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Quantifying Nitrogen Use Efficiency in Wheat Using High-Precision Phenotyping

By

Nicholas John Sitlington Hansen

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"I'll about it this evening,
and I will presently pen down my dilemmas,
encourage myself in my certainty,
put myself into my mortal preparation,
and by midnight look to hear further from me."

Shakespeare
All's Well That Ends Well
Act III Scene VI

From sunrise till sunset, may the name of Grain be praised.
People should submit to the yoke of Grain.
Whoever has silver, whoever has jewels, whoever has cattle,
whoever has sheep shall take a seat at the gate of whoever has grain,
and pass his time there.

Sumerian myth: *Debate between Sheep and Grain*

Wheat, Wheat, Wheat! Oh, the sound of it is sweet!
I've been praisin' it an' raisin' it in rain an' wind an' heat.
Since the time I learned to toddle, till it's beatin' in my noddle,
Is the little song I'm singin' you of Wheat, Wheat, Wheat!

CJ. Dennis - *Wheat*

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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.

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Abstract

Modern cereal cultivars rely heavily on nitrogenous inputs to reach their yield potential. However, the nitrogen use efficiency (NUE) of wheat is poor and the recovery of applied nitrogen (N) in cereal production is low, between 30-50 %. This inefficiency results in the N pollution of natural ecosystems, and an economic loss to producers. Improving NUE in wheat (*Triticum aestivum* L.) has so far proved difficult due to the complexity of NUE and a lack of phenotyping resolution to identify superior NUE genotypes.

Three knowledge gaps are addressed in this research. Firstly, the ability of high throughput phenotyping (HTP) to help us define the NUE of Australian wheat cultivars into differences that are apparent across a range of environments and managements. Central to this is the interaction between N and water, one of the major environmental determinants of N uptake. Secondly, although shoot tissue is one of the main reservoirs of plant N, and essential to understanding N uptake and utilisation, knowledge regarding tissue N response to changing N availability is limited. These processes are known to be dynamic and must be observed temporally in order to differentiate their responses to changes in N availability. Lastly, can a combination of these two phenotyping methods help explain plant responses to variable nutrient supply?

Growth responsiveness to N supply in a selection of bread wheat cultivars with varying water provision was measured using HTP. Cultivar differences were discovered in their ability to increase shoot area in response to N, in absolute growth rate response to changes in water availability as well as the ability to convert this into yield.

In order to differentiate N uptake in real time, a hyperspectral reflectance method utilising a field spectrophotometer and leaf clip was adapted to Australian bread wheats using partial least squares regression. The robustness of the method was established by

regressing tissue N analysis with reflectance spectra readings, giving an R^2 of the predictions at 0.83. The sensitivity of the method was determined to detect changes in leaf N % in a hydroponics system with alternating high/low N availability. The cultivars responded to the change in N by readjusting their leaf-N content to an equivalent steady-state N level within two days.

The final part of this project was to incorporate HTP and the hyperspectral leaf N measurements to determine how wheat growth and N uptake responded to split applications of N. When N was added at stem elongation and booting growth stages, the plants delayed their point of maximal shoot area by six days, and increases in leaf N concentration were observed the day after application. The increases in N harvest index and the grain protein content found at destructive harvest were linked to growth and leaf N concentration differences during the experiment.

Overall, the research presented here has measured NUE and cultivar differences repeatedly and with high resolution. These protocols show promise for the selection of improved NUE phenotypes, which could be combined with forward genetics to differentiate NUE and its component processes and identify the underlying genetic control.

List of Abbreviations

AGR	absolute growth rate
AlaAT	alanine aminotransferase
C	carbon
d	day
df	degrees of freedom
DAP	days after planting
DD	drought
DW	drought/well watered
GS	glutamine synthetase
GWAS	genome wide association study
GxExM	genotype, environment and management interaction
h	hour
ha	hectare
HATS	high affinity transporters
HI	harvest index
HN	high nitrogen
HTP	high throughput phenotyping
IR	thermal infrared
kg	kilogram
LAI	leaf area index
LATS	low affinity transporters
LIDAR	light detection and ranging
LN	low nitrogen
N	nitrogen
NDVI	normalised difference vegetation index
NHI	nitrogen harvest index
NIR	near infrared
NO_3^-	nitrate
NNI	nitrogen nutrition index
NR	nitrate reductase
NRT1/NPF	nitrate reductase transporter family
NUE	nitrogen use efficiency
NUpE	nitrogen uptake efficiency

NUtE	nitrogen utilisation efficiency
PLSR	partial least squares regression
Ppd	photoperiod sensitivity
PSA	projected shoot area
QTL	quantitative trait loci
RGB	red green blue
RGR	relative growth rate
Rht	reduced height gene
SE	south eastern
SECV	standard error of cross validation
TKW	thousand kernel weight
TUE	transpiration use efficiency
UAV	unmanned aerial vehicles
Vrn	vernalisation requirement
WW	well watered
YEB	youngest emerged blade

Chapter 1: Literature Review

1.1 Introduction

Nitrogen (N) is one of the most important plant mineral nutrients, essential for numerous biochemical and physiological processes. Greater use of N fertiliser played an important role in increasing yields during the 'green revolution' (Evenson and Gollin 2003). In recent times, however, cereal yield increases have stagnated, especially in developing countries (Lin and Huybers 2012; Ray et al. 2013; Ray et al. 2012). There has been a call for a second 'green revolution' to address flattening yields in a sustainable fashion, and increasing the nitrogen use efficiency (NUE) of cereal crops can play an important role.

Nitrogen use efficiency is important because inefficient N use is deleterious to the environment, expensive, and reduces the yield potential of crops. Cereal production utilises 60 % of all agricultural nitrogen applications but unfortunately, cereals generally recover less than half of the supplied N, causing wastage and pollution (Fageria and Baligar 2005; Peoples et al. 1995; Raun and Johnson 1999; Sylvester-Bradley and Kindred 2009). Therefore, increasing the NUE of cereals would have a significant environmental and economic impact (Ladha et al. 2016).

1.2 NUE

For cereal production, NUE was defined by Moll et al. (1982) as grain production per unit N available in the soil. Nitrogen use efficiency is the combination of plant uptake efficiency (NUpE), how effectively the plants capture N, and utilisation efficiency (NUtE), how well the plants use the N that is taken up (Good et al. 2004; Sadras and Richards 2014).

1.2.1 NUpE

Nitrogen uptake efficiency can be defined as the amount of N taken up by the plant as a proportion of the N available (both residual and added N) (Good et al. 2004). Nitrogen

uptake efficiency is influenced by mass flow of soil water to the root, root morphology, transporter activity on the root surface, timing of N application, and microbial competition (Garnett et al. 2009).

Nitrogen is available to plants primarily as nitrate (NO_{3-}) and ammonium (NH_4^+). In cropping soils, N is predominantly available as NO_{3-}, with NH_4^+ being generally 10 % of the NO_{3-} concentration (Wolt 1994); however, plants have been shown to perform better with a combination of NO_{3-} and NH_4^+ available (Forde and Clarkson 1999). Nitrate is readily mobile in the soil and moves to the root surface via mass flow, after which it is taken up by high and low affinity transporters (HATS and LATS, respectively) which are part of the NRT2 and NRT1/NPF gene families (Léran et al. 2014; Plett et al. 2010), whose expression regulates the activity of the transporters. Ammonium is much less mobile in soil than NO_{3-} and is taken into the root by the AMT transporter family (Gu et al. 2013; Howitt and Udvardi 2000; Ludewig et al. 2007). Transporters on the root surface have been targets for transgenic or genetic upregulation in order to increase uptake capacity (Fan et al. 2016); however, thus far, results have been mixed. They may have been unsuccessful as tissue N concentration is tightly controlled and negative feedback mechanisms prevent increased uptake (Garnett et al. 2015).

Altering root morphology has had limited success in improving N uptake. As NO_{3-} moves readily to the root via mass flow, changing root architecture is more effective for immobile nutrients such as phosphorus, than for NO_{3-} (Burns 1980). Burns (1980) showed that, due to the plastic nature of the root system, plants can cope with only 15 % of their roots being exposed to NO_{3-}, leaving little imperative to increase root biomass from an NUpE perspective. Increased rooting depth may be useful in deep sandy soils to intercept highly mobile NO_{3-} being leached through the profile, or in deep soils with stored water and N at

depth, however, in less porous soils increased root growth may be an inefficient use of carbon (C) (Garnett and Rebetzke 2013).

1.2.2 NUtE

Nitrogen utilisation efficiency is the proportion of aboveground biomass N which is converted to grain (Good et al. 2004). This grain N is derived from tissue N that has been assimilated pre-anthesis and N that is taken up post-anthesis (Hawkesford 2017). Prior to anthesis the biomass acts as an N-sink; however post-anthesis those resources are remobilised to the grain as well as N that is assimilated post-anthesis (wheat/barley) (Martre et al. 2003) and post-silking (maize) (Rajcan and Tollenaar 1999). Harvest index (HI) is the ratio between the harvestable and shoot biomass and represents how efficient the plant is at assimilation and translocating resources to the grain (Sinclair 1998). Harvest index has increased greatly over the last 50 years through the development of semi-dwarf varieties (Fischer 2011; Sinclair 1998). Nitrogen harvest index (NHI), the ratio of grain N to aboveground biomass N, is synonymous with NUtE. In wheat, improvements in NUtE have been mainly due to improvements in the HI (Fischer and Wall 1976; Foulkes et al. 1998; Ortiz-Monasterio et al. 1997). However, in modern varieties NHI is high and consistent irrespective of N fertilisation (Barraclough et al. 2010). In wheat, the remobilisation of N can be quite efficient, with little residual N remaining in the straw (Hawkesford 2017).

The manipulation of remobilisation has been shown to be possible via changes to the 'stay-green' traits which either reduce the rate, or delay the onset, of senescence (Thomas and Smart 1993). For crops such as sorghum, stay-green traits can be advantageous when they are grown with access to stored soil moisture as they can benefit from a longer period of photosynthesis, assimilating greater amounts of N into tissue, providing a greater source for grain filling (Borrell et al. 2001). However, for wheat/barley, this is not always ideal, for example, in Mediterranean climates which experience a hot-dry finish to the season with

limited stored soil water. In these conditions, a rapid remobilisation is preferable to shorten the period between anthesis and maturity (Garnett and Rebetzke 2013).

1.3 Efforts to Improve NUE, But No Progress

Efforts to improve NUE have ranged from improving agronomic practices, identifying significant QTL affecting NUE component traits (uptake and utilisation) and transgenic approaches; however, these efforts have so far not resulted in NUE improvements. Breeding has historically taken place under plentiful N conditions, and it was hypothesised that this produced germplasm with reduced NUE, especially under low N conditions (Kamprath et al. 1982). More recently, this has been disproven by studies showing that newer varieties are more N efficient under low N conditions than older varieties in both wheat (Ortiz-Monasterio et al. 1997) and maize (Ding et al. 2005; Echarte et al. 2008). These improvements may have been incidental when breeding for yield; however, these gains are minimal and must now be improved using a more targeted approach.

Nitrogen use efficiency can be improved agronomically via better management practices including matching N fertilisation to plant requirement, management of surface runoff, improving acidic soils, avoiding waterlogging to reduce anaerobic denitrification, and canopy management (Fageria and Baligar 2005; Herwaarden et al. 1998; Keeney and Nelson 1982). Although agronomic improvements will continue to play a central role in improving NUE, without improving the plant NUE progress will always be limited.

Genetic mapping in order to identify the QTL associated with NUE is an important step in its improvement and gains have been made in wheat (An et al. 2006; Quraishi et al. 2011; Xu et al. 2014), maize (Agrama et al. 1999; Gallais and Hirel 2004), rice (Cho et al. 2007; Wei et al. 2012), and barley (Mickelson et al. 2003). In one example in wheat, 11 major chromosomal regions responsible for NUE were identified (Quraishi et al. 2011). The loci identified in wheat are co-located with Ppd (photoperiod sensitivity), Vrn (vernalisation

requirement), and Rht (reduced height), which are all developmental genes, possibly controlling the amount of time that the plants can take-up and utilise N (Quraishi et al. 2011). For an extensive investigation of the genes identified, there are a number of recent reviews (Cormier et al. 2014; Garnett et al. 2015; Han et al. 2015; Quraishi et al. 2011).

The genetic variability of NUE within cereals has been shown to be high; especially under low N conditions (Dhugga and Waines 1989; Le Gouis et al. 2000; Ortiz-Monasterio et al. 1997), however, conventional breeding of elite lines has not resulted in NUE-improved germplasm. This is possibly because there is a large number of QTL influencing NUE (Garnett et al. 2015). This in turn requires a large population of backcrossed individuals in order to observe segregation at loci of interest and repeated measurements to assure confidence in the QTL measured, as the environmental impact is often more significant than the genotypic difference observed (Han et al. 2015).

Transgenic attempts to improve NUE have targeted amino acid biosynthesis, translocation/remobilisation, signalling and N regulation, and C/N storage proteins for reviews consult (Garnett et al. 2015; McAllister et al. 2012). Some of the most promising transgenic approaches have overexpressed the genes responsible for glutamine synthetase (GS) (Brauer et al. 2011), glutamate dehydrogenase (Abiko et al. 2010), the rice nitrate transporter (NRT2.3/2.5) (Fan et al. 2016), and alanine aminotransferase (AlaAT) (Good et al. 2007). However, despite the concerted effort, neither transgenic nor conventional breeding has resulted in the commercial release of cereals with dramatically improved NUE.

1.4 Why Has No Progress Been Made?

Nitrogen use efficiency is a complex trait determined by a group of processes which transport the N molecules into the root, assimilate and utilised that N to produce biomass, and finally remobilise N to the grain. A large number of QTL are believed to be responsible for NUE but there has been very little overlap between mapping studies (Garnett et al. 2015).

Large numbers of QTL require large mapping populations and their repeated study to verify results. Furthermore, studies investigating NUE genetic variability have often been undertaken in single years, which does not take into account the significant environmental effects that are obvious in multi-year experiments (Barraclough et al. 2010; Hawkesford 2017). A minimum of three years of data per variety is recommended to account for the genotype x environment x management interaction (GxExM) previously noted (Hawkesford 2017), suggesting some QTL studies may be compromised in this way.

Compounding the difficulty in identifying NUE QTL has been the use of inappropriate phenotyping methods. Ideally, NUE performance should be measured as the difference in plant growth and yield between high and low N. However, some QTL mapping studies investigating NUE have only utilised a single N level of fertilisation, potentially missing QTL which are present at one or the other (Cormier et al. 2016). As described above, it is suggested there should be multiple years of field trials to reduce the E component in GxExM. Studies in controlled environments, although having more control over E, need to be rigorous and repeatable. This has not always been the case. Pot experiments in controlled environments are criticised as sometimes having little bearing on field performance (Passioura 2006), and this may in part be due to poor experimental setup, e.g., small pots, inappropriate watering levels, or poor growth conditions (Poorter et al. 2016). Hydroponics experiments allow tighter control of N levels but are further removed from the field than pots and results derived from these need to be validated in soil. A large number of studies reporting progress with NUE in transgenic plants have never advanced beyond the very basic phenotyping carried out in the initial publication. If controlled environment experiments were designed to be as comparable as possible to the field, their relevance to the field may be enhanced and field relevant progress made. However, often the methods used are poorly described in publications, and as with many field studies, there is an incomplete description

of the growth environment. This is a critical oversight when trying to understand such an environmentally affected trait.

1.5 Can Modern Phenomics Help?

Modern phenomics, the study of plant growth, performance, and composition, utilises new technologies to better characterise plant responses to the environment and also better describes the growth environment itself (Furbank and Tester 2011). Phenomics can aid in the phenotyping of NUE performance via non-destructive measurements of biomass, growth rates, and transpiration rates to observe germplasm differences over the course of their life cycle, adding a temporal dimension to the phenotype and providing more opportunities to understand final yields. Phenomics can also provide a platform wherein non-invasive biological data can be collected on a large number of plants simultaneously, providing observations of plant behaviour that have been unavailable via traditional phenotyping techniques and destructive harvests, e.g., chlorophyll fluorescence for photosynthetic performance or hyperspectral imaging for measuring leaf constituents. Finally, modern technologies allow much better quantification of the environment in which plants grow. In combination, these advances may enable progress in dissecting NUE that until now has been lacking.

1.5.1 Phenomics in Controlled Environments

Nitrogen use efficiency is a difficult trait to phenotype because the interaction with the environment can obscure genetic gains. Therefore, one way to improve the characterisation of the genetic component of NUE is to provide a controlled, quantifiable and replicable environment within which to 'fine dissect' the component traits of NUE (Furbank and Tester 2011). Controlled environments provide this to different degrees, ranging from growth rooms, and glasshouses, to field-based installations such as rainout shelters (Rebetzke et al. 2012). To maximise value and allow replication of experiments, the controlled environment

conditions should be well characterised and published with the phenotypic data (Billiau et al. 2012; Krajewski et al. 2015).

Controlled environment NUE phenotyping is often reliant on artificial illumination, the quantity and quality of which can vary significantly and is not often accounted for (Cabrera-Bosquet et al. 2016). In controlled environments, light quality varies greatly depending on the light source (Hogewoning et al. 2010). Given that light quality, not just intensity, can have major impact on plant growth, it needs to be quantified (Dueck et al. 2016; Max et al. 2012; Ugarte et al. 2010). It is now viable and relatively cheap to measure light quality, not just the intensity, and this should be done routinely and reported.

In addition to light quality, if experiments are to reflect field performance, the daily light incidence (mol m^{-2} d^{-1}) and temperature settings in controlled environments should reflect those of the target environment as much as possible. Meta-analysis of controlled environment experiments has demonstrated that experimental conditions often fall significantly outside desired climactic ranges, causing differences in specific leaf area and tillering among others, compared to the field and may affect NUE performance (Poorter et al. 2016). For example, daily light incidence settings would be crucial when trying to tease apart the role of Ppd on NUE in wheat (Quraishi et al. 2011).

In addition to illumination and climate variation, controlled environment experiments can provide some control over soil homogeneity. Achieving uniform N across a field trial is nearly impossible and requires careful soil reserve depletion in previous seasons, but even then there can be considerable variation (Shaw et al. 2016). Controlled environment experiments can ensure a consistent level of soil structure and N content in all pots within the experiment, resulting in more precise N fertilisation than in the field. Automatic watering systems in greenhouse phenotyping platforms also offer greater control over water application than the field or conventional pot experiments which, often suffer

from excessive watering levels causing hypoxia, affecting root growth (Passioura 2006). These conditions could affect NUE phenotypes dramatically and are avoided in modern phenomics systems via the use of gravimetric watering systems, which can maintain soil water contents at levels more closely mirroring field conditions (Passioura 2006).

When effective environmental monitoring is undertaken in controlled environments, it can become obvious that there are spatial differences that need to be taken account of in order to reduce error. The statistical design of experiments is crucial to achieve this (Brien et al. 2013). In order to account for the spatial variance within a greenhouse, a statistical design and analysis approach (blocked design) was more accurate than continually alternating the position of the plants within the experiment (Brien et al. 2013).

1.6 HTP Platforms

High-throughput phenotyping (HTP) platforms are specifically designed to automate the collection of plant biometric data. Most controlled environment HTP platforms are comprised of individual pots on conveyor belts which deliver the plants to a series of imaging cabinets and watering stations (although some HTP platforms are now based on moving whole benches of plants). Basic imaging is usually undertaken via red-green-blue (RGB) cameras but systems can also include fluorescence, thermal infrared (IR), near infrared (NIR), and hyperspectral imaging. The accurate estimation of biomass from digital images has been demonstrated in various crops, including barley (Honsdorf et al. 2014), rice (Yang et al. 2014), wheat (Golzarian et al. 2011), and sorghum (Neilson et al. 2015). Accurate growth curves can be derived from these images.

Forward genetics screens using HTP have become a powerful tool to identify relevant QTL and phenotype germplasm. The efficacy of genetic analysis of HTP data to identify relevant QTL has been demonstrated in maize (Muraya et al. 2017), barley (Chen et al. 2014; Honsdorf et al. 2014), rice (Campbell et al. 2015), and wheat (Parent et al. 2015).

High-throughput phenotyping has been used in a forward genetics approach to investigate the genetic basis of maize growth traits and 988 QTL have subsequently been identified (Zhang et al. 2017). These traits include morphological traits, leaf architecture, biomass, and colour. The use of HTP was crucial in these studies because many of the phenotypes studied were dynamic metrics, such as growth or transpiration rates, which could only be obtained on large populations via non-destructive HTP. High-throughput phenotyping has also been used to assess the response of sorghum to N and water limitation, via their growth, composition, and shape in a dose–response experiment (Neilson et al. 2015). This study aimed to optimise the use of HTP for the identification of plant phenotypes that correlated with performance under water and N stress. In addition to water stress and N treatment, HTP platforms have also allowed the phenotyping of salinity tolerance in barley (Meng et al. 2017) and rice (Al-Tamimi et al. 2016). Al-Tamimi et al. (2016) demonstrated that the growth curve analysis available in the HTP platform allowed the comparison of transpiration (gravimetrically), transpiration use efficiency (TUE), and relative growth rates (RGR) 1–13 days after salt application in 553 accessions. These phenotypes were then associated with specific genomic loci via genome-wide association study and have become targets for further research. The quantification of these phenotypes would not have been practical prior to HTP and genes with relatively small effects can now be identified for potential use in genomic selection approaches (Campbell et al. 2017). The use of HTP with genome wide association studies (GWAS) shows promise for the identification of candidate genes for NUE improvement (Brown et al. 2014).

The same approach used for these complex and dynamic traits could also be used to fine dissect the component traits of NUE: NUtE and NUpE. The resolution of the growth observations allows for a dissection of growth rates at specific times during experiments and in response to changes in N or water availability. For example, the comparison of RGRs

under specific N levels can identify germplasm which is able to rapidly establish biomass. Early biomass is advantageous for N uptake as N fertiliser application commonly occurs at seeding and early utilisation minimises losses. Furthermore, a major advantage of being able to measure growth is the temporal aspect of the response to N in plants. Growth analysis allows the timing of N response to be determined, this being important in matching growth to fertiliser availability.

Nitrogen, specifically NO_{3}-, is freely mobile in moist soil but in drying soil its movement is restricted. The ability to control water availability in HTP systems allows the application of combined water and N stress. This can help identify which genotypes are able to respond to different N levels under drought conditions. Specifically, HTP platforms can identify germplasm which are N responsive under Mediterranean field conditions, the 'hot-dry finish' commonly experienced in wheat and barley production areas (Van Herwaarden et al. 1998).

Currently, measuring N uptake in cereals is dependent on destructive harvests or proxies such as chlorophyll content, which are limiting as they remove plant material from the experiment or in the case of chlorophyll, are inaccurate at high concentrations (Ecarnot et al. 2013). The interaction of electromagnetic radiation with molecules in the leaf makes spectral reflectance measurements a suitable method to assess leaf chemistry accurately and non-destructively (Kokaly 2001). Leaf or canopy spectrometry is versatile and has been demonstrated to estimate N in maize (Yendrek et al. 2016), wheat (Ecarnot et al. 2013), and rice (Sun et al. 2017). Such non-invasive methods of phenotyping over time are ideal to tackle the dynamics of nitrogen partitioning throughout cereals (Garnett et al. 2013). Non-invasive phenotyping allows the observation of N uptake and partitioning as well as how these are affected by N availability and interactions with water. Comparing leaf-N contents between cultivars under changing N supply may provide insights into their respective N

response capacities, i.e., germplasm that are able to maintain their leaf N content and growth under N scarcity. During remobilisation, being able to measure leaf N directly would show the speed and efficiency of translocation, the different contribution of individual leaves and the interaction with water availability, and how this differs between germplasm.

1.7 Phenomics in the Field

Although controlled environment phenotyping systems provide extensive information on plant performance and allow the selection of material with putatively enhanced NUE, field performance is vital for translating research into commercial outcomes. As discussed, while field trials are essential, they are also problematic because of the inconsistent environmental conditions within one site, let alone between field environments. They are also challenging in terms of measuring growth parameters beyond yield at harvest. Advances in measurement technology and environmental monitoring mean that modern phenomics could have a major impact on phenotyping of NUE in the field.

Harvest yield is currently the standard measurement for NUE evaluation in breeding trials. Material being evaluated for NUE must have higher yields under the nitrogen treatments tested and, in the case of cereals such as wheat, maintain grain quality (Foulkes et al. 2009). Huge efforts globally have been expended on purely yield-based field evaluation of NUE with limited or no success in delivering higher NUE crops. Success may be improved with better environmental monitoring to better understand the E component of GxExM. However, even if the environment is described to the best practice standards, if NUE performance is just based on yield, large amounts of potentially useful information is lost.

Modern field phenomics technologies facilitate the collection of this non-invasive range of plant characteristics such as leaf N, providing alternatives to destructive harvests. Total nitrogen uptake and remobilisation can be ascertained from final biomass harvests and

tissue N determination. However, as this is costly, time consuming and can compromise harvest yield measurement, they are not commonly carried out. Even if final biomass is measured, it provides no indication of the temporal nature of N uptake and remobilisation. This can be important, for example, if early uptake of nitrogen is a major determinant of yield. Being better able to measure component traits that contribute to yield, and that may have greater heritability than yield per se (Rebetzke et al. 2016), has the potential to facilitate real improvement in NUE in the future.

As with controlled environments, field phenotyping will be most effective if combined with environmental monitoring. Climate data associated with field evaluations have often been lacking, relying on the nearest meteorological stations rather than weather stations onsite (Lovett et al. 2007). When phenotyping is undertaken in a site without adequate environmental observation, results may be attributed to genetic difference, when in fact they may be due to environmental conditions. Encouragingly, like in controlled environments, environmental monitoring in the field is becoming ubiquitous with decreasing cost and size of instruments. Ideally, each field site should have its own weather station that can also measure solar radiation. Nitrogen use efficiency and plant performance could then be normalised for weather conditions, solar irradiance, soil water, or tissue N content to provide better comparisons of phenotypes between research sites.

Soil greatly influences plant phenotypes; however, it is heterogeneous within and between field sites, resulting in environmental variation which needs to be accounted for (Lovett et al. 2007). An ideal field trial site would have a homogenous N and soil structure across the site. Achieving this would require resources beyond the scope of most research trials and so a compromise needs to be made between field preparation and variation. As field trial site uniformity cannot be achieved, effort should be concentrated in monitoring and evaluation. Regular soil testing should be undertaken during each experiment, and

ideally the spatial variation characterised (Shaw et al. 2016). An idea of the soil disease load is important and is often available from local area mapping (Heap and McKay 2009). Field sites should also be mapped for salt, clay, and soil water via electromagnetic conductance EM38 measurements (Araus and Cairns 2014). Where possible, the heterogeneity of field sites should be quantified and the differences taken into account in experimental design.

The non-destructive phenotyping of modern phenomics allows the acquisition of much more information on plant performance compared to destructive harvests alone, allowing a much better understanding of the dynamics of traits. For this reason, numerous groups are working on improving field phenotyping capabilities with a variety of approaches being utilised to increase the precision, resolution, and throughput of phenotyping in situ by the conveyance of sensors over the crop canopy (Araus and Cairns 2014; Virlet et al. 2016).

1.8 Field HTP Technologies

The capacity of HTP in the field to characterise the performance of thousands of plants rapidly in situ is already available and the amount of data that can be collected can be challenging (White et al. 2012). The difference in the rate of data collection between HTP and conventional phenotyping is significant. A tractor boom-operated sensor bank containing multispectral cameras, ultrasonic sensors, and environmental monitoring instruments is able to collect height, canopy temperature, and reflectance ratios, which correlate well with yield, biomass, flowering time, and N status at a throughput of 3,000 plots an hour. In contrast, the rate of manual phenotyping done by two people for the simple trait of 'plant height' is about 45 plots hour^{-1} (Tanger et al. 2017).

The sensors used in field phenotyping must be conveyed across the top of the plant canopy and many methods have been developed or utilised to do this. Systems range from ground-based gantry structures (Virlet et al. 2016), unmanned aerial vehicles (UAVs) (Sankaran et al. 2015), 'phenobuggies' (Crain et al. 2016; Rebetzke et al. 2016), or modified

agricultural vehicles (Tanger et al. 2017), each having their own issues around sensor payload, resolution, cost, and speed. UAV drones and blimps fly above the canopy with sensor payloads generally weighing less than 5 kg, carrying RGB and multispectral cameras (Burger and Geladi 2006; Chapman et al. 2014). Field buggies or 'phenobuggies' range in complexity from a manually pushed trolley to larger motor and GPS-assisted vehicles (Crain et al. 2016; Deery et al. 2014) and are a convenient compromise between large payload and low-tech solutions. Agricultural vehicles such as tractors and quadbikes can be utilised with sensors attached to booms (Tanger et al. 2017). Ground-based methods can provide high spatial resolution observations due to the proximity of the sensor to the canopy, albeit at a lower throughput than UAVs. Unlike UAVs, ground-based platforms are not as restricted in their sensor payload and can carry heavier sensors such as short-waved infrared (SWIR) hyperspectral cameras (Eitel et al. 2014). Ground-based systems are disadvantaged under waterlogged conditions and may cause soil compaction after repeated measurements. A permanent gantry structure avoids soil disturbance and can operate under wet conditions while maximising the number of sensors conveyed, resulting in permanent high spatial resolution, where the detection of individual wheat ears in a plot is possible (Virlet et al. 2016). However, the disadvantages are cost, limited number of plots, and the fixed location requiring compromises between repeat experiments and necessary crop rotation (Andrade-Sanchez et al. 2013; Virlet et al. 2016).

For NUE phenotyping, RGB and multispectral cameras can be used to assess plant biomass, architecture, and chlorophyll-based indices such as normalised difference vegetation index (NDVI) (Holman et al. 2016). Spectral reflectance indices from multispectral cameras have demonstrated good correlation with wheat yield under irrigation (Babar et al. 2006) as well as many other physiological parameters (Peñuelas and Filella 1998). Although multispectral cameras cannot give a direct measure of plant N status, they

can greatly expand the physiological parameters which can be collected non-destructively and that may correlate with NUE performance. Hyperspectral reflectance can also be utilised in the field to measure N directly in leaf tissue (Ecarnot et al. 2013). Light detection and ranging (LIDAR) (with and without a red laser) has also been used to simultaneously measure biomass and nitrogen distribution in the canopy (Eitel et al. 2014; Rebetzke et al. 2016).

Recent examples of high-throughput NUE phenotyping in the field have included the categorisation of sorghum growth in response to N fertilisation in order to assist with genomics-assisted breeding selection (Watanabe et al. 2017). When UAVs fitted with near-infrared green-blue (NIR-GB) cameras were used to predict canopy height, r^2 values of 0.678 at high-N and 0.842 at low N were found for correlations with actual canopy height. Alternatively in rice, ground-based HTP was utilised on a population of 1,516 recombinant inbred lines (RILs) to assess canopy height, temperature, and reflectance ratios which correlate well with biomass, leaf area index, flowering time, and nitrogen status (Tanger et al. 2017). These methods were able to identify the genomic regions associated with yield and yield-related traits in this large mapping population. High-throughput phenotyping facilitated this research and allowed it to be done significantly faster and at a lower cost than conventional phenotyping. More importantly, it allowed measurement of parameters that would have been impractical using manual measurement, and allowed them to be measured non-destructively on multiple occasions.

1.8 Conclusion

Little progress has been made in improving NUE of cereals (Garnett et al. 2015). This is despite the fact that the genomes of important cereal crops have been sequenced. Furthermore, genes, loci of interest, and regulatory networks influencing NUE have been identified but, as yet, no improved NUE cereals have been released commercially.

Deepening genetic understanding may have provided false hope that improving cereal NUE could be easily achieved. The NUE research carried out in the field and in controlled environments, although not yet leading to germplasm with improved NUE, has helped us better understand the complexity of the trait and, in particular, the major GxExM interaction. Modern phenomics as detailed here gives us the opportunity to better characterise the environment, plant responses to the environment and, combined with continually increasing genetic information, offers the opportunity to make real progress in improving NUE.

1.9 Aims and Objectives

The aims and objectives of this thesis are:

i) To quantify NUE phenotypes in Australian bread wheats using high-throughput phenotyping;

ii) To establish a hyperspectral reflectance protocol in order to measure leaf N with the aim of distinguishing N uptake and utilisation differences;

iii) To use these technologies in combination to measure and help explain the growth and leaf N responses of wheat cultivars under varying N availability.

Chapter 2 quantifies NUE phenotypes via growth over time using high-throughput phenotyping by comparing differences under three N levels and four water availability schedules. Growth differences were illuminated in high resolution and connected to the subsequent yield results. Physiological differences in cultivars were observed over two years of experimentation indicating a stability required for the dissection of NUE and its component traits.

Chapter 3 examines how a hyperspectral method for the leaf N concentration of wheat leaves could be established using partial least squares regression combined with destructive analysis.

Chapter 4 contains the use of this protocol to observe leaf N differences in wheat under varying N availability in a hydroponic system.

Chapter 5 combined these techniques to determine whether they could quantify and explain growth and leaf N differences between two wheat cultivars in response to split N applications. The growth and leaf N differences were used to explain the conventional destructive harvest differences observed at the conclusion of the experiment.

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Chapter 2: Nitrogen use efficiency and nitrogen response under drought phenotypes revealed in wheat via high resolution growth analysis

TITLE

Nitrogen use efficiency and drought resistance phenotypes revealed in wheat via high resolution growth analysis

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ABSTRACT

The improvement of nitrogen use efficiency (NUE) in wheat is a major priority in cereal research due to environmental and economic imperatives. Broadly speaking, NUE is the grain yield per unit nitrogen (N) added and is comprised of N uptake efficiency and N utilisation efficiency. Improving NUE has proved an intractable problem due to the interaction between such a complex trait and the environment, paired with ineffective phenotyping methods dependent on destructive harvest. Technology is now available to greatly increase the phenotyping power of cereal growth using high throughput phenotyping with high resolution growth imaging. The work presented here utilises these methods to quantify differences in shoot area and growth rate in response to different levels of N and water availability, including the interaction between these, for ten commercial bread wheat (*Triticum aestivum*) cultivars.

The ability to accurately measure growth rate revealed cultivar differences in N response (to 25, 75 and 150 mg N kg soil^{-1}). Cultivar differences in their ability to increase shoot area with increasing N application were observed as well as cultivar differences in N response under drought, with Excalibur, Gladius and Mace demonstrating an increase in shoot area under high N. The shoot area at 52 days after planting (DAP) was correlated with yield at harvest and the relationship became even stronger when separated into water availability treatments. The restriction of water at 48 DAP demonstrated which cultivars suffered from 'haying-off, as they were unable to convert their shoot area into grain yield, resulting in incomplete grain filling. The phenotyping results were repeatable over two years of experimentation, addressing one of the main issues facing NUE improvement, the genotype x environment x management interaction (GxExM). This protocol offers stability, control over abiotic factors and treatment combinations that are not practical to achieve in

the field (precise water availabilities). This protocol would be suitable for use with forward genetic approaches and represents a step forward in the improvement of NUE in wheat.

INTRODUCTION

Modern bread wheat (*Triticum aestivum*) is reliant on nitrogenous fertiliser additions in order to reach its yield potential. However, wheat generally recovers less than half of the applied nitrogen (N), causing ecosystem pollution and economic losses (Fageria and Baligar 2005; Peoples et al. 1995; Raun and Johnson 1999; Sylvester-Bradley and Kindred 2009). As food production requirements increase over the next 50 years, increasing the nitrogen use efficiency (NUE) of wheat could have a major impact on the sustainability and cost of production, as well as help in mitigating stagnating yields (Ladha et al. 2016; Nathaniel et al. 2014).

In wheat production, NUE is defined as the grain yield per unit of N added as fertiliser (Good et al. 2004). NUE includes both the uptake of N from the soil (N uptake efficiency; NUpE), and its efficient usage within the plant and transfer to the grain (N utilisation efficiency; NUtE). NUE is comprised of complex processes, ranging from whole plant physiology to the enzymatic level and can be dissected into an array of interacting traits (Sadras and Lemaire 2014). In addition to these well-established NUE assessments, are the comparison of performance (yield) under low and high N (Hawkesford 2017). This is because the plants may express different traits at different input levels, i.e., N uptake efficiency traits at low N. These phenotypes, which have been described as ‘low N tolerant’ in sorghum (*Sorghum bicolor*) (Veley et al. 2017), but have not been well described in wheat, would be desirable in low input production areas. Alternatively, cultivars which are able to continue responding to increasing N levels, ‘N responsive’ cultivars, would be desirable in high input production areas of Australia.

Although no wheat genotypes with significantly improved NUE have been released commercially, variation in NUE does exist, especially under low N conditions (Barraclough et al. 2010; Dhugga and Waines 1989; Le Gouis et al. 2000; Ortiz-Monasterio et al. 1997). Barraclough et al. (2010) characterised 39 elite commercial winter wheat varieties and found a range in NUE of between 24-42 % between cultivars under different N application rates. Although NUE has not historically been selected for by breeders, NUE has improved in CIMMYT varieties since the 1950s (Ortiz-Monasterio et al. 1997). The genetic improvement in terms of yield and NUE from 1950-1985 was between 1.1-1.9 % yr^{-1}, with newer varieties being more N responsive and yielding more when supplied with equivalent N. These improvements are believed to have been incidental when selecting for yield (Ortiz-Monasterio et al. 1997). Experiments undertaken on Australian wheat varieties, released between 1958-2007, identified that the N uptake capacity of roots had increased over time, due to increased uptake per unit root length rather than increased root length (Aziz et al. 2016). It was suggested this was an unintended consequence of selecting for yield, reduced root:shoot ratio and time to booting, requiring the plants to compensate via roots with a higher affinity for nitrate (NO_3^-) (Pang et al. 2015) as well as thinner roots more able to access soil water and therefore N (Aziz et al. 2016).

NUE improvement via the selection of genetically superior cultivars in the field is difficult because NUE encompasses a series of complex biological processes. Many of these processes and their underlying quantitative trail loci (QTL) are influenced strongly by environment and management (GxExM) (Cooper et al. 1997; Garnett et al. 2015; Xu et al. 2012), making NUE improvements difficult to measure and, consequently, difficult to attribute to specific genetic loci (Barbottin et al. 2005). There have been significant efforts to characterise the NUE performance of wheat around the world (Gaju et al. 2011; Garnett

et al. 2015; Hawkesford 2017), as well as decades of yield trials, however these have not led to significantly improved NUE cultivars.

One of the most important environmental interactions in an Australian context is between soil and water, as NO_3^-, the main available form of N in soil, is water soluble and moves to the root surface passively via mass flow. As a result, under drought conditions, the plant may also experience N deprivation, further compounding drought effects (Hofer et al. 2017). Indeed water and N have been found to 'co-limit' the productivity of plants, with water being the limiting factor under dry conditions and N being the limiting factor under wetter conditions (Cossani et al. 2010; Hooper and Johnson 1999). Cultivars which can more efficiently access N, even under water stress, will be at an advantage in Mediterranean conditions (Sadras 2002). Conversely, a negative interaction between soil N and water leads to 'haying off' caused by incomplete grain filling due to vigorous early vegetative growth followed by spring drought (Van Herwaarden et al. 1998). Nitrogen budgeting, accounting for water availability and plant requirements, are necessary to manage this complex relationship. Any effective phenotyping system for SE Australian conditions must take into account differences in water availability and replicate those as effectively as possible in order to understand the interactions between genotype x water x nitrogen.

Efforts are now underway to identify cereal NUE related traits non-destructively both in controlled environments (Garnett et al. 2015; Neilson et al. 2015) and in the field (Araus and Cairns 2014; Nguyen et al. 2016), using automated imaging platforms. These high throughput phenotyping (HTP) facilities have been utilised for the non-invasive measurement and analysis of cereal growth over time in response to abiotic stresses (Al-Tamimi et al. 2016; Parent et al. 2015; Tilbrook et al. 2017). In rice (*Oryza sativa* L.), growth rates under salt-application were observed via RGB imaging over time, facilitating the collection of growth rates responses for 553 genotypes simultaneously, an achievement

which was previously impossible (Al-Tamimi et al. 2016). From these, salt tolerant accessions, which could maintain growth under salt application, could be identified. Similarly, barley (*Hordeum vulgare)* lines were assessed for their tolerance to 50, 150 and 250 mM NaCl (Tilbrook et al. 2017) to identify cultivars which were able to continue growing in the presence of salt. This HTP growth analysis led to the identification of cultivars with different shoot Na^+ tolerance mechanisms (Tilbrook et al. 2017). In wheat, growth analysis from HTP was utilised to assess the impact of certain QTL on drought tolerance (Parent et al. 2015). As a result, co-located QTLs were found for growth, transpiration rate and water use efficiency, demonstrating the utility of HTP when used in combination with genetic analysis (Parent et al. 2015).

Since HTP has successfully been used to identify plant growth phenotypes in response to abiotic stresses within a stable environment, it is a promising approach to quantifying and characterising N responsiveness phenotypes. Furthermore, in order to be field relevant these N response phenotypes must be assessed for potential interaction with water availability. In this research biomass, growth rates and yield were compared under three N fertilisation levels and four water availability schedules, chosen as they reflect those commonly found in South East Australian rain fed growing conditions. The growth response phenotypes to these abiotic factors demonstrated cultivar difference in growth response from low to high N fertilisation, N uptake efficiency at low N as well as cultivars which performed better under certain water stress schedules.

METHODS AND MATERIALS

Two high-throughput phenotyping experiments were conducted beginning in August in consecutive years (2014 and 2015, hereby referred to as Exp. 1 and Exp. 2). Ten Australian elite wheat cultivars were used in both experiments (Table. 1). These cultivars were chosen as they represent different maturity profiles and are commercially widely used in Australia.

Table 1. Company, year of registration and maturity profile on the ten cultivars utilised in this research

Cultivar	Company	Year of registration	Maturity profile
Axe	AGT	2007	Early
Espada	AGT	2008	Mid
Excalibur	AGT/RAC	1991	Early
Gladius	AGT	2006	Mid
Kukri	RAC	2000	Early/Mid
Mace	AGT	2007	Early/Mid
RAC875	RAC	NA (breeding line)	Early/Mid
Scout	Longreach	2004	Mid
Wyalkatchem	Intergrain (GRDC)	2001	Early/Mid
Yitpi	Waite Institute	1999	Late

Plant growth conditions

Wheat plants were grown in the south-eastern glasshouse on the Lemnatec Scanalyzer 3D System (LemnaTec GmbH, Aachen, Germany) at The Plant Accelerator (Australian Plant Phenomics Facility, University of Adelaide, Adelaide, Australia; -34.97113°, 138.63989°) in August 2014 (Exp. 1) and August 2015 (Exp. 2). For Exp. 1, plants were imaged from 24-76 days after planting (DAP) and then grown in a glasshouse until maturity. For Exp. 2, plants were imaged from 19-54 DAP and then destructively harvested. The glasshouse temperature was set to 15/22 °C night/day, with a 24 h diurnal SIN-shape temperature curve.

For both experiments, four seeds were planted into 2.6 kg moist soil (equivalent to 2.2 kg oven dry soil) into 2.6 L white plastic pots containing equal parts (v/v) cocopeat, clay/loam, UC Davis mix (a combination of sand, peatmoss and lime). There were three N treatments of 25, 75 and 150 mg N kg soil^{-1} (N1, N2 and N3 respectively). The N was mixed into the soil before potting as urea (CH_4N_2O). Plants were grown on benches in the glasshouse and at the three leaf stage (14 DAP) the seedlings were thinned out to a single

uniform specimen. The plants were manually watered on benches until 24 DAP in Exp. 1, and 19 DAP in Exp. 2 before being placed onto the Lemnatec Scanalyser 3D phenotyping platform. Each plant was supported by a blue wire frame and pots were placed into individual carriers with square 170 mm saucers.

In Exp. 1, the four water treatments were well-watered (WW) (23.5 % (g/g) gravimetric soil water content), drought through-out (DD) (13 % (g/g)), drought then well-watered (DW) from 48 DAP and well-watered then drought (WD) from 48 DAP (Fig. S1). In Exp. 2, the two water treatments were well-watered (23.5 % (g/g)) and drought (13 % (g/g)). Every two days the plants were moved by conveyer system to the imaging hall where an RGB camera system captured top and side images (see below). The plants were also watered to weight, then returned back to their position in the glasshouse.

In Exp. 1, after being on the phenotyping system from 24-76 DAP, the plants were moved to a bench in a glasshouse until maturity. In Exp. 2 the plants were on the phenotyping system from 19-54 DAP and then destructively harvested.

Design

The experiments were a split-plot design. The 24 lanes of the conveyer system were divided into four replicates, consisting of six lanes x 20 positions, which contained a complete set of the combinations of genotypes x nitrogen x drought. Each replicate was divided into three main plots, consisting of three pairs of lanes and the N treatments were randomised to these. Within each pair, the lanes formed subplots to which the drought treatments were randomised. The genotypes were assigned to the 20 carts within each subplot using a row-and-column design. The genotype assignment was generated using DiGGer (Coombes, 2009) and the N and drought treatments were randomised using DAE (Brien, 2011), packages for the R statistical computing environment (R Development Core Team, 2014).

Destructive harvest

In Exp. 1, plants were destructively harvested at maturity and height, tiller number, head number and head length were measured. The plants were dried at 70° C for three days and then stem weight, grain weight and total plant biomass were measured. Subsequently, spikelet number, seed number and thousand kernel weight (TKW) were calculated. After weighing, the samples were separated into grain and shoot and ground to a fine powder using a Genogrinder (SPEX, Metuchen, NJ, USA). Approximately 1.5-2.0 mg of the ground sample was placed in tin capsules (Elemental Microanalysis Pty Ltd, Devon, United Kingdom) and N and carbon (C) content were assessed by Dumas combustion to give N and C concentration (%) in tissue (Elementar, Mt. Laural, NJ, USA) (Muñoz-Huerta et al. 2013). The plants were visually scored using the Zadoks physiology system at 50 DAP in Exp. 1 using the images as the source (Zadoks et al. 1974).

In Exp. 2 the entire plants were harvested and fresh weight was measured at 54 DAP. They were dried for three days at 70° C and dry weight was measured as well as height, number of tillers and Zadoks growth stage assessed. They were then ground to a powder, for N analysis as with Exp. 1.

Image Acquisition and Analysis

Images of the plants were collected by a top and side view RGB camera. In Exp. 1 the cameras had a resolution of 2056 x 2454 pixels (5 megapixel). One top view image and two side view images were captured in each imaging round. In Exp. 2, a 2472 x 3296 pixel (8 megapixel) camera captured top and two side views were captured with a 90° horizontal rotation. The images were processed using LemnaGrid software (LemnaTec GmbH, Aachen, Germany). Plant tissue was separated from background using a nearest-neighbour colour classification. Noise was removed via erosion and dilatation procedures prior to the

confirmation that all of the parts of the plant were one object (Neilson et al. 2015). Projected shoot area (PSA) was calculated as the average number of pixels from the three images.

Statistical analysis

A longitudinal analysis was performed to estimate the trend in the projected shoot area over time for each treatment. This was achieved by fitting a mixed model using ASReml-R (Butler et al. 2007), a package for the R statistical computing environment (R Core Team 2018), that included terms allowing for (i) main-plot variability for each day of observation, (ii) random linear trends for each plant, (ii) correlation between observations on different days for the same plant, the correlation decreasing exponentially, and (iv) splines with 10 knot points fitted to the trend over the days for each combination of a Genotype with a N and a Drought treatment (Fig. S5).

The smoothed PSA is obtained by using the R function smooth.spline to fit a spline with five degrees of freedom to the PSA values for each plant for all days of imaging. The smoothed absolute growth rate (AGR) is obtained as the first derivative of the fitted spline for each day and the smoothed relative growth rate (RGR) is the smoothed AGR divided by the smoothed PSA for each day. The values of these traits for 52 and 62 DAP were selected. Also calculated from the smoothed AGR for each plant is the mean of its AGR values, the maximum of its AGR values and the day that the maximum AGR is achieved.

The average slope of smoothed AGR between 40 and 50 DAP is obtained by (i) using the R function smooth.spline to fit a spline with five degrees of freedom to the PSA values for each plant between 40 and 50 DAP and to obtain the second derivative of the fitted spline for each day between 40 and 50 DAP, and (ii) calculating the mean of the values of the second derivative, the second derivative being the slope of the AGR.

The average smoothed AGR (i) between day 39 and day 40, (ii) between 52 and 62 DAP, and (iii) past 50 DAP is obtained by (a) using the R function smooth.spline to fit a spline with five degrees of freedom to the projected shoot area values in one of the intervals each plant and to obtain the first derivative of the fitted spline for each day in the interval, and (b) calculating the mean of the values of the first derivative values in the interval, the first derivative being the AGR. Also calculated from the fitted splines for between 30 and 40 DAP are the maximum smoothed AGR and the day of maximum smoothed AGR.

RESULTS

Using the automated imaging system, it was possible to measure changes in PSA, which is equivalent to biomass prior to senescence (Golzarian et al. 2011), over time and in response to different N fertilisations and water availability treatments in Exp. 1 (Fig. 1). Each of the four water treatments produced a unique PSA response. PSA under WW conditions increased to 50 DAP until maximal PSA after which there was a decrease in PSA continuing until the conclusion of imaging (72 DAP). Conversely, the DW water treatment initially suppressed PSA until rewatering at 48 DAP, after which PSA increased. Under DD, PSA was suppressed for the entire imaging period. Under WD treatment, the PSA increase mirrored the WW treatment until 48 DAP, when water was restricted, after which PSA decreased sharply.

N responsiveness was determined as the increase in production of shoot area in response to increasing N availability. In terms of PSA, differences in cultivar N responsiveness were observed under WW conditions. Excalibur, Gladius, Kukri, Mace, Wyalkatchem and Yitpi responded to an increase in N fertilisation from N2 to N3 by increasing PSA, whereas Axe, RAC875 and Scout were non-responsive. The PSA increased by approximately 33 % from N1 to N2 for all cultivars, whereas from N2 to N3 there was a smaller increase in those that were high N responsive (Gladius increased by 16 %).

Furthermore, differences in the PSA response to N had a strong interaction with the different water availability treatments (Table S2). Under DW there was a suppression of PSA until rewatering (48 DAP), after which there was a recovery under N2 and N3, whereas under N1DW there was only a slight increase in PSA. Compared to N2DW, under N3DW Kukri and Mace were significantly N responsive in terms of their PSA increase, however for the other cultivars there was no increase in PSA, as shown by the longitudinal analysis of the PSA curves (Fig. S2). Under WD, there was an even separation between the PSA curves representing the three N fertilisation treatments, which mirrored the PSA under WW before the limitation of water (48 DAP). After the limitation of water (N3WD) there was a decrease in the size of the PSA by approximately 30 % from maximal PSA until the conclusion of imaging (76 DAP). Under WD, the PSA did not decrease to the size of those under DD treatment. Under DD, N1 and N2 had an equivalent PSA, whereas some cultivars were able to respond to N3 (Gladius, Kukri, Mace and Excalibur). Compared to N1DD, under N2DD Scout and Yitpi were significantly N responsive reaching a PSA of 85.2 and 86.3 kpixels respectively, which was almost equivalent to the PSA of Kukri under N3DD (89.2 kpixels).

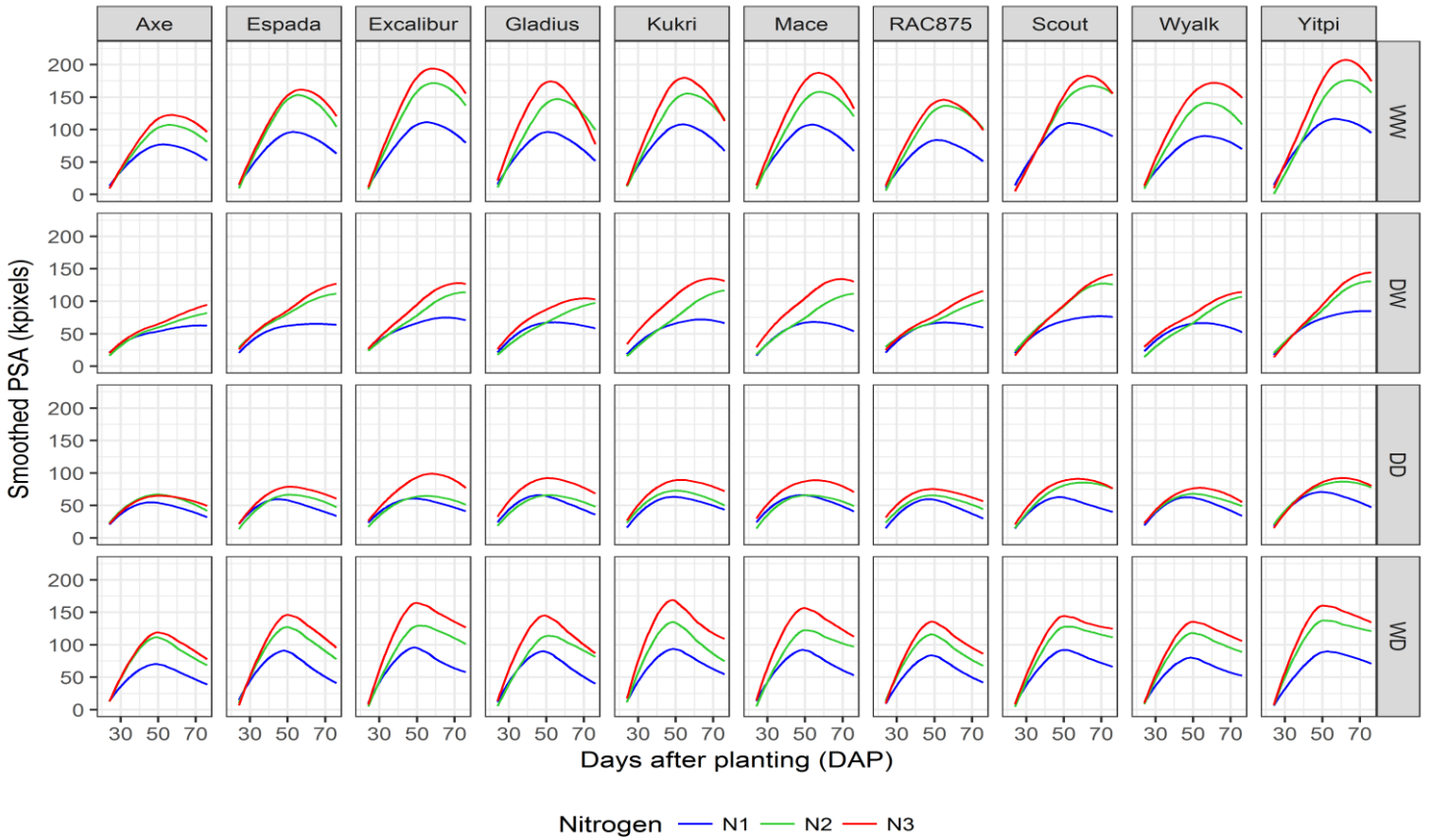

Figure 1 Smoothed projected shoot area (pixels '000) curve for each cultivar and their combination of water treatment and nitrogen level. The N levels are within each panel (25, 75 and 150 mg N kg soil^{-1} as N1 (blue), N2 (green) and N3 (red) respectively). Each row is a water treatment schedule (well-watered (WW), drought (DD), drought until day 48 DAP and then well-watered (DW), and well-watered until day 48 DAP and then drought (WD)). The y-axis represents smoothed projected shoot area (pixels '000) and the x-axis represents DAP. n=4.

The tendency to change the length of time until maximal growth in response to treatments was not consistent for all cultivars. The number of days until maximal PSA is shown for each cultivar compared to the WW conditions (Table 1). The number of days until maximal PSA was reached increased for all cultivars from WW to DW, whilst under WD the cultivar difference was suppressed, with maximal PSA occurring between 48.3 to 50.3 DAP. For example, the point of maximal PSA for Axe changed from 52 (+/-1.7 SEM) DAP under N3WW conditions to 72 (+/- 2.0 SEM) DAP under N3DW, an extension of 20 d. Wyalkatchem and Yitpi were the least plastic, extending time until maximal PSA by 6.0 and 5.6 d respectively.

Table 1 The days until maximal projected shoot area as compared to well-watered conditions. The N levels are (25, 75 and 150 mg N kg soil^{-1} as N1, N2 and N3 respectively). The water treatments (well-watered (WW), drought (DD), drought until 48 DAP and then well-watered (DW), and well-watered until 48 DAP and then drought (WD)). In bold is the time until maximal projected shoot area under WW conditions, with the following three columns being the difference in days it took for that treatment to reach maximum projected shoot area, n=4.

	N1WW	N1DW	N1DD	N1WD	N2WW	N2DW	N2DD	N2WD	N3WW	N3DW	N3DD	N3WD
Axe	**53**	16	-7	-2	**52**	19	-1	-3	**53**	19	0	-3
Espada	**54**	12	-5	-5	**55**	15	-2	-5	**53**	19	0	-2
Excalibur	**53**	11	-1	-1	**60**	10	-4	-10	**56**	9	3	-8
Gladius	**51**	2	0	0	**55**	15	-2	-5	**52**	11	1	-3
Kukri	**53**	9	0	-2	**53**	17	0	-5	**52**	15	1	-4
Mace	**52**	5	0	0	**58**	11	-6	-8	**56**	10	0	-7
RAC875	**52**	7	-1	-1	**55**	16	-7	-5	**52**	20	-4	-3
Scout	**52**	15	-5	0	**64**	3	0	-14	**60**	8	-2	-9
Wyalkatchem	**57**	1	-7	-6	**57**	12	-5	-7	**63**	6	-9	-13
Yitpi	**55**	12	-2	-2	**62**	7	-1	-12	**62**	6	1	-12

The PSA curves detail the shoot area, however cultivar growth is shown by the conversion of PSA into AGR curves (Fig. 2). The interaction between the water treatment and the AGR is shown in the different shapes of the curves (Fig. 2). Under WW, the initial positive growth was clear until the point of maximal growth when the plants continued increasing PSA at a decreasing rate until reaching 'apparent' negative growth. Prior to the AGR becoming negative there was a correlation between PSA and biomass, however at 'apparent negative growth' this relationship decoupled as the plants entered their reproductive stage, leaves senesced and resources were translocated to grain development. The apparent negative growth does not indicate a loss of biomass, but rather is a reduction in leaf area and also a change in the fresh:dry weight ratio. The imaging data has been shown to be highly correlated with cereal biomass until 42 DAP (Honsdorf et al. 2014) and 48 DAP (Neilson et al. 2015), but becomes less accurate after the onset of senescence. Under DW, the AGR decreased from the start of imaging until rewatering at 48 DAP when there was a recovery of growth rate amongst the N2 and N3 treatments. Under DD, the AGR decreased throughout the experiment and apparent negative PSA growth was reached earlier compared to WW. Under WD, the AGR curves mirrored the WW until water was restricted at 48 DAP, after which there was a rapid movement into apparent negative growth until the conclusion of imaging at 76 DAP.

The AGR curves allow for the direct observation of cultivar differences with regard to growth (Fig. 2). Under WW, Espada, Kukri and Gladius cultivars have a SIN-wave shaped growth curve which was much more pronounced than for Axe and Wyalkatchem, which had a flatter AGR curve. Under N1DW, Axe and Excalibur were the only cultivars that increased AGR after rewatering at 48 DAP, whilst the other cultivars entered negative growth. Under DD, the only cultivar which appeared to be N responsive was Excalibur, as there was an increase in its AGR between N2 and N3. Under WD, Kukri had negative growth after the

restriction of water, exacerbated under higher levels of N, this was unique amongst the cultivars.

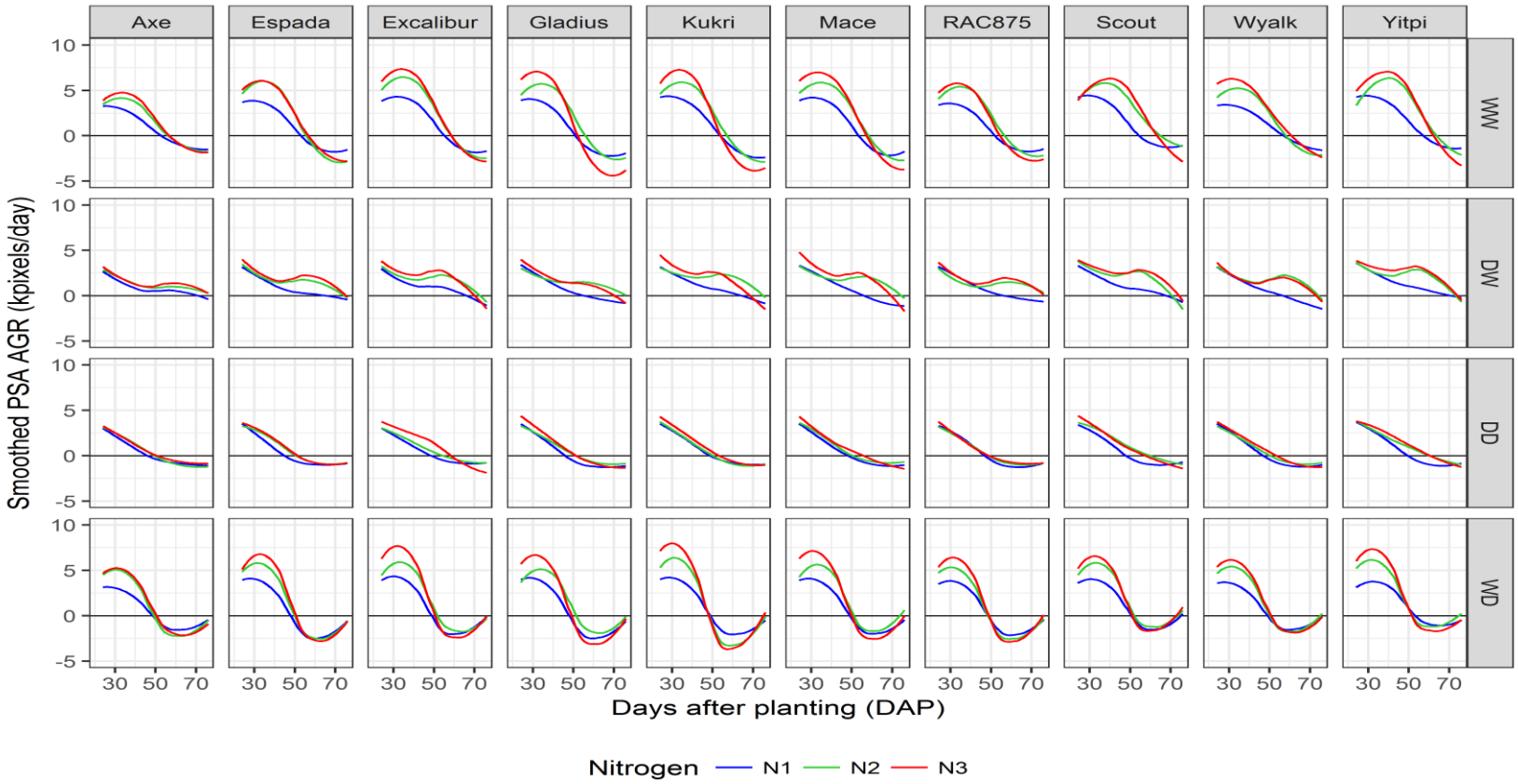

Figure 2 Absolute growth rates from 24-76 DAP. Within each panel are the 3 N levels (25, 75 and 150 mg N kg soil^{-1} as N1 (blue), N2 (green) and N3 (red) respectively), each row being a water treatment (well-watered (WW), drought (DD), drought until 48 DAP and then well-watered (DW), and well-watered until 48 DAP and then drought (WD)) and all of the ten cultivars shown, n=4.

In order to highlight growth behaviour held within the AGR curves, specific periods of growth were dissected into windows representing key transitions from vegetative to reproductive phenological stages. The period 40-50 DAP was selected as there were cultivar differences observed for the point of maximal growth rate under WW (Fig. 3). There was a significant higher order interaction between cultivar x N x water treatment for all treatment combinations ($P < 0.001$) (Table S2). Under the WW and WD treatment, most cultivars reached their maximum AGR at approximately 40 DAP. Under WW, there was a negative growth rate during this period which increased in magnitude with higher N availability. Gladius had the most negative AGR under N3WW (-0.51 kpixels day^{-1}), contrasting with Scout and Yitpi, which had a growth rate closer to zero (-0.15 and -0.20 kpixels day^{-1} respectively). Under the DW treatment, there was a decreasing growth trajectory under N1, neutral growth trajectory under N2 and positive growth trajectory under N3. Under DD, there was a negative growth rate of approximately –0.2 kpixels day^{-1} which was not influenced by cultivar or N interaction ($P > 0.05$). Conversely, under the WD treatments there was a strong negative growth rate which had an N interaction and cultivar differences, increasing from -0.3 to -0.7 kpixels day^{-1}. Kukri, which had the most negative growth trajectory under N2WD and N3WD (-0.7 and -0.8 kpixels day^{-1} respectively), contrasted with Axe which had the lowest (-0.5 and -0.4 kpixels day^{-1} respectively).

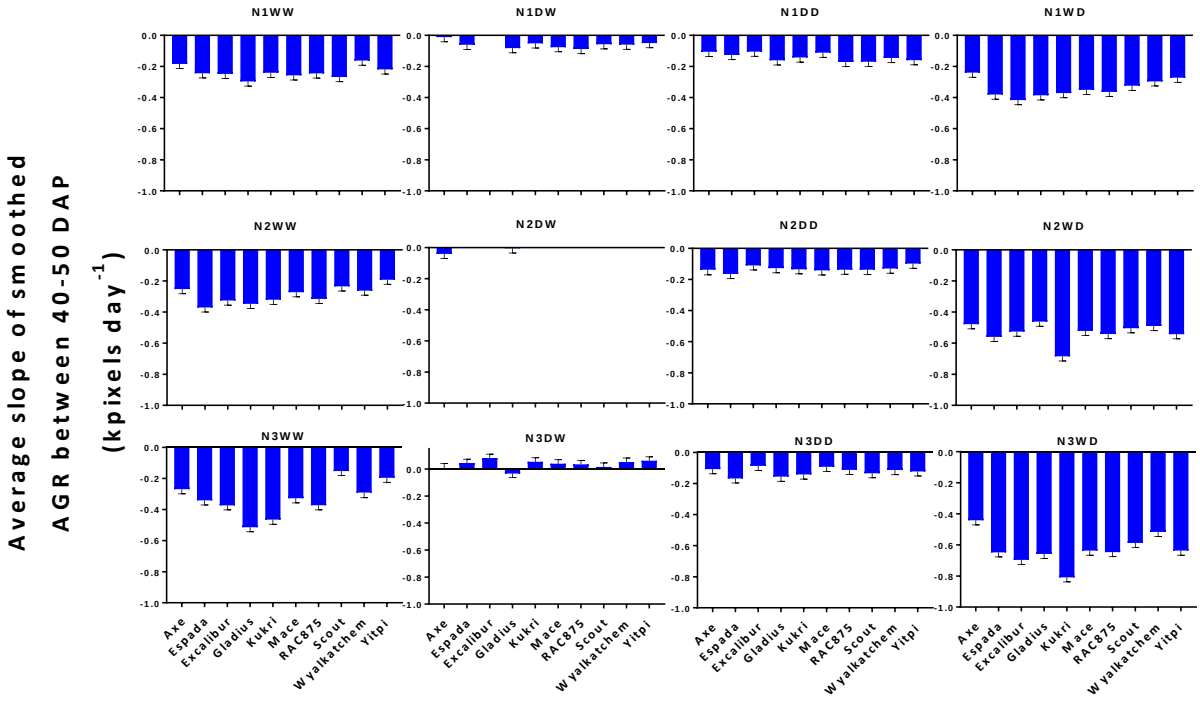

Figure 3 Three-way interaction predicted means of average slope of smoothed AGR between 40-50 DAP. Each row represents an N level (25 (N1), 75 (N2) and 150 (N3) mg N kg soil-1). Each column is a water treatment (well-watered (WW), drought (DD), drought until 48 DAP and then well-watered (DW), and well-watered until 48 DAP and then drought (WD)). Error bar is SEM, n=4.

Absolute growth rate differences between cultivars also occurred when the plants entered the reproductive stages (50 – 76 DAP), exacerbated by water availability treatments (Fig. 4). Under WW, there were large cultivar differences in average AGR. Most of the cultivars had a negative average AGR with the exception of Scout and Yitpi under N2 (0.29 and 0.00 kpixels day^{-1} respectively). Gladius was the most negative in its AGR under N3WW (-3.98 kpixels day^{-1}), whereas the AGR of Scout, Wyalkatchem and Yitpi was close to zero during this period. Under N2DW and N3DW all cultivars had positive average AGR, whereas under N1DW treatment

Axe and Yitpi were the only two cultivars which had a positive AGR (0.18 and 0.31 kpixels day^{-1} respectively). The remaining cultivars had neutral or negative AGR in this period. Under N3DW, Scout and Yitpi had the highest rate of positive AGR (1.57 and 1.71 kpixels day^{-1} respectively), whilst Gladius and Kukri had the lowest (0.32 and 0.54 kpixels day^{-1} respectively). The negative AGR of Gladius and Kukri under N3WW was significantly more negative than the other cultivars. Under WD there was a consistent negative AGR at approximately -2 kpixels day^{-1} under each of the N levels. Under N3WD, similar to N3WW, Scout and Wyalkatchem were the least negative AGR, whilst Gladius had the most negative AGR.

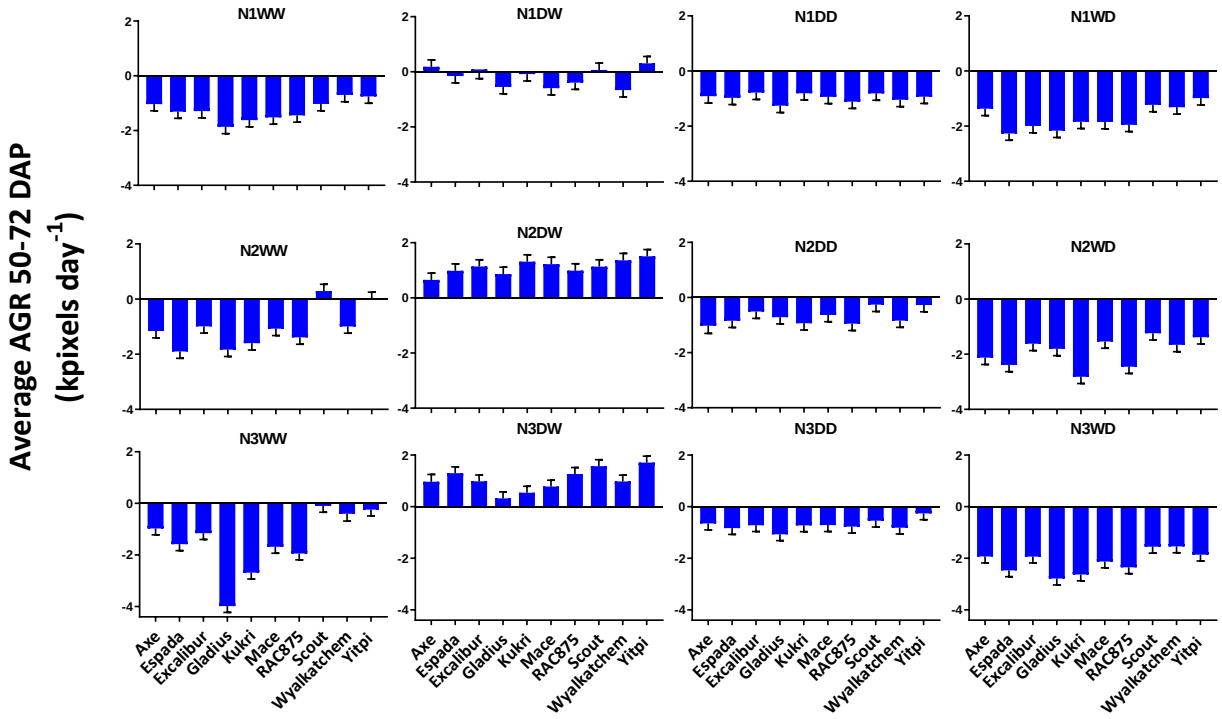

Figure 4 Three-way interaction predicted means of average AGR from 50-76 DAP. The y–axis is the kpixels day^{-1} change. Each panel shows the ten cultivars within each combination of N level (25, 75 and 150 mg N kg soil^{-1} as N1, N2 and N3 respectively) and water treatments (well-watered (WW), drought (DD), drought until 48 DAP and then well-watered (DW), and well-watered until 48 DAP and then drought (WD)). Error bar is SEM, n=4.

One of the most important aspects of this research was observing the interplay between yield, PSA and the response of these to N and water availability. The results shown here demonstrate that PSA at 52 DAP correlate to yield at harvest (Fig. 5) and that this correlation became stronger when the results were separated into water treatments. Under the WD treatment, there was a decrease in yield compared to WW, which was greater than the decrease in PSA. Conversely, those plants under the DW treatment, had a similar PSA to DD, but the yield results were higher than DD. Under the DD treatment both yield and PSA at 52 DAP were diminished compared to WW. Under DD, there was no cultivar where the N3 treatment exceeded the PSA or yield found under the N1WW treatment highlighting the impact of water availability on yield.

There were differences in the cultivar response to N and water availability as seen in the relationship between PSA at 52 DAP and yield. Gladius was the most N responsive cultivar under WW conditions, demonstrated by the distance between the N2 and N3 points for both PSA and yield. Conversely, Yitpi and Wyalkatchem were not N responsive with respect to yield under WW conditions, with only a slight increase in PSA between N2 and N3. The slope of each water treatment line highlights the cultivar differences in responsiveness to N in terms of PSA at 52 DAP and yield. The more vertical the line, the more the cultivar was able to increase PSA but not yield with increasing N, whereas the more horizontal the line the more able the cultivar was to increase yield, compared to WW. For example, comparison between Scout WD and Axe WD shows that yield increased more significantly under Axe than Scout. As well as the slope of the curves the distance between the points also indicates how N responsive the cultivar is to an increase in N availability.

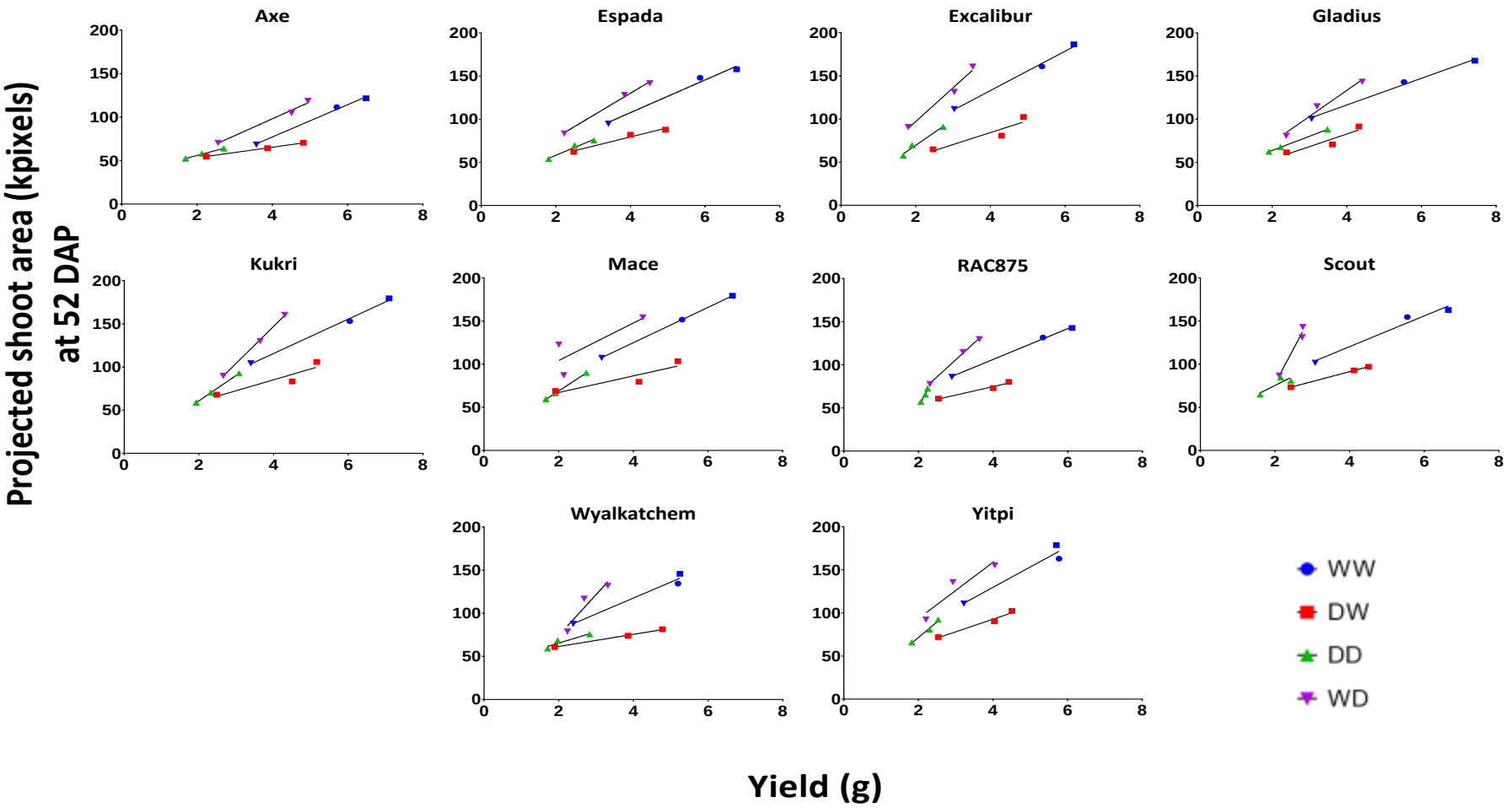
Axe
Espada
Excalibur
Gladius
Kukri
Mace
RAC875
Scout
Wyalkatchem
Yitpi
WW
DW
DD
WD
Projected shoot area (kpixels) at 52 DAP
Yield (g)

Figure 5 The relationship between the projected shoot area (pixels ‘000) at 52 DAP on the y-axis and the grain yield at maturity (g plant^{-1}) on the x-axis. Each panel represents one cultivar within which the four water regimes are represented (well-watered (WW), drought (DD), drought until 48 DAP and then well-watered (DW), and well-watered until 48 DAP and then drought (WD), and on each of those lines are the three N levels (25 (triangle), 75 (circle), 150 (square) mg N kg soil^{-1}). The lines are lines of best fit, n=4.

Under N1, the total shoot N was equivalent across the four water treatments between 50-75 mg N plant^{-1} (Fig. S3). Whereas under N2 and N3, the WW and DW treatments had a slightly higher total shoot N compared to under the WD, whilst the lowest total shoot N was under DD. There were cultivar differences amongst each of the treatment combinations. Under N3DW, Mace and RAC875 took up more N than the other cultivars. Under N3DD, Axe had the least amount of N uptake, whilst Excalibur, Kukri and Mace had the most uptake. Under N3WD, Yitpi had the highest N uptake, followed by Scout.

The NUE measurements were determined by destructive harvest and combined both of the components: NUpE and NUtE (Fig. S4). Both the NUE and NUpE decrease with increasing N fertilisation, with the highest NUpE being under WW, and the lowest under DD. There are cultivar differences in NUE, with a 20 % difference between the highest and lowest NUE results under N1WW, Axe had the highest NUE at 65 %, with the lowest being Wyalkatchem at 44 % (Fig. S4). This variation is reduced under the N2WW and N3WW treatments. A similar pattern emerged with NUpE as under N1WW there was the most variation. In this instance, Yitpi had the highest N uptake efficiency, with Excalibur the lowest. Under N3, the cultivars did not differ from one another and were able to take up only 50 % of the applied N, with the exception of N3DD which took up approximately 37 % of the applied N.

The NUtE was calculated as the grain weight divided by the amount of N in the aboveground plant tissue and is highest under N1WW, and decreased as N increased, but not to the same degree as NUE. The dry finish treatments (e.g. N3DD, N3WD) had the lowest efficiency at converting plant N into grain weight, managing between 20-30 % compared to under the WW treatment of 30-50 %. The ability of the plants to allocate their tissue N to the grain is the N harvest index (NHI). The NHI results ranged from 55.6 % (Scout N3WD) to 78.9 % (Axe N2DD) however there was no consistent cultivar difference

in the ability to allocate N to the grain, nor was there a difference between N fertilisation or water treatment (data not shown).

The maturity profiles of the cultivars differed and as such the growth stages of the plants were assessed at 50 DAP using the Zadoks decimal code for maturity estimation (Zadoks et al. 1974) (Fig. S5). The cultivars varied from Zadoks 60 for Axe regardless of water treatment to Zadoks 30 and 35 for Yitpi under N3WW and N3DD, respectively. There did not appear to be a consistent interaction with water treatment.

The ten cultivars from Exp. 1 were grown again the following year in Exp. 2 using the same protocols to ensure the repeatability of the method and the stability of the phenotyping platform, one of the main impediments to the improvement of NUE. The PSA at 54 DAP from the two experiments were related, and this relationship became stronger when the cultivars where separated into N and water treatments (Fig. 6). There was a high association (R^2 = 0.82) between the PSA results of the two experiments and within the treatment groups. The discrepancy between the number of pixels between the two experiments is due to an increase in the resolution of the cameras used for image collection in the second experiment.

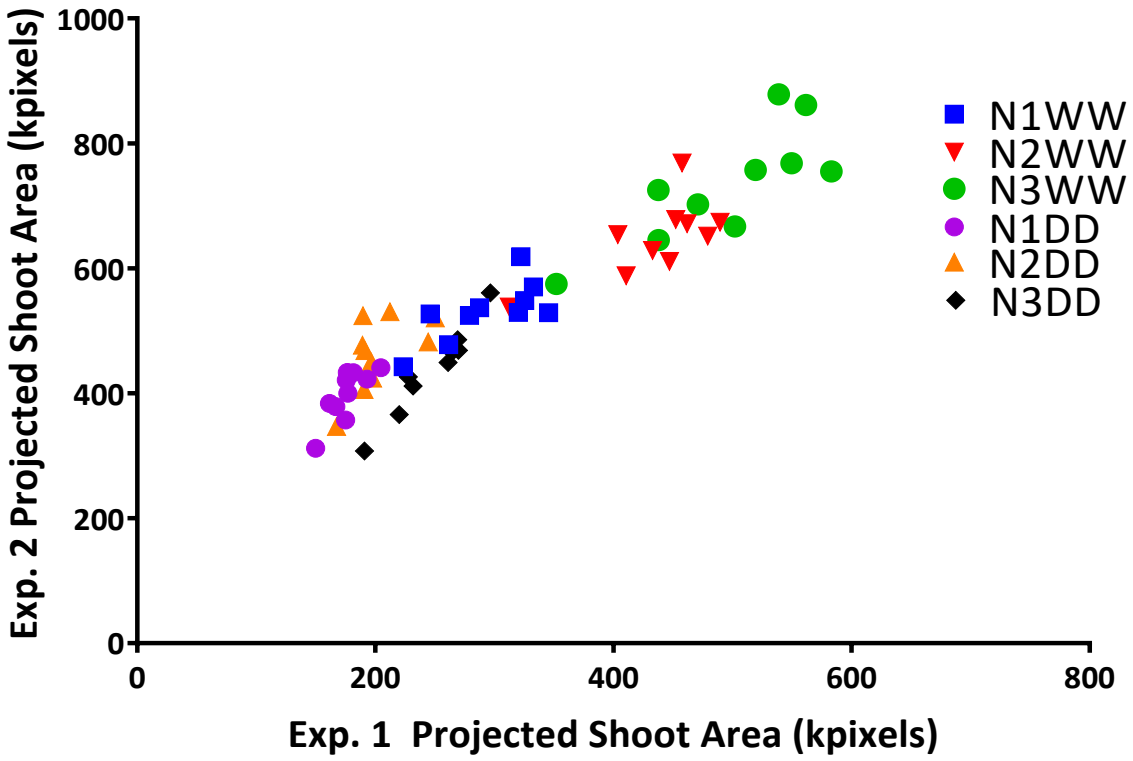

Figure 6 The correlation of projected shoot area at 54 DAP between the two experiments: Exp. 1 on the x-axis and Exp. 2 on the y-axis. The data is grouped by N fertilisation (25, 75 and 150 mg N kg soil^{-1} which were N1, N2 and N3 respectively) and water availability (WW: well-watered or DD: drought) (25 % and 13 % water content (g/g)), $R^2 = 0.82$.

DISCUSSION

High resolution growth phenotyping was utilised to observe differences in wheat cultivars in terms of their growth response to N and water availability. The ability to analyse growth phenotypes, rather than making NUE determinations based on yield only, greatly increases the amount of information about the genetic difference between cultivars (Tardieu et al. 2017). As a result, we found significant differences in growth behaviour that would not have been possible to quantify, prior to high resolution (non-destructive) phenotyping. The effectiveness of the phenotyping protocol, seen in the differences in growth between cultivars may contribute to NUE improvement, as it can determine genetic differences easily on a large number of plants. The effectiveness of the HTP system in measuring growth

dynamics and how these correlate with yield is novel, and indicate the ability to predict yield accurately from PSA and also measure N responsiveness.

The improvement of NUE is one of the most intractable problems in modern agriculture due to the complexity of the trait, and its interaction with the environment (Garnett et al. 2015; Han et al. 2015). The results presented here showed that there were differences in PSA observed, but more importantly that the PSA was related closely to yield, and that these yield predictions became even more accurate when separated into the four field-relevant water treatments. Regressions could be undertaken between PSA at any day during the experiment and final yield. This was undertaken in this experiment and 52 DAP had the highest correlation with these water availability treatments. The improvement of predictions due to the separation of the results into the water availability treatments is important as it highlights the relevance of well-characterised phenotyping conditions and for the attribution of genetic gain in response to abiotic conditions (Araus et al. 2018). Further than this, one of the main impediments to improving NUE has been a stable phenotyping environment, the results presented here demonstrate that this protocol undertaken over two years has successfully replicated the PSA shoot area results of ten cultivars, a significant complement to the unpredictability of the field.

As well as relating yield to PSA at single time points, the growth phenotypes of cultivars were differentiated and advantages under certain water availabilities identified. The phenotype of Gladius, Kukri and Espada under WW conditions seemed to be advantageous when water restrictions were implemented at 48 DAP under the WD treatment. The rapid increase in biomass (PSA), early switch to reproductive stage and the apparent negative AGR in order to reduce leaf area liability (more leaf area than can be can be supported by the available water) observed in these cultivars may have contributed to their high yields. The ability of a plant to respond to water shortage rapidly by shortening vegetative phase,

give it the best chance of avoiding a severe yield penalty (Shavrukov et al. 2017). The ability to analyse the AGRs via HTP in response to water availability illuminated differences which would otherwise have gone undetected. Once differences can be detected, they can be linked to higher N or water use efficiencies and ultimately yield. This reflects similar successful studies in other cereals measuring growth responses to changes in abiotic stress conditions: salt tolerance in barley (Tilbrook et al. 2017) and rice (Al-Tamimi et al. 2016) and drought tolerance in wheat (Parent et al. 2015). In other words, the resolution of the observations allows for the separation of cultivars by growth characteristics in response to changes in abiotic conditions (Meng et al. 2017).

The NUE results (derived from destructive harvest) support previous research demonstrating NUE variation amongst cultivars (Hawkesford 2017; Ortiz-Monasterio et al. 1997). Under N1WW, there was a 30 % difference between the highest and lowest NUE results (Fig. S4). Furthermore, NUE had an inverse relationship with N fertiliser supply which supports previous studies of N saturation curves (Lemaire et al. 2008). The plants demonstrated a diminishing return from the application of N, as well as the inability to take up all the available N during growth. The NHI remained constant at approximately 60-70 %, regardless of N fertilisation level or cultivar, which has been previously established by Barraclough et al. (2010). This is significant as it supports the theory that cereals are reaching the biological limit of resource recovery during senescence and that greater amounts of harvestable N are to be gained via larger amount of biomass (Hay 1995), where water availability permits.

The maturity profiles of commercially used wheat cultivars differ, allowing for the selection of the appropriate profile for the environment (Flohr et al. 2017; GRDC 2018), and in this research they could be determined from the high resolution growth images. A meta-analysis of 223 field trials over 12 years across Australia also showed that maturation time

was one of the key determinants of yield variation (McDonald et al. 2013). This meant that cultivars flowering earlier had an advantage in Western Australia, whilst late flowering was advantageous in South Australia, Victoria and New South Wales (McDonald et al. 2013). The results presented here showed a link between maturity profile and high N yield. The highest yielding cultivars under N3 were the early maturing cultivars, whereas lower yielding cultivars were still in the vegetative stage at 50 DAP. This protocol can incorporate maturity assessment due to the high resolution images of the plants and further experimentation could construct growth curves that were normalised for growth stage in order to account for different maturity profiles influence on results.

The ability to measure AGR via high resolution growth phenotyping also allows for the comparison of growth plasticity, the ability of plants to adjust their physiology in response to environmental conditions (DeWitt et al. 1998). Plasticity is important to the NUE of wheat as it increases the period of photosynthetic production when conditions improve, maximising potential N uptake and photosynthetic production, as well as increasing adaptability to increasingly unpredictable climactic patterns (Anwar et al. 2015). Plasticity was observed in response to water availability and cultivar difference. These differences can be observed in more detail if they are deemed to be a desirable phenotype. Cultivar difference was a range of 14 days under N3DW and 11 days under N3WD, with cultivars behaving differently under different water availabilities. The level of plasticity observed in this research emphasised the effectiveness of utilising field relevant water availability conditions to differentiate cultivar performance (Martre et al. 2015).

Nitrogen application requires careful management in Mediterranean growing conditions because of the negative effects of ‘haying-off’, the incomplete grain filling due to an overinvestment in biomass during the vegetative stage (Van Herwaarden et al. 1998). This is a problem in Australia, as wheat growing areas often experience a ‘hot-dry finish’.

This response is clearly demonstrated here as the plants transitioning from well-watered to water limiting conditions were unable to convert biomass to grain yield. Cultivar differences in response to decreased water availability combined with high N availability were clearly evident in the PSA at 52 DAP and grain yield comparisons. Cultivars such as Axe were more resilient to these conditions, whereas Scout and Yitpi were less able to convert biomass to yield under these conditions.

The ability to effectively and repeatedly recreate this protocol in a semi-controlled environment is important as it would be nearly impossible to recreate this many treatments in a single field season, as well as be able to replicate it season after season. The environmental interaction of this trait is significant, so in order to replicate this in the field, trials would need to be established in numerous locations in order to ensure the best probability of experiencing both an 'ideal' and a 'hot-dry finish' season. Additionally, more N treatments would be required in the field to achieve a smooth N response increase, and numerous biomass estimates would be required throughout the season. Lastly, the work of Van Herwaarden et al. (1998) investigating 'haying-off' and other work in phenotyping N response in the field has generally been based on a single destructive biomass harvest, whilst having the dynamics of biomass development from the image analysis, rather than a single time point harvest, allows us to better understand the cultivar difference within the haying off response. The complexity, expense and difficulty of running these experiments in the field reinforces the advantageous nature of the non-destructive growth measurements and control of water and N available in HTP facilities.

CONCLUSIONS

One of the biggest challenges to the genetic improvement of NUE in wheat has been the lack of precision and repeatability available for phenotyping this trait. Because of the complexity of the NUE trait and the high level of interaction with the environment, the

dissection of the genetic component of NUE is difficult. The precision and repeatability of this method shows promise in identifying material with improved NUE, particularly in relation to water availability. Measuring growth using image based HTP clearly differentiates N response characteristics of wheat cultivars. This research made it possible to measure interactions between water and N, through the precise control of water availability. This was leveraged to observe the haying-off effect which is common to wheat growing areas with Mediterranean climates. Equivalent research would not be practical in the field. Furthermore, the HTP platform was shown to produce consistent results over two years of experimentation, a major barrier to phenotyping NUE. This technology would be an ideal tool to investigate NUE at the population level in wheat.

SUPPLEMENTARY MATERIAL

Table S1 Company, year of registration and maturity profile on the ten cultivars utilised in this research

Cultivar	Company	Year of registration	Maturity profile
Axe	AGT	2007	Early
Espada	AGT	2008	Mid
Excalibur	AGT/RAC	1991	Early
Gladius	AGT	2006	Mid
Kukri	RAC	2000	Early/Mid
Mace	AGT	2007	Early/Mid
RAC875	RAC	NA (breeding line)	Early/Mid
Scout	Longreach	2004	Mid
Wyalkatchem	Intergrain (GRDC)	2001	Early/Mid
Yitpi	Waite Institute	1999	Late

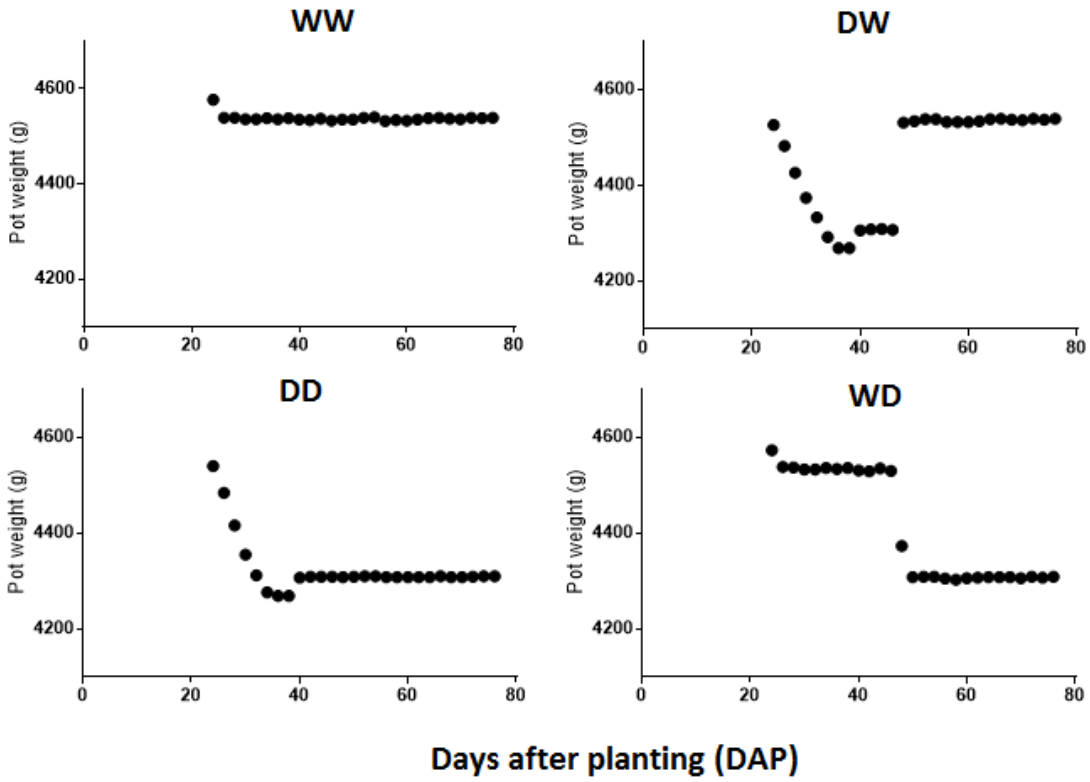

Figure S1 The four water availability treatments used in experiment 1 (well-watered (WW: 23 % w/w), drought (DD: 13 % w/w)), drought until 48 DAP and then well-watered (DW), and well-watered until 48 DAP and then drought (WD)). Each point represents a pot weight.

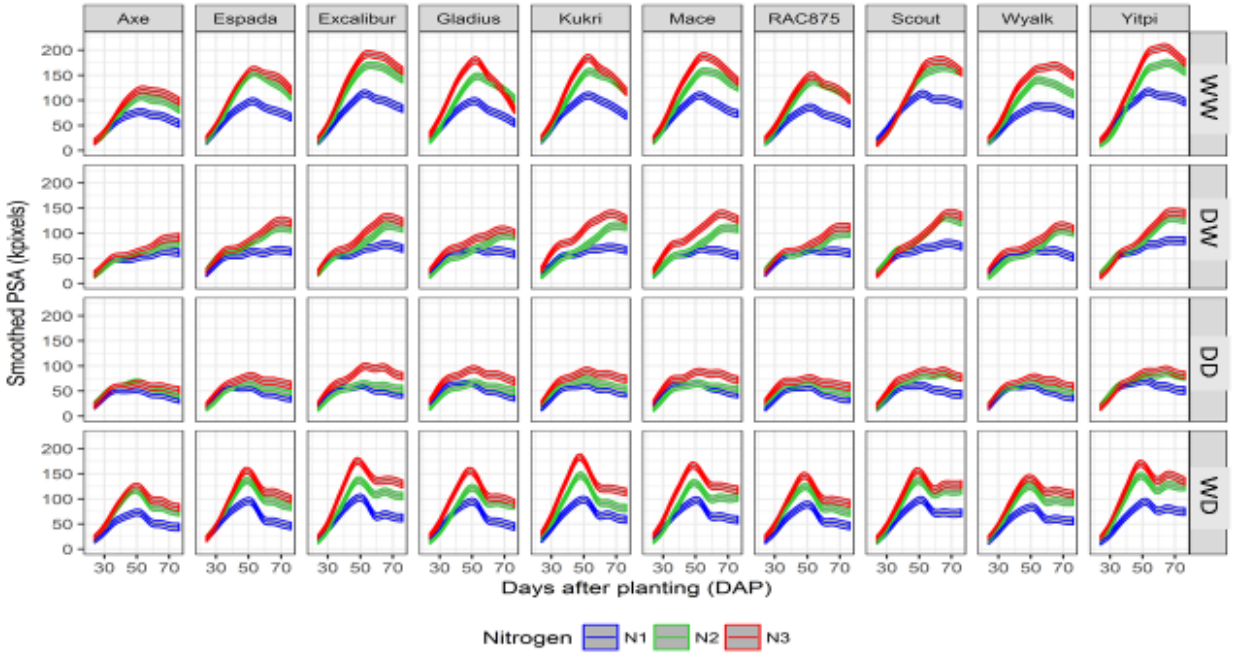

Figure S2 The longitudinal analysis of the growth curves captured from RGB image analysis over the course of Exp. 1 from 24-76 DAP. The treatments are 25, 75 and 150 mg N kg soil^{-1} as N1, N2 and N3 respectively and water treatments (well-watered (WW), drought (DD), drought until 48 DAP and then well-watered (DW), and well-watered until 48 DAP and then drought (WD)). The dark shaded area around each curve represents the boundary of significant difference, n=4.

Table S2 The statistical interactions between genotype, nitrogen and water availability. P-values are shown with * denoting significant difference

	Genotype:Treatment 1: Treatment 2	Genotype: Nitrogen	Genotype: Water availability	Nitrogen: Water availability
Average slope of smoothed AGR between day 40 and day 50	<.001*	NA	NA	NA
Average smoothed absolute growth rate between day 30 and day 40	0.105	<.001*	<.001*	<.001*
Point of maximum pixels	<.001*	NA	NA	NA
Days to maximum pixels	<.001*	NA	NA	NA
Average AGR	<.001*	NA	NA	NA
Average AGR past day 50	<.001*	NA	NA	NA

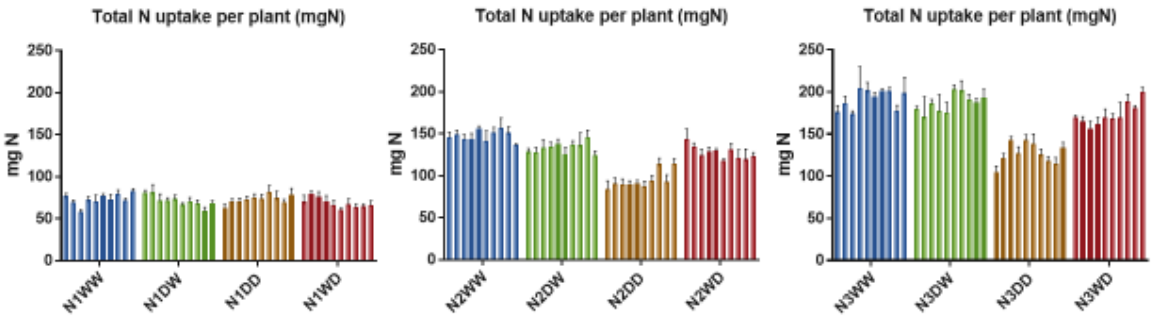

Figure S3 Total shoot uptake (mg N plant^{-1}). The 3 panels represent the 3 N levels (25, 75 and 150 mg N kg soil^{-1}), the x-axis shows the water treatments (well-watered (WW), drought (DD), drought until 48 DAP and then well-watered (DW), and well-watered until 48 DAP and then drought (WD)). Each bar is a cultivar and the 10 cultivars within each colour are in the following order: Axe, Espada, Excalibur, Gladius, Kukri, Mace, RAC875, Scout, Wyalkatchem and Yitpi. The error bars shown represent the SEM, n=4

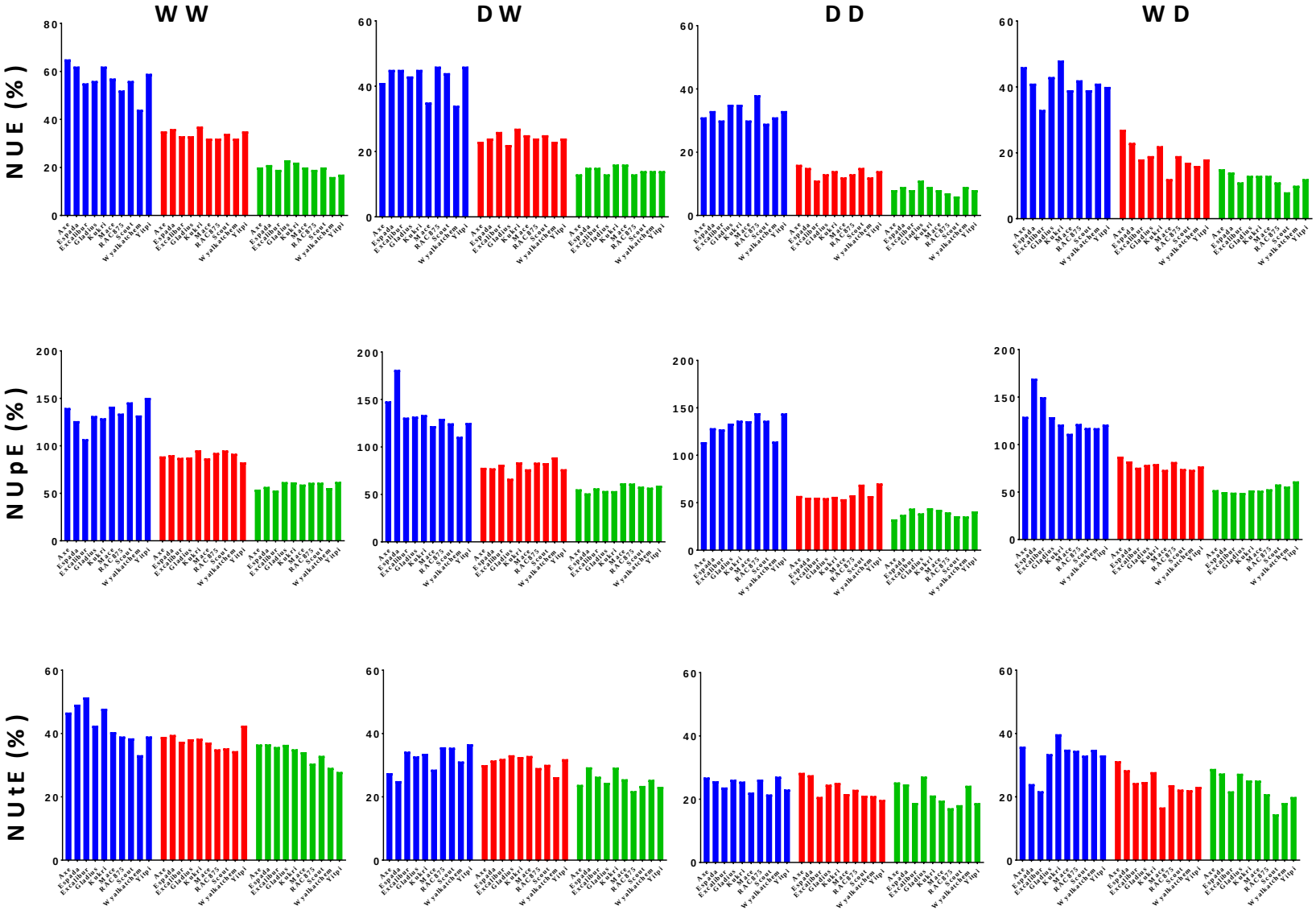
W W
D W
D D
W D
NUE (%)
NUpE (%)
NUtE (%)
Axe
Espada
Excalibur
Gladius
Kuivi
MACE
RAC875
Scout
Wyalkatchem
Yitpi

Figure S4 The NUE results for Exp. 1. The calculations are as described in Good et al. (2004). The treatments are 25, 75 and 150 mg N kg soil^{-1} as N1, N2 and N3 respectively and water treatments (well-watered (WW), drought (DD), drought until 48 DAP and then well-watered (DW), and well-watered until 48 DAP and then drought (WD)), n=4.

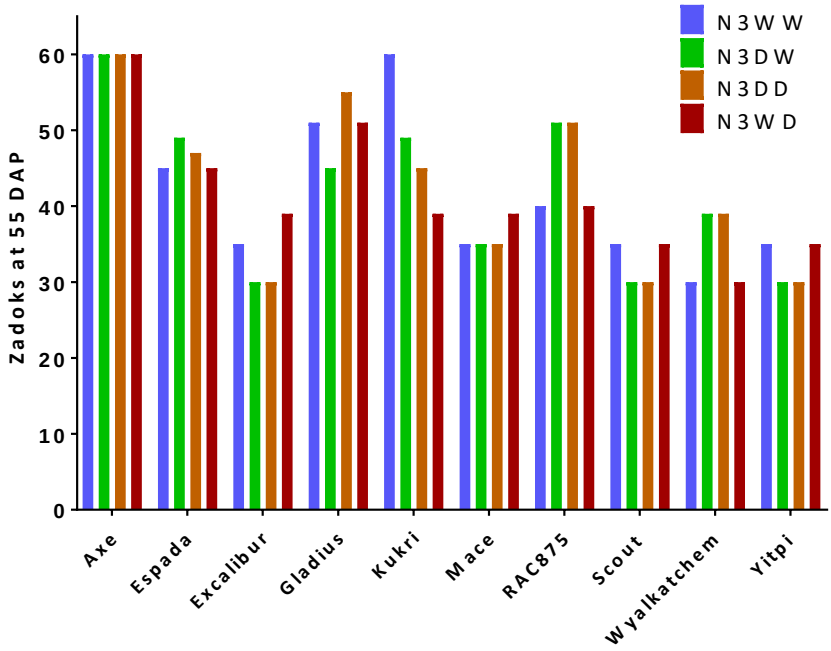

Figure S5 Zadoks growth stages (y-axis) for each of the ten cultivars under N3 (150 mg N kg soil^{-1}) at 50 DAP. The four water treatments are shown (well-watered (WW), drought (DD), drought until day 48 DAP and then well-watered (DW), and well-watered until day 48 DAP and then drought (WD)). Zadoks growth stage of 60 represents anthesis.

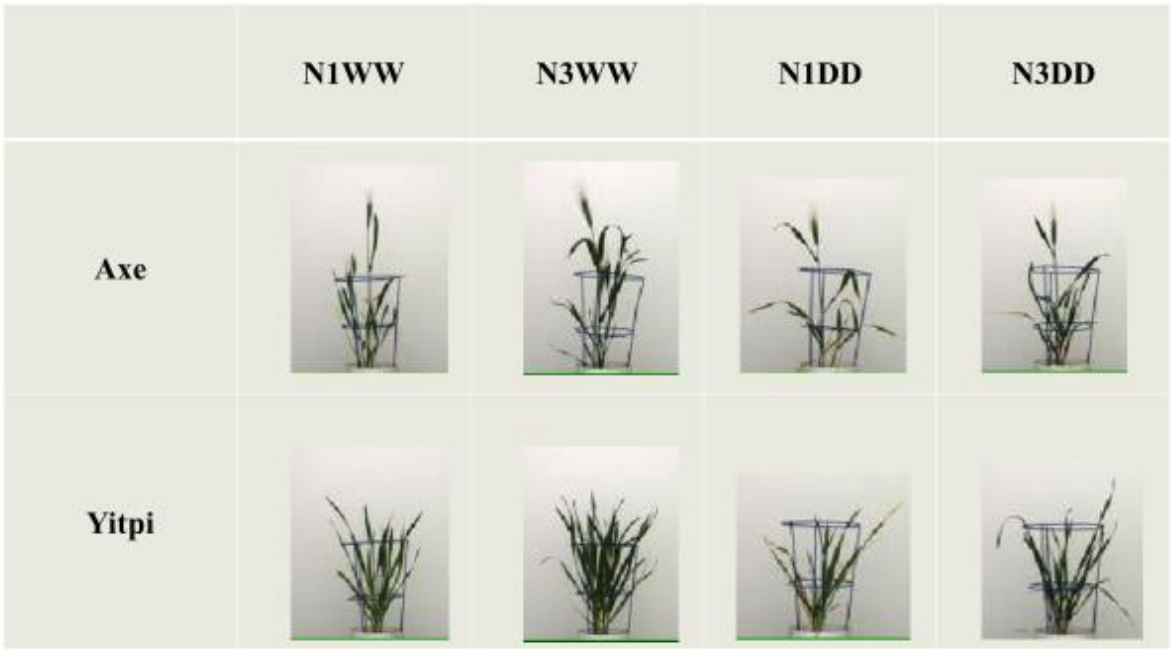

Figure S4 Images of Axe and Yitpi under four experimental treatments at 50 days after planting. The contrasting growth of the cultivars is accurately quantified by image analysis. These images demonstrate the cultivar and treatment effects on shoot biomass and growth stage.

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Chapter 3: Non-destructive determination of leaf nitrogen concentration in bread wheat using hyperspectral reflectance

TITLE

Non-destructive determination of leaf nitrogen concentration in bread wheat using hyperspectral reflectance

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ABSTRACT

Accurately phenotyping the nitrogen use efficiency (NUE) of wheat plants is currently one of the challenges facing wheat improvement. Understanding the dynamics of nitrogen (N) within wheat plants via its non-destructive measurement would greatly assist in selecting superior NUE lines. Hyperspectral reflectance has been shown to accurately predict leaf N % in European durum wheats, this has also shown promise in predicting leaf N % in Australian wheats. To evaluate this approach, eighty plants were grown in a semi-controlled environment under well-watered and drought conditions with high and low N treatments. Reflectance spectra were collected from two leaves per plant using a field spectrophotometer and the leaves were subsequently destructively harvested between 24-56 days after planting. A total of 129 destructive harvests were associated with their respective spectra and regressed using partial least squares regression with the method having a predictive value of $R^2 = 0.85$. The accuracy of the method was not as high as desired, as the standard error of cross validation was 0.78. This was potentially due to the small calibration set and the narrowness of the leaves causing background interference with the spectral reflectance instruments. Improvements are required to increase the accuracy of the method including the addition of more destructive harvests to the calibration set. These results nevertheless demonstrate that leaf N % can be assessed non-destructively in individual wheat leaves using hyperspectral reflectance, opening up possibilities for quantifying whole plant N dynamics over time.

INTRODUCTION

Nitrogen (N) is one of the most important plant mineral nutrients and is a major component of chlorophyll, thus vital to photosynthetic efficiency (Parry et al. 2010). Therefore, the efficient uptake and utilisation of N is integral to increasing yield per unit N (Garnett et al. 2015). In order to achieve this, an understanding of, and selection for, superior N uptake and remobilisation dynamics is essential (Nguyen et al. 2016; Sanchez-Bragado et al. 2016). Previous work investigating the movement of N within the plant have relied on destructive harvests, in order to determine N content and concentration (Dhugga and Waines 1989; Dreccer et al. 2000; Evans 1983). This has been problematic because it is slow, labour intensive, expensive and requires the removal of plants from the experiment. A non-destructive method to analyse leaf N % would allow the measurement of real time N uptake and the dynamics of N partitioning within the plant.

The use of reflectance properties to estimate the properties of plant tissue has already been established using a limited number of spectral bands (Curran 1989; Peng et al. 1993). Multispectral based vegetation indices such as normalised difference vegetation index (NDVI) are regularly used to predict crop vigour, yield (Babar et al. 2006) and nitrogen status (Hansen and Schjoerring 2003), primarily to inform management decisions. The SPAD-502© chlorophyll meter (Minolta Camera Co., Ltd, Japan), which measures light transmittance at two wavelengths (650 nm, 940 nm) has been used to infer N concentration from chlorophyll content (Follett et al. 1992), however the method is limited by the relationship between N and chlorophyll, decoupling above 125 mmol m^{-2} N (Ecarnot et al. 2013; Evans 1983).

Expanding the wavelength collection range from a limited number of bands only to the entire spectrum between 350-2500 nm greatly increases the amount of reflectance data

that can be obtained. Using this approach the reflectance properties related to specific chemical bonds, such as N-bonds, can be used to determine tissue N (Kokaly 2001). Work by Ecarnot et al. (2013) showed that leaf N % could be accurately measured regardless of leaf phenology or age, in both fresh and dried wheat leaf tissue, with the predictive power of the leaf spectra readings being between 0.932 to 0.958, with a standard error of cross-validation (SECV) of 0.30 N %. The calibration set established by Ecarnot et al., (2013) used European durum wheat varieties with samples harvested at late phenological stages (anthesis/post-anthesis) from five field sites, with the amount of N applied varying considerably (from 80-180 kg ha^{-1}) and several applications per season. This approach produced a robust model for leaf N % determination in durum wheat in a European environment. This has been expanded upon in work undertaken by Silva-Perez et al. (2018) which found high correlation using hyperspectral reflectance and gas exchange chromatography (0.93 N per unit leaf area (g/m^2).

The genetic differences between durum and bread wheat, combined with the different growing conditions in Australia may impact upon the reflectance characteristics of the leaves and subsequent N prediction, therefore a custom model must be developed (Cozzolino 2014). In this study, ten commonly used bread wheat cultivars were grown under different N and water levels to develop a robust model for Australian wheat.

MATERIALS AND METHODS

Plant Material

Ten common SE Australia wheat cultivars were used in this study: Axe, Corack, Gladius, Grenade CLplus, LrpbTrojan, Mace, RAC875, Shield, Beckom (V06008-14) and Yitpi (Table S1). These cultivars were chosen because they have diverse genetic pedigrees and are widely grown in SE Australia. The plants were grown in a glasshouse at the Plant Accelerator (Australian Plant Phenomics Facility, University of Adelaide, Adelaide, Australia; -

34.97113°, 138.63989°). The glasshouse was maintained at approximately 23/16 °C day/night. Seeds were planted in substrate which had equal parts (v/v) coco peat, clay/loam and UC Davis Mix (a combination of sand, peatmoss and lime). There were two N treatments: low (LN) and high (HN) (25 and 150 mg N kg^{-1} dry soil, respectively), mixed as urea (CH_4N_2O) into the soil prior to potting. The pots were round 2.5 L white pots containing 2.3 kg dry soil. Four seeds were planted in each pot, at a depth of 2 cm. Seedlings were then thinned to a single uniform sized seedling at the three leaf stage (11 days after planting: DAP). After establishment, the pots were placed on an automated watering to weight system which maintained two water treatments: well-watered (WW) set at 23.5 % gravimetric soil water content and drought stress (DD), set at 13 % gravimetric soil water content.

Collecting spectra and harvesting leaves

Hyperspectral reflectance data was collected with a Field Spec 3 (Analytical Spectral Devices, Inc. (ASD), Boulder, CO, USA) in combination with a leaf clip and white background reflectance panel (Fig. S1). The spectral range of the spectrometer was 350-2500 nm, its spectral resolution was 3 nm in the region of 350-1000 nm and 10 nm in the 1000-2500 nm region. The spectral information was collected on the adaxial side of the leaf. Four readings were collected moving a quarter of the way along the leaf blade each time from the base to the tip. The four readings were averaged. The leaf blade was then excised at the stem and dried for three days at 60 °C. The collection of spectra occurred every fourth day from 28 to 56 DAP. In the first four harvest dates, the blade below the youngest emerged blade (YEB-1) was harvested, and in the last four harvest dates, the youngest emerged blade (YEB) was harvested.

N analysis

There were a total of 80 plants (10 cultivars x 2 N treatments x 2 water treatments x 2 replicates) from which two leaves were sampled for destructive N analysis. The dried leaves

were milled to a fine powder in a tissue homogeniser (SPEX, Metuchen, NJ, USA) in 2 mL PCR tubes with 2 mm ball bearings. The samples were then weighed into tin capsules (Elemental Microanalysis, Devon, United Kingdom) at weights of between 1-2 mg and samples were analysed for N content via continuous-flow direct combustion and mass spectrometry using a Europa Scientific SL-2020 system (Stable Isotope Lab, Utah State University, Utah, USA).

Spectra processing

The collected leaf spectra were pre-processed in R statistical software (R Core Team 2018) using the following approach. The collected leaf spectra were adjusted for the switch in detectors which occurred at 1000 nm and 1830 nm. The moving average was then taken into account by the standard normal variate being applied to each spectrum individually to remove scatter. The spectra were then smoothed using Salitzky-Golay smoothing filter to maintain line shape (Savitzky and Golay 1964). Outliers were removed where there was an obvious jump in the reflectance at the switch in detector at 1830 nm. These were the result of insufficient recalibration of the Field Spec 3. After these errors were noticed the protocol was adjusted to recalibrate the instrument with a white reflectance panel every 30 minutes. These outliers contributed to the reduction in samples processed from 160 to 129. The destructive N analysis data were regressed with the four averaged reflectance spectra collected on that leaf via partial least squares regression (PLSR). PLSR was used for the SECV which determined that 8 latent variables would be utilised in the model. Values were treated as outliers if they varied by more than 1.5 % leaf N from the regression vs predicted line as per Ecarnot et al. (2013). The model and calibration set developed by Ecarnot et al. (2013) was also utilised to process the collected spectra to predict leaf N % as a comparison.

RESULTS

When the model developed for durum wheat by Ecarnot et al. (2013) was used to predict leaf N % in Australian bread wheats there was no relationship with measured leaf N % (Fig. 1A), despite the wide range of leaf N %. The range of leaf N was 2.2 - 6.8 %. On the other hand, the model developed in this study using bread wheat varieties showed a relationship between predicted and measured N with an R^2 of 0.8528 (Fig. 1B).

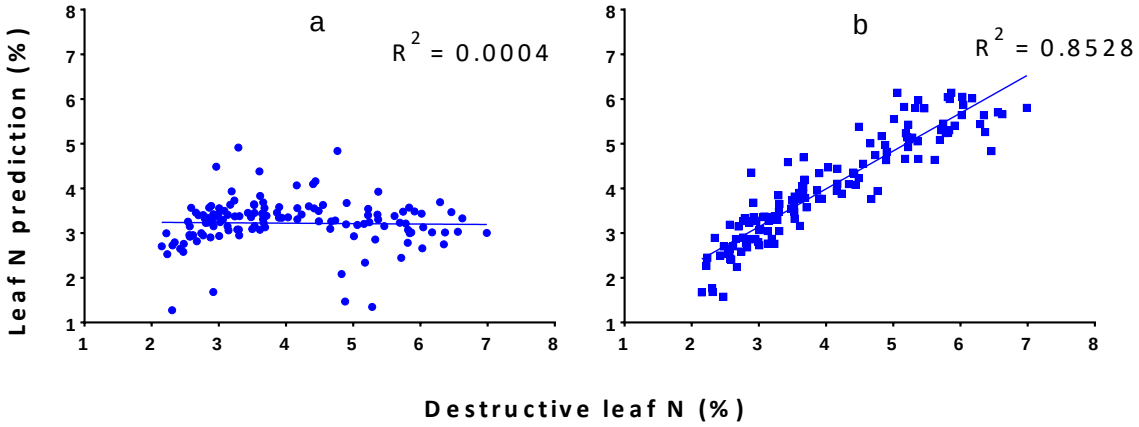

Figure 1 The correlations between the destructive leaf N % values from Australian wheat varieties (x-axis) and (A) the leaf N (%) predictions (y-axis) using the European calibration set of 601 samples of durum wheat varieties from Ecarnot et. al., (2013), and (B) the correlation between the predicted N % values from wheat leaf blades from the Australian bread wheat calibration set (129 samples) utilised in this experiment.

The leaf N % predictions garnered from Australian bread wheats were dissected into the different N treatments and watering levels to test whether these had an obvious impact on the predictive ability of the protocol (Fig. 2). The predictions were not significantly affected by the cultivar, N or water treatment as shown by the relationships in Fig. 2. The date of sampling had a significant effect on the N level ($P < 0.001$). The date of sampling (Fig. 2A)

shows the earlier dates have higher leaf N %, gradually decreasing over time. The correlation values appear to vary at 28 and 32 DAP much more than the subsequent sampling dates. The grouping by cultivar (Fig. 2B) shows an even distribution of leaf N % across the different cultivars with no cultivar having an obvious effect on leaf N % prediction. Similarly, the level of N (Fig. 2C) and the level of water treatment (Fig. 2D) did not overtly impact the predicative capability of the model. This is important to have confidence that the method was accurate over the different experimental variables.

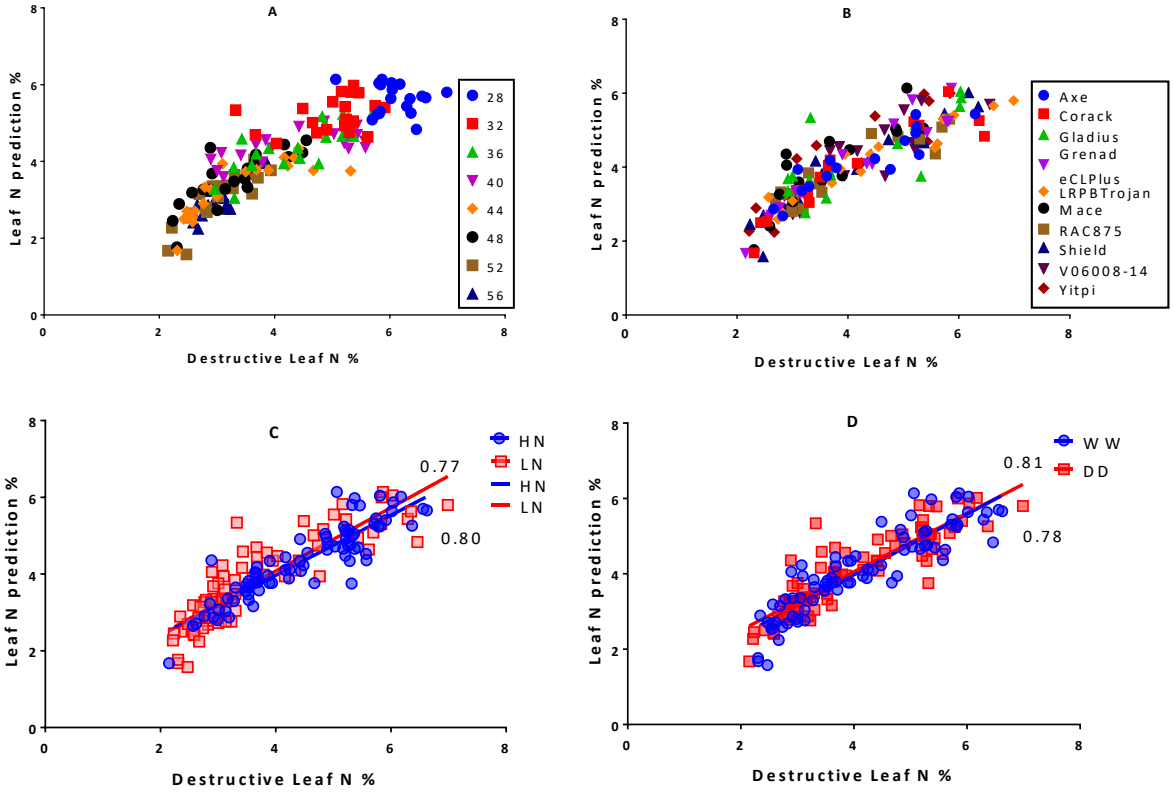

Figure 2 Relationships between predicted leaf N % (y-axis) and destructive leaf N % analysis (x-axis) using the calibration model developed using ten common Australian wheat varieties. The calibrations are grouped by (A) sampling day (DAP), (B) cultivar, (C) N level: HN (150 mg N kg^{-1}; R^2 = 0.77) and LN (25 mg N kg^{-1}; R^2 = 0.80) and (D) water treatment: WW (23.5 % soil water content; R^2 = 0.81) and DD (13% soil water content; R^2 = 0.78).

In order to correlate the reflectance spectra, the leaves had to be excised for destructive N analysis (data no shown). On average the YEB-1 had a higher leaf N % than the YEB, due to the fact that the YEB-1 were harvested at an earlier time in the experiment (from 28-40 DAP). There was a more obvious difference under the HN and LN treatments later in the experiment from 44-56 DAP compared to those harvested earlier. There was an overall range in leaf N % from 2.22 (Yitpi YEB under LN-DD) to 6.62 leaf N % (LRPB Trojan YEB-1 under HN-WW), providing the desired range of leaf N % to develop an accurate leaf N % prediction over a wide array of samples. There was a wide range of leaf N % in the calibration set used to develop the model with leaf N % varying according to treatment but also other factors, such as the DAP and leaf rank. The HN treatment was hypothesised to result in a higher leaf N %, but there was also an impact of the date of sampling ($P < 0.001$) (Fig. 2A). The leaves harvested at a younger age had significantly higher N content than the older leaves and the relationship between spectra and leaf N % in younger plants may have less predictability, as suggested by the grouping by harvest days (Fig. 2A).

The discrepancy between the two calibration sets and the subsequent affect this had on the quality of the observations is demonstrated in Fig. 3. The European calibration set appeared to underestimate the leaf N % compared to the Australian wheat calibration set. Furthermore, there was a much greater amount of variation in the European calibration set when compared to the Australian.

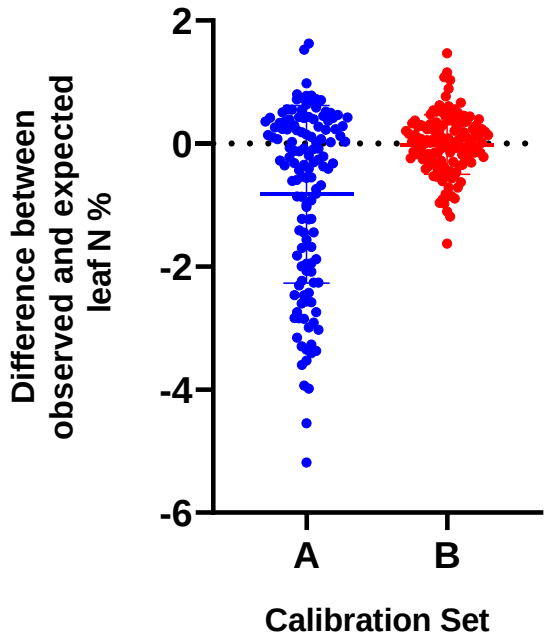

Figure 3 The difference between expected and observed leaf N % between the two calibration sets: A) the European calibration set of durum wheats (Ecarnot et al. 2013) and B) the calibration set of ten commercial Australian wheat varieties

DISCUSSION

The leaf N % predictions were consistent over cultivar, water and N treatment indicating the method is robust enough to operate over a range of water and N conditions that can be found in the Mediterranean conditions of Australian wheat experiments. The ability to differentiate leaf N % non-destructively would increase the power of NUE phenotyping as one of the main reservoirs of N within the plants could be measured and compared. This protocol demonstrates that hyperspectral reflectance is effective in this regard due to the expanded spectral range, which can analyse the interaction of light with N compounds within the leaf tissue (Yao et al. 2010).

The calibration set from Ecarnot et al. (2013) was demonstrated to be inaccurate when used to predict leaf N % for Australian bread wheats (R^2 = 0.0004). The Ecarnot et al. (2013) method established a calibration set of 601 leaves (349 fresh leaves, 252 dried leaves)

which were destructively sampled and regressed with their associated spectra. For the leaf N % in fresh leaves, they achieved a standard error of cross validation (R^2cv) of 0.92 using a number of pre-treatments to reduce the noise from 350-400 nm and standard normal variate (subtraction of mean, divided by standard deviation) to account for panchromatic light variations reaching the detector (Barnes et al. 1989). However, the lack of relationship between the Ecarnot calibration data and the spectra collected on Australian bread wheat in this experiment can possibly be explained by a combination of factors. The original calibration is comprised of durum wheats which have reached anthesis. The leaves harvested at earlier time points in this experiment had a higher leaf N %, above that reported by Ecarnot et al. (2013) of 0.48-4.85 leaf N %. Indeed, it appears as if the relationship between the two prediction models decouples at 4 N %, supporting this hypothesis. This can potentially be explained by the younger leaves utilised in this experiment which had higher leaf N % when harvested. Leaf N % diminished over time as the leaves became bigger, and assimilated more carbon (C) in what is known as the 'dilution effect' (Ata-Ul-Karim et al. 2017; Bertheloot et al. 2008; Taub and Wang 2008)

One of the main differences between the protocol employed here and that of Ecarnot et al. (2013) was the physical dimensions of the leaves sampled. All of the leaves obtained by Ecarnot et al. (2013) would have been wider than some of those sampled in this research, as they were sampled at anthesis, limiting the interference caused by the leaf not covering the entire leaf clip aperture, as occurred in this calibration. This may explain some of the variation observed with leaf samples from younger plants. Collecting spectra using the leaf clip requires the lining up of the leaf with the aperture of the leaf clip. However, during sampling in this experiment many of the leaves were too narrow to cover the leaf clip's entire white background resulting in differences in white background reflectance between samples. This may be problematic as the white background of the leaf clip (instead of a black panel

measuring transmittance) has been theorised to increase variance and inaccuracy (Silva-Perez et al. 2018).

As the samples obtained by Ecarnot et al. (2013) were from the field whereas the plants in this study were from a pot experiment in a semi-controlled environment there may also be differences in the leaf tissue caused by environmental factors, such as planting density, solar radiation, biotic stressors and nitrogen application. Conditions in the field vary significantly to those found in pot based experiments, and as such *in situ* calibrations must be completed before having confidence using the method in the field (Vigneau et al. 2011).

The ten cultivars used had different leaf characteristics (waxiness, leaf hairs, etc) in order to ensure these would not affect the robustness of the model developed. RAC875 was chosen as it has waxy crystals on its leaf surface which give the leaf a blue tinge, contributing to its drought tolerance (Izanloo et al. 2008). Mace was selected because of its 'hairy' leaf physiology. The correlation results show no difference for cultivars with differing leaf surface properties.

Although the leaf N % prediction method works effectively, the results have a variance level between prediction and their associated destructive harvest that is high (inaccurate by absolute error of up to 1.1 leaf N %). This is not sufficient when the range for leaf N is between 2-7.5 %. The SECV achieved by Ecarnot et al. (2013) is between 0.3-0.4 % absolute error, and therefore this should be the aim of this calibration going forward. Although the amount of error achieved in this current research represents an improvement on SPAD-502 based protocols, the method used in this research should be improved through the addition of more samples to the calibration set. As well as additional destructive harvests, changes to the protocol can be made such as observing leaf N % in leaves that are at or past anthesis as these are wider and cover the aperture of the leaf clip.

CONCLUSIONS

The non-destructive prediction of leaf N % using hyperspectral reflectance, first used in durum wheats in France, was shown to work well for Australian bread wheats. A combination of chemometric analysis with regressions of hyperspectral reflectance readings is an effective way to predict leaf N %. Although this method requires further development it is still superior to chlorophyll based inferences (Ecarnot et al. 2013), as it is flexible across different experimental conditions, does not become saturated at high leaf N concentrations and provides a greater number of spectral bands which can be analysed and adjusted using statistical methods. The method is able to be constantly improved if more destructive harvests are added to the calibration set, resulting in a gradual accumulation of samples, increasing the accuracy (Yuan et al. 2016). This hyperspectral reflectance method can be used to phenotype N uptake differences more accurately and in a non-destructive manner in a controlled environment, and potentially in the field, greatly assisting in the selection of superior NUE wheat lines in the future.

SUPPLEMENTARY MATERIAL

Table S2 List of cultivars utilised in this research with company, year of registration and maturity profile

Cultivar	Experiment	Company	Year of registration	Maturity profile
Axe	2014	AGT	2007	Early
Corack	2015	AGT	2011	Early/Mid
Gladius	2014	AGT	2006	Mid
Grenade CLPlus	2015	AGT	2012	Mid
LRPB Trojan	2015	Longreach	2013	Mid/Long
Mace	2014	AGT	2007	Early/Mid
RAC875	2014	RAC	NA (breeding line)	Early/Mid
Shield	2015	AGT	2012	Early/Mid
V06008-14 (Beckom)	2015	AGT	2015	Mid
Yitpi	2014	Waite Institute	1999	Late

Figure S2 The Field Spec 3 with the leaf clip taking a reading from a leaf that has just been excised.

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Chapter 4: Real time dynamics of wheat leaf N content observed in response to fluctuating N supply

TITLE

Real time dynamics of wheat leaf N content observed in response to fluctuating N supply

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ABSTRACT

Improving nitrogen (N) uptake in wheat requires the selection of superior lines via accurate phenotyping. Differences in leaf N concentration may reflect different N uptake and utilisation phenotypes, but are challenging to find because of the nature of destructive sampling techniques. However, direct observation of leaf N % in individual leaves via non-destructive hyperspectral reflectance is now possible. In experiments detailed here, this method was utilised to differentiate N uptake within a hydroponic system which allowed fluctuating N supply. Two wheat varieties (Gladius and Yitpi) were observed under four N treatments: high N (5 mM NO_3^-), low N (0.5 mM NO_3^-), high N to low N switch and low N to high N switch (the switch occurred at 31 days after germination). The varieties differed in leaf N % and both cultivars maintained more N in their younger leaves compared to older leaves. Yitpi's older leaves had the greatest decrease in leaf N % after a switch from high to low-N supply. Amongst all leaves there was a general decline in leaf N % even under steady state N levels, possibly due to the dilution effect as the leaves increased in biomass. Cultivar differences were observed in this research suggesting that the non-destructive phenotyping of N uptake using hyperspectral reflectance based measurements techniques may improve the ability to select superior N uptake varieties in the future.

INTRODUCTION

The current agricultural system is heavily reliant on nitrogen (N) fertiliser to maximise production in cereals (Ladha et al. 2016). Despite considerable effort, no varieties with improved N use efficiency (NUE) have been commercially released, perhaps reflecting an inability to identify, phenotype and select desirable NUE traits (Garnett et al. 2015). Desirable phenotypes to increase the N use dynamics of wheat would include a greater capacity to respond quickly to available N by increasing leaf N %, maintenance of leaf N % under low N availability as well as an ability to partition N to the younger leaves under low N availability. A significant portion of N within a wheat plant is located in shoot tissue

(Sanchez-Bragado et al. 2016), and therefore being able to measure genotypic differences in tissue N in response to changing N supply could increase NUE.

The improvement of NUE may have been hampered by the inability to accurately and rapidly measure the N concentration in leaf tissue. Destructive harvest based measurements are time consuming and laborious (Masclaux-Daubresse et al. 2010). Chlorophyll based proxies for N are rapid but inaccurate (Debaeke et al. 2006). One of the impediments to quantifying this has been the reliance on destructive harvests for leaf N % data, on what are known to be dynamic processes (Guo et al. 2016), making the repeated observation of the tissue chemistry of a single plant over time impossible because leaves have been destructively sampled and can't be measured again. Novel methods for the non-destructive observation of leaf N % are now available using hyperspectral reflectance (Ecarnot et al. 2013). This facilitates the repeated observation of N tissue concentrations, which may allow the selection of cultivars with superior uptake and remobilisation dynamics required for improved NUE (Garriga et al. 2017).

If observations of leaf N can be made repeatedly over a time course, the response of wheat plants to changing N availability can be investigated. In South East Australia, wheat plants are often grown in low yielding environments with limited provision of soil N (Sadras and Lawson 2013). Additions of N are often made at sowing or during growth as split applications (López-Bellido et al. 2005). This fact, combined with the rapid movement of nitrate (NO_3^-) through the soil profile means that plants need to respond rapidly to available N in order to maximise uptake (Sadras and Lawson 2013). Understanding the response of plants to changing N availability may also help in identifying cultivars with greater remobilisation efficiency, those better able to utilise tissue N (Martre et al. 2003), or increase uptake capacity (Aziz et al. 2016).

The goals of this research were to understand and quantify the N uptake and leaf N % of two Australian bread wheat cultivars in response to fluctuating N availability in a hydroponic system as well as evaluate the accuracy of the spectral reflectance leaf N % measurements. The results showed differences between cultivars exist in leaf N %, movement between leaf ranks and tested the feasibility of the method for use in larger scale pot screening.

MATERIALS AND METHODS

Plant material

Wheat seeds (*Triticum aestivum* L. var. Gladius and Yitpi) were imbibed with RO water on paper towels for 48 h at 5 °C, after which they were washed in ethanol and RO water and placed in germination trays on moist paper towel in a growth room (5 d, 22 °C). These cultivars were chosen as they had shown differing tillering behaviour in previous experiments; Gladius having fewer tillers than Yitpi. Post emergence, they were transferred to mesh collars within hydroponic PVC tubes (H 300 mm x D 50 mm) (Garnett et al. 2013). These tubes were then allocated to one of eight 120 L ebb and flow hydroponic systems (two systems for each of the four N availability treatments), which had a fill/empty cycle of 30 min. Inert volcanic rocks were added to the tubes in order to prevent ambient light from affecting the exposed roots.

The controlled environment room which housed the hydroponic system had a day:night cycle of 12 h:12 h, 22 °C:16 °C, with a flux density at canopy level of *c.* 650 µmol m^{-2-} s^{-1} and relative humidity of 60 %. The nutrient solution was a modified Johnson's solution (Johnson et al. 1957) which contained 0.5 NO_3^-, 0.8 K, 0.1 Ca, 0.5 Mg, 1 S and 0.5 P (mM) for the 0.5 mM NO_3^- treatment, and 5 NO_3^-, 0.8 K, 0.1 Ca, 0.5 Mg, 1 S and 0.5 P (mM) for the 5 mM NO_3^- treatment. The hydroponic solutions also contained (in µM): 2 Mn, 2 Zn, 25 B, 0.5 Cu, 0.5 Mo, 100 Fe (as FeEDTA and ethylendiamine-*N,N'*-bis(2-

hydroxyphenylacetic acid) FeEDDHA)). Iron was topped up twice weekly as $Fe(NH_4)_2(SO_4)_2.6H_2O$ (8 mg L^{-1}) to avoid deficiency. The solutions were maintained at between 19-21 °C using a chiller. The pH of the solutions was monitored daily and maintained between 5.8 and 6.0. The solutions were completely replaced every seven days to avoid nutrient deficiency.

There were four N solution treatments: HN (5 mM NO_3^-), LN (0.5 mM NO_3^-), HN solution initially then transferred to LN solution at 31 days after planting (DAP) in the hydroponic system (hereafter referred to as 'HN to LN') and LN solution initially then transferred to HN solution at 31 DAP (hereafter referred to as 'LN to HN'). The experimental layout could have been improved, as there were not three replicates in each hydroponic tub, but rather there were two replicates in one tub and one in the second tub. Although two replicates is sometimes common, the aim was to have three.

Reflectance spectra

Hyperspectral reflectance data was collected using a Field Spec 3 (Analytical Spectral Devices, Inc. (ASD), Boulder, CO, USA) in combination with a leaf clip and white background reflectance panel (outlined in Chapter 3). The spectral range of the spectrometer was 350-2500 nm, its spectral resolution was 3 nm in the region of 350-1000 nm and 10 nm in the 1000-2500 nm region. The spectral information was collected on the adaxial side of the leaf. Two readings were collected at 1/3rd and 2/3rd the distance along the leaf from the base to tip and were averaged. Reflectance spectra were collected on two leaves from a single tiller on 29, 30, 31, 32, 33, 34, 36, 39 and 40 DAP. The leaves were labelled in order of emergence, the 2nd and 4th to emerge (known hereafter as leaf 2 and leaf 4), with most plants having four leaves on the tiller measured. The leaves were marked so that repeated measurements could be taken from a single leaf.

The collected leaf spectra were pre-processed using R statistical software prior to their regression with destructively harvested leaf N % measurements (R Core Team 2018). The first step in the analysis of the collected leaf spectra was to adjust for the switch in detectors which occurred at 1000 and 1830 nm. The moving average was then taken into account by the standard normal variate being applied to each spectrum individually to remove scatter. The spectra were then smoothed using Salitzky-Golay smoothing filter to maintain line shape (Savitzky and Golay 1964). The leaf N % were predicted using the partial least squares regression (PLSR) derived method that was outlined in detail in Chapter 3.

RESULTS

Leaf N % measurements from 29-40 DAP were collected on both leaf 2 and leaf 4 of Gladius and Mace cultivars. Leaf N % decreased for all treatments from 29 to 40 DAP (Fig. 1). At 40 DAP, both cultivars had a higher leaf N % in their leaf 4 than their leaf 2 under both HN and LN. Furthermore, both cultivars' leaf N % was equivalent for the LN leaf 4 and the HN leaf 2 at 40 DAP. For Yitpi at 34 DAP, HN leaf 4 was equivalent to HN leaf 2, however by 40 DAP leaf 4 leaf N % was higher than leaf 2. At 30 DAP, there was a sudden decrease in leaf N % for all treatments, except Yitpi LN leaf 2, and there was a convergence in leaf N % between the treatments or leaf ranks on that day. The trends of younger leaves having higher leaf N %, and a gradual decrease over time appear consistent. There was no difference between the cultivars with respect to leaf N (%) at 40 DAP.

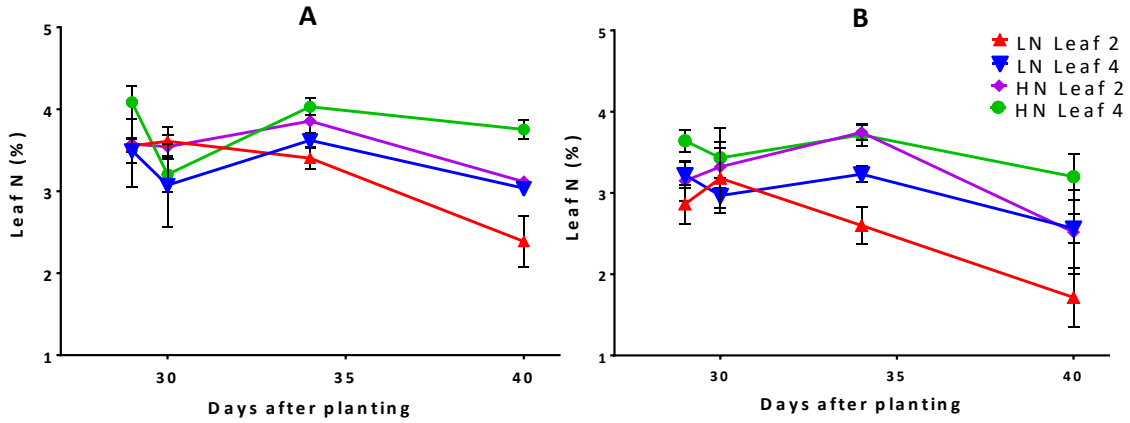

Figure 1 Leaf N % (y-axis) of (A) Gladius and (B) Yitpi leaf 2 and leaf 4 under stable high N (HN) (5 mM NO_3^-) and low N (LN) (0.5 mM NO_3^-). 'Leaf 2' and 'leaf 4' refer to the leaf positions on the first day of sampling (29 DAP). The leaf N % was obtained via the use of hyperspectral reflectance on the individual leaves from 29-40 DAP after emergence (x-axis). Means are presented +/- the SEM, n=3.

As well as the comparison between cultivars, the comparison between the equivalent leaves under changing N availability enabled the measurement of the dynamic nature of leaf N concentration (Fig. 2 and 3). The measurement of leaf N % over the course of the research demonstrated changing leaf N % values in response to the change in N availability. Each panel of the two figures compares a steady state to a fluctuating N availability treatment. For leaves initially under HN but switched to LN (HN to LN), there was a decrease of the leaf N % to a lower level that that under steady state LN (Fig 2A & B). For Gladius (Fig 2), the leaf N % after moving plants from LN to HN exceeded the leaf N % found under the steady state HN (occurring at the dashed vertical line) (Fig. 2C & D). Leaf N % decreased at the greatest rate for LN leaf 2 (Fig. 2D), whilst the LN to HN leaf 2 maintained a stable leaf N %.

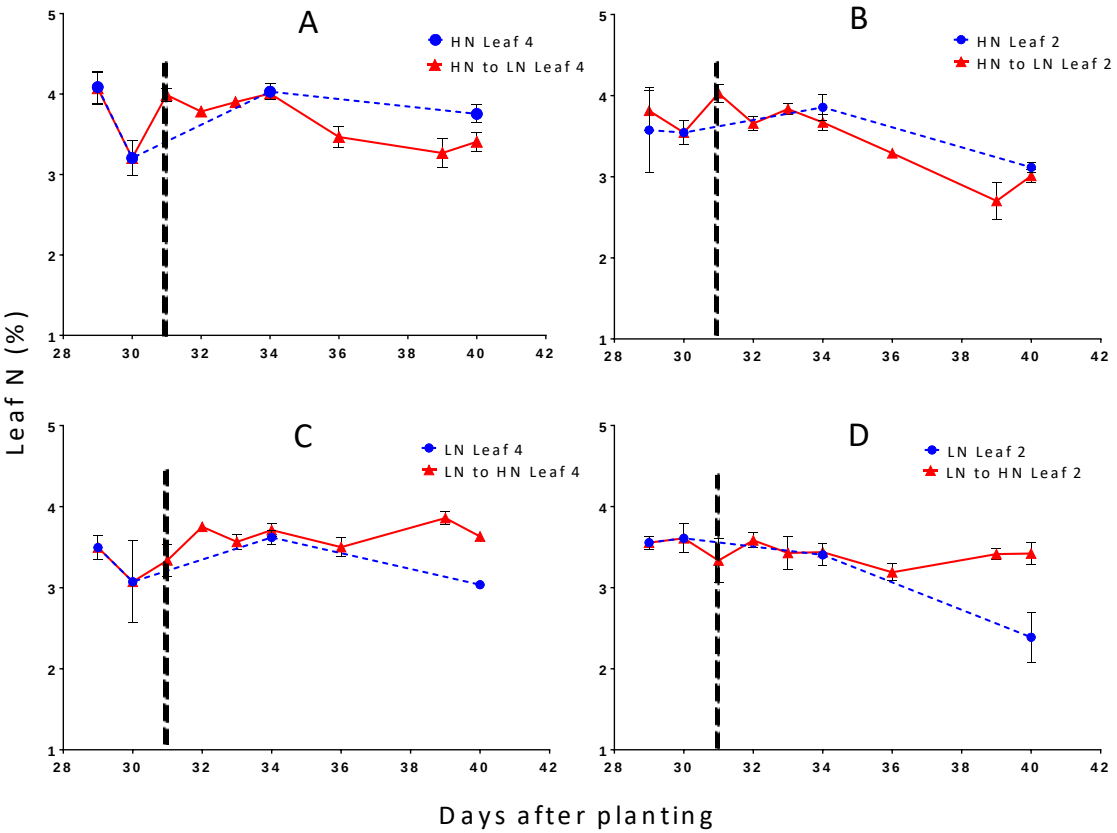

Figure 2 Gladius leaf 2 and leaf 4 N % under the four N treatments; (A) HN (5 mM NO_3^- solution), (B) HN (5 mM NO_3^- solution) switched to LN (0.5 mM NO_3^- solution), (C) LN (0.5 mM NO_3^- solution) and (D) LN (0.5 mM NO_3^- solution) switched to HN (5 mM NO_3^- solution). The vertical dashed line represents the point of switching the N solutions (31 DAP) (HN to LN, LN to HN). Means are presented +/- the SEM, n=3.

Unlike Gladius, Yitpi maintained leaf N % in leaf 4 after the change from HN to LN (Fig. 3A). However, the leaf 2 of Yitpi had a decrease in leaf N % after the shift from HN to LN (Fig. 3B). Under both treatments, the leaf 2 had a lower leaf N % than leaf 4, especially at the final time point. Under a switch from LN to HN, the YEB was able to respond and increased its leaf N % beyond that of the steady state LN, however YEB-1 only transiently had a higher leaf N % than LN YEB-1 by 40 DAP. There is a consistent downward trend in leaf N % amongst all cultivar combinations over the observation window.

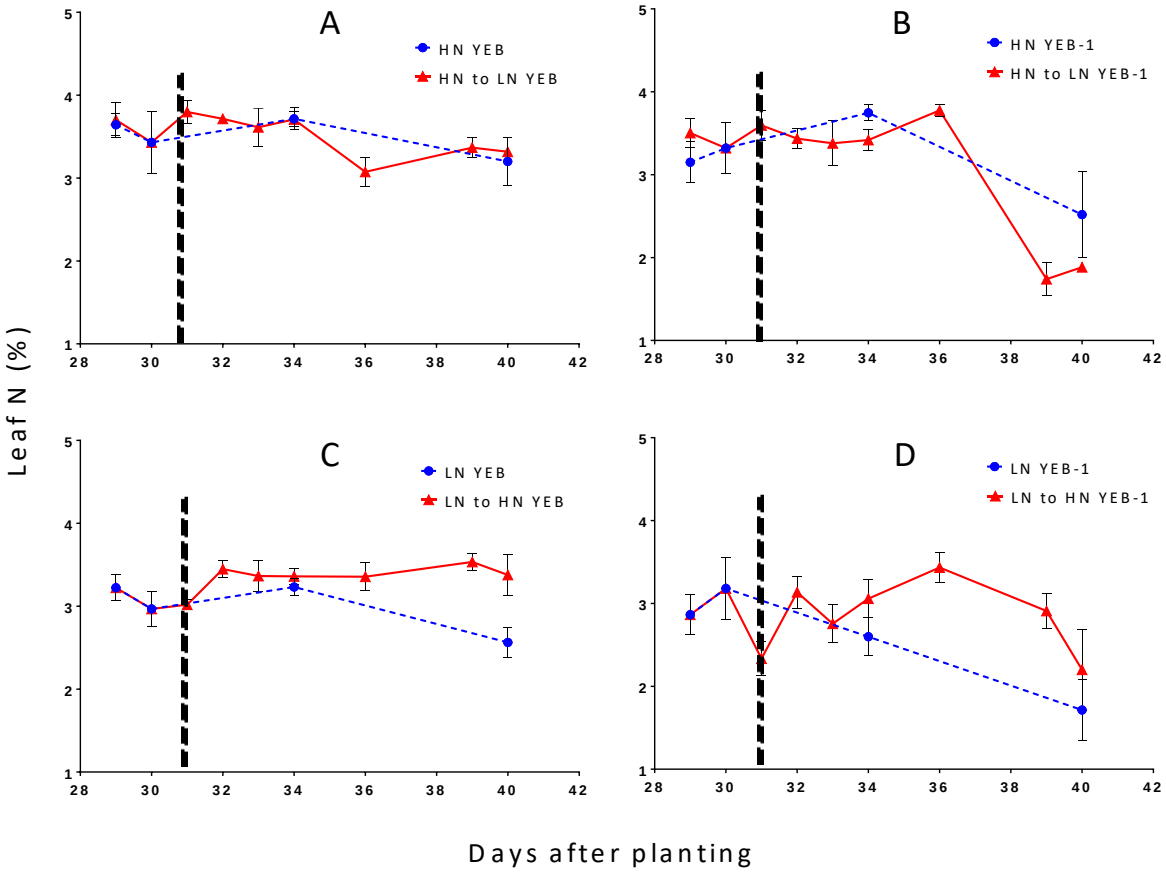

Figure 3 Yitpi YEB and YEB-1 leaf N % under the four N availability schedules; (A) (HN (5 mM N solution), (B) HN (5 mM N solution) switched to LN (0.5 mM N solution), (C) LN (0.5 mM N solution) and (D) LN (0.5 mM N solution) switched to HN (5 mM N solution). The vertical dashed line represents the point of switching the N solutions (31 DAP) (HN to LN, LN to HN). Means are presented +/- the SEM, n=3.

DISCUSSION

The results of this research demonstrate that leaf N % is dynamic over time and responds to changing N availability. The use of proximal sensing in this experiment is based on the work done by Ecarnot et al. (2013) who established the chemometric analysis combined with spectral reflectance to predict leaf N %. This research shows the effectiveness of the hyperspectral reflectance method in identifying hypothesised phenotypes in response to changing N availability. These included the speed of uptake (increase in leaf N %) in

response to increasing N availability and the maintenance of leaf N % when faced with a sudden loss of N availability. Both of these phenotypes could contribute to improved NUE, via more efficient N uptake when it is available (Sadras and Lawson 2013), as well as the ability to maintain leaf N % in order to maintain photosynthetic production (Parry et al. 2010). The method was effective in identifying cultivar differences, it also showed that for both cultivars the leaf N % changed according in N availability, either increasing or decreasing leaf N %, in the same direction as the treatment.

Physiologically, if N is in short supply, it is efficient to partition N to the youngest and highest leaves which will have access to the most solar radiation in a closed canopy (Field 1983). Whereas, if N is not in short supply then it would be efficient to leave N in older leaves that may not have as much access to sunshine. As the N concentration of leaves reflects their photosynthetic capacity (Evans 1983), the prioritising of younger leaves closer to the grain ear, may maximise carbon fixation per unit N within the plant, by both photosynthetic productivity and proximity to the ear (Bertheloot et al. 2008). The methods used in this experiment allow direct observation of the partitioning of limited N resources between leaf ranks and the potential of this to distinguish between cultivars (Gaju et al. 2014). Overall, leaf 4 was prioritised over the leaf 2 for both cultivars in terms of leaf N %. This was despite there not being significant senescence by the conclusion of the observation period.

The dynamic nature of individual leaf N concentration over time was observable in this research. There was a dip in leaf N % across the HN treatments at 30 DAP possibly due to high vegetative growth and leaf expansion, and thus the plants may not have been able take up enough N to maintain leaf N %, although this has not been established in the literature. Leaf N % increased again for both cultivars by 34 DAP, potentially as growth slowed as the plants moved out of their vegetative stage. There was a general decrease in

leaf N % across all cultivars and treatments which supports the assessment of wheat tissue N dilution over time conducted by Pilar et al. (1983). These results suggests that this method needs to be combined with high-throughput image based growth analysis in order to understand these dynamics.

The protocol utilised in this research shows promise for understanding dynamics of N uptake and is a significant improvement on the destructive analysis of tissue (Oscarson et al. 1995) or assessment via chlorophyll based proxies (e.g. SPAD-502), previously the only methods available to determine or approximate tissue N content. High throughput phenotyping (HTP) provides high resolution growth analysis over time on hundreds of plants simultaneously (Neilson et al. 2015). The combination of leaf N % observations with growth analysis would be an extremely powerful phenotyping protocol, collecting many orders of magnitude more data than has been previously possible, in order to differentiate N uptake phenotypes. The inclusion of more genetic diversity, leaf ranks and observations over a greater amount of time could facilitate in identification of genetic differences in uptake and N dynamics useful in breeding and improving NUE of wheat (Barraclough et al. 2010).

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Chapter 5: High resolution growth analysis combined with hyperspectral nutrient analysis to understand nitrogen response in wheat

TITLE

High resolution growth analysis combined with hyperspectral nutrient analysis to understand nitrogen response in wheat

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ABSTRACT

Efficient phenotyping can help in characterising nitrogen use efficiency (NUE) and the identification of promising bread wheat (*Triticum aestivum*) lines. In order to phenotype NUE accurately, nitrogen (N) uptake and N allocation need to be measured in conjunction, including the dynamics of these processes. Previously, obtaining this data has been challenging because it relied on destructive sampling. However, using hyperspectral reflectance and high-throughput image based growth phenotyping we can now measure the growth and N concentration of leaves over time and simultaneously. This research determined how two wheat cultivars, Mace and Gladius, responded to different levels of N fertilisation.

This study found significant cultivar differences in growth rate and leaf N concentration in response to a high and low steady state N treatment and two split application treatments. When N was added at stem elongation both Mace and Gladius delayed the point of maximal growth by six days compared to the steady state low N treatment. Split application of N increased the total N uptake of Mace by 57 % above the high N treatment. Grain protein increased by 45 % for Gladius when N was added at booting. Nitrogen addition resulted in an increase in upper leaf N content (g N m^{-2}) shortly after application and the leaves remained viable for longer, compared to steady state N treatments. Different NUE phenotypes were observed which combined growth and leaf N concentration, greatly increasing the observation power of N dynamics which may lead to the dissection of NUE phenotypes in wheat in the future.

INTRODUCTION

The demand for cereals is increasing year on year with global population growth (Eickhout et al. 2006). However, nitrogen (N), a major component of cereal protein is not utilised efficiently within the agricultural system (Nathaniel et al. 2014), limiting potential yields

(Garnett et al. 2015). Nitrogen use efficiency (NUE), a combination of N capture from the soil (N uptake efficiency: NUpE) and the subsequent conversion of N in plant tissue to grain yield (N utilisation efficiency: NUtE), remains low, at 35 % globally (Good et al. 2004). This is problematic, as leakage of N into the ecosystem causes environmental damage and is an economic loss for producers. The improvement of NUE in cereals has become a priority, however little progress has been made.

The improvement of NUE in bread wheat (*Triticum aestivum* L) varieties requires the assessment of genotypic differences via the observation and classification of their phenotypes (Furbank and Tester 2011). However, this is a challenge as NUE encompasses a myriad of biological processes from the cellular to whole plant physiology (Sadras and Richards 2014). All of these processes interact with the environment and management (genotype x environment x management interaction: GxExM) influencing plant phenotypes and obscuring the causes of performance variation. So far, the assessment of NUE has mostly been via yield trials and limited destructive harvests (Barraclough et al. 2014; Ortiz-Monasterio et al. 1997; Sadras and Lawson 2013). However, perhaps due to the complexities of the GxExM, thus far these approaches have made limited progress towards the commercial release of improved NUE cultivars (Nguyen and Kant 2018).

Understanding the growth dynamics which contribute to yield may help in understanding the GxExM interactions underlying NUE in wheat, which until recently, could only be observed with a series of destructive harvests throughout the growth season. This is problematic as destructive harvests are laborious, expensive and require large numbers of replicates in order to accommodate biomass removal from the experiment. Improved sensor technology and image analysis techniques have meant that non-destructive alternatives to measure growth in cereals have now become available in both the field (Araus et al. 2018) and controlled environments (Al-Tamimi et al. 2016; Honsdorf et al. 2014). Automated high-

throughput phenotyping systems (HTP) can now measure growth non-destructively on hundreds of plants simultaneously (Campbell et al. 2017; Erica et al. 2017; Muraya et al. 2017), illuminating growth differences in response to N availability and environment, an approach used successfully by Nguyen et al. (2016), to compare wheat growth under high and low N.

The measurement of growth dynamics in response to N availability is a major development but in order to really understand and thus improve NUE, phenotyping needs to also include N uptake, utilisation and remobilisation behaviour. This will also allow investigation into the importance of the different components of NUE (uptake versus utilisation) for grain yield and how these vary according to N levels, an area with conflicting findings in the literature. Ortiz-Monasterio et al. (1997) found that the contribution of the two components varied with increasing N availability. When N application increased, the contribution to NUE shifted from NUpE to NUtE (Ortiz-Monasterio et al. 1997). Dhugga and Waines (1989), on the contrary, found that NUpE was a more important determinant of NUE at higher N fertilisations. Kichey et al. (2007) found in field trials that NUtE contributed the majority of final grain N and that there were significant genotypic differences. Furthermore, they showed that NUtE greatly depended on N availability at flowering and N uptake post-anthesis.

In order to differentiate N uptake phenotypes, the N concentration of shoot area must also be observed. Using recently developed spectral reflectance techniques, the N content of individual leaves can now be measured non-destructively over time (Ecarnot et al. 2013; Silva-Perez et al. 2018), providing an accurate and rapid alternative to destructive harvests. Spectral reflectance based techniques to measure leaf N combined with high resolution growth analysis may facilitate the phenotyping of growth and leaf N behaviour in response to different levels of N.

In results presented here, we profiled the growth and leaf N concentration of two common Australian commercial wheat cultivars, Mace and Gladius, under steady state and split N applications, over an extended period of time. The N was applied in split applications, in order to both reflect common practice in wheat production and to test whether the phenotyping techniques employed here could observe plant response to changing N. The use of high resolution growth analysis was combined with spectral reflectance to better understand the relationship between growth, N uptake and allocation in wheat in response to N supply.

METHODS AND MATERIALS

Two common Australian bread wheat cultivars were utilised: Gladius (a RAC875, Excalibur, Kukri, Krichauff and Trident derivative) and Mace (Wyalkatchem derivative), which are both early/mid-season maturing and developed by Australian Grain Technologies (Adelaide, Australia). These cultivars were chosen because they have similar maturity profiles, different genetic pedigrees and are widely grown in SE Australia. The plants were grown in a glasshouse at the Plant Accelerator (Australian Plant Phenomics Facility, University of Adelaide, Adelaide, Australia; -34.97113°, 138.63989°). The glasshouse temperature was maintained at 23/16 °C day/night. The pots used were round 2.5 L white pots containing 2.225 kg of dry soil, which was equal parts (v/v) cocopeat, clay loam and U.C. Davis mix (a combination of sand, peatmoss and lime).

There were four N treatments: 1. Low N (LN) fertilisation (50 mg N/kg soil), 2. High N (HN) fertilisation (150 mg N/kg dry soil), 3. Low N until an N addition at Zadoks growth stage 30 (LN-30) (LN initially, with an addition of 170 mg N per pot as ammonium nitrate ($N_2H_4O_3$), equating to a total of 115 mg N kg soil^{-1}) and 4. Low N until an N addition at Zadoks growth stage 40 (LN-40) (LN initially, with an addition of 170 mg N as ammonium nitrate, equating to a total of 115 mg N kg soil^{-1}). Initial N additions were mixed into the soil

as urea (CH_4N_2O) prior to potting, with additional N being added as ammonium nitrate dissolved in 200 ml RO-H_2O. Steady state N treatments (LN and HN) had 200 ml water added at the same time as the ammonium nitrate additions. The physiological developmental and maturity of the two cultivars did not differ and as such the point of N addition was made when the majority of the plants were at Zadok's 30 and 40.

Four seeds were planted in each pot, at a depth of 2 cm. The four seedlings were then thinned to a single seedling of a uniform size at the three leaf stage (11 days after planting (DAP)). At 14 DAP the plants were transferred to a Lemnatec Scanalyzer 3D HTP System (LemnaTec GmbH, Aachen, Germany) for automated watering and imaging. The plants were maintained at 22 % gravimetric soil water content whilst they were on the automated phenotyping system.

Image Acquisition and Analysis

Images of the plants were collected automatically every two days from 16-74 DAP on the HTP system by 8 megapixel (2472 x 3296 pixel) RGB cameras (Prosilica GT3300, Allied Vision, Stadtroda, Germany) which captured one top and two side views with a 90° horizontal rotation. The images were processed using LemnaGrid software (LemnaTec, GmbH, Aachen, Germany). Plant pixels were separated from background using a nearest-neighbour colour classification. Noise was removed via erosion and dilatation procedures prior to combining all of the parts of the plant to one object (Neilson et al. 2015). Projected shoot area (PSA) was calculated via the sum of plant pixels from the three images and smoothed PSA curves were calculated using this data over a time series (Campbell et al. 2017). The smoothed PSA curves were computed using the splitSplines routine in R package 'imageData', after which a standard R routine for fitting cubic splines to data was applied (Brien 2018). The absolute growth curves (AGR) were computed from smoothed PSA (splitContGRdiff routine within imageData package), by differencing consecutive smoothed

PSA values and dividing by the time interval. The operation of smooth.spline can be controlled by several parameter settings, the most important of which is degrees of freedom (df). Higher (lower) df values correspond to weak (strong) smoothing. Values ranging from df = 4 (strong) to df = 7 (moderately weak) were tried during the preliminary analysis of this research (with the aid of probed function in imageData package), and the graphical output was compared subjectively for smoothed PSA and AGR. On this basis, it was decided to use df = 6 for the main analysis and graphs.

Collecting spectra

Hyperspectral reflectance data was collected with a Field Spec 3 (Analytical Spectral Devices, Inc. (ASD), Boulder, CO, USA) in combination with a leaf clip and black background reflectance panel. The spectral range of the spectrometer was 350-2500 nm, its spectral resolution was 3 nm in the region of 350-1000 nm and 10 nm in the 1000-2500 nm region. The spectral information was collected on the adaxial side of the leaf. Two readings were collected at one-third and two-thirds along the leaf from the base to tip and were averaged. The leaves from the main tiller were marked, and had their spectra collected, as they emerged, with the first emerged labelled ‘leaf 1’.

The hyperspectral method utilised in Chapters 3 & 4 of this thesis has been updated in this chapter, as three main issues have been addressed. The improvements in the protocol include changing the background for the reflectance readings from white to black which reduces the interference from the background panel, the addition of a ‘leaf mask’ which reduces the width of the aperture to account for thin leaves (Fig. S1) and a change in the calibration set from which the tissue N predictions were calculated. The calibration set utilised to calculate the leaf N predications and the updated protocol are outlined fully in Silva-Perez et al. (2018).

Spectra processing

The collected leaf spectra were processed using the calibration set established by Silva-Perez et al. (2018) with a break in the reflectance detectors at 1000 and 1830 nm. The change in the protocol from the previous method outlined in Ecarnot et al. (2013), to the method developed by Silva-Perez et al. (2018) improved the power of N prediction from 0.82 (Chapter 3) to 0.93 (Silva-Perez et al. 2018). The predicted N measurement utilised in this research provided values in 'N per unit leaf area' (R^2 = 0.93) (g N m^{-2}) rather than N (%) (R^2 = 0.70), which was calculated using leaf mass area (g m^{-2}), accounting for leaf thickness, and is theorised to improve accuracy (Silva-Perez et al. 2018).

Destructive harvest

At 77 DAP, the plants were taken off the automated imaging system and placed on a bench in a glasshouse and grown until maturity under well-watered conditions. The plants were harvested when dry. The grain and shoot were harvested at the soil surface and weighed before and after being dried for three days at 60 °C. The shoot samples were shredded and a subsample of 1.0 g of material per shoot was further ground to a fine powder using a Genogrinder (SPEX, Metuchen, NJ, USA) within centrifuge tubes using ball bearings. The grains were threshed and then also ground to a fine powder using the same Genogrinder protocol.

Nitrogen analysis

Between 100-200 mg of dried sample was weighed into nitrogen-free paper (Thomas Scientific), which was folded and pressed into a tablet using a pill press. This was then analysed for N content using a 'rapid N exceed' N analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany) which utilises the Dumas combustion method (Matejovič 1996).

NUE calculations

Grain protein was calculated by multiplying the N (%) of the grain by 5.4 (Mosse 1990). NUE calculations were adapted from Good et al. (2004). The NUE was calculated as the grain weight/N supplied as fertiliser. NUpE (%) was the N in the total plant (mg) divided by the N supplied (mg). NUtE (%) was equal to the grain weight (g) divided by the total N in the aboveground biomass (g). Nitrogen harvest index (NHI) was calculated as the N content of the grain as a proportion of the total N in the aboveground biomass.

Statistical analyses

The experiment was a randomized complete-block design with 5 replicates. The design was randomized using dae (Brien 2017), a package for the R statistical computing environment (R Core Team 2018). All other statistical analysis carried out within this research utilised analysis of variance (ANOVA).

RESULTS

Non-destructive imaging was used to measure the PSA of the two wheat cultivars, Mace and Gladius, in the four N treatments (Fig. 1). While the shape of the PSA curve was comparable under HN and LN, under HN the mean maximal PSA was 930 kpixels compared to LN PSA of 790 kpixels for the Mace cultivar. The maximum mean PSA of Gladius under HN was approximately 30 % higher than under LN, while Mace had 20 % more mean maximum PSA under HN compared to LN. The DAP at which the maximal PSA was reached was equivalent under HN and LN fertilisation, between 58-60 DAP.

The addition of N to LN treatments (LN-30 and LN-40) had a major effect on PSA. Under the LN-30 treatment the PSA was similar to LN until the point of N addition (corresponding vertical bar), at which point PSA increased until reaching a maximal point higher than LN, but not as high as HN. Under the LN-30 treatment, Gladius increased its PSA by 17 % compared to that under LN (749 and 616 kpixels respectively), whereas the

PSA of Mace only marginally increased. Under LN-40, the N addition increased maximum PSA to 850 kpixels for Mace, increasing PSA above that observed under LN (797 kpixels) and close to the maximal PSA point as observed under the LN-30 treatment (844 kpixels). For Gladius under LN-40, there was no increase in maximum PSA compared to LN. Mace had a larger PSA than Gladius under each of the four N treatments. The difference between the two under HN was approximately 12 %, whereas under LN Mace was 30 % larger than Gladius.

After the application of N under the LN-30 treatment both cultivars finish the imaging period (74 DAP) with PSA measurements equivalent to those under HN. Whereas under LN-40 treatment, both cultivars finish the imaging period with a higher PSA than under LN, 30 % and 25 % larger than under the LN treatment for Gladius and Mace respectively.

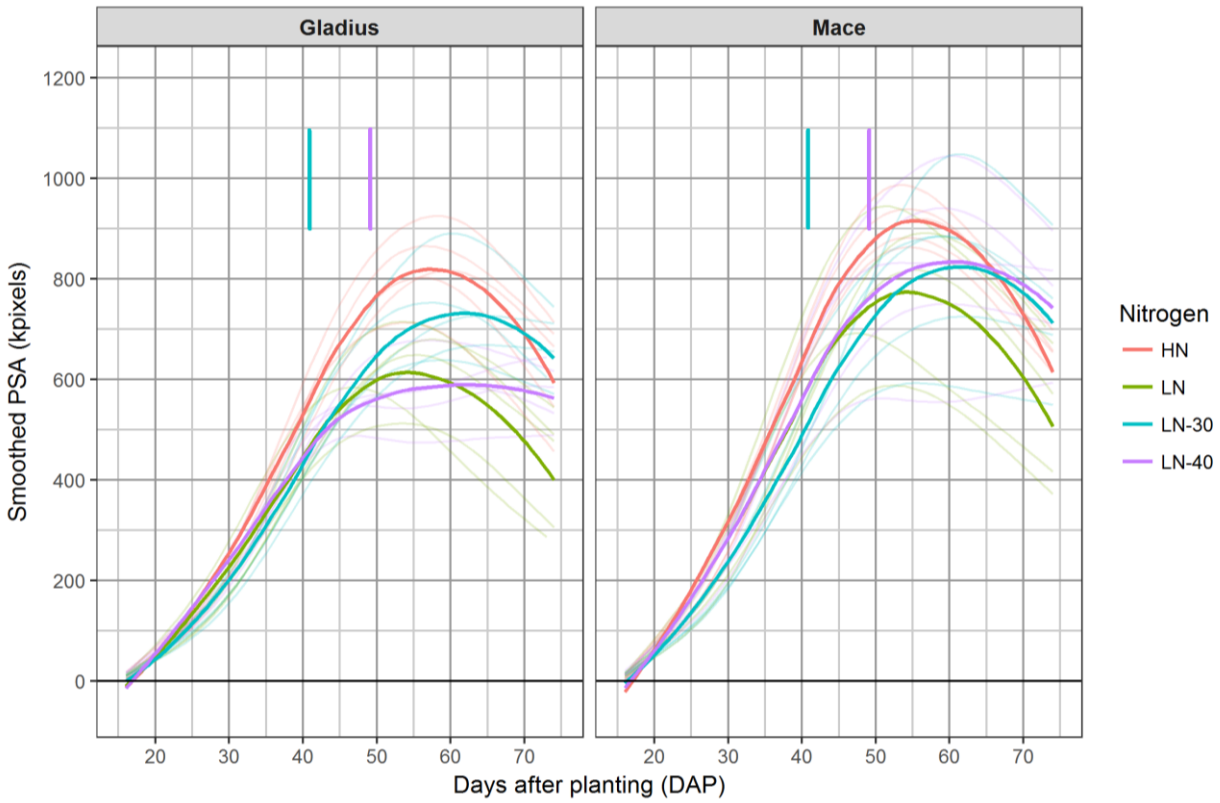

Figure 1 The smoothed projected shoot area growth curves for both Gladius and Mace with five replicates (thin curves) and the Loess curves (bold curve). The four bold curves represent the four nitrogen treatments: HN (red; 150 mg N kg soil^{-1}), LN (green; 50 mg N kg soil^{-1}), LN-30 (blue; LN until growth stage 30 (41 DAP), at which ammonium nitrate was added equating to 115 mg N kg soil^{-1}) and LN-40 (purple; LN until growth stage 40 (49 DAP), at which ammonium nitrate was added equating to 115 mg N kg soil^{-1}). The vertical lines represent the point of ammonium nitrate addition for LN-30 and LN-40 treatments (blue and purple respectively), n=5.

The conversion of PSA into an absolute growth rate (AGR) provides greater detail on the growth response to the four N treatments (Fig. 2). The AGR curves demonstrate a typical growth cycle for a wheat plant: an initially increasing rate of growth, reaching maximal growth rate between 35-40 DAP, a subsequent reduction in growth rate reaching zero growth between 55-65 DAP, after which AGR decreases into negative growth rate (reduction of

apparent leaf area) (Fig. 2). From these curves, the day at which zero growth was reached can be ascertained, after which PSA decreases, potentially caused by a remobilisation of resources from leaf tissue into the grain and a possible change in the fresh:dry weight ratio (Gregersen et al. 2008). The shift often occurs before drying, usually around maximum AGR, at the commencement of booting. A figure demonstrating this change in PSA using the images captured with this HTP system is provided (Fig. S2).

Differences between the AGR under HN and LN treatments were observable in the amplitude of the curves, AGR increases and decreases being less pronounced under LN. There was an influence on plant growth under LN-30, with a prolonged period of positive growth and a greater number of days to reach a growth rate of zero kpixels day^{-1}. Zero growth was reached at 56 DAP under LN, but increased to 62 DAP under LN-30 and 60 DAP under LN-40 for both cultivars. At the conclusion of imaging (74 DAP) the AGR followed the N treatments, with HN having the lowest AGR (negative 20 kpixels day^{-1} for both cultivars), followed by LN at approximately negative 15 kpixels day^{-1}, LN-30 at negative 10 kpixels day^{-1} and finally LN-40 with a growth rate of zero and negative 7 kpixels day^{-1} for Gladius and Mace, respectively.

After the addition of N the AGR curves show a response that is not seen under the steady state treatments. After N addition at 41 DAP under LN-30, both cultivars increased their AGR, equating and then exceeding the AGR under HN. For both cultivars this occured between 46-50 DAP. Under LN-40, after N was added at 49 DAP Gladius exceeded the AGR of plants under HN at 57 DAP, whereas Mace responded more rapidly and exceeded the AGR under HN at 53 DAP (Fig. 2).

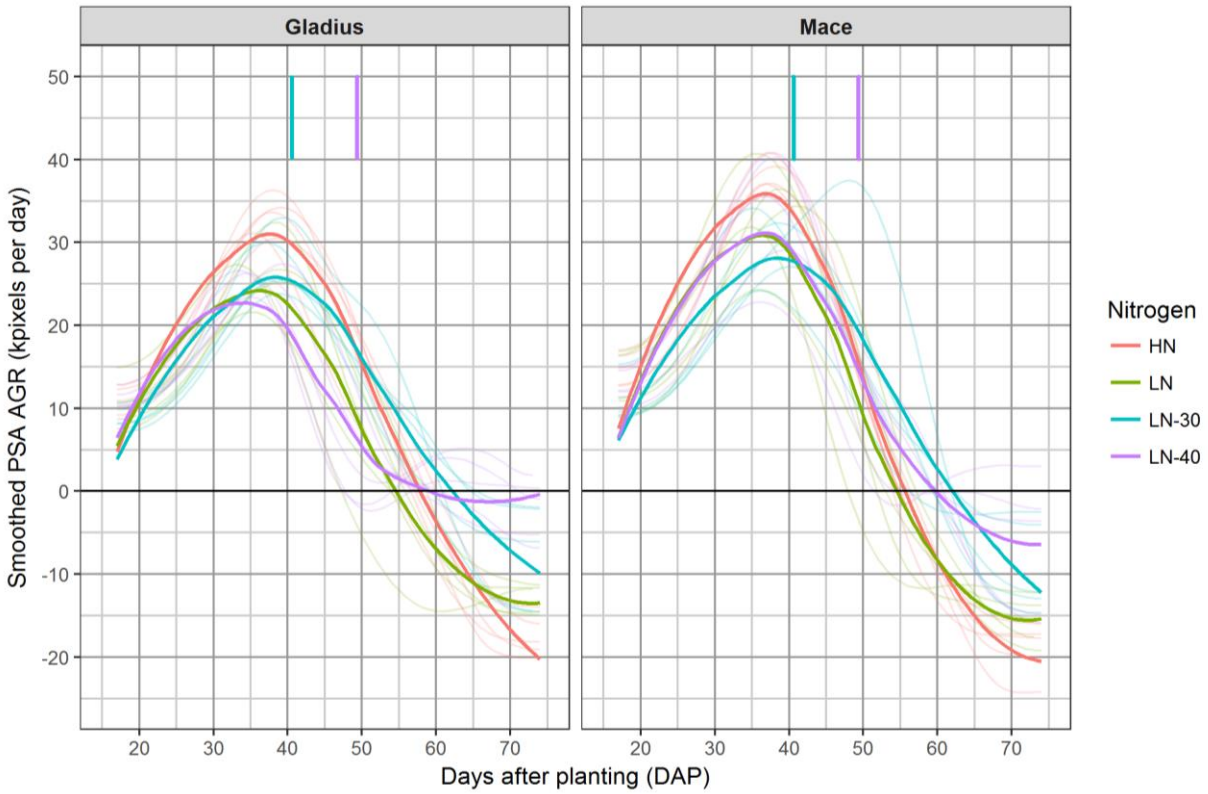

Figure 2 The smoothed projected shoot area derived absolute growth curves of Gladius and Mace cultivars with five replicates (thin curves) and the Loess curve (bold curves). The four curves represent the four nitrogen treatments: HN (red; 150 mg N kg soil^{-1}), LN (green; 50 mg N kg soil^{-1}), LN-30 (blue; LN until growth stage 30 (41 DAP), at which ammonium nitrate was added, equating to 115 mg N kg soil^{-1}) and LN-40 (green; LN until growth stage 40 (49 DAP), at which ammonium nitrate was added equating to 115 mg N kg soil^{-1}). The vertical lines represent the point of ammonium nitrate addition for LN-30 and LN-40 treatments (blue and purple respectively), n=5.

The differences in PSA and AGR demonstrated the real time responses of the cultivars to changing N availability. These differences were given perspective via the destructive analysis at the conclusion of the experiment. The grain yield (g per plant^{-1}) and the grain protein (%) both increased with higher N availability (Fig. 3). There was no cultivar effect

on yield under any of the four treatments. However, there was a significant treatment effect, with yield under LN being lower than the other treatments. Under LN the yield was approximately 5.8-7.4 g plant^{-1}. Under HN, LN-30 and LN-40 there was also no significant difference between treatment or cultivar, with yield ranging from 8.6-9.4 g plant^{-1}. The late additions of N increased the yield of both cultivars from their yield under LN level to an equivalence with HN yield results, regardless of time of application (Fig. 3A).

The split addition of N greatly increased the grain protein (%) compared to that under LN and HN (Fig. 3B). There was no difference in grain protein for either cultivar under LN or HN, both at approximately 10 %. However there was an increase for both cultivars under LN-30 and LN-40 treatments, resulting in an approximately 50 % increase in grain protein compared to the LN and HN treatment.

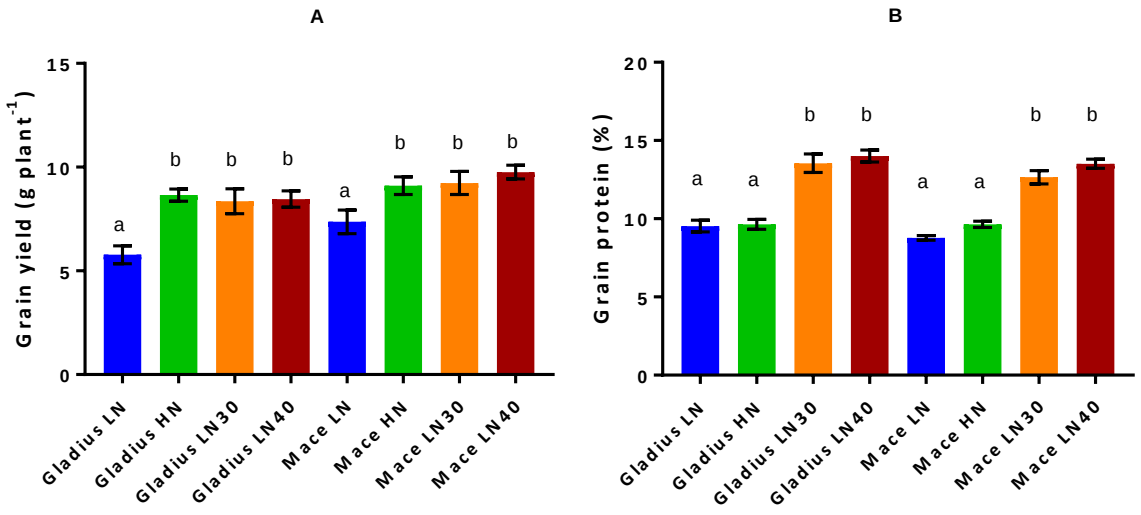

Figure 3 (A) Average grain yield (g plant^{-1}) and (B) protein concentration of the grain (%) for Gladius and Mace cultivars under four nitrogen treatments: LN (50 mg N kg soil^{-1}), HN (150 mg N kg soil^{-1}), LN-30 (LN until growth stage 30 (41 DAP), at which ammonium nitrate was added equating to 115 mg N kg soil^{-1}) and LN-40 (LN until growth stage 40 (49 DAP), at which ammonium nitrate was added equating to 115 mg N kg soil^{-1}). Different letters above columns represent significantly different results ($P < 0.05$). The error bars are SEM, n=5.

The N content (mg N plant^{-1}) of the aboveground biomass at maturity was also greatly increased by the split application of N fertiliser (Fig. 4). In the split applications, both Gladius and Mace doubled the N content compared to the LN treatment (from 150 to 300 mg N plant^{-1}). For Gladius, compared to the N content under HN, the N contents under LN-30 and LN-40 increased by 37 % and 36 %, respectively. For Mace, under LN-30 the N content increased by 41 % compared to the HN treatment, whilst under LN-40 N content increased by 57 % compared to content under HN. There were no cultivar differences under each of the treatments ($P < 0.05$).

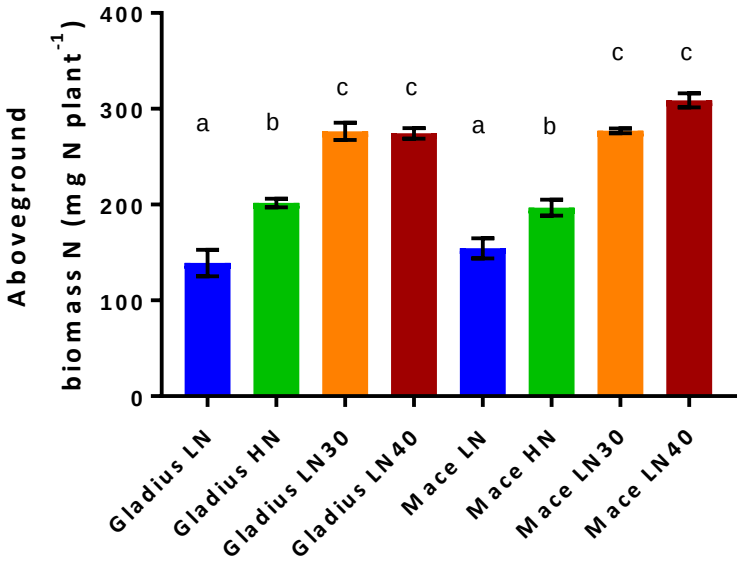

Figure 4 The total aboveground nitrogen content (mg N plant^{-1}) for both Gladius and Mace under the four nitrogen treatments. The four nitrogen treatments: HN (150 mg N kg soil^{-1}), LN (50 mg N kg soil^{-1}), LN-30 (LN until growth stage 30 (41 DAP), at which ammonium nitrate was added equating to 115 mg N kg soil^{-1}) and LN-40 (LN until growth stage 40 (49 DAP), at which ammonium nitrate was added equating to 115 mg N kg soil^{-1}). Different letters above columns represent significantly different results ($P < 0.05$). The error bars are SEM, n=5.

The calculation of NUE as well as its component processes are required in order to assess and understand the usage of N resources in response to N addition (Fig. 5). For NUE, as described by Good et al. (2004), a number of calculations are required to assess this complex phenotype. NUE, defined as grain production per N applied, was highest under LN, with a cultivar difference between Mace (57 %) and Gladius (44 %). NUE was lower under the HN, LN-30 and LN-40 treatments, with no significant difference between them at between 22-32 % (Fig. 5A). For NUpE, one of the two components of NUE, both cultivars under LN and Mace LN-40 treatments had the highest NUpE (>100 % of applied N) (Fig. 5B). The

plants under LN-30 and LN-40, had a significant increase in NUpE compared to HN (from 50 % under HN to 95 % under LN-30 and LN-40). The second component of NUE, NUtE, was 30 % higher under the steady state treatments HN and LN, than under the LN-30 or LN-40 treatments (Fig. 5C). There was no cultivar difference for NUtE results. Nitrogen harvest index was between 70-80 % for all treatments and cultivars and there was no difference in the efficiency of N recovery (Fig. 5D).

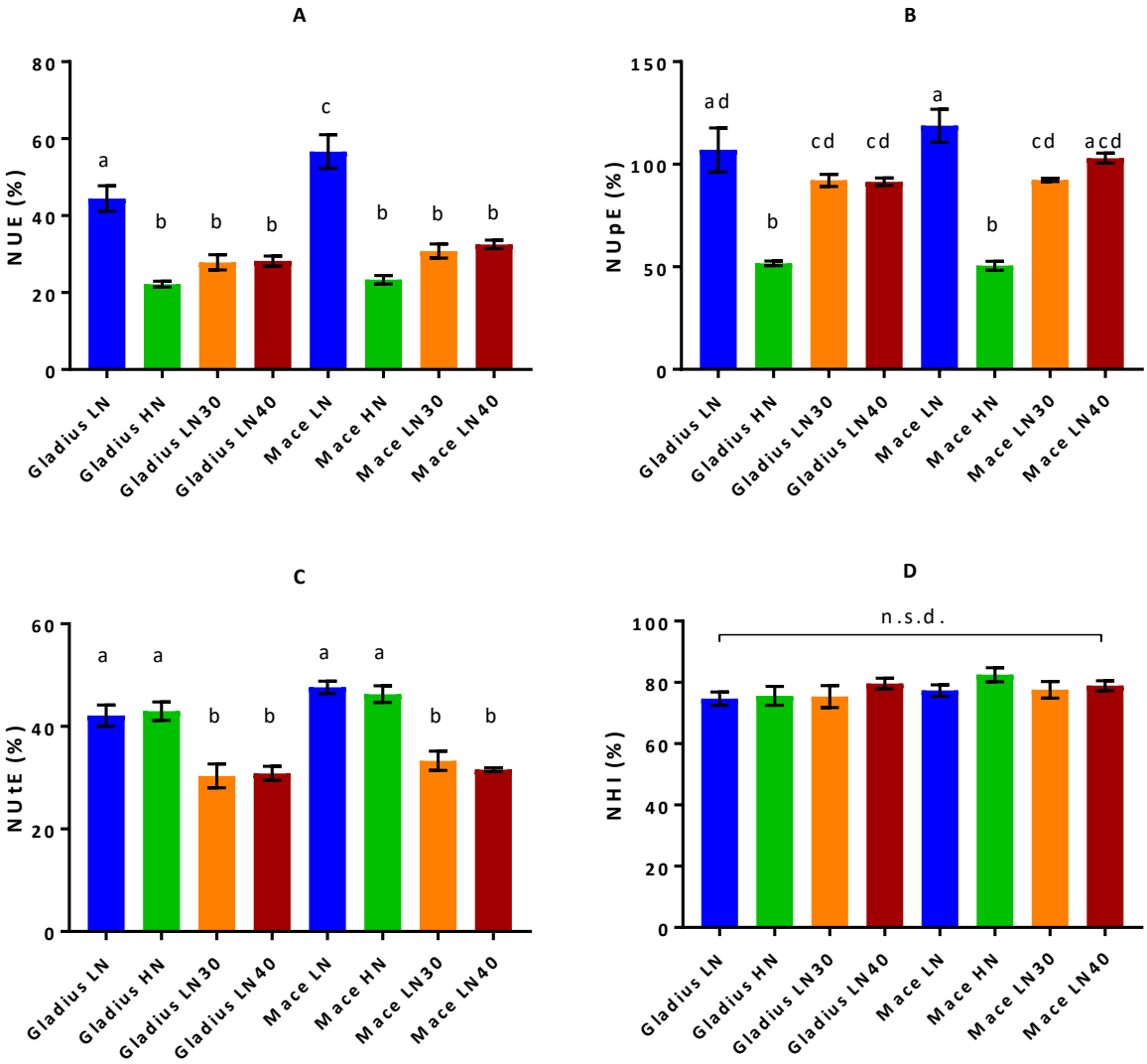

Figure 5 Destructive endpoint analysis: (A) NUE of grain production (%), (B) NUpE (%), (C) NUtE (%) and (D) NHI (%). The four nitrogen treatments were: HN (150 mg N kg soil^{-1}), LN (50 mg N kg soil^{-1}), LN-30 (LN until growth stage 30 (41 DAP), at which ammonium nitrate was added equating to 115 mg N kg soil^{-1}) and LN-40 (LN until growth stage 40 (49 DAP), at which ammonium nitrate was added equating to 115 mg N kg soil^{-1}). Different letters above columns represent significantly different results ($P < 0.05$). The error bars are SEM, n=5.

In order to understand the N dynamics of wheat, non-destructive measurements were conducted to assess the leaf N concentration (g N m^{-2}) of the leaves over time. The leaf N concentration in each of the leaves of the main tiller, labelled in order of emergence, is shown under each of the four N treatments (Fig. 6). Each leaf was tracked between 28-68 DAP, and as long as they were green. The leaves became unviable in the same order as they emerged, when they became dry through senescence, once the leaves were dry spectra could not be collected using the protocol presented here. Dry tissue is able to have spectra collected, but requires the excision and grinding of the leaf into a fine powder (Ecarnot et al. 2013), not practical in this research.

The use of non-destructive observation of leaf N concentration was utilised to differentiate NUE phenotypes i.e., differences in N uptake and allocation. The leaf N concentrations under steady state N treatments had their highest N contents in younger leaves with a gradual decrease in concentration from 57 DAP. There was a decrease in leaf N concentration at 37 DAP for all treatment combinations followed by an increase at day 39, and then a gradual decrease in leaf N. Both Mace and Gladius under LN and HN treatments, followed a similar pattern. The Mace HN treatment showed a higher leaf N concentration in younger leaves (leaf 4) compared to leaf 4 under the LN treatment.

The N addition under LN-30 and LN-40 was observed in leaf N concentration shortly after addition. For Gladius, the addition of N under LN-30 corresponded with the emergence of leaf 5 on the main tiller, and resulted in that specific leaf N concentration being 25 % higher than under the HN treatment (Fig. 6). Contrastingly, Mace did not have a leaf 5 under LN-30, and the leaf 4 did not mirror the increase in leaf N concentration seen under Gladius LN-30. Both Gladius and Mace had a leaf 5 under LN-40, and the N concentration increased post N application, until a decrease at 66 DAP. This is in contrast to the leaf N concentration under LN and HN which reached its maximum at 54 DAP. The leaves under

LN-30 and LN-40 had higher leaf N concentration for a longer period of time, compared to the HN and LN treatments. The addition of N resulted in a more stable leaf N concentration, rather than the peak and decrease that was observed under the steady state N treatments. In terms of number of leaves, Gladius always had five leaves on the main tiller, whereas Mace alternated between four and five.

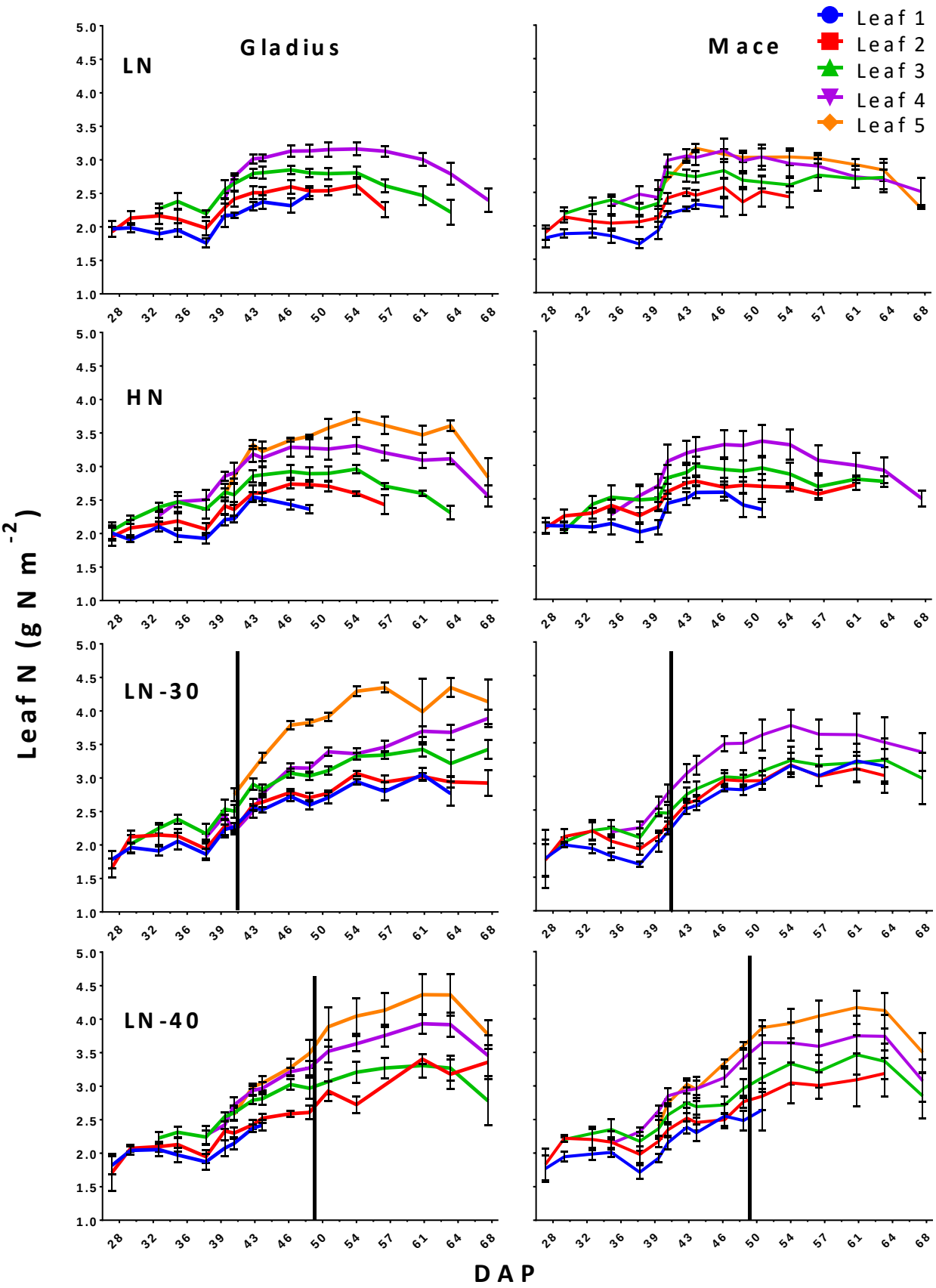
Gladius
Mace
LN
HN
LN-30
LN-40
Leaf 1
Leaf 2
Leaf 3
Leaf 4
Leaf 5
Leaf N (g N m-2)
DAP
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
28
32
36
39
43
46
50
54
57
61
64
68

Figure 6 Leaf N concentration (g N m^{-2}) of Gladius and Mace obtained via hyperspectral reflectance on single leaves. Leaves are numbered in order of their emergence on the main tiller, and were measured from 28-67 DAP under four N treatments. The four nitrogen treatments were: HN (150 mg N kg $soil^{-1}$), LN (50 mg N kg $soil^{-1}$), LN-30 (LN until growth stage 30 (41 DAP), at which ammonium nitrate was added equating to 115 mg N kg $soil^{-1}$) and LN-40 (LN until growth stage 40 (49 DAP), at which ammonium nitrate was added equating to 115 mg N kg $soil^{-1}$). The addition of N is represented by the solid vertical line. The error bars are SEM. Each point represents 3-5 replicates.

DISCUSSION

In this study, high resolution growth analysis, hyperspectral leaf N measurement and destructive end-point harvests were able to differentiate the N responses of two wheat cultivars subjected to a split application of N. In a novel protocol, two key components of NUE were addressed; the shoot area and leaf N concentration over time. The non-destructive phenotyping of these two components greatly expands the amount of data available regarding NUE in wheat, and differences between these two cultivars were observed over time. Absolute growth rate responses to added N were precisely measured and differed depending on the cultivar and time of application. Achieving this level of detail via destructive harvest in the field would require many biomass cuts and there would not be the same control over soil N content and environmental interactions (Garnett et al. 2015). Additionally, the HTP system addresses one of the main barriers to advancing NUE performance, which is GxExM (Gastal et al. 2015). Although the HTP system is within what is categorised as a 'semi-controlled environment' (natural sunlight), the conditions are closely monitored by an array of sensors, enabling accounting of conditions from year to year (Tardieu et al. 2017). This level of environmental monitoring is far from commonplace or comprehensive in the field. The HTP platform combined with hyperspectral reflectance

estimations of leaf N concentration represent a contribution to the quantification of NUE phenotypes in wheat (Gastal et al. 2015).

Split N application is a common strategy used by cereal producers in order to increase the protein content of grain (Blandino et al. 2015; Fischer 1993; López-Bellido et al. 2005), as was observed here. As well as increased grain protein, the plants under split N application also had a full yield recovery compared to those under HN, as well as equivalent NUpE and total aboveground N content, from a total of 30 % less N applied. Under split N application, the NUtE decreased however, attributable to the definition of NUtE by Good et al. (2004), which only takes grain weight, but not grain N content, into account.

The question of which NUE components account for NUE performance under different N levels remains unresolved (Liao et al. 2004). Contrary opinions exist in the literature: that N uptake is controlled by plant growth (Pang et al. 2014), or that N availability is the main determinant of N uptake (Rao and Rains 1976). In this work, NUpE differed between HN and LN, whilst NUtE did not. Furthermore, under LN and HN the leaf N concentrations appeared to be equivalent. The growth recovery and subsequent destructive harvest under split application of N would suggest that N availability is more responsible for N uptake and growth. N uptake was influenced by the time of application, as the plants under split N application treatments had higher total uptake than HN. As well as higher N uptake, leaf N concentration results appeared to show more effective utilisation of N under split application treatments, with higher leaf N concentration of upper leaves over a longer period of time compared to those plants under HN.

The ability of this protocol to analyse growth rate differences and recovery revealed a relevant NUE phenotype, ‘plasticity’, that is the ability to respond to abiotic changes (in this case N addition) by increasing growth (DeWitt et al. 1998). This phenotype is relevant to NUE as the maintenance of viable leaf area in response to an improvement in abiotic

conditions increases the photosynthetic production per unit N, increasing NUtE (Cormier et al. 2016). Previous work has shown that at stem elongation N allocation has prioritised leaf area to boost photosynthetic production (Palta and Fillery 1995). The cultivars did not differ in this regard, both extended the period of time before AGR reached zero (kpixels day^{-1}), which possibly indicated an ability to delay a change in the fresh weight:dry weight ratio, although this was not determined in this research. Kichey et al. (2007) found that late applications of N did not result in increased leaf area, but rather reduced the rate of senescence and prioritised late N uptake resources to the grain. This was supported by the length of leaf viability in the hyperspectral results and the increases in grain protein under the split N applications.

Additionally, the leaf N concentrations were observed to increased markedly at approximately day 38. This is hypothesised to be due to a change in growth behaviour and possibly a switch from vegetative to reproductive stages. The increase in leaf N corresponds with the highest AGR observations regardless of N application. This may suggest that at the conclusion of the highest growth period the leaf N concentration increases, possible as N uptake catches up with vegetative growth.

The results presented here suggest that split application of N is advantageous, especially in Mediterranean climates, supporting field work indicating it is a more efficient use of N resources (Papakosta and Gagianas 1991), whilst avoiding the dangers of 'haying off' associated with high initial N applied at sowing. A large application of N at sowing may be detrimental to production as plants overinvest in biomass production, which, if faced with a hot-dry finish, may be unsustainable, resulting in the incomplete filling of grains (Van Herwaarden et al. 1998). Split application gives producers flexibility, as the plants shown here responded rapidly in terms of growth and leaf N concentration, the producer can adapt to weather predictions and soil water contents to inform N application rates (Ahrens et al.

2010). Less N could be applied if the end of season was predicted to be hot and dry in order to avoid yield penalty.

The results presented here demonstrate that this protocol allows for the simultaneous phenotyping of hundreds of individual plants and therefore could be combined with forward genetic studies, such as genome wide association studies (GWAS) (Cormier et al. 2014) or with genetic approaches, laid out in more detail in Garnett et al. (2015) and Han et al. (2015). Alternatively the protocol would be well suited to complement a breeding program as it would be able to illuminate differences between cultivars of interest and measure genetic gain, in a way that is not yet possible in the field.

CONCLUSIONS

The combination of phenotyping techniques utilised in this research was effective at linking two essential components of NUE, growth and leaf N concentration. Even with only two cultivars, there was significant variation in response to split N application, given context by the destructive harvest derived yield and NUE measures. The improvement of NUE will only be addressed via the increased capacity of phenotyping to quantify relevant traits (Cooper et al. 2014), and the measurement of biochemical and physiological leaf level traits are required for a deeper understanding (Yendrek et al. 2017). There was a rapid growth response to N additions of the cultivars as well as the partitioning of N into the leaves. This protocol was successful at differentiating N response phenotypes in a novel way. Even amongst the two cultivars utilised in this study, there were phenotypic differences, suggesting there is potential for the use of these protocols for forward genetic studies.

SUPPLEMENTARY MATERIAL

Fig. S3 The hyperspectral reflectance leaf clip attached to the FieldSpec 3. The leaf mask is seen on the left and the leaf clip with leaf mask collecting a spectrum from a wheat leaf on the right. This leaf clip was one of the changes to the hyperspectral reflectance protocol utilised in Chapter 3 and 4 which reduced the aperture of leaf clip, reducing background interference.

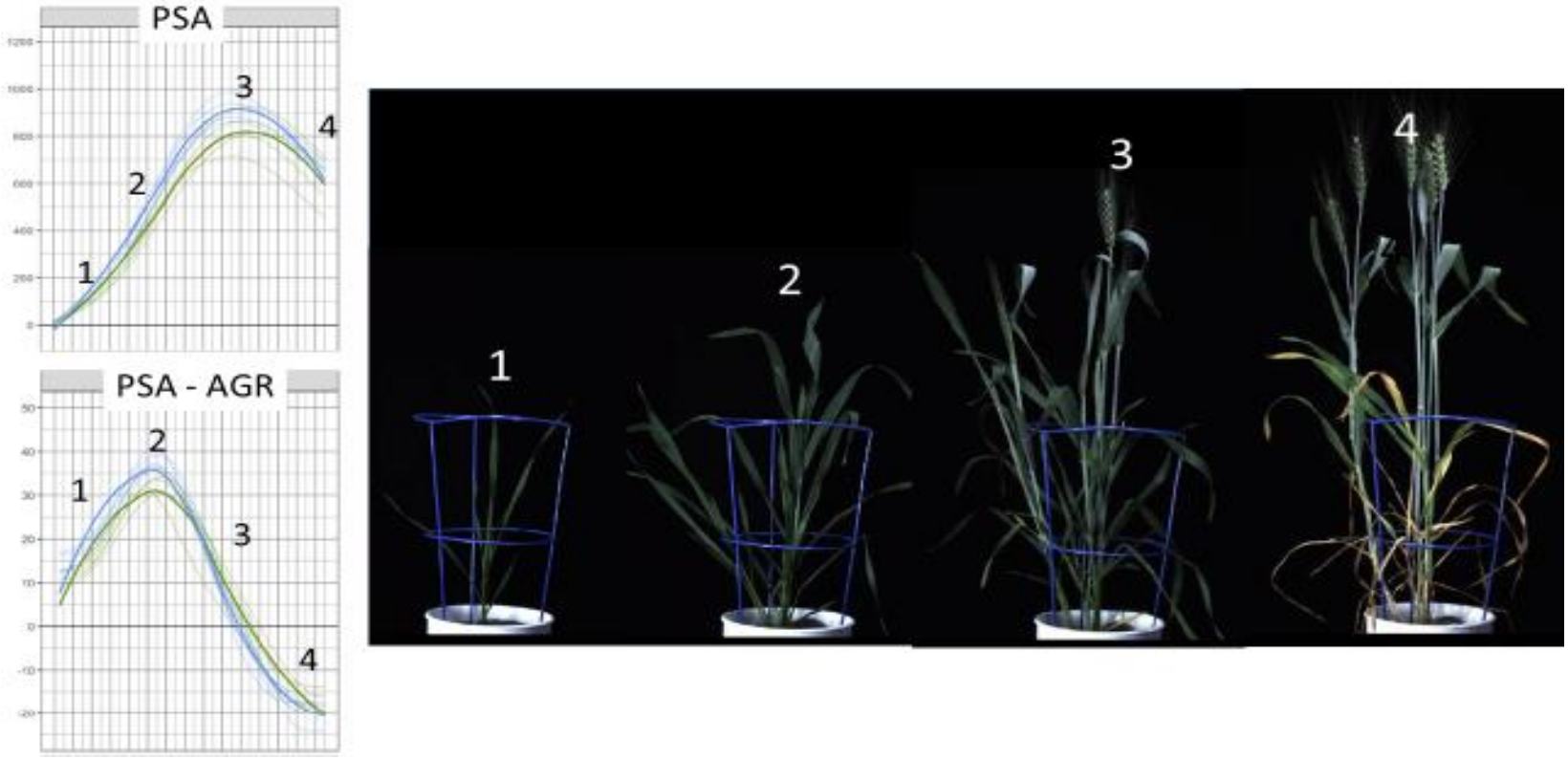

Fig. S4 The corresponding time points between the PSA and AGR curves and the images of the plants. This figure highlights the physiology of the plants when they have "apparent negative growth" (at time point '4'). As is illustrated by the images, the plants are senescing and the lower leaves are becoming shrivelled, which does not indicate a decrease in biomass but rather a shift in the fresh weight: dry weight ratio of the plants as some lower leaves senescence and resources are relocated to the grain ears.

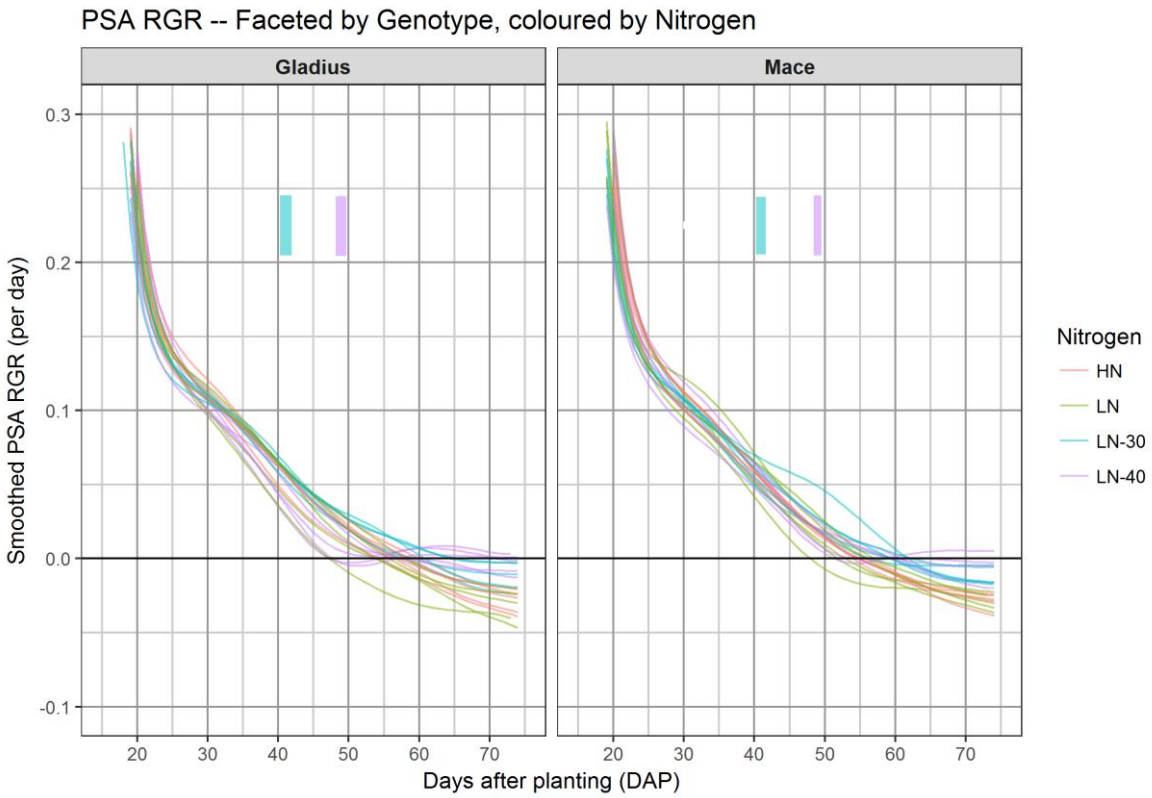

Fig. S5 The smoothed projected shoot area derived relative growth curves of Gladius and Mace cultivars with five replicates (thin curves). The four curves represent the four nitrogen treatments: HN (red; 150 mg N kg soil^{-1}), LN (green; 50 mg N kg soil^{-1}), LN-30 (blue; LN until growth stage 30 (41 DAP), at which ammonium nitrate was added, equating to 115 mg N kg soil^{-1}) and LN-40 (green; LN until growth stage 40 (49 DAP), at which ammonium nitrate was added equating to 115 mg N kg soil^{-1}). The vertical lines represent the point of ammonium nitrate addition for LN-30 and LN-40 treatments (blue and purple respectively), n=5.

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Chapter 6: General discussion

The imperative to improve NUE has increased because of the excessive loading of N into the environment, the flattening yields in developed countries and the desire to reduce input costs. The purpose of this research was to utilise novel non-destructive phenotyping technologies, to quantify NUE and N dynamics within bread wheats. Determinations of NUE via destructive harvests have been carried out for decades (Moll et al. 1982). However this knowledge has not been leveraged into improved NUE germplasm. This may be due to the complexity of the trait and the inability to accurately observe NUE phenotypes, and thus select for, improved NUE. The development of technologies which greatly increase the data collected from plants throughout their lifecycle, rather than just at destructive harvest, provides the opportunity to understand plant behaviour in response to N.

Advances in knowledge from this research

Non-destructive N responsive growth phenotyping

The first component of this research (Chapter 2) was the quantification of N responses in wheat via high throughput phenotyping. Even from a small selection of commercially available cultivars, different N response phenotypes were observed clearly. Image derived shoot area curves showed cultivar difference in the ability to increase shoot area in response to increasing N availability. This in itself greatly improves on knowledge gained via destructive harvest. The automation of the high throughput phenotyping platform enabled the simulation of water availability scenarios that are commonly found in South Eastern Australia. The correlation between projected shoot area (PSA) at 52 DAP correlated closely with final yield, indicating the predictive power of this phenotyping. When the yield and PSA correlations were separated into water treatments the relationship became stronger. The link between yield and water availability treatments and their impact on growth could be observed in growth rate changes over the course of the experiments. Further cultivar differences were identified including cultivars susceptible to ‘haying-off’, the yield penalty

incurred as a result of the over investment in shoot biomass followed by water scarcity, a common issue in Mediterranean climates.

The level of control over the environmental conditions in the study presented (Chapter 2) addressed one of the main hurdles of improving NUE, the GxExM interaction. Conducting a similar experiment in the field would be a huge logistical challenge, with no guarantee of the desired conditions. In order to observe similar water availability treatments in the field, many field trials would need to be planned in different areas to provide the best chance that the desired climactic conditions would eventuate. After which, obtaining growth data would require HTP in the field over many days or destructive biomass cuts. It would be possible to achieve the same resolution as that presented in Chapter 2, however the effort and expense involved would increase by many magnitudes. The protocol presented in Chapter 2 could be utilised with forward genetics approaches, including genome wide association studies and bi-parental mapping populations of divergent parents, to make progress in improving NUE. This protocol is the ideal method with which to undertake these approaches as we have demonstrated consistency over two years within a defined environment. Whole populations can be phenotyped with the high throughput phenotyping protocol outlined in Chapter 2, as opposed to undertaking field trials which have limited control over conditions and have risks regarding weather.

Non-destructive leaf N measurement

The measurement of growth alone does not provide any information on the content of N within plants, a problem when ascribing differences in N uptake and utilisation. The second part of this research developed a protocol to measure the N content of wheat leaf tissue via non-destructive hyperspectral reflectance (Chapter 3). The method is a partial least squares derived method combining a calibration set of destructive harvests with chemometric analysis. This method was more accurate than chlorophyll based indices, and to its further

advantage, models developed can be consistently improved via the addition of destructive harvests to the calibration set. Knowledge regarding the changes in shoot tissue N composition may lead to the identification of desirable N uptake or utilisation phenotypes. As leaf tissue is one of the main reservoirs of N within a plant, its dynamics are key to building a working model of uptake and utilisation. These may help identify genetic differences in leaf N concentration, speed of uptake, stay green phenotypes and differences in the length of viability of leaves, maximising photosynthetic productivity. This protocol was an improvement on limited reflectance spectra methods (i.e., SPAD-502), as it could provide absolute values of leaf N % and could compare these under high and low N fertilisation, to observe whether any cultivars are able to maintain high leaf N % under low N availability.

The ability to measure leaf N % was deemed promising and so the protocol was tested in a hydroponic experiment to observe the leaf N % dynamics within bread wheats under alternating N availability (Chapter 4). The research succeeded in observing leaf N % changes in response to increasing or decreasing N availability. There were also differences in leaf N between the leaves depending on their order of emergence, with younger leaves having higher leaf N %. The experiment did not show cultivar differences but this may have been due to the use of only two cultivars. The method showed promise for differentiating N uptake phenotypes, as well as partitioning differences, as it would be able to measure every leaf of the plant to follow total N uptake.

Combination of growth and leaf N measurements for a fuller picture of NUE

The final experimental chapter (Chapter 5) leveraged the knowledge gained in the first four chapters to combine HTP growth analysis, non-destructive leaf N measurement with split application of N to quantify NUE and N dynamics over time. The protocol was sensitive enough to measure growth responses to added N within a few days of N application.

Differences in growth responses to N were clearly observed and differed between cultivars and time of application. The results demonstrated that the N applied during vegetative and stem elongation growth stages was observed in leaf N concentration, but was also prioritised to the grain. Grain protein in plants with split-application treatment increased by approximately 30 % compared to plants under high N fertilisation at sowing. The leaf N measurements showed that leaf N was prioritised to the younger leaves after the split application, supporting the results of leaf N measurements in Chapter 4. High resolution growth analysis showed that the cultivars differed in their growth rate responses to the addition of N, phenotypes that have not been described before. This is important as the improvement of NUE may rely on the dissection and selection of phenotypes which have not yet been characterised because of technological limitations. The analysis of new germplasm can be undertaken using the protocol presented in Chapter 5, incorporating N uptake and N responsive phenotypes.

Future directions

The classification of germplasm within the field environment will remain the goal of NUE evaluation. However, due to the difficulties of field trials previously explained, high throughput phenotyping in semi-controlled environments will remain important in dissecting NUE into its component traits. Both the growth phenotyping and the hyperspectral reflectance will be further developed in the field and the protocols presented here may be adapted to field conditions. However, complementary to field phenotyping, the methods presented here will aid in the selection of desirable phenotypes, by undertaking experiments that are difficult in the field such as the growth responses to interactions between N and water and split N applications.

The information contained within the growth response of plants to abiotic stress conditions is only beginning to be explored (Al-Tamimi et al. 2016; Araus et al. 2018; Veley

et al. 2017). Using the growth analysis presented here in a forward genetics approach now offers the possibility to identify the underlying genetics of N specific growth responses. The observations presented demonstrate a baseline against which newly developed varieties of wheat can be compared and hopefully improved upon.

The use of hyperspectral reflectance has been demonstrated as effective in complementing growth analysis in this research. Its use in future experimentation could be expanded from the leaf to the whole plant using hyperspectral imaging within HTP platforms. Eventually a three dimensional N distribution model will become feasible utilising HTP and hyperspectral imaging, to measure the N content of each pixel of the plant captured as well as its overall biomass. This will illuminate genetic differences more clearly especially in response to water and N interactions. Hyperspectral imaging would be very useful in determining leaf N concentration responses to split N applications in the field. Split applications are commonly utilised and have been shown to increase productivity per unit N applied, and as such hyperspectral reflectance could greatly aid in identifying germplasm with increased N uptake and utilisation to match genotype with management strategies.

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