a consequence of the ubiquitous existence of a nickel-activated urea-hydrolyzing enzyme, urease that breaks down urea to ammonium (Wang *et al.* 2012, Watson *et al.* 1994).

Unfortunately, most plants including cereals have a poor root N uptake efficiency, especially later in the growing season, translating to a low N use efficiency with approximately 33% of N that is taken up from the soil ending up in the grain (Hirel *et al.* 2007, Raun and Johnson 1999, Sylvester-Bradley and Kindred 2009). The calculated N use efficiency for wheat ranges from 14 -59% (López-Bellido *et al.* 2005, Melaj *et al.* 2003). The unused N is a financial cost to farmers and can cause devastating effects on the environment through nitrate leaching, eutrophication and air pollution from ammonia volatilization and nitric oxide gas (Giles 2005, Tilman 1999). With the projected N fertiliser use increasing to feed a rapidly growing population, it is necessary to find alternative N management strategies to improve N uptake and

minimize wastage. A split application of N, involving an initial basal soil N application followed by a complimentary top-up using foliar application of liquid N fertilisers, could be effective in availing N only when needed by the plant (Ercoli *et al.* 2013). This way, farmers could maintain high-yielding crops with high quality while minimizing wastage (Rossmann *et al.* 2019).

Foliar N fertilization may offer a more efficient uptake option where there is inefficient root uptake in dry conditions during late growth stages in wheat (Bly and Woodard 2003, Bogard *et al.* 2010, Ellen and Spiertz 1980). Since liquid N fertilisers are used in foliar applications, it is important to identify the proper product formulation for efficient foliar uptake. Previous research concluded that liquid fertilisers applied as foliar sprays can cause leaf damage and increase susceptibility to diseases, thus decreasing total yields. For example, Wylie *et al.* reported a case where foliar urea ammonium nitrate (UAN) increased susceptibility to yellow spot disease and also attracted armyworms (Wylie *et al.* 2003).

Overall, there is little information on the time taken for different N forms to traverse the leaf cuticle barrier into plant cells, and which, if any, of the three N forms may be preferentially taken up and accumulated in the plant. The leaf cuticle has hydrophobic properties due to the presence of long-chain fatty acids and soluble waxes made from hydrophobic compounds (Pollard et al. 2008). This hydrophobicity makes it possible for non-ionic molecules such as urea to penetrate than for charged ionic species. Cuticular hydrophobicity is thought to make ionized nutrient diffusion difficult (Fernández *et al.* 2013). However, previous research indicates ionic molecules like ammonium and nitrate may enter the plants through aqueous pores, which arise from the hydration of membrane-bound polar functional groups (Schönherr 2000, Liu et al. 2015). UAN is a commonly used foliar fertiliser (FAO 2017) and contains urea,

ammonium, and nitrate in unequal proportions. In Australia, commercially available UAN formulation includes EasyN® that has 42.5% N w/v (21.5% urea, 10.5% ammonium and 10.5 % nitrate) (Incitec Pivot Fertilisers) and N42® containing 42% N w/v (21. 5% urea, 10.25% ammonium and 10.25 % nitrate) (Yara Australia Pty. Ltd.).

Most studies of N uptake by plants have focused on roots, looking specifically at the N forms preferentially taken up by roots, and N transporters involved in the uptake of the different N forms, and the transporter uptake kinetics (Crawford and Glass 1998, Gazzarrini *et al.* 1999, Griffiths and York 2020, Miller *et al.* 2007, Plett *et al.* 2010, Yin *et al.* 2007, Zanin *et al.* 2014). The major N transporters in wheat are *TaNRT1.1, TaNRT2.2* and *TaNRT2.2* (Guo *et al.* 2014, Wang *et al.* 2011, Yin *et al.* 2007). Also, previous studies have identified morphological root traits for efficient N uptake (Melino *et al.* 2015). Research on N uptake through the leaf is limited and could be vital for the proper selection and effective application of foliar N fertilisers with minimal N losses. This study aimed to investigate the time taken for foliar N entry and accumulation into wheat plants and the N form preferentially taken up through the leaf. Root N uptake is highly regulated and feedback signals may come from shoots. N absorbed by leaves from a foliar application could produce signals from the nitrogenous compounds, such as amino acids and small peptides, which could move to roots regulating the root transport system and N uptake (Zheng *et al.* 2018). Therefore, the final aim was to identify feedback regulation of foliar N on root N uptake, focusing on nitrate accumulation in root tissue and effects on root nitrate transporters.

Materials and method

Plant material

Triticum aestivum L. cv. Gregory (Pelsart/2*Batavia); and Spitfire (Drysdale/Kukri), were grown in glasshouse conditions in the Plant Phenomic Facility (The Plant Accelerator), Waite Campus. The day/nighttime temperatures and humidity were controlled at 22/15 °C and 54/64%, respectively. Repeated experiments were carried out in glasshouse conditions at the Plant Research Centre (Waite Campus). The controlled day/night temperatures were 20/15 °C. The plants were grown in pots with 1.5 kg soil containing a mix of loam, clay, and cocopeat (a multipurpose growth medium made from coconut husks) in a 1:1:1 ratio. Granular urea at 50 mg N per kg of soil was added into the soil at sowing.

Determining foliar N uptake time in wheat

Gregory and Spitfire wheat seedlings (five replicates) at the fifth leaf growth stage (Zadoks growth stage 15) were used to investigate wheat leaf N uptake. The seedlings were treated with a mix of the three N forms: urea ($CH_4{}^{15}N_2O$), ammonium ($^{15}NH_4^+$), nitrate($^{15}NO_3^-$), using stock solutions of urea as $CO(^{15}NH_2)_2$, ammonium sulfate $(^{15}NH_4)_2SO_4$), and potassium nitrate ($K^{15}NO_3$) (10 atom % for each). The total N concentration of the combined N forms was 6 M (equivalent to the N concentration in commercial UAN fertiliser that has urea (21.5% N (w/v), ammonium (10.5% N (w/v) and nitrate (10.5% N (w/v), in its formulation). As an adjuvant, 0.5% (v/v) SpreadWet 1000 wetting agent (SST AUSTRALIA, Bayswater, VIC, Australia) was added to the mix. Five leaves were each treated with 20 µl of the mixed ^{15}N label. A 10 µl micropipete was used to dispense 1 µl droplets evenly onto the adaxial and abaxial sides of the flag leaf. The total volume of mix applied was 100 µl, containing 0.6 mmoles N per

plant. Control plants were treated with 0.5% SpreadWet 1000 only, hereafter referred to as control. Above-ground plant parts were harvested at 2 h, 4 h, 24 h, 48 h and 72 h after treatment, rinsed in four different tubs with reverse osmosis (RO) water to remove the excess label on the leaf surface. The samples were oven-dried and ground by mechanical disruption with ball bearings (4 mm and 8 mm chrome steel) using a GenoGrinder (SPEX Sample Prep 2010, Metuchen, NJ, USA) at 1100 – 1500 rpm. The ground powder was subsampled at 1.5 – 1.6 mg per sample and capsulated in tin foils for ^{15}N analysis (Sydney University Mass Spectrometry Laboratory).

The ^{15}N accumulated in the grains was calculated using the ^{15}N natural abundance method, from which differences in ^{15}N is used to trace the N transfer to plant tissues (He *et al.* 2009). The formula used is $\delta^{15}\mathrm{N}(‰) = [(R_{\mathrm{sample}} - R_{\mathrm{standard}}) - 1] \times 1,000$, where ^{15}N natural abundance expressed as delta (∂) in parts per thousand ‰, per mil (Paul *et al.* 2012).To further explain the formula, R = $^{15}N/^{14}N$ (atom%) calculated for the sample and standard. The standard is atmospheric N_2, which has a $\delta^{15}N$ value (0‰) = (0.00366295 ^{15}N abundance) (Mariotti 1983). The amount of ^{15}N in the grain tissue is therefore calculated as $\delta^{15}N_{(grains)} = \delta^{15}N_{(rgrains)} \times$ mg N in grains (Handley and Scrimgeour 1997).

Foliar N uptake of individual ^{15}N forms (urea, ammonium, and nitrate)

To investigate the N form preferentially taken up through leaves, plants of cv. Gregory were grown to maturity in glasshouse conditions as described above. The experimental design was a randomised complete block design with two basal soil N levels; 50 mg/kg soil (LN) and 100 mg/kg soil (HN) supplied as granular urea. This was followed by foliar treatments with single N forms ($CH_4{}^{15}N_2O$, $^{15}NH_4^+$ or $^{15}NO_3^-$) (10 atom % each), each as a 5 M total N stock concentration. The individual ^{15}N forms were applied at 7 days post-anthesis (7 DPA) on the flag leaf (adaxial and abaxial sides) of the main tiller in a

total volume of 50 µl and totalling 0.25 mmol N per plant. For the repeat experiment, the concentration of the stocks of the individual N forms was reduced to 3 M N, applied in a total volume of 50 µl and totalling 0.15 mmol N per plant. There were four and five replicates per treatment in the first and repeat experiments, respectively. Control SpreadWet solution was also applied at 7 DPA. All applications were made mid-morning (between 10 am - 12 pm) to minimise diurnal effects. At maturity, the main tiller of each plant was harvested. The treated flag leaf was separated from the rest of the plant and not used for further analysis. This was because we did not have a leaf surface cleaning protocol that we could ascertain removed all the surface N to avoid off-target N quantification. N accumulation was quantified in the main tiller devoid of the flag leaf. Samples were oven-dried, separated into grain, leaf, stem, and chaff, which were processed for biomass and grain weight. This was followed by grinding to fine powder, weighing and capsulation for ^{15}N analysis (Sydney University Mass Spectrometry Laboratory for the first experiment and University of California (UC) Davis Mass Spectrometry Laboratory for the repeat experiment). The ^{15}N accumulated in the grains was calculated using the ^{15}N natural abundance method, from which differences in ^{15}N is used to trace the N transfer to plant tissues (He *et al.* 2009).

Foliar N uptake from combined urea, ammonium, and nitrate (UAN)

A pot experiment was carried out using cv. Gregory, in conditions akin to those described above, with foliar N applied as UAN from different combinations of ^{15}N-labelled urea, ammonium and nitrate. The experimental design was a randomised complete block design with two basal soil N levels; 50mg/kg soil (LN) and 100mg/kg soil (HN) supplied from granular urea, followed by foliar ^{15}N treatments (mix 1: $CH_4{}^{15}N_2O$ + $^{15}NH_4^+$ + $^{15}NO_3^-$; mix 2 (CH_4N_2O + NH_4^+ + $^{15}NO_3^-$), mix 3 ($CH_4{}^{15}N_2O$ + NH_4^+ + NO_3^- and mix 4 (CH_4N_2O + $^{15}NH_4^+$ + NO_3^-) (10 atom % for each), prepared to

a total N concentration of 3 M. There were five replicates per treatment. A total volume of 50 µl containing 1.5mmol N (mix 1) and 0.5 mmol N in (mix 2, mix 3 and mix 4) was applied at 7 DPA on the flag leaf (adaxial and abaxial sides) of the main tiller. At maturity, the plants were harvested, and the treated flag leaf removed as before. The grain was dried, ground to a fine powder, weighed and capsulated for ^{15}N analysis (UC Davis Mass Spectrometry Laboratory).

Effects of foliar nitrate on root N uptake and expression of root *NRT* transporter genes at different time points

Seedlings of cv. Gregory were grown in hydroponics for four weeks, in glasshouse conditions as described above. Seeds were germinated in Petri dishes containing wet filter paper. Germinated seedlings were transferred to a mesh collar inserted into hydroponic tubes (300 mm x 500 mm). The meshed tube allowed independent root growth without intertwining with those of other plants and also allowed access to the hydroponics solution. The tubes were arranged in four plastic boxes with holes accommodating each tube (Fig.1). The boxes were connected to a tank containing 100 L of hydroponic solution. Each tank had a water pump that pumped the solution in and out of the tubs at intervals of 15 min. The nutrient solution used was a modified Johnson's solution (Johnson *et al.* 1957) containing nutrients in mM concentration as follows: $MgSO_4$ (2.0), KH_2PO_4 (0.1), H_3BO_3 (0.025), $MnSO_4$ (0.02) $ZnSO_4$ (0.02), $CuSO_4$ (0.0005), Na_2MoO_4 (0.0005), KCl (0.05), Fe-EDTA (0.05), $Ca(NO_3)_2$ (1.5), KNO_3 (2.0), K_2SO_4 (1.0) and $CaCl_2$ (2.45). The final N concentration in the hydroponic solution was 0.5 mM NO_3^-. The solution was adjusted to a pH of 5.9 and changed weekly.

Foliar fertiliser, UAN (42.5% w/v N), was applied after four weeks of growth. The treatment was applied to three leaves (youngest fully emerged leaf (leaf 1) and the two

leaves below (leaf 2 and leaf 3)) by applying a total volume of 80 µl (40 µl on leaf 1, 20 µl on leaf 2 and 20 µl leaf 3). Half of the volume was applied to each leaf on both the adaxial and abaxial sides. The experiment was set up as a randomized complete block design, with eight different harvest times (10 min, 30 min, 1 h, 3 h, 12 h, 24 h, 48 h and 72 h post-treatment) with five replications. Two controls were also included, a no-treatment (nothing applied on the leaf surface) and a 0.5% SpreadWet control. Plants were used for a nitrate flux experiment and analysis of nitrate transporter gene expression, as described in the following section.

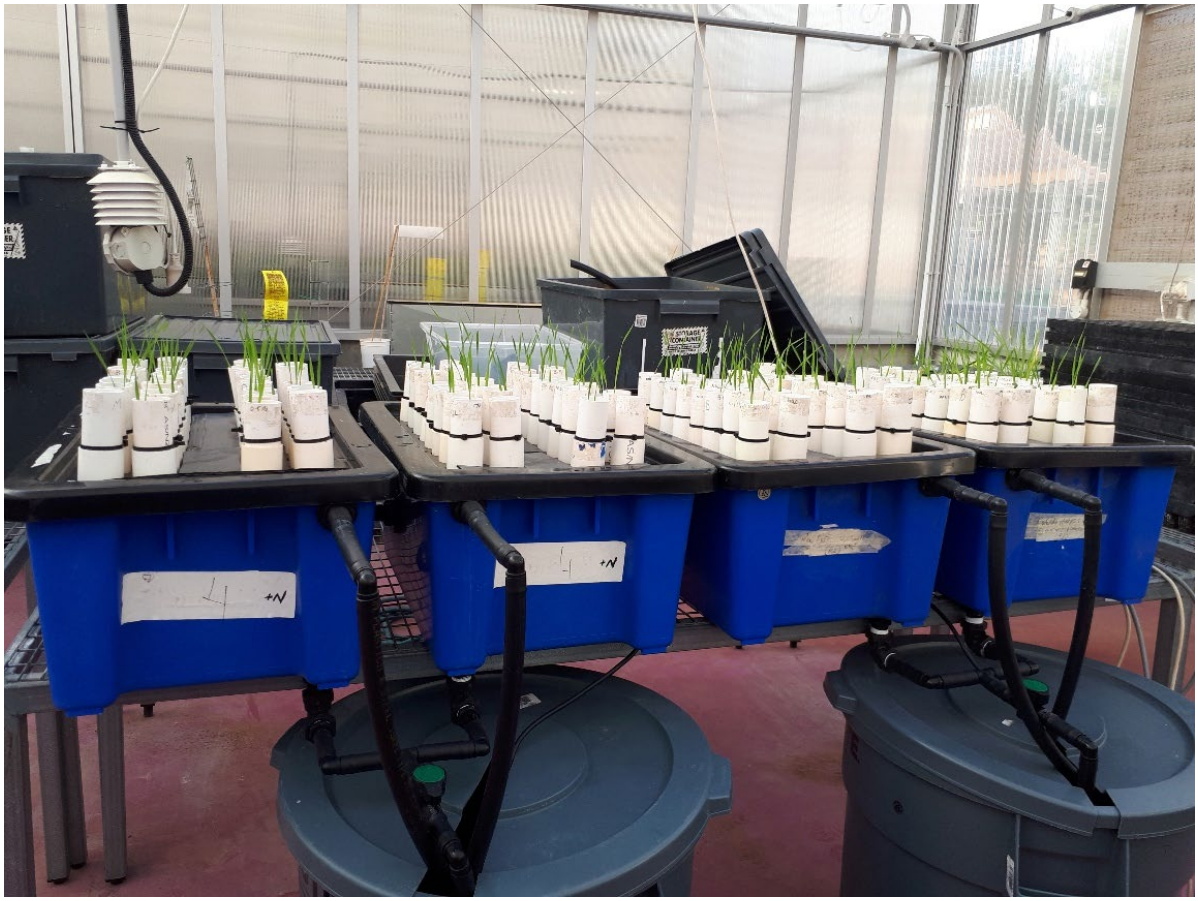

Figure 1: A hydroponics set up containing 1-week-old Gregory seedlings after germination. The large grey tanks contain hydroponic solution. The tanks each contain a submersible pump which moves the solution through the connecting pipes to the tub containing mesh collared tubes housing the seedlings.

UAN-treated hydroponically grown seedlings were used for a nitrate flux experiment, essentially as conducted as described by Garnett and colleagues (Garnett *et al.* 2013). Foliar-treated and control plants were immersed in 5 L closed pots containing nutrient solution with 0.1 mM KNO_3 for 5 min, then moved to pots with growth solution containing 0.1 mM $K^{15}NO_3$, (99 atom %) for 10 min. Plants were then rinsed by transferring to third and fourth 5 L closed pots for 2 min each, containing nutrient solution without added N, to remove the excess label on the root surface. Roots were then blotted on paper towel, weighed, snap frozen in liquid N_2 and later sub-sampled for ^{15}N analysis. The sub-samples were weighed, freeze-dried, ground to a fine powder and capsulated for ^{15}N analysis (UC Davis Mass Spectrometry Laboratory).

The ^{15}N accumulated in the roots was calculated using the ^{15}N natural abundance method.

RNA was also extracted from frozen root tissues sampled from the root flux experiment, using a Direct-zol™ RNA Miniprep Kit (ZYMO RESEARCH). A DNase I column digestion treatment was included to remove any genomic DNA contamination in the RNA sample, and a final elution was done using DNase/RNase-Free water. The concentration of RNA was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, USA). Approximately 1 μg RNA was then used for the synthesis of cDNA using SuperScript III™ reverse transcriptase (ThermoFisher SCIENTIFIC). The cDNA was stored at −20 °C until qPCR analysis.

The primers for *TaEFα, TaNRT1.1* and *TaNRT2.1* were based on work by Melino and colleagues (Melino *et al.* 2015). *Ta2991*, encoding for an ADP-ribosylation factor, was used as a reference gene (Paolacci *et al.* 2009).

For qPCR, 10 μl master mix was prepared, containing 5 μl Precision Fast 2x qPCR Master Mix (Low Rox) with SYBR Green (PRIMER DESIGN), 0.5 μl forward primer and

0.5 µl reverse primer, 2 µl water and 2 µl (approximately 2.5 ng/µL) cDNA. Real-time qPCR was carried out using the QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher SCIENTIFIC). The thermal cycling consisted of an initial enzyme activation (hot start) at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 20 s and a final extension at 95 °C for 15 s. Each reaction per treatment was performed using four biological replicates and three technical replicates.

The average threshold cycle (CT) value was calculated for each sample. *Ta2991* and *TaEFα* (Elongation Factor α) were the two reference genes used (Table 1). The CT values obtained for each sample were normalized using the two reference genes. The control treatment sample (no treatment) was used as a calibrator of gene expression. The qPCR data were extracted and analysed in LinRegPCR, a program that analyses quantitative PCR (qPCR) data based on the PCR efficiency of amplification curves (Ramakers *et al.* 2003). Non-baseline corrected data, was used, whereby the software made a baseline correction for each sample and determined a window-of-linearity followed by linear regression analysis to determine the PCR efficiency from the slope of the regression line (Ramakers *et al.* 2003). Finally, the mean PCR efficiency per amplicon was calculated (Peirson *et al.* 2003).

The formula used to calculate the relative expression ratio of the gene of interest is

$E^{-\Delta\Delta CT}$, with E= real-time PCR efficiency of target gene transcript, $\Delta CT = CT_{\text{target gene}}$ minus $CT_{\text{reference genes}}$, and $E^{-\Delta\Delta CT}$, where $\Delta\Delta CT = [(CT_{\text{target, treated sample}} - CT_{\text{reference gene, treated}})-(CT\ CT_{\text{target, untreated sample}} - CT_{\text{reference gene, untreated}})]$. The standard error was computed from the average of the $E^{-\Delta\Delta CT}$ values for each treatment (Schmittgen and Livak 2008).

Table 1: List of primers used for gene expression analysis

Genes	Primer sequences (5′–3′)	Primer source
TaNRT1.1	Forward: CACGGGAGCAACGACGGCTG Reverse: ATGCGTTTCTCCTTGTACACGTAG	Made in this study
TaNRT2.1	Forward: GCTGCTCWTAGTTGTGAGTGYAAC Reverse: TGAAGGACTTGGCCTTGT GCT C	(Melino *et al.* 2015)
TaEFα	Forward: CAGATTGGCAACGGCTACG Reverse: CGGACAGCAAAACGACCAAG	(Melino *et al.* 2015)
Ta2991	Forward: GCTCTCCAACAACATTGCCAAC Reverse: GCTTCTGCCTGTCACATACGC	(Paolacci *et al.* 2009)

Statistical analyses

A hypothesis of no differences between means was tested using one-way ANOVA to analyse ^{15}N uptake of the different N forms and gene expression data. The means of treatment effects were analysed using Graphpad Prism^{TM}. When differences were identified from the ANOVA analysis, multiple comparisons with Tukey's significant differences test was used at $p \leq 0.05$. Gene expression analysis was done using GraphPad PrismTM using one-way ANOVA, multiple comparisons comparing means of treatment with control and a Tukey's significant differences test was used at $p \leq 0.05$.

Results

Foliar N accumulation in Gregory and Spitfire

To identify the time taken for foliar N to accumulate in wheat, Gregory and Spitfire at the Zadok 15 growth stage were treated with a combined mix of the three N forms ($CH_4{}^{15}N_2O$ + $^{15}NH_4^+$ + $^{15}NO_3^-$) as foliar and analyzed for the accumulation of the

labeled N at different time points (2 h, 4 h, 24 h, 48 h, and 72 h). Accumulation of the foliar-applied N in Gregory wheat seedlings was evident, with the maximum accumulation observed at 2 h after foliar application (Fig. 2), the earliest sampling point. The ^{15}N accumulation did not change between 2 h – 72 h for this variety. Spitfire accumulated most N in the first 2 h but continued to accumulate ^{15}N as seen from an increased accumulation between the 24 h – 72 h time point. Overall, Spitfire had a significantly higher foliar N uptake compared to Gregory (Fig.2), which is also observed in the previous research (Chapter 3, Figure 1). The proportion of foliar N applied that was taken up in Spitfire was (21.4% - 26.9%) while Gregory had (17.7% - 19.9%), at all-time points which was statistically significant (Fig. 2).

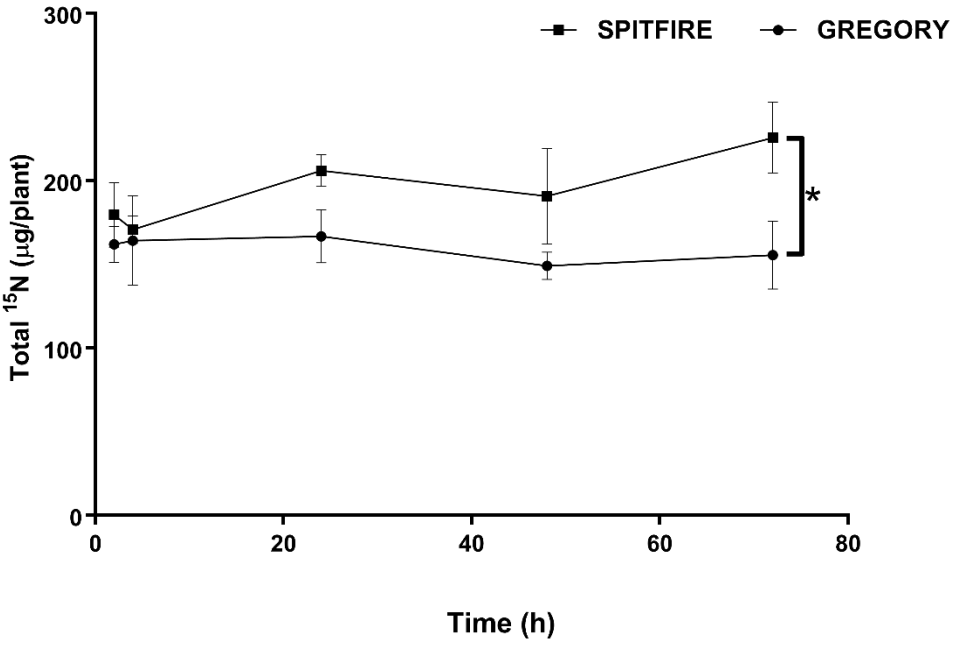

Figure 2: Gregory and Spitfire foliar ^{15}N accumulation in plants at the fifth leaf stage seedlings after foliar treatment with labeled UAN at different time-points (2 h, 4 h, 24 h, 48 h, and 72 h). Error bars indicate the standard error of five replicates.

Urea, ammonium, and nitrate have different translocation rates

Gregory was grown in two basal soil N levels (low basal N and high basal N), followed by a foliar ^{15}N treatment with the three N forms; nitrate ($^{15}NO_3^-$), urea ($CH_4{}^{15}N_2O$) and ammonium ($^{15}NH_4^+$) applied individually. The amount of N applied from the individual

N forms was 2.5 mmoles on the flag leaf at 7 DPA. This experiment was repeated, applying 1.5 mmoles of the individual N forms. Gregory was chosen due to its responsiveness to foliar N applied at 7 DPA in terms of increasing grain N concentration (Chapter 2). The plants were harvested at maturity and measured for biomass, grain weight and grain N concentration. In the first experiment, biomass was significantly higher in plants foliar-treated with ammonium under low basal N treatment and those foliar-treated with urea under high basal N when compared to control (SpreadWet) under low basal N (Fig 3a). In the same experiment, grain weight was significantly higher in urea-treated plants under high basal N compared to control (SpreadWet) under high basal N (Fig. 3b). Grain N concentration was higher under high basal N treatment for all treatments compared to low basal N (Fig. 3c). In the repeat experiment, grain N concentration was significantly higher in nitrate-treated plants under high basal N treatment compared to control under low basal N treatment (Fig. 3f). Also, in the repeat experiment, biomass, grain weight and grain N concentration were higher in the foliar nitrate treatment compared to all other treatments under low basal N treatment, (Fig 3d – 3f). The grain N concentration was also higher under high basal N treatment in all treatments compared to the low basal N treatments (Fig. 3f).

To identify which of the three N forms applied as foliar had the highest tissue accumulation, we carried out ^{15}N analysis and quantified accumulation in grain, leaf, stem, and chaff of the main tiller harvested in the first experiment. The ^{15}N accumulation in the leaf, stem and chaff was very low (results not shown). In the repeat experiment, N accumulation in the main tiller was quantified in the grains only. In the first experiment, foliar treatment with labelled urea resulted in the highest grain ^{15}N accumulation, at 70 µg and 85 µg N per tiller for plants grown with low and high basal

N treatments, respectively (Fig. 4). The grain ^{15}N accumulation from urea treatment was significantly higher than nitrate treatment in low and high basal N treatments. Foliar treatment with ammonium had the second-highest grain N accumulation at 51 µg and 65 µg per tiller under low and high basal N treatments, respectively (Figure 4). Grain ^{15}N accumulation from ammonium treatment was significantly higher than nitrate treatment in high basal N treatments. Foliar nitrate treatment had the least grain ^{15}N accumulation at 29 µg and 10 µg per tiller under low and high basal N treatments, respectively.

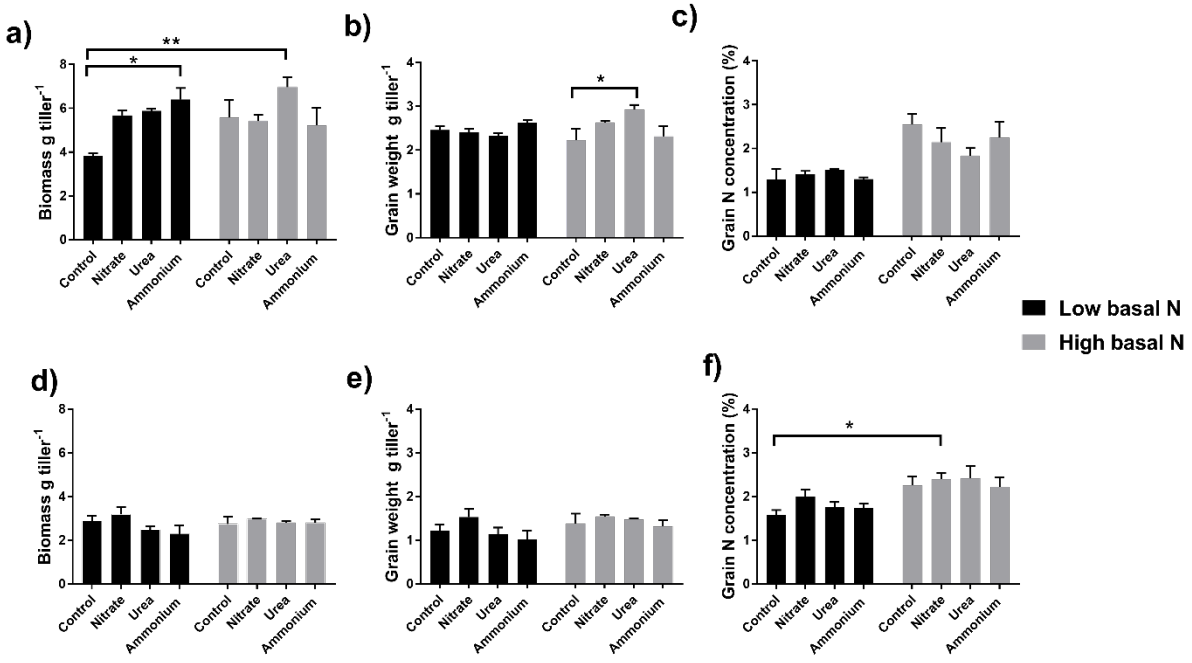

Figure 3: Biomass (g tiller^{-1}; a and d), grain weight (g tiller^{-1}; b and e) and grain N concentration (%; c and f) for Gregory after foliar treatment of the flag leaf at 7 DPA with single N forms (urea, ammonium, and nitrate) and control (SpreadWet). For the first experiment, (a-c), N was applied as 0.25 mmoles ^{15}N. A repeat experiment (d-f)used 0.5 mmoles ^{15}N. Error bars indicate the standard error of four (a-c) or five (d-e) replicates, and asterisks (*) and (**) indicate significant differences at ($P<0.01$) and ($P<0.001$), respectively.

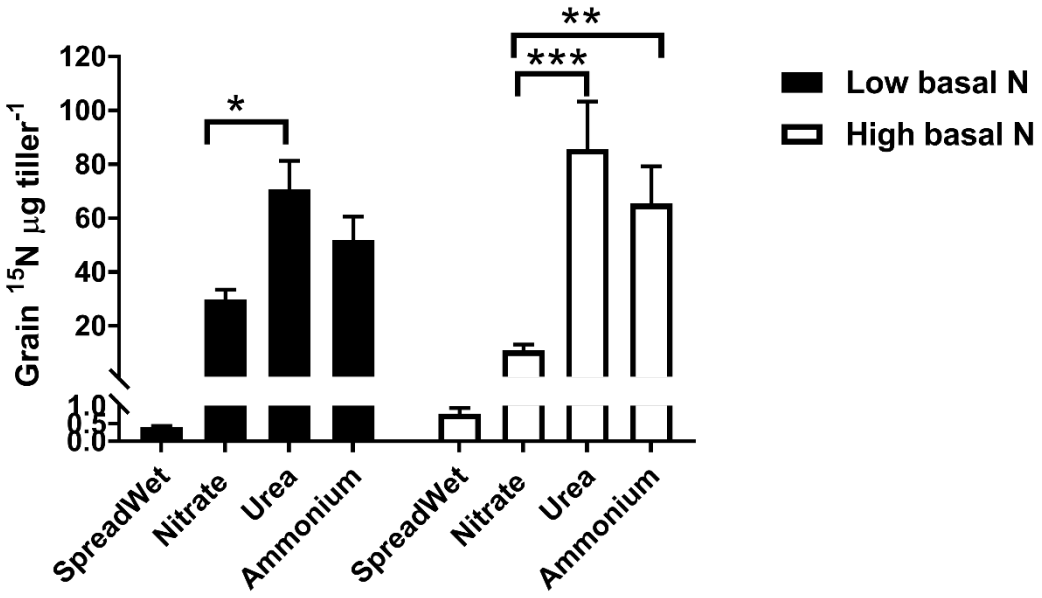

Figure 4. Accumulation of ^{15}N in the grain of the main tiller of cv.Gregory at maturity, derived from foliar application of 0.25 mmol of three different forms of ^{15}N-labelled N (nitrate ($^{15}NO_3^-$), urea ($CH_4{}^{15}N_2O$) and ammonium ($^{15}NH_4^+$)) at 7DPA on the flag leaf, compared to a control treatment using unlabelled SpreadWet. Plants were grown at two basal soil N treatments (low basal N and high basal N). There were four replicates per treatment, and error bars indicate the standard error. (*P<0.01, **P<0.001, ***P<0.0001).

In the repeat experiment, urea and nitrate application contributed similar ^{15}N to the grain at 25 µg and 24 µg per tiller respectively in low basal N treatment (Fig. 5). Ammonium treatment had a significantly low grain ^{15}N accumulation at 9 µg and 14 µg per tiller under low and high basal N treatments, respectively. In the high basal N treatment, urea treatment contributed a significantly high amount of ^{15}N to the grain compared to the nitrate at 47 µg per tiller. On the hand, nitrate treatment contributed a significantly high amount of ^{15}N to the grain compared to the ammonium at 37 µg per tiller. In this experiment, basal N treatment significantly increased grain ^{15}N accumulation under the high basal treatment in all the three ^{15}N form treatments ($p < 0.0001$).

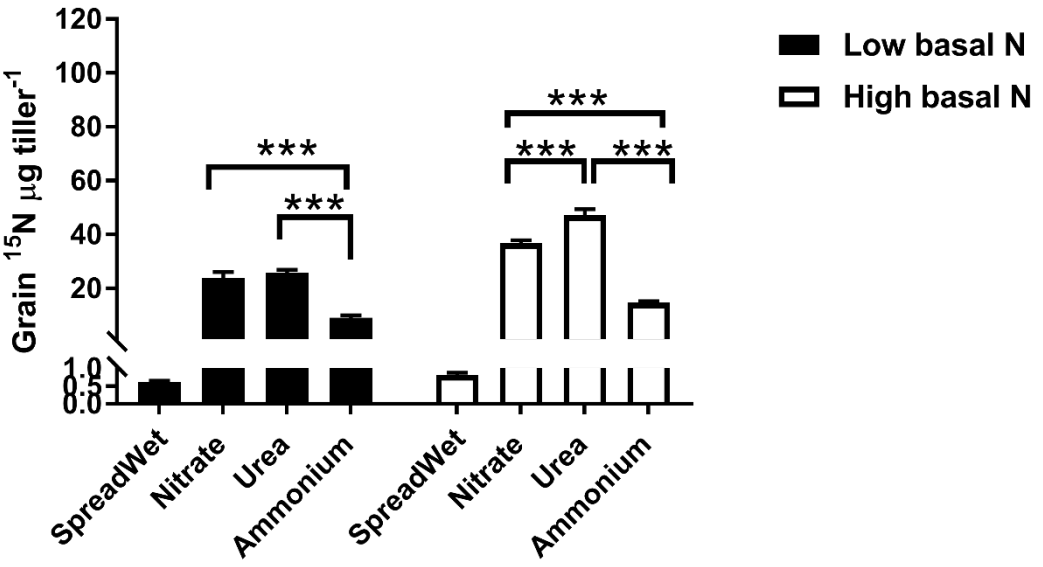

Figure 5: Gregory main tiller grain ^{15}N accumulation (repeat) after foliar treatment with 0.15 mmoles of the different N forms (nitrate ($^{15}NO_3^-$), urea ($CH_4{}^{15}N_2O$) and ammonium ($^{15}NH_4^+$)). Accumulation is quantified after foliar application 7DPA on the flag leaf and control treatment using SpreadWet. There were five replicates per treatment. Error bars indicate the standard error (SE) of five replicates and asterisks (*) indicate significant differences between genotypes; (*P<0.01, **P<0.001, ***P<0.0001).

Foliar N uptake of combined ^{15}N forms (urea, ammonium, and nitrate)

Several labelled combinations of the three N forms were prepared at a total N amount of 1.5 mmol N (mix 1) and 0.5 mmol N (mix 2, mix 3 and mix 4) applied to the flag leaf of Gregory at 7 DPA. The ^{15}N grain accumulation in the mix 1 where all the N forms were labelled ($CH_4{}^{15}N_2O$ + $^{15}NH_4^+$ + $^{15}NO_3^-$), contributing 35 µg and 70 µg ^{15}N under low basal N and high basal N treatments (Fig. 6), respectively. There were no significant differences between mix 2 (CH_4N_2O + NH_4^++ $^{15}NO_3^-$), mix 3 ($CH_4{}^{15}N_2O$ + NH_4^++ NO_3^- and mix 4 (CH_4N_2O + $^{15}NH_4^+$+ NO_3^-) that had similar N amount applied. Overall, the % of foliar N applied that was translocated to the grain ranged between 3% to 24% from all the single N form experiments, and between 14% and 35% from the combined N forms.

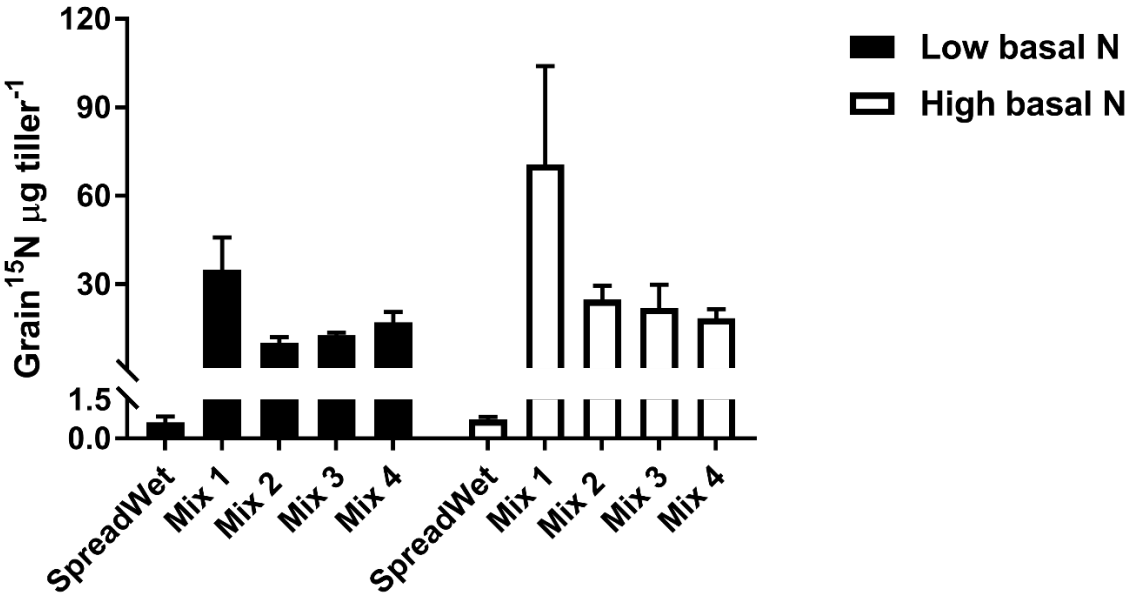

Figure 6: Gregory main tiller grain ^{15}N accumulation after foliar treatment with 0.5mmoles N in the combinations of the three N forms; mix 1 ($CH_4{}^{15}N_2O$ + $^{15}NH_4^+$ + $^{15}NO_3^-$), mix 2 (CH_4N_2O + NH_4^+ + $^{15}NO_3^-$), mix 3 ($CH_4{}^{15}N_2O$ + NH_4^+ + NO_3^-) and mix 4 (CH_4N_2O + $^{15}NH_4^+$ + NO_3^-) at 7DPA on the flag leaf and control treatment using SpreadWet. There were five replicates per treatment. Error bars indicate the standard error of five replicates and asterisks (*) indicate significant differences between genotypes; (*P<0.01 and **P<0.001).

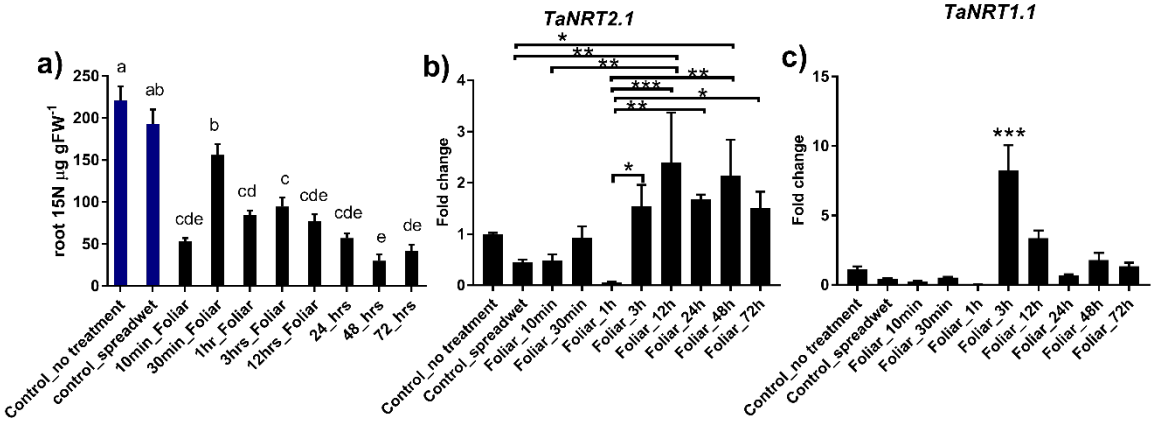

Figure 7: a) Gregory a) root flux showing root ^{15}N uptake. b) and c) root nitrate transporters (TaNRT2.1 and TaNRT1.1) gene expression in four weeks old seedlings following foliar treatment using UAN fertilizer applied on the youngest fully emerged leaf (leaf 1) and the two leaves below (leaf 2 and leaf 3))at different time points. a) Error bars indicate the standard error (SE) of five replicates and letters represent significant differences where the same letter shows no significance. b) and c) Error bars indicate the standard error of four biological replicates and three technical replicates, and asterisks (*) indicate significant differences between genotypes; (*P<0.01, **P<0.001 and ***P<0.0001).

Effects of foliar-applied nitrate on root N accumulation and expression of root *NRTs* at different time points

The objective of this experiment was to establish whether foliar-applied N generated a feedback regulation on root NO_3^- uptake. Foliar UAN solution was applied at different time points (10 min, 30 min, 3 h, 12 h, 24 h, 48 h, and 72 h) before measurement of root $^{15}NO_3^-$ fluxes. For the controls, a nil application, and SpreadWet applied at the 72-h time point before flux measurement, were used. Root ^{15}N accumulation was affected by foliar treatment (Fig. 7*a*). The highest root N accumulation was in the control (nil application) and the second-highest accumulation in control (SpreadWet). There was a decrease in ^{15}N root accumulation at 10 min post-treatment. This was followed by a significant increase in ^{15}N uptake at 30 min post-treatment. The uptake then gradually decreased between 1 h post-treatment and 72 h post-treatment. ^{15}N uptake was significantly less than the controls for all times after foliar UAN application except at 30 min post-treatment.

To elucidate the effect of foliar N on roots through feedback regulation, the expression pattern of two major root-expressed nitrate transporter genes, *TaNRT2.1* and *TaNRT1.1,* was analysed. The expression of the two nitrate transporter genes was affected by foliar treatment (Fig. 7 b-c). *TaNRT2.1* expression was significantly downregulated at 1 h post-treatment. Furthermore, the expression of this gene increased between 3 h post-treatment and 72 h post-treatment. The highest differential expression was at 12 h and 48 h post-treatment, as compared to the control (SpreadWet) and 1 h post-treatment. *TaNRT1.1*, like *TaNRT2.1*, was significantly downregulated at 1 h post-treatment. *TaNRT1.1* expression level increased significantly at 3 h post-treatment.

Discussion

N fertiliser formulation and the time taken to enter the plant may affect the success rate of foliar N application. Understanding how these two factors enhance foliar N uptake in wheat may help to explain the variability observed in the performance of foliar N fertilisers (Gooding and Davies 1992). In the experiment to decipher time taken for foliar ^{15}N to accumulate into Gregory and Spitfire seedlings, the maximum ^{15}N accumulation was reached at 2 h after application, especially in Gregory (Fig. 2). These results indicated that this time point or even earlier is a crucial period for foliar N uptake through leaf tissue after application. Foliar N fertilisers applied may, therefore, be taken up rapidly for resolving nutrition deficiencies. The highest proportion of foliar N applied that accumulated in the wheat seedlings from the combined N forms, was 20% and 27% in Gregory and Spitfire, respectively, similar to previously reported values. Oosterhuis showed a rapid entry of foliar ^{15}N in cotton with a 30% absorption within one hour and translocation to the nearest boll within 6 to 48 hours after application (Oosterhuis 2009). Spitfire seedling responded significantly to foliar compared to Gregory as observed in Figure 2 as well as previous research (Chapter 3, Figure 1). The explanation could be from the wax quantity which is significantly low in Spitfire compared to Gregory as observed from the previous research carried out (Chapter 3, Figure 2)

To understand the N formulation that may be involved in efficient foliar N application, the uptake of different ^{15}N forms through the leaf tissue and their accumulation in different plant parts was examined. At maturity, the highest ^{15}N accumulation after flag leaf foliar pre-treatment at 7 DPA was in the grain tissues, similar to previous research (Blacklow 1982). Previous research shows that after anthesis, the wheat spike is a strong sink organ (Egle *et al.* 2008). Of the different forms of foliar-applied N, ^{15}N-

labelled urea showed the highest accumulation to the grain under high and low basal N treatment (Fig. 4 and Fig. 5). Urea is a neutral molecule that easily traverses the cuticle through a lipoidal route, entering the cuticle in a nonpolar undissociated form (Oosterhuis 2009, Qi-rong and Guo-hua 2001), which may explain its more effective accumulation in the grain than other forms of foliar N. The entry of the other two N forms, ammonium, and nitrate, which are positively and negatively charged, respectively, is thought to be via membrane-bound aqueous pores that are formed from hydration of polar functional groups. Consequently, ammonium and nitrate may have slow penetration and may be dependent on a saturated atmosphere (Liu *et al.* 2015, Oosterhuis 2009, Schönherr 2000). Another possible path of entry is through leaf cell-localised N transporters, especially those categorised as low-affinity transporters that are induced by N levels and active when N concentration is high, or those constitutively expressed (Miller et al., 2007).

Lowering the foliar N amount in the single N forms repeat experiment resulted in lower grain ^{15}N accumulation (Figure 5), as expected. Overall, N accumulation was basal N-dependant with differences in grain N accumulation between the low basal N and high basal N treatments (Fig. 4 and Fig. 5). In the repeat experiment, a higher level of basal N consistently resulted in a greater accumulation of grain N from foliar-applied ^{15}N. To explain these results, under a high basal N, there are more grain numbers, resulting in a stronger sink for foliar applied ^{15}N. It can be concluded that the basal N applied at sowing and amount of foliar N applied may influence the uptake and accumulation of the three N forms when applied as single N forms. However, a repeat of the experiment varying the two aspects to ascertain correlation to foliar N uptake and accumulation, may be necessary.

To follow on from the above experiment, the three N forms were combined in different mixes and applied as foliar ^{15}N. Uptake and translocation occurred as seen from the accumulated grain ^{15}N (Fig. 6). Mix 1, where all the N forms were labelled (i.e. ($CH_4{}^{15}N_2O$ + $^{15}NH_4^+$ + $^{15}NO_3^-$)) with 1.5mmol ^{15}N applied, had a high ^{15}N accumulation in the grain. This is expected as it had the highest ^{15}N applied coming from the three N forms. Since the other combined N form mixes (mix 2, mix 3 and mix 4) with similar ^{15}N applied (0.5 mmol) were not significantly different between each other (Fig. 6), we speculate that the three N forms combined have a similar uptake and tissue accumulation regardless which of the three N forms is labelled.

Like previous research, this study confirmed that commercial urea for the single N form or UAN (Urea Ammonium Nitrate), which has a high urea N % (21.5%) for the combined N forms may be good choices for foliar N application (Qi-rong and Guo-hua 2001). The proportion of applied foliar N that accumulated in the grain was not very high, ranging between approximately 3% and 35% across all treatments, but could contribute effectively to increasing grain N and maintaining high grain N concentrations as observed from previous research (chapter 2). The timing of application of the different N forms is crucial as it affects the grain quality and N fertilizer efficiency (Tran and Tremblay 2000). Previous research showed that proportions of grain N derived from ^{15}N labelled fertilizer, applied in the soil, were significantly high at booting stage (40% - 60%) compared to seedling stage (20% - 40%) in spring (Tran and Tremblay 2000). A good root development/activity in good soil conditions in later wheat growth stages explain efficient N absorption (Tran and Tremblay 2000). Folair N fertiliser efficiency, though low could be due to leaf morphological features that are yet to be understood and improved. Identification of the leaf morphological features directly correlated to foliar N uptake together with proper N formulation applied in suitable

environmental conditions, could increase the proportion of foliar N taken up and translocated to the grain. These could improve the foliar N absorption efficiency.

In addition, assimilation of foliar-applied nitrogen is not well understood, while phloem translocation of nitrogen may, rather than uptake, be a bottleneck for foliar nitrogen fertilization. Future foliar N experiments would be vital especially on reduction rate of foliar N after absorption, activity of nitrate reductase in flag leaf and old leaves. Also, the transport of nitrogen compounds from source to sink leaves specialising on phloem loading could be incooprated in future studies to further understand phloem nitrate translocation by phloem nitrate transporters (Hsu and Tsay 2013, Lezhneva *et al.* 2014).

Experiments on root nitrate flux and gene expression experiments were conducted to investigate feedback regulation of foliar N on root N accumulation. There was a time-dependent effect of foliar N on root ^{15}N accumulation (Fig. 7a). Root accumulation was highest in controls (nil treatment and SpreadWet), intimating that foliar N application reduces root nitrate uptake. The 10 min post-treatment had the least root nitrate uptake which could be due to a very rapid N signal sent by the plant on available N entering through the leaf tissue, momentarily inhibiting any root N accumulation. Nitrogenous compounds (nitrate, amino acids, and ammonium) may be transported from cells, when there is an excess N supply (Forde and Clarkson 1999). These compounds move to the root as signals that could regulate N uptake and accumulation in the root tissues. Also, expression levels of two root nitrate transporters, *TaNRT2.1* and *TaNRT1.1*, were low at this time point, which could similarly be explained by a negative regulation by the rapid N signal (Fig. 7b and 7c). Because there are limited studies on foliar N regulation of the root transporter system, the expression profiles from this study were compared to regulation after root N provision. Similar expression profiles have been

observed for the two genes in Arabidopsis, where there was a strong induction upon root nitrate provision and a downregulation when N supply was increased (Nazoa *et al.* 2003, Wang *et al.* 2011, Zhuo *et al.* 1999). In the present study, *TaNRT2.1* was downregulated by foliar N treatment at 10 min to 1 h post-treatment, and upregulated at between 3 h and 72 h. On the contrary, previous research showed an increased accumulation of *TaNRT2.1* mRNA from transient induction by root nitrate, with a rapid increase by 1 h post-treatment. The transcript levels reached a maximum at 4 h post-treatment and decreased to the original levels by 24 h (Yin *et al.* 2007). There was no increase in transcript levels by ammonium induction (Yin *et al.* 2007). As a response to foliar application of N, the nitrogenous signal would be sent from the shoot to the root and could be mostly ammonium and amino acids. The increased uptake observed in this study at 30-minute post-treatment does not correlate with the expression levels of the two genes, supporting the idea that correlating nitrate transporter gene expression to nitrate uptake genes does not accurately give the N-uptake capacity in wheat (Melino *et al.* 2015). There may be post-translational regulation as observed from previous research studies on NRTs (Liu and Tsay 2003). Hence, quantifying the protein amount is essential too (Li *et al.* 2010).

From this study, efficient uptake of foliar N could depend on the basal N applied at sowing. As mentioned above, the % foliar N accumulation in the grain from the amount applied to the leaf is not high (3 -35%), meaning the decision of applying foliar N to increase grain yield and protein needs to be carefully considered to include an economic evaluation. However, since wheat only accesses an average of 30 - 50% N applied to the soil (Sylvester-Bradley and Kindred 2009), the foliar top-up could be beneficial. Foliar N fertilization has a regulatory effect on root nitrate uptake by decreasing root N accumulation. Depending on the growth stage of wheat when foliar

fertiliser is applied, it could adversely affect root N uptake capacity. In early growth stages the root activity is high to provide N necessary for growth, while in later growth stages there appears to be a decreasing amount of root N uptake activity (Bogard *et al.* 2010). Hence, foliar application may be most beneficial and most effective at later growth stages when there will be minimal effects on the root uptake capacity. Future research should consider the effect of foliar N on root ammonium and urea uptake, and on root uptake at later stages of development.

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References

ABS (2018). Land Management and Farming in Australia, 2016-2017. 4627.0. Canberra.

Blacklow, W. (1982). "15n Moved to the Grain of Winter Wheat When Applied as Nitrate to Senescing Flag Leaves." Functional Plant Biology 9(6): 641-646.

Bly, A. G. and H. J. Woodard (2003). "Foliar Nitrogen Application Timing Influence on Grain Yield and Protein Concentration of Hard Red Winter and Spring Wheat." Agronomy Journal 95: 335-338.

Bogard, M., V. Allard, M. Brancourt-Hulmel, E. Heumez, J. M. Machet, M. H. Jeuffroy, P. Gate, P. Martre and J. Le Gouis (2010). "Deviation from the grain protein concentration-grain yield negative relationship is highly correlated to post-anthesis N uptake in winter wheat." J Exp Bot 61(15): 4303-4312.

Crawford, N. M. and A. D. Glass (1998). "Molecular and physiological aspects of nitrate uptake in plants." Trends in plant science 3(10): 389-395.

Egle, K., H. Beschow and W. Merbach (2008). "Assessing post-anthesis nitrogen uptake, distribution and utilisation in grain protein synthesis in barley (Hordeum vulgare L.) using 15N fertiliser and 15N proteinogenic and non-proteinogenic amino acids." Annals of Applied Biology 152(2): 209-221.

Ellen, J. and J. Spiertz (1980). "Effects of rate and timing of nitrogen dressings on grain yield formation of winter wheat (T. aestivum L.)." Fertilizer research 1(3): 177-190.

Ercoli, L., A. Masoni, S. Pampana, M. Mariotti and I. Arduini (2013). "As durum wheat productivity is affected by nitrogen fertilisation management in Central Italy." European journal of agronomy 44: 38-45.

FAO (2017). Fertilizers by Product. DATA. Rome, Italy, Food and Agriculture Organization of the United Nations (FAO).

Fernández, V., T. Sotiropoulos and P. H. Brown (2013). "Foliar fertilisation: Principles and practices." International Fertiliser Industry Association (IFA): Paris, France: 140.

Forde, B. G. and D. T. Clarkson (1999). Nitrate and ammonium nutrition of plants: physiological and molecular perspectives. Advances in botanical research, Elsevier. 30: 1-90.

Garnett, T., V. Conn, D. Plett, S. Conn, J. Zanghellini, N. Mackenzie, A. Enju, K. Francis, L. Holtham, U. Roessner, B. Boughton, A. Bacic, N. Shirley, A. Rafalski, K. Dhugga, M. Tester and B. N. Kaiser (2013). "The response of the maize nitrate transport system to nitrogen demand and supply across the lifecycle." New Phytologist 198(1): 82-94.

Gazzarrini, S., L. Lejay, A. Gojon, O. Ninnemann, W. B. Frommer and N. von Wirén (1999). "Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into Arabidopsis roots." The Plant Cell 11(5): 937-947.

Giles, J. (2005). "Nitrogen study fertilizes fears of pollution." Nature 433(7028): 791-791.

Gooding, M. J. and W. P. Davies (1992). "Foliar urea fertilization of cereals: A review." Fertilizer Research 32(2): 209-222.

Griffiths, M. and L. M. York (2020). "Targeting root ion uptake kinetics to increase plant productivity and nutrient use efficiency." Plant Physiology 182(4): 1854-1868.

Guo, T., H. Xuan, Y. Yang, L. Wang, L. Wei, Y. Wang and G. Kang (2014). "Transcription Analysis of Genes Encoding the Wheat Root Transporter NRT1 and NRT2 Families During Nitrogen Starvation." Journal of Plant Growth Regulation 33(4): 837-848.

Handley, L. and C. Scrimgeour (1997). "Terrestrial plant ecology and 15N natural abundance: the present limitation for uncultivated systems with original data from a Scottish old field." Adv Ecol Res 27: 134-212.

He, X., M. Xu, G. Y. Qiu and J. Zhou (2009). "Use of 15N stable isotope to quantify nitrogen transfer between mycorrhizal plants." Journal of Plant Ecology 2(3): 107-118.

Hirel, B., J. Le Gouis, B. Ney and A. Gallais (2007). "The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches." Journal of Experimental Botany 58(9): 2369-2387.

Hsu, P.-K. and Y.-F. Tsay (2013). "Two phloem nitrate transporters, NRT1. 11 and NRT1. 12, are important for redistributing xylem-borne nitrate to enhance plant growth." Plant Physiology 163(2): 844-856.

IFA, I. F. A. (2019). Executive Summary Fertilizer Outlook 2019-2023. 87th IFA Annual Conference. Montreal, Canada.

Jackson, W. and R. Volk (1992). "Nitrate and ammonium uptake by maize: adaptation during relief from nitrogen suppression." New Phytologist 122(3): 439-446.

Johnson, C., P. Stout, T. C. Broyer and A. B. Carlton (1957). "Comparative chlorine requirements of different plant species." Plant and soil 8(4): 337-353.

Lezhneva, L., T. Kiba, A. B. Feria-Bourrellier, F. Lafouge, S. Boutet-Mercey, P. Zoufan, H. Sakakibara, F. Daniel-Vedele and A. Krapp (2014). "The Arabidopsis nitrate transporter NRT2.5 plays a role in nitrate acquisition and remobilization in nitrogen-starved plants." Plant J 80(2): 230-241.

Li, X.-J., M.-F. Yang, H. Chen, L.-Q. Qu, F. Chen and S.-H. Shen (2010). "Abscisic acid pretreatment enhances salt tolerance of rice seedlings: proteomic evidence." Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1804(4): 929-940.

Liu, D., L. Yang, Q. Zheng, Y. Wang, M. Wang, X. Zhuang, Q. Wu, C. Liu, S. Liu and Y. Liu (2015). "Analysis of cuticular wax constituents and genes that contribute to the formation of 'glossy Newhall', a spontaneous bud mutant from the wild-type 'Newhall'navel orange." Plant molecular biology 88(6): 573-590.

Liu, K. H. and Y. F. Tsay (2003). "Switching between the two action modes of the dual-affinity nitrate transporter CHL1 by phosphorylation." The EMBO journal 22(5): 1005-1013.

López-Bellido, L., R. J. López-Bellido and R. Redondo (2005). "Nitrogen efficiency in wheat under rainfed Mediterranean conditions as affected by split nitrogen application." Field crops research 94(1): 86-97.

Mariotti, A. (1983). "Atmospheric nitrogen is a reliable standard for natural 15N abundance measurements." Nature 303(5919): 685-687.

Melaj, M. A., H. E. Echeverría, S. C. Lopez, G. Studdert, F. Andrade and N. O. Bárbaro (2003). "Timing of nitrogen fertilization in wheat under conventional and no-tillage system." Agronomy Journal 95(6): 1525-1531.

Melino, V. J., G. Fiene, A. Enju, J. Cai, P. Buchner and S. Heuer (2015). "Genetic diversity for root plasticity and nitrogen uptake in wheat seedlings." Functional Plant Biology 42(10): 942-956.

Miller, A. J., X. Fan, M. Orsel, S. J. Smith and D. M. Wells (2007). "Nitrate transport and signalling." Journal of experimental Botany 58(9): 2297-2306.

Nazoa, P., J. J. Vidmar, T. J. Tranbarger, K. Mouline, I. Damiani, P. Tillard, D. Zhuo, A. D. Glass and B. Touraine (2003). "Regulation of the nitrate transporter gene AtNRT2. 1 in Arabidopsis thaliana: responses to nitrate, amino acids and developmental stage." Plant molecular biology 52(3): 689-703.

Oosterhuis, D. (2009). Foliar fertilization: mechanisms and magnitude of nutrient uptake. Proceedings of the fluid forum.

Paolacci, A. R., O. A. Tanzarella, E. Porceddu and M. Ciaffi (2009). "Identification and validation of reference genes for quantitative RT-PCR normalization in wheat." BMC Molecular Biology 10(1): 11.

Paul, E., J. Melillo, R. Knowles and H. Blackburn (2012). Nitrogen isotope techniques, Elsevier.

Peirson, S. N., J. N. Butler and R. G. Foster (2003). "Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis." Nucleic acids research 31(14): e73-e73.

Plett, D., J. Toubia, T. Garnett, M. Tester, B. N. Kaiser and U. Baumann (2010). "Dichotomy in the NRT gene families of dicots and grass species." PloS one 5(12): e15289.

Qi-rong, S. and X. Guo-hua (2001). "Foliar absorption and translocation of labelled urea-^ 1^ 5N in corn and wheat." Acta Pedologica Sinica 38(1): 74-79.

Ramakers, C., J. M. Ruijter, R. H. L. Deprez and A. F. M. Moorman (2003). "Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data." Neuroscience Letters 339(1): 62-66.

Raun, W. R. and G. V. Johnson (1999). "Improving nitrogen use efficiency for cereal production." Agronomy journal 91(3): 357-363.

Rossmann, A., B. Pitann and K.-H. Mühling (2019). "Splitting nitrogen applications improves wheat storage protein composition under low N supply." Journal of Plant Nutrition and Soil Science 182(3): 347-355.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C T method." Nature protocols 3(6): 1101.

Schönherr, J. (2000). "Calcium chloride penetrates plant cuticles via aqueous pores." Planta 212(1): 112-118.

Sylvester-Bradley, R. and D. R. Kindred (2009). "Analysing nitrogen responses of cereals to prioritize routes to the improvement of nitrogen use efficiency." Journal of Experimental Botany 60(7): 1939-1951.

Tilman, D. (1999). "Global environmental impacts of agricultural expansion: the need for sustainable and efficient practices." Proceedings of the National Academy of Sciences 96(11): 5995-6000.

Tran, T. and G. Tremblay (2000). "Recovery of 15 N-labeled fertilizer by spring bread wheat at different N rates and application times." Canadian Journal of Soil Science 80: 533-539.

Wang, P., Z.-l. Wang, R.-g. Cai, Y. Li, X.-g. Chen and Y.-p. Yin (2011). "Physiological and Molecular Response of Wheat Roots to Nitrate Supply in Seedling Stage." Agricultural Sciences in China 10(5): 695-704.

Wang, W.-H., B. Köhler, F.-Q. Cao, G.-W. Liu, Y.-Y. Gong, S. Sheng, Q.-C. Song, X.-Y. Cheng, T. Garnett, M. Okamoto, R. Qin, B. Mueller-Roeber, M. Tester and L. Liu (2012). "Rice DUR3 mediates high-affinity urea transport and plays an effective role in improvement of urea acquisition and utilization when expressed in Arabidopsis." New Phytologist 193(2): 432-444.

Watson, C., M. H, P. Poland, D. Kilpatrick, M. Allen, M. Garrett and C. Christianson (1994). "Soil properties and the ability of the urease inhibitor N-(n-butyl) thiophosphoric triamide (nBTPT) to reduce ammonia volatilization from surface-applied urea." Soil Biology and Biochemistry 26(9): 1165-1171.

Wylie, P., A. Mayfield, F. Harrop and A. Roe (2003). The potential of fluid fertilisers for broadacre cropping in Australia HOR 00002 Australia, Grains Research and Development Corporation.

Yin, L.-P., P. Li, B. Wen, D. Taylor and J. O. Berry (2007). "Characterization and expression of a high-affinity nitrate system transporter gene (TaNRT2.1) from wheat roots, and its evolutionary relationship to other NTR2 genes." Plant Science 172(3): 621-631.

Zanin, L., N. Tomasi, C. Wirdnam, S. Meier, N. Y. Komarova, T. Mimmo, S. Cesco, D. Rentsch and R. Pinton (2014). "Isolation and functional characterization of a high affinity urea transporter from roots of Zea mays." BMC plant biology 14(1): 222.

Zheng, C., P. Li, M. Sun, C. Pang, X. Zhao, H. Gui, S. Liu, Y. Qin, H. Dong and X. Yu (2018). "Effects of Foliar Nitrogen Applications on the Absorption of Nitrate Nitrogen by Cotton Roots." Cotton Science 30(4): 338-343.

Zhuo, D., M. Okamoto, J. J. Vidmar and A. D. Glass (1999). "Regulation of a putative high-affinity nitrate transporter (Nrt2; 1At) in roots of Arabidopsis thaliana." The Plant Journal 17(5): 563-568.

Chapter 5

Genetic variation in nitrogen dynamics and physiological traits related to yield and grain quality

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
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Abstract

Grain Protein Content (GPC) determines the export market value of wheat and its end-use in the food and feed industry. However, there is a negative correlation between GPC and grain yield, complicating the identification of wheat varieties that have the potential to produce both high yield and high GPC. One approach to identify wheat genotypes with higher GPC than expected from their grain yield has been the use of Grain Protein Deviation (GPD). Identifying physiological traits that are associated with positive GPD (high GPC without a reduction in grain yield) is necessary. This study aimed to decipher the nitrogen utilization in genotypes contrasting in GPD and identify physiological traits associated with both high grain yield and GPC in field and glasshouse conditions. Wheat genotypes with a high yield but contrasting GPD were selected from two years (2014 and 2015) from field trials in Tarlee, South Australia. The selected genotypes and two Australian elite cultivars were then grown in the field and greenhouse conditions under different nitrogen (N) treatments. There was an influence of genotype and N treatment on grain yield and GPC both under field and greenhouse conditions. Most of the pre-selected positive GPD genotypes were consistently high yielding with high GPC in the two environments. Total N at anthesis, post-anthesis N uptake, biomass at maturity and total N at maturity had large effects on grain weight (grain yield) and GPC. The genotypes RAC875 and 6HRWSN98 that had a high yield and GPC (positive GPD) in different environments (field and control), could be used in the breeding programs targeting the two important agronomic traits. Also, the physiological traits strongly correlated with both a high yield and high GPC under different N treatments including grain N content, total N at anthesis, total N at maturity and post-anthesis N uptake could aid in understanding the negative correlation of yield and GPC.

Introduction

Wheat is an important cereal crop used as food and feed, with global production estimated at 765 million tonnes (Mt) in 2019 (FAO, 2019). Australia is among the top five wheat exporters in the world, with 25 Mt produced annually, accounting for 3 - 4% of world wheat production (ABARES 2019). The total wheat exported from Australia is 65-75%, with Western Australia and New South Wales, having the largest export share (ABARES 2019, Wang *et al.* 2018). There has been an increase in wheat yield and production quantity over the years because of successful breeding and other agricultural strategies including the application of exogenous nitrogen (N) fertiliser. The increase is not linked to an increase in the area of harvest.

The amount of synthetic N fertilizers produced from 2002 to 2017 has increased from 86 Mt to 120 Mt, respectively (FAO 2019). The agricultural uses of the produced N fertilisers have also increased in this 15-year timeline from 83 Mt to 110 Mt (FAO 2019). Australia's total N fertiliser use is estimated to be over 1 MT each year (ABARES 2013). However, Nitrogen Use Efficiency (NUE), defined as grain dry matter produced for every N unit available from the soil, is low in cereal crops, including wheat. The N uptake and utilization efficiency in fertilised cereal crops is estimated to be between 30-50%, resulting in fertiliser wastage and financial losses (Raun and Johnson 1999, Sylvester-Bradley and Kindred 2009). It is, therefore, necessary to find ways to improve N uptake and utilization for stable yield and grain quality in wheat production. In Australia, Grain Protein Content (GPC) is the most common measure of grain quality and determines the export market value and end-use for wheat in the food and feed industries. Growers receive premiums for higher GPC grain. However, it is difficult to produce wheat with both high yield and high GPC. This is due to a negative correlation between the two important agronomical traits. The cause of the negative correlation is

hypothesised to be either a consequence of competition for energy from photosynthesis between N assimilation and carbon acquisition or due to dilution of N allocated to the grain as a result of increased grain number (Acreche and Slafer 2009, Munier *et al.* 2005). Previous studies have proposed the use of the deviation from the regression line between grain yield and GPC, termed as grain protein deviation (GPD), to identify genotypes with higher GPC than expected from their grain yield (Monaghan *et al.* 2001). Previous research findings indicate that genotypes with positive GPD tend to have higher levels of N uptake after flowering and also greater nitrogen remobilization, (the movement N from vegetative tissue to the grain0 (Barbottin *et al.* 2005).

Generally, N taken up before anthesis is invested in biomass production and later remobilised to developing grain, while N uptake post-anthesis is invested in preformed grains increasing grain N concentration (Hirel *et al.* 2007). The contribution of post-anthesis N uptake on GPD is largely independent of anthesis date and total N at anthesis. However, some research has shown that there is genetic variation in GPD which is also related to post-anthesis N uptake in association with anthesis date (Monaghan *et al.* 2001).

The underlying physiological traits possibly relate to genotypic differences in access to soil N, regulation of N uptake depending on plant N status, or maintenance of root activity during the grain-filling period (Bogard *et al.* 2010). The selection of cultivars that can take up and use N efficiently and break the negative correlation between grain yield and GPC, is a significant challenge for breeders today (Kichey *et al.* 2007). This research aimed to study N use and identify physiological traits in genotypes selected from a wheat diversity panel contrasting for GPD. Contrasting genotypes were grown under different N treatments in field and glasshouse conditions. N treatment was varied

at the vegetative stage and post-anthesis, to mimic different N conditions at the beginning and the end of the wheat-growing season. The yield components and grain N were measured to elucidate N utilization in the wheat diversity panel and to identify the physiological traits correlated to grain yield and GPC.

Materials and methods

Selection of wheat varieties contrasting for GPC from a genetic diversity panel

Field trials for a genetic diversity panel were conducted in 2014 and 2015 in Tarlee (34.281295^{o} S, 138.772695^{o} E), South Australia, as previously described (Garcia *et al.* 2019). Briefly, 568 spring wheat genotypes from 36 countries were selected to include elite cultivars, synthetic wheat, and landraces. Sowing was done in June of both years and one N fertiliser rate was added at sowing. The rainfall and temperature conditions were as previously described (Garcia *et al.* 2019).

At maturity, wheat was harvested and scored for yield components and GPC. The selection of extreme genotypes was made on the regression between average grain yield and average GPC calculated from the two-year field data as explained from previous research (Oury and Godin 2007). Briefly, the average grain yield and average GPD for all the genotypes for trials in both years were calculated. The values were then plotted in a quadrant and genotypes with high yield and contrasting GPD were selected. From both years, 28 genotypes with high yield and high GPC (positive GPD) and 32 genotypes with high yield and low GPC (negative GPD) were initially selected.

Field experiment at Balaklava under different N conditions

Subsequently, thirteen genotypes (six positive GPD and seven negative GPD) and two elite Australian cultivars, Spitfire and Gregory, that are both high yielding varieties with contrasting GPC, were grown in 2018 in Balaklava, South Australia (34.3391° S, 138.4755° E). The trial was sown on 15 May. Rainfall in 2018 was low at the start of the growing season at 33 mm and 21 mm, for June and July, respectively, with a wetter August at 54 mm, while the rest of the season was drier at 8 mm, 9 mm, 34 mm and 12mm for September, October, November and December. Monthly temperatures recorded during the growing season ranged from 14.1°C to 23.1°C at the start of the season, with a high of 30°C very late in the season (BOM 2019a; b).

The experimental design was a randomised complete block with each genotype replicated thrice and grown in three rows under two fertiliser treatments: no fertiliser application due to high residual N (30 mg N/kg of soil; CSBP Soil and Plant Analysis Laboratory) termed as low N; and a high N treatment consisting of two applications of urea (30 kg N/ha at tillering stage and 30 kg N/ha at booting stage). Superphosphate fertiliser was applied to all treatments at 100kg P/ha at sowing.

Semi-hydroponics experiment under different N concentration

The thirteen genotypes and two elite Australian cultivars Spitfire and Gregory were grown in a semi-hydroponic set-up in a greenhouse with controlled day/night temperatures of 22/15°C and relative humidity of 54/64%. The plants were grown in pots filled with diatomaceous rocks and housed in eight hydroponics trays, each connected to an 80-liter tank containing nutrient solution. Each tank had a water pump that pumped the solution in and out of the pots at intervals of 15 minutes. The nutrient solution used was a modified Johnson's solution (Johnson *et al.* 1957). There were

two N concentrations, low N (LN, 0.5 mM N) and high N (HN, 5 mM N). The LN solution contained the following nutrients in mM concentration (in brackets): $MgSO_4$ (2), KH_2PO_4 (0.1), H_3BO_3 (0.025), $MnSO_4$ (0.02), $ZnSO_4$ (0.02), $CuSO_4$ (0.0005), Na_2MoO_4 (0.0005), KCl (0.05), Fe-EDTA (0.05), $Ca(NO_3)_2$ (0.2) and KNO_3 (0.1). The HN solution contained, $MgSO_4$ (2), KH_2PO_4 (0.1), H_3BO_3 (0.025), $MnSO_4$ (0.02), $ZnSO_4$ (0.02), $CuSO_4$ (0.0005), Na_2MoO_4 (0.0005), KCl (0.05), Fe-EDTA (0.05), $Ca(NO_3)_2$ (1.5), KNO_3 (2), K_2SO_4 (1), $CaCl_2$ (2.45). The solutions were maintained at a pH of 5.9 and changed weekly.

The experimental design was a randomised complete block design with fifteen genotypes replicated four times, in two N concentrations: four LN trays and four HN trays until anthesis. At anthesis (when 50% of the main tiller's head had anthers extruded), individual plants (four replicates) from each initial concentration were switched, creating two additional treatments denoted as LN-HN and HN-LN. Switching was done across several days since genotypes reached anthesis at different times. Half of the plants (four replicates) were maintained in their original treatments of LN and HN. Switching allowed an independent study of the effects of LN and HN during vegetative and post-anthesis growth stages. The first harvest (four replicates) was done at anthesis.

Sampling protocol and physiological measurements

For field material, harvesting was done at maturity, where two 50 cm rows were harvested per plot. The heads were threshed, and the grain was cleaned and weighed. Grain was subsampled, dried in the oven at 65 °C for 72 hrs and ground using ball bearings (4 mm and 8 mm chrome steel) in a Geno Grinder (SPEX Sample Prep 2010, Metuchen, NJ, USA) at between 1000 rpm – 1400 rpm. A subsample of the fine powder

(100 mg) was encapsulated in N-free paper for N analysis using a nitrogen/protein analyser (Elementer Rapid N exceed®, Elementar Analysensysteme GmbHanalyser, Germany). Aspartic acid (100 mg, 10.52% N) and blank paper were used as standards to calibrate the analyser. The theoretical N% of aspartic acid (10.52) was divided by the N analyser's N% output to determine the N factor. The N% measured for each sample was corrected using this calculated N factor. Total N content in different plant tissues was calculated as plant part N% multiplied by dry weight (DW) $Ntotal = N\% \times DW$.

In the greenhouse, for the semi-hydroponics experiment, destructive harvests were made at anthesis and maturity for each genotype. Plants were separated into the main tiller and other tillers. The samples were then oven-dried at 65 °C for 72 hrs and separated into spike, leaf, and stem. Weights of each component were recorded (termed as biomass at anthesis), as well as tiller number and spikelets per spike. At maturity, the spike was further separated into chaff, rachis, and grain. The samples from both growth stages were ground to a fine powder using the method described above and analysed for N concentration. Translocation, defined as the N moved to the grain at maturity from vegetative parts, was calculated as *Total above-ground N at anthesis (plant N% × DW) – vegetative N at maturity (vegetative N%× DW)*, and in this definition, the post-anthesis plant N uptake and net N losses were not considered (Kong *et al.* 2016). Post-anthesis N uptake defined as the N taken up after anthesis was calculated as *Total N at maturity (plant N% × DW) – Total N at anthesis (plant N% × DW)* (Taulemesse *et al.* 2016).

Statistical analysis

The null hypothesis between means was tested using two-way ANOVA to analyse genotype and treatment effects, using the *Asreml* function in R version 3.6.0 and GraphPad Prism software. Tukey's Honest Significant Differences test was used to correct for multiple comparisons when differences were observed in ANOVA. Pearson product-moment correlations between different physiological traits were performed in R with data normality testing using a D'Agostino & Pearson normality test.

Results

Field yield components and grain N content

Selection of contrasting genotypes for GPC

Two field trials were conducted in 2014 and 2015 using a genetically diverse wheat panel comprising of 568 spring wheat genotypes from 36 countries including elite cultivars, synthetic wheat, and landraces (Garcia *et al.* 2019). The average grain yield from all genotypes in the field trial in 2014 and 2015 was 2.56±0.74 t/ha and 2.01±0.87 t/ha, respectively (Fig 1). In 2014, the average GPC from all genotypes was 12.1±1.16%, while in 2015, the average GPC was 14.7±1.70%. There was a negative correlation between grain yield and GPC in both years (Fig 1). The regression analysis carried out for consistently high yielding genotypes with contrasting GPC resulted in 28 genotypes with high GPC and 32 genotypes with low GPC in both years. Principal component analysis (PCA) from the 28 and 32 genotypes based on grain yield and GPC shows two distinct genotype clusters (Fig 2). A further selection was made using the means, standard deviation for both grain yield and GPD across both years. The

scaled values of grain yield and GPD were then calculated, averaged and plotted. From this plot, 13 genotypes with high yields and contrasting GPD were selected. The selected genotypes (Table 1) had values of GPD ranging from -1.73 to +1.45; these included both breeding lines and varieties, originating from Australia as well as other countries.

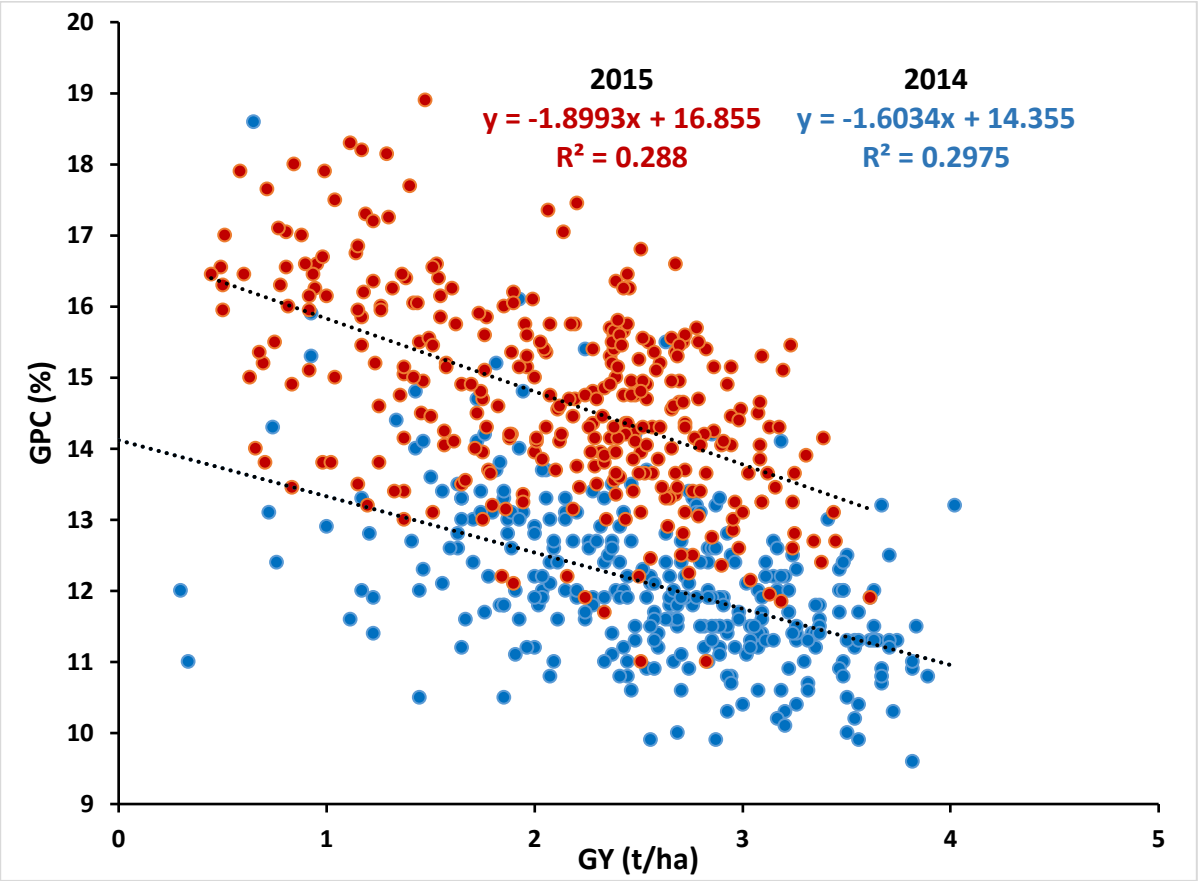

Figure 1: Field trial data for a set of 568 diverse spring wheat genotypes grown in two seasons at Tarlee, South Australia (Garcia et al. 2019). There was a negative correlation between GY (Grain Yield) and GPC (Grain Protein Content) for both years at R2= 0.29 in 2014 (blue) and R2= 0.28 in 2015 (red). Each data point represents the average yield and GPC values (n=2) for a specific genotype.

Table 1: Thirteen genotypes of wheat contrasting for GPD (ordered from the highest to the lowest GPD), selected from a genetic diversity field trial carried out in 2014 and 2015 in Tarlee, SA. GY (Grain Yield), GPC (Grain Protein Content), GPD (Grain Protein Deviation), type of wheat and the country of origin.

NAME	GY (T/HA)	GPC (%)	GPD	TYPE	COUNTRY OF ORIGIN	
6HRWSN98	3.04	14.20	1.45	Variety	Mexico	Positive GPD
RAC655	3.03	14.10	1.42	Breeding line	Australia	
KING ROCK	3.02	14.27	1.31	Variety	Australia	
ESPADA	3.46	13.67	1.06	Variety	Australia	
GENARO	3.05	13.90	0.87	Variety	Mexico	
CID473259	2.96	13.15	0.34	Breeding line	Mexico	
YECORA 70	3.64	12.07	-0.26	Variety	Chile	Negative GPD
H742A	3.44	11.55	-0.71	Variety	Israel	
KENNEDY	3.13	11.63	-0.98	Variety	Australia	
CID399062	3.15	11.38	-1.17	Breeding line	Mexico	
SILVERSTAR	3.17	11.50	-1.31	Variety	Australia	
CID82996	3.17	11.03	-1.56	Breeding line	Mexico	
N46	3.34	10.87	-1.73	Variety	Israel	

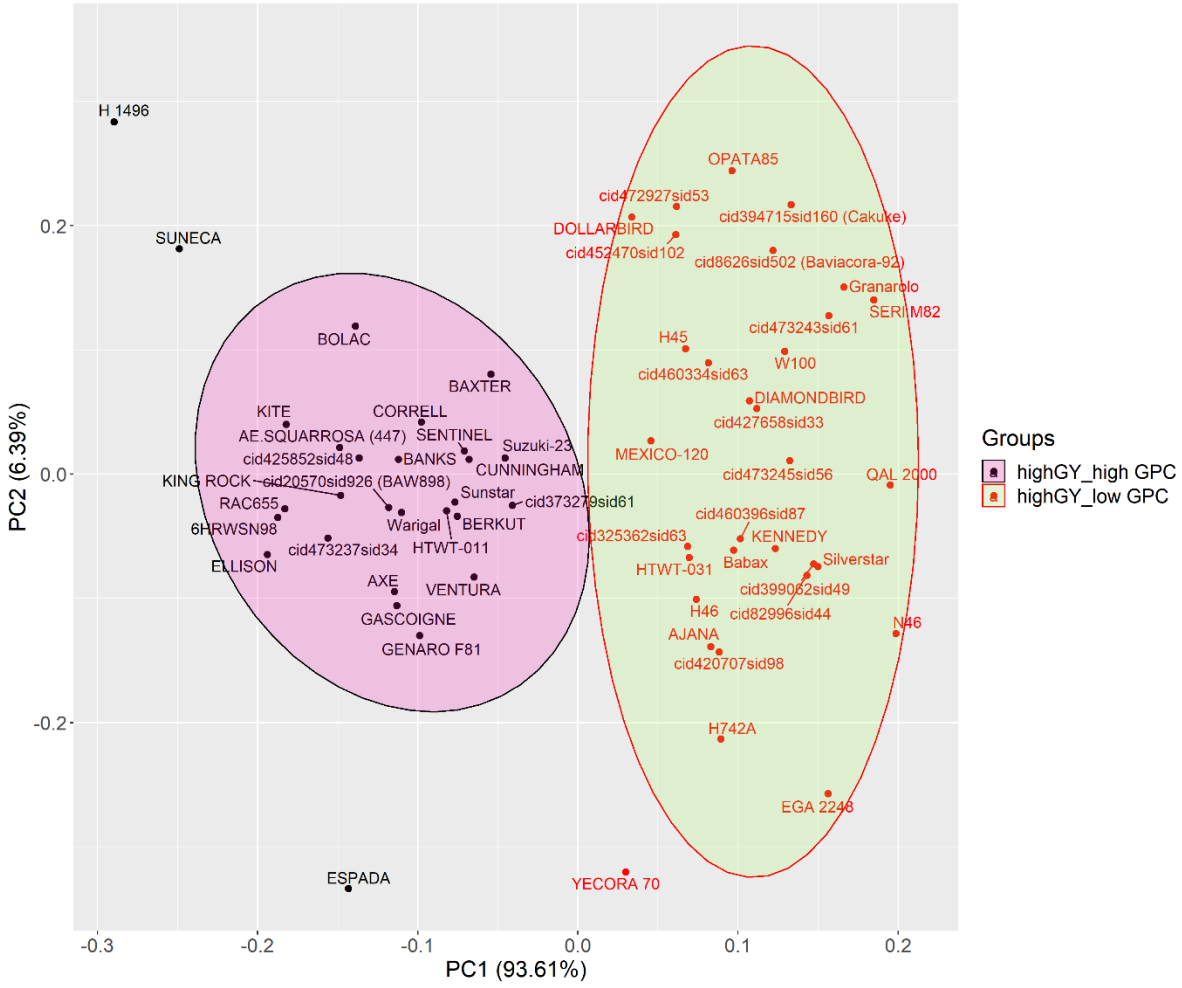

Figure 2: Dimension reduction using Principle Component Analysis (PCA) distinctly partitioned the genotypes with high yield and contrasting GPC identified in the Tarlee field trials in Table 1. The first principal component explains 93.61% of the variance among genotypes.

Grain yield and GPC in selected genotypes grown in the field

Thirteen high-yielding genotypes contrasting for GPD and two elite Australian cultivars (Gregory and Spitfire) were grown in field conditions under two N treatments in Balaklava, South Australia. In 2018, the wheat growing season was particularly dry, with winter rainfall below average for the northern and eastern areas of South Australia. (ABARES 2018). The average yield for 2018 was 0.70 t/ha (Fig 3).

The grain yield in YECORA was statistically lower compared to KINGROCK and GREGORY (Fig. 3a) under LN. Overall, at LN treatment, both the positive and negative GPD genotypes maintained a high yield. Under the HN treatment, there was no yield difference between the positive GPD and the negative GPD genotypes (Fig. 3b). Overall, there was no N response as seen from the lack of significant differences in yield between the two N treatments. Gregory, an Australian elite cultivar, maintained a high yield under both LN and HN.

For Grain N concentration, there were statistical differences between the genotypes in both N treatments. Under LN, 6HRWSN98 had a significantly high grain N concentration compared to SILVERSTAR, CID 82996sid44 and Gregory. Also, Spitfire had a significantly high grain N concentration compared to Gregory (Fig 3c). Under HN, 6HRWSN98 had a significantly high grain N concentration compared to SILVERSTAR, H472A and N46 (Fig 3d). In both N treatments, 6HRWSN98 and RAC655 maintained high grain N concentration for the positive GPD group (Fig 3c and d). Spitfire maintained a higher grain N concentration under both LN and HN treatments compared to Gregory. Genotypes that were significantly different for grain N concentration in both treatments are listed in Supplementary Table 1. Overall, there was no statistical difference between the LN and HN treatments for grain N concentration. However, in the two treatments, the two genotypes with the highest

grain N concentration in the positive GPD group, 6HRWSN98 and RAC655, also ranked the highest for GPC in the field trials in 2014 and 2015 (Table 1).

A regression analysis was done for grain yield and GPC for both the positive and negative GPD genotypes under LN and HN treatment (Fig. 4). Most of the positive GPD genotypes were above the regression line, while the negative GPD lines were under the regression line in both LN treatment (Fig. 4b) and HN treatment (Fig. 4b). 6HRWSN98, RAC655 and the elite Australian cultivar Spitfire are circled, and all three fall well above the regression line, in both LN and HN treatments. The regression though not high illustrates that in the Balaklava field trial the genotypes maintained contrasting GPD, which was similar to field results.

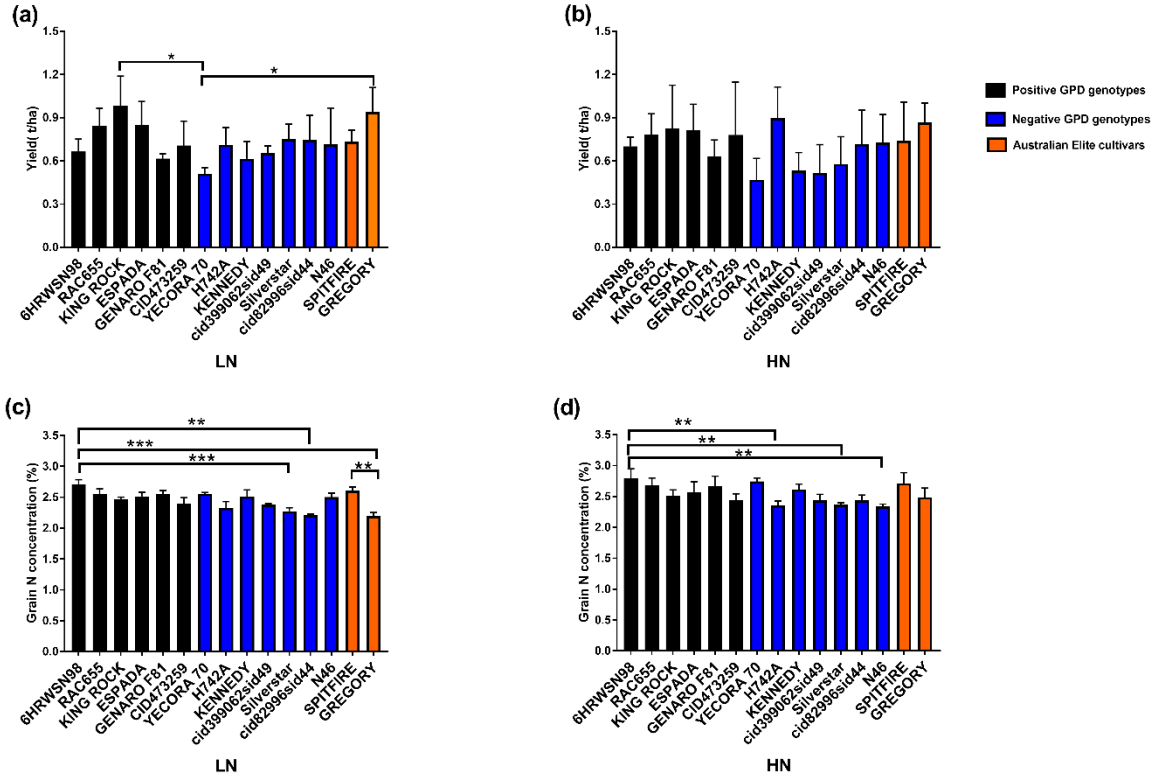

Figure 3: Yield and grain N concentrations for thirteen genotypes grown in a field trial at Balaklava, SA (2018). Data are shown for six genotypes identified in initial screening with positive GPD (black bars) and seven genotypes with negative GPD (blue bars), and two Australian elite cultivars (orange bars), each having three replicates. (a) Yield (t/ha) under LN treatment, (b) Yield (t/ha) under HN treatment, (c) Grain N concentration(%) under LN and (d) Grain N concentration(%) under HN. Error bars indicate the SEM of three replicates, and asterisks (*) indicate significant differences between the genotypes, (* P<0.01, ** P<0.001, *** P<0.0001).

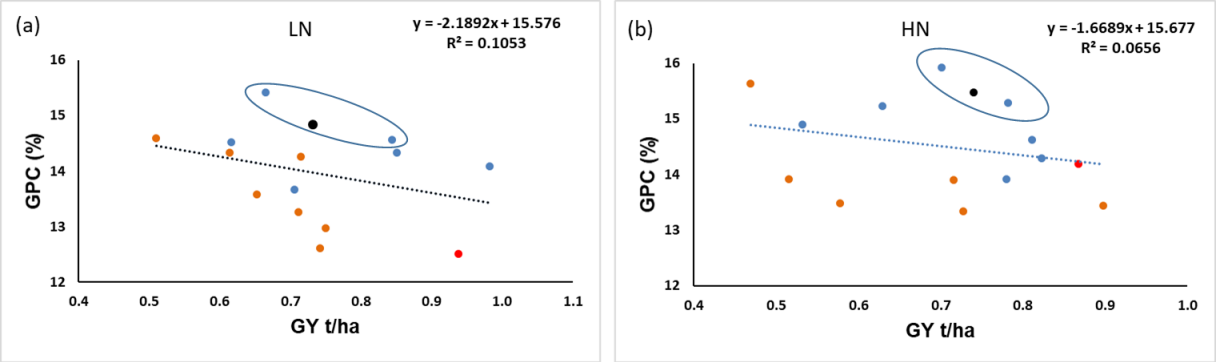

Figure 4: Relationship between GY (Grain yield) and GPC (Grain Protein Concentration) in a) LN, R2= 0.10, and b) HN, R2= 0.06 treatments from Balaklava field trial 2018. Blue dots represent, positive GPD genotypes, orange dots represent negative GPD genotypes, while black and red dots represent the two Australian elite cultivars Spitfire and Gregory respectively.

N utilization at anthesis stage, in contrasting genotypes grown in a controlled environment

To study the effects of N on yield and GPC in a more controlled way, a semi-hydroponics experiment was carried out in a greenhouse with the fifteen genotypes contrasting for GPD that had been grown prior, in field condition. Two N concentrations (LN (0.5mM) and HN (5mM)) were employed during vegetative growth. At anthesis, half of the plants were switched to create four N treatments: LN, LN-HN, HN, and HN-LN. Plants were harvested at anthesis and maturity and scored for yield components and grain N yield.

The biomass components (tiller numbers, total plant N and biomass) were significantly affected by the genotype and N treatments (Table 2). Biomass was significantly different between the genotypes and ranged from 2.34 g to 14.42 g in LN treatment and 2.18 g to 33.1 g under HN treatment. SILVERSTAR and 6HRWSN98 had the lowest biomass in both N treatments. Gregory was the most N responsive genotype, with an increase in biomass in response to HN of 2-fold (Table 2). The average biomass for the positive and negative GPD genotypes excluding the two elite cultivars

was 5.35 g and 6.23 g, respectively under LN, and 7.44 g and 8.58, g respectively under HN. The negative GPD genotypes had slightly higher biomass at anthesis compared to the positive GPD genotypes. Overall, biomass was significantly affected by the genotype by N treatment interaction. Spikelet number was significantly affected by genotype and N treatments (Table 2). There were significant differences in spikelet number between the fifteen genotypes Spikelet number ranged from 14.25 to 24.25 (LN) and 15.25 to 25.33 (HN). Gregory and GENARO had the highest spikelet number for both treatments. SILVERSTAR, 6HRWSN98, and ESPADA had the lowest spikelet number under both LN and HN treatments.

Overall, the negative GPD genotypes had a slightly higher spikelet number compared to the positive GPD genotypes.

Tiller numbers were significantly affected by both genotype and N treatment, and the interaction between the two (Table 2). Gregory and N46 had the highest tiller numbers, while SILVERSTAR, CID82996sid44, and 6HRWSN98 had the lowest tiller numbers. Genotypes with the largest differences for the tiller numbers between LN and HN treatments were YECORA 70 (□11), Genaro (□9.6), and Cid473259 (□8.2). Tiller number differences for the other genotypes between the two treatments ranged between 0.25 to 6.67. Overall, the positive GPD genotypes had slightly higher tiller numbers compared to the negative GPD genotypes.

Total N at anthesis significantly affected by genotype, N treatment, and the interaction between the two (Table 2). Total N at anthesis was N-responsive and increased in the HN treatment compared to LN for all the genotypes except for SILVERSTAR. Gregory had the highest total N under the two treatments: 397.52 mg N under LN, and 1035.97 mg N under HN. 6HRWSN98 had the lowest total N (79.12 mg N) under LN and second lowest (126.82 mg N) after SILVERSTAR under HN. Total N for the rest of the

genotypes ranged from 100.66 mg N to 301.63 mg N under LN, and 170.92 mg N to 531.82 mg N in HN. Overall, the negative GPD genotypes had a slightly higher total N compared to the positive GPD genotypes at anthesis

Grain yield and grain N concentration at maturity

Two additional N treatments were created at anthesis, with some plants transferred from LN to HN and vice versa, termed as LN-HN and HN-LN. This was done to create different N conditions early and late in the growing season. A crop may have high N (basal N from previous deposited soil N or high fertilization) early in the season and low N conditions post-anthesis, or start with a low basal N treatment and get a top-up with high N (post-anthesis fertilisation) later in the season. The treatments where plants were exposed to the same N concentrations throughout the growing period are termed LN and HN.

The measurements including grain weight, biomass, and harvest index (HI: the ratio of harvested grain to total shoot dry matter) for each treatment are presented in Table 3. Grain weight and biomass were significantly affected by genotype, N treatment, and the interaction between genotype and N treatment. CID473259 had the highest grain weight in LN and HN-LN treatments. Gregory had the highest grain weight in HN and LN-HN treatments. For biomass in all N treatments, RAC655 and CID473259 had the highest biomass. Gregory also maintained very high biomass across all N treatments. Overall, the positive GPD genotypes had higher grain weight and biomass across all the N treatments compared to the negative GPD genotypes. The negative GPD genotypes had a higher HI across all the N treatments compared to the positive GPD genotypes.

Table 2: Spikelet number, tiller number, total plant N and biomass at anthesis for fifteen wheat genotypes grown in semi-hydroponics under two N treatments (LN (0.5mM N) and HN (5mM N)). The values are means of four biological replicates ± standard error (SE). Statistical groups are ranked by post-ANOVA multiple comparison Tukey HSD test for p ≤0.05 and ranked using letters

Genotype	Biomass (g plant^{-1})		Spikelet number		Tiller number		Total plant N (mg plant^{-1})	
	LN	HN	LN	HN	LN	HN	LN	HN
6HRWSN98	2.70±0.42c	3.80±0.77cd	14.33±0.33gh	15.25±1.10e	2.66±0.66ef	4.75±1.65fg	79.12±11.64c	126.82±32.17cd
RAC655	7.28±0.93b	6.98±1.15c	16.75±0.62ef	16.66±0.76e	5.50±0.64bc	12.00±0.86ab	216.53±30.41bc	278.33±50.07bcd
KING ROCK	3.31±0.41c	6.95±1.04c	14.75±0.75gh	15.25±0.85e	5.00±0.91cd	10.00±1.92bc	110.00±9.35c	305.51±51.82bcd
ESPADA	5.43±0.77b	4.40±0.96cd	15.50±0.28fg	15.75±0.75e	4.00±0.40de	4.00±1.35gh	177.00±23.92bc	170.92±39.67cd
GENARO F81	5.97±1.07b	7.73±1.27c	24.25±0.65a	22.33±0.88ab	2.75±0.25ef	12.33±0.33ab	178.05±33.40bc	415.10±63.42bc
CID473259	7.41±1.22b	14.82±0.94bc	22.00±0.57ab	22.75±0.47ab	3.33±0.33ef	11.50±1.84b	251.63±38.03ab	524.48±83.04b
AVERAGE	**5.35**	**7.44**	**17.93**	**17.99**	**3.87**	**9.09**	**168.72**	**303.52**
YECORA	5.06±0.93b	9.04±0.65c	17.50±0.25de	18.25±0.25de	4.25±0.21de	15.25±0.47a	179.13±46.90bc	355.63±20.79bcd
H742A	3.36±0.43b	4.95±0.92c	16.66±0.33ef	18.33±0.66cd	3.33±0.33ef	10.00±1.52bc	100.66±15.02c	182.55±24.52cd
KENNEDY	5.09±0.92b	7.60±1.10c	20.00±0.57cd	19.50±0.28bc	3.00±0.00ef	6.50±0.86ef	146.33±25.67bc	263.65±42.81bcd
CID399062sid49	8.63±0.85b	9.79±1.78c	21.66±0.66b	21.75±1.03ab	4.33±0.88de	7.50±1.02de	269.30±32.86ab	411.50±70.69bc
SILVERSTAR	2.34±0.66c	2.18±0.32cd	14.25±0.62h	15.50±0.64e	1.75±0.47f	2.00±0.00fh	84.17±21.69c	80.28±11.19d
CID82996sid44	4.37±0.33b	9.22±1.39c	21.33±0.88b	23.75±0.478ab	2.00±0.00f	8.25±0.25cd	130.83±10.69bc	348.84±54.59bcd
N46	14.81±1.88a	17.316±0.51b	20.66±1.45bc	21.75±1.49ab	7.33±0.33ab	11.50±1.84b	301.63±23.16ab	531.82±59.17b
AVERAGE	**6.23**	**8.58**	**18.86**	**19.83**	**3.71**	**8.71**	**173.15**	**310.61**
SPITFIRE	5.94±1.04b	9.19±0.83c	18.25±1.03cd	19.66±0.88bc	5.00±0.57bc	11.33±0.66bc	177.88±34.91bc	343.65±37.63bcd
GREGORY	14.42±1.06a	33.15±5.02a	24.50±1.19a	25.33±2.02a	8.75±1.65a	14.33±1.88ab	397.52±61.72a	1035.97±68.94a
TOTAL AVERAGE	**6.40**	**9.80**	**18.82**	**19.45**	**4.19**	**9.41**	**186.65**	**358.33**
Genotype	***		***		***		***	
N Treatment	***		*		***		***	
Genotype × N Treatment			ns		***		***	

Treatments with the same letter are not significantly different. ns; not significant *Significant at the 0.05 probability level **Significant at the 0.01probability level ***Significant at the 0.001 probability level

Grain N concentration, total N and N harvest index (NHI: N in the grain as a proportion of total plant N) were significantly affected by both genotype and N treatment, while the interaction between the genotypes and N treatment had a significant effect on for total N and NHI (Table 4). Spitfire had the highest grain N concentration under LN and LN-HN treatments. YECORA and N46 had the highest grain N concentration under HN treatment, while Gregory had the highest grain N concentration under HN-LN treatment. Total plant N was high in RAC655 and Gregory under all N treatments. Kennedy had the highest NHI under LN treatment (0.86), Genaro had the highest NHI in LN-HN and HN (0.80 in both treatments) and HN, while Gregory had the highest NHI under HN-LN treatment (0.89) (Table 4). Grain N concentration and total N were higher in positive GPD varieties across all the N treatments compared to the negative GPD genotypes. The negative GPD genotypes had a higher NHI across all the N treatments compared to the positive GPD genotypes.

N partitioning at anthesis and maturity

At anthesis, N partitioning differences were observed in the tissues of the main tiller; stem, leaf, and head of the fifteen genotypes grown under LN and HN treatments (Fig. 5). Under LN, the leaf had the highest proportion of N ranging from 48.7% to 72.1%, followed by the stem (14.9% to 34.8%) (Fig. 5a). The spike had the least N (9.2% to 23.9%). CID473259, KING ROCK, and CID399062sid49 had more leaf N compared to other genotypes and comparatively lower stem and head N. Similar tissue N partitioning trends were observed for the HN treatment, where the leaf tissue had the highest proportion of N ranging from 34.1% to 68.3%, followed by the stem (21.3% to 43.7%) and head (8.1% to 22.1%) (Fig. 5b). SILVERSTAR had the lowest fraction of leaf N under both LN and HN treatments.

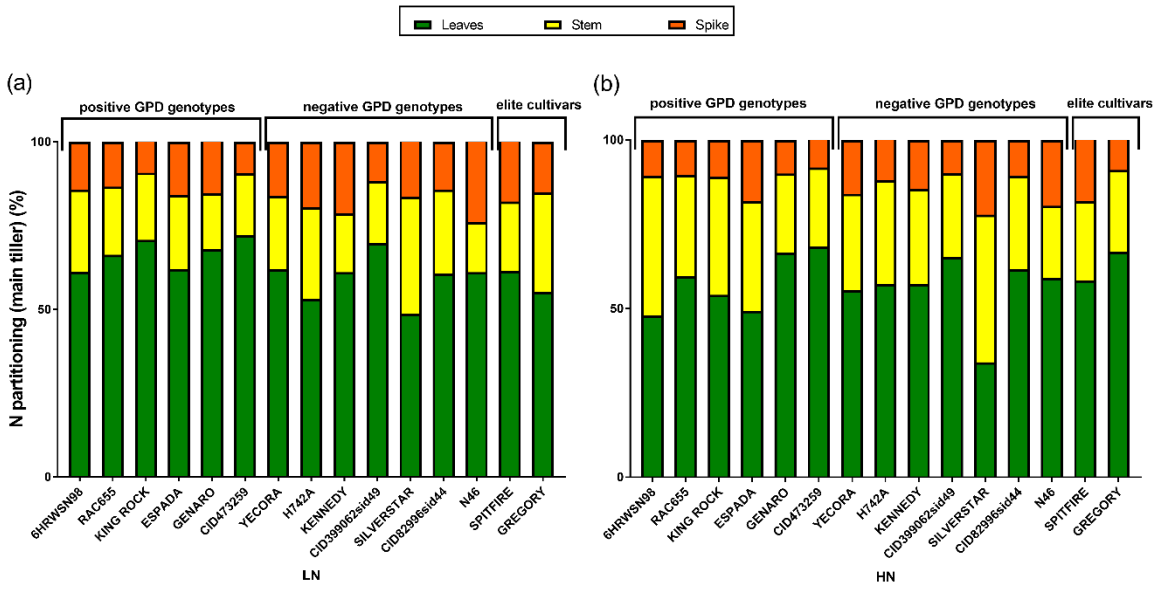

Figure 5: N partitioning at anthesis for the main tiller of fifteen genotypes, in three tissues: leaf (green bars), stem (yellow bars) and head (brown bars), under two N treatments. (a) LN treatment (0.5mM N) and (b) HN treatment (5mM N). Actual data values are provided in Supplementary Table 2.

Under LN, the positive GPD genotypes tended to have a slightly greater proportion of leaf N than the negative GPD genotypes, although this difference was not significant (Fig. 5a). Positive GPD genotypes had a smaller proportion of spike N, while the negative GPD genotypes, had a higher proportion of spike N. Overall, there was more consistency in the partitioning of N between the positive GPD varieties. Under HN, both positive and negative GPD genotypes had an increase in their stem N proportion compared to the LN treatment (Fig. 5b). The actual values of the N partitioning in the different tissues are in Supplementary table 2.

N partitioning in the main tiller was analysed at maturity for each of the four N treatments (Fig. 6). In the LN treatment, the proportion of total N in the grain ranged from 45.0% (SILVERSTAR) to 82.2% (GENARO) (Fig. 6a). There was a greater proportion of total N remaining in the chaff for this treatment compared to the HN, LN-HN and HN-LN treatments, with genotypes containing between 7% and 40% total N in the chaff component. Grain N partitioning in the LN-HN treatment ranged from 59.6% to 82.2% (Fig. 6b). Here, ESPADA (59.2%) and Gregory (59.6%) had the least grain

N partitioning, while Kennedy (82.2%) had the highest grain N partitioning. For the HN treatment, grain N partitioning range was 42.5% to 63.7% (Fig. 6c), whereby Spitfire (42.5%) and RAC655 (42.6%) had the least grain N partitioning, while CID473259 (63.7%) had the highest grain N partitioning. The rachis tissue had accumulated more N under HN treatment compared to all other N treatments, with between 7% and 33% of total N found in the rachis for HN plants. Under HN-LN the grain N partitioning range was 63.5% to 85.1% (Fig. 6d), with genotypes in this treatment overall accumulating a greater proportion of total N in the grain compared to the other three treatments. N46 (63.5%) and 6HRWSN98 (67.4%) had the least grain N partitioning while ESPADA (85.1%) and Kennedy (84.5%) had the highest grain N partitioning.

Overall, for the two switch treatments, LN-HN and HN-LN, there was a similar N partitioning in the different tissues and a consistently higher grain N partitioning across all the genotypes (Fig. 6b and d). There was no consistency in tissue partitioning in r the LN and HN treatments. Under HN treatment, there is more N remaining in the stem and rachis that is not remobilised to the grain. However, when plants are switched from HN-LN treatment, there was less rachis N and stem N. The N partitioning in the different tissues (grain, chaff, rachis, stem, and leaf) showing the exact amount in each tissue type are in supplementary table 3.

Post-anthesis N uptake (PANU) and translocation

Grain N comes from two sources: from vegetative tissues to the grains via remobilization/translocation and PANU directly to the grains. In this chapter as described above, the term translocation is used and defined as *Total above-ground N at anthesis (plant N% × DW) – vegetative N at maturity (vegetative N%× DW)*, and in.

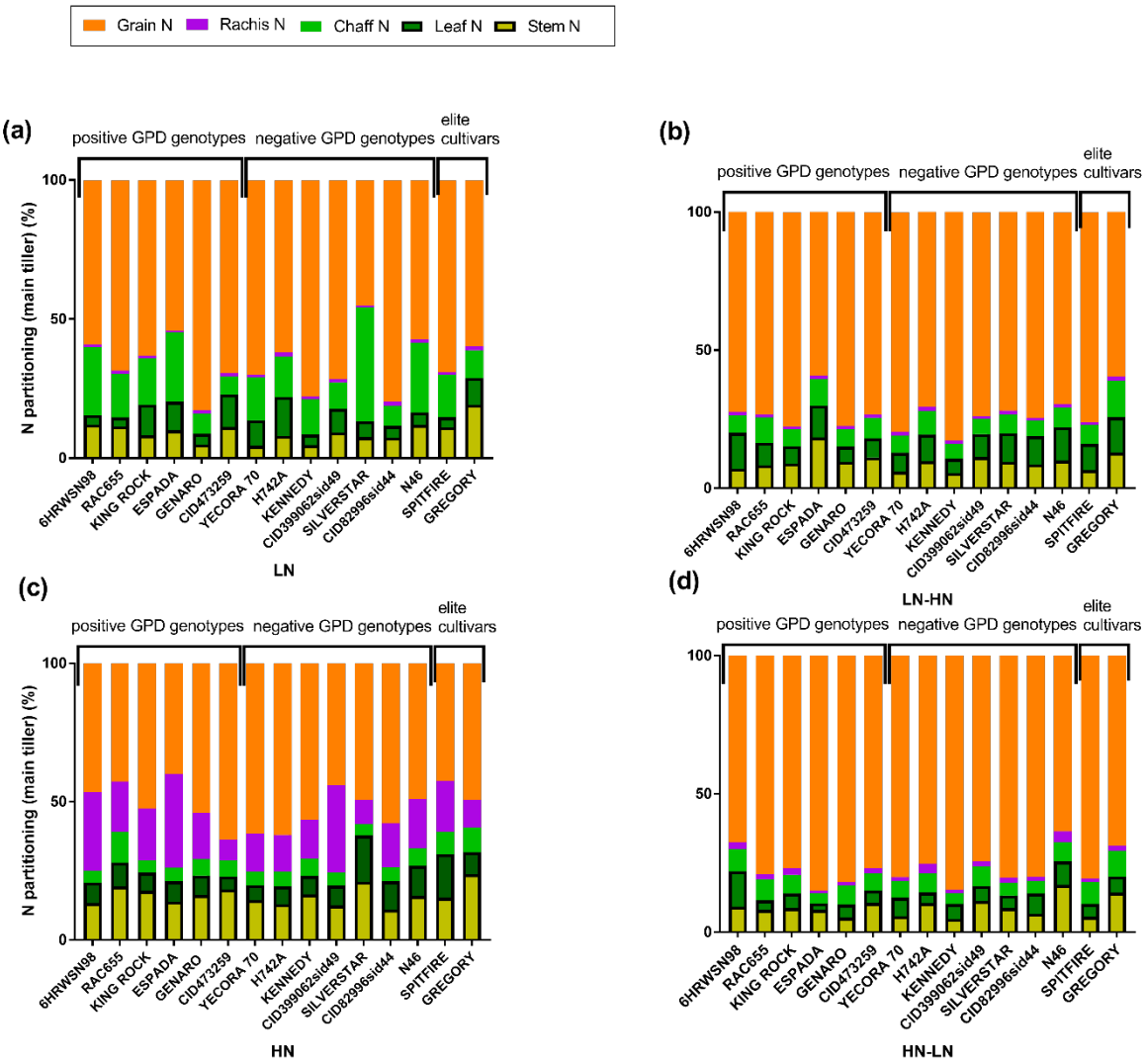

Figure 6: N partitioning in the grain, stem, leaf, chaff and rachis of the main tiller from fifteen genotypes of wheat grown in semi-hydroponics at (a) low N (LN; 0.5 mM N), (b) low N to high N (LN-HN; 0.5 mM – 5mM N, (c) high N (HN; 5 mM N), and (d) high N to low N (HN-LN; 5 mM – 0.5mM N), to maturity. Plants in LN-HN and HN-LN were grown in the first N treatment to anthesis, after which they were changed to the second N treatment. Actual data values are provided in Supplementary Table 3.

this definition, the post-anthesis plant N uptake and net N losses were not considered (Kong *et al.* 2016, Wu *et al.* 2018). PANU is defined as $Total\ N\ at\ maturity\ (N\% \times DW) - Total\ N\ at\ anthesis$ (Taulemesse *et al.* 2016, Wu *et al.* 2018). The calculations were made from whole plants. As shown in Table 5, for all four N treatments, positive GPD genotypes had a higher average PANU compared to negative GPD genotypes. Conversely, the average translocation for the negative genotypes was greater than for the positive GPD genotypes. There were some negative values for PANU, in some

genotypes which may have been due to coincidentally harvesting plants with higher biomass at anthesis (with higher total N) and harvesting smaller plants at maturity (with less total N) resulting in negative values. There were also some negative values for translocation for some genotypes which could be explained by the continued accumulation of non-grain tissue N after anthesis (which was assumed to be negligible in this definition of translocation)

Correlation of yield components and N components in different N treatments at maturity

To identify physiological traits that could be associated with an increase in both yield and GPC (grain N concentration (%) ×5.7), correlation analysis was carried out. The strength of the correlation between yield components and N yield components showed variation across the different N treatments (Figure 7, Supplementary Tables 4 - 8).

GPC under LN and LN-HN was highly correlated to total grain N content (grain N concentration (%) × grain weight), at r= 0.60, and r = 0.56, respectively, while under HN and HN-LN the correlation between GPC and total grain N content was much lower, at r = 0.13 and r = 0.33, respectively. Furthermore, GPC at LN and LN-HN was highly correlated to total plant N at maturity at r= 0.56 and r = 0.54, respectively (Fig. 7a and b). In the LN-HN treatment, GPC was also highly correlated with the sum of chaff and rachis weight, head weight at anthesis and total plant N at anthesis r = 0.69, r = 0.74, and r = 0.65, respectively) (Fig. 7b). Both GPC and grain weight were negatively correlated with HI and NHI in the LN and LN-HN treatments. However, this negative correlation was weaker in HN treatment for HI and positive for NHI, while under HN-LN treatment, the correlations were positive for both HI and NHI (Fig. 7c and d). Finally, under HN-LN treatment, GPC was correlated with shoot N concentration and positively correlated to NHI, at r = 0.57 and r = 0.75, respectively (Fig. 7d).

Total grain N content in the four treatments was strongly correlated with grain weight, biomass at maturity, total N at maturity and PANU (Fig. 7). Also, in HN and HN-LN, there was a high correlation between grain N content and chaff and rachis weight. For the LN-HN and HN-LN treatments, there was a high correlation between grain N content and biomass N, at r = 0.77 and r = 0.81, respectively.

Grain weight for all four N treatments was highly correlated with biomass at maturity, total plant N at maturity and PANU. However, for the HN and HN-LN treatments, grain weight was also highly correlated with chaff and rachis weight and total N at anthesis r=0.86 and r = 0.76 for HN, and r = 0.87 and r = 0.82, for HN-LN, respectively. Under HN-LN, there was also a high correlation between grain weight and N translocation r = 0.67. Overall, there was a high positive correlation between GPC and total grain N content, except in HN, as well as grain weight and biomass for the different N treatments.

Biomass was strongly negatively correlated with HI and NHI at LN r = - 0.68 and r = - 0.68, respectively (Fig. 7a). These correlations were weaker for the other N treatments but still tended to be negative. There was a strong negative correlation between post-anthesis N uptake and translocation for all the N treatments r = - 0.74, r = - 0.76, r = - 0.80 and r = - 0.43 for LN, LN-HN, HN and HN-LN respectively.

Table 5: Mean for the translocation and post anthesis N uptake (PANU) (whole plant)) of fifteen wheat genotypes grown in semi-hydroponics in four different N treatments (LN, LN-HN, HN, and HN-LN; see main text for treatment explanation). Translocation (N mg plant−1) is calculated as (Total above-ground N at anthesis – vegetative N at maturity). PANU (N mg plant−1) is calculated as (Total above-ground N at maturity – Total above-ground N at anthesis).

Genotype	**LN**		**LN-HN**		**HN**		**HN-LN**	
	Translocation (N mg plant^{-1})	**PANU (N mg plant^{-1})**	**Translocation (N mg plant^{-1})**	**PANU (N mg plant^{-1})**	**Translocation (N mg plant^{-1})**	**PANU (N mg plant^{-1})**	**Translocation (N mg plant^{-1})**	**PANU (N mg plant^{-1})**
Positive GPD genotypes	38.10	215.16	25.71	247.59	-35.78	413.04	148.58	303.33
Negative GPD genotypes	118.01	94.88	35.12	216.62	148.54	198.97	190.71	212.36

Table 3: Yield components; Grain weight, Biomass and HI (Harvest index) of fifteen wheat genotypes (whole plant) grown in semi-hydroponics under four N treatments (LN, LN-HN, HN and HN-LN; see main text for treatment explanation). The values are means of four biological replicates ± standard error (SE).

Genotype	Grain weight (g plant^{-1})				Biomass (g plant^{-1})				HI			
	LN	LN-HN	HN	HN-LN	LN	LN-HN	HN	HN-LN	LN	LN-HN	HN	HN-LN
6HRWSN98	3.87±0.20ef	6.69±0.31cd	5.70±0.34bc	7.61±0.64de	6.93±0.40fg	11.01±0.57d	10.39±1.01de	14.69±0.00de	0.56±0.04ab	0.65±0.02a	0.56±0.07a	0.51±0.04bc
RAC655	8.77±1.04bc	6.70±2.29cd	11.61±2.22bc	19.43±3.71abcd	28.56±1.07a	15.05±4.10cd	47.16±9.79ab	44.49±7.23abc	0.30±0.03c	0.42±0.03de	0.24±0.02b	0.42±0.02c
KINGROCK	8.78±0.36b	7.15±0.82cd	8.78±0.40bc	9.98±0.51cde	17.85±1.29cd	14.91±2.15cd	20.29±0.43cde	21.70±1.08cde	0.49±0.02abc	0.48±0.01bcd	0.43±0.01ab	0.46±0.05c
ESPADA	6.04±0.78de	4.63±0.49 d	6.50±2.42bc	10.22±0.43cde	11.86±1.11ef	10.51±2.23d	13.52±4.08cde	19.15±0.44de	0.53±0.11ab	0.46±0.06cd	0.46±0.04ab	0.53±0.01bc
GENARO	5.24±0.58de	7.06±0.84cd	10.69±1.21bc	12.45±2.00abcde	11.30±0.01ef	12.09±1.19cd	19.40±2.17cde	23.77±5.02bcde	0.51±0.05abc	0.52±0.05bcd	0.61±0.13a	0.52±0.02bc
CID473259	11.39±0.19a	12.77±1.51ab	18.43±1.63ab	25.77±1.75ab	23.07±3.01b	25.57±1.22b	42.00±6.61ab	46.72±4.08ab	0.51±0.05abc	0.50±0.04bcd	0.49±0.12a	0.55±0.01b
AVERAGE	**7.35**	**7.50**	**10.29**	**14.24**	**16.60**	**14.86**	**25.46**	**28.42**	**0.48**	**0.51**	**0.47**	**0.50**
YECORA	6.79±2.18bcd	5.34±1.17d	5.94±1.20bc	15.24±3.43abcde	12.60±4.05e	9.07±1.65d	12.19±1.26cde	26.79±5.50bcde	0.54±0.02ab	0.57±0.02ab	0.47±0.05ab	0.56±0.01bc
H742A	6.29±1.08cde	4.30±0.51d	7.95±0.55bc	7.53±0.35e	11.51±2.50ef	9.65±1.52d	14.05±2.41cde	14.04±0.88cde	0.55±0.02ab	0.45±0.02cde	0.62±0.02a	0.54±0.02bc
KENNEDY	3.79±0.19ef	6.48±1.10cd	10.60±2.29bc	10.17±1.41cde	6.30±0.32fg	11.31±2.17d	20.79±3.51cde	18.67±1.34de	0.60±0.00a	0.57±0.01ab	0.50±0.02a	0.53±0.03bc
CID399062sid49	7.49±0.19bcd	13.07±1.09ab	15.67±0.93ab	20.82±0.59abc	15.16±1.91de	23.65±1.36b	35.21±3.80abc	38.78±1.95abcd	0.50±0.05abc	0.55±0.11abc	0.45±0.11ab	0.53±0.01bc
SILVERSTAR	2.70±0.31f	7.09±0.99cd	3.80 ±1.18c	7.42±1.02e	5.01±0.62g	12.20±0.82cd	6.66±1.27e	13.16±1.51e	0.54±0.00abc	0.57±0.04ab	0.54±0.07a	0.56±0.03b
CID82996sid44	7.87±0.46bcd	7.35±0.29cd	8.48±0.41bc	13.78±2.57bcde	15.67±0.80cde	14.74±1.39cd	16.96±0.63cde	21.04±2.68de	0.50±0.04abc	0.50±0.03bcd	0.50±0.03a	0.64±0.04a

N46	7.59±0.18bcd	6.18±0.42cd	7.47±1.09bc	7.98±1.04de	15.29±0.26de	11.85±0.86d	17.27±2.37cde	18.45±1.59de	0.49±0.00abc	0.52±0.04bcd	0.43±0.02ab	0.43±0.04c
AVERAGE	**6.62**	**7.30**	**9.38**	**12.99**	**13.85**	**13.98**	**21.28**	**24.81**	**0.51**	**0.52**	**0.49**	**0.52**
SPITFIRE	7.71±0.47bcd	9.59±0.88bc	8.22±1.00bc	12.95±0.91cde	15.47±0.58cde	19.27±2.30bc	20.26±1.52cde	25.42±1.49bcde	0.49±0.02abc	0.50±0.02bcd	0.43±0.02ab	0.50±0.01bc
GREGORY	8.05±0.29bcd	13.37±1.73a	24.20±4.30a	24.86±4.73a	20.81±1.66bc	36.42±3.81a	53.55±5.95a	57.18±10.78 a	0.39±0.01bc	0.36±0.01e	0.45±0.03ab	0.43±0.02c
TOTAL AVERAGE	**6.662308**	**7.293077**	**9.355385**	**12.95385**	**13.93154**	**13.97**	**21.22231**	**24.72692**	**0.509231**	**0.52**	**0.484615**	**0.521538**
Genotype			***				***				***	
N Treatment			***				***				ns	
Genotype×N Treatment			***				***				ns	

Treatments with the same letter are not significantly different. HI = N Harvest Index. ns; not significant *Significant at the 0.05 probability level **Significant at the 0.01probability level ***Significant at the 0.001 probability level

Table 4: N components: Grain N concentration, Total plant N and NHI (N Harvest index) of fifteen wheat genotypes (whole plant) grown in semi-hydroponics under four N treatments (LN, LN-HN, HN and HN-LN; see main text for treatment explanation). The values are means of four biological replicates ± standard error (SE).

Genotype	Grain N concentration (%)				Total N (mg plant^{-1})				NHI			
	LN	LN-HN	HN	HN-LN	LN	LN-HN	HN	HN-LN	LN	LN-HN	HN	HN-LN
6HRWSN98	2.86±0.09 bc	3.31±0.06 bc	2.76±0.05 c	2.98±0.03ab	145.30±6.72cd	316.34±30.12d	234.62±22.2cd	301.81±24.56c	0.76±0.03a	0.71±0.07a	0.67±0.01ab	0.75±0.01b
RAC655	3.37±0.35 abc	3.75±0.10 ab	3.61±0.02 abc	3.43±0.08ab	629.12±53.46a	406.76±27.66cd	1373.31±261.37a	1007.97±177.47ab	0.46±0.03b	0.60±0.03 ab	0.30±0.03c	0.65±0.02b
KINGROCK	3.69±0.22 ab	3.60±0.22 bc	3.50±0.06 abc	3.44±0.71ab	488.90±31.96ab	394.81±42.54cd	564.45±14.83 bcd	513.04±13.50 bc	0.66±0.01a	0.65±0.03 ab	0.54±0.02abc	0.66±0.06b
ESPADA	3.55±0.88 abc	3.61±0.14 abc	3.59±0.08 abc	3.30±0.08ab	281.65±34.80bcd	288.59±55.16d	376.65±26.31 bcd	443.20±26.45c	0.71±0.01a	0.60±0.07 ab	0.60±0.04abc	0.76±0.02b
GENARO	3.42±0.52 abc	3.82±0.16 ab	3.70±0.13 abc	4.10±0.86ab	233.58±7.36bcd	337.32±47.22d	528.15±39.16 bcd	469.93±98.17 bc	0.84±0.01a	0.80±0.05 a	0.80±0.00a	0.81±0.02a
CID473259	2.91±0.25 bc	3.72±0.00 ab	3.52±0.36 bc	3.07±0.03ab	479.39±62.98ab	717.70±66.73b	1151.43±161.52ab	985.37±60.15 ab	0.70±0.03a	0.66±0.01ab	0.57±0.04abc	0.80±0.04b
AVERAGE	3.30	3.64	3.45	3.39	376.32	410.25	704.77	620.22	0.69	0.67	0.58	0.74
YECORA	3.25±0.16 abc	3.35±0.07 bc	4.42±0.79 a	3.54±0.07ab	474.89±68.46ab	233.04±43.85d	368.10±17.40 bcd	541.48±48abc	0.78±0.01a	0.75±0.04 a	0.66±0.02ab	0.78±0.01b
H742A	3.02±0.04 abc	3.41±0.07 bc	3.21±0.73 bc	3.09±0.08ab	253.97±52.96bcd	230.57±36.60d	368.15±68.09 bcd	303.65±9.03c	0.76±0.02a	0.61±0.01 ab	0.67±0.01ab	0.76±0.01b
KENNEDY	2.79±0.07 bc	3.40±0.08 bc	3.56±0.13 abc	3.33±0.21ab	122.20±5.54cd	287.01±61.39d	521.40±96.21 bcd	435.34±32.31 c	0.86±0.01a	0.78±0.02 a	0.71±0.01ab	0.76±0.01b
CID399062sid49	2.83±0.27 bc	3.19±0.10 c	2.99±0.62 bc	2.73±0.08b	275.33±39.16bcd	584.71±11.38bc	955.56±102.94ab	705.66±34.15 bc	0.78±0.03a	0.71±0.04 a	0.51±0.12ab	0.80±0.02b
SILVERSTAR	2.81±0.03 bc	3.57±0.31 bc	3.14±0.16 bc	3.00±0.11ab	108.89±15.25d	362.06±56.17cd	167.61±36.19 d	304.15±41.14 c	0.70±0.01a	0.71±0.05 a	0.68±0.07ab	0.73±0.03b

CID82996sid44	2.53±0.15 c	3.19±0.09 c	3.21±0.50 bc	2.83±0.28ab	267.03±5.24bc d	351.31±40.33cd	490.42±91.22 bcd	471.56±108.64bc	0.75±0.07a	0.67±0.04 a	0.70±0.03a b	0.83±0.03 b
N46	3.02±0.03 abc	3.56±0.18 abc	3.85±0.46 ab	4.11±0.30a	352.60±12.93a bd	342.77±32.05d	574.80±3943 bcd	443.01±58.90 c	0.65±0.01a	0.64±0.05 ab	0.47±0.02a bc	0.74±0.07 b
AVERAGE	**2.87**	**3.35**	**3.42**	**3.09**	**250.39**	**341.45**	**478.54**	**460.31**	**0.77**	**0.71**	**0.66**	**0.78**
SPITFIRE	3.96±0.58 a	3.95±0.19 a	3.65±0.22 abc	3.22±0.18ab	438.37±67.86a bc	582.03±55.73bc	503.37±21.72 bcd	566.81±56.44 bc	0.70±0.01a	0.65±0.03 ab	0.58±0.02a bc	0.73±0.01 b
GREGORY	3.42±0.50 abc	3.91±0.20 a	3.19±0.05 bc	4.28±0.36a	513.68±61.76a b	942.07±27.80a	1487.22±80.51a	1178.54±208.53a	0.53±0.04b	0.45±0.07 c	0.52±0.01a bc	0.89±0.07 b
TOTAL AVERAGE	**3.16**	**3.56**	**3.46**	**3.36**	**337.66**	**425.14**	**644.35**	**578.10**	**0.71**	**0.67**	**0.60**	**0.76**
Genotype	***				***				***			
NTreatment	**				***				***			
Genotype×NTreatment	ns				***				*			

Treatments with the same letter are not significantly different. NHI = N Harvest Index. ns; not significant *Significant at the 0.05 probability level **Significant at the 0.01probability level ***Significant at the 0.001 probability level

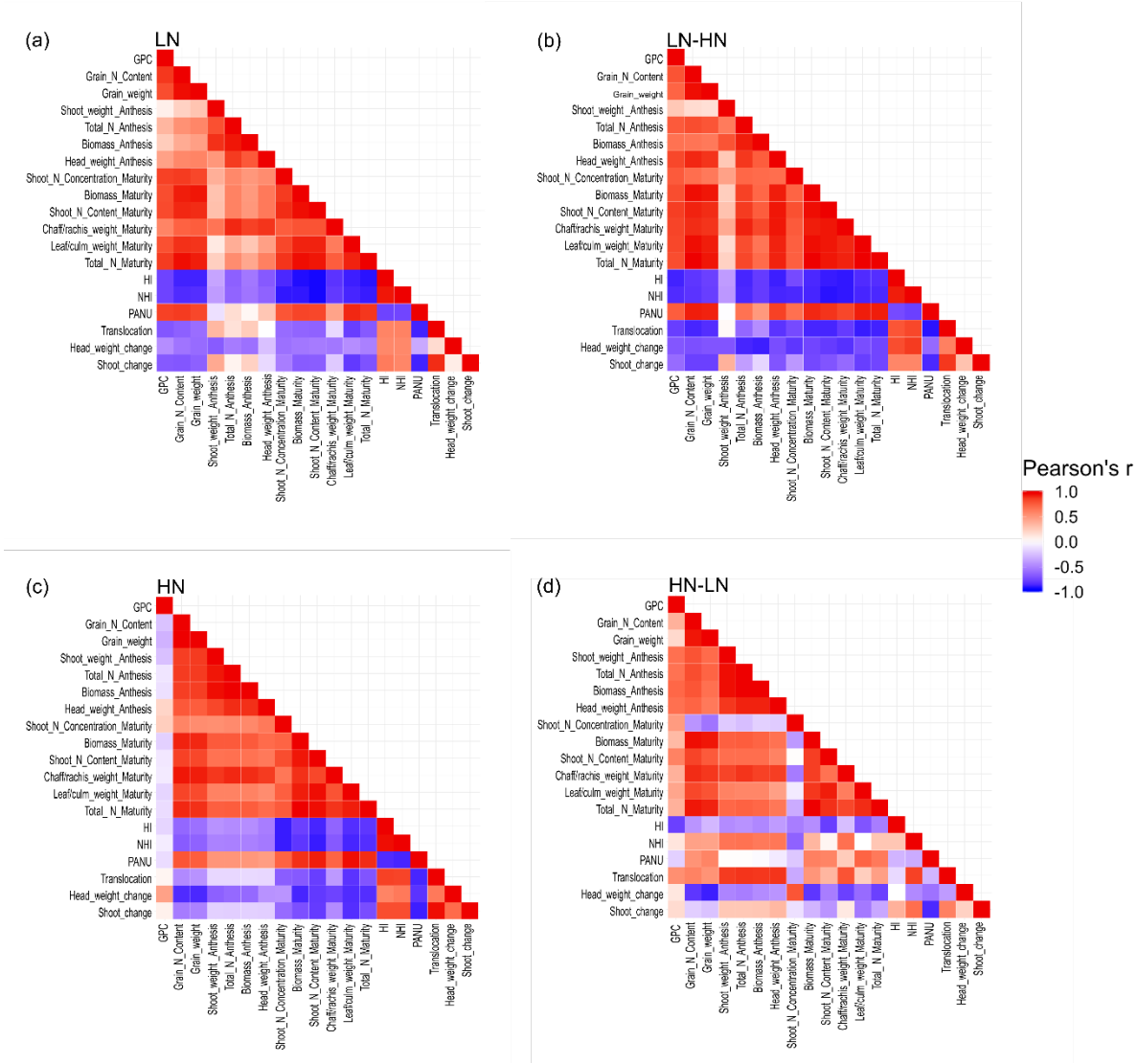

Figure 7: Correlation matrices of different physiological traits measured for fifteen wheat genotypes grown in semi-hydroponics under (a) LN, (b) LN-HN, (c) HN and (d) HN-LN treatments (see main text for treatment and trait explanations); Grain Protein Content (GPC)(%), Grain N content (mg plant-1), Grain weight (g), Shoot weight at anthesis (g), Total N at anthesis (mg plant-1), biomass at anthesis (g), spike weight at anthesis (g) shoot N concentration at maturity (%), biomass at maturity (g), shoot N content at maturity (mg plant-1), chaff/rachis weight (g), leaf/culm weight at maturity (g), total N at maturity (mg plant-1), HI, NHI, PANU (mg plant-1) and translocation (mg plant-1), spike weight change between anthesis and maturity (g), shoot weight change between anthesis and maturity (g). The red gradient colouration represents a positive correlation while the blue gradient colouration represents the negative correlation between traits. Stronger colours indicate stronger correlations, as indicated in the legend.

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Discussion

The selection of genotypes that have both high grain yield and high GPC (positive GPD) has been a difficult target to achieve in wheat breeding due to a negative correlation between the two traits (Slafer *et al.* 1990). Previous studies have tried to explain the physiological basis of the negative relationship (Bhatia and Rabson 1976, Bogard *et al.* 2010, Dhugga and Waines 1989), without universal acceptance (Simmonds 1995). The reasons for this lack of a consensus view of the underlying physiological traits include a poor understanding of the negative relationship and a lack of single trait measurement for both grain yield and GPC (Pushman and Bingham 1976). However, some wheat cultivars have a higher GPC than is expected from their grain yield (Law and Payne 1983) (Monaghan *et al.* 2001). This study aimed to identify physiological traits associated with positive GPD using genetically diverse wheat grown in the field and controlled conditions. Field trials in 2014 and 2015 at a single location showed differences in average yield and average GPC between seasons (Garcia *et al.* 2019). These differences likely resulted from the different rainfall and temperature conditions during the two growing seasons. There was a negative correlation between grain yield and GPC for both trial years, similar to previous studies (Fig. 1) (Simmonds 1995).

An initial selection of 28 genotypes and 32 genotypes contrasting for GPD but with consistently higher grain yield was done from a two-year field trial. Further analysis using PCA confirmed that indeed the genotypes were clustering into two distinct groups (Fig. 2). Thirteen high yielding genotypes that were contrasting for GPC and GPD were finally selected for more in-depth experiments (Table 1). The genotypes were mostly from countries with low rainfall, including Australia.

N effects on contrasting GPD wheat genotypes

The yield from a field trial conducted at Balaklava in 2018 was particularly low compared to the 2014 and 2015 field trials in Tarlee, due to very low rainfall during the growing season (BOM 2019a). There was no N treatment effect on either grain yield or grain protein content. Since the residual soil N analysis showed a high soil N at 30 mg/kg of soil, it is assumed that high residual N may have obscured an N treatment effect. Furthermore, water stress might have obscured the effect of N treatment (BOM 2019a). Plant-available soil N is mobile in water, and dry conditions will be unaccessible for uptake (Bogard *et al.* 2010).

Under the two N treatments, both the positive and negative GPD genotypes maintained similar yields (Fig. 3a and b). This is as expected since the initial selection was based on similarity in high yielding genotypes with contrasting GPC. For GPC, some genotypes in both positive and negative GPD genotypes maintained a high GPC across multiple field trials. From the Balaklava field trial, the genotypes with the highest GPC in the positive GPD group; 6HRWSN98 and RAC655 also ranked the highest for GPC in the field trials in 2014 and 2015. In the negative GPD group, YECORA 70 and KENNEDY with the highest grain N concentration in the Balaklava field trial, similar to the Tarlee 2014 and 2015 field trials. Overall, from the regression analysis (Fig 4), the genotypes remained within their classification (positive or negative GPD) when grown in Balaklava, confirming their earlier selection and categorization into the two groups.

We also grew the contrasting genotypes using a semi-hydroponic set up to better control N availability. This also allowed for the switching of N treatments at anthesis, presenting different N levels in the post-anthesis stage, a difficult undertaking in a soil setup (Taulemesse *et al.* 2016). Previous studies have shown that available plant N

before anthesis is invested towards yield, while N taken up after anthesis increases grain N concentration (Brown and Petrie 2006, Ottman *et al.* 2000). The regulation of N uptake is driven by plant N demand in addition to soil N availability (Feil 1997, Imsande and Touraine 1994, Sadras and Rodriguez 2010).

In the semi-hydroponic experiment, total N at anthesis was heavily influenced by N supply. Plants in the HN treatment had a greater total N compared to the LN treatment, which was similar to previous findings whereby N status at anthesis is determined by N supply (Dhugga and Waines 1989, Gaju *et al.* 2014). Total N at anthesis was higher in negative GPD genotypes, and these genotypes also had a greater biomass accumulation compared to positive GPD varieties (Table 3). N partitioning at anthesis was more consistent in positive GPD genotypes compared to negative GPD genotypes, with proportionally more N accumulation in vegetative parts (leaf and stem) compared to the spike (Fig. 5). This may be due to the positive GPD group having fewer spikelets (Table 3; although the difference between groups was not significant). In previous research, both leaf, and stem had similarly high N for both tissues at anthesis (Gaju *et al.* 2014).

At maturity, CID473259 and Gregory maintained a high grain weight and biomass across each of the four treatments, (LN, LN-HN, HN, and HN-LN) (Table 3). These two genotypes also had the highest biomass and total N at anthesis, which likely contributed to the grain weight (Table 3). Previous research also describes the effect of total N at anthesis remobilised from non-grain plant parts on grain yield (Taulemesse *et al.* 2016). Grain weight was highly correlated with total N at anthesis, PANU and total N at maturity (Fig 7). The correlations between grain weight and total N at anthesis under LN LN-HN, HN and HN-LN treatments were r = 0.46, r = 0.76, r = 0.76 and r = 0.82, respectively, and suggest an effect of pre-anthesis N treatment on the total N

accumulated up to anthesis, followed by the use of this N in determining grain weight (Barbottin *et al.* 2005, Cox *et al.* 1985, Taulemesse *et al.* 2016).

There was a strong correlation between PANU and grain weight in LN (r=0.71) and LN-HN (r=0.69) treatments, compared to HN (r=0.44) and HN-LN (r=0.35). This could be explained by the availability of pre-anthesis N reducing the need for N uptake of post-anthesis N under HN and HN-LN treatments. Plants absorbed more N under HN treatment before anthesis resulting in a high N remobilization that gradually decreased N uptake capacity in the post-flowering period as observed by Oscarson and colleagues in wheat grown in a hydroponic set-up (Oscarson *et al.* 1995). The correlation of total N at maturity with grain weight was also high in all N treatments between r=0.78 and r=0.89). From the correlation analysis, important physiological traits that may be useful in the selection of high yielding genotypes, are elucidated. Overall, grain weight, biomass, grain N concentration and total N at maturity was generally higher in positive GPD genotypes compared to negative GPD varieties. Interestingly, HI and NHI tended to be higher in negative GPD varieties (Table 3 and 4). This may be due to the genotypes investing more assimilates into grain yield relative to biomass, with slightly more bias towards carbon than N.

N partitioning at maturity was quite consistent for the positive and negative GPD genotypes in the LN-HN and HN-LN treatments (Fig. 6). However, in the LN treatment there was a higher proportion of chaff N compared to other N treatments, while in HN, there was a higher proportion of rachis N. These high levels of rachis N may be due to excess N deposition, while the high levels of chaff N in LN treatment could be due to restricted remobilization of N from the chaff to the grain tissue also reflected in the low grain N concentration (%) in LN (Table 5).

The positive GPD genotypes generally had a higher PANU compared to negative GPD genotypes (Table 5). These genotypes had low biomass which likely rules out efficient translocation as the source of high GPC. PANU may therefore, explain the high grain N concentration (Table 4). These results confirm earlier findings whereby, PANU had a positive effect on grain N compared to grain yield (Monaghan *et al.* 2001) (Kichey *et al.* 2007) (Bogard *et al.* 2010) (Gooding and Davies 1992). Also, previous findings suggested that despite the low correlation between GPC and PANU, there was a strong correlation between early PANU and GPC (Taulemesse *et al.* 2016). In this experiment, only one harvest was conducted at maturity, and a correlation with early PANU could not be tested. PANU, having shown correlation to GPC may be vital in the improvement of GPC translating to grain quality in modern wheat varieties.

The wheat gene *NAM-B1* discovered in previous research was linked to high GPC, as a positive regulator of nutrient translocation leading to increased N remobilised to grain tissue (Distelfeld *et al.* 2006, Uauy *et al.* 2006). However, the functional allele of the gene is rare in elite wheat cultivars (Tabbita *et al.* 2017). The selected lines used in this experiment were also negative for the *NAM-B 1* gene (data not shown).

At maturity, the greatest proportion of the tissue N was found in grains, except under HN treatment, where there was lower grain N proportion due to an increased N partitioning into rachis and chaff. Moreover, under HN, grain N content was highly correlated to chaff and rachis weight. This could be due to the HN available before anthesis increasing chaff and rachis tissue weight and tissue N (Cox *et al.* 1985). Chaff and rachis may be important tissues of N deposition that determine grain N in the different genotypes.

Genotypic differences in N utilization for grain yield and grain N

There were genotypic differences for grain yield and GPC in field-grown wheat. Comparing results from field trials in Tarlee in 2014 and 2015 and Balaklava in 2018, some genotypes maintained a consistently high grain yield and grain N concentration. Among the positive GPD genotypes, 6HRWSN98 and RAC655 maintained a high grain N concentration in the three field trials in Tarlee and Balaklava. Interestingly, in the controlled semi-hydroponics condition, 6HRWSN98 had low biomass, tiller number, and spikelet number at anthesis and had a lower yield compared to other positive GPD varieties.

Previous research has shown that some high GPD genotypes may tend to invest in less biomass, therefore the N is distributed in a small plant reducing the N dilution factor (Rahimi Eichi *et al.* 2019). Interestingly, positive GPD genotypes had fewer spikelets at anthesis compared to the negative GPD genotypes, which could explain the high grain N concentration from less N investment on yield components. RAC655, on the other hand, maintained a high spikelet number at anthesis, while still producing a high grain N concentration. 6HRWSN98 and RAC655 genotypes had higher PANU compared to translocation across the four N treatments. The two genotypes could therefore be potential candidates to further study the different N utilization before anthesis and after anthesis for a high yield and high GPC.

YECORA 70 had the lowest yield under LN and HN treatments in the field, and, on average, high grain N concentration. This, as per previous findings (Gallagher *et al.* 1983), is likely due to high remobilisation and efficient translocation of N from shoot to the grain after anthesis. In this study, translocation (Table 3) in YECORA 70 was higher

in all N treatments compared to PANU except under LN treatment where PANU was higher than translocation.

Overall, the yield difference between the positive and negative GPD varieties was not significant, which is as expected as their selection was based on high yields (Fig. 3a and b). Gregory, an Australian elite cultivar, was high yielding in both field and controlled conditions (Fig. 3 and Table 3). In the field, Spitfire had a significantly high grain N concentration under LN treatment compared to HN treatment, similar to previous research (Rahimi Eichi *et al.* 2019).

In the controlled semi-hydroponics experiment, the genotypes with the highest yield components at anthesis (biomass, tiller numbers, and spikelet number) (Table 2) did not necessarily have the highest yield at maturity (Table 3), indicating that PANU may be important in the final determination of grain yield. However, since in a semi-hydroponic setup there are ideally no water limitations, this conclusion needs confirmation in a Mediterranean environment where moisture stress is sometimes a limiting factor, especially in the post-anthesis growth stage of wheat. Similar N partitioning at anthesis may be due to a similarly high yield potential of the fifteen genotypes, creating a similar tissue N allocation pre-anthesis in the positive and negative GPD genotypes. The biomass of RAC655 and CID473259 was not very high at anthesis but these two genotypes had the greatest biomass at maturity. This may be an indication that the two genotypes can take up more N after anthesis for accumulation in biomass as observed from previous studies and this study (Borrell *et al.* 2001). The biomass in the HN treatment at anthesis ranged between 2.18 g to 33.1 g (Table 2). This wide range could have been due to the differences in development among the contrasting genotypes, with those accumulating high biomass also having delayed heading and flowering. In this experiment, a wide duration of the anthesis

dates among the genotypes before the switch to different N treatments was observed (data not shown).

In conclusion, these findings highlight the complexity in identifying key physiological traits associated with both high grain yield and grain N concentration. However, some physiological traits were strongly correlated with the two important agronomic traits (grain yield and grain N concentration) in multiple environments, and further studies on the traits using elite cultivars and genetically diverse wheat could bring us closer to demystifying the negative correlation between grain yield and GPC.

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References

ABARES (2013). Agricultural commodity statistics 2013.

ABARES (2018). Agricultural Commodities_2018_seasonal conditions.

ABARES (2019). Agricultural commodities report 2019.

Acreche, M. M. and G. A. Slafer (2009). "Variation of grain nitrogen content in relation with grain yield in old and modern Spanish wheats grown under a wide range of agronomic conditions in a Mediterranean region." The Journal of Agricultural Science 147(06): 657-667.

Barbottin, A., C. Lecomte, C. Bouchard and M.-H. Jeuffroy (2005). "Nitrogen remobilization during grain filling in wheat." Crop science 45(3): 1141-1150.

Bhatia, C. and R. Rabson (1976). "Bioenergetic considerations in cereal breeding for protein improvement." Science 194(4272): 1418-1421.

Bogard, M., V. Allard, M. Brancourt-Hulmel, E. Heumez, J. M. Machet, M. H. Jeuffroy, P. Gate, P. Martre and J. Le Gouis (2010). "Deviation from the grain protein concentration-grain yield negative relationship is highly correlated to post-anthesis N uptake in winter wheat." J Exp Bot 61(15): 4303-4312.

BOM (2019a). Rainfall Data 2017 and 2018. 47086292. South Australia, Australian Government.

BOM (2019b). Temperature Data 2017 and 2018. 47086221. South Australia, Australian Government. IDCJAC0010

Borrell, A., G. Hammer and E. Van Oosterom (2001). "Stay-green: A consequence of the balance between supply and demand for nitrogen during grain filling?" Annals of Applied Biology 138(1): 91-95.

Brown, B. D. and S. Petrie (2006). "Irrigated hard winter wheat response to fall, spring, and late season applied nitrogen." Field Crops Research 96(2-3): 260-268.

Cox, M. C., C. O. Qualset and D. W. Rains (1985). "Genetic variation for nitrogen assimilation and translocation in wheat. I. Dry matter and nitrogen accumulation." Crop Sci 25(3): 430-435.

Dhugga, K. S. and J. G. Waines (1989). "Analysis of Nitrogen Accumulation and Use in Bread and Durum Wheat." Crop Science 29(5): 1232-1239.

Distelfeld, A., C. Uauy, T. Fahima and J. Dubcovsky (2006). "Physical map of the wheat high-grain protein content gene Gpc-B1 and development of a high-throughput molecular marker." New Phytol 169(4): 753-763.

FAO (2019). Global fertilizer production and agricultural use 2002 - 2017. Rome, Food and Agriculture Of The United Nation.

Feil, B. (1997). "The inverse yield-protein relationship in cereals: possibilities and limitations for genetically improving the grain protein yield." Trends Agron 1(103): e119.

Gaju, O., V. Allard, P. Martre, J. Le Gouis, D. Moreau, M. Bogard, S. Hubbart and M. J. Foulkes (2014). "Nitrogen partitioning and remobilization in relation to leaf senescence, grain yield and grain nitrogen concentration in wheat cultivars." Field Crops Research 155: 213-223.

Gallagher, L. W., K. M. Soliman, D. Rains, C. Qualset and R. Huffaker (1983). "Nitrogen Assimilation in Common Wheats Differing in Potential Nitrate Reductase Activity and Tissue Nitrate Concentrations 1." Crop science 23(5): 913-919.

Garcia, M., P. Eckermann, S. Haefele, S. Satija, B. Sznajder, A. Timmins, U. Baumann, P. Wolters, D. E. Mather and D. Fleury (2019). "Genome-wide association mapping of grain yield in a diverse collection of spring wheat (Triticum aestivum L.) evaluated in southern Australia." PloS one 14(2): e0211730.

Gooding, M. J. and W. P. Davies (1992). "Foliar urea fertilization of cereals: A review." Fertilizer Research 32(2): 209-222.

Hirel, B., J. Le Gouis, B. Ney and A. Gallais (2007). "The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches." Journal of Experimental Botany 58(9): 2369-2387.

Imsande, J. and B. Touraine (1994). "N demand and the regulation of nitrate uptake." Plant physiology 105(1): 3.

Johnson, C., P. Stout, T. C. Broyer and A. B. Carlton (1957). "Comparative chlorine requirements of different plant species." Plant and soil 8(4): 337-353.

Kichey, T., B. Hirel, E. Heumez, F. Dubois and J. Le Gouis (2007). "In winter wheat (Triticum aestivum L.), post-anthesis nitrogen uptake and remobilisation to the grain correlates with agronomic traits and nitrogen physiological markers." Field Crops Research 102(1): 22-32.

Kong, L., Y. Xie, L. Hu, B. Feng and S. Li (2016). "Remobilization of vegetative nitrogen to developing grain in wheat (Triticum aestivum L.)." Field Crops Research 196: 134-144.

Law, C. and P. Payne (1983). "Genetical aspects of breeding for improved grain protein content and type in wheat." Journal of Cereal Science 1(2): 79-93.

Monaghan, J. M., J. W. Snape, A. J. S. Chojecki and P. S. Kettlewell (2001). "The use of grain protein deviation for identifying wheat cultivars with high grain protein concentration and yield." Euphytica 122(2): 309-317.

Munier, J., N. G and C. Salon (2005). "Are the carbon costs of seed production related to the quantitative and qualitative performance? An appraisal for legumes and other crops." Plant, Cell & Environment 28(11): 1388-1395.

Oscarson, P., T. Lundborg, M. Larsson and C.-M. Larsson (1995). "Genotypic differences in nitrate uptake and nitrogen utilization for spring wheat grown hydroponically." Crop Science 35(4): 1056-1062.

Ottman, M. J., T. A. Doerge and E. C. Martin (2000). "Durum Grain Quality as Affected by Nitrogen Fertilization near Anthesis and Irrigation During Grain Fill." Agronomy Journal 92(5): 1035-1041.

Oury, F.-X. and C. Godin (2007). "Yield and grain protein concentration in bread wheat: how to use the negative relationship between the two characters to identify favourable genotypes?" Euphytica 157(1): 45-57.

Pushman, F. M. and J. Bingham (1976). "The effects of a granular nitrogen fertilizer and a foliar spray of urea on the yield and bread-making quality of ten winter wheats." The Journal of Agricultural Science 87(2): 281-292.

Rahimi Eichi, V., M. Okamato, S. M. Haefele, N. Jewell, C. Brien, T. Garnett and P. Langridge (2019). "Understanding the Interactions between Biomass, Grain Production and Grain Protein Content in High and Low Protein Wheat Genotypes under Controlled Environments." Agronomy 9(11): 706.

Raun, W. R. and G. V. Johnson (1999). "Improving nitrogen use efficiency for cereal production." Agronomy journal 91(3): 357-363.

Sadras, V. and D. Rodriguez (2010). "Modelling the nitrogen-driven trade-off between nitrogen utilisation efficiency and water use efficiency of wheat in eastern Australia." Field Crops Research 118(3): 297-305.

Simmonds, N. W. (1995). "The relation between yield and protein in cereal grain." Journal of the Science of Food and Agriculture 67(3): 309-315.

Slafer, G. A., F. H. Andrade and S. E. Feingold (1990). "Genetic improvement of bread wheat (Triticum aestivum L.) in Argentina: relationships between nitrogen and dry matter." Euphytica 50(1): 63-71.

Sylvester-Bradley, R. and D. R. Kindred (2009). "Analysing nitrogen responses of cereals to prioritize routes to the improvement of nitrogen use efficiency." Journal of Experimental Botany 60(7): 1939-1951.

Tabbita, F., S. Pearce and A. J. Barneix (2017). "Breeding for increased grain protein and micronutrient content in wheat: Ten years of the GPC-B1 gene." Journal of Cereal Science 73: 183-191.

Taulemesse, F., J. Le Gouis, D. Gouache, Y. Gibon and V. Allard (2016). "Bread Wheat (Triticum aestivum L.) Grain Protein Concentration Is Related to Early Post-Flowering Nitrate Uptake under Putative Control of Plant Satiety Level." PLoS ONE 11(2): e0149668.

Uauy, C., A. Distelfeld, T. Fahima, A. Blechl and J. Dubcovsky (2006). "A NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat." Science 314(5803): 1298-1301.

Wang, B., D. L. Liu, G. J. O'Leary, S. Asseng, I. Macadam, R. Lines-Kelly, X. Yang, A. Clark, J. Crean, T. Sides, H. Xing, C. Mi and Q. Yu (2018). "Australian wheat production expected to decrease by the late 21st century." Global Change Biology 24(6): 2403-2415.

Wu, H., J. Xiang, Y. Zhang, Y. Zhang, S. Peng, H. Chen and D. Zhu (2018). "Effects of Post-Anthesis Nitrogen Uptake and Translocation on Photosynthetic Production and Rice Yield." Scientific Reports 8(1): 12891.

Supplementary Tables

Supplementary Table 1: Significant grain N concentration (%) differences under LN and HN treatment for thirteen selected genotypes grown in a field experiment in Balaklava in 2018. A Tukey's multiple comparison test shows the statistical significance between genotypes at $p \leq 0.05$.

Grain N concentration (%) LN	
Tukey's multiple comparisons tests	**P-value**
SPITFIRE vs. cid82996sid44	*
GREGORY vs. YECORA 70	*
GREGORY vs. GENARO F81	*
GREGORY vs. RAC655	*
YECORA 70 vs. cid82996sid44	*
Grain N concentration (%) HN	
Tukey's multiple comparisons tests	**P-value**
SPITFIRE vs. H742A	*
SPITFIRE vs. N46	*
SPITFIRE vs. SILVERSTAR	*
YECORA 70 vs. H742A	*
YECORA 70 vs. N46	*
YECORA 70 vs. SILVERSTAR	*

Supplementary Table 2: N (mg per plant) in different parts of the main tiller sampled at anthesis: leaf, stem, and spike, for fifteen wheat genotypes grown semi-hydroponically in two N treatments. (a) LN treatment (0.5mM N) and (b) HN treatment (5mM N)

LN					HN			
Genotype	**Leaves N (mg)**	**Stem N (mg)**	**Spike N (mg)**		**Genotype**	**Leaves N (mg)**	**Stem N (mg)**	**Spike N (mg)**
6HRWSN98	26.34	10.54	6.11		6HRWSN98	20.02	4.43	17.32
RAC655	53.14	16.32	10.69		RAC655	45.76	7.91	23.13
KINGROCK	40.90	11.60	5.33		KINGROCK	36.93	7.47	23.84
ESPADA	32.97	11.68	8.48		ESPADA	31.26	11.52	20.52
GENARO	67.40	16.60	15.19		GENARO	80.24	11.94	28.14
CID473259	111.73	28.82	14.45		CID473259	106.17	12.69	36.39
YECORA	58.03	20.46	15.12		YECORA	34.39	9.90	17.73
H742A	22.87	11.70	8.42		H742A	36.27	7.56	19.39
KENNEDY	52.44	15.04	18.36		KENNEDY	50.95	12.91	25.00
CID399062sid49	83.29	22.01	13.98		CID399062sid49	88.91	13.34	33.85
SILVERSTAR	22.18	15.85	7.49		SILVERSTAR	12.65	8.22	16.23
CID82996sid44	51.40	21.19	12.05		CID82996sid44	68.52	11.87	30.76
N46	52.94	12.98	20.72		N46	61.10	20.16	22.07
SPITFIRE	44.34	14.87	12.86		SPITFIRE	46.62	14.49	18.70
GREGORY	62.75	33.75	17.06		GREGORY	113.21	15.06	40.98

Supplementary Table 3: N (mg per plant) in parts of the main tiller for fifteen wheat genotypes grown semi-hydroponically in different N treatments and sampled at maturity: grain, stem, leaf, chaff, and rachis. (a) low N (LN; 0.5 mM N) treatment, (b) low N to high N (LN-HN; 0.5 mM – 5mM N treatment, (c) high N (HN; 5 mM N) treatment, and (d) high N to low N (HN-LN; 5 mM – 0.5mM N treatment). The LN and HN plants were grown in the same N concentration up to maturity while the switched plants in LN-HN and HN-LN were grown in the same concentration (either LN or HN) up to anthesis when the switch was made.

LN						**LN-HN**					
	Stem N (mg)	**Leaf N (mg)**	**Chaff N (mg)**	**Rachis N (mg)**	**Grain N (mg)**		**Stem N (mg)**	**Leaf N (mg)**	**Chaff N (mg)**	**Rachis N (mg)**	**Grain N (mg)**
6HRWSN98	0.73	0.21	1.48	0.07	3.60	**6HRWSN98**	0.45	0.82	0.40	0.07	4.57
RAC655	0.87	0.24	1.19	0.09	5.22	**RAC655**	0.66	0.64	0.73	0.09	5.80
KING ROCK	0.68	0.92	1.38	0.08	5.25	**KING ROCK**	0.64	0.45	0.45	0.07	5.61
ESPADA	0.94	0.96	2.36	0.05	5.10	**ESPADA**	0.95	0.59	0.50	0.06	3.04
GENARO	0.50	0.39	0.75	0.11	8.45	**GENARO**	1.17	0.68	0.77	0.12	9.47
CID473259	1.56	1.65	0.89	0.20	9.74	**CID473259**	1.61	1.00	1.08	0.17	10.58
YECORA 70	0.33	0.70	1.16	0.08	5.30	**YECORA 70**	0.44	0.50	0.46	0.09	5.88
H742A	0.59	1.02	1.07	0.11	4.55	**H742A**	0.63	0.61	0.54	0.11	4.49
KENNEDY	0.37	0.31	1.03	0.08	6.34	**KENNEDY**	0.54	0.50	0.54	0.10	8.12
CID399062 sid49	0.98	0.91	1.02	0.13	7.62	**CID399062 sid49**	1.03	1.10	0.73	0.15	7.69
SILVERSTAR	0.63	0.48	3.46	0.06	3.80	**SILVERSTAR**	0.85	0.63	0.42	0.06	5.56
CID82996sid44	0.62	0.35	0.62	0.12	6.67	**CID82996sid44**	0.91	1.07	0.59	0.11	7.82
N46	0.73	0.28	1.54	0.08	3.53	**N46**	0.79	0.95	0.57	0.10	5.51
SPITFIRE	1.01	0.33	1.39	0.09	6.27	**SPITFIRE**	0.65	0.95	0.69	0.10	7.60
GREGORY	1.97	0.99	1.03	0.13	6.14	**GREGORY**	1.33	1.30	1.35	0.15	6.11

HN						HN-LN					
	Stem N (mg)	Leaf N (mg)	Chaff N (mg)	Rachis N (mg)	Grain N (mg)		Stem N (mg)	Leaf N (mg)	Chaff N (mg)	Rachis N (mg)	Grain N (mg)
6HRWSN98	0.73	0.41	0.23	1.57	2.56	**6HRWSN98**	0.44	0.61	0.38	0.12	3.21
RAC655	1.39	0.62	0.81	1.31	3.06	**RAC655**	0.59	0.25	0.57	0.13	5.81
KING ROCK	1.29	0.49	0.32	1.36	3.84	**KING ROCK**	0.54	0.33	0.43	0.15	4.79
ESPADA	0.92	0.49	0.33	2.26	2.67	**ESPADA**	0.67	0.20	0.31	0.07	7.14
GENARO	1.25	0.53	0.46	1.27	4.14	**GENARO**	0.54	0.49	0.71	0.12	8.39
CID473259	2.85	0.73	0.92	1.16	9.97	**CID473259**	1.33	0.57	0.79	0.24	9.71
YECORA 70	1.05	0.39	0.36	1.01	4.49	**YECORA 70**	0.42	0.49	0.45	0.09	5.85
H742A	0.96	0.49	0.40	0.99	4.63	**H742A**	0.53	0.19	0.36	0.17	3.80
KENNEDY	1.61	0.66	0.60	1.39	5.54	**KENNEDY**	0.58	0.65	0.47	0.15	10.18
CID399062 sid49	2.44	1.94	0.48	1.03	5.73	**CID399062 sid49**	1.09	0.58	0.59	0.22	10.13
SILVERSTAR	0.78	0.44	0.30	1.96	2.74	**SILVERSTAR**	0.64	0.30	0.40	0.10	4.22
CID82996sid44	0.93	0.86	0.42	1.35	4.88	**CID82996sid44**	0.63	0.69	0.45	0.13	7.61
N46	1.34	0.90	0.55	1.48	4.12	**N46**	1.04	0.52	0.43	0.24	3.87
SPITFIRE	1.36	1.38	0.72	1.63	3.77	**SPITFIRE**	0.52	0.44	0.74	0.13	7.57
GREGORY	2.71	0.91	1.01	1.14	5.62	**GREGORY**	1.60	0.65	1.03	0.22	7.71

Supplementary Table 4: Correlation matrix of different physiological traits under LN treatment; GPC (%), Grain N content (mg plant^{-1}), Grain weight (g), Shoot N%, Shoot N (mg plant^{-1}), Biomass Maturity (g), Chaff/rachis weight (g), HI, NHI, Total plant N Maturity (mg plant^{-1}), Total plant N Anthesis (mg plant^{-1}), Biomass Anthesis (g), Shoot weight Anthesis (g), Head weight Anthesis (g), PANU (mg plant^{-1}) and Translocation (mg plant^{-1}). See Supplementary table 8 for correlation p-values.

LN	GPC (%)	Grain N content (mg plant^{-1})	Grain weight (g)	Shoot N%	Shoot N (mg plant^{-1})	Biomass Maturity (g)	Chaff/rachis weight (g)	HI	NHI	Total plant N Maturity	Total plant N Anthesis	Biomass Anthesis (g)	Shoot weight Anthesis (g)	Head weight Anthesis (g)	PANU
GPC (%)															
GRAIN N CONTENT (MG PLANT^{-1})	0.60														
GRAIN WEIGHT (G)	**0.14**	**0.87**													
SHOOT N%	0.46	0.49	0.31												
BIOMASS N (MG PLANT^{-1})	0.41	0.70	0.60	0.68											
BIOMASS MATURITY (G)	0.31	**0.84**	**0.85**	0.41	**0.88**										
CHAFF/RACHIS WEIGHT (G)	0.19	0.56	0.52	0.47	0.71	0.69									
HI	**-0.50**	-0.43	-0.23	-0.41	-0.76	-0.68	-0.46								
NHI	**-0.14**	-0.35	-0.34	-0.75	**-0.86**	-0.65	-0.63	0.70							
TOTAL PLANT N MATURITY	0.56	0.92	0.78	0.64	**0.91**	**0.92**	0.66	0.65	0.65						
TOTAL PLANT N ANTHESIS	0.18	0.48	0.46	0.33	0.54	0.57	**0.92**	0.38	0.46	0.54					
BIOMASS ANTHESIS (G)	0.15	0.38	0.35	0.35	0.48	0.47	0.85	0.36	0.45	0.45	0.95				
SHOOT WEIGHT ANTHESIS (G)	0.04	0.17	0.19	0.30	0.25	0.24	0.52	0.21	0.33	0.22	0.68	**0.86**			
HEAD WEIGHT ANTHESIS (G)	0.27	0.48	0.37	0.27	0.58	0.55	**0.87**	0.42	0.41	0.56	**0.81**	0.66	0.18		
PANU	0.19	0.76	0.71	0.62	0.68	0.75	0.18	0.32	0.59	0.76	-0.04	-0.13	-0.12	0.05	
TRANSLOCATION	-0.09	-0.21	-0.12	-0.42	-0.41	-0.28	0.24	0.26	0.43	-0.32	0.51	0.51	0.41	0.34	**-0.74**

Supplementary Table 5: Correlation matrix of different physiological traits under LN-HN treatment; GPC (%), Grain N content (mg plant^{-1}), Grain weight (g), Shoot N%, Shoot N (mg plant^{-1}), Biomass Maturity (g), Chaff/rachis weight (g), HI, NHI, Total plant N Maturity (mg plant^{-1}), Total plant N Anthesis (mg plant^{-1}), Biomass Anthesis (g), Shoot weight Anthesis (g), Head weight Anthesis (g), PANU (mg plant^{-1}) and Translocation (mg plant^{-1}). See supplementary table 8 for correlation p-values.

LN-HN	GPC (%)	Grain N content (mg plant^{-1})	Grain weight (g)	Shoot N%	Shoot N (mg plant^{-1})	Biomass Maturity (g)	Chaff/rachis weight (g)	HI	NHI	Total plant N Maturity	Total plant N Anthesis	Biomass Anthesis (g)	Shoot weight Anthesis (g)	Head weight Anthesis (g)	PANU
GPC (%)															
Grain N content (mg plant^{-1})	0.56														
Grain weight (g)	0.39	**0.97**													
Shoot N%	0.11	0.14	0.10												
Biomass N (mg plant^{-1})	0.47	**0.77**	0.72	0.44											
Biomass Maturity (g)	0.47	**0.93**	**0.92**	0.19	**0.92**										
Chaff/rachis weight (g)	0.69	0.90	**0.84**	0.05	**0.93**	**0.92**									
HI	**-0.39**	-0.21	-0.14	-0.19	-0.62	-0.46	-0.44								
NHI	**-0.25**	-0.26	-0.22	-0.62	-0.73	-0.51	-0.44	0.82							
Total plant N maturity	0.54	**0.94**	**0.89**	0.31	0.94	**0.98**	**0.94**	0.45	0.54						
Total plant N anthesis	0.65	0.78	0.76	0.00	0.75	**0.79**	**0.90**	0.37	0.36	**0.79**					
Biomass Anthesis (g)	0.56	0.66	0.64	-0.06	0.62	0.65	**0.81**	0.20	0.20	0.66	**0.95**				
Shoot weight Anthesis (g)	0.24	0.27	0.27	-0.11	0.20	0.24	0.42	0.11	0.06	0.24	0.68	**0.86**			
Head weight Anthesis (g)	0.74	**0.87**	**0.80**	0.09	0.91	0.89	0.93	0.54	0.51	0.92	**0.81**	0.65	0.18		
PANU	0.36	0.72	0.69	-0.02	0.67	0.73	0.59	0.18	0.17	0.72	0.35	0.18	0.13	0.54	
Translocation	-0.30	-0.50	-0.41	-0.31	-0.69	-0.56	-0.51	0.27	0.42	-0.61	-0.11	-0.01	0.29	-0.47	**-0.76**

Supplementary Table 6: Correlation matrix of different physiological traits under HN treatment; GPC (%), Grain N content (mg plant^{-1}), Grain weight (g), Shoot N%, Shoot N (mg plant^{-1}), Biomass Maturity (g), Chaff/rachis weight (g), HI, NHI, Total plant N Maturity (mg plant^{-1}), Total plant N Anthesis (mg plant^{-1}), Biomass Anthesis (g), Shoot weight Anthesis (g), Head weight Anthesis (g), PANU (mg plant^{-1}) and Translocation (mg plant^{-1}). See supplementary table 8 for correlation p-values.

HN	GPC (%)	Grain N content (mg plant^{-1})	Grain weight (g)	Shoot N%	Shoot N (mg plant^{-1})	Biomass Maturity (g)	Chaff/rachis weight (g)	HI	NHI	Total plant N Maturity	Total plant N Anthesis	Biomass Anthesis (g)	Shoot weight Anthesis (g)	Head weight Anthesis (g)	PANU
GPC (%)															
Grain N content (mg plant^{-1})	0.13														
Grain weight (g)	-0.07	0.96													
Shoot N%	0.40	0.21	0.19												
Biomass N (mg plant^{-1})	0.00	0.63	0.64	0.55											
Biomass Maturity (g)	-0.01	**0.85**	**0.86**	0.36	0.91										
Chaff/rachis weight (g)	-0.14	**0.84**	**0.84**	0.13	0.45	0.63									
HI	**-0.10**	0.02	0.11	-0.30	-0.44	-0.30	0.11								
NHI	**0.31**	-0.01	-0.05	-0.14	-0.61	-0.37	-0.14	0.72							
Total plant N maturity	0.06	**0.86**	**0.86**	0.45	**0.93**	**0.98**	0.66	0.27	0.39						
Total plant N anthesis	-0.13	**0.75**	**0.76**	0.19	0.28	0.50	**0.90**	0.07	0.10	0.51					
Biomass Anthesis (g)	-0.09	**0.76**	**0.78**	0.25	0.30	0.52	**0.87**	0.11	0.05	0.52	**0.99**				
Shoot weight Anthesis (g)	0.27	0.63	0.66	0.10	0.21	0.43	**0.76**	0.01	0.16	0.41	**0.93**	**0.95**			
Head weight Anthesis (g)	-0.01	0.67	0.66	0.22	0.26	0.43	**0.87**	0.11	0.06	0.46	**0.86**	**0.82**	0.64		
PANU	-0.23	0.47	0.44	0.07	0.79	0.72	0.43	0.29	0.53	0.75	0.18	0.17	0.18	0.14	
Translocation	0.10	-0.01	0.04	0.11	-0.69	-0.47	0.11	0.42	0.52	-0.49	0.37	0.36	0.32	0.32	**-0.80**

Supplementary Table 7: Correlation matrix of different physiological traits under HN-LN treatment; GPC (%), Grain N content (mg plant-1), Grain weight (g), Shoot N%, Shoot N (mg plant^{-1}), Biomass Maturity (g), Chaff/rachis weight (g), HI, NHI, Total plant N Maturity (mg plant^{-1}), Total plant N Anthesis (mg plant^{-1}), Biomass Anthesis (g), Shoot weight Anthesis (g), Head weight Anthesis (g), PANU (mg plant^{-1}) and Translocation (mg plant^{-1}). See supplementary table 8 for correlation p-values.

HN-LN	GPC (%)	Grain N content (mg plant^{-1})	Grain weight (g)	Shoot N%	Shoot N (mg plant^{-1})	Biomass Maturity (g)	Chaff/rachis weight (g)	HI	NHI	Total plant N Maturity	Total plant N Anthesis	Biomass Anthesis (g)	Shoot weight Anthesis (g)	Head weight Anthesis (g)	PANU
GPC (%)															
Grain N content (mg plant^{-1})	0.33														
Grain weight (g)	0.08	0.95													
Shoot N%	0.57	-0.10	-0.23												
Biomass N (mg plant^{-1})	0.40	**0.81**	**0.71**	0.25											
Biomass Maturity (g)	0.17	**0.96**	**0.96**	-0.15	0.86										
Chaff/rachis weight (g)	0.19	**0.82**	**0.87**	-0.12	0.56	0.82									
HI	**0.14**	0.04	0.12	0.15	-0.30	-0.08	0.28								
NHI	**0.75**	0.30	0.21	0.12	0.09	0.23	0.20	0.64							
Total plant N maturity	0.22	**0.90**	**0.88**	-0.04	**0.85**	**0.93**	**0.81**	0.06	0.20						
Total plant N anthesis	0.23	**0.81**	**0.82**	0.00	**0.73**	**0.83**	**0.84**	0.08	0.16	**0.78**					
Biomass Anthesis (g)	0.25	**0.79**	**0.79**	0.04	**0.77**	**0.82**	**0.78**	0.04	0.14	**0.76**	**0.99**				
Shoot weight Anthesis (g)	0.17	**0.77**	**0.77**	-0.06	**0.75**	**0.81**	0.64	0.01	0.13	0.69	0.93	**0.95**			
Head weight Anthesis (g)	0.31	0.64	0.66	0.11	0.47	0.57	**0.86**	0.19	0.04	0.59	0.85	**0.82**	0.64		
PANU	-0.01	0.37	0.35	-0.16	0.33	0.31	0.11	0.06	0.01	0.29	-0.15	-0.17	-0.16	-0.18	
Translocation	0.14	0.63	0.67	-0.07	0.47	0.67	0.78	0.16	0.18	0.61	0.93	0.90	0.85	0.82	**-0.43**

Supplementary Table 8: p values for Pearson's correlations of different physiological traits under LN, LN-HN, HN and HN-LN treatment

LN Treatment	GPC (%)	Grain N content (mg plant-1)	Grain weight (g)	Shoot N%	Shoot N (mg plant-1)	Biomass Maturity (g)	Chaff/rachis weight (g)	HI	NHI	Total plant N Maturity	Total plant N Anthesis	Biomass Anthesis (g)	Shoot weight Anthesis (g)	Head weight Anthesis (g)	PANU
GPC (%)															
Grain N content (mg plant-1)	2E-06														
Grain weight (g)	2E-01	1E-16													
Shoot N%	3E-04	1E-04	1E-02												
Shoot N (mg plant-1)	1E-03	4E-09	2E-06	2E-08											
Biomass Maturity (g)	1E-02	9E-15	1E-15	1E-03	0E+00										
Chaff/rachis weight (g)	3E-01	2E-02	2E-02	4E-02	2E-03	2E-03									
HI	1E-04	1E-03	5E-02	2E-03	8E-11	4E-08	4E-02								
NHI	2E-01	6E-03	9E-03	3E-10	9E-16	2E-07	6E-03	8E-09							
Total plant N Maturity	2E-05	0E+00	2E-11	5E-07	0E+00	0E+00	4E-03	2E-07	2E-07						
Total plant N Anthesis	3E-01	3E-02	4E-02	1E-01	2E-02	1E-02	5E-07	8E-02	4E-02	2E-02					
Biomass Anthesis (g)	3E-01	8E-02	1E-01	1E-01	4E-02	4E-02	2E-05	1E-01	5E-02	5E-02	4E-08				
Shoot weight Anthesis (g)	4E-01	3E-01	2E-01	1E-01	2E-01	2E-01	2E-02	2E-01	1E-01	2E-01	3E-03	2E-05			
Head weight Anthesis (g)	2E-01	3E-02	9E-02	2E-01	1E-02	2E-02	1E-05	6E-02	6E-02	2E-02	1E-04	4E-03	3E-01		
PANU	2E-01	5E-04	1E-03	7E-03	3E-03	6E-04	3E-01	1E-01	1E-02	5E-04	4E-01	3E-01	2E-01	4E-01	
Translocation	4E-01	2E-01	3E-01	6E-02	7E-02	2E-01	2E-01	2E-01	5E-02	1E-01	3E-02	3E-02	6E-02	1E-01	9E-04

LN -HN Treatment	GPC (%)	Grain N content (mg plant-1)	Grain weight (g)	Shoot N%	Shoot N (mg plant-1)	Biomass Maturity (g)	Chaff/rachis weight (g)	HI	NHI	Total plant N Maturity	Total plant N Anthesis	Biomass Anthesis (g)	Shoot weight Anthesis (g)	Head weight Anthesis (g)	PANU
GPC (%)															
Grain N content (mg plant-1)	1E-05														
Grain weight (g)	2E-03	0E+00													
Shoot N%	2E-01	2E-01	3E-01												
Shoot N (mg plant-1)	3E-04	2E-11	2E-09	7E-04											
Biomass Maturity (g)	3E-04	0E+00	0E+00	9E-02	0E+00										
Chaff/rachis weight (g)	2E-03	3E-06	5E-05	4E-01	2E-07	7E-07									

LN -HN Treatment	GPC (%)	Grain N content (mg plant-1)	Grain weight (g)	Shoot N%	Shoot N (mg plant-1)	Biomass Maturity (g)	Chaff/rachis weight (g)	HI	NHI	Total plant N Maturity	Total plant N Anthesis	Biomass Anthesis (g)	Shoot weight Anthesis (g)	Head weight Anthesis (g)	PANU
HI	2E-03	7E-02	2E-01	9E-02	7E-07	3E-04	5E-02								
NHI	4E-02	3E-02	6E-02	6E-07	4E-10	6E-05	5E-02	1E-13							
Total plant N Maturity	2E-05	0E+00	0E+00	1E-02	0E+00	0E+00	1E-07	5E-04	3E-05						
Total plant N Anthesis	5E-03	3E-04	6E-04	5E-01	7E-04	3E-04	3E-06	9E-02	9E-02	3E-04					
Biomass Anthesis (g)	1E-02	4E-03	5E-03	4E-01	7E-03	4E-03	1E-04	2E-01	2E-01	4E-03	5E-08				
Shoot weight Anthesis (g)	2E-01	2E-01	2E-01	3E-01	2E-01	2E-01	6E-02	4E-01	4E-01	2E-01	3E-03	2E-05			
Head weight Anthesis (g)	9E-04	1E-05	2E-04	4E-01	1E-06	4E-06	2E-07	2E-02	3E-02	9E-07	1E-04	4E-03	3E-01		
PANU	1E-01	1E-03	2E-03	5E-01	3E-03	1E-03	1E-02	3E-01	3E-01	1E-03	1E-01	3E-01	3E-01	2E-02	
Translocation	1E-01	3E-02	6E-02	1E-01	2E-03	1E-02	3E-02	2E-01	6E-02	8E-03	3E-01	5E-01	1E-01	4E-02	5E-04

HN Treatment	GPC (%)	Grain N content (mg plant-1)	Grain weight (g)	Shoot N%	Shoot N (mg plant-1)	Biomass Maturity (g)	Chaff/rachis weight (g)	HI	NHI	Total plant N Maturity	Total plant N Anthesis	Biomass Anthesis (g)	Shoot weight Anthesis (g)	Head weight Anthesis (g)	PANU
GPC (%)															
Grain N content (mg plant-1)	0.37														
Grain weight (g)	0.63	0.00													
Shoot N%	0.00	0.15	0.18												
Shoot N (mg plant-1)	1.00	0.00	0.00	0.00											
Biomass Maturity (g)	0.95	0.00	0.00	0.01	0.00										
Chaff/rachis weight (g)	0.63	0.00	0.00	0.66	0.10	0.02									
HI	0.50	0.87	0.44	0.04	0.00	0.03	0.72								
NHI	0.03	0.94	0.76	0.00	0.00	0.01	0.64	0.00							
Total plant N Maturity	0.67	0.00	0.00	0.00	0.00	0.00	0.01	0.06	0.01						
Total plant N Anthesis	0.63	0.00	0.00	0.51	0.32	0.07	0.00	0.80	0.74	0.06					
Biomass Anthesis (g)	0.75	0.00	0.00	0.37	0.30	0.06	0.00	0.70	0.87	0.06	0.00				
Shoot weight Anthesis (g)	0.32	0.01	0.01	0.71	0.47	0.13	0.00	0.98	0.58	0.15	0.00	0.00			
Head weight Anthesis (g)	0.98	0.01	0.01	0.43	0.36	0.13	0.00	0.70	0.84	0.10	0.00	0.00	0.01		
PANU	0.41	0.07	0.10	0.82	0.00	0.00	0.11	0.32	0.05	0.00	0.52	0.55	0.53	0.62	
Translocation	0.72	0.98	0.89	0.68	0.01	0.09	0.70	0.13	0.06	0.08	0.18	0.19	0.25	0.25	0.00

HN-LN Treatment	GPC (%)	Grain N content (mg plant-1)	Grain weight (g)	Shoot N%	Shoot N (mg plant-1)	Biomass Maturity (g)	Chaff/rachis weight (g)	HI	NHI	Total plant N Maturity	Total plant N Anthesis	Biomass Anthesis (g)	Shoot weight Anthesis (g)	Head weight Anthesis (g)	PANU
GPC (%)															
Grain N content (mg plant-1)	0.02														
Grain weight (g)	0.58	0.00													
Shoot N%	0.00	0.49	0.11												
Shoot N (mg plant-1)	0.00	0.00	0.00	0.07											
Biomass Maturity (g)	0.24	0.00	0.00	0.30	0.00										
Chaff/rachis weight (g)	0.51	0.00	0.00	0.67	0.03	0.00									
HI	0.35	0.77	0.42	0.31	0.03	0.59	0.35								
NHI	0.00	0.03	0.14	0.40	0.53	0.11	0.50	0.00							
Total plant N Maturity	0.12	0.00	0.00	0.79	0.00	0.00	0.00	0.70	0.15						
Total plant N Anthesis	0.42	0.00	0.00	1.00	0.00	0.00	0.00	0.80	0.59	0.00					
Biomass Anthesis (g)	0.39	0.00	0.00	0.90	0.00	0.00	0.00	0.90	0.63	0.00	0.00				
Shoot weight Anthesis (g)	0.55	0.00	0.00	0.83	0.00	0.00	0.01	0.97	0.65	0.01	0.00	0.00			
Head weight Anthesis (g)	0.28	0.01	0.01	0.71	0.08	0.03	0.00	0.52	0.88	0.03	0.00	0.00	0.01		
PANU	0.96	0.20	0.22	0.57	0.23	0.29	0.69	0.86	0.98	0.32	0.59	0.54	0.57	0.53	
Translocation	0.63	0.02	0.01	0.81	0.08	0.01	0.00	0.60	0.54	0.02	0.00	0.00	0.00	0.00	0.11

Chapter 6

General discussion and future direction

This study focussed on ways to improve nitrogen utillisation in wheat. Nitrogen (N) is an important macronutrient that determines yield and grain quality. It is vital to recognise the poor uptake and use efficiency in cereal crops, including wheat. This necessitates the identification of ways to improve N use to maximise yield and grain quality while minimizing losses of excess N that can cause harm to the environment. One way to do this is through better fertiliser N management strategies, including split N application. The rationale is to provide N in wheat only when required, by matching the N supply with plant growth demands across different stages. For a successful split N application, fertiliser application at sowing in the soil can be complemented with foliar application later in development to improve grain N quality. Therefore, the first aim of this study was to determine the most suitable growth stage to apply foliar N fertiliser to ensure a stable yield and an increase in grain protein content (GPC) in wheat (Chapter 2).

Two Australian bread wheat varieties, Spitfire and Gregory, are contrasting for GPC and were compared in this study to investigate the effectiveness of foliar N in increasing GPC. Foliar N was applied at various stages under two basal N treatments (low and high). The results indicated a positive effect of foliar N applied at heading and 7 days post-anthesis (DPA) through an increased GPC in Gregory, a high yielding but low GPC variety, across different environments (field and controlled). However, there was no effect of foliar N in Spitfire, a high yielding and high GPC variety. This suggests the existence of genetic variability in the uptake of foliar N in wheat, which may be dependent on leaf morphological features, (investigated for Spitfire and Gregory in Chapter 3), leaf N transporter activities, N status-driven feedback responses, and/or the form of foliar N applied (Chapter 4). Spitfire had significantly more total plant N per unit biomass at anthesis (Chapter 2, Fig.2). Thus, the capacity for N uptake early during

development may influence foliar N uptake after anthesis. Furthermore, a high remobilisation efficiency of vegetative tissue N already taken up, and a reduced sink capacity in the developing grains may limit foliar N uptake after anthesis. Spitfire had fewer spikes and grains per head than Gregory (Chapter 2, Supplementary Table 1), reducing the capacity for this genotype to acquire N from foliar applications post-anthesis. Basal N is also an important factor affecting the efficiency of foliar N uptake (Chapter 2, Fig. 5 and 6). Low basal N creates N limiting conditions that may lead to increased N uptake post-anthesis, as N is the limiting factor. However, increasing the basal N supply may lower uptake rates post-anthesis, due to factors limiting growth and grain production other than N (Cassman *et al.* 2003).

Overall, N utilisation pre- and post-anthesis was genotype-dependent and were important factors determining the success rate of foliar N as a top-up to improve grain quality. Interestingly, granular N application at 7 DPA did not improve grain quality (Chapter 2, Fig. 5 - 6). There was no significant effect of foliar N application on grain yield in the two genotypes, which is as expected as the N taken up after anthesis is mainly translocated to the already formed grains, contributing primarily to an increase in grain N.

Low adoption of foliar N application by grain growers in Australia may be due to a limited understanding of the factors that determine successful foliar N application translating to increased grain yield and grain N. Also, the minimal knowledge of these factors may contribute to the reported variability in the performance of foliar nutrient application in wheat, further discouraging its use.

The second aim of this study was to investigate wheat foliar N uptake at a physiological and molecular level. The main area of focus was on leaf surface properties that may

be involved in foliar N uptake (Chapter 3), the penetration/uptake rates of different forms of N using ^{15}N-labeled species of N (Chapter 4), and N transporters (nitrate, ammonium and/or urea transporters found through *in silico* analysis to be expressed in leaf tissues) involved in foliar N uptake (Chapters 3 and 4).

To elucidate aspects affecting foliar N uptake, I studied leaf morphological features using microscopy, wax quantification, and chemical composition analysis (Chapter 3). I also undertook a gene expression study of the leaf-expressed N transporters in flag leaves of cv. Gregory after foliar N treatment (Chapter 3). The four genotypes used for the leaf morphological study were Spitfire, Gregory and two additional wheat varieties, RAC875 and Kukri, that contrast for glaucousness. Glaucousness is a blue-white leaf colouration caused by cuticle wax reflection of visible light, and a characteristic that has been related to drought susceptibility or resistance (Bi *et al.* 2016). Gregory and Kukri showed an increase in grain N concentration in response to foliar N applied at 7 DPA (Chapter 3, Fig. 1). This was correlated with low primary alcohol levels in Gregory and high alkanes in both varieties (Chapter 3, Fig. 3).

A further explanation that may only apply to Kukri is the presence of the inhibitor wax 1 *(iw1)* gene that is responsible for decreased diketones and increased alkanes, resulting in a non-glaucous phenotype and drought susceptibility (Adamski *et al.* 2013). The gene or the phenotype produced may be correlated with effective foliar N uptake in Kukri. It is not known if Gregory possesses the *iw1* gene variant, but would be of interest to determine. A possible initial hypothesis was that the high levels of diketones measured in wax from the leaves of Gregory, which was correlated with a characteristic tubular-shaped wax structure, may encourage adherence of foliar N solution to the leaf for better retention and uptake (Chapter 3, Fig. 4). However, RAC875 also had high levels of leaf wax diketones at 7 DPA (Chapter 3, Fig. 3, Supplementary Table 2) and

numerous tubular structures (Chapter 6, Supplementary Fig.3), but showed no grain N response to foliar-applied N (Chapter 3, Fig. 1). The RAC875 phenotype has been associated with drought tolerance (Bi *et al.* 2016). Gregory had a minimal change in total wax load between stem elongation and 7 DPA, with the least primary alcohol amount at 7 DPA compared to other genotypes, which could also be responsible for the foliar N uptake (Chapter 3, Fig. 2, Fig. 3, Supplementary Table 2). Another possible explanation for the foliar N uptake in Gregory could be the observed high trichome density compared with Spitfire (Chapter 3, Fig. 5, Table 1). Previous research has suggested that aqueous pores at the base of trichome cells may provide a pathway for the entry of solutes into leaf tissue (Schönherr 2006). Trichome density, the presence and amount of primary alcohols and alkanes in leaf surface waxes, may all be characteristics that contribute to efficient foliar N uptake. Further investigation to elucidate the roles of each of these in foliar N uptake is necessary. Targeting these traits could be considered for available bi-parental mapping populations of Spitfire Gregory, Kukri, and RAC875.

To investigate the entry time taken for foliar-applied N to enter the leaf tissue, ^{15}N-labelled urea ($CH_4{}^{15}N_2O$), ammonium ($^{15}NH_4^+$) and nitrate ($^{15}NO_3^-$) (10 atom % each) were combined to create a mix of urea ammonium nitrate (UAN) ($CH_4 15N_2O$ + $15NH_4^+$ +$15NO_3^-$), which was applied to leaves of Gregory and Spitfire seedlings. The wheat seedlings were then harvested at different time points. The results show a maximum ^{15}N accumulation in the seedlings after 2 h and no increase thereafter in Gregory (Chapter 4, Fig. 2). In contrast, Spitfire, continued to take up foliar N between 24 h and72 h post-treatment, suggesting genetic variation in rates of uptake of foliar N at the wheat seedling stage. In this experiment, the earliest harvest was made at 2 h post-treatment. Earlier sampling times may have more clearly demonstrated differences in

uptake between the two genotypes. Future experiments should include a 0 h time point and time points between 0 h and 2 h. To identify which of the N forms are preferentially taken up through wheat leaf tissue, ^{15}N-labelled single species, as well as a combination of the three N forms, were used (Chapter 4). The highest quantities of ^{15}N accumulating in grain tissue were derived from labelled urea, while the mix of the three N forms was also effectively accumulated (Chapter 4, Fig 4, 5 and 6). A high uptake of urea suggests the relative ease of entry of non-ionic molecules through the cuticle, possibly without the assistance of transporter(s). The proportion of foliar N applied that was translocated to the grain calculated as being derived from the single N forms and combined N forms across these experiments ranged between approximately 3% and 35% (Chapter 4). These contributions may be effective in increasing grain N and maintaining high grain N concentrations. Furthermore, since wheat can only access an average of 30% -50% N applied to the soil (Sylvester-Bradley and Kindred 2009), a foliar top-up could be beneficial complementing soil N application. The conclusion from these results was that urea- and UAN-based liquid fertilisers can be effectively used as foliar sprays in wheat.

The uptake of foliar nutrient solutions localised on the leaf surface is a physicochemical aspect dependant on leaf morphology, as well as the fertiliser chemical formulation that includes adjuvants and surfactants (Peirce *et al.* 2016). The use of Spreadwet in this experiment reduced surface run-off. Previous research showed that at zero splashing and run-off, spreading (from reduced surface tension by adjuvants) had minimal effect on the uptake efficiency of wheat leaf (Peirce *et al.* 2016). In this experiment, we did not investigate correlation of Spreadwet and foliar N uptake, therefore may not ascertain its effectiveness. The role of adjuvants in improving droplet spreading by a low contact angle, therefore, may not guarantee foliar N uptake, but

improves the chances of entry when the solution stays on the leaf. Oil-based adjuvants have been found to improve foliar absorption of fungicides (Gent *et al.* 2003). In future research, the success rate of the various types of adjuvants and surfactants in modifying the chemical composition and application characteristics of foliar nutrient solutions to improving uptake efficiency should be considered on a case by case.

The expression of N transporter genes has been widely studied in roots but not in shoots in plants. I sought to identify leaf-expressed N transporters in wheat for the three N forms, nitrate, urea, and ammonium, and from their expression try to elucidate their possible roles in foliar N uptake. Leaf-expressed transporters were initially identified from literature (i.e. *NRT1.1, NRT2.4, NRT2.5, AMT1.1, AMT2.1, AMT2.2* and *DUR3*) (Chapter 3, Supplementary Table 3). Further *in silico* analysis suggested the leaf expression of *NRT1.1*, *DUR3, AMT1.1* and *AMT2.1* in wheat (Chapter 3, Supplementary Fig. 6). Expression analysis was performed using flag leaf tissue sampled from cv. Gregory, a genotype that was consistently responsive to foliar-applied N (Chapter 2; Fig. 3, Fig. 4, Fig. 5, and Chapter 3, Fig.1). Two basal N treatments were included to investigate the effects of basal N status on the response of the transporters to foliar-applied N. The nitrate transporter *NRT1.1* has been previously found to be expressed in guard cells of *Arabidopsis thaliana* and is proposed to be responsible for stomatal opening (Guo *et al.* 2003). In wheat, *TaNRT1.1* expression was confirmed in leaf and showed responses to foliar N application (Chapter 3, Fig. 6).

The gene was largely down-regulated after foliar N treatment, indicating a sensitivity of *TaNRT1.1* to the N status of the plant and suggesting a possible role in regulating the entry of foliar N into leaf tissue cells. *TaDUR3*, a singleton symporter responsible for the uptake of urea, had very low expression in leaf tissue from previous findings in

rice and Arabidopsis (Liu *et al.* 2003, Wang *et al.* 2012). In this study, the gene was expressed in leaf tissue (Chapter 3, Fig. 7). These findings of *TaDUR3* expression in leaf tissue suggest wheat has evolved to utilise urea that may be available as foliar. *TaDUR3* was significantly downregulated in response to all foliar N treatments except at 2 h after urea application, under both low and high basal N (Chapter 3, Fig. 6d). Given the apparent entry of foliar-applied N at 2 h or earlier (Chapter 4), foliar urea may be rapidly causing negative feedback on TaDUR3. Another explanation as explained above could be that urea enters leaf cells independently of any transporter (passively, across the lipid bilayer, since it is a non-ionic molecule), or via aquaporins (Wang *et al.* 2008).

Expression of the ammonium transporters *TaAMT1.1* and *TaAMT2.1* was also analysed. Both genes were responsive to foliar N treatment as seen in either a downregulation or upregulation in various treatments (Chapter 3, Fig. 7). The expression patterns of *TaAMT1.1* and *TaAMT2.1* were suggestive of sensitivity to plant N status and negative feedback from foliar N uptake. Overall, there was no strong correlation between gene expression and foliar N uptake in leaves of cv. Gregory, and it was concluded that other factors, such as post-translational modification of the transporter proteins, could be involved in the regulation of foliar uptake of N through transporters. This has also been concluded in previous wheat N transporter studies undertaken in root tissues (Melino *et al.* 2015). Other explanations could be that there are other leaf-expressed N transporters, not identified here, that have an active role in leaf N uptake. Furthermore, since the qPCR primers were designed from all three homeologs for each gene, this may obscure the ability to detect a correlation between homeolog-specific gene expression and foliar N uptake. The time points chosen for assessment of gene expression in this study may also not have been optimal, despite

the use of labelled N uptake experiments (Chapter 4, Fig 1) to guide in the selection of an appropriate sampling time.

Another aim was to identify feedback effects of foliar N on root N uptake and accumulation, as well as on root-expressed nitrate transporter gene expression (Chapter 4). In this experiment, labelled nitrate ($^{15}NO_3^-$) was used in a flux experiment to quantify root N accumulation after a foliar UAN pre-treatment. Root N accumulation was lower in foliar-treated plants, suggesting negative feedback regulation of root uptake (Chapter 4, Fig 7). For the root nitrate transporters, the expression of *TaNRT1.1* and *TaNRT2.1* was downregulated by foliar treatment in the early time points and upregulated at later time points (Chapter 4, Fig 7). Contrary to our results, previous research showed an increased accumulation of *TaNRT2.1* mRNA from transient induction by nitrate applied to roots in early time-points and a decrease at later time points, and no change in transcript levels with ammonium (Yin et al. 2007). A possible explanation for the expression pattern of *TaNRT2.1* in the current study could be that the nitrogenous signal sent from the shoot after a foliar UAN treatment to the root, could be mostly ammonium and amino acids as the N signals. Since the expression of *TaNRT1.1* and *TaNRT2.1* did not show a strong correlation with root N accumulation, the reason could be that expression studies do not always give a direct indication of the active role of genes due to the interplay of other factors, including post-translational modifications and gene redundancy.

The final aim was to understand genetic differences in N utilisation in wheat genotypes selected from a diversity panel that were contrasting for GPC (Chapter 5). The negative correlation between grain yield and GPC, and a limited understanding of the underlying genetic and physiological traits contributing to GPC, have made breeding simultaneously for yield and GPC difficult. Wheat farmers would appreciate gaining

from both traits, for better returns and increased premiums. Previous research has explained the negative correlation between the two traits as a result of the strong interplay of nitrogen and carbon competition, and N dilution (Acreche and Slafer 2009, Munier *et al.* 2005). The use of grain protein deviation (GPD) has been suggested as a tool to aid selection, with this measure identifying genotypes with GPC higher than expected based on their yield (Monaghan *et al.* 2001). In the current study, fifteen wheat genotypes from a genetic diversity panel were selected using data from two field trials, and only consistently high yielding genotypes with contrasting GPC and GPD were selected. The selected panel of wheats was re-assessed in field conditions and found to maintain differences in GPD (Chapter 5, Fig. 3). Regression analysis showed the positive GPD and negative GPD genotypes were always above or below the regression line respectively (Chapter 5, Fig. 4). In controlled, semi-hydroponics conditions, the panel was grown in four N treatments with low N (LN) and high N (HN) up until anthesis, after which half of the plants were switched to either LN or HN treatments. There were minimal differences in the uptake and partitioning of N up to anthesis between the LN and HN treatments (Chapter 5, Fig. 5, Supplementary Table 4). This may be explained by the consistently high-yielding phenotypes of the wheat panel, promoting efficient uptake of the supplied N under both treatments. At maturity, partitioning of N between tissue types was slightly different between the genotypes under different N treatments (Chapter 5, Fig.6), which could reflect differences in remobilisation and post-anthesis uptake of N. The positive GPD genotypes at anthesis tended to have a lower proportion of total N in the head, resulting in a greater sink capacity for N during the post-anthesis period. This was further confirmed with a measure of higher post-anthesis N uptake in positive GPD genotypes compared to negative GPD genotypes (Chapter 5, Table 5).

The negative GPD genotypes tended to have higher harvest index (HI) and nitrogen harvest index (NHI) compared to positive GPD genotypes (Chapter 5, Table 3 - 4). This may be due to these genotypes investing more assimilates into grain yield relative to biomass, with slightly more bias towards carbon than N. In correlation studies, the physiological traits strongly correlated to GPC included grain N content, total N at anthesis, total N at maturity and post-anthesis N uptake (PANU), but only in the LN and LN-HN treatments. A lack of correlation in the HN and HN-LN treatments suggest that N treatment conditions determine how physiological traits correlate with GPC (Chapter 5, Fig.7). Grain weight in all N treatments was highly correlated with grain N content, total N at anthesis, biomass at maturity, total N at maturity and PANU (Chapter 5, Fig.7, Supplementary Table 4-8). These traits also correlated with grain weight in an N treatment-dependant manner, whereby traits like total N at anthesis, biomass at maturity and total N at maturity had the highest correlations with grain weight in the LN-HN and HN-LN treatments (Chapter 5, Fig.7, Supplementary Table 5, 7 and 8). PANU had the highest correlations with grain weight in the LN treatment (Chapter 5, Fig.7, Supplementary Table 4). This is as expected since N limiting conditions should result in more N uptake after anthesis. Both GPC and grain weight were negatively correlated with HI and NHI in the LN treatment and LN-HN treatments (Chapter 5, Fig.7, Supplementary Table 4, 5 and 8). However, in the HN-LN treatment, GPC and grain yield were positively correlated to HI and NHI (Chapter 5, Fig.7, Supplementary Table 7 and 8). From these results, it can be concluded that the N treatment throughout the growing season is an important determiner of the HI and NHI that will then translate to grain yield and GPC. Further studies of these traits using elite cultivars and genetically diverse wheat could bring us closer to resolving the negative correlation between grain yield and GPC. In conclusion, the findings from the correlation studies

highlight the complexity in identifying key physiological traits associated with both high grain yield and high GPC.

The findings in this thesis highlight the benefits of foliar N when applied at the appropriate stage. This knowledge could help farmers to make better decisions around when to apply foliar N. Furthermore, because the % foliar-applied N that ended up in the grain was estimated at between 3% – 35%, a grower can calculate the cost-benefit of applying expensive liquid fertilisers as a top-up to increase GPC. The success rate of foliar N uptake in wheat seems to be determined by genetic variability in the uptake of N pre- and post-anthesis, the leaf surface morphology and the form of N fertiliser applied. This knowledge can be used as a basis to evaluate the variability in the performance of foliar-applied solutions for broader applications, including other fertilisers, herbicides, and pesticides. Breeders may take into consideration the leaf morphological traits to breed for wheat with improved uptake of foliar spray solutions. Further work is required, however, across more wheat genotypes, to confirm that the leaf morphological features found in this study are strongly correlated to foliar uptake of solutions. Furthermore, the physiological traits correlated to high yield and high GPC from contrasting GPD genotypes indicates the complexity involved in finding a single specific component trait strongly correlated to both agronomic traits. Also, the few physiological traits found in this study to correlate to high yield and high GPC, while N treatment-dependent, should be studied further in genetically diverse wheat panels contrasting for GPD to prove if they are useful in breaking the negative correlation between grain yield and GPC. Studies need also to be done in more field-related conditions to try and identify a set of traits that correlate better to high yield and high GPC. Overall, this research has highlighted the importance of N utilisation in wheat

plants and N management in wheat production in ensuring yield and grain quality are maintained.

References

Acreche, M. M. and G. A. Slafer (2009). "Variation of grain nitrogen content in relation with grain yield in old and modern Spanish wheats grown under a wide range of agronomic conditions in a Mediterranean region." The Journal of Agricultural Science 147(06): 657-667.

Adamski, N. M., M. S. Bush, J. Simmonds, A. S. Turner, S. G. Mugford, A. Jones, K. Findlay, N. Pedentchouk, P. von Wettstein-Knowles and C. Uauy (2013). "The I nhibitor of wax 1 locus (I w1) prevents formation of β-and OH-β-diketones in wheat cuticular waxes and maps to a sub-c M interval on chromosome arm 2 BS." The Plant Journal 74(6): 989-1002.

Bi, H., S. Luang, Y. Li, N. Bazanova, S. Morran, Z. Song, M. A. Perera, M. Hrmova, N. Borisjuk and S. Lopato (2016). "Identification and characterization of wheat drought-responsive MYB transcription factors involved in the regulation of cuticle biosynthesis." Journal of Experimental Botany.

Cassman, K. G., A. Dobermann, D. T. Walters and H. Yang (2003). "Meeting cereal demand while protecting natural resources and improving environmental quality." Annual Review of Environment and Resources 28(1): 315-358.

Gent, D. H., H. F. Schwartz and S. J. Nissen (2003). "Effect of Commercial Adjuvants on Vegetable Crop Fungicide Coverage, Absorption, and Efficacy." Plant Disease 87(5): 591-597.

Guo, F.-Q., J. Young and N. M. Crawford (2003). "The Nitrate Transporter AtNRT1.1 (CHL1) Functions in Stomatal Opening and Contributes to Drought Susceptibility in Arabidopsis." The Plant Cell 15(1): 107-117.

Liu, L.-H., U. Ludewig, W. B. Frommer and N. von Wirén (2003). "AtDUR3 encodes a new type of high-affinity urea/H+ symporter in Arabidopsis." The Plant Cell 15(3): 790-800.

Melino, V. J., G. Fiene, A. Enju, J. Cai, P. Buchner and S. Heuer (2015). "Genetic diversity for root plasticity and nitrogen uptake in wheat seedlings." Functional Plant Biology 42(10): 942-956.

Monaghan, J. M., J. W. Snape, A. J. S. Chojecki and P. S. Kettlewell (2001). "The use of grain protein deviation for identifying wheat cultivars with high grain protein concentration and yield." Euphytica 122(2): 309-317.

Munier, J., N. G and C. Salon (2005). "Are the carbon costs of seed production related to the quantitative and qualitative performance? An appraisal for legumes and other crops." Plant, Cell & Environment 28(11): 1388-1395.

Peirce, C. A., C. Priest, T. M. McBeath and M. J. McLaughlin (2016). "Uptake of phosphorus from surfactant solutions by wheat leaves: spreading kinetics, wetted area, and drying time." Soft Matter 12(1): 209-218.

Sylvester-Bradley, R. and D. R. Kindred (2009). "Analysing nitrogen responses of cereals to prioritize routes to the improvement of nitrogen use efficiency." Journal of Experimental Botany 60(7): 1939-1951.

Wang, W.-H., B. Köhler, F.-Q. Cao, G.-W. Liu, Y.-Y. Gong, S. Sheng, Q.-C. Song, X.-Y. Cheng, T. Garnett, M. Okamoto, R. Qin, B. Mueller-Roeber, M. Tester and L. Liu (2012). "Rice DUR3 mediates high-affinity urea transport and plays an effective role in improvement of urea acquisition and utilization when expressed in Arabidopsis." New Phytologist 193(2): 432-444.

Wang, W.-H., B. Köhler, F.-Q. Cao and L.-H. Liu (2008). "Molecular and physiological aspects of urea transport in higher plants." Plant Science 175(4): 467-477.