Analysing the shoot and root response of wheat in a soil environment under variable water and nitrogen supply

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Abstract

Increasing water variability and the cost and sustainability of nitrogen (N) fertiliser use are of growing concern globally. Soil water supply directly and indirectly regulates soil N availability. As a result, plants have to adapt their shoots and roots in order to optimise water and N uptake. This thesis seeks to investigate the interactive effects of water and N supply on important soil properties and the growth and physiology of two Australian wheat varieties, Gladius and Kukri, both possessing phenotypic traits and water/N use efficiencies. This research seeks to explore and discuss how soil moisture affects soil N dynamics, and subsequently how different root traits affect N and water acquisition in a complex soil environment.

Overall, water and N supply affected root plasticity, shoot growth, soil N dynamics and microbial biomass carbon (C). Soil mineral N availability was strongly influenced by soil moisture, with the availability of ammonium and nitrate decreasing with low soil moisture. Changes to soil physiochemical properties were associated with changes in root architecture, C allocation to roots and shoots, and aboveground physiology. Moreover, the differing physiological responses of wheat varieties Kukri and Gladius to variable water and N supply have provided insights into the phenotypic responses that could potentially aid in enhancing water productivity, nutrient use efficiency and yields.

The use of an automated gravimetric watering platform allowed the precise measurement of plant weight in real-time and irrigation of pots to a pre-programmed water level. This allowed three harvests to be conducted over a period of three months, with each harvest representing a different growth stage of wheat. The results highlighted that plants were more responsive to N, with low N negatively affecting plant growth. Additionally, moderate water encouraged plant growth with medium and high N, whereas plants were not well adapted to variable watering (wet/dry cycling).

From this, we wanted to further investigate whether plants were capable of reusing nutrients in previously used soil. This led to 36 pots having undergone the same treatments as those in Experiment 1, left to dry down and the wheat heads were harvested. After three months, the pots were re-watered and re-planted with wheat (the old wheat root systems from the previous harvest remained in the pot). Results from both harvests showed a clear legacy effect, with wet/dry cycling producing biggest plants in the second crop season, with a flush of mineral N.

The idea that frequency and quantity of watering would impact plant growth and soil nutrition differently led to cv. Gladius and Kukri being subjected to three water treatments and two N treatments. Results showed that water had a greater impact on plant growth than N, with frequency of water more detrimental to plant growth. However, plant recovery or adaptability was seen with the wet/dry cycles. Additionally, there was a phenotypic response difference between genotypes.

Further investigation into root architectural response, soil N dynamics and N uptake in response to variable water and N treatments were important to test the hypothesis that under low N and water supply, plant C allocation changed. Under low N and low water, results showed that root properties, such as total root length and root tip number, increased, but root volume decreased. The average ^{15}N uptake in roots was also measured by exposing excised roots to different forms of labelled N, with root uptake preference for nitrate-^{15}N over ammonium-^{15}N or glycine-^{15}N.

The results presented in this thesis highlight trade-offs between wheat shoots and roots in order to maintain growth. These trade-offs include increasing root growth under low N (trade-off: less shoot growth); and producing longer, thinner roots under low water and/or low N (trade-off: fewer roots). By understanding how these trade-offs affect water and N uptake, and ultimately growth efficiency, would help develop more precise water and nutrient application strategies and overall crop management strategies. These improvements would boost crop productivity, especially under abiotic stresses.

Publications from this work

Olivia H. Cousins, Trevor P. Garnett, Amanda Rasmussen, Sacha J. Mooney, Ronald J. Smernik, Chris J. Brien, Timothy R. Cavagnaro (20120) Variable water cycles have a greater impact on plant and soil nitrogen response than constant watering. *Plant Science* 290
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Thesis Structure

This thesis is composed of a mixture of published and submitted papers in 'paper format', but also includes chapters that have not been submitted for publication.

Chapter 1 is a summary of the literature relating to water and nitrogen stress/variability/use efficiency and outlines the objectives of this project.

Chapter 2 details an experiment conducted at the University of Adelaide on the effect of water and nitrogen variability on wheat growth. This has been published in *Plant Science*.

Chapter 3 is an experimental chapter which outlines the impact of growing a second harvest of wheat into a previous pot trial (post-harvest), and how this affects wheat biomass allocation and mineral nitrogen pools. This was also conducted at the University of Adelaide.

Chapter 4 outlines an experiment on the role of water variability on both nitrogen cycling and wheat biomass allocation. It seeks to address whether frequency or quantity has a bigger impact on growth and soil nitrogen transformations. This experiment was also undertaken at the University of Adelaide. This paper has been submitted to Agricultural Water Management.

Chapter 5 is combination of data from two experiments conducted at the University of Nottingham, one of which uses X-ray Computed Tomography to understand the effect of different water and nitrogen treatments on root distribution and growth, and the other which utilises microdialysis and ^{15}N to understand nitrogen depletion zones and nitrogen uptake in wheat grown in rhizoboxes.

Chapter 6 summarises the overall outcomes of the project.

Chapter 1

Review of the literature

1.1. Introduction

The work herein is presented as a collection of journal manuscripts, a published paper and one short chapter, each of which contain a detailed literature review. However, in order to provide context for the project as a whole, a short literature review is presented in Chapter 1.

Increasing frequency of erratic rainfall (form of water stress) and the cost and sustainability of nitrogen (N) fertiliser use are of growing concern. Soil N availability is strongly regulated by soil moisture. Over time, plants have adapted to numerous abiotic stresses, and these beneficial traits have been bred into modern-day crops, for example, shoot and root traits which optimize water and N uptake. This project seeks to understand how different root traits affect N and water acquisition in a complex soil environment. Root traits observed under different abiotic stresses include: deep rooting, shallow rooting (Lynch, 2011), lots of branching, little branching (Gao and Lynch, 2016), thick roots, thin roots (Lynch, 2014).

The emphasis of this thesis is on interactions between wheat genotypes (of two wheat varieties), water and N. By understanding plant growth behaviour in response to water and N uptake and how this affects growth efficiency, it is hoped crop management strategies can be developed to help improve crop productivity during abiotic stresses.

1.1.1. Water

Water is important for all aspects of life, including agriculture. With rainfall patterns across the globe predicted to become more erratic due to climate change (Black, 2016, Loo *et al.*, 2015, Monjo and Martin-Vide, 2016), this poses a major problem to crop husbandry. It is projected that in many regions of the world there will be a shift from more predictable rainfall to less frequent but more intense rainfall events, followed by increasingly long periods of no or very little rainfall (Borken and Matzner, 2009, Coumou and Rahmstorf, 2012).

Plant growth is affected by the amount, seasonality and frequency of water supply (Austin *et al.*, 2004, Gibson-Forty *et al.*, 2016, Izanloo *et al.*, 2008). For example, Gibson-Forty *et al.* (2016) found a reduction in rainfall significantly reduced the biomass of grasslands. Abid *et al.* (2016) also demonstrated that both moderate and severe water stress can significantly reduce plant dry weight, and as a response, plants started anthesis at an earlier stage and reached maturity quicker to avoid reproductive failure (Abid *et al.*, 2016, Chaves *et al.*, 2002). Such changes are expected to have profound impacts on plants and cropping systems.

Additional studies have demonstrated that timing or frequency of watering events is also important for a healthy or high-yielding crop. When water stress occurs at important developmental stages, crop establishment and final productivity can be severely hampered due to the interference with plant physiological processes (Abid *et al.*, 2016). For example, in one study, the total dry weight produced from wheat was only significantly reduced when moderate drought stress was applied at the jointing

stage but not the tillering stage (Abid *et al.*, 2016). In contrast, other studies have shown that grain yields and water use efficiency (the ratio of water used in plant upkeep to water lost by the plant through transpiration) was highest when additional irrigation was applied at the jointing stage, and even the heading stage (Bian *et al.*, 2016). These results suggested higher grain yields could be due to an increase in tillers because of reduced water frequency.

Reducing a simulated rainfall treatment in a grassland system by 50% has been shown to reduce total plant biomass, when compared to a watering frequency of every 8 days (Gibson-Forty *et al.*, 2016). However, reduced watering frequency, i.e. providing the same amount of water once a week (pulsed) as opposed to thrice weekly (regular), increased specific root length and total root length in a grassland system (Padilla *et al.*, 2013). This is possibly due to substantial water stress at the end of a pulsed water cycle, which subsequently improves water use efficiency with the immediate replenishment of water at the end of the pulsed water cycle. This pulse of water encourages root growth for accessing the available soil water and newly released N (and other nutrients; Ješko *et al.*, 1997, Padilla *et al.*, 2013).

The ability of plants to adapt their phenotype in response to changes in water and/or N availability highlights the importance and complexity of plant plasticity. Although the ultimate aim may be to maximise crop yields under variable water conditions, sometimes a more practical goal may be to achieve stable crop yields under a variety of conditions. Under water stress, increasing a crop's water use efficiency will help to maintain efficiency and maximise productivity per unit of water, thus stabilising crop yields (Geerts and Raes, 2009). A plant's potential to recover and adapt upon re-watering after water stress is known as drought recovery (Fang and Xiong, 2015). If we can understand the mechanisms behind increasing water use efficiency in response to variable water availability, then it may be possible to tailor management techniques to different crop systems under different stresses or at the very least, model and predict future crop yields under different rainfall scenarios.

1.1.2. Nitrogen

Although variability in both quantity and frequency of irrigation or rainfall impacts crop physiology and water use efficiency, it also plays a major role in soil N turnover and availability (Cui and Caldwell, 1997, Fierer and Schimel, 2002, Hoogmoed *et al.*, 2016).

Soil water and stress have a negative impact on crops every year worldwide. Most previous research has focused on measuring water or crop N efficiency separately (Foulkes *et al.*, 2009, Manschadi *et al.*, 2006, York *et al.*, 2015), while relatively few studies have investigated the trade-offs between water and N uptake in crops. The N cycle is heavily dependent on water availability, since certain microbes that carry out the conversion or decomposition of N forms (Figure 1.1) require either aerobic or anaerobic conditions.

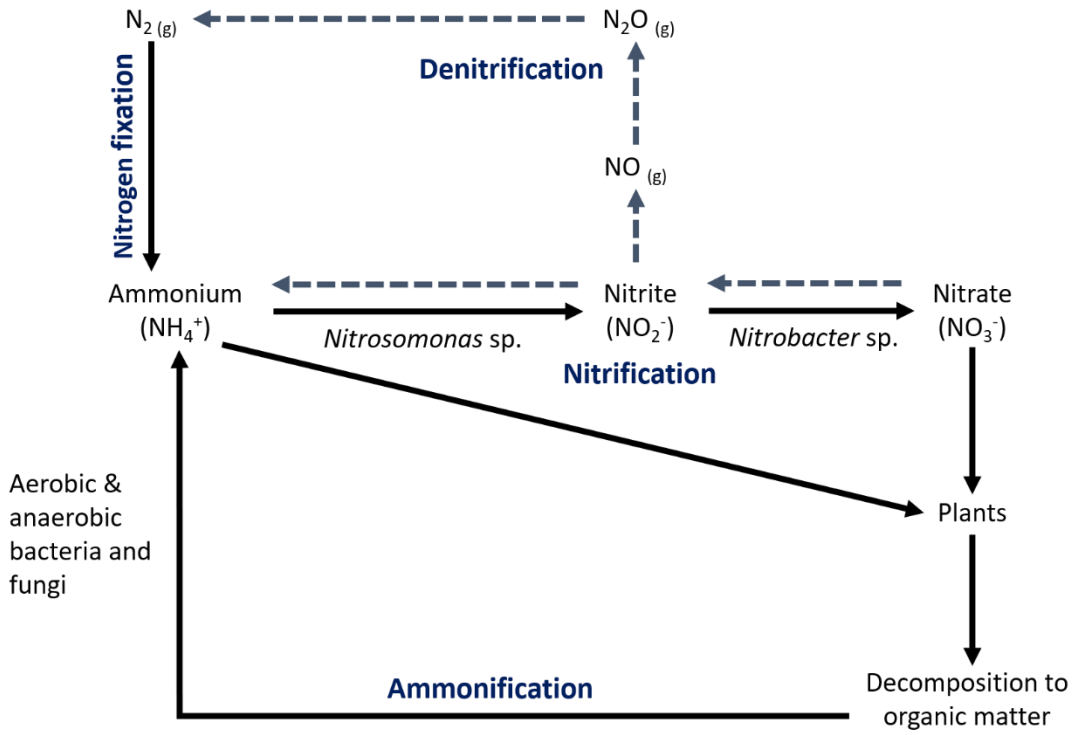

Figure 3.1 Nitrogen cycle showing sequence of events for N conversion processes, leading to N forms that are available to plants in relation to plant N uptake; examples of important microorganisms are in italics, solid arrows represent aerobic conditions and dotted arrows represent anaerobic conditions (Galloway *et al.*, 2008, Gruber and Galloway, 2008). When soil is saturated, microbes that respire anaerobically become more dominant, due to lack of O_2. When soil becomes dry, aerobically respiring microbes are dominant.

Nitrogen assimilation in plants is essential for healthy plant growth. Despite $N_{(g)}$ comprising approximately 78% of the atmosphere, plants rely on lightning and microbes to help convert $N_{2(g)}$ into NH_4^+ or NO_3^-. Therefore, N is often a limiting nutrient for plant growth. Both the amount, form and behaviour of soil N are strongly affected by soil water supply (Burger *et al.*, 2005). Plant N uptake relies on water to solubilise N species, particularly NH_4^+ and NO_3^-, and move them to root surfaces for absorption (Davidson *et al.*, 1987). In basic terms, N uptake is dependent on three factors: a) the plant's N requirements; b) root length distribution; c) the concentrations of NH_4^+ -N and NO_3^- -N in the rooting zone (Davidson *et al.*, 1987). As a result, modern farming typically utilises high quantities of N fertiliser to maximise yield and encourage faster growth, by ensuring the plant gets its optimum N requirements and increasing chances of mineralisation of plant-available N forms.

Due to the high mobility of dissolved N species, particularly NO_3^-, there is a high risk of run-off into surrounding water or leaching through the soil into the water table. Also, denitrification can lead to production of the undesirable greenhouse gas nitrous oxide (N_2O), which has a long atmospheric lifetime and destroys stratospheric ozone. Production of N fertilisers is highly energy intensive and environmentally costly, which leads to unsustainable agricultural practices. Therefore, fertiliser use

efficiency is important to consider when improving environmental quality (Letey *et al.*, 1982). There is potential to produce plants with higher N uptake efficiency, diminishing the need for high rates of fertilisation, subsequently reducing NO_3^- pollution into the environment. This could be done through novel breeding methods where crops are better at taking up naturally low levels of N. Better crop management strategies could also be implemented, such as using fewer or lower applications of N or changing the timing of N applications. This could help ensure plants take up N before it can leach, thus minimising waste (Le Gouis *et al.*, 2000). Better use or uptake of N would aid in N use efficiency (the ratio of N given to a plant compared to the amount of N used by the plant).

One of the challenges of studying N in soil and its uptake by plants, is its highly dynamic and mobile nature. This makes it harder to get an accurate measurement of N in a soil through time, most techniques only give a snapshot of N at a specific time point. To help overcome this, several experimental approaches and techniques have been developed. I will now introduce two of these, namely, microdialysis and stable N isotopes, that were used in this project.

1.1.2.1. Microdialysis

Microdialysis involves the use of needle-size probes, placed adjacent to specific roots within a soil column or rhizobox (Figure 1.2). The probes contain a semi-permeable membrane that allows passage of ions with the passing of water, therefore, it is possible identify which ions are present in the soil without affecting soil water content. A probe has two piping tubes attached to it, one is attached to a syringe pump which delivers deionised water to the point of soil contact. The second piping tube connects the probe to an Eppendorf tube to collect the solution with nutrients from the point of soil contact. The creation of a concentration gradient enables the deionised water to cross the probe's semi-permeable membrane into the soil, and then pick up ions from the soil. From this, it is possible to simulate what plant roots could absorb, in theory (Brackin *et al.*, 2017).

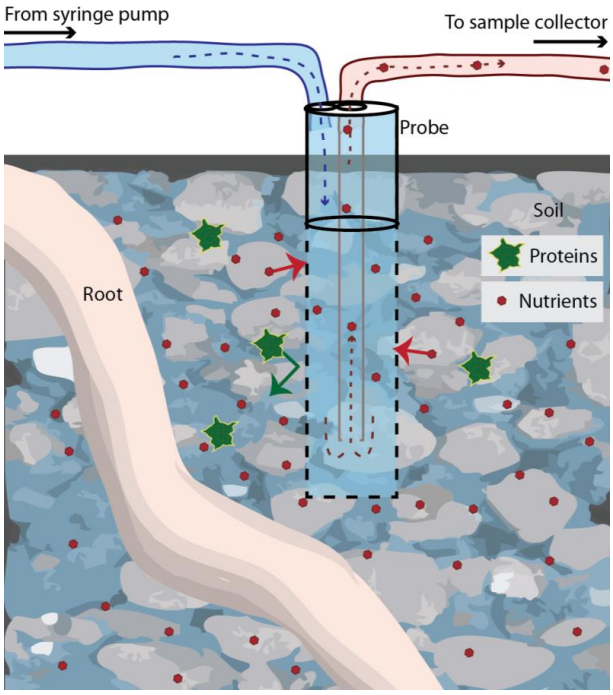

Figure 1.2. Microdialysis for analysing nutrient environment within a soil system. Location of probe can be used to identify nutrient hotspots and depletion zones in relation to probe and root proximity (Brackin *et al.*, 2017).

1.1.2.2. Stable N isotopes

One useful way to quantify N uptake by roots and soil N fluxes is with ^{15}N stable isotopes. There are two stable isotopes of N: ^{14}N, which is much more naturally abundant and ^{15}N, which is rare, making it a good tracer. Using mass spectrometry, samples can be analysed for percentage N, percentage C, $^{14}N/^{15}N$, $^{12}C/^{13}C$. For the purpose of this research, values for percentage N and $^{14}N/^{15}N$ were used to calculate the amount of ^{15}N taken up by the root samples (Godwin laboratory, University of Cambridge, UK). Studies have used stable isotopes of ^{15}N to compare root uptake of different N forms under optimal and stress conditions (limited water and/or N), because it allows N to be tracked through either a soil or plant system through time, or at specific time point (Brackin *et al.*, 2015, Buljovcic and Engels, 2001, Seligman *et al.*, 1986, Wuest and Cassman, 1992a).

1.1.3. Interactions of water and N

Studies on combined water stress and re-watering regimes are not common, even less so when combined with N stress (Izanloo *et al.*, 2008). Not only do water and N variability result in crop physiological changes within shoots, they can also change carbon (C) allocation with roots, thus changing root architecture. Root architecture is important for nutrient and water uptake (Atkinson *et al.*, 2014, Atkinson *et al.*, 2015, Hochholdinger and Zimmermann, 2008). Root system architecture can vary between plant genotypes, and phenotypes can vary within a genotype (a phenomenon known as plasticity), and these changes in phenotypes can positively affect the efficiency of water and nutrient uptake (Manschadi *et al.*, 2006). Identifying different root system architecture under different combinations of stress will help with finding solutions for improving root system architecture. By

manipulating root system architecture, absorption and uptake in roots may be increased, creating plants better adapted to their changing environment. Figure 1.3 highlights the differences in root system architecture in four crop species. Roots can be separated into two classes, those that form from the embryo (found in dicots) and those that form from existing roots or non-root tissues (found in monocots) (Atkinson et al., 2014). Root systems of dicots, such as *Arabidopsis* (Figure 1.3A) and tomato (Figure 1.3C) are primarily made up of a primary root or taproot, with first order lateral roots forming off the primary root, then second and third order lateral roots forming successively. On the other hand, monocots, such as maize (B) and wheat (D), produce seminals then first order lateral roots. In addition, monocots produce crown and/or brace roots (e.g. wheat and maize respectively), with crown roots forming belowground and brace roots aboveground. Studies have shown crown and brace roots to aid in water and nutrient uptake especially under water or nutrient stress (Atkinson *et al.*, 2014, Mattsson *et al.*, 1993, Sebastian *et al.*, 2016).

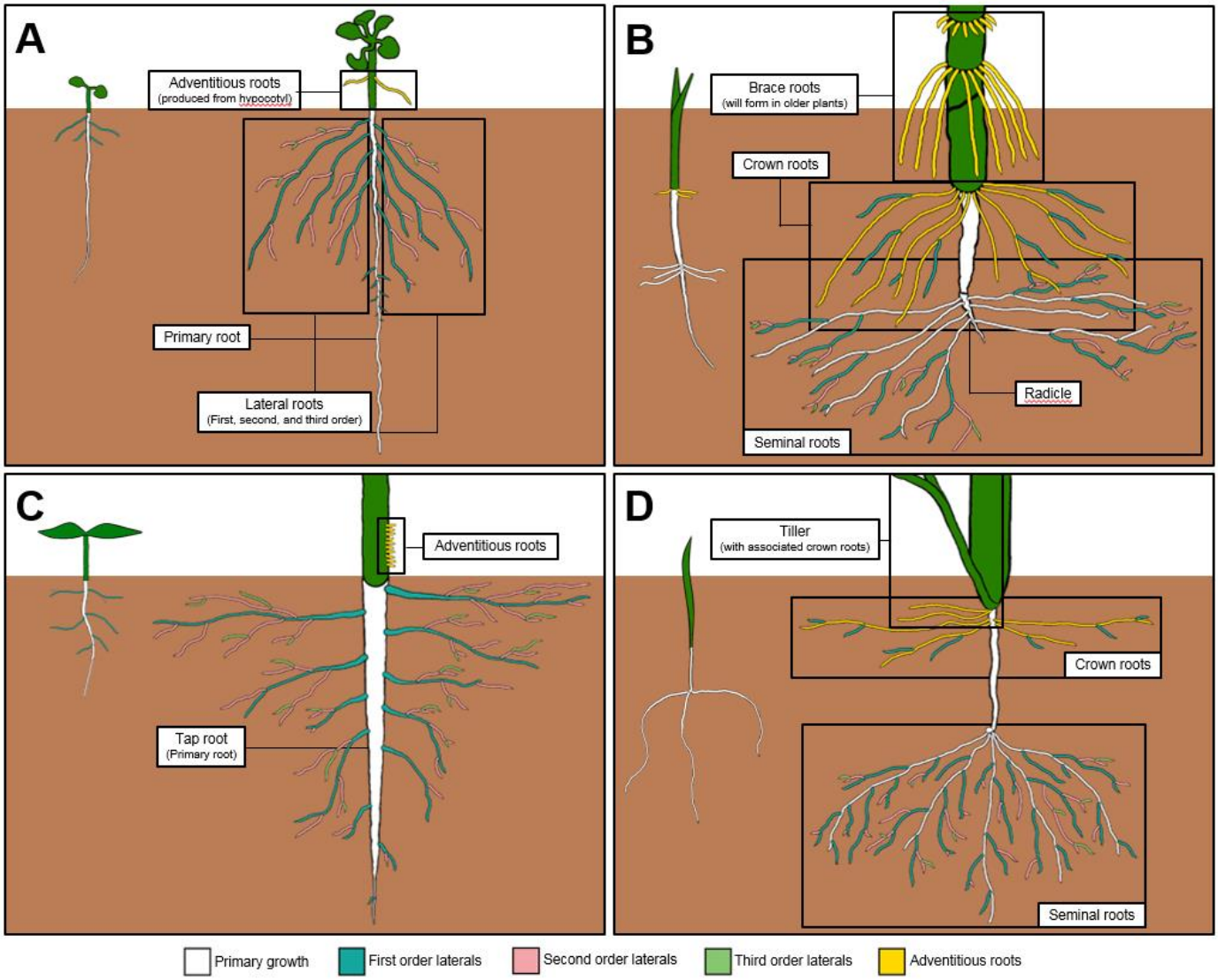

Figure 1.4. Seedling and mature growth stages of root system architecture in four crop species, distinguishing between dicots A) *Arabidopsis* C) tomato, and monocots B) maize, D) wheat (Atkinson *et al.*, 2014, Foulkes *et al.*, 2009).

1.1.3.1. Root traits: nitrogen uptake efficiency

Studies show that inadequate N supply reduces plant growth, but it can also change biomass allocation. Lower soil N availability has been shown to encourage more root biomass in wheat (López-Bellido *et al.*, 2005). On the other hand, too much soil N can be detrimental to growth, resulting in a decrease in root length in wheat (Comfort *et al.*, 1988). The root architecture subsequently is impacted by N variability. During a crop's lifespan, its uptake rate can differ depending on growth stage. Mattsson *et al.* (1993) showed that NO_3^--N uptake decreased from the vegetative to reproductive growth stages in barley root systems, but the uptake rates remained constant within the nodal root system of barley (Mattsson *et al.*, 1993, Pierret *et al.*, 2007). Lazof *et al.* (1992) also concluded that in comparison to uptake in the nodal root, uptake from the apex of the primary root tip was low. This is because fewer

cells within the primary root apex have a high accumulation capacity for NO_3^--N ions. Also, efficiency of nutrient uptake pathways decreases with age, slowing or disrupting ion uptake (Pierret *et al.*, 2007).

Regions within the roots have differences in nutrient uptake and assimilation. In maize plants, NO_3^--N accumulation is most prominent in the lateral roots (roots that develop from either the primary root or seminals), with high translocation through the xylem to the shoots (Lazof *et al.*, 1992). As the lateral roots comprise approximately 70% of total root surface area, the rate of translocation is high. Studies have shown when barley roots encounter a NO_3^--N hotspot, there is increased lateral root formation within that nutrient hotspot, as the plant does not waste extra energy to find nutrients further afield (Drew, 1975, Pierret *et al.*, 2007). An even NO_3^--N distribution results in a higher number of roots throughout the whole soil profile; roots have to branch out further and deeper in order to access available nutrients (Pierret *et al.*, 2007).

1.1.3.2. Root traits: water uptake efficiency

Plants have varying tolerance to drought stress. Obvious signs of drought susceptibility in crops are wilting and reduced crop yields. In wheat in particular, when the seminal roots (roots that develop from the radicle) are heavily affected by drought, it causes extreme reduction in leaf expansion, reducing leaf size (Pierret *et al.*, 2007).

Several root traits have been identified as being desirable for improving drought resilience. In crops such as wheat, rooting depth, root elongation rate, root angle and distribution all help with access and uptake of water. Studies have shown that crops with larger root system develop a better drought tolerance, resulting in a higher grain yield. Early development of extensive roots can help the plant to store water and nutrients for times of stress, even if certain crops are not naturally highly tolerant to water stress (Hurd, 1974). Root traits vary across crops, for example, under drought conditions a deep and thick rooting system has shown to improve wheat and rice performance and yields (Liu *et al.*, 2013, Price and Courtois, 1999). Increasing the extent of the root system increases the surface area, also allowing deeper access to subsoil soil moisture, therefore determining the water and nutrient uptake rate per unit of root length (O'Toole and Bland, 1987).

How roots distribute themselves greatly depends on the soil environment and (micro)climate. Under water stress, having a fast root elongation rate may improve the plant's ability to assimilate any available water more quickly. Root angle can also influence water uptake during drought. Wheat genotypes with a wider angle in their roots have longer seminal roots than wheat genotypes with narrow angled roots, especially in the top 10 cm of the soil profile. Increased lateral branching of roots increases root surface area, and so a higher uptake of water is possible (O'Brien, 1979). Navara (1987) showed that seminal roots were most important in water uptake in maize, particularly during important periods of the crop's lifespan, such as grain filling and shoot development (Navara, 1987). Wheat tends to produce finer roots per unit of soil when under water and nutrient stress. However, the subsequent production of grain is

adversely affected (Mankse and Vlek, 2002). Studies by Manschadi *et al.* (2006) appear to show that root architecture and the vertical pattern of root development are very important in helping crops, such as wheat and barley, to adapt to environments exhibiting water stress. However, there are contradicting results with regards to root traits most beneficial for abiotic stress tolerance, highlighting the importance of understanding the local conditions to select the most advantageous root system architecture.

In addition, little research has been carried out on the effects of combination stresses, or how microbe interactions impact water and nutrient uptake efficiency. Therefore, understanding how crops behave under a combination of stress conditions can help to identify physiological responses and genes responsible for drought or nutrient stress tolerance or susceptibility. Such information will be important for efforts seeking to manipulate the root architecture of crops with an enhanced ability to resist extreme environmental changes.

1.1.4. Role of microbes

Water quantity and frequency also plays a role in soil microbial activity. Under low water or drought conditions, soil microbial activity is reduced, slowing down N cycling, thus soil N accumulation decreases (Jensen *et al.*, 2003). However, microbial activity has been shown to increase under certain conditions, particularly under variable soil moisture, i.e. wetting-drying cycles, with the water pulses encouraging the breakdown of N forms that are locked up in organic matter, therefore increasing N availability (López-Bellido *et al.*, 2005, Schwinning and Sala, 2004, Wang *et al.*, 2015).

Microbes have the potential to aid plants in adapting to water and/or N variability. A range of plant growth promoting bacteria, and mycorrhizal associations (such as arbuscular mycorrhizal fungi) have the ability to help plants adapt to stress, by stimulating production of plant growth hormones (phytohormones) (Glick, 2012, Loper *et al.*, 2012). Plant growth promoting bacteria, such as *Pseudomonas, Enterobacter, Arthrobacter* and *Azotobacter* sp., are known to enhance plant growth (Donn *et al.*, 2015, Saharan and Nehra, 2011). *Azospirillum* sp. are free-living bacteria that are capable of fixing N, i.e. converting N_2 into NH_4^+-N ions (Glick, 2012). Studies conducted by Dalla Santa *et al.* (2004) showed a significant increase in wheat grain yield when inoculated with *Azospirillum* sp. (RAM-7 strain), as an indirect result of increased N fixation by the bacteria ($N_{(g)}$ to NH_4^+-N, which is then converted to NO_3^-N by nitrifying bacteria), in addition to bacterial production of phytohormones, such as auxins. Ultimately, these phytohormones stimulate shoot and root growth, NH_4^+-N and NO_3^--N transport systems, thereby increasing in N uptake (Dalla Santa *et al.*, 2004). Because many soils lack vital essential nutrients at concentrations sufficient for optimum plant growth, the addition of plant growth promoting bacteria can help plants to tolerate nutrient stresses and even improve uptake of nutrients (Glick, 2012).

Better understanding of the interactions between water, N and microbes will enable us to maximise water and N use efficiency. Ultimately, this will help to create a more targeted approach to crop

management, where plant physiological responses to water and N can be measured, and subsequently supplemental irrigation and fertiliser applications optimised.

1.1.5. Wheat

Wheat is the second most important crop worldwide (Bian *et al.*, 2016), with Australia and the UK the 6th and 13th top producers of wheat, respectively (not counting the EU as a collective). The FAO presents data for wheat production as three different categories: area harvested, yield and tonnes of total crop production (this includes non-edible parts). In the UK for 2017, the UK had a harvest area of 1,792,000 ha; yields of 82,796 hectograms/ha; and 14,837,000 tonnes of total crop produced. The UK yield per ha in 2017 was three times higher than Australia's (26,100 hectograms/ha). However, Australia had a much larger cropped area of 12,191,153 ha, and hence a larger total crop of 31,818,744 tonnes (FAO, 2019). These statistics say a lot about the growing conditions in Australia compared to the UK. Crops need to survive harsh climatic conditions, including extreme drought and intermittent rainfall.

1.1.6. Root imaging

Phenomic studies can be carried out in two ways, using both 2-D and 3-D techniques. Various systems can be used to grow plants for two-dimensional measurements, such as hydroponics and rolled-germination paper, and these systems can be used to monitor root traits and their growth, without damaging the root system. From here, measurements can be further analysed using different software. One such system is WinRhizo, which allows a 2-D visualisation of root systems and some measurements include total root length, estimated root volume, tip number, root diameter (Atkinson *et al.*, 2015). For three-dimensional phenotyping, methods such as X-ray Computed Tomography (CT) have revolutionised how root dynamics are studied. This method is a non-invasive, non-destructive method of visualising the internal structure of soils including particles, pores and roots present within the soil (Helliwell *et al.*, 2013).

Using both 2-D and 3-D phenotyping platforms allows for a more extensive and comprehensive analysis and allows comparisons to be made between analyses. The current phenotyping methods are highlighted along with their advantages and disadvantages in Table 1.1.

Table 1.1. The advantages and disadvantages of using 2-D and 3-D advanced phenomic approaches to visualise and quantify root traits.

Methods	Advantages	Disadvantages
2-D analysis		
Germination paper method	Screens large number of lines	Not for screening a soil system
Rhizotron/Gel growth chamber	Visualisation of roots without soil (Bengough *et al.*, 2006)	Limitation of the window structure Different porosity and moisture near wall/window
	Screens a soil system	More difficult for big screening experiments
	Non-destructive Easy to measure root growth rate without plant disturbance	
	Not affect plant growth comparably to field-grown plants	
	Possible to experiment with other factors, i.e. mineral uptake (Liu *et al.*, 2013)	
	Software (WinRHIZO®, RootNav) can measure: Root number Total root length Project root area Root surface area Root angles Root volume (Atkinson *et al.*, 2015)	
	Verifies accuracy of X-ray CT images (Tracy *et al.*, 2012b)	

Hydroponics	Visualisation of roots in water or other medium	Destructive harvest
Root washing	Quick and easy observation of: Root distribution Root length and size (Mairhofer *et al.*, 2012, Smit *et al.*, 2000)	Destructive (fine roots and root hairs susceptible)
		Impossible to know root spatial distribution after harvest (Mairhofer *et al.*, 2013, Tracy *et al.*, 2012b)
3-D analysis		
X-ray Computed Tomography (CT)	Non-destructive, non-invasive	Walls limiting root spread
	Visualisation of the internal structure of soils including particles, pores, roots and organisms within the soil (Helliwell *et al.*, 2013)	
	Observe: Size Density Shape Texture (Tracy *et al.*, 2010)	Temperature difference to field soil
	Can achieve high resolutions from 1µm to 1mm	Increase in sample size lowers scanning resolution
	Software follows all cross-sectional image slices and identifies each greyscale	Software can be difficult to navigate Image stacks slow to process

	value/voxel pertaining to that root material. Can calculate: Root number Total root length Root volume Root angles (Mairhofer *et al.*, 2012, Tracy *et al.*, 2010)	

1.2. Research Aims

The overarching question that underpins this research is: what are the combined effects of variable water and N supply on wheat shoot and root biomass accumulation, root architecture and soil N dynamics?

Key gaps mentioned above include how a combination of water stress, either through quantity or frequency of watering, interacts with soil N to affect wheat phenotypic plasticity, plant nutrition status and soil nutrition. This project specifically aimed to investigate shoot and root physiological responses to combinations of water and N variability of two Australian wheat varieties, quantifying biomass allocation, plant water and N use efficiency, soil N concentrations, and root N uptake. The aims were to:

1. **Chapter 2**

   Quantify the impacts of variable water and N supply on wheat biomass, soil mineral N pools and soil microbial biomass at three developmental stages (tillering, flowering and early grain milk development).

   Hypotheses:

   - More root growth under high N and high water

   - Low water and variable water would result in less biomass

2. **Chapter 3**

   Measure soil water and N legacy effect on a second crop of wheat grown in soil that previously had a crop of wheat grown in under three different soil moisture regimes and three rates of N supply.

   Hypotheses:

   - A previous set of water and N treatments would leave a soil moisture legacy effect, resulting in less biomass for the second crop due to lowered nutrient resources

   - Re-wetting of soil for second crop would remobilise N after a long dry period

3. **Chapter 4**

   Identify phenotypic differences in shoots and roots between two Australian wheat varieties, and determine if plants prefer changes in frequency or quantity of water.

   Hypotheses:

   - The frequency of watering would have a greater impact on plant growth than quantity

- The water regimes imposed would affect N availability, uptake and subsequently create differences in carbon allocation

- Based on the different water and N-use efficiencies of both wheat cultivars, there would be a difference in growth between Gladius and Kukri

4. Chapter 5

Experiment A identified differences in root architecture between two wheat varieties (Kukri and Gladius) in response to the water and N treatments by utilising X-ray Computed Tomography (CT) to image the root systems.

Experiment B investigated N uptake, by measuring rate of uptake through microdialysis and uptake preference of N forms with ^{15}N stable isotopes, and measuring photosynthetic capacity of Kukri under variable water and N.

Hypotheses:

Experiment A

- Kukri would produce more biomass (shoots and roots) and would grow more vigorously than Gladius

- Low water and low N would result in smaller root biomass.

Experiment B

- Low N particularly would result in a higher photosynthetic stress response

- Kukri roots would preferentially take up ammonium or glycine over nitrate.

1.3. Contribution to the discipline

Identifying and quantifying shoot and root responses of wheat to varying degrees of water and N stress will provide better insight into how crops and the environment interact. Specifically, this thesis highlights several contributions to the discipline of agriculture and crop management. Firstly, the different watering treatments imposed in the following experiments showcase the impact on soil N cycling (mineralisation, nitrification). Secondly, soil moisture (high, low, wet/dry cycling) can change C allocation in both shoots and roots, with wet/dry cycling affecting plant growth more than constant watering (either high or low water). This highlights how frequency can have a more detrimental impact on plant growth than quantity. Thirdly, measuring root N uptake distinguished preferences of N forms (ammonium, nitrate or glycine). Finally, the photosynthetic capacity of wheat is primarily affected by

N variability (which is also driven by soil moisture), and measuring different photosynthesis parameters can be used to quantify plant stress. From all of this knowledge, we can potentially use less water and N without negatively impacting plant growth, therefore reducing the need for excessive N fertilisers and water (crop optimisation). Lowering agricultural N inputs will likely help to mitigate climate change (i.e. less N_2O released with better N uptake efficiency, benefiting both agriculture and society). This project gives further understanding to the whole plant-soil-water dynamics, and has the potential to be expanded for other cropping systems.

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Chapter 2

Variable water cycles have a greater impact on wheat growth and soil nitrogen response than constant watering

The work contained in this chapter has been published in *Plant Science*, and is presented in 'published manuscript format'.

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Author Contribution

Project supervision given by TP Garnett, A Rasmussen, SJ Mooney, RJ Smernik, and TR Cavagnaro. All practical work and analysis was done by OH Cousins. OH Cousins was also lead author for this paper, and therefore was largely responsible for all text. Statistical advice was given by CJ Brien.

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Submitted to **Plant Science** on 25th October, 2018 are the undersigned, and there are no other authors. The order in which the authors' names appear in the submitted paper is acceptable to all authors. All authors agree that they have approved the submitted version of the paper.

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Variable water cycles have a greater impact on wheat growth and soil nitrogen response than constant watering

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Key words

Biomass allocation; nitrogen stress; roots; *Triticum aestivum*; variable water; water use efficiency

2.1. Abstract

Current climate change models project that water availability will become more erratic in the future. With soil nitrogen (N) supply coupled to water availability, it is important to understand the combined effects of variable water and N supply on food crop plants (above- and below-ground). Here we present a study that precisely controls soil moisture and compares stable soil moisture contents with a controlled wetting-drying cycle. Our aim was to identify how changes in soil moisture and N concentration affect shoot-root biomass, N acquisition in wheat, and soil N cycling. Using a novel gravimetric platform allowing fine-scale control of soil moisture dynamics, a 3 × 3 factorial experiment was conducted on wheat plants subjected to three rates of N application (0, 25 and 75 mg N/kg soil) and three soil moisture regimes (two uniform treatments: 23.5 and 13% gravimetric moisture content (herein referred to as Well-watered and Reduced water, respectively), and a Variable treatment which cycled between the two). Plant biomass, soil N and microbial biomass carbon were measured at three developmental stages: tillering (Harvest 1), flowering (Harvest 2), and early grain milk development (Harvest 3). Reduced water supply encouraged root growth when combined with medium and high N. Plant growth was more responsive to N than the water treatments imposed, with a 15-fold increase in biomass between the high and no added N treatment plants. Both uniform soil water treatments resulted in similar plant biomass, while the Variable water treatment resulted in less biomass overall, suggesting wheat prefers consistency whether at a Well-watered or Reduced water level. Plants did not respond well to variable soil moisture, highlighting the need to understand plant adaptation and biomass allocation with resource limitation. This is particularly relevant to developing irrigation practices, but also in the design of water availability experiments.

2.2. Introduction

To achieve global food security we must adapt to climate change and develop resilient crop varieties. Rainfall patterns in many regions of the world are predicted to become more erratic in the near future (Black, 2016, Loo *et al.*, 2015, Monjo and Martin-Vide, 2016). A shift from more predictable rainfall to less frequent but more intense rainfall events, followed by long periods of no or low rainfall, is already being observed globally (Borken and Matzner, 2009, Coumou and Rahmstorf, 2012). Such changes in the quantity and timing of rainfall not only affect crop water use efficiency, but also other key soil ecosystem services, including soil N cycling (Hoogmoed *et al.*, 2016).

Nitrogen is the nutrient that most often limits plant production globally (Ågren *et al.*, 2012, LeBauer and Treseder, 2008, Vitousek *et al.*, 2010). Consequently, N fertilisers, such as urea are commonly used to enhance yields. Plant N uptake is influenced by many factors, including, but not limited to, the concentrations of ammonium (NH_4^+-N) and nitrate (NO_3^--N) in the rooting zone, the plant N requirements, root length distribution, activity and mycorrhizal status, and the movement of N in the soil solution (Davidson *et al.*, 1987, Jackson *et al.*, 2008, Veresoglou *et al.*, 2012). Furthermore, the soil N cycle is tightly coupled to water availability (Burger *et al.*, 2005). In saturated soils, N can be lost from the soil as N_2 and as the potent greenhouse gas N_2O (Butterbach-Bahl *et al.*, 2013), while NO_3^--N and NH_4^+-N leaching and surface runoff during high water flow events (Bijay *et al.*, 1995, Carstensen *et al.*, 2014) can result in annual N losses of up to 160 kg per hectare in some systems (Herzog *et al.*, 2008). Together, these losses not only impact the environment, but result in reduced N fertiliser efficiency, affecting agricultural productivity, profitability and sustainability (Jackson *et al.*, 2008).

Previous work has sought to investigate the separate impacts of variable N and water on cereal crops (Foulkes *et al.*, 2009, Manschadi *et al.*, 2006, York *et al.*, 2015) and plant traits associated with maximising acquisition of either resource (Lynch, 1995a). However, in the field, variable water conditions and N-resource limitations often co-occur (Araus *et al.*, 2013, Dijkstra *et al.*, 2016, Elazab *et al.*, 2016). As soil N availability is strongly regulated by soil moisture, and plant adaptations to variable water and nutrients, such as N, may differ, there is a need for factorial studies specifically investigating plant responses to combinations of different levels of water and N availability. To this end, Ayad *et al.* (2010) demonstrated that rain-fed wheat and barley grown with and without supplemental watering at three soil N levels had reduced root biomass and length under low N with supplemental water. Similarly, water uptake efficiency of durum wheat was found to be higher with high N, regardless of water regime (Araus *et al.*, 2013, Cabrera-Bosquet *et al.*, 2007). In contrast, Shen *et al.* (2013) showed that water uptake efficiency was increased in winter wheat under high N but only when combined with a low water availability. This variability in plant response to simultaneous changes in water and N availability highlights the need for studies that investigate plant above- and below-ground responses to combinations of water and N variability.

Rainfall variability is becoming increasingly common, but watering within pot trial/controlled experiments, even on a daily basis, can result in repeated intermittent dry-downs which may impact soil

nutrient cycling, microbial activity and plant growth (Burger *et al.*, 2005, Cavagnaro, 2016, Xiang *et al.*, 2008, Yu and Ehrenfeld, 2009). One way of controlling soil water and reducing effects of wetting and drying cycles is through the use of wicking beds or tension tables, where pots are placed onto a bed of sand equilibrated to a precise matric potential allowing plants to uptake water according to use (Araya *et al.*, 2010, Semananda *et al.*, 2016, Tinklin and Weatherley, 1968). Although the particle size distribution of the sand can be selected to achieve a desired water potential, it can be difficult to establish variable cycles in this manner. Alternatively, precise soil moisture and wetting-drying cycles can be achieved through the use of fully automated, gravimetric, lysimeter-based, plant growth platforms (Vadez *et al.*, 2015). In these systems, plants are placed on individual lysimeters and watering occurs when soil moisture (as determined by pot weight) falls below a pre-determined level. In addition, these systems make it possible to establish pre-determined patterns of water supply, including wetting-drying cycles. Finally, they can also be used to record water-use throughout the entire plant growth cycle.

Here we report on the combined effects of variable water and N supply on wheat biomass (above- and below-ground), soil mineral N pools and soil microbial biomass carbon. A gravimetric platform was used to precisely control soil moisture, to understand how variability of water and N can impact plant growth. We focused on two levels of constant water supply and a third treatment where soil moisture cycled between the two levels. Important interactions between water and N in wet soils have been identified (Helliwell *et al.*, 2014, Parent *et al.*, 2015), but further understanding of these interactions is needed to improve water by N management strategies. These water regimes were combined with three soil N addition treatments in a fully factorial design.

2.3. Materials and Methods

2.3.1. Plant growth experiment

General growth conditions: A soil mix (composed of a mixture of clay loam, UC (University of California; Baker, 1957) mix and cocopeat (1:1:1 W:W:W) referred to as 'soil' hereafter) was used in this experiment. This medium has been used extensively in previous experiments, and as a result is well-characterised for N and water responses of wheat (Honsdorf *et al.*, 2014, Takahashi *et al.*, 2015). Basal nutrients were added to all treatments to ensure that N was the only limiting nutrient in the experiment, as follows: dolomite lime 0.98 g/L, ag lime 2.72 g/L, hydrated lime 0.63 g/L, gypsum 0.98 g/L, superphosphate 1.96 g/L, iron sulphate 2.45 g/L, iron chelate 0.163 g/L, micromax (ICL Australia & New Zealand, New South Wales, Australia) 0.98 g/L. Plastic, free-draining pots (4.5 L, 185 mm deep × 195 mm diameter), were filled with 4.7 kg of nutrient-amended soil (including N – see *Experimental treatments*), and packed to a bulk density of 1.2-1.3 g/cm^3.

Pots were left undisturbed for a week to equilibrate at room temperature, then transferred to an automated gravimetric watering system (DroughtSpotter, Phenospex, Heerlen, The Netherlands). The system is an automated watering and water-use monitoring system composed of 168 individual lysimeters, arranged in two 3 × 28 grids, one on each side of an aisle. Each lysimeter is connected to a separate watering spigot, which waters from the top. The system recorded the weight of individual pots (and thus water use) every 10 minutes. These data were used to precisely control soil water additions, and quantify water use in each pot.

Two days after the commencement of the individual watering treatments (see *Experimental treatments*), two seeds of wheat *Triticum aestivum* (variety Gladius) were sown directly into each pot. After seedling emergence (3 days), seedlings were thinned to one per pot. To minimise evaporative losses from the soil, a mesh was placed on the soil surface of all pots. Conditions in the glasshouse were 22/15°C day/night, and light levels were supplemented at 400 µmol/m^2/s using LEDs (GreenPower LED toplighting module DR/B HB 400V, Philips Electronics Australia Ltd, New South Wales, Australia) with a 12/12 h day/night photoperiod. To help measure evaporative loss, an additional 11 pots were set up as plant-free controls.

Experimental treatments: The experiment included three N addition treatments (Table 1) established by adding urea to the soil as follows: N1 to which no urea was added to the soil; N2 to which urea was added to the soil at a rate of 25 mg of N/kg of soil; and N3 to which urea was added at a rate of 75 mg of N/kg of soil. The gravimetric moisture content of the soil was 14.5% at the time of urea addition.

Table 1. Treatment combinations of water and N regimes.

Water	**Nitrogen**
Reduced water 13% gravimetric moisture	N1 (no urea) N2 (25 mg N/kg soil) N3 (75 mg N/kg soil)
Well-watered 23.5% gravimetric moisture	N1 (no urea) N2 (25 mg N/kg soil) N3 (75 mg N/kg soil)
Variable water 7 days at 23.5%, dried down to 13%, then re-wet to 23.5% for 7 days, and repeated (see Figure 1)	N1 (no urea) N2 (25 mg N/kg soil) N3 (75 mg N/kg soil)

The concentrations of soil NH_4^+ -N and NO_3^- -N were measured on 2 M KCl extracts (see below) at the start of the experiment, which was 2 days after the addition of urea. While concentrations of NO_3^--N at the start of the experiment were relatively similar, NH_4^+-N concentrations increased with increasing supply of urea (Table 2). Although the addition of different amounts of urea to the different N treatments are likely to have an influence on osmotic potential, it is only likely to have a small effect on total osmotic potential due to the presence of much higher concentrations of other soluble species, such as Ca^{2+}, Mg^{2+} and SO_4^{2-}. Additionally, the urea added will have an even smaller effect on total water potential due to the contribution of matric potential, especially for the lower water content treatment.

Table 2. Measured concentrations of soil mineral N under each treatment regime (as NH_4^+ -N and NO_3^- -N) at the start of the experiment.

Nitrogen treatment	Amount of N in potting soil (mg of N/kg of soil)	NH_4^+ -N (µg/g dry soil)	NO_3^- -N (µg/g dry soil)
N1	0	0	12.0 ± 1.9
N2	25	15.2 ± 0.7	9.4 ± 1.2
N3	75	62.3 ± 0.1	10.8 ± 1.2

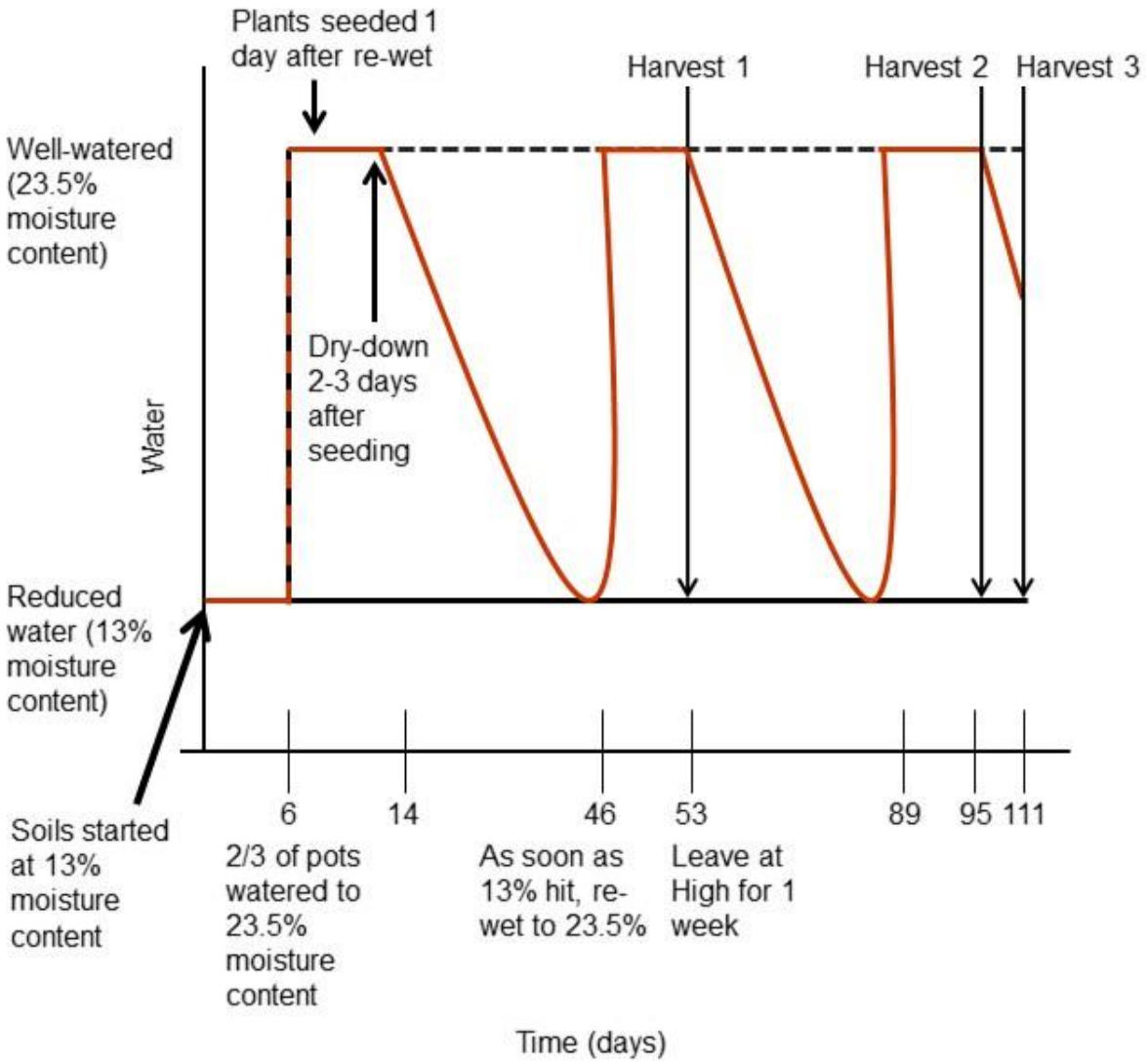

Figure 1. Watering regimes (schematic) applied to wheat as a percentage of the soil's gravimetric moisture content: 13% of gravimetric moisture content (solid line); 23.5% of gravimetric moisture content (dashed line) and variable (wetting-drying) cycle (watered to 23.5%, dried to 13%, then watered up to 23.5%, solid line peak and troughs).

The gravimetric platform was used to establish three soil watering regimes. The treatments were 13% of gravimetric moisture content (referred to as "Reduced water" hereafter), 23.5% ("Well-watered" hereafter) and a "Variable" treatment which cycled between 23.5% and 13% gravimetric moisture content (Figure 1 and Table 2). Throughout this paper, we will refer to the water content of our treatments as a percentage of the field capacity of the potted system, even though we are aware that the pot itself affects the notional 'field capacity' of potted soil. Field capacity was determined from a subsample of soil at 1 m suction and the water content for our media at field capacity was 13%. Therefore, the Reduced water treatment was maintained at field capacity, and the Well-watered water treatment was near-saturation (making it very well-watered). The Well-watered water treatment of

23.5% is 1.8x the moisture content of the Reduced water treatment and thus representative of significantly contrasting water regimes.

These water conditions were described as adequate to support plant growth, with plant growth expected to increase under wetter soil conditions, as well as increased microbial activity, thus impacting microbial biomass carbon levels and N mineralisation (based on Helliwell *et al.*, 2014, Parent *et al.*, 2015). To calculate the field capacity of the soil, a 1 m head of suction was imposed on glass funnels containing approximately 182 g of air-dried soil. The soil was completely saturated with water, the funnel covered with Clingfilm and left to drain for 48 h. A sub-sample of soil was taken from the funnel and oven-dried at 105°C for 24 h; at a higher temperature, organic matter is at risk of being burned off, and any lower, hygroscopic water will not be completely removed (Klute and Gardner, 1986, Rowell, 1994). In addition, both total porosity and water filled pore space was calculated. To calculate total porosity, bulk densities of 1.2 g/cm^3 and 1.3 g/cm^3 were used, with a particle density of 2.1 g/cm^3 (based on a soil mixture that is two-thirds mineral content (clay loam, UC mix) and one-third organic (cocopeat) that is packed to a bulk density of 1.2-1.3 g/cm^3; Table 3).

From this, the water filled pore space was calculated as:

$$((\text{gravimetric moisture content x bulk density})/\left(1-\frac{\text{bulk density}}{\text{particle density of quartz}}\right)) \times 100$$

Since water filled pore space is the ratio between soil water content and soil porosity, soils in the Reduced water treatment had an average water filled pore space of 36.4%, whereas the Well-watered water treatment had an average water filled pore space of 65.8% (Table 3). Because the soil used had a high organic matter content, the water filled pore space did not take into account the water within microsites, therefore the water filled pore space values are only approximate.

Table 3. Calculated soil porosity and water filled pore space with an approximate particle density of 2.1 g/cm^3, based on a soil mixture that is two-thirds mineral content (clay loam, UC mix) and one-third organic (cocopeat) that is packed to a bulk density of 1.2-1.3 g/cm^3.

		Bulk density 1.2 g/cm^3			Bulk density 1.3 g/cm^3		
Water treatment	Gravimetric moisture content/g dry soil	Total porosity (cm^3/cm^3)	Volumetric water content (cm^3/cm^3)	Average water filled pore space (% of total pore space)	Total porosity (cm^3/cm^3)	Volumetric water content (cm^3/cm^3)	Average water filled pore space (% of total pore space)
Reduced water	0.13	0.429	0.156	36.4	0.381	0.169	44.4
Well-watered water	0.235	0.429	0.282	65.8	0.381	0.306	80.3

Initially all pots (including Well-watered and Variable) were maintained at 13% gravimetric moisture content for six days before sowing. After this period, pots for Well-watered and Variable water treatments were watered up to the Well-watered soil moisture content (23.5%). The Variable treatment pots were maintained at the Well-watered soil moisture content (Figure 1) for seven days and then allowed to dry down until all pots had reached 13% (Reduced water) soil moisture content, with the last Variable treatment pot reaching 13% soil moisture at day 32. The Variable pots were then re-wet to 23.5% moisture content and maintained at this moisture content for seven days. Four pots from each treatment were harvested at this time, except for Reduced water × N1 treatment with only three pots harvested (day 53; Harvest 1, Figure 1) (35 pots in total). This watering sequence for the Variable treatment was then repeated, with 13% moisture reached at day 89, when pots were re-wet and maintained at 23.5% for a week before a second set of four plants were harvested from each treatment, except for the Reduced water × N3 treatment where only three pots were harvested (Harvest 2 day 95, Figure 1) (35 pots in total). The drying cycle was repeated once more for 15 days but did not reach 13% gravimetric moisture content. At this time, all remaining plants were harvested, a total of 36 plants with four replicates per treatment (day 111, Harvest 3). Each harvest corresponded to a different

developmental stage: Harvest 1 plants at the start of tillering, Harvest 2 plants at mid-flowering (50% of spikes flowering), and Harvest 3 at early milk development of grains (Zadoks *et al.*, 1974).

2.3.2. Plant sampling and analysis

For Harvest 1 and 2 (see Figure 1), plants were carefully removed from their pots, the roots washed of any adhering soil using reverse osmosis (RO) water and then above- and below-ground biomass were separated. Total root length was calculated for whole root systems using WinRhizo (Harvests 1 and 2). Specific root length was only calculated for the N1 treatment (but for all water treatments), because clean WinRhizo scans could not be obtained for roots under N2 or N3 treatment. The quality of root images for N1, N2 and N3 treatments can be seen in Supplementary Figure 1 and Figure 2. Roots were not collected at Harvest 3 as plants were severely pot-bound making it impossible to wash roots completely free from soil. The above-ground biomass was separated into vegetative biomass (shoots hereafter) and heads. All plant biomass was oven-dried (60°C) until a constant weight was achieved (72 h), and dry weights recorded. The dried shoot material was homogenised by grinding to a fine powder for 1-2 min using a ring mill (Standard Ring Mill, SRM-RC-3P; Rocklabs Ltd, Auckland, New Zealand) with a stainless steel head (CHRO-40-BLP or CHRO-200-BLRP depending on the size of plant biomass). Shoot samples were analysed for total nitrogen (TN) by dry combustion, with sample weight standardised across all treatments (http://www.apal.com.au/; Rayment and Lyons, 2011).

2.3.3. Soil sampling and analysis

At each harvest for every pot, the soil was homogeneously mixed and a soil sample was taken from each pot (approximately 100 g) and divided into two sub-samples. The first sub-sample was extracted with 2M KCl, and the extracts analysed for NH_4^+-N (Forster, 1995) and NO_3^--N (Miranda *et al.*, 2001). The second sub-sample was analysed for microbial biomass carbon using the fumigation-extraction method (Vance *et al.*, 1987).

2.3.4. Statistical design and analysis

The experiment used a split-split-plot design to assign the 36 treatments to 144 plants, one per lysimeter. The soil watering treatments (three levels) were assigned to whole-plots (main-units) using a randomized complete block design with four replicates (blocks). Each whole-plot was split into four subplots (areas) for which there were four randomised harvest times; each subplot contained three plants each in a pot (sub-subplots) to which N additions (three levels) were randomised. As different plants were used for each harvest time, this is not a repeated measures experiment. For this experiment, only three harvests were analysed, therefore with nine treatments and three harvest times there was a total of 108 pots.

Over the course of the experiment, two plants were lost due to equipment malfunction; consequently, for Harvest 1 the Reduced water/N1 treatment had three replicates, as did the Well-watered water/N3

treatment at Harvest 2. The data for each harvest was analysed separately using a split-plot analyses of variance (factorial ANOVA) that was performed with GenStat (VSN International, 2012). Response variables included in the analysis were shoot and root biomass, soil NH_4^+-N and NO_3^--N content, shoot total N, and MBC. Where the ANOVA revealed a significant treatment effect ($p<0.05$), significant differences between individual treatments were identified using least significant differences of means at the 5% level, i.e. Fisher's protected LSD procedure was used. Tukey's HSD was considered, but was rejected as the results are dependent on treatment number included (thus making it inconsistent (Saville, 2015)) and the number of comparisons used in this experiment was not large. All data was checked for a normal distribution in R (version 3.2.5).

2.4. Results

2.4.1. Plant growth and nutrients

Soil watering and N addition treatments impacted plant biomass both above- and below-ground at all harvest times (Figure 2). The main effects of water and N are present for Harvest 1; this means that water had an effect on cumulative water uptake irrespective of N, and nitrogen had an effect regardless of the water treatment. Significant interactions are present for Harvest 2 (shoots and roots) and for Harvest 3 (shoots), showing that both water and nitrogen had an effect on shoot or root growth due to each abiotic factor, simultaneously.

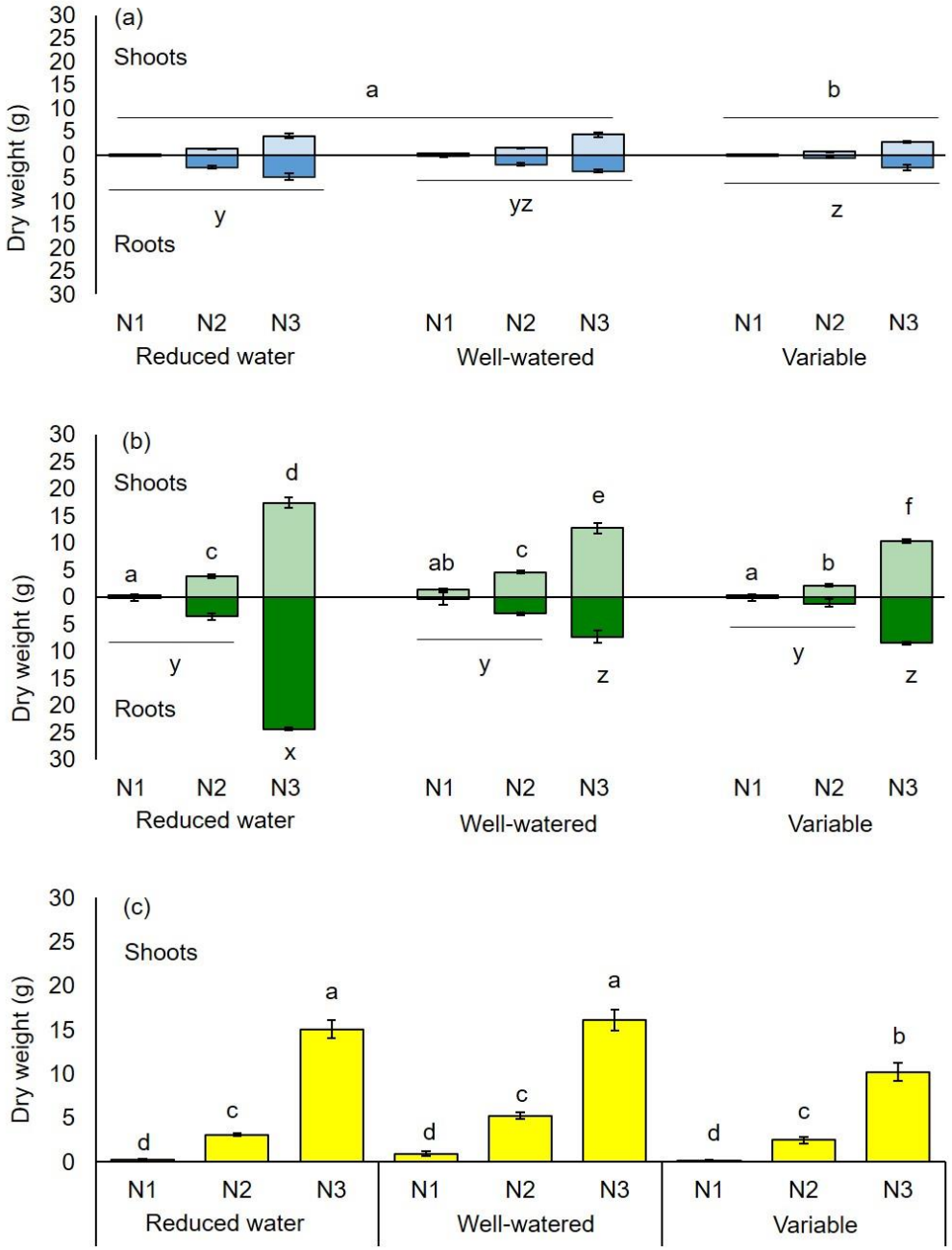

Figure 2. Shoot dry weight (pale shading, above x-axis) and root dry weight (dark shading, below x-axis) for Harvest 1 (a) and Harvest 2 (b), shoot dry weight only for Harvest 3 (c); with three water treatments (Reduced water, Well-watered, Variable) and three N treatments: N1 (0 mg/kg of N), N2 (25 mg/kg of N), N3 (75 mg/kg of N). Values are presented as mean values ± SE, n=4 except n=3 for treatments Harvest 1 Reduced water/N1 and Harvest 2 Well-watered/N3. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$ or 0.001). Harvest 1 (Panel a) shows significant main effects (Shoots: main effect of water $p<0.05$ with letters above bars, and main effect of N $p<0.05$ with $N1^{a}$ $N2^{b}$ $N3^{c}$; Roots: for Harvest 1 (panel a) main effect of water $p<0.001$ with letters above bars; and main effect of N $p<0.001$ with $N1^{a}$ $N2^{b}$ $N3^{c}$). Significant interactions are present for Harvest 2 (water × N $p<0.001$ for shoots; water × N $p<0.01$ for roots; (panel b)) and Harvest 3 (water × N $p<0.01$ for shoots; (panel c)).

At Harvest 1, both shoot and root dry weights increased with increased N supply (Panel 2a), with the main effect of N showing N treatments were significantly different ($N1^a$ $N2^b$ $N3^c$). Shoot and root response were also modulated by soil moisture treatment. For example, shoot dry weight was lower for the Variable water/N3 treatment compared to both the Reduced water and Well-watered treatments at N3 ($p<0.05$). Root dry weights were also lower in the Variable water treatment, compared to the Reduced water treatment ($p<0.05$). At Harvest 2 (Panel 2b), roots were especially responsive to N under the Reduced water treatment, with a 16-fold increase in root dry weight between N1 and N2, and a 10-fold increase between N2 and N3, with plants subjected to the Reduced water N3 treatments producing the highest root dry weight. Root dry weights for Well-watered and Variable water treatments were not different. The shoot biomass of plants at Harvest 3 (Panel 2c) increased significantly with increasing N, except for a reduction in shoot biomass in the N3 plants with Variable watering compared to the other water treatments. There were no other significant impacts of water supply on plant growth.

In order to further characterise root responses to the treatments, specific root length (i.e. root length per unit root mass) was calculated (Figure 3). Specific root length did not differ between Reduced water, Well-watered and Variable water for N1 treatments at Harvest 1 and Harvest 2 ($p>0.05$), but roots had higher specific root lengths, suggesting thinner roots at Harvest 2 than Harvest 1.

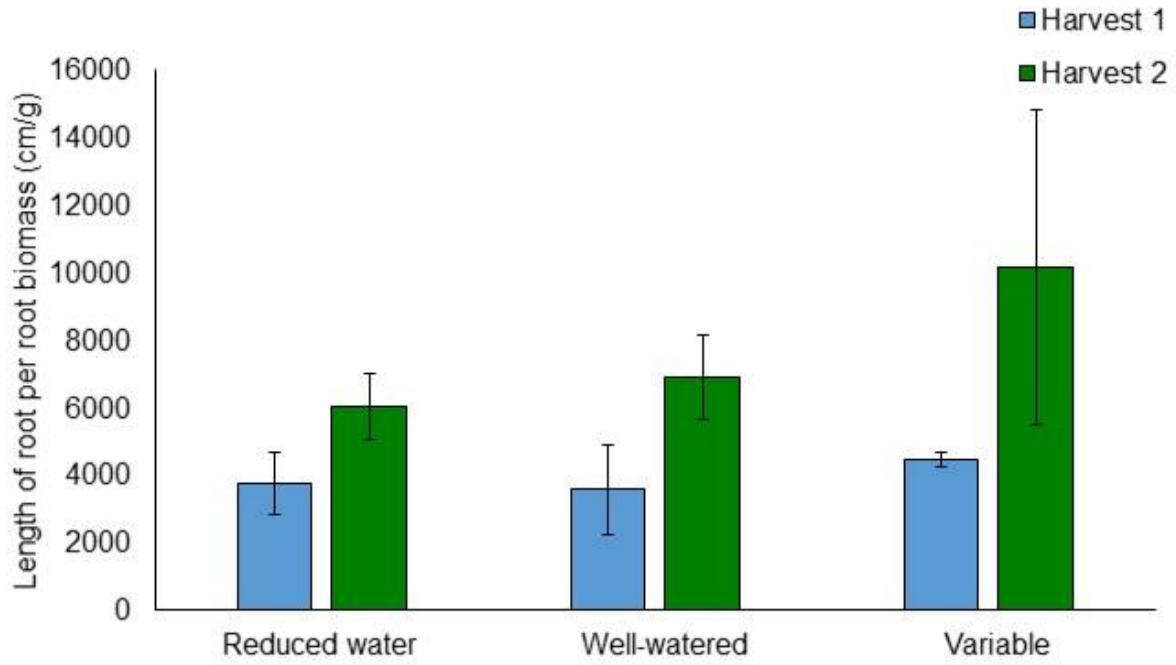

Figure 3. Mean specific root length of plants ± SE at Harvest 1 and Harvest 2 with three water treatments (Reduced water, Well-watered, Variable) with N1 treatment; SE, n=4 except n=3 for treatment Harvest 1 Reduced water/N1. Using ANOVA and LSD of means 5% level, no significant main effects or interactions of water and N were found ($p>0.05$).

The N concentration of shoot tissues generally decreased with each successive harvest (Supplementary Figure A.3). At Harvest 1, shoot N concentrations were highest in three of the combination treatments: Reduced water N3, Well-watered N1 and Variable N3 (Supplementary Panel A.3a). At Harvest 2, plants under Variable water had higher shoot N concentrations than plants under Reduced water, irrespective of N treatment (main effects of water ($p<0.05$) and N ($p<0.001$; $N1^a$ $N2^b$ $N3^c$)) (Supplementary Panel A.3b). Finally, at Harvest 3, there were no differences in shoot N concentrations among any of the treatments (Supplementary Panel A.3c).

2.4.2. Soil properties

Mineral N pools were dominated by NO_3^--N, with NH_4^+-N concentration very low at all harvest times (data not shown); accordingly, the sum of NO_3^--N and NH_4^+-N is presented as mineral N (Figure 4). At Harvest 1, there was a greater concentration of soil mineral N under N3 supply, with the Reduced water treatment having the highest concentration (Panel 4a). Water did not affect soil mineral N concentration, whereas mineral N concentrations were higher in N3 than N1 and N2 ($p<0.001$). A similar trend was seen at Harvest 2 (Panel 4b), but the only significant differences were in the Variable treatment, where mineral N in the N3 treatment was higher than in all other treatments at this harvest. At Harvest 3, mineral N was highly variable, especially in the Well-watered treatment. Only N1 and N3 treatments were significantly different from each other across all water treatments ($N1^a$ $N2^{ab}$ $N3^b$, Panel 4c).

Microbial biomass C was also measured to determine the impact of watering and N regimes on microbial abundance (Panel 4d-f). Microbial biomass C increased with N supply in most water and N regimes and harvests, except for Harvest 1 Reduced water and Harvest 3 Well-watered. An interaction between water and N was present, so microbial biomass C was affected by both water and N simultaneously (Panel 4d). At Harvest 2, Reduced water and Variable water treatments encouraged microbial biomass C, irrespective of N treatment ($N1^a$ $N2^b$ $N3^c$, Panel 4e). For Harvest 3, Reduced water and Well-watered treatments had significantly different microbial biomass C regardless of N, but Variable water did not differ from the other two treatments ($N1^a$ $N2^a$ $N3^b$, Panel 4f).

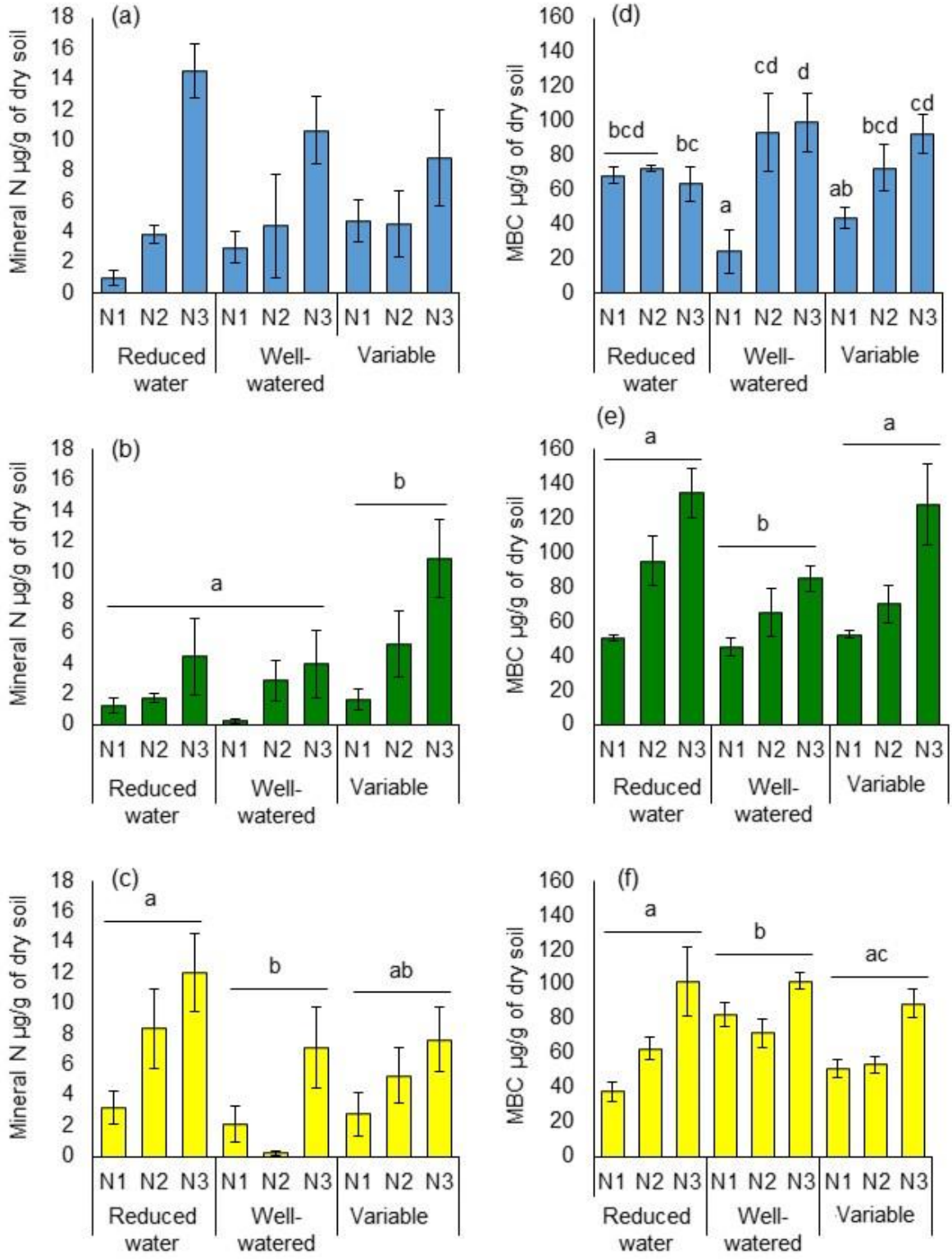

Figure 4. Panels a-c are mineral N and show the total mineral N concentrations comprising of NO_3^--N and NH_4^+-N µg/g dry soil from Harvest 1 (a), Harvest 2 (b) and Harvest 3 (c); Panels d-f are microbial biomass carbon (MBC) at Harvest 1 (d), Harvest 2 (e) and Harvest 3 (f). Error bars represent the standard error of the mean (SE), with n=4 except n=3 for treatments Harvest 1 Reduced water/N1 and Harvest 2 Well-watered/N3. Bars with different letters are significantly different ($p<0.05$ or 0.001, LSD of means 5% level). Significant main effects of

either water or N are calculated using ANOVA and LSD of means. Mineral N statistical analysis: main effects are described here. Panel (a) no significance shown for water; main effect of N $p<0.001$, $N1^{a}$ $N2^{a}$ $N3^{b}$. Panel (b) main effects of water $p<0.05$, letters above bars; main effect of N $p<0.05$, $N1^{a}$ $N2^{a}$ $N3^{b}$. Panel (c) main effects of water $p<0.05$, letters above bars; main effect of N $p<0.05$, $N1^{a}$ $N2^{ab}$ $N3^{b}$. MBC statistical analysis: both main effects and interactions of water and N are described here. Panel (d) significant interactions between water and N shows water × N interaction $p<0.05$ (letters of significance above bars). Panel (e) main effects of water $p<0.05$, letters above bars; and main effect of N $p<0.001$, $N1^{a}$ $N2^{b}$ $N3^{c}$. Panel (f) main effects of water $p<0.05$ letters above bars; and main effects of N $p<0.001$, $N1^{a}$ $N2^{a}$ $N3^{b}$.

2.4.3. Water use

Cumulative water applied to the pots was recorded by the gravimetric platform over the course of the experiment (Figure 5).

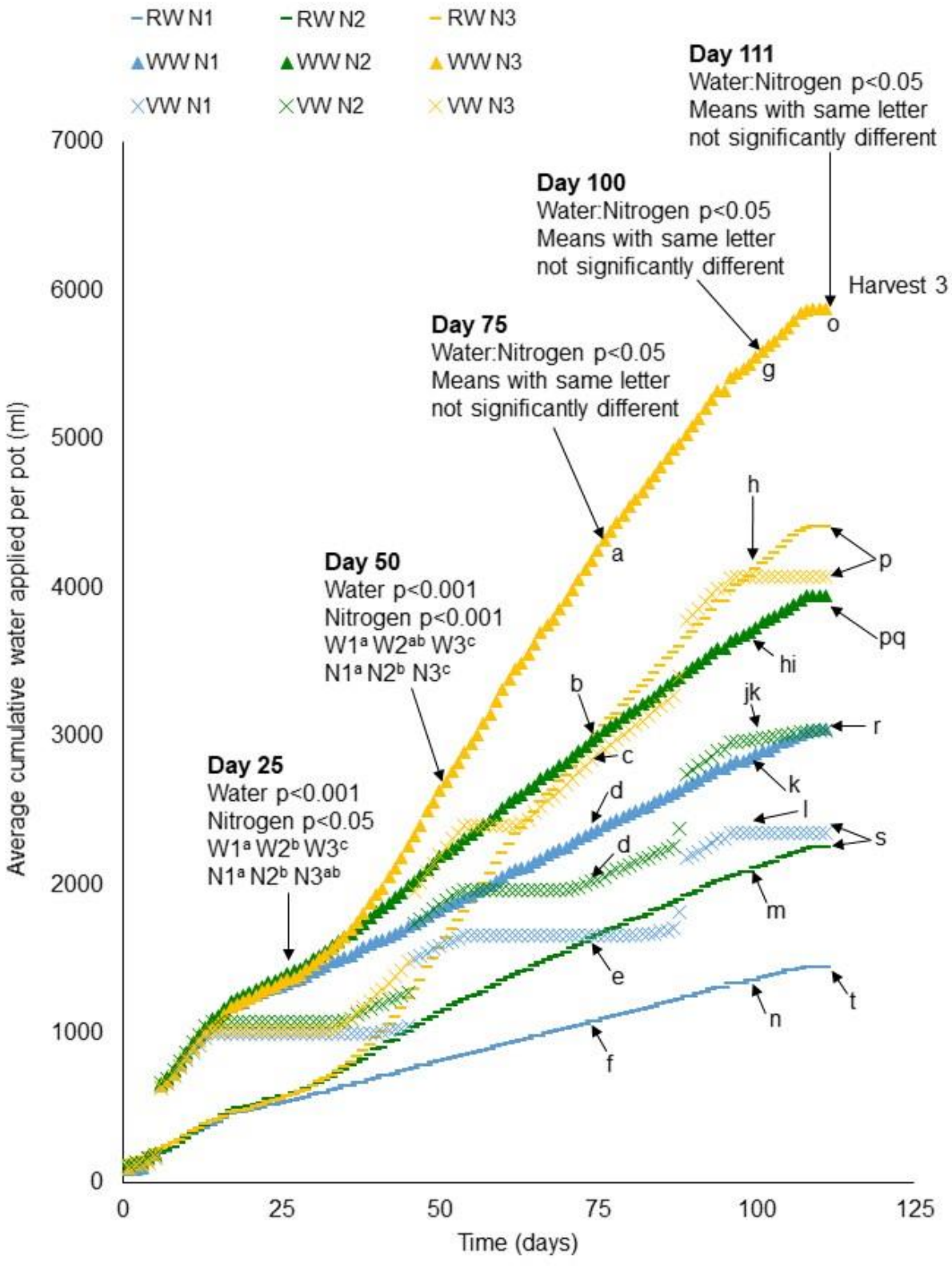

Figure 5. Average cumulative water applied per pot (mL) at Harvest 3, n=4 except n=3 for treatments Harvest 1 Reduced water/N1 and Harvest 2 Well-watered/N3. Data points show three water treatments: Reduced water (RW, dash), Well-watered (WW, triangle), Variable water (VW, cross); and three nitrogen treatments: N1 (0 mg/kg of N), N2 (25 mg/kg of N), N3 (75 mg/kg of N). Outcomes of significant values from ANOVA and LSD of means 5% level are shown in the graph. Significant main effects of water or N are shown for Day 25 and Day 50, where

both water and nitrogen have an effect on cumulative water use regardless of each other. Significant interactions between water and nitrogen are shown as letters above Day 75, Day 100 and Day 111. Data points with different letters are significantly different ($p<0.05$, LSD of means 5% level). The statistical comparisons are made within the same time point, not between time points. In the interest of clarity, error bars are not shown.

As the amount of N applied to the pots increased, the amount of water applied to each pot also increased. Plants under Reduced water with N1 used less water over time than plants under Reduced water and N2 or N3 treatments. There was also an incremental increase in water applied with increasing N supply for the Well-watered treatment. This pattern was also seen in the Variable water treatment. Until day 30, the cumulative water use was similar for all plants within a given water regime, irrespective of N treatment. By day 50, applied cumulative water had separated out between most treatments; however, only the main effects for water or N were significant. From day 75 to day 111 (Harvest 3), both Well-watered N3 and Reduced water N1 consistently showed a significant interaction between water and N, resulting in the highest and lowest cumulative water applied, respectively. From day 100 to day 111, cumulative water applied was similar between Reduced water N3 and Variable water N3. At harvest, water was the main driver for cumulative water use for the N3 treatment. However, for N1 and N2 treatments, cumulative water applied was affected more by N. Water use efficiency (g plant dry weight/L of water applied) was calculated, and differed between N addition treatments, but not watering regime (Supplementary Figure A.4). Water use efficiency increased from N1 to N3, with N3 resulting in higher water use efficiency than N1 or N2 across all harvests. There was a consistent, but non-significant trend of higher water use efficiency for Reduced water and Well-watered water treatments at N3 compared to plants in the Variable/N3 treatment; this trend was strongest at Harvest 2 ($p>0.05$). Water use efficiency was lower at Harvest 3 than Harvest 2, corresponding to plants nearing grain maturity.

2.5. Discussion

2.5.1. Effects of water and N supply on plant biomass

Plant growth was affected by both the watering and N treatments. In general, plants were responsive to soil N supply, with an increase in shoot and root growth, thereby affecting mineral N concentrations in the soil (see Sections 4.2, 4.3) and plant N concentrations (see Supplementary data). While shoot growth was similar in the Reduced water and Well-watered treatments, in the Variable water treatment (which cycled between Reduced water and Well-watered), shoot and root growth were generally reduced. Overall, root growth did not differ between Well-watered and Variable water treatments, which in hindsight was expected. Plants in the Reduced water treatment produced more root biomass than shoot biomass, particularly with the Reduced water/N3 treatment (resulting in a higher root:shoot ratio) (Figure 2b). This is consistent with previous work showing greater allocation to roots under relatively drier conditions (Elazab *et al.*, 2012, Sharp *et al.*, 1990, Wang *et al.*, 2014).

It was unclear what changes would occur in plant shoot and root phenotypes as a result of the imposed water and N treatments. However, plants subjected to less N and Reduced water produced larger root systems than plants under higher N and Well-watered or Variable water, possibly resulting in an increase in N uptake as well as capturing more available soil water at earlier growth stages. With water being the most limiting resource in the Reduced water/N3 treatment, this is indicative of the optimal partitioning theory, which suggests that resources are allocated to the plant organ that is experiencing resource limitations. Because the shoots are mostly likely the organ experiencing stress of nutrient/water limitations, this encourages root proliferation in order to capture more nutrients and/or water for productive shoot growth (Ledo *et al.*, 2017). This increased root production suggests that, in this experiment, having a larger root system helped maintain overall plant growth.

Lower plant growth for Variable watering treatment than for either stable watering treatment (Well-watered and Reduced) suggests that even when water is a non-limiting factor (reflected by Well-watered and Reduced water treatments not resulting in significant differences in shoot biomass), a lack of consistency in water supply can adversely affect plants, particularly in terms of biomass. This is especially important in the context of current projections of greater variability in rainfall patterns with climate change (Black, 2016, Monjo and Martin-Vide, 2016). It is well-established that plants can adapt their root architecture in response to different water conditions (Lynch, 1995a, Manschadi *et al.*, 2006, Wasson *et al.*, 2012). Plants in the Variable water treatment may have acclimatised to the Well-watered conditions, resulting in root proliferation. However the resulting stress from the intermittent dry-downs could then create less pressure to produce even longer roots to find water. Also, the addition of a dry-down stress could cause the new roots amassed during the seven days of Well-watered conditions to die back. Both these reasons could explain a smaller, shorter root system, and smaller shoot dry weight. However, when under nutrient or water variability, an increase in root proliferation can aid plant survival with root phenotypic plasticity increasing the capacity of plants to acquire water and nutrients (López-Bucio *et al.*, 2003, Manschadi *et al.*, 2006). Other studies have shown plants subjected to

alternate wetting-drying or partial root-zone irrigation had an increase in root growth, which subsequently encouraged shoot growth (Kang and Zhang, 2004, Zhang *et al.*, 2009). Additionally, a limited N supply in soil could also increase root proliferation at the expense of shoot growth, resulting in a higher root:shoot ratio, thereby increasing the N capture (Evans *et al.*, 1975, Sims *et al.*, 2012). It is also well-known that by the end of flowering, N is remobilised from root, to stem, to seeds, and this is well explained by Schjoerring *et al.* (1995), who discovered 30-40% of plant N moving into oilseed rape pods after flowering. In addition, Barraclough *et al.* (2014) found that the percentage of N remobilised from vegetative parts of wheat to the grain increased with plant maturity.

The increase in specific root length from Harvest 1 to Harvest 2 suggests roots became 'cheaper' per length, focusing resources and energy on becoming longer (Figure 4). Although we did not assess the internal root morphology of these plants, this energy efficiency could have come from thinner roots or through changes in aerenchyma and cell size as a result of water or N stress. Other studies support this hypothesis with several reporting that thinner roots occurred with an increase in specific root length (length:dry biomass ratio) (Eissenstat, 1992, Liao *et al.*, 2006, Ostonen *et al.*, 2007, Poorter and Ryser, 2015). Plants grown in low-nutrient soils have been shown to have higher specific root lengths compared to those grown under optimal nutrient conditions (Fitter, 1985), with more energy conserved for producing deep roots to search for nutrients or water. In this experiment, root dry weight increased with increased levels of N; however, other studies have reported that higher levels of N discouraged large root dry weight in wheat, but encouraged root branching, subsequently resulting in an increase of thinner roots with a higher specific root length (Elazab *et al.*, 2016, Herrera *et al.*, 2007).

2.5.2. Effects of water and N on plant nutrition

Over the course of this experiment, plants became increasingly N deficient. With total N concentrations in plant biomass dropping from a maximum of 2.5% at Harvest 1 to below 1% at Harvest 3, the plants were at or below the critically N deficient range of 1.28-1.39% suggested by Reuter and Robinson (1997). However, as no additional N was added and plant growth dilutes plant tissue N concentration, we expected and saw a decrease in shoot N concentration at each harvest.

Adequate N is necessary for optimal plant growth, stimulating production of tillers, and, subsequently, increasing spike number (Frederick and Camberato, 1995). Combined with adequate water, this can result in a high yield; however, a combination of low water and low N could encourage early senescence thus affecting the plant's ability to photosynthesise and produce grain (Gregory *et al.*, 1992, Silla and Escudero, 2004). High N levels in combination with low water can also have a detrimental effect, with N promoting excessive leaf growth which encourages faster water uptake and transpiration (Evans *et al.*, 1975).

2.5.3. Effect of water and N on soil mineral N and microbial C

There was a clear increase in mineral N with increasing supply of urea to the soil. There was no significant effect of water and there were no significant interactions between water and N at any harvest. However, mineral N was particularly high (relative to the other treatments) in the Variable N3 treatment at Harvest 2. Given that N mineralisation can be increased under Variable regimes, this is not unexpected. In addition, the N content was higher in plants in this treatment than in the N1 and N2 treatments with Variable water, suggesting plant N uptake was not reduced, but rather the Variable water affected soil water uniformity, affecting soil N nitrification and mobility. Repetition of wetting and drying cycles has been shown to affect N mineralisation, since water is not only important for movement of N throughout the soil profile, but also microbial populations responsible for mineralisation of N, i.e. conversion of urea to NH_4^+-N, and thence, NO_3^--N (Birch, 1958, Fierer and Schimel, 2002, Mikha *et al.*, 2005). It is worth noting that a soil moisture probe was used on a similar experiment to look at water distribution in the pots (data not shown) and showed water moved down through the soil profile, with wetter soil towards the bottom of the pot. This water distribution could have affected the distribution of N pools, and subsequently mineralisation of N and MBC pools. As the climate becomes more variable, it will be important to take into consideration impacts on soil moisture and soil N cycling. This is further complicated by impacts of variable moisture on plant growth.

Not only does quantity and frequency of rainfall affect plant growth, but N stress can cause changes to biomass allocation below-ground and root structure in a range of crops (Bonifas and Lindquist, 2009, Garnett *et al.*, 2009, Palta *et al.*, 2011, Postma *et al.*, 2014, Walch-Liu *et al.*, 2006). Low levels of NO_3^--N in Harvest 2, particularly under Reduced water and Well-watered treatments, highlight that the growth stage at which plants take up N is important, especially when under variable or cyclic wet-dry water conditions. Plants at Harvest 2 and 3 were post-anthesis, a stage shown by others to be when N uptake becomes variable (Harper *et al.*, 1987, Kichey *et al.*, 2007, Wuest and Cassman, 1992b). Much of the plant N is remobilised to the grain from stems and leaves, diminishing the need for soil mineral N uptake (Harper *et al.*, 1987). It has been suggested that N uptake after anthesis may not contribute much to the overall N budget, since it only amounts to 7-11% of the total plant N content above-ground (Wuest and Cassman, 1992b).

Soil microbial biomass carbon was variable across the experiment (especially at Harvest 1); however, some patterns were observed. For example, at Harvest 2 there was a clear increase in microbial biomass carbon with N supply (irrespective of watering treatment), consistent with earlier studies (Meyer *et al.*, 2017, Wardle, 1992). Moreover, at Harvest 2, microbial biomass carbon was lower in the Well-watered treatment than the Reduced water treatment. At Harvest 3, water availability may have slowed microbial growth, as N (particularly the N1 and N2 treatments) seemed to have less effect on microbial biomass carbon, especially in the Well-watered and Variable water treatments. It is well established that the soil microbial communities are affected by drying and rewetting of soils (Fierer and Schimel, 2002). Gordon *et al.* (2008) showed microbial biomass carbon decreased significantly through

dry-rewetting cycles, thus suggesting increased variability of soil moisture creates a cascade effect, affecting the soil microbiome and nutrients, and subsequently, plant growth.

2.5.4. Effect of water and N on plant water use

The use of the gravimetric system allowed soil moisture dynamics to be measured in real-time, unlike many comparable experiments, specifically by measuring and controlling the amount of water added to each plant over time. Cumulative water use was initially driven by the water treatments, after which it was driven by water and N separately. Towards the end of the experiment, water and N began to have an interactive effect on the plants, thus affecting cumulative water use. An increase in cumulative water applied would be expected with high N and Well-watered treatments due to increased plant growth increasing transpiration losses. However, studies have demonstrated that under lower soil water availability plant water use can increase when coupled with high N (Elazab *et al.*, 2016, Shen *et al.*, 2013). In addition, the presence of either a small or large soil N pool would affect plant size regardless of water availability, with an N deficiency potentially exacerbating water stress response (Passioura, 2002), reducing the water use efficiency.

Regarding plant water use efficiency (Supplementary Panel A.4), the wheat variety Gladius is known for its drought tolerance (Fleury *et al.*, 2010), so it is possible that the plants were not stressed enough between the Reduced water and Well-watered treatments, leading to similar water use efficiencies for plants under those conditions. However, what is interesting is the change in water use efficiency between harvests; smaller plants at Harvest 1 compared to Harvest 2 and Harvest 3 resulted in less water consumption. Additionally, an increase in biomass and water use efficiency from Harvest 1 to Harvest 2 suggests the plants were able to produce more biomass with less water. Increased access to the available N pool would result in root proliferation, encouraging further root growth and potentially above-ground biomass (Araus et al. 2013; Ayad et al. 2010; Passioura 2002). By Harvest 3, plants began to senesce which could explain the decrease in water use efficiency over time (Passioura and Angus, 2010, van Herwaarden *et al.*, 1998).

These findings suggest that quantities of water available at the start of a cropping season or at later growth stages can have important consequences on plant health and yield. Crops subjected to a dry seasonal start have been shown to have improved crop water use efficiency through the adjustment of irrigation applications after compensating for soil and plant evapotranspiration rates. This could be a preferential strategy to improve water use efficiency or N use efficiency, in response to reductions in soil moisture contents. This ultimately maximises productivity per unit of water, stabilising crop yield rather than maximising it (Geerts and Raes, 2009). However, a wet seasonal start or additional irrigation can be beneficial for stronger crop establishment and improved grain quality, especially during critical growth stages, such as tillering, booting or heading (the emergence of extra shoots, flag leaf, and grain head, respectively (Poole *et al.*, 2015, Salter and Goode, 1967)). Although timing of irrigation or rainfall plays a role in plant growth, crop acclimatisation to water regimes will always depend on nutrient

availability. Therefore, to maximise grain yields, it is important to find the right application of N in relation to variable rainfall or irrigation patterns.

Overall, the combinations of different N and watering regimes affected soil N (ammonium and nitrate concentrations) and microbial biomass C, which in turn affected plant growth and plant tissue N concentrations. Plant growth was limited under low N conditions. However, Reduced water conditions encouraged significant plant growth at Harvest 2 and Harvest 3, particularly below-ground in combination with high N (N3), maintaining shoot growth; whereas Variable water resulted in reduced biomass overall (most likely due to cyclic watering affecting N mineralisation and subsequent movement through the soil). Although rate of urea mineralisation was not calculated, it is clear to see that water frequency did have an impact on mineralisation. Particularly at Harvest 2, Variable water resulted in greater soil mineral N pools, possibly due to water flushing the soil system during re-wetting, thus encouraging more urea mineralisation. Soil N affected microbial biomass C levels, with soil microbial biomass C decreasing under low N supply across all harvests. There was no difference in microbial biomass C between Reduced water, Well-watered and Variable water supply.

2.5.5. Conclusions

This study provides further evidence concerning the interactions between water and N availability. Low levels of soil N negatively affected both shoot and root growth; however, Reduced water particularly encouraged root growth when combined with medium and high N levels, whilst maintaining shoot growth. In addition, plants did not respond well to the Variable water treatment (cycling between Well-watered and Reduced water), regardless of N treatment, consistently producing smaller plants and affecting N mineralisation and uptake.

Understanding resource variability is becoming more important for both irrigated and non-irrigated systems as weather patterns become increasingly erratic. Optimising crop productivity in these conditions requires an understanding of how plants respond to the combined impact of variable supply of both water and N. This knowledge can also inform breeding programs targeting adaptable crops and cultivars, with root systems optimised for N and water acquisition. The watering regime is not only important for crop growth, but also on soil N cycling. This idea of resource variability was addressed in this experiment, by comparing perfectly regulated water regimes to a more typical irrigation cycle. The intent was to mirror current climate change weather patterns, and focus on the irregularity of rainfall and supplemental irrigation. We also identified the importance of root growth in helping the plant to acclimatise to resource limitations. Identifying genetic differences of plants that can increase root growth under variable water conditions would allow more targeted breeding programs to be developed. Plants can adapt to extreme environments, whether extreme heat, cold, drought, flooding, or nutrient-poor soil; however, if environmental conditions regularly change, plants may be slow to adapt, making it hard for them to recover from these abrupt environmental changes. Understanding trade-offs between water and N uptake efficiency, as a result of variable water regimes, can lead to the development of

crop management strategies to help improve crop productivity and improve the environmental and economic sustainability of food production.

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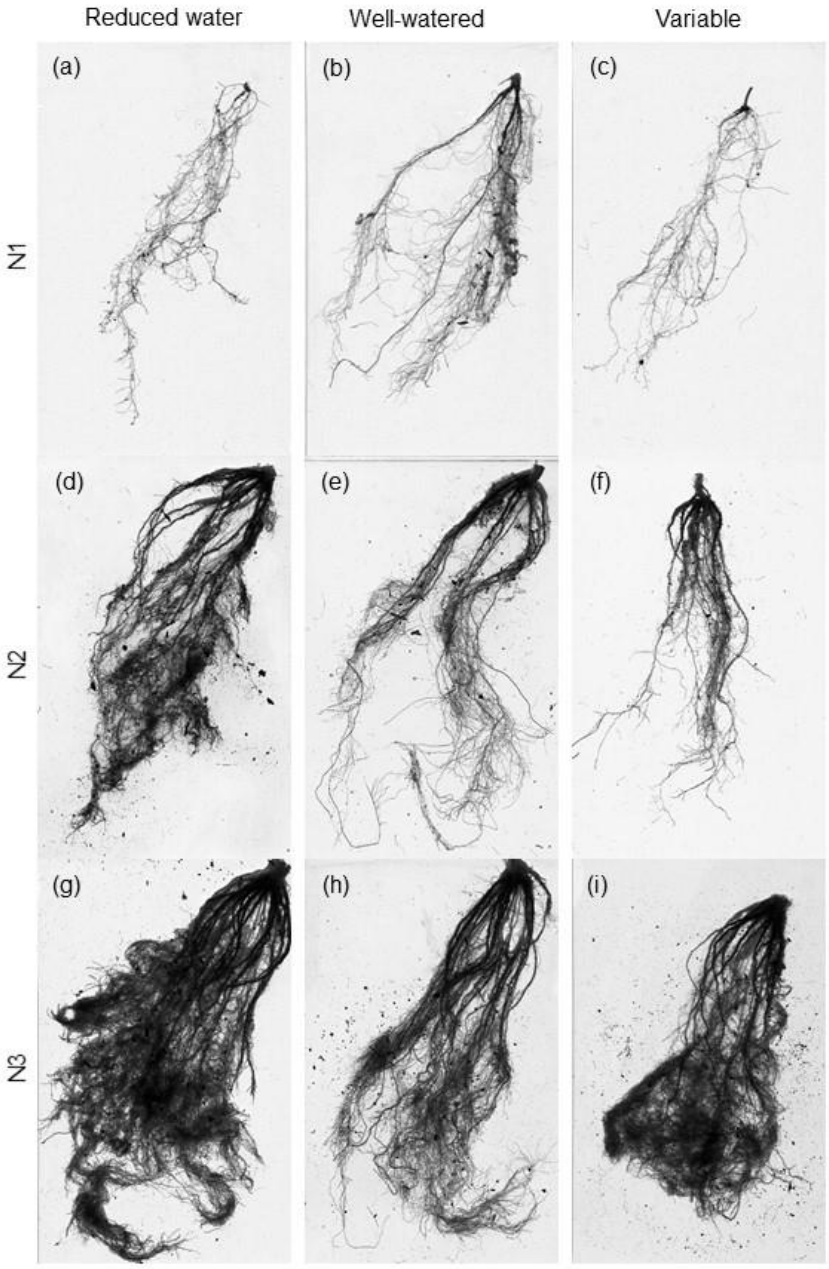
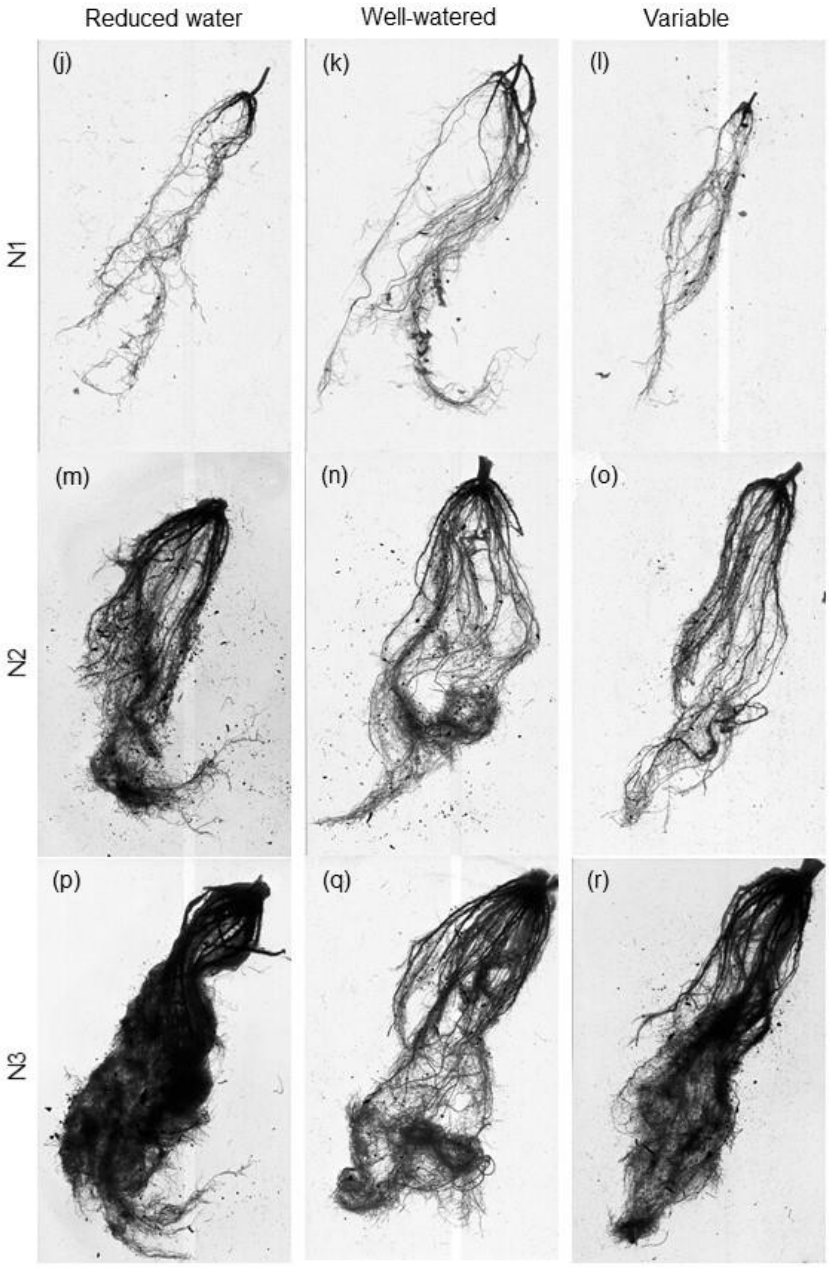
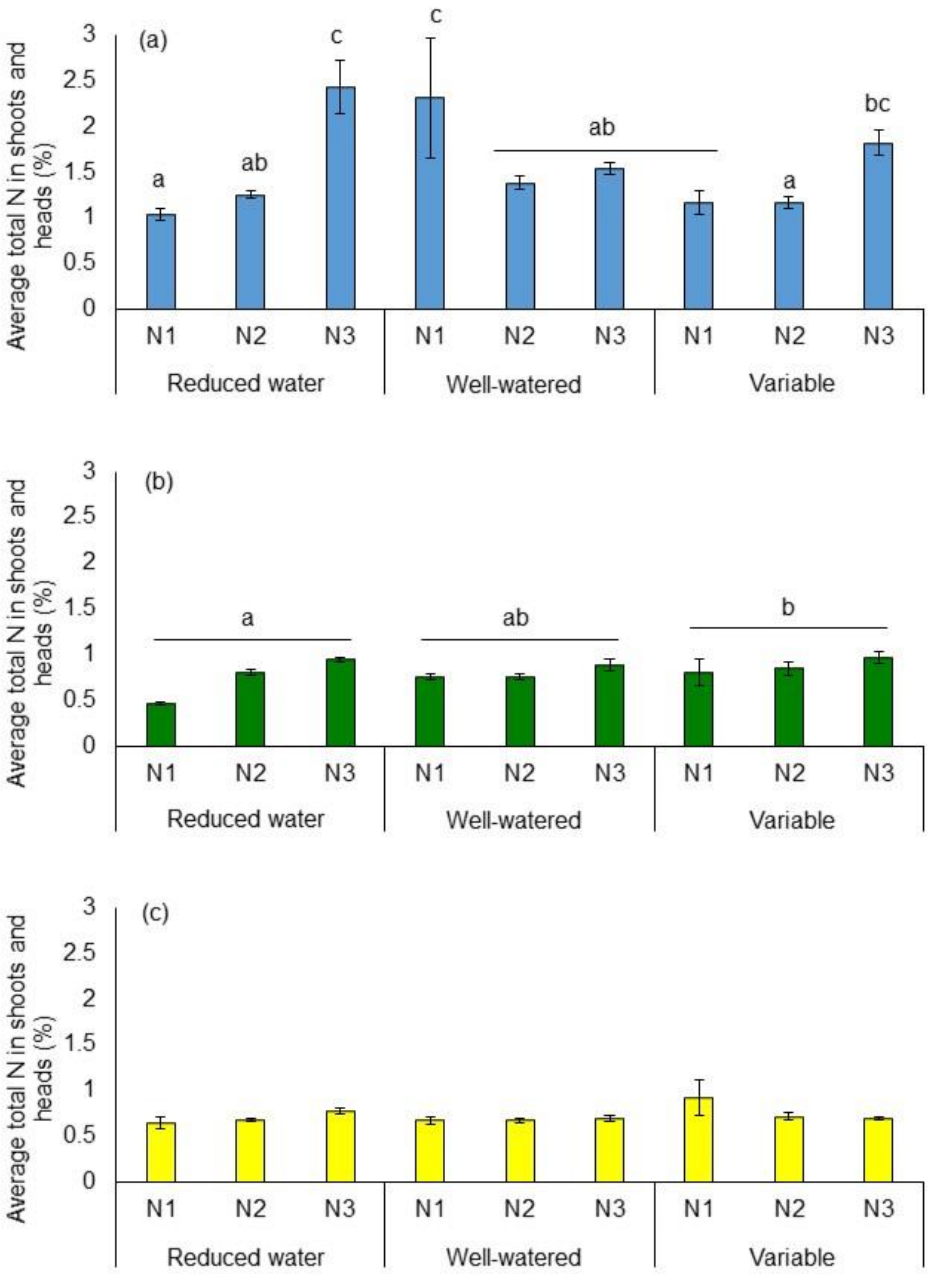
2.7. Supplementary Material



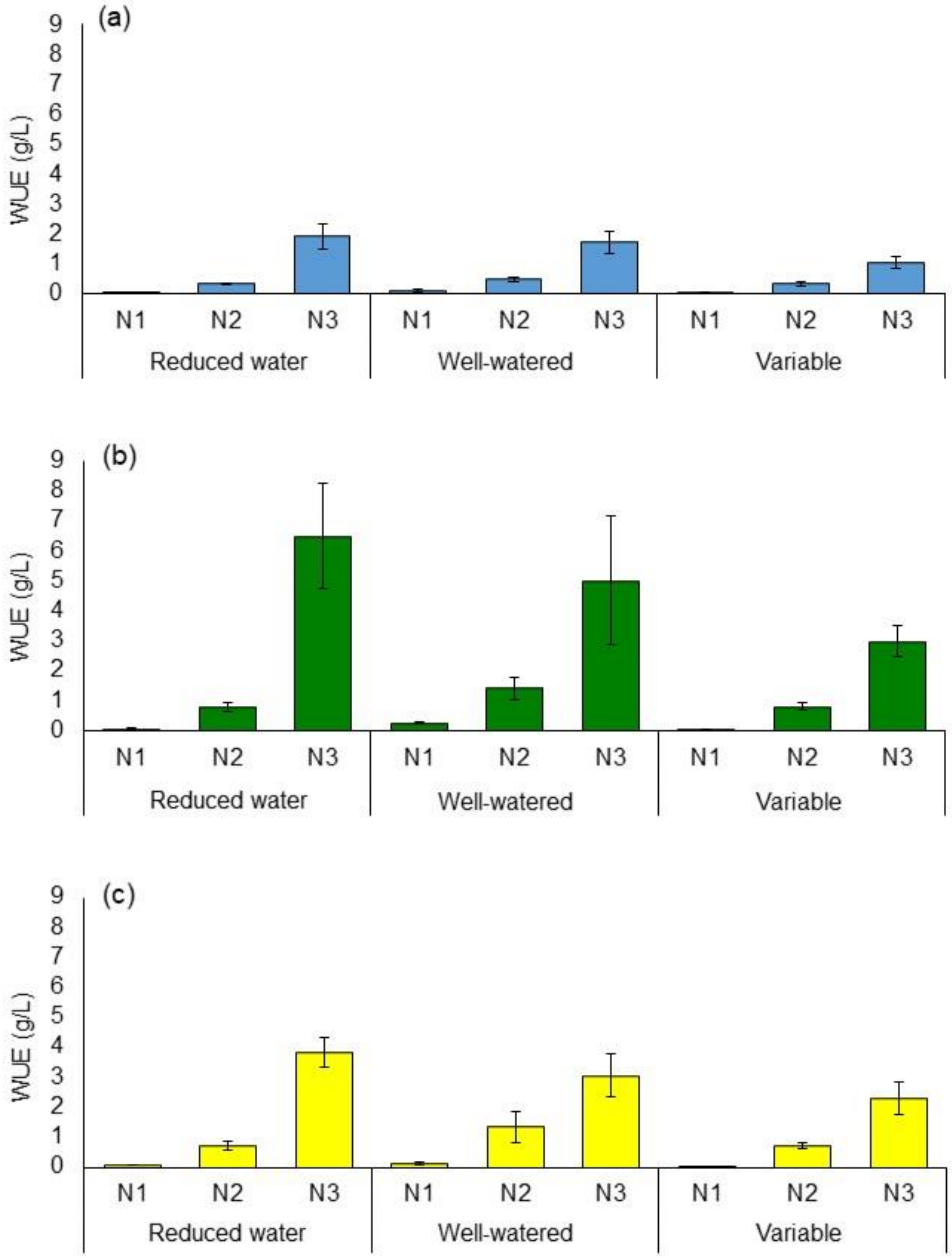
Supplementary Figure A.1. Roots for plants subjected to Reduced water (a, d, g), Well-watered water (b, e, h) and Variable (c, f, i) water treatments with N1 (0 mg of N/kg), N2 (25 mg/kg of N) and N3 (75 mg/kg of N) across Harvest 1; scanned using WinRhizo in a tray 200 cm by 300 cm.



Supplementary Figure A.2. Roots for plants subjected to Reduced water (j, m, p), Well-watered (k, n, q) and Variable (l, o, r) water treatments with N1 (0 mg of N/kg), N2 (25 mg/kg of N) and N3 (75 mg/kg of N) across Harvest 2; scanned using WinRhizo in a tray 200 cm by 300 cm.



Supplementary Figure A.3. Percentage of plant total N for Harvest 1 (a), Harvest 2 (b) and Harvest 3 (c); with three water treatments (Reduced water, Well-watered, Variable) and three nitrogen treatments: N1 (0 mg/kg of N), N2 (25 mg/kg of N), N3 (75 mg/kg of N). Significant interactions between water and N (Panel a) are represented by letters above bars. Error bars represent the standard error of the mean (SE), with n=4 except n=3 for treatments Harvest 1 Reduced water/N1 and Harvest 2 Well-watered/N3. Means with different letters are significantly different ($p<0.05$ or 0.001, LSD of means 5% level). Harvest 1 (Panel a) shows water × N interaction of $p<0.01$; Harvest 2 (b) shows main effects of water ($p<0.05$) and N ($p<0.001$; $N1^{a}$ $N2^{b}$ $N3^{c}$); and Harvest 3 (c) shows no significant differences between water and N ($p>0.05$).



Supplementary Figure A.4. Average water use efficiency (WUE) at Harvest 1 (a), Harvest 2 (b) and Harvest 3 (c) for three water treatments (Reduced water, Well-watered, Variable) and three nitrogen treatments: N1 (0 mg/kg of N), N2 (25 mg/kg of N), N3 (75 mg/kg of N). Error bars represent the standard error of the mean (SE), with n=4 except n=3 for treatments Harvest 1 Reduced water/N1 and Harvest 2 Well-watered/N3. Significant main effects of either water or N are shown in the legend using ANOVA and LSD of means 5% level. For all three harvests, the main effect of N is $p<0.001$, with N3 being significantly different from N1 and N2 for Harvest 1 and Harvest 2, and all N treatments different for Harvest 3.

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Chapter 3

Confirmation of soil moisture and nitrogen legacy effect on a second crop of wheat

The work contained in this chapter was conducted at the University of Adelaide, in collaboration with The Plant Accelerator, University of Adelaide (part of the Australian Plant Phenomics Facility).

3.1. Introduction

Increased variability in precipitation is becoming more frequent, and is only set to become more extreme (Jentsch *et al.*, 2007, Rebetzke *et al.*, 2009). These changes in frequency or quantity of precipitation can not only impose some level of stress on plant growth, but can also indirectly affect both soil nutrient status, by changing N properties and distribution.

Research has shown that soil moisture conditions prior to planting can have a profound impact on plant growth and soil properties (Burger *et al.*, 2005, Cavagnaro, 2016). This 'soil moisture legacy effect' has been documented (Burger *et al.*, 2005, Cavagnaro, 2016, Cui and Caldwell, 1997). Periods of soil wetting and drying, whether excessive (flooding) or not (seasonal rainfall), have been shown to impact soil nutrient availability. In the case of N, rates of mineralisation, ammonification and denitrification are strongly influenced by soil moisture (Burger *et al.*, 2005), with a 46% reduction of initial plant N in moist soil to 29% in repeatedly dried and wetted soil (Franzluebbers *et al.*, 1994).

Soil moisture conditions during a first crop growth not only impact the soil moisture legacy effect, but also impact biomass allocation of the first crop (directly) and the second crop (indirectly). Root biomass from the first crop over time can break down becoming part of the soil organic matter. This soil organic matter typically contains approximately 5% N and 50% C (Jackson *et al.*, 2008). This in turn drives the soil N cycle and affects the amount of labile N available. Another factor to consider is that the N and C cycles are closely linked, in that soil C availability from both soil organic matter and root exudates can drive microbial processes, releasing more plant-available N (Jackson *et al.*, 2008). However, it does mean that both soil C and soil N are in high demand by microbes, and soil N is in high demand by plants.

In Chapter 2 of this thesis, the effect of variable soil moisture and nitrogen (N) concentrations on biomass and N acquisition in wheat, and soil N cycling was studied. When that experiment was established, the intention was to include four harvest points at which destructive sampling would take place. However, plants had reached maturity by the time of the third harvest. Consequently, there was a fourth set of experimental plants surplus to the requirements of that work. Because of the predictions of increased variability in rainfall with climate change in many regions of the world, understanding soil moisture legacy effects and their impacts on plants will be important. Therefore, the aim of this experiment was to quantify the growth of a second crop of wheat grown in soil that had previously had a crop of wheat grown in under three different soil moisture regimes and three rates of N supply (i.e. the remaining soil cores from the work presented in Cousins *et al.* (2020) (Chapter 2)). Two hypotheses were established, (1) that a previous set of water and N treatments would leave a soil moisture legacy effect, resulting in less biomass for the second crop due to lowered nutrient resources; and (2) that re-wetting of soil for second crop would remobilise N after a long dry period. Above-ground biomass was quantified for both the first and second crop growth, with soil mineral N pools measured simultaneously.

3.2. Materials and Methods

3.2.1. Soil conditions

The soil medium used in this experiment was composed of a 1:1:1 ratio of clay loam, UC mix and cocopeat. This was the same soil used in a previous wheat experiment observing wheat growth response to variable water and N treatments (Cousins *et al.*, 2020; Chapter 2).

3.2.2. Plant sampling and analysis

The pots with full grown plants were taken off the automated gravimetric watering system (DroughtSpotter, Phenospex, Heerlen, the Netherlands) after Harvest 3 ((Figure 3.1; Cousins *et al.*, 2020; Chapter 2) was completed, and moved to a glasshouse (School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, PMB1, Glen Osmond, SA, 5064, Australia). In terms of the experiment described in this chapter, this is referred to as Time point 1 (DroughtSpotter removal) hereafter. These pots had initially gone through different watering treatments, shown in Figure 1, under three different N treatments.

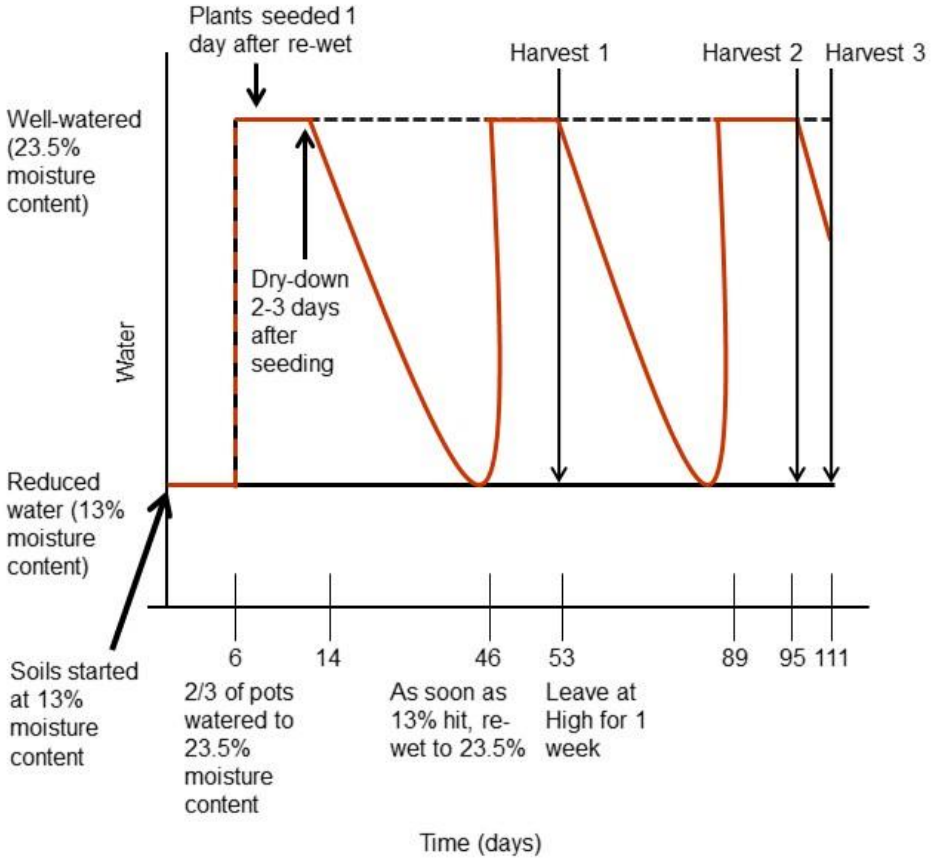

Figure 3.1. Watering regimes (schematic) applied to wheat as a percentage of the soil's gravimetric moisture content: 13% of gravimetric moisture content (solid black line); 23.5% of gravimetric moisture content (dashed black line) and variable (wetting-drying) cycle (watered to 23.5%, dried to 13%, then watered up to 23.5%, solid red line peak and troughs).

At Time point 2 (Crop 1 harvest, Table 3.1), shoots of the first crop of wheat were harvested. Watering was stopped from this point, and pots were left undisturbed for 7 months, then soil moisture measurements were taken (Time point 3). Additionally, at Time points 4, soil cores were taken from the pots to determine NH_4^+-N and NO_3^--N concentrations. At Time point 5, Crop 2 of Gladius was sown into the same soil from which Crop 1 had been harvested. These plants were grown in the same glasshouse and watered thrice weekly to 23.5% of gravimetric soil moisture content.

Table 3.1. Length of time (in days) between important experimental stages (labelled as Time points 1-6).

	Description of experimental stages and analysis	Months after DroughtSpotter removal
Time point 1	Pots taken off DroughtSpotter.	0
Time point 2	Crop 1 harvested, shoots and heads only.	4
Time point 3	Soil taken after Crop 1 harvest, before watering recommenced, and soil moisture measured on these samples.	7
Time point 4	Soil cores taken as soon as soil moisture reached 23.5% moisture content. Soil was tested for NH_4^+-N and NO_3^--N concentrations.	8
Time point 5	Sowing of Crop 2.	8
Time point 6	Crop 2 harvested, shoots only. Soil cores were taken for N analysis.	9 ¾

At Time point 6, the shoots from Crop 2 were harvested. Roots were not collected at either harvest time point, because it was impossible to wash roots completely from soil and separate the two root systems. All plant biomass was oven-dried (60 °C) until a constant weight was achieved (72 h), and dry weights recorded. The dried shoot material was homogenised by grinding to a fine powder for 1-2 min using a ring mill (Standard Ring Mill, SRM-RC-3P; Rocklabs Ltd, Auckland, New Zealand) with a stainless-steel head (CHRO-40-BLP or CHRO-200-BLRP depending on the size of plant biomass).

The glasshouse conditions were 22°C day and 17°C night with supplemental lighting (1000 W metal halide lamps) for a 16/8 hours day/night photoperiod. The weights of all the pots were taken thrice

weekly (Monday, Wednesday, Friday), and the weights of the pots, saucers, anti-evaporative mesh were subtracted from the final pot weight. Additionally, the weights of the intact drying shoot and heads for Crop 1 were estimated from shoot biomass values at Harvest 3 (Cousins *et al.*, 2020; Chapter 2). These estimated weights were then subtracted from the final pot weight to give the estimated soil weight over time (Figure 3.2). This figure shows the dry-down of the soil over time.

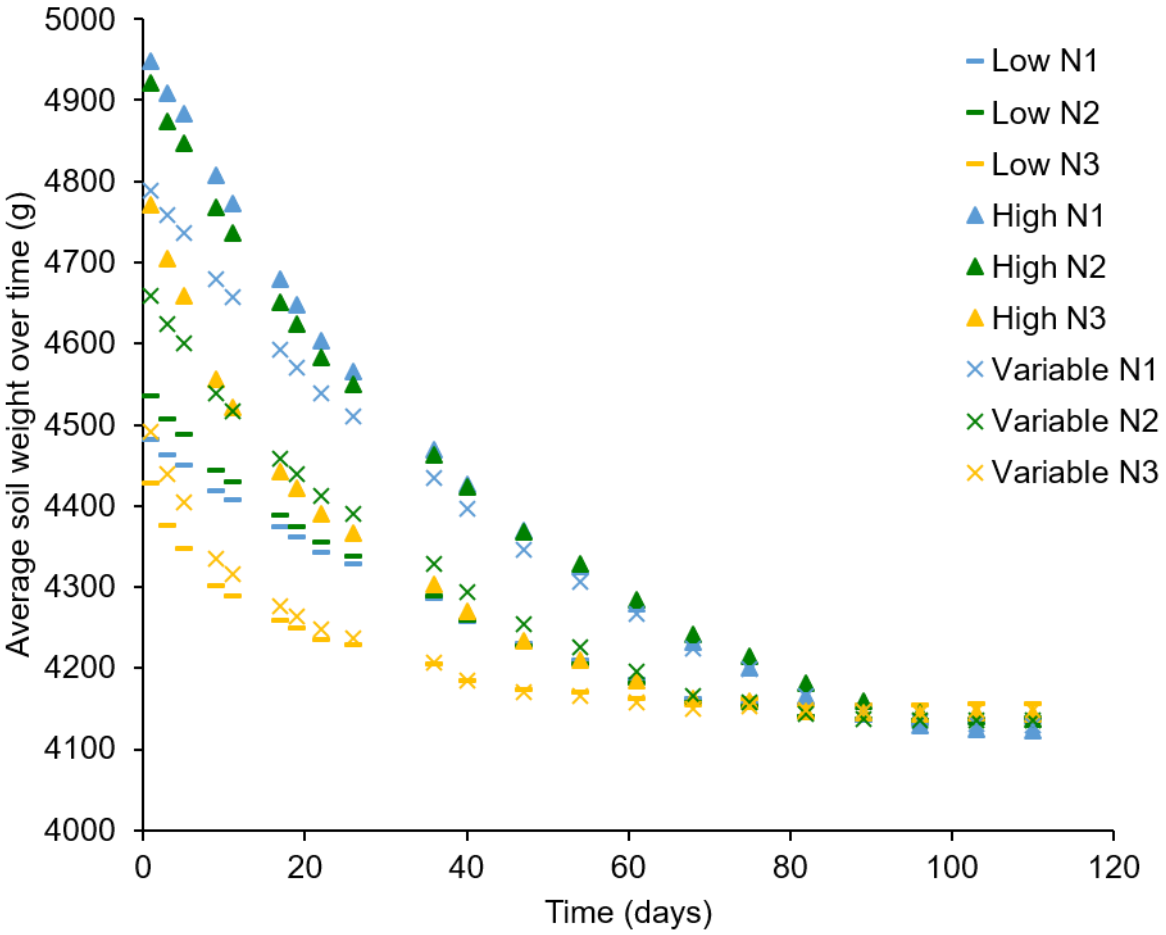

Figure 3.2. Average moist soil weight (minus weight of pot, saucer, anti-evaporative mesh and estimated shoot dry weight, based on shoot weight values from Harvest 3, Cousins et al., 2020) during the post-harvest dry-down.

3.2.3. Water treatment set-up

Following the harvesting of Crop 1 shoots and heads (Time point 2), the soil was slowly rewet (Time points 2- 4). For the first week, this was done be placing ice cubes on the surface of the soil; this approach allows for a slower distribution of water across the soil (which was already very dry). After this, water was applied to the top of the soil until a gravimetric moisture content of 23.5% was achieved; this moisture content was identified as optimal for plant growth in Experiment 1 (Cousins *et al.*, 2020; Chapter 2).

3.2.4. Soil sampling and analysis

At both harvests (Crop 1 and Crop 2) for every pot, a soil core was taken from each pot (approximately 100 g) and divided into two sub-samples. The first sub-sample was extracted with 2M KCl (shaken for 20 min, centrifuged), and the extracts (supernatant from centrifugation) analysed for NH_4^+-N (Forster, 1995) and NO_3^--N (Miranda et al., 2001). For NH_4^+-N analysis, two reagent mixes were made and added to a sub-sample of KCl-soil extract. Reagent A used 6.5 g sodium salicylate, 5 g sodium citrate, 5 g sodium tartrate dibasic dihydrate and 0.025 g sodium nitroprusside in 100 mL milliQ water (bottle wrapped in aluminium foil). Reagent B used 6 g sodium hydroxide and 2.4 mL bleach (4% sodium hypochlorite) in 100 mL milliQ water (bottle wrapped in aluminium foil). Standards of NH_4^+ NO_3^- were made up according to Table 3.2. Into a 96-well plate, 127 µL of Reagent A and B were added to 45 µL of extract or standards, and samples were left to develop for minimum 1 h. The plate was read at 650 nm in a spectrophotometer.

For NO_3^--N analysis, two reagent mixes were made and added to a sub-sample of KCl-soil extract. To make Solution 1, 0.4 g of vanadium chloride and 50 mL of 1 M hydrochloric acid (bottle wrapped in aluminium foil). For Solution 2, 0.2 g of sulphanilamide and 0.01 g of NED (N-(1-naphthyl)ethylenediamine dihydrochloride) were dissolved in 400 mL of milliQ water (bottled wrapped in aluminium foil). Solution 1 was added to Solution 2 using a ratio of 5:40 (Solution 1:Solution 2). Into a 96-well plate, 295 µL of Solution1:Solution 2 mixture was added to 6 µL of extract or standards, and samples were left to develop overnight. The plate was read at 540 nm in a spectrophotometer.

Table 3.2. Standards used for NH_4^+-N and NO_3^--N analysis, made from a $NH_4^+NO_3^-$ solution.

Standards of $NH_4^+NO_3^-$ used for NH_4^+-N analysis (ppm)	Standards of $NH_4^+NO_3^-$ used for NO_3^--N analysis (ppm)
0	0
0.1	1
0.2	2
0.5	4
1	5
2	6
4	8
5	10
6	12
8	16
10	18
20	22
	25

The second sub-sample was analysed for gravimetric moisture content, by weighing out a measured amount of wet soil, baked at 105°C, and weighed post-drying. In order to calculate soil moisture loss, the following calculation was used:

$$\frac{(weight\ of\ wet\ soil - weight\ of\ dry\ soil)}{weight\ of\ dry\ soil}$$

3.2.5. Statistical analysis

Data were analysed by two-way ANOVA, with N addition and watering treatment as factors in the model. Response variables included in the analysis were shoot and head dry weights, soil NH_4^+-N, NO_3^--N content, and total mineral N. Where the ANOVA revealed a significant treatment effect, significant differences between individual treatments were identified using Tukey's HSD/LSD tests. All statistical analysis was performed in R, with data also checked for normal distribution; additional R packages used were 'agricolae' and 'car' (version 3.2.5).

3.3. Results

Biomass and soil mineral N data are presented for Crop 1 and for Crop 2.

3.3.1. Biomass allocation

The shoot dry weight for Crop 1 increased with increasing N, with little difference between the water treatments (Figure 3.3a). For head dry weights, yield was the greatest in the Reduced water N3 (75 mg N/kg of soil) treatment. Growing a (second) wheat plant in soil that have previously had a wheat plant grown in it under various soil moisture and N treatments, produced some interesting results. Specifically, for Crop 2 harvest (Time point 6), plant biomass was much lower than Crop 1 (Figure 3.3b). The most striking result was the large biomass of the plants in the Variable N3 (75 mg of N/kg of soil) treatment compared to all other treatments in Crop 2.

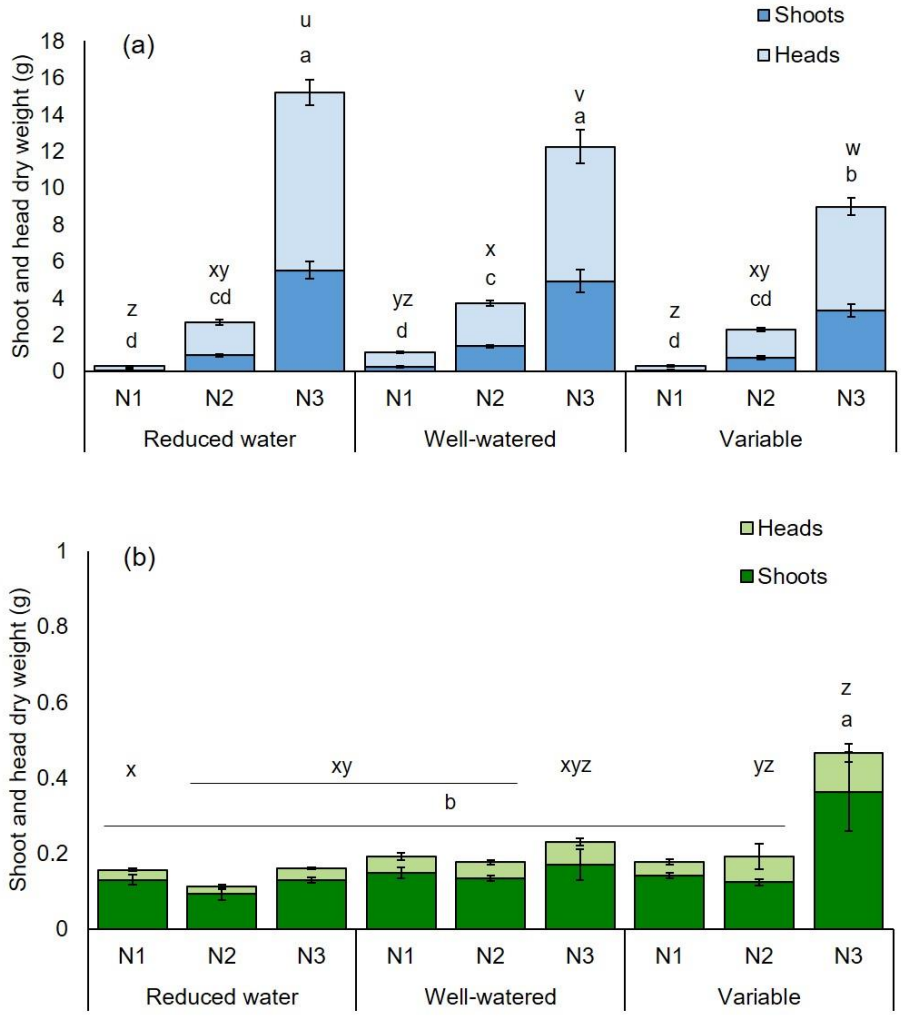

Figure 3.3. Average shoot and head dry weight of Gladius for Crop 1 (Time point 2; 4a) and Gladius Crop 2 (time point 5, b), with three water treatments (Reduced water, Well-watered, Variable) and three N treatments: N1 (0 mg/kg of N), N2 (25 mg/kg of N), N3 (75 mg/kg of N). Values are presented as mean values ± SE, n=4. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$). Crop 1 harvest (a) shows a significant interaction, with letters a-d for shoot dry weight and u-z for head dry weight values. Crop 2 harvest (b) shows a significant interaction between water and N, with letters a-b for shoot dry weight and x-z for head dry weight.

3.3.2. Nitrogen dynamics

Soil collected at the time of Crop 2 sowing contained significantly (eight times) more mineral N (and in particular NO_3N, with very little NH_4^+-N) in the N3 (75 mg of N/kg soil) treatments than all other N addition treatments, irrespective of soil moisture treatment (Figure 3.4a and c). When Crop 2 was harvested, mineral N (NO_3^--N and NH_4^+-N) was substantially lower than when Crop 2 was sown (note the smaller scale) and did not differ between any of the treatments (Figure 3.4b, d).

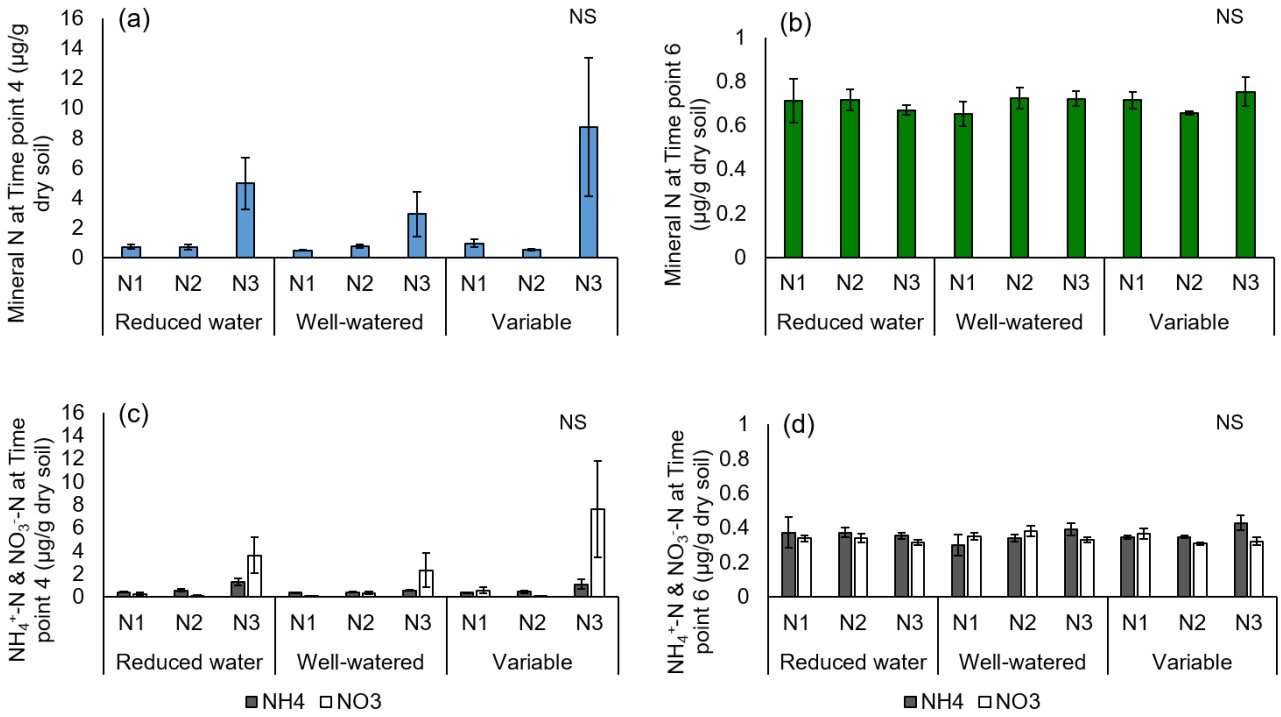

Figure 3.4. Average total concentrations of NH_4^+-N and NO_3^--N µg/g dry soil for Crop 2 (pre-sowing (a) and harvest (b)); total mineral N concentrations comprising of NO_3^--N and NH_4^+-N µg/g dry soil are shown for Crop 2 (pre-sowing (c) and harvest (d)), with three water treatments (Reduced water, Well-watered, Variable) and three N treatments: N1 (0 mg/kg of N), N2 (25 mg/kg of N), N3 (75 mg/kg of N). Values are presented as mean values ± SE, n=4.

3.3.3. Water dynamics

After pot removal from DroughtSpotter, pots were watered to weight every other day (Monday, Wednesday, Friday) to 23.5% gravimetric water content (Table S1.). The average cumulative water use remained similar across all combinations of treatments, other than Reduced water N3 (75 mg of N/kg soil), for which average cumulative water was 1.5x more than other treatments, indicating greater plant demand for water.

3.4. Discussion

The aim of the work presented in this chapter was to explore the potential legacy effects that might arise from the experimental treatments imposed in Cousins *et al.* (2020) (Chapter 2). This work is preliminarily in nature, but highlights the impact of the soil moisture legacy effect on future seasons of crop growth.

The results show the existence of a legacy effect of previous watering and N supply treatments. Specifically, the previous soil moisture and N regimes and a previous crop harvest did affect growth of a second crop in addition to soil N dynamics. This is consistent with previous work showing that pre-

planting moisture regimes alter soil nutrients and subsequent plant growth (Burger *et al.*, 2005, Cavagnaro, 2016, Meisner *et al.*, 2013). The response of the second crop to the soil moisture and N legacy effect are discussed, alongside the potential implications of these results in a climate with increasing rainfall variability.

3.4.1. Soil moisture and N legacy effect on plant physiology

As expected, the biomass of the first crop was strongly affected by the treatments they were exposed to in Experiment 1(Cousins *et al.*, 2020; Chapter 2). In contrast, in the second crop, the Variable N3 treatment only had a much higher shoot dry weight than all other treatments. There are several possible explanations for this. Firstly, the Variable water treatment in the first crop would have created both nutrient hotspots and nutrient-poor zones, due to some N being trapped in dry soil patches (Cousins *et al.*, 2020, Cui and Caldwell, 1997, Harrison-Kirk *et al.*, 2013). Nitrogen is heavily dependent on soil moisture for movement and conversion to plant-accessible ions, i.e. NH_4^+-N and NO_3^--N (Burger *et al.*, 2005, Ivans *et al.*, 2003). Secondly, with the flush of soil moisture in the second crop, this would have likely encouraged microbial activity by the release of accessible N from previously dry microsites (Burger *et al.*, 2005, Cui and Caldwell, 1997), allowing N (in the form of urea) that was previously locked in dry patches to be mineralised into NH_4^+-N and NO_3^--N. The regular addition of water during the second crop – equivalent to the Well-watered treatment of the first crop – would have allowed regular movement of N through the soil system, subsequently allowing the plant roots to access mineral N present (Cui and Caldwell, 1997).

With regards to the dry head weights (Crop 1), it is possible that Reduced water encouraged more shoot growth to increase flowering (Abid *et al.*, 2016, Chaves *et al.*, 2002). An earlier flowering time and a shorter vegetative phase could be a water stress coping mechanism/strategy (Shavrukov *et al.*, 2017). From an evolutionary perspective, this would enhance the survival and fecundity (i.e. increase fitness) (Abid *et al.*, 2016, Cossani *et al.*, 2010). An increase in flowering has the potential to increase grain yield, but the presence of water or nutrient stress can increase the risk of sterile flowers, resulting in grain yield reduction (Ferrante *et al.*, 2012, Shavrukov *et al.*, 2017).

3.4.2. Soil moisture and N legacy effect on soil N dynamics

The previous water treatments and first crop growth resulted in the mineral N levels present at the first crop. For the second crop, the highest water treatment from the first crop (Well-watered, Cousins *et al.*, 2020) was used; having a higher soil moisture content could have flushed out any remaining mineral N (Cavagnaro, 2016, Cavagnaro *et al.*, 2015, Cui and Caldwell, 1997). Additionally, it could have increased microbial activity, which in turn could have increased rates of mineralisation (from the decomposition of roots from the first crop) and/or denitrification, thus increasing ammonium and decreasing NO_3^--N concentrations, respectively (White, 2005). Another possibility is that the previous Well-watered treatment from the first crop could have fully recycled most, if not all, N available to

plants (Cossani *et al.*, 2010, Ivans *et al.*, 2003). However, with the Reduced and Variable water treatments from the first crop, it is possible that some N would still be locked up in dry microsites of soil and could potentially be flushed out by the addition of water in the second crop (Cui and Caldwell, 1997, Harrison-Kirk *et al.*, 2013).

In the first crop, soil concentrations of NO_3^--N under N3 treated plants were higher than concentrations of NH_4^+-N, which could suggest that rates of nitrification were higher than rates of ammonification (Cavagnaro, 2016). However, by the second crop, both NO_3^--N and NH_4^+-N levels had drastically decreased and did not differ significantly from each other. This is possibly due to the second crop utilising nutrients remaining in the soil. After the first crop harvest, the soil moisture drastically decreased due to the enforced dry-down, subsequently, whatever N remained in the soil was most likely immobilised without water and remobilised upon re-watering of the soil in preparation for the second crop (Cui and Caldwell, 1997). By measuring both NH_4^+-N and NO_3^--N concentrations, it is possible to see how the different N forms responded to the legacy soil moisture and N treatments. At the first crop harvest, NO_3^--N did not differ from NH_4^+-N, however, under N3 treatments, there was more NO_3^--N present. This pattern was not observed at the second crop harvest, with similar concentrations present of both NH_4^+-N and NO_3^--N. A 20-fold decrease in NH_4^+-N and NO_3^--N concentrations at the second crop harvest compared to the first crop harvest, suggests the role of legacy effect, and gives credence to the hypothesis that N was immobilised during the period of low soil moisture between the first crop and second crop.

3.4.3. Soil moisture and N legacy effect on plant water use

The increase in cumulative water applied under the legacy Variable water is not completely unexpected, because other studies have confirmed an increase in plant water use, especially when coupled with high N (Elazab *et al.*, 2016, Shen *et al.*, 2013). Because the soil moisture content increased from 0% to 23.5% for the second crop, it was likely that the increased soil moisture infiltrated areas of dry soil which mmight contain immobilised N forms. The flush of water could encourage remobilisation of N ions, or mineralisation of N into plant-available forms (Burger *et al.*, 2005, Jackson *et al.*, 2008). Both the soil moisture and unlocked N encouraged plants under the legacy Variable water x N3 (75 mg of N/kg of soil) treatment to grow bigger, thus encouraging higher water use (Elazab *et al.*, 2016).

3.4.4. Conclusions

In this experiment, a legacy effect was visually apparent in the second crop growth, with less biomass for the second crop; this supports the first hypothesis. This was likely due to changes in soil N (and possibly other nutrients) and microbial activity associated with the different amounts of N added in Experiment 1 and the watering (which in turn impacted N availability). The re-wetting of the soil for the second crop did show a flush of N mineralisation, thus supporting the second hypothesis. In addition, the soil moisture legacy effect would have affected microbial activity, which in turn would drive the soil N cycle. The poor growth of the second crop, irrespective of the legacy effect, would most likely

be overcome with N fertiliser addition, but in an increasingly more variable climate, it will be important to better understand these processes, by understanding crop response to changes in environment both before and during planting. Moreover, in natural systems such processes may be important too. Although this was a preliminary experiment, it does suggest there is some interesting and promising future work to be done in this field.

3.5. Supplementary Material

Table S3.1. Water and nitrogen treatments for Crop 1 and Crop 2 of Gladius, with average total water added per pot (mL) for Crop 2. Crop 1water treatments were: Reduced water, Well-watered, Variable; and nitrogen treatments: N1 (0 mg of N/kg of soil), N2 (25 mg of N/kg of soil), N3 (75 mg of N/kg of soil). Crop 2 water treatment was Well-watered, with no additional nitrogen added.

	Crop 1		**Crop 2**		
Wheat cultivar	**Water treatment**	**Nitrogen treatment**	**Water treatment**	**Nitrogen treatment**	**Total water added per pot (mL)**
Gladius	Reduced water	N1 (0 mg of N/kg soil)	Well-watered	No added N	3284
		N2 (25 mg of N/kg of soil)	Well-watered	No added N	3141
		N3 (75 mg of N/kg soil)	Well-watered	No added N	4579
	Well-watered	N1 (0 mg of N/kg soil)	Well-watered	No added N	3453
		N2 (25 mg of N/kg of soil)	Well-watered	No added N	3487
		N3 (75 mg of N/kg soil)	Well-watered	No added N	3261
	Variable	N1 (0 mg of N/kg soil)	Well-watered	No added N	3310
		N2 (25 mg of N/kg of soil)	Well-watered	No added N	3202
		N3 (75 mg of N/kg soil)	Well-watered	No added N	3327

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Chapter 4

Frequency vs quantity: phenotypic response of two wheat varieties to water and nitrogen stress

The work contained in this chapter has been resubmitted to Agricultural Water Management, and is presented in ‘manuscript format’.

Author Contribution

Project supervision given by TP Garnett, A Rasmussen, SJ Mooney, RJ Smernik, and TR Cavagnaro. All practical work and analysis was done by OH Cousins. OH Cousins was also lead author for this paper, and therefore was largely responsible for all text. Statistical advice was given by CJ Brien.

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Frequency vs quantity: phenotypic response of two wheat to water and nitrogen variability

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Key words

Biomass allocation; nitrogen stress; nitrogen use efficiency; plant physiology; roots; *Triticum aestivum*; variable water; water stress; water use efficiency

4.1. Abstract

Due to climate change, water availability will become increasingly variable, in turn affecting nitrogen (N) availability. This increases pressure on plant plasticity, as plants must adapt quickly to these variations. Therefore, understanding the combined effects of variable water and N supply on biomass allocation is vital. Here we used a novel platform allowing fine-scale control of soil moisture dynamics to precisely compare the impact of quantity and frequency of water supply under variable N levels. Two wheat genotypes (Kukri and Gladius) were used in a factorial experiment with three N application rates (25, 75 and 150 mg N/kg soil) and five soil moisture regimes (either changing frequency or quantity of water). Water use, plant biomass and soil N were measured. Higher water content encouraged biomass, particularly root growth under medium N. Watering less frequently resulted in a greater biomass reduction than providing less water. Lower water contents encouraged an increase in root thickness, even when root biomass remained the same across N treatments, subsequently affecting mineral N use. Ultimately, reduced frequency of water was more detrimental to growth than reduced quantity of water. The preference in wheat for water consistency highlights an unexplored opportunity for optimising yield by identifying plant phenotypic responses.

4.2. Introduction

Providing global food security sustainably is one of the great challenges of our time. That we need to do so in a time of significant environmental change, makes achieving this goal all the more difficult. Current climate projections indicate increased variability in precipitation in many regions of the world (Rebetzke *et al.*, 2009). This is likely to have a profound impact on plants grown in rain-fed systems. Similarly, in irrigated systems as rainfall frequency becomes more variable, there will be increased competition for water resources. Taken together, these projected changes are likely to have an important impact on global food production systems. Without doubt, improving our understanding of crop responses to limited and/or variable rainfall and irrigation is a key priority for future efforts to enhance sustainable intensification of agriculture.

Plant growth is affected by amount, seasonality and frequency of water supply (Austin *et al.*, 2004, Gibson-Forty *et al.*, 2016, Izanloo *et al.*, 2008). It is well established that a reduction in rainfall significantly reduces grassland biomass compared to reduced rainfall frequency (Gibson-Forty *et al.*, 2016). In contrast, Padilla *et al.* (2013) found specific root length and total root length was higher when grassland species received the same amount of water once a week (pulsed) as opposed to thrice weekly (regular). There was substantial drought stress at the end of the pulsed watering cycle, however, it did improve water use efficiency, most likely due to the immediate replenishment of water, which in turn encouraged further root growth as plants search for water and/or nutrients. In other research, a soil water deficit can encourage roots to grow longer and deeper (Ješko *et al.*, 1997). These differences in plant trait responses highlight the importance and complexity of plant phenotypic plasticity. Taken together changes in the amount and frequency of supply can have both direct and indirect impacts on plants.

Water availability affects not only plant growth, but the plant available nutrients too. Plant growth is tightly coupled to soil nitrogen (N) supply. Lower soil N availability has been shown to encourage more root biomass in wheat (López-Bellido *et al.*, 2005). In contrast, too much soil N can be detrimental to growth, resulting in a decrease in root length in wheat (Comfort *et al.*, 1988).

Both the amount, form and behaviour of soil N are strongly affected by soil water supply (Burger *et al.*, 2005). Under drought conditions, N accumulation in soil is reduced due to a decrease in microbial activity, which slows N cycling (Jensen *et al.*, 2003). High rainfall or irrigation (water pulses) could encourage breakdown of N forms locked up in organic matter, thus increasing N availability (López-Bucio *et al.*, 2003, Schwinning and Sala, 2004, Wang *et al.*, 2015). These water pulses also increase the risk of N-fertiliser leaching and surface run-off into bodies of water (Bijay *et al.*, 1995, Carstensen *et al.*, 2014), resulting in more fertiliser being applied to compensate for low N availability. Therefore, it is necessary to understand how N behaves under different water conditions (mirroring erratic rainfall patterns), and how we can maximise nitrogen use efficiency under such water conditions. Moreover, a

more targeted approach to crop management is needed, where the plant response to water and N can be measured, and subsequently irrigation and fertiliser applications optimised.

Since weather events are becoming more extreme than what the plasticity of any one variety can cope with, genotypic diversity in crops is becoming increasingly important. Many plant varieties differ in their responses to water and nutrients, particularly adapting their roots to optimise growth (Hurd, 1964, 1974, Lynch, 1995b, Ober *et al.*, 2014).

Central to optimising water and nitrogen use efficiencies is root biomass allocation. Under any one condition, a root system that allows foraging for nutrients or water, but not at the expense of crop yield, is ideal (Elazab *et al.*, 2016). These root foraging strategies can be different dependent on depth of soil and water or nutrient hotspots. Because of the complexity of root plasticity in response to environmental conditions, identification of the different root traits beneficial to plant growth are still in progress. These results indicate a clear need for detailed understanding of plant responses to variable water and N resource supply if we are to identify target traits in plant breeding programs.

Understanding how water and N create variability in plant plasticity is an important but complex undertaking. Surprisingly few studies have quantified the combined impact of variable water and N supply on crops. This is in part due to difficulties associated with being able to precisely control and monitor soil water conditions in real time. It is possible to use wicking beds or tension tables, where pots are placed onto a bed of sand equilibrated to a precise matric potential allowing plants to take up water according to use (Araya *et al.*, 2010, Semananda *et al.*, 2016, Tinklin and Weatherley, 1968). However, such an approach does not lend itself well to establishing cyclic watering patterns which persist in the field. One way to overcome this problem is to use an automated, lysimeter, plant growth platform. This system (DroughtSpotter, Phenospex, Heerlen, Netherlands) is an automated gravimetric platform which allows very fine-scale control of soil moisture dynamics, by weighing each individual pot and measuring water added and therefore, can monitor plant adaptability by measuring the amount of water used over time due to environmental change, i.e. water or N treatments (Cousins *et al.*, 2020). The ability of the gravimetric system to simulate possible rainfall or irrigation outcomes in the field make it a unique tool to answer questions related to precision agriculture. Importantly, the gravimetric system records water use on a very fine temporal scale, thereby providing valuable insights into the water use of plants over their entire growth cycle.

Here we present results of an investigation into the combined effects of variable water and N supply on two cultivars of wheat (Gladius and Kukri). We hypothesised (1) that the frequency of watering would have a greater impact on plant growth than quantity, and (2) the water regimes imposed would affect N availability, uptake and subsequently create differences in carbon allocation. Based on the different water and N use efficiencies of both wheat cultivars, we also hypothesised that (3) there would be a

difference in growth between Gladius and Kukri. We specifically quantified both above- and below-ground biomass allocation and soil mineral N pools.

4.3. Materials and Methods

4.3.1. Plant growth experiment

The growth medium used for this experiment was a mixture of clay loam, UC (University of California; Baker, 1957) sand mix and cocopeat (1:1:1 W:W:W) (referred to as 'soil' henceforth). This medium has been used extensively in previous experiments on N and water responses of wheat (Cousins *et al.*, 2020, Honsdorf *et al.*, 2014, Takahashi *et al.*, 2015). Basal nutrients were added to all treatments, as follows: dolomite lime 0.98 g/L, ag lime 2.72 g/L, hydrated lime 0.63 g/L, gypsum 0.98 g/L, superphosphate 1.96 g/L, iron sulphate 2.45 g/L, iron chelate 0.163 g/L, micromax (ICL Australia & New Zealand, New South Wales, Australia) 0.98 g/L. This ensured N was the only limiting nutrient. The pots used were 2.5 L free-draining pots, with 2.2 kg of the air-dried soil added to each pot.

Seeds (two per pot) were sown directly into pots; half of the pots were sown with *Triticum aestivum* cv. Gladius, and others with cv. Kukri. Gladius was used because of its high yields under drought conditions, whereas Kukri was chosen because of its drought susceptibility (Bennett *et al.*, 2012, Izanloo *et al.*, 2008), and its high N use efficiency (Mahjourimajd *et al.*, 2016). After seedling emergence (five days after sowing), seedlings were thinned to one per pot and the soil surface was covered with a semi-permeable mesh to allow water to filter through but minimise evaporative loss. To help measure evaporative loss, an additional 21 pots were set up as plant-free controls. These pots were only watered to the field capacity (FC) of this soil.

This experiment utilised an automated gravimetric watering system (DroughtSpotter, Phenospex, Heerlen, Netherlands). This system allowed for constant monitoring of water use and uptake over the whole experiment. Conditions in the glasshouse were 22/15°C day/night, and light levels were supplemented with 400 µmol/m2/s LEDs (GreenPower LED toplighting module DR/B HB 400V, Philips Electronics Australia Ltd, New South Wales, Australia) with a 12/12 h day/night photoperiod.

Experimental treatments: The experiment included three N addition treatments (Table 1). Urea was added to the soil as follows: 25N – urea added at a rate of 25 mg of N/kg of soil; 75N – urea added at a rate of 75 mg of N/kg of soil; 150N – urea added at a rate of 150 mg of N/kg of soil. Concentrations of ammonium (NH_4^+-N) and nitrate (NO_3^--N) at the start of the experiment were measured on 2 M KCl extracts, as described by Forster (1995) and Miranda *et al.* (2001). The starting soils of 25, 75, 150 mg of N/kg of soil had 28 ± 1.8; 88 ± 0.1; 116 ± 2.0 of NH_4^+-N (mg/kg dry soil); and 464 ± 66.4; 394 ± 50.7; 443 ± 30.5 of NO_3^--N (mg/kg dry soil), respectively (Supplementary Table S1). Concentrations of NH_4^+-N increased with increasing urea, whereas NO_3^--N remained at similar levels.

Table 4.1. Combinations of water and nitrogen treatments.

Water	**Nitrogen**
FC 16% gravimetric moisture	N1 (25 mg N/kg soil) 75N (75 mg N/kg soil) 150N (150 mg N/kg soil)
FC 48h Watered every 48h from 13% to 16%	25N (25 mg N/kg soil) 75N (75 mg N/kg soil) 150N (150 mg N/kg soil)
0.5FC 8% gravimetric moisture	25N (25 mg N/kg soil) 75N (75 mg N/kg soil) 150N (150 mg N/kg soil)
0.5FC 48h Watered every 48h from 5% to 8%	25N (25 mg N/kg soil) 75N (75 mg N/kg soil) 150N (150 mg N/kg soil)
Wet/Dry cycle Dried down to 8%, then re-wet to 16% for 7 days (see Fig. 1)	25N (25 mg N/kg soil) 75N (75 mg N/kg soil) 150N (150 mg N/kg soil)

The gravimetric system was used to establish five carefully controlled and monitored watering regimes (Table 4.2). As the starting soil was wetter than the initial target moisture content, all pots were placed in the glasshouse and dried down until they reached a gravimetric soil moisture content of 16% (field capacity for this soil; FC) then held at this water content until water regimes were implemented between 12-14 days (with the exception of FC water treatment plants, see Table 4.2). Each pot took varying amounts of time to dry down to the required moisture content of 16%, before they started their specific water treatments. Once a pot had reached the required moisture content, the gravimetric system maintained its weight at that required target weight, until all pots had reached their target weights. Seeds of wheat cv. Gladius and Kukri were sown on day one after potting. The five water treatments were

implemented and included two kept at field capacity: one (FC) was watered as required every 10 minutes and one (FC 48h) was watered only every 48 hrs. A further two were kept at half field capacity: one (0.5FC) was watered as required every 10 minutes and one (0.5FC 48h) was watered only every 48 hours. The final treatment was a Wet/Dry treatment where pots were allowed to dry down to half of field capacity, then re-wet to field capacity and maintained there until harvest (Table 4.1). With the gravimetric system, every time the pot weighed 0.5% below the target weight equal to the target soil moisture content (16% for FC, 8% for 0.5FC), it was watered back to that soil moisture content. With the 48h watering treatments, pots were watered to target soil moisture content (13% or 5%) every 48 hours (FC 48h, 0.5FC 48h respectively) (Table 4.1).

Table 2.2. Water treatment start days.

Genotype	**Water treatment**	**Treatment start**	**Re-water treatment start**
Gladius	FC	Day 8-13	-
	FC 48h	Day 12-14	-
	0.5FC	Day 12-13	Day 26-32
	0.5FC 48h	Day 11-13	Day 28-38
	Wet/Dry	Day 12-13	Day 27-30
Kukri	FC	Day 2-8	-
	FC 48h	Day 12-14	-
	0.5FC	Day 12-13	Day 22-27
	0.5FC 48h	Day 12-13	Day 24-26
	Wet/Dry	Day 12-13	Day 23-26

4.3.2. Plant sampling and analysis

After 41 days of growth, plants were destructively harvested by carefully removing the soil from the pots (a sub-sample of soil was retained for N analysis – see below), and roots washed of any adhering soil using reverse osmosis (RO) water. Roots and above-ground biomass were then separated, and fresh weights determined. Total root length was measured (on a sub-sample of roots of a known fresh weight) using the gridline intersection method (Newman, 1966).

All plant biomass was oven-dried (60°C) until a constant weight was achieved and dry weights recorded and root:shoot ratios calculated. The dried shoot material was homogenised and ground to a fine

powder, using a ring mill (Standard Ring Mill, SRM-RC-3P; Rocklabs Ltd, Auckland, New Zealand) with a stainless-steel head (CHRO-40-BLP or CHRO-200-BLRP depending on the size of plant biomass) for 1-2 min. The ground samples were analysed for total nitrogen (TN) by dry combustion (http://www.apal.com.au/; Rayment and Lyons, 2011).

4.3.3. Soil sampling and analysis

At harvest, after root excavation, a soil sample was taken from each pot (approximately 100 g) and divided into two sub-samples. A sub-sample was used for analysis of NH_4^+ -N and NO_3^- -N, as described previously (see Section 4.3.1.).

4.3.4. Statistical analysis

The experiment used a split-plot design for each genotype, each genotype being located on different sides of the gravimetric system. For each genotype there were four replicates of the 15 combinations of N addition (three levels) and soil watering treatments (five levels), so that there were 120 pots in total. The Water treatments were assigned to the Main Plots and the Nitrogen treatment to the Subplots. A split-plot factorial ANOVA was performed on the 60 values of each variable for each genotype. In these analyses Blocks, Whole Plots and Subplots were represented by Replicates, Water treatment and Plants (detailing Nitrogen treatment) respectively. The data for each of the variables were tested for normality; some of the data was left as original and some were treated by applying either log base e or square root functions (Supplementary Table S2). Response variables included in the analysis were shoot and root biomass, soil NH_4^+-N, NO_3^--N content, shoot total N and soil total N. Where the ANOVA revealed a significant treatment effect ($p<0.05$), significant differences between individual treatments were identified using least significant differences of means at the 5% level (LSD of means) tests. All data were checked for normal distribution prior to analysis, and subsequent statistical analyses performed using GenStat 19th Edition.

4.4. Results

4.4.1. Plant biomass

There was a large genotypic difference in biomass accumulation and distribution between shoots and roots (Figure 4.1).

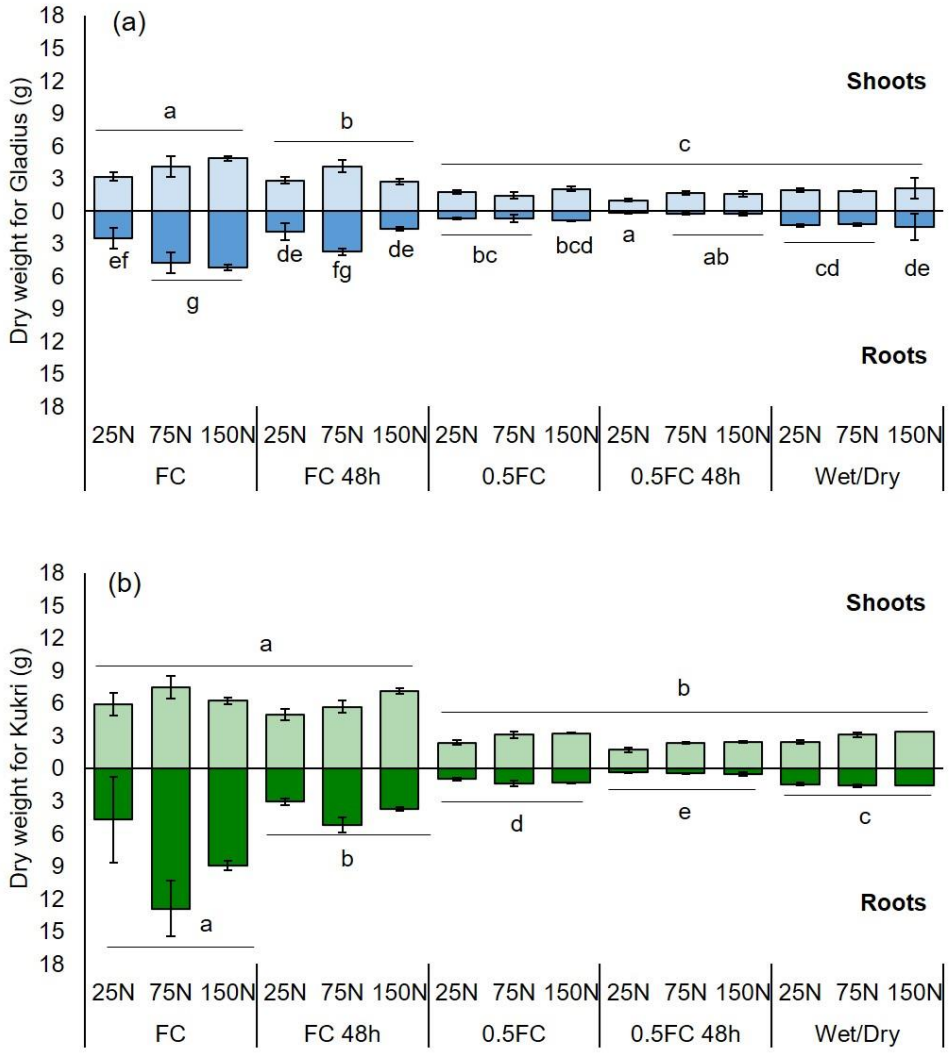

Figure 4.1. Average shoot dry weight (pale shading, above x-axis) and root dry weight (dark shading, below x-axis) for Gladius (a) and Kukri (b); with five water treatments (FC, FC 48h, 0.5FC, 0.5FC 48h, Wet/Dry) and three nitrogen treatments: 25N (25 mg/kg of N), 75N (75 mg/kg of N), 150N (150 mg/kg of N). Values are presented as mean values ± SE. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$ or 0.001). Gladius (a) shows significant main effect for shoots, letters above bars (water $p<0.05$; N not significant); for roots, water x N $p<0.05$, letters above bars. For Kukri shoots (b), main effects present for both water and N, letters for water above bars (water $p<0.001$ with N $p<0.05$, $25N^a$ $75N^b$ $150N^c$). For Kukri roots, main effects were present for water $p<0.001$ (letters above bar) and N ($p<0.05$; $25N^a$ $75N^b$ $150N^a$).

For Gladius, the shoot dry mass (Figure 4.1a) differed significantly between watering treatments irrespective of N addition treatment. Plants with access to full soil water capacity (FC and to a lesser extent FC 48h) had significantly higher above-ground biomass than plants with access to half the soil water capacity (0.5FC and 0.5FC 48 h) and the Wet/Dry cycling treatment. In contrast, Gladius root dry

weights were influenced by both water and N (significant interaction, $p<0.05$). Belowground biomass increased (to varying extents) with increasing N in all water treatments, except the FC 48h treatment where root dry weights were greatest in the intermediate N addition treatment (75N). For Kukri, both water and N addition treatments had a significant main effect on both shoot and root dry weights (Figure 4.1b). Kukri shoot dry weight differed between the wetter treatments (FC and FC 48h) and drier treatments (0.5FC, 0.5FC 48h, Wet/Dry) irrespective of N treatment, with more shoot biomass in the wetter treatments. For nitrogen, 25N differed from 75N and 150N (nitrogen $p<0.01$, $25N^{a}$ $75N^{b}$ $150N^{b}$). With Kukri roots, all water treatments resulted in differences in root systems regardless of the N treatments. The FC treatment resulted in particularly large root systems, and 0.5FC 48h treatment had the smallest root systems.

Specific root length (Figure 4.2) was highly variable among water treatments for both varieties. For Gladius, there was a significant interaction between water and N addition treatments, with specific root length generally greatest in the low N addition treatment, and especially so in the 0.5FC 48h and Wet/Dry treatments (Figure 4.2a). While Kukri showed a similar pattern overall, only the main effect of N addition treatment was significant with specific root length highest in the 25N treatment, lowest in 150N and intermediate in the 75N treatment (Figure 4.2b). However, the error bars for Kukri are small confirming little variation within treatments.

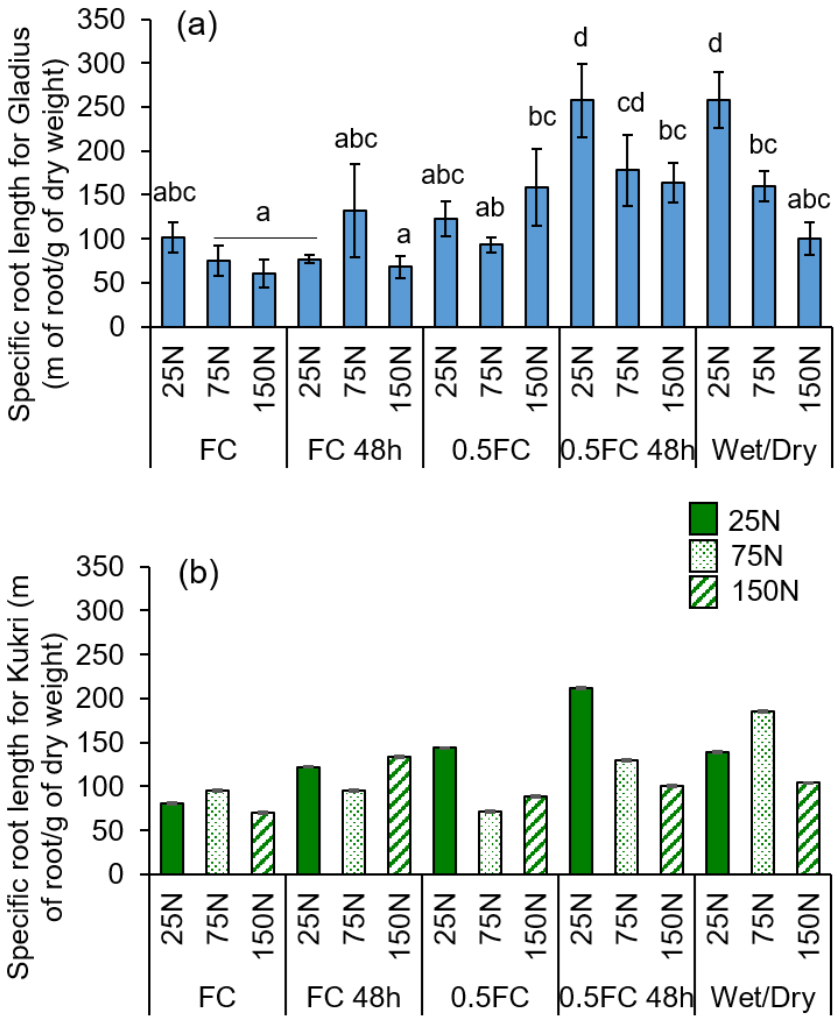

Figure 4.2. Mean specific root length for Gladius (a) and Kukri (b) under five water treatments (FC, FC 48h, 0.5FC, 0.5FC 48h, Wet/Dry), and three N treatments: 25N (25 mg/kg of N), 75N (75 mg/kg of N), 150N (150 mg/kg of N). Values are presented as mean values ± SE, n=4. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different. For Gladius (a), water x N $p<0.05$. For Kukri (b), only main effect of N present ($p<0.05$, $25N^{b}$ $75N^{ab}$ $150N^{a}$), where bars of a different pattern are different from each other.

4.4.2. Plant water use

In addition to providing a precise watering regime, the DroughtSpotter platform can also be used to record water applied (by weight). The amount of water applied per day was used to calculate cumulative water appled per pot for both varieties, with average total cumulative water shown in Table S4.1. The plant-free controls (watered to FC) received a total of 12.6 L water over 6 weeks, compared to 19.2 L water for the pots with wheat. From this, we calculated that the wheat pots had approximately an extra 6.7 L of water over the course of the experiment.

Water use efficiency, that is, the amount of plant biomass (g dry weight) produce per unit water (L) applied, was calculated (Figure 4.3). For Gladius, there was a significant interaction between water and N, with water use efficiency significantly lower in the 0.5FC 48h 25N treatment compared to the rest of the treatments (Figure 4.3a). However, with Kukri (Figure 4.3b), only water was significant as the main effect (regardless of N). FC water treatment resulted in the highest water use efficiency ($p<0.05$).

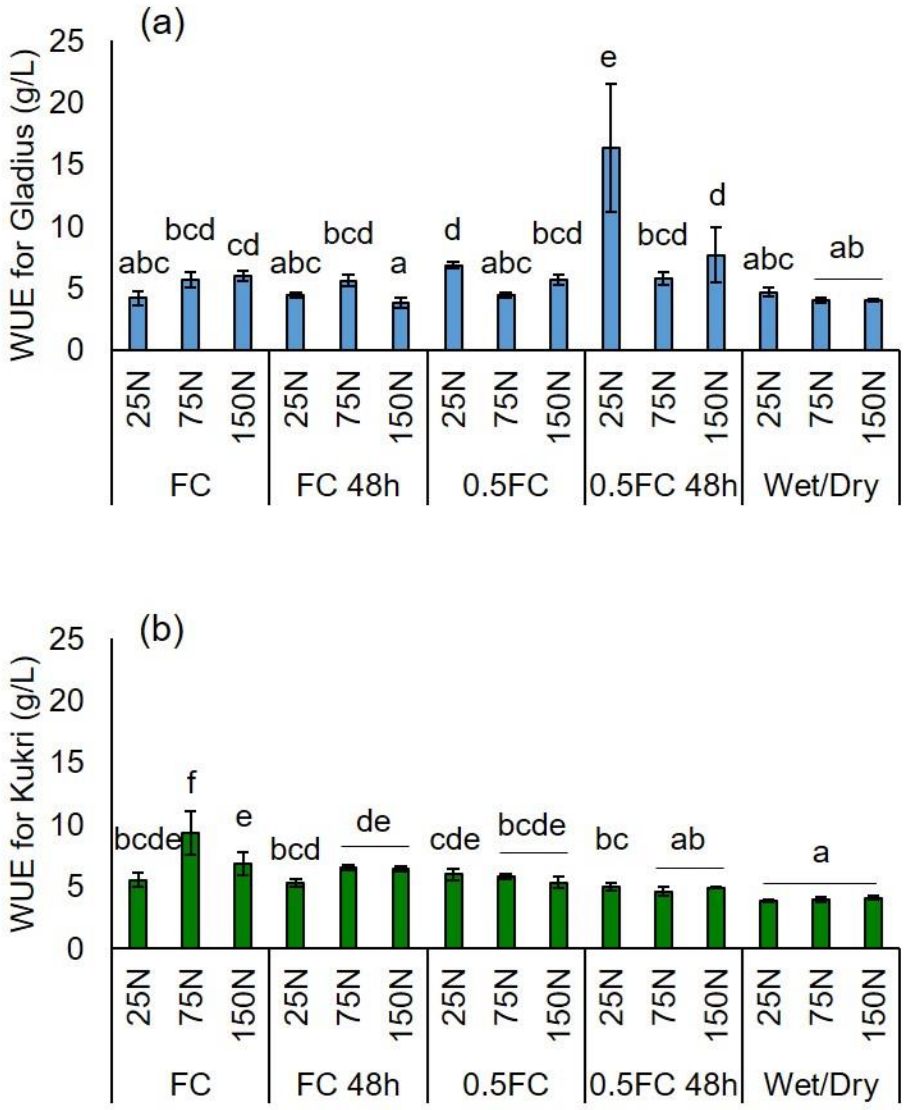

Figure 4.3. Average water use efficiency (WUE) for five treatments (FC, FC 48h, 0.5FC, 0.5FC 48h, Wet/Dry) and three nitrogen treatments: 25N (25 mg/kg of N), 75N (75 mg/kg of N), 150N (150 mg/kg of N). Error bars represent the standard error of the mean (SE), n=4. Bars with different letters are significantly different ($p<0.05$ or 0.001, LSD of means 5% level). For Gladius (a) and Kukri (b) significant interaction of water x N $p<0.05$ (letters of significance above bars).

4.4.3. Nitrogen dynamics

Plant nitrogen concentrations were affected by both water and N addition treatments (Figure 4.4). Overall, shoot total N did not differ greatly between treatments. However, there was a significant water × N interaction for Gladius (Figure 4.4a). With Kukri, the water treatments FC, 0.5FC and 0.5FC 48h did not differ from each other in terms of total N (regardless of N addition treatment), but were lower than for FC 48h and Wet/Dry treatments, regardless of N addition treatment (Figure 4.4b).

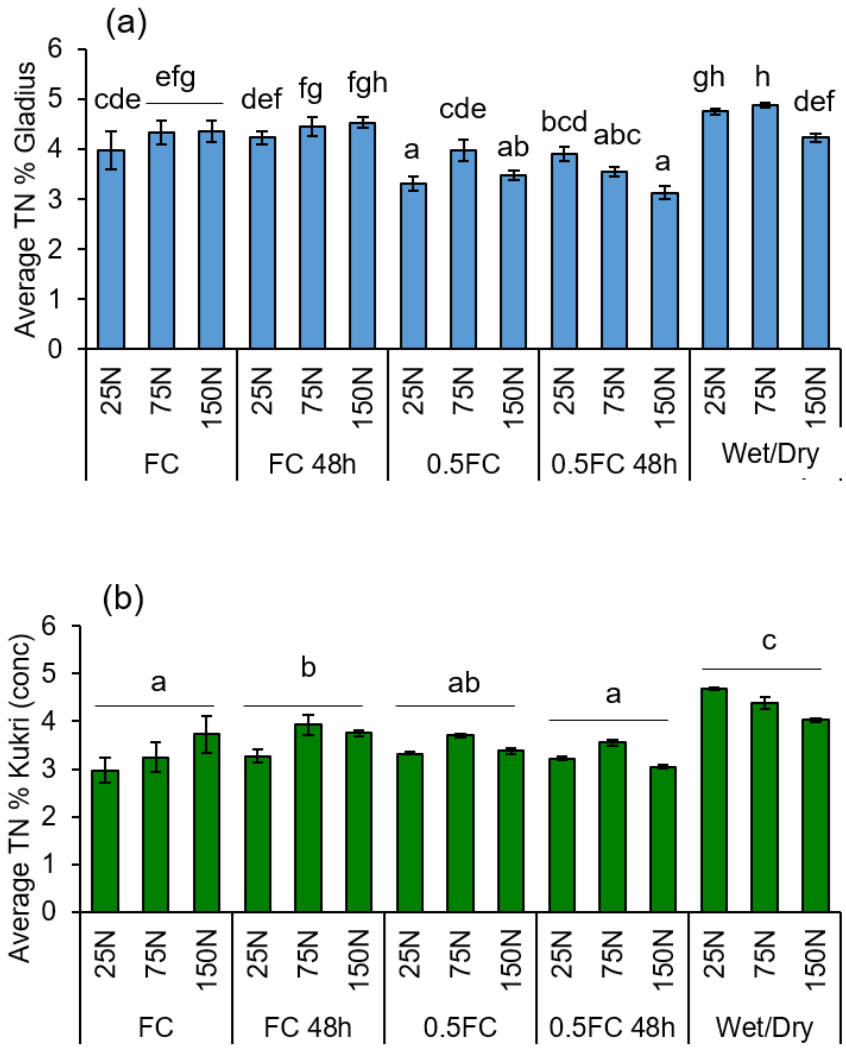

Figure 4.4. Average shoot total N as a percentage of dry weight for Gladius (panel a) and Kukri (panel b) under five water treatments (FC, FC 48h, 0.5FC, 0.5FC 48h, Wet/Dry), and three N treatments: 25N (25 mg/kg of N), 75N (75 mg/kg of N), 150N (150 mg/kg of N). Values are presented as mean values ± SE, n=4. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different. For Gladius (a), water x N $p<0.05$, letters above bars. For Kukri (b), main effects of water $p<0.001$, letters above bars, no main effect for N.

Mineral N in the soil at harvest (Figure 4.5) was calculated by adding NO_3^--N and NH_4^+-N concentrations; the soils were NO_3^- dominated with NO_3^--N values up to 13 times greater than NH_4^+-N concentrations (Supplementary Table S4.2).

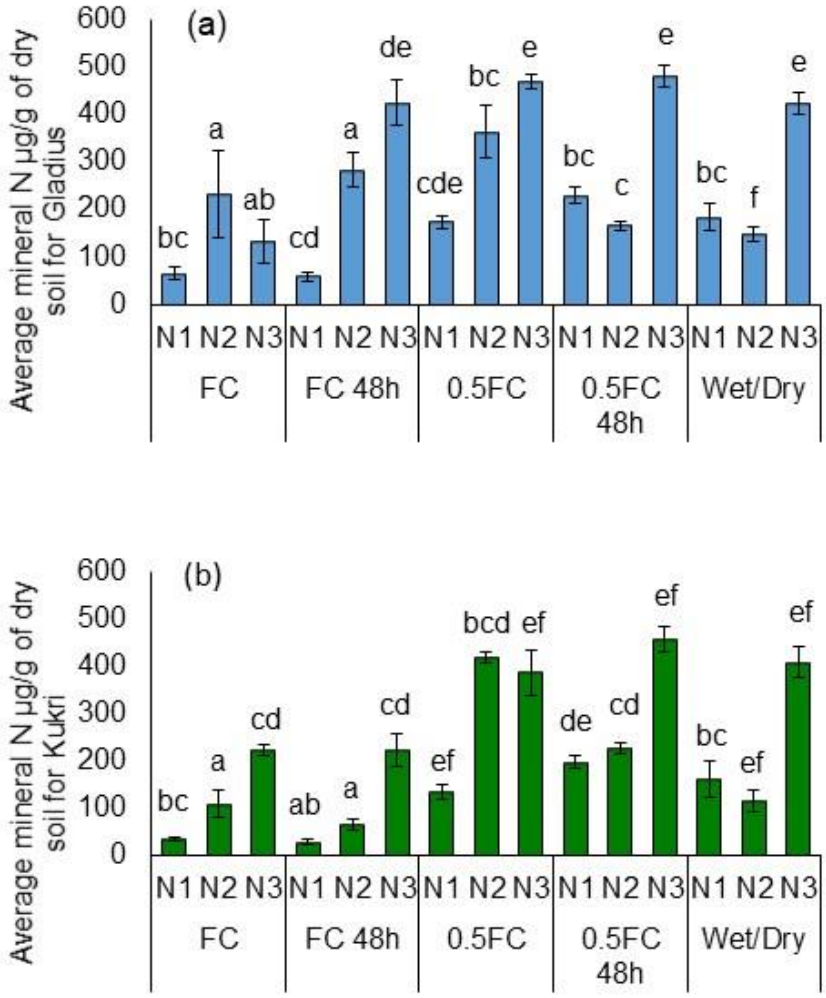

Figure 4.5. Total mineral N concentrations comprising of NO_3^--N and NH_4^+-N µg/g dry soil for Gladius (a) and Kukri (b). Error bars represent the standard error of the mean (SE), n=4. Significant interactions of water x N are represented as letters above bars, calculated using ANOVA and LSD of means. Means with different letters are significantly different ($p<0.001$, LSD of means 5% level). Gladius (a; $p<0.05$) and Kukri (b; $p<0.01$) showed a significant interaction of water × N ($p<0.05$), letters above bars.

Mineral N was highly variable within water and N treatments, and between genotypes. For both genotypes there was a significant interaction between water and N addition treatments. In the case of Gladius (Figure 4.5a) mineral N was generally highest in the 150N addition treatments, with the FC treatment the exception. Mineral N for Kukri (Figure 4.5b) was similar to that of Gladius, and was also generally higher in the 150N treatment. Overall mineral N for Kukri was generally lower in the FC and FC 48h treatments.

4.5. Discussion

4.5.1. Overview

Water and N treatments, whether in combination or alone, had significant impacts on plant water use, biomass accumulation, N uptake and soil N dynamics. There were also significant differences in genotypic response to these variables, with Kukri exhibiting greater N use efficiency and Gladius showing greater tolerance to water stress. Results are now discussed in the context of variability in water and N supply.

4.5.2. Plant physiological responses to water and nitrogen

Crops perform better with co-limitation of resources (both water and N limited). For example, Cousins *et al.* (2020) found that reduced water coupled with medium concentration of soil N encouraged plant growth, particularly increasing root growth. In other work, grain yield, NUE and water use efficiency increased with the increase in water and N co-limitation (Cossani *et al.*, 2010). However, the greatest yield gap (difference between maximum attainable yield and actual yield) was present with the highest stress. As a result, it is necessary to minimise the gap between potential yields and actual yields under stress. This concept of resource allocation to priority organs was suggested by Bloom *et al.* (1985), whereby plants adjust their carbon allocation particularly between shoot and roots until the ratio of produce made (sugars) to the cost of producing these sugars is equal.

Under optimal conditions, wheat response can differ depending on genotype. Gladius root biomass increased with increasing N when water content was held at FC. This is not unexpected, considering these would be classed as optimal conditions, with N as the only limiting factor. However, this pattern was not observed for Kukri. Instead, more root (and shoot) growth was observed under 75N. Due to Kukri's higher NUE, this has enabled it to adapt and produce more root and shoot growth under lower N levels. Interestingly, this pattern (greatest biomass at 75N) was present under FC 48h for both varieties. Nitrogen was the limiting factor for Kukri FC treatment, however, for the FC 48h treatment, N and water have the potential to limit growth for both varieties. It is possible that because FC 48h is a stress condition as water is not frequent, the plant response was to produce longer roots (not necessarily thinner) to access both N and water. Another possibility is that water pulses every 48h flushes the system, potentially saturating the soil and allowing for pockets of previously inaccessible urea to become mobile again and encourage mineralisation (Cui and Caldwell, 1997, Ivans *et al.*, 2003).

It is not surprising that 0.5FC 48h had the smallest root:shoot ratio for both varieties, because both the quantity and frequency of watering imposed a stress. The plants were smaller than Wet/Dry plants and also 0.5FC plants for Kukri. Not only was water a limiting factor, but also the timing of the watering played a huge part in whether the plants were able to adapt. There have been several studies that explore the pulsed water effect, frequency and quantity of water. Ivans *et al.* (2003) showed that with just one simulated rainfall event, plant acquisition of N was stimulated thus encouraging a higher root uptake of

N. There is the possibility that plants under the Wet/Dry treatment had more roots before the dry-down happened (especially at 25N when compared to Gladius). This could be surmised from the fact that the root biomass was significantly lower for 0.5FC plants than those under Wet/Dry. Gibson-Forty *et al.* (2016)showed that a reduction in rainfall quantity had a more severe impact on growth than a reduction in frequency. However, different magnitudes or frequencies of pulsed water supply would impact plants differently, even if when water quantity remains the same (Padilla *et al.*, 2013). Not only did the frequency and quantity of water affect plant growth, but it also impacted soil moisture and soil drying dynamics. This is consistent with other studies, where wetting-drying events or pulsed water events changed the way soil dries or re-wets (Fierer and Schimel, 2002, Padilla *et al.*, 2013). Having frequent watering but reduced quantity actually resulted in water deficit over the short period of time between watering, whereas less frequent watering resulted in wetter soil conditions for a longer period of time, most likely due to the larger water pulses (Padilla *et al.*, 2013).

Under variable water conditions (0.5FC, 0.5FC 48h, Wet/Dry), specific root length increased – with roots more prone to getting longer and thinner in order to access water or N pools further down the soil pot. Interestingly, although root biomass of both varieties stays relatively the same across 0.5FC treatments or Wet/Dry, the specific root lengths tell a different story. For Gladius and Kukri 0.5FC 48h and Gladius Wet/Dry, there was a decrease in specific root length with increasing N. Changes to specific root length can be explained by the idea of C allocation. Producing longer and thinner roots and reducing the mean root diameter creates a larger root surface (Fitter, 2002). This enables the plant to maximise water and N uptake, particularly in situations where these resources are limited. Ultimately, plant resource use comes at a cost to their biomass (Tataw *et al.*, 2016). Although specific root length does not quantify the actual thickness of the root, there are two assumptions that must be made: the length of the root is proportional to resource acquisition, and root biomass or the weight is proportional to the maintenance of the root/plant (Eissenstat and Yanai, 1997, Ostonen *et al.*, 2007). If a plant has thinner and longer roots, it becomes less expensive for the plant to maintain these, than if they were thicker (Withington *et al.*, 2006).

As the plants under more optimal conditions were larger, they had greater leaf area for transpiration to occur, and larger root systems to provide water to the leaves, and therefore these plants also required the most water added to each pot to replace transpired water. Although Gladius has previously performed better than Kukri under drought conditions (Bennett *et al.*, 2012, Izanloo *et al.*, 2008), Kukri was larger in this experiment. As Kukri has a higher NUE than Gladius (Mahjourimajd *et al.*, 2016), it is possible that this compensated for its drought susceptibility, by encouraging root growth to aid in nutrient and water uptake. Although Kukri was larger, this also meant it used more water than the smaller Gladius plants.

4.5.3. Soil and plant nitrogen dynamics

Despite the water and N variability imposed on both Gladius and Kukri, the average N uptake did not differ hugely between treatment combinations. The presence of an interaction between water and N for Gladius suggests it is better adapted to taking up N regardless of the stress imposed. However, with Kukri, water was the main driver for N uptake. These might be important considerations when deciding which genotype to grow in the field.

Mineral N analysis showed that the NO_3^--N values were up to 13 times greater than NH_4^+-N concentrations. Mineral N is also highly variable within water and N treatments, and between genotypes. The amount of mineral N left in the soil differs greatly between genotypes, with less mineral N available under FC or FC 48h conditions for Kukri compared to Gladius. Soil type is important with respect to nutrient availability. For example, a soil with a fine texture will have a greater flush of mineralised N, due to the greater water holding capacity (Austin *et al.*, 2004). In addition, a soil with a higher organic matter content, will have more pools of 'untapped' nutrients. A pulse of water could encourage movement of microbes and N solutes and unlock nutrient pools for roots to access (Cui and Caldwell, 1997, Gordon *et al.*, 2008).

Plant tissue N was generally uniform across the treatments, which suggests good internal N homeostasis. Overall, plant total N ranged between 3 and 6%, which is out of the critically N deficient range of 1.28-1.39% (as suggested by Reuter and Robinson (1997)). Our study highlights how carbon allocation in roots is also affected by N availability. Under high soil NO_3^--N concentrations, the shoots dominate assimilation of NO_3^--N, but this is an energy-intensive process. Under low soil NO_3^--N concentrations, root C:N ratio becomes higher, so most of the NO_3^--N is taken up and functions as a signal molecule to nitrate transporters, affecting auxin production which regulates root growth (Krouk *et al.*, 2010). Such conditions are likely to favour formation of lateral roots (Zhang *et al.*, 1999). As a result, very little of the assimilated NO_3^--N is translocated to the shoots. Obviously, the C:N ratio and what plants determine as a low or high NO_3^--N level differs between species (Wang and Ruan, 2016, Zheng, 2009). If soil NO_3^--N concentrations increased (root C decreases overall), more NO_3^--N could be translocated to the shoots. This result mirrors what was discovered by Bloom *et al.* (1993), who showed that with higher concentrations of NO_3^--N and NH_4^+-N root growth was significantly lowered, due to a smaller root being able to acquire enough N for its root function as well as send enough to the shoot. Therefore, it would be unnecessary to produce more root, as this would require extra energy.

Overall, Kukri plants had a greater shoot biomass than Gladius. Kukri has shown the potential to be better adapted to pulsed or variable watering, by producing higher specific root length under lower N (25-75 mg of N/kg of soil) coupled with less water (0.5FC 48h) or wet/dry cycling, which represent the most field-like conditions. Kukri also produced longer and deeper roots under the more roots under lower N (75 mg of N/kg of soil). It is possible that with just one water pulse, root development was

encouraged, allowing further exploration into more N pools. Studies have shown that pulses of water, either through rainfall events or scheduled irrigation timing, can both directly and indirectly control belowground process (Austin *et al.*, 2004). Direct control would result in biochemical changes to N, such as increase in C or nutrient pools, as a result of heightened microbial activity, which in turn encourages mineralisation or denitrification of N substrates. Indirect control could be explained by root proliferation and increased shoot growth as a result of increased pools of plant-available N (Austin *et al.*, 2004, Cui and Caldwell, 1997). This highlights how water is tightly coupled to nutrient cycling and how timing of water applications changes a plant's response.

4.5.4. Conclusions

The aim of this study was to not only compare highly regulated water regimes, but to identify whether frequency or quantity of resource, i.e. water, had a greater impact on plant growth, C allocation and soil N mineralisation. Here, water had a greater impact on plant growth than N, with biomass encouraged most under the FC treatment. In addition, less frequent watering had a negative effect on plant growth compared to quantity of water, thus supporting our first hypothesis. However, there was evidence for an interaction between water and N, especially when combining FC water with 75N treatment, resulting in the highest root:shoot ratio, agreeing with our second hypothesis. By measuring specific root length, it was possible to identify differences in C allocation as a result of water variability, with changes to root thickness even when root biomass remained the same across N treatments. We were able to accept our third hypothesis, with both varieties having major differences in overall plant growth. As more extreme weather events increase in frequency, this will put more pressure on plants to adapt, posing agricultural challenges globally, especially if food yields are to be met or increased. To optimise plant production, understanding plant plasticity under variable and combined abiotic stresses, such as water and N, becomes increasingly important. As these environmental stresses often occur simultaneously, in-depth knowledge of plant response to combination stresses is the priority. Key questions for future research include how does water or nitrogen use efficiency change, and how does C allocation differ in relation to the abundance or limitation of resources? From this, both root and shoot traits can be identified that would aid plant survival under moderate to extreme resource limitation, and they can be implemented into breeding programs.

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Christina Asanopoulos, Binh Thi Thanh Tran and Rebecca Stonor for their assistance with harvesting. This research was funded via the award of a Beacon of Enlightenment Scholarship to OHC as part of a partnership between the University of Adelaide and the University of Nottingham.Supplementary Material

4.6. Supplementary Material

Table S4.1. Average total water added per pot (mL) for Gladius and Kukri, with five water treatments: FC, FC 48h, 0.5FC, 0.5FC 48h, Wet/Dry; and three nitrogen treatments: 25N (25 mg of N/kg of soil), 75N (75 mg of N/kg of soil), 150N (150 mg of N/kg of soil).

Wheat cultivar	Water treatment	Nitrogen treatment	Total water added per pot (mL)
Gladius	FC	25 mg of N/kg of soil (25N)	1653
		75 mg of N/kg of soil (75N)	1278
		150 mg of N/kg of soil (150N)	1535
	FC 48h	25 mg of N/kg of soil (25N)	1101
		75 mg of N/kg of soil (75N)	1055
		150 mg of N/kg of soil (150N)	1392
	0.5FC	25 mg of N/kg of soil (25N)	508
		75 mg of N/kg of soil (75N)	348
		150 mg of N/kg of soil (150N)	472
	0.5FC 48h	25 mg of N/kg of soil (25N)	303
		75 mg of N/kg of soil (75N)	120
		150 mg of N/kg of soil (150N)	331
	Wet/Dry	25 mg of N/kg of soil (25N)	896
		75 mg of N/kg of soil (75N)	689
		150 mg of N/kg of soil (150N)	762
Kukri	FC	25 mg of N/kg of soil (25N)	2126

	75 mg of N/kg of soil (75N)	1871
	150 mg of N/kg of soil (150N)	2127
FC 48h	25 mg of N/kg of soil (25N)	1697
	75 mg of N/kg of soil (75N)	1509
	150 mg of N/kg of soil (150N)	1663
0.5FC	25 mg of N/kg of soil (25N)	852
	75 mg of N/kg of soil (75N)	562
	150 mg of N/kg of soil (150N)	778
0.5FC 48h	25 mg of N/kg of soil (25N)	599
	75 mg of N/kg of soil (75N)	419
	150 mg of N/kg of soil (150N)	598
Wet/Dry	25 mg of N/kg of soil (25N)	1214
	75 mg of N/kg of soil (75N)	990
	150 mg of N/kg of soil (150N)	1184

Table S4.2. Soil and plant N concentrations pre- and post-experiment.

			Start			End	
Genotype	Water	N	Starting soil NH_4^+-N mg/kg	Starting soil NO_3^--N mg/kg	Starting soil mineral N mg/kg	Average TN ppm mg/kg	End soil average mineral N mg/kg
Gladius	FC	25N	28	464	492	3975	66
		75N	88	394	482	4325	233
		150N	116	443	559	4350	132
	FC 48h	25N	28	464	492	4225	59
		75N	88	394	482	4450	282
		150N	116	443	559	4525	425
	0.5FC	25N	28	464	492	3300	173
		75N	88	394	482	3975	364
		150N	116	443	559	3475	469
	0.5FC 48h	25N	28	464	492	3900	230
		75N	88	394	482	3550	165
		150N	116	443	559	3125	480
	Wet/Dry	25N	28	464	492	4750	183
		75N	88	394	482	4875	149
		150N	116	443	559	4225	425
Kukri	FC	25N	28	464	492	2975	35
		75N	88	394	482	3250	109
		150N	116	443	559	3725	223
	FC 48h	25N	28	464	492	3275	28
		75N	88	394	482	3925	65
		150N	116	443	559	3750	222
	0.5FC	25N	28	464	492	3325	134
		75N	88	394	482	3700	421

	150N	116	443	559	3375	388
0.5FC 48h	25N	28	464	492	3225	198
	75N	88	394	482	3550	227
	150N	116	443	559	3050	457
Wet/Dry	25N	28	464	492	4675	161
	75N	88	394	482	4375	114
	150N	116	443	559	4025	410

Table S4.3. Treatments used to normalise data for statistical analysis.

		Data treatment		
	Variable	Raw	Log base e	Square root
Gladius	Shoot biomass	✓		
	Root biomass			✓
	Specific root length	✓		
	Water applied	✓		
	WUE		✓	
	Total N %	✓		
	Mineral N		✓	
Kukri	Shoot biomass	✓		
	Root biomass	✓		
	Specific root length		✓	
	Water applied	✓		
	WUE		✓	
	Total N %	✓		
	Mineral N			✓

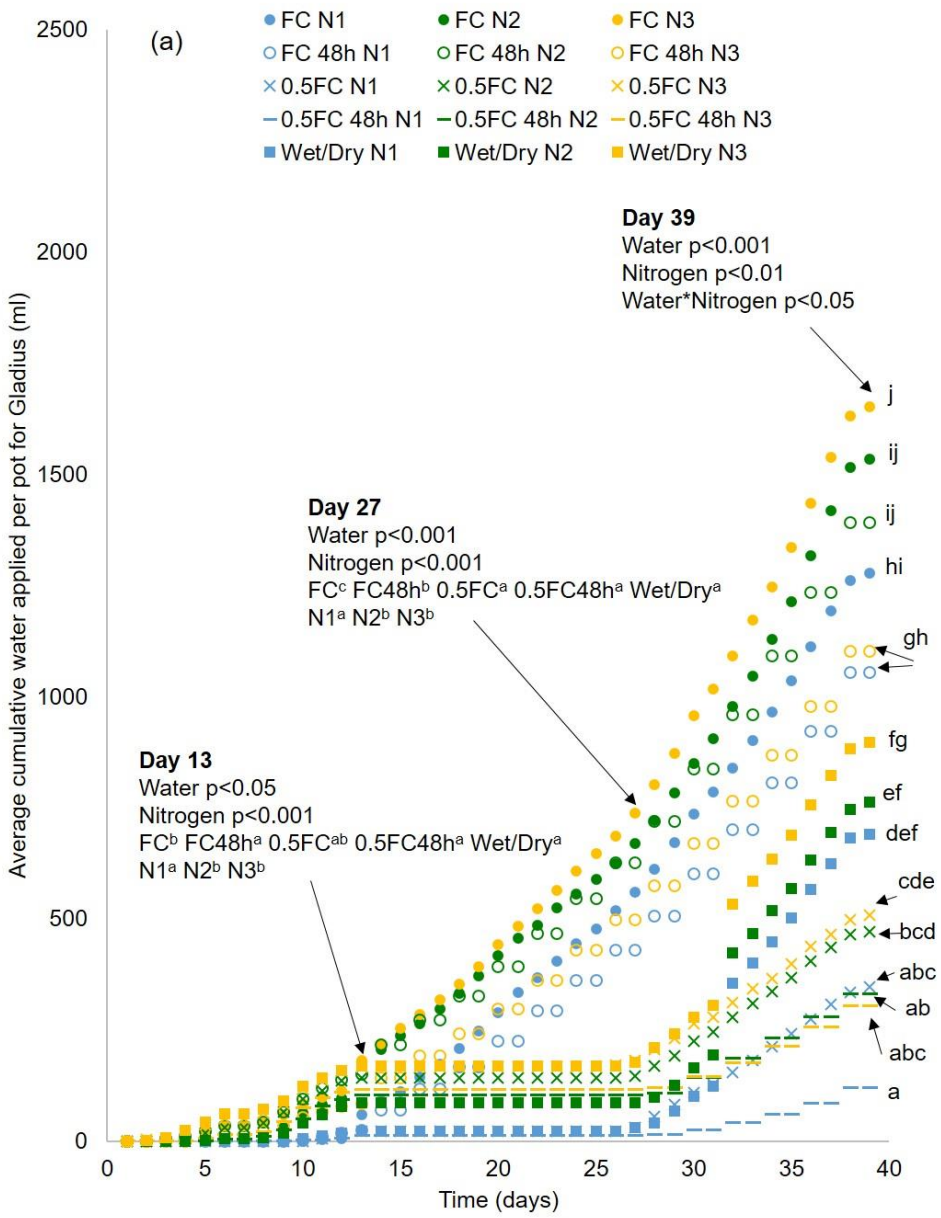

Figure S4.1. Average cumulative water applied per pot (mL) for Gladius, with five water treatments: FC (solid circle), FC 48h (line circle), 0.5FC (cross), 0.5FC 48h (dash), Wet/Dry (square); and three nitrogen treatments: 25N (25 mg/kg of N), 75N (75 mg/kg of N), 150N (150 mg/kg of N). Significant interactions between water and nitrogen are shown as letters on the right side of the graph.

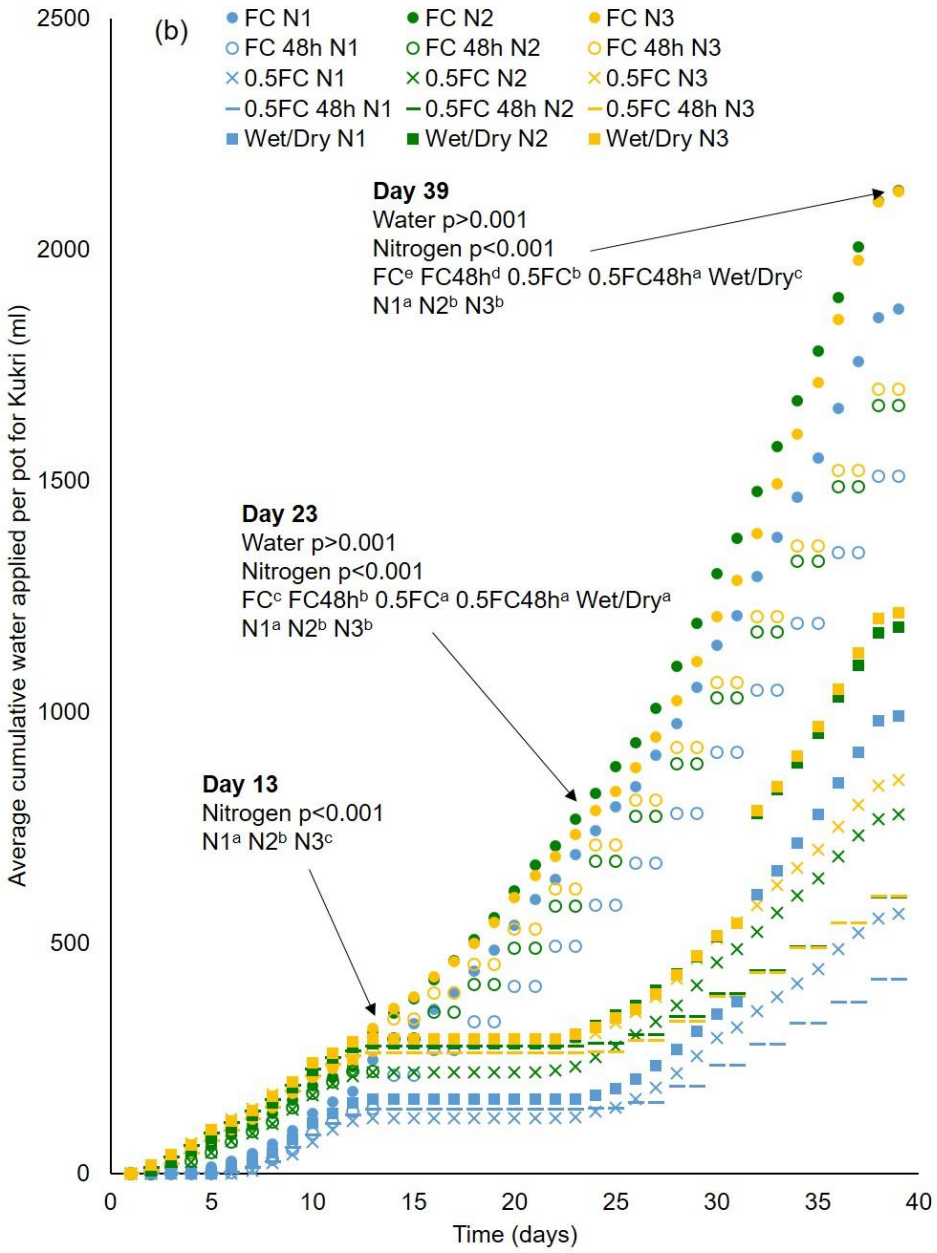

Figure S4.2. Average cumulative water applied per pot (mL) for Kukri, with five water treatments: FC (solid circle), FC 48h (line circle), 0.5FC (cross), 0.5FC 48h (dash), Wet/Dry (square); and three nitrogen treatments: 25N (25 mg/kg of N), 75N (75 mg/kg of N), 150N (150 mg/kg of N). Outcomes of significant values from ANOVA are shown in the graph, with the significant main effects of water or N are shown for Day 13, 23 and 39, where both water and N have an effect on cumulative water use regardless of each other. No significant interactions between water and N were found. In the interest of clarity, error bars are not shown.

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Chapter 5

The effect of soil water and N supply on shoot photosynthetic capacity, biomass accumulation, root architecture, soil N dynamics and uptake

The work contained in this chapter combines data from two experiments conducted at University of Nottingham, in collaboration with Dr Stéphanie Swarbreck and the Godwin lab at the University of Cambridge for the use of their mass spectrometer for ^{15}N analysis.

5.1. Introduction

With extreme weather events, such as drought and variable rainfall, the need to produce more food from less water is becoming more urgent. By increasing crop water efficiency, it is possible to increase yields with minimised losses under low or variable water supply (Zwart and Bastiaanssen, 2004). Crop water efficiency is affected by a range of factors, namely, climate, irrigation or water management and nutrient management (Zwart and Bastiaanssen, 2004).

5.1.1. Water

One way in which crop water productivity (crop yield compared to input of water and/or nutrients needed) can be improved is through deficit irrigation (water applied during drought-sensitive crop growth stages). Studies have shown that deficit irrigation reduces water consumption of the crop but does not adversely affect yield (Ali *et al.*, 2007, Davies *et al.*, 2010, Dodd, 2009, Dodd *et al.*, 2006). However, in contrast, some studies have demonstrated that a reduction in rainfall quantity has a greater negative effect on crop productivity, especially when compared to frequency of rainfall (Gibson-Forty *et al.*, 2016).

In understanding crop performance in response to reduced water, it is important to note the crop growth stage. For example, mild stress at any point of growth can improve water use efficiency, distribution of carbon (C) and other nutrients from root to grain, and ultimately increases yields (Kashiwagi *et al.*, 2015). On the other hand, if deficit water is imposed after anthesis, this negatively impacts photosynthesis and encourages early senescence (Kashiwagi *et al.*, 2015).

Although aboveground biomass is important for farmers in terms of grain yield, the driver for good grain C assimilation and yields is the root system (Asseng *et al.*, 1998, Bakhshandeh *et al.*, 2016, Carvalho *et al.*, 2014). Under deficit water conditions, several root traits have been identified that aid in maintaining plant productivity: increased root to shoot ratio, smaller root diameter, deeper root biomass, high specific root length and root length density (Asseng *et al.*, 1998, Carvalho *et al.*, 2014, Comas *et al.*, 2013, Feng *et al.*, 2017). Also, the rooting depth changes depending on soil moisture levels and stage of plant growth, with greater root density and/or distribution in deeper soil layers to maximise water uptake (Asseng *et al.*, 1998, Feng *et al.*, 2017). Gibson-Forty *et al.* (2016) also found a 50% reduction in water resulted in a shift of root growth from a deep rooting profile to a shallower rooting profile. A reduction in watering fails to increase soil moisture at depth, therefore having a shallower root system allows the plant to access soil moisture under reduced rainfall. Alternatively, large and infrequent rainfall events tend to increase soil moisture at depth; this can result in plants producing deeper roots to cope with the frequent water deficits in the shallow soil layers (Gibson-Forty *et al.*, 2016, Schwinning and Sala, 2004, Xu and Li, 2006). Root plasticity in response to different quantities of water highlights an adaptive trait optimising plant productivity and water use efficiency.

5.1.2. Nitrogen

Although water is an important resource for optimal plant growth, nitrogen (N) is fundamental for grain production. Soil N availability affects biomass partitioning (Cambui *et al.*, 2011), leading to decreased root to shoot ratios as soil N increases (Bonifas and Lindquist, 2009). Generally, an increase in N results in a larger root system (but more shoots to roots), with an increase in N uptake per unit of root length (Aziz *et al.*, 2017); but too much N can result in a decrease in root length in wheat (Comfort *et al.*, 1988).

Soil nutrient status not only impacts overall root architecture, it can also affect growth of different types of roots. Under low nutrient conditions, uptake differs between contrasting root types, with crown roots taking up the least amount of nutrients compared to primary roots or seminal roots (Steffens and Rasmussen, 2016). However, a shallow rooting profile can aid nutrient uptake regardless of root type (Feng *et al.*, 2017), because mineralisation of N happens most often in the topsoil especially since fertiliser is added to the soil surface (Bakhshandeh *et al.*, 2016). Thus, understanding root distribution, branching and depth is very important in maximising water and N uptake especially under limited soil water and N supply. The 'ideal' root system would be able to maximise nutrient uptake and water uptake, proliferate through a soil profile and maximise grain yield without negatively impacting overall shoot growth.

5.1.3. Water-nitrogen interaction

Water affects N solubility, movement and availability, as some N forms are highly water soluble, i.e. nitrate (Burger *et al.*, 2005). An increase in soil moisture content encourages breakdown of N from fertilisers or organic matter into forms plants can take up, thus increasing N availability (López-Bucio *et al.*, 2003, Schwinning and Sala, 2004, Wang *et al.*, 2015). At the same time, high rainfall or irrigation levels increase the risk of N-fertiliser leaching and surface run-off (Bijay *et al.*, 1995, Carstensen *et al.*, 2014), resulting in more fertiliser being applied to compensate for low N availability. However, under deficit water, accumulation of N in both plants and soils is drastically reduced due to decreased microbial activity which aids in N cycling (Jensen *et al.*, 2003).

Cossani *et al.* (2010) hypothesised that crops perform better when a moderate limitation of water and N is imposed together on a cropping system; this combination of stress can be defined as co-limitation. Plants under multiple moderate limitations, i.e. water and N, are better able to compensate than under one severe limitation (Cossani *et al.*, 2010). Elazab *et al.* (2016) theorised that water is the main constraint limiting plant productivity, regardless of N level. Their research showed that with high water and N, shoot production increased, but root production did not necessarily follow this pattern. There is evidence that root production decreases under high water and increases under lower water and N levels (Cousins *et al.*, 2020). Root biomass and length increased with improved water but decreased with higher N (Elazab *et al.*, 2016). A low nutrient environment also affected specific root length, a decrease

mass of root per length could be due to a reduction in root diameter and/or root tissue density (Elazab *et al.*, 2016). This was also confirmed with studies done by Ayad *et al.* (2010), where rain-fed wheat and barley grown with and without supplemental watering at three soil N levels had reduced root biomass and length under low N with supplemental water. The possible advantage of having longer but thinner roots as a result of high water and low N could help plants increase nutrient uptake by increasing root surface area and enlarging the absorption area (Elazab *et al.*, 2016). The level of stress of both water and N affecting a plant can affect water use efficiency and N use efficiency, with research suggesting both water use efficiency and N use efficiency increases with the degree of co-limitation (Cossani *et al.*, 2010). Water use efficiency also increased when water supply was low and nutrient supply high in tree species, whereas N use efficiency increased when water supply was high and nutrient supply low (Dijkstra *et al.*, 2016). The concept of co-limitation is not new, but further research is needed to understand the trade-offs between water and N uptake in crops.

Here I present results from two experiments which both aimed to quantify the impact of two water and N treatments on biomass allocation and soil N concentrations. Experiment A helped to identify differences in root architecture between two wheat varieties (Kukri and Gladius) in response to the water and N treatments by utilising X-ray Computed Tomography (CT) to image the root systems. I hypothesised that (1) Kukri would grow bigger and more vigorously than Gladius, and (2) low water and low N would result in smaller root biomass. Experiment B investigated N uptake, by measuring rate of uptake through microdialysis and uptake preference of N forms with ^{15}N stable isotopes, and measuring photosynthetic capacity of Kukri under variable water and N. The hypotheses explored were that (3) low N particularly would result in a higher photosynthetic stress response, and (4) Kukri roots would have a preference for ammonium or glycine uptake.

5.2. Materials and Methods

5.2.1. X-ray Computed Tomography experiment (Experiment A)

5.2.1.1. Soil and column preparation

A sandy loam (66.4% sand 18% silt, 15.6% clay; (Burr-Hersey, 2019) was collected from the University of Nottingham experimental farm at Sutton Bonington, Leicestershire, UK. The soil was then air-dried and sieved to <2 mm. Columns (165 mm height x 80 mm diameter) were uniformly packed to a bulk density of 1.2 g cm^{-3}.

Seeds of *Triticum aestivum* cv. Kukri and Gladius seeds were pre-germinated by soaking in a Petri dish in a dark enclosed space at 21°C. After two days, they were transplanted into the columns. Columns were placed in a growth chamber at 22/15°C day/night with a 12h/12 day/night photoperiod. These two varieties have been used in previous water and N stress studies (Cousins *et al.*, 2020; Chapter 4) and in

single-stress experiments Kukri is more drought susceptible (Bennett *et al.*, 2012, Izanloo *et al.*, 2008), and has a higher NUE (Mahjourimajd *et al.*, 2016) compared to Gladius.

For this experiment, two water treatments were established. One water treatment consisted of columns watered to field capacity (25.5% moisture content, FC); the second treatment consisted of columns watered to half of field capacity (12.75% moisture content, 0.5FC). In addition, two N treatments were included. Ammonium nitrate ($NH_4^+NO_3^-$-N; Nitram®, CF Fertilisers UK Limited, Cheshire, U.K.) was added to the soil as follows: for N1 treatment, no $NH_4^+NO_3^-$-N was added (this treatment is called Field N henceforth); for N2 treatment, $NH_4^+NO_3^-$-N was added at a rate of 120 mg of N/kg of soil (this treatment is called Elevated N henceforth). The rate of 120 mg of N/kg of soil was chosen based on previous research conducted at The Plant Accelerator (University of Adelaide), where 120-150 mg of N/kg of soil were described as optimal for healthy plant growth and yield.

5.2.1.2. Image processing and analysis

After two weeks of plant growth, all columns were scanned at a resolution of 61 μm using a Phoenix V|Tome|X m X-ray 240kV microCT system (GE Measurement & Control Solutions, Wunstorf, Germany) at the Hounsfield Facility at the University of Nottingham. The X-ray source settings were 170 kV and 190 μA, with a 0.1 mm copper filter on the soil column to reduce detector saturation. Each column was scanned in two segments to obtain the full column length, and the segments digitally stitched together following data reconstruction. Each scan acquired 2520 projection images over a 360° rotation of the sample using a detector exposure time of 200 ms, integrated over three averaged images resulting in a total scan time of 1 hour and 8 minutes min for both scans.

5.2.1.3. Plant sampling and analysis

After scanning, plants were carefully removed from their columns; roots were separated from the shoots and the soil washed from roots. WinRhizo was used to measure total root length for each root system and the specific root lengths, diameter and root tip number. All plant biomass was oven-dried (60°C) until a constant weight was achieved (48 h). Fresh and dry weights of shoots and roots were recorded.

5.2.2 Rhizobox experiment (Experiment B)

5.2.2.1. Soil and rhizobox preparation

A sandy loam topsoil (68% sand, 18% silt, 14% clay; British Sugar (2019)) was supplied by British Sugar (British Sugar plc, Newark Factory, Newark, Nottinghamshire, NG24 1DL, UK). Seeds of *Triticum aestivum* cv. Kukri seeds were pre-germinated as described for Experiment A. After two days, they were transplanted into 50 L rhizoboxes situated in a glasshouse with 22/15°C day/night and a 12/12 h day/night photoperiod.

The field capacity of the soil was calculated as 22% moisture content, and two water treatments were established. Half of the rhizoboxes were watered to field capacity (FC, 10 L water per box); the other

half were watered to half of field capacity (11% moisture content, 0.5FC, 5 L water per box). Watering was conducted on alternate days, based on growth rates and water use of Kukri in Chapter 3 and Chapter 4. Each watering period, 10 or 5 L was added.

In order to establish the N treatments, NH_4^+-N and NO_3^--N concentrations were measured in a subsample by 2M KCl extracts and colorimetrically analysed for NH_4^+-N (Forster, 1995) and NO_3^--N (Miranda *et al.*, 2001). The residual mineral N (NH_4^+-N plus NO_3^--N) was measured as 29.6 mg/kg of dry soil. Subsequently, additional ammonium nitrate ($NH_4^+NO_3^-$-N; Nitram®, CF Fertilisers UK Limited, Cheshire, U.K.) was added to the soil in the water at two rates: N1 treatment, no $NH_4^+NO_3^-$-N added (termed Field N); N2 treatment, $NH_4^+NO_3^-$-N was added to ensure soil contained 120 mg of N/kg of soil (termed Elevated N). These treatments were chosen to be as similar to the Experiment A soil as possible.

On the fifth day post sowing, a Hortimix Standard solution (Hortifeeds Direct, Lincoln, LN1 2LD, UK) containing phosphorus, potassium and trace elements was added to the rhizoboxes (phosphorus pentoxide soluble in water 36%, potassium oxide soluble in water 36%, magnesium oxide 2%, iron chelated by EDTA 1533 mg/kg, manganese 1000 mg/kg, boron 300 mg/kg zinc 270 mg/kg, copper 200 mg/kg, molybdenum 120 mg/kg). Hortimix Standard was dissolved at the rate of 10 kg per 100 L of stock solution to achieve a 10% stock solution. Each rhizobox received 5 L of the diluted 10% stock solution.

5.2.2.2. Growth measurements

Every week, the tip of the longest root was marked with a marker on the outside of the rhizobox window. At the end of the experiment, a photograph was taken of each window, to measure the rate of root growth over the course of the experiment (Figure 5.1).

Figure 5.1. Photograph of rhizobox used to measure rate of root growth over time. The units on the ruler to the left of the rhizobox are centimetres.

A week before harvest, physiology measurements including leaf temperature differential, FvP/FmP, NPQt (chlorophyll fluorescence), linear electron flow, Phi2, PhiNO, PhiNPQ and relative chlorophyll were measured using PhototsynQ MultispeQ v2.0 (PhotosynQ LLC, East Lansing, Missouri, USA).

Phi2 is the photosynthetic efficiency of Photosystem II, (amount of energy used for sugar production); phiNPQ is the proportion of energy quenched in the leaf (normal protection); and phiNO is the proportion released as free energy (energy that can potentially cause damage to photosystems) (Kramer *et al.*, 2004, Maxwell and Johnson, 2000).

5.2.2.3. Microdialysis

Microdialysis was conducted across two days on 12 (half) of the rhizoboxes four days before harvest. A microdialysis probe with a semipermeable membrane at its tip was placed directly into the soil. The probe was connected to a microdialysis pump (CMA 4004, CMA Microdialysis AB, Solna, Sweden) at a flow rate of 5 µl/min for 1 hour (see Chapter 1, Figure 1.2). Six probes were used per rhizobox, three probes placed directly next to three different roots (close to root), the other three probes placed 10 cm away from the original roots (away from root). Microdialysis sampling provides information about the amount of N available to plants. It also allows positional nutrient levels to be identified based on probe proximity to a root (in this case, far was the equivalent to 10 mm and near was equivalent to 5 mm).

5.2.2.4. Stable isotopic analysis

Three different forms of N - $K^{15}NO_3$, $(^{15}NH_4)_2SO_4$, ^{15}glycine - were labelled with ^{15}N to measure relative uptake and root uptake preference of these N forms using a mass spectrometer (Table 5.1).

Table 5.1. Compositions of each stable isotope tracer mix used to measure ^{15}N uptake in wheat roots.

	Ingredients	**Define**	**Concentration**
Batch 1	$K^{15}NO_3$	Stable isotope	1mM
	$(NH_4)_2SO_4$	Normal	0.5mM
	Glycine	Normal	0.5mM
Batch 2	$(^{15}NH_4)_2SO_4$	Stable isotope	1mM
	KNO_3	Normal	0.5mM
	Glycine	Normal	0.5mM
Batch 3	^{15}Glycine	Stable isotope	1mM
	KNO_3	Normal	0.5mM
	$(NH_4)_2SO_4$	Normal	0.5mM

Approximately 100-200 mg (fresh weight) of roots (with similar age) were carefully extracted from the soil and placed into a 50 ml Falcon tube containing one of the three ^{15}N solutions and left to sit in

solution for 30 minutes. Each root sample was transferred into another 50 ml Falcon tube containing 15 ml of 10 mM of KCl and shaken for 30 seconds, then transferred to the final tube containing 15 ml of RO water and shaken again for 30 seconds. The root was removed, and fresh weight determined. All root samples were dried at 60°C for 48 hours, then ground using a mortar and pestle. They were then transferred to 2 ml Eppendorfs and two steel balls (one 3 mm and one 4 mm) were put into each tube and ground further using a tissue lyser II (Retsch, Qiagen) for a total of 12 minutes. Approximately, 500 µg of ground sample was weighed into foil boats, which were folded into closed capsules and placed into wells of a 96-well plate. The plate was placed into the mass spectrometer (Godwin laboratory, University of Cambridge, UK) to measure the total amount of ^{15}N. Samples were analysed for percentage N, ratios of $^{14}N/^{15}N$, using a Costech Elemental Analyser attached to a Thermo DELTA V mass spectrometer in continuous flow mode. The sample is introduced into the combustion chamber, and flash combustion occurs, releasing the sample's elemental components. Water was removed by passing the gases through a tube containing magnesium perchlorate. The gases were then flowed through the gas chromatographic separation column which is kept at a constant temperature of 45 +/- 0.1 °C. As they pass through the GC column the gases are separated and transported sequentially through a Thermo Conflo IV interface and "open split" into the mass spectrometer for analysis. The mass spectrometer software measures the $^{14}N/^{15}N$ ratio. Reference standards from IAEA in Vienna were also run at intervals throughout the sequence and these values were used to calibrate to the international standards for $^{14}N/^{15}N$ (delta ^{15}N air). Precision of analyses is better than 0.1 per mille for $^{14}N/^{15}N$ (Godwin laboratory, University of Cambridge, UK).

5.2.3. Soil sampling (Experiments A and B)

At harvests of both Experiment A and B, the soil was homogeneously mixed and a soil sample was taken from each column and rhizobox. The soil was extracted with 2M KCl, and both the KCl and microdialysis extracts analysed for NH_4^+ -N (Forster, 1995) and NO_3^- -N (Miranda *et al.*, 2001). Analysis of KCl extraction shows the total amount of N present in the soil extraction.

5.2.4. Statistical analysis (Experiments A and B)

Data were analysed by a two-way ANOVA, with N addition and watering treatment as factors in the model. Statistical analysis was separated for the two experiments (Supplementary Table S5.2). Where the ANOVA revealed a significant treatment effect, significant differences between individual treatments were identified using Tukey's HSD/LSD tests. All statistical analysis was performed in R, with data also checked for normal distribution in both Genstat (VSN International, 2012; 19th edition) and R (version 3.2.5); additional R packages used were 'agricolae' and 'car'.

5.3. Results

5.3.1. Plant biomass allocation (Experiment A and B)

For Experiment A, there was no significant difference for dry weight of shoots or roots for either Gladius or Kukri (Figure 5.2a, b). However, Gladius had slightly more root and shoot biomass under Field N x 0.5.FC (compared to other water and N treatments; Figure 5.2a). For Kukri (Figure 5.2b), the lowest root biomass was under Field N x FC, and the largest root biomass under Field N x 0.5FC. When analysing root:shoot ratios for Gladius in Experiment A (Figure 5.2c), there was no significant difference between treatments. The large standard error bars were due to loss of replicates (low germination rate). Biomass of Kukri was significantly higher in 0.5FC x Field N compared with both FC treatments (Figure 5.2d).

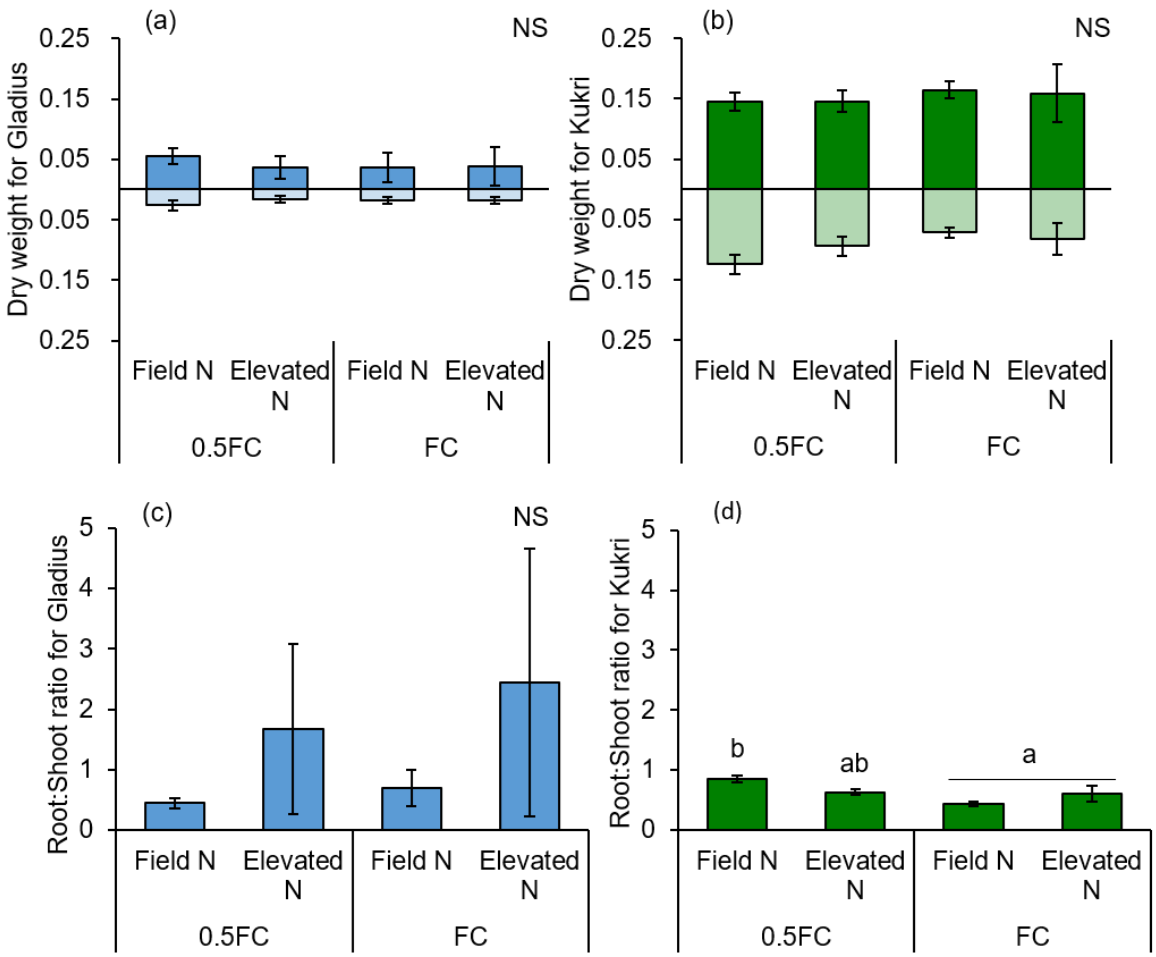

Figure 5.2. Shoot and root biomass for Experiment A. Mean shoot (dark shading, above x-axis) and root (pale shading, below x-axis) dry weight and root:shoot ratio ± SE for 2-week old Gladius (a) and Kukri (b) plants grown in soil columns under two water treatments (0.5FC, FC) and two N treatments (Field N, 0 mg of N/kg of soil; and Elevated N, 120 mg of N/kg of soil). Mean values of root:shoot ratios for Gladius (c) and Kukri (d) shown. Mean values are presented ± SE; Kukri shoots n=4, Gladius shoots n=3 for all 0.5FC treatments, n=2 all FC treatments; Kukri roots n=4, Gladius roots n=3 for all 0.5FC treatments, FC/Elevated N, n=2 for FC/Field N. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$). Gladius dry weight (a) shows no significance (NS) $p=0.802$; Kukri dry weight (b) shows no significance (NS), $p=0.859$; Gladius root:shoot ratio (c) shows no significance (NS), $p=0.673$; Kukri root:shoot ratio (d) shows significant interactions of water by N, $p=0.0223$.

For experiment B, there were no differences in root biomass for 5-week old Kukri grown in rhizoboxes (Figure 5.3). Shoot biomass differed between treatments, with the highest under Field N x 0.5FC, and the lowest biomass under Field N x FC.

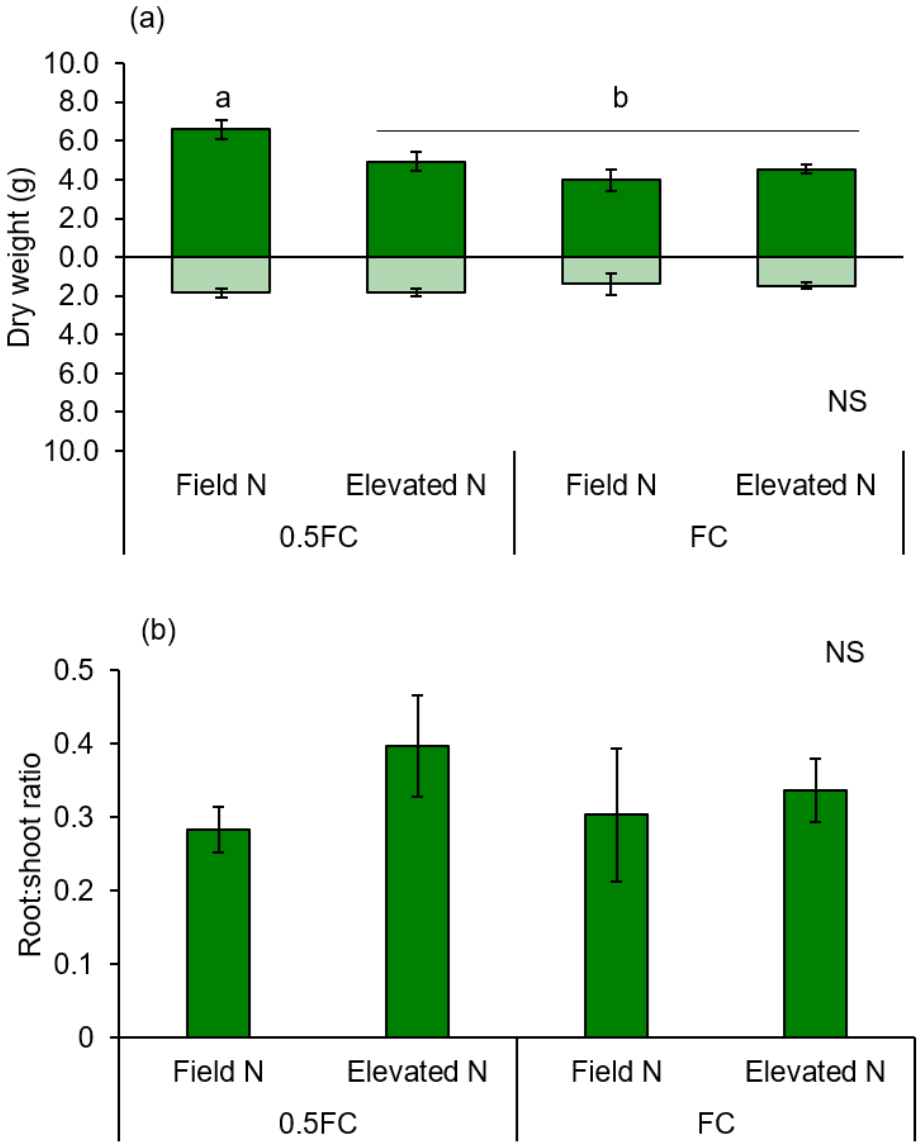

Figure 5.3. Shoot and root biomass for Experiment B. Mean shoot (dark shading, above x-axis) and root (pale shading, below x-axis) dry weight ± SE for 5-week old Kukri grown in 50 L rhizoboxes under two water treatments (0.5FC and FC) and two N treatments (Field N, 29.6 mg of N/kg of soil; and Elevated N, 120 mg of N/kg of soil). Mean values are presented ± SE n=6, except n=5 for FC/Field N. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$). Kukri shoot dry weight shows an interaction water by N $p=0.023$; root dry weight shows no significance (NS), $p=0.204$.

5.3.2 Root architecture (Experiment A)

From the X-ray CT (Experiment A), the root system architecture was captured for both Gladius and Kukri (Figures 5.4, 5.5). Overall, the CT images suggest that 0.5FC and Field N resulted in the greatest number of seminals and roots in general (Figure 5.4a), with seminal number decreasing with increased water (Figure 5.4). For Kukri, from the root segmentation, it was possible to see a lot of roots at the lower part of the soil column, which could suggest possible leaching of the Elevated N due to watering (Figure 5.5c, d). The root system with the highest volume is Kukri grown under 0.5FC and Elevated N (Figure 5.5c). Additionally, the root system for Field N and 0.5FC does not appear much smaller than that of Field N and FC, which could suggest that although a limited supply of N is the common factor between the two, the water treatments did not negatively impact growth (Figure 5.5a, b).

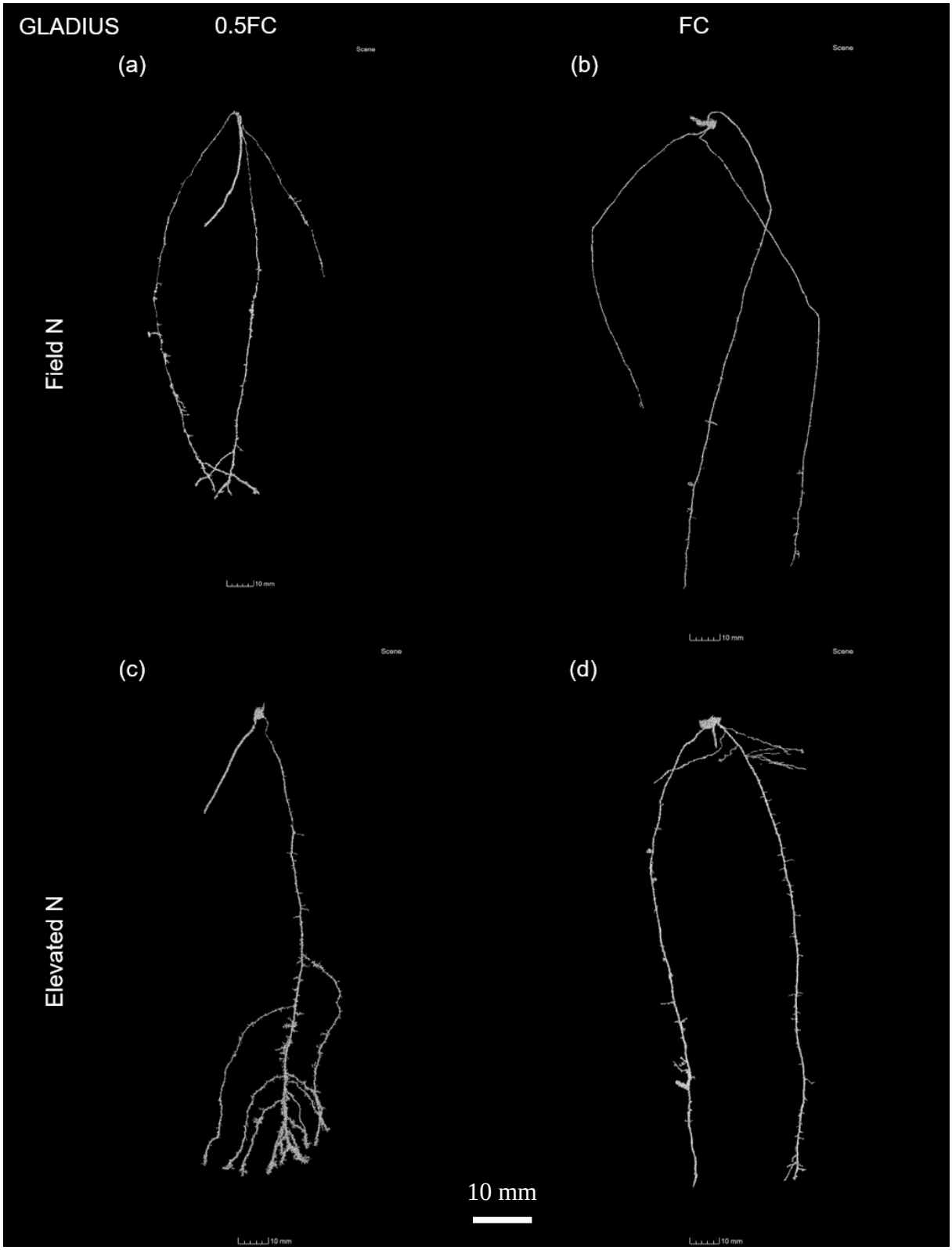

Figure 5.4. Root system architecture for Gladius (Experiment A) under two water treatments (0.5FC and FC) and two N treatments (Field N, 0 mg of N/kg of soil; Elevated N, 120 mg of N/kg of soil).

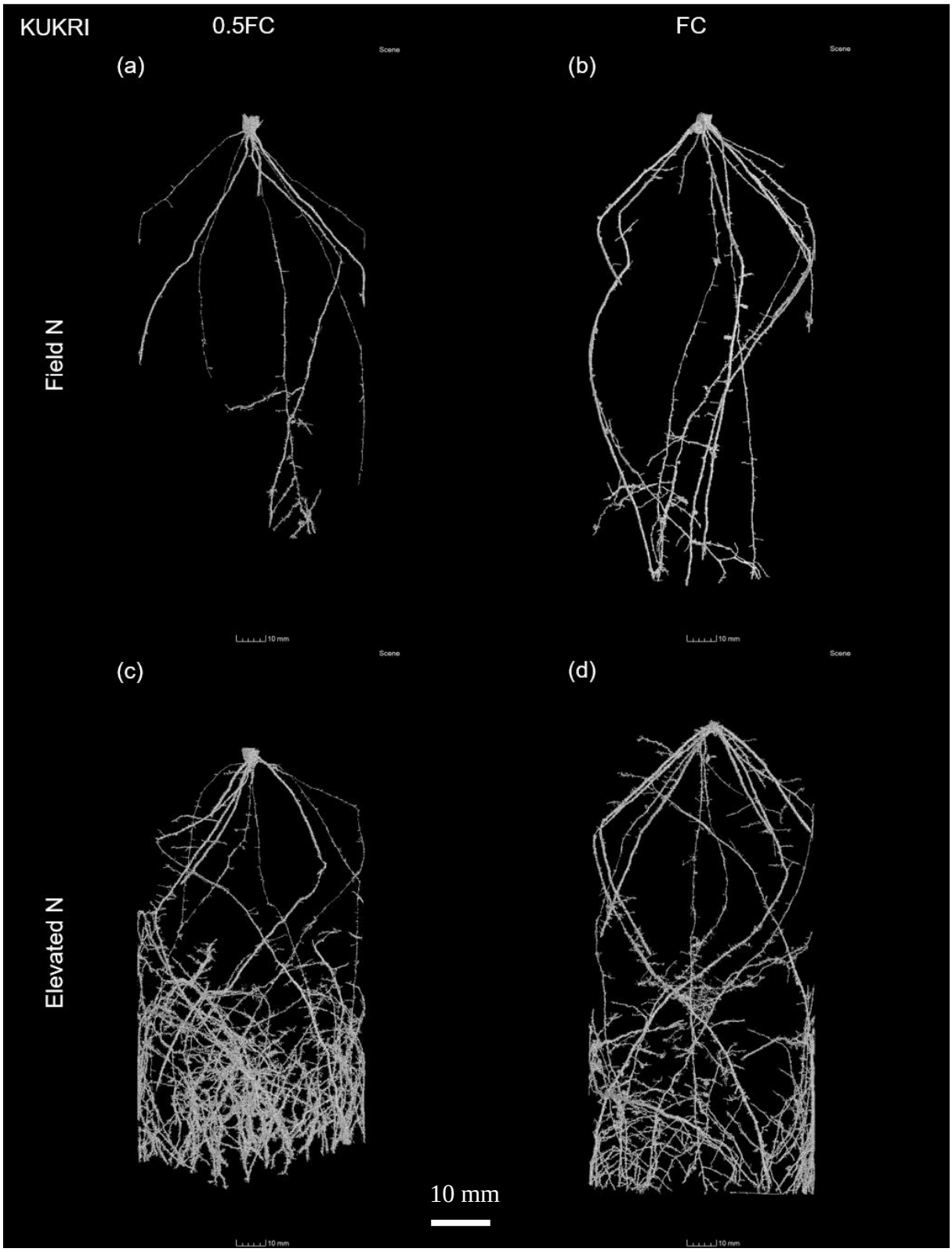

Figure 5.5. Root system architecture for Kukri (Experiment A) under two water treatments (0.5FC and FC) and two N treatments (Field N, 0 mg of N/kg of soil; Elevated N, 120 mg of N/kg of soil).

Number of seminal roots identified from the X-ray CT images differed between treatments for both Gladius and Kukri (Figure 5.6a, b). Seminal numbers were highest with Field N x 0.5FC and Elevated N x FC (Figure 5.6a). For Kukri, seminal numbers differed between water treatments but not N treatments (Figure 5.6b). Root tip number did not hugely differ between Elevated N and Field N of 0.5FC and FC in Gladius (Figure 5.6c). In Kukri, the highest tip number was found in plants under Field N x 0.5FC; overall, root tip number decreased with increasing N and increasing water (Figure 5.6d).

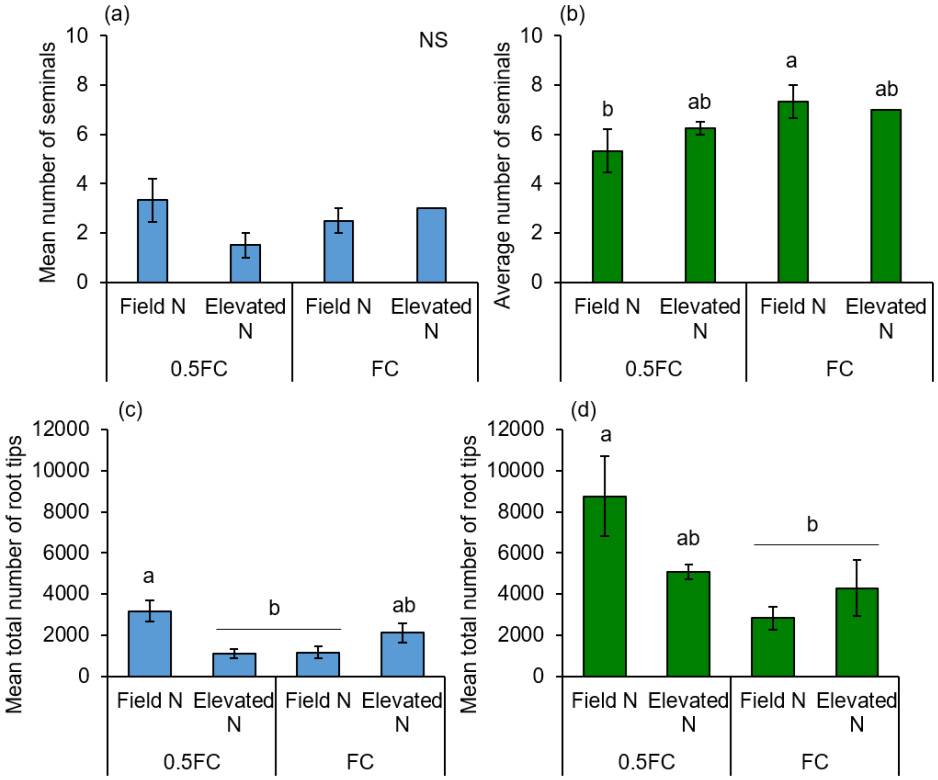

Figure 5.6 Mean seminal number (from CT images) and root tip number (from WinRhizo scans) after 2 weeks growth for Gladius (a,c) and Kukri (b, d), with two water treatments (0.5FC, FC) and two N treatments (Field N, 0 mg of N/kg of soil; and Elevated N, 120 mg of N/kg of soil). Mean values are presented ± SE; n=4, except n=3 for treatments 0.5FC/Elevated N, 0.5FC/Field N and n=2 FC/Elevated N, FC/Field N. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$). Seminal number for Gladius (a) shows no significance (NS), and Kukri (b) shows main effect of water, $p=0.035$. Number of root tips for Gladius (c) shows a significant interaction of water:N, $p=0.009$, and Kukri (d) shows a main effect of water, $p=0.012$.

For Experiment A, the highest root length observed was under Field N x 0.5FC (Figure 5.7a). The roots of both varieties had reached the bottom of the soil columns by the harvest. There was no difference between FC treatments. Both Gladius and Kukri, however, showed highest root length under Field N x 0.5FC (Figure 5.7a, b). With Kukri, the general trend showed root length decreasing with increased N and increased water (Figure 5.7b). In Gladius, the highest specific root length was observed with the lowest water and lowest N (0.5FC and Field N; Figure 5.7c), but under the FC treatment, specific root length increased with Elevated N. With Kukri, there was no difference between treatments; neither water nor N resulted in different specific root length (Figure 5.7d). The average root diameter did not differ significantly between treatments in Gladius (Figure 5.7e), but in Kukri (Figure 5.7f), the lowest root diameters were for both Field N treatments.

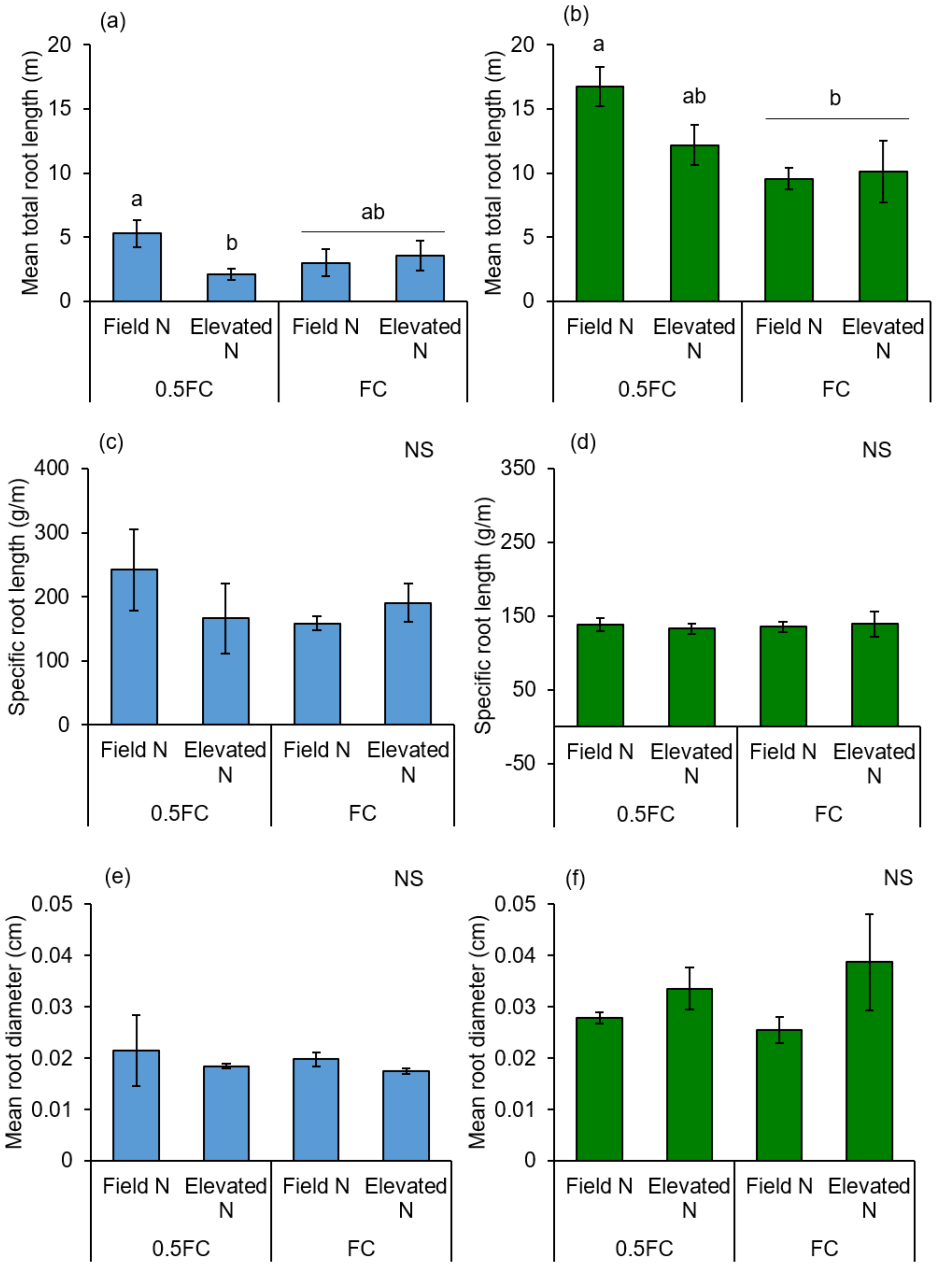

Figure 5.7. Mean root length, specific root length and root diameter for Experiment A, 2-week old Gladius (a, c) and Kukri (b, d) grown in soil columns under two water treatments (0.5FC, FC) and two N treatments (Field N, 0 mg of N/kg of soil; and Elevated N, 120 mg of N/kg of soil). Mean values ± SE n=4, except n=3 for treatments 0.5FC/Elevated N, 0.5FC/Field N and n=2 FC/Elevated N, FC/Field N. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$). Gladius total root length (a) shows main effect of N, $p=0.049$; Kukri total root length (b) shows main effect of water, $p=0.028$; Gladius specific root length (c) shows no significance (NS), $p=0.649$; Kukri specific root length (d) shows no significance (NS), $p=0.975$.). Root diameter for Gladius (e) and Kukri (f) shows no significance (NS).

Gladius had the lowest root volume with 0.5FC and Elevated N, with the biggest difference in root volume between Field N and Elevated N under 0.5FC (Figure 5.8a). However, Kukri had the lowest root volume under Field N x FC water (Figure 5.8b). The total volume from the X-ray CT analysis of

Gladius roots did not differ between treatments (Figure 5.8c), but in Kukri roots, Field N x 0.5FC produced the lowest volume compared to the other treatments (Figure 5.8d).

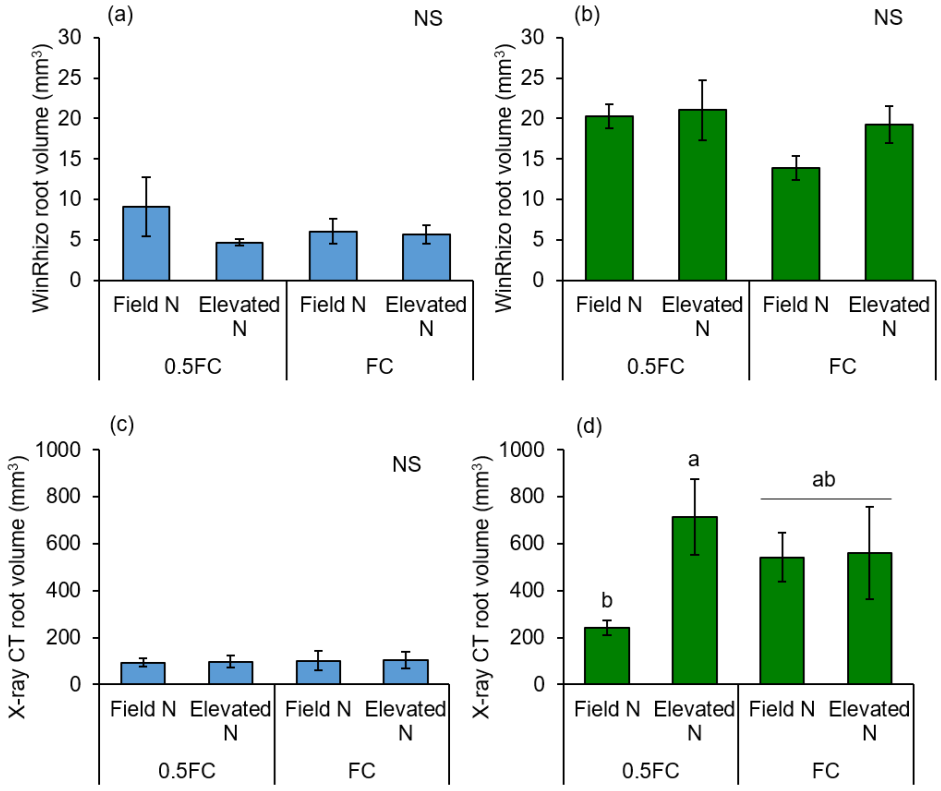

Figure 5.8. Average total root volume from WinRhizo and X-ray CT analysis of Gladius and Kukri grown under two water treatments (0.5FC, FC) and two N treatments (Field N, 0 mg of N/kg of soil; and Elevated N, 120 mg of N/kg of soil). Mean values are presented ± SE; n=4, except n=3 for treatments 0.5FC/Elevated N, 0.5FC/Field N and n=2 FC/Elevated N, FC/Field N. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$). WinRhizo root volume for Gladius (a) and Kukri (b) show no significance (NS); CT root volume for Gladius (c) shows no significance (NS), and Kukri (d) shows possible main effect of N, p=0.06 LSD, p=0.022 ANOVA.

5.3.3. Root growth rate (Experiment B)

The first root length measurements were taken on 10-Jun 2019 (Figure 5.9). The rate of root growth differed between treatments at the first measurement. Treatments FC x Field N and FC x Elevated N had the longest and shortest root growth respectively, by 10-Jun. On the second measurement day (14-Jun), rate of root growth decreased overall for all treatments, with Field N treatments having less centimetres per day than Elevated N treatments. By the third measurement (21-Jun), there was a decrease in root growth for all treatments. By the final measurement, the 0.5FC treatment encouraged increased rate of root growth than FC.

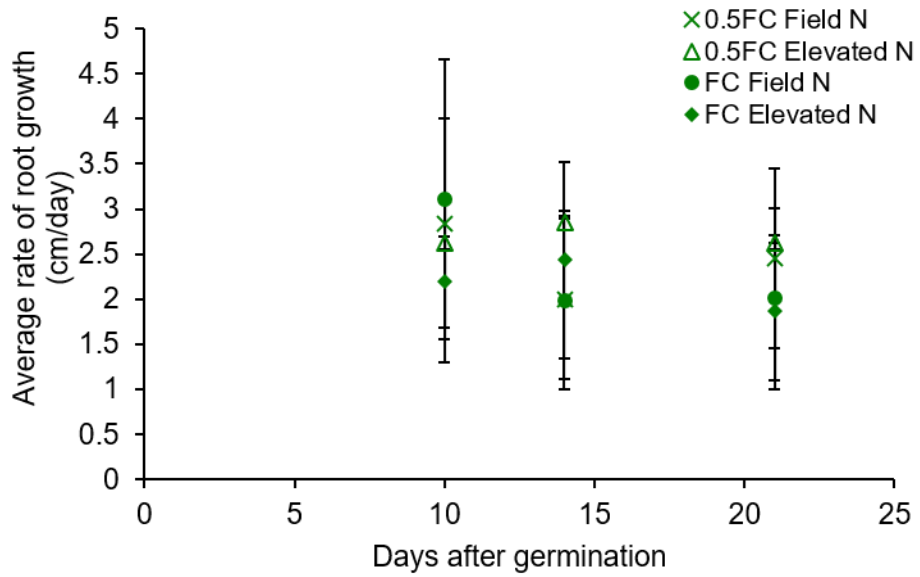

Figure 5.9. Average root growth rate for Kukri between 10 June and 21 June for a combination of two water and two N treatments: 0.5FC x Field N (cross); 0.5FC x Elevated N (open triangle); FC x Field N (closed circle); FC x Elevated N (closed diamond)), where Field N equals 29.6 mg of N/ kg of soil, and Elevated N equals 120 mg of N/kg of soil. Mean values are presented ± SE; n=6, except n=5 for 0.5FC/Field N/Day14, n=4 FC/Field N all days.

5.3.4. Plant physiology measurements (Experiment B)

To monitor how the plants were responding to the water x N treatments, PhotosynQ was used to quantify photosynthetic efficiency (FvP/FmP, NPQt, linear electron flow, Phi2, PhiNO), transpiration (via leaf temperature differential), and chlorophyll levels. Figure 10 highlights results from PhotosynQ (PhotosynQ LLC, 2019, v0.80), helping to define the stress level of the plants. When a leaf is transpiring the evaporation of water cools the leaf resulting in negative leaf temperature differential values (Figure 5.10a). The more negative leaf temperature differential was observed in plants under FC x Field N, and the least negative leaf temperature differential found under FC x Elevated N.

Light energy enters the leaf and can be dissipated in several ways, one of which is fluorescence of excess energy. FvP/FmP is defined as the maximum quantum yield of chlorophyll fluorescence (the ratio between the minimal amount of fluorescence from the leaf to the maximum fluorescence) in the dark-adapted state of Photosystem II (the quantum efficiency of open Photosystem II centres) (Maxwell and Johnson, 2000, Sheng *et al.*, 2008). The lowest ratio (stress indicator) was shown in plants treated with FC x Elevated N, whereas the highest ratio was observed for Field N treatments.

Energy in the system can also be expressed in three ways: energy dissipated by Non-photochemical Quenching (NPQt), energy used to make sugars via the linear electron flow (LEF) and energy released as free energy (phi2, phiNO, phiNPQ). NPQt levels were highest under both Elevated N treatments (Figure 5.10c), with Elevated N x FC producing the highest level of NPQt. Figure 10d demonstrates the linear electron flow (LEF) for Kukri. LEF is the number of micromoles of electrons flowing out of Photosystem II per second. It highlights the energy used to make sugars in photosynthesis. LEF numbers are similar for the Elevated N treatments, irrespective of water. Phi2 is the photosynthetic efficiency of Photosystem II, so how much energy is used for sugar production. PhiNPQ is the proportion being quenched in the leaf (normal protection). PhiNO is the proportion released as free energy (energy that can potentially cause damage to photosystems). PhiNO (Figure 5.10e) shows a significant interaction of water:N, $p=0.032$, whereas both phi2 and phiNPQ show marginal main effects of water and N respectively. The largest phiNO was observed for FC x Field N. There was also a slight increase in phiNPQ from increasing water and N. Under FC x Field N, there was a decrease in phi2. In addition, the PhotosynQ measures relative chlorophyll; this is measured as its level of greenness. Relative chlorophyll levels were similar across treatments (Figure 5.10f), but the lowest chlorophyll level was observed in plants under FC x Elevated N. Plants under FC x Field N produced the highest chlorophyll levels (marginally).

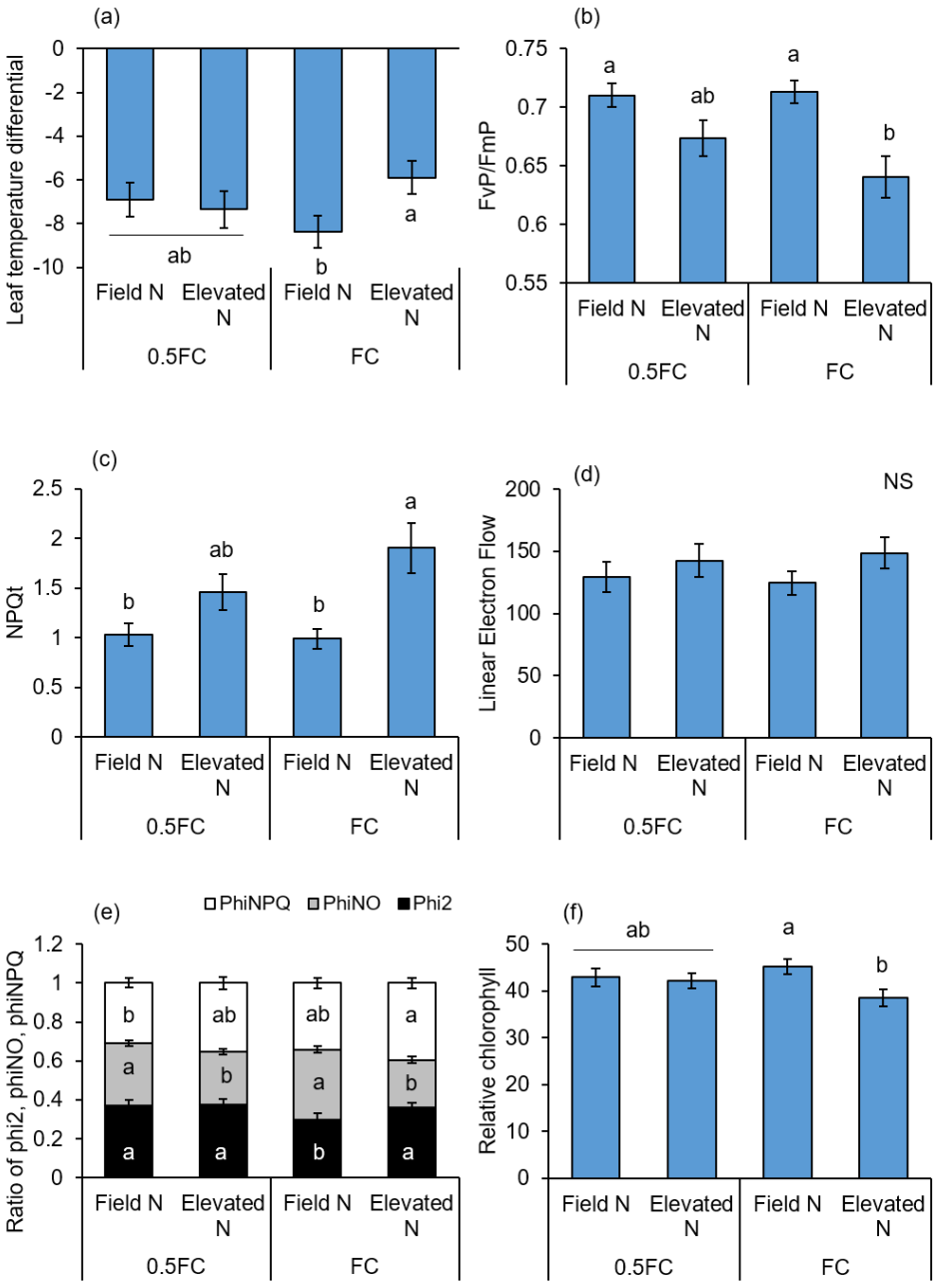

Figure 5.10. Photosynthetic data highlighting stress levels of 5-week old Kukri under water (0.5FC and FC) and N (N1, 29.6 mg/kg of dry soil; N2, 120 mg of N/kg) treatments. Values are presented as mean values ± SE; n=6, except n=5 for treatment FC/Field N. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$). Leaf temperature differential (a) shows marginal interaction of water:N, p=0.069. FvP/FmP (b) shows main effect of N, p=0.0003. NPQt (c) shows main effect of N, p=0.0005. Linear electron flow (d) shows no significance (NS). Phi2 (panel e) shows marginal main effect of water p=0.09; PhiNO shows a significant interaction of water:N, p=0.032; PhiNPQ shows marginal main effect of N, p=0.082.

5.3.5. Nitrogen dynamics (Experiments A and B)

5.3.5.1. Soil N (Experiment A and B)

Mineral N (NH_4^+-N and NO_3^--N) concentrations are presented below for both the X-ray CT (Experiment A; Figure 5.11) and rhizobox experiments (Experiment B; Figure 5.12). Focusing on experiment A, for Gladius, mineral N concentrations were highest with the Field N treatment, regardless of water treatment (Figure 5.11a). This pattern was also observed in Kukri, although concentrations were higher in Field N x FC treatments (Figure 5.11b). Kukri had very little mineral N left in Elevated N soils compared to Gladius.

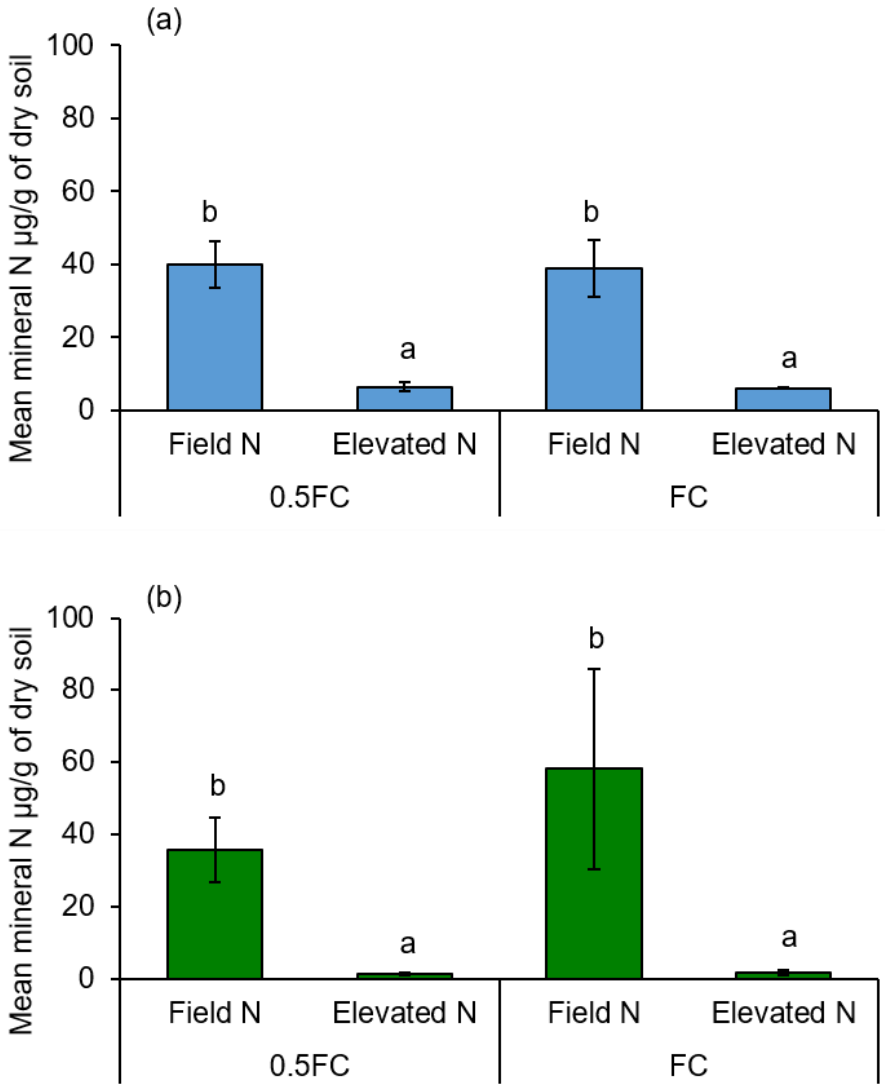

Figure 5.11. KCl extracted mineral N (NH_4^+-N and NO_3^--N) concentrations for Experiment A. Soils treated with two water treatments (0.5FC; FC) and two N treatments (Field N, 29.6 mg/kg of dry soil; Elevated N, 120 mg of N/kg). Mean values are presented ± SE; n=3 for all 0.5FC treatments and n=2 for all FC treatments. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$). Mineral N from Gladius samples (a) show main effect of N, $p=0.0007$; and Kukri (b) show main effect of N, $p<0.001$.

Looking at the soil in the rhizoboxes (Experiment B), two techniques were used: microdialysis (Figure 5.12a) and KCl extractions (Figure 5.12b). The highest nitrate concentrations were found in the Elevated N x 0.5FC treatment in soil far from the root tip. There was no significant difference in any of the treatments, but with Field N soil under 0.5FC, samples at 5 mm appeared to have more nitrate. The same trend was found with higher amounts of nitrate in the 0.5FC and lower levels in the FC treatments although these differences are also not significant (Figure 5.12b).

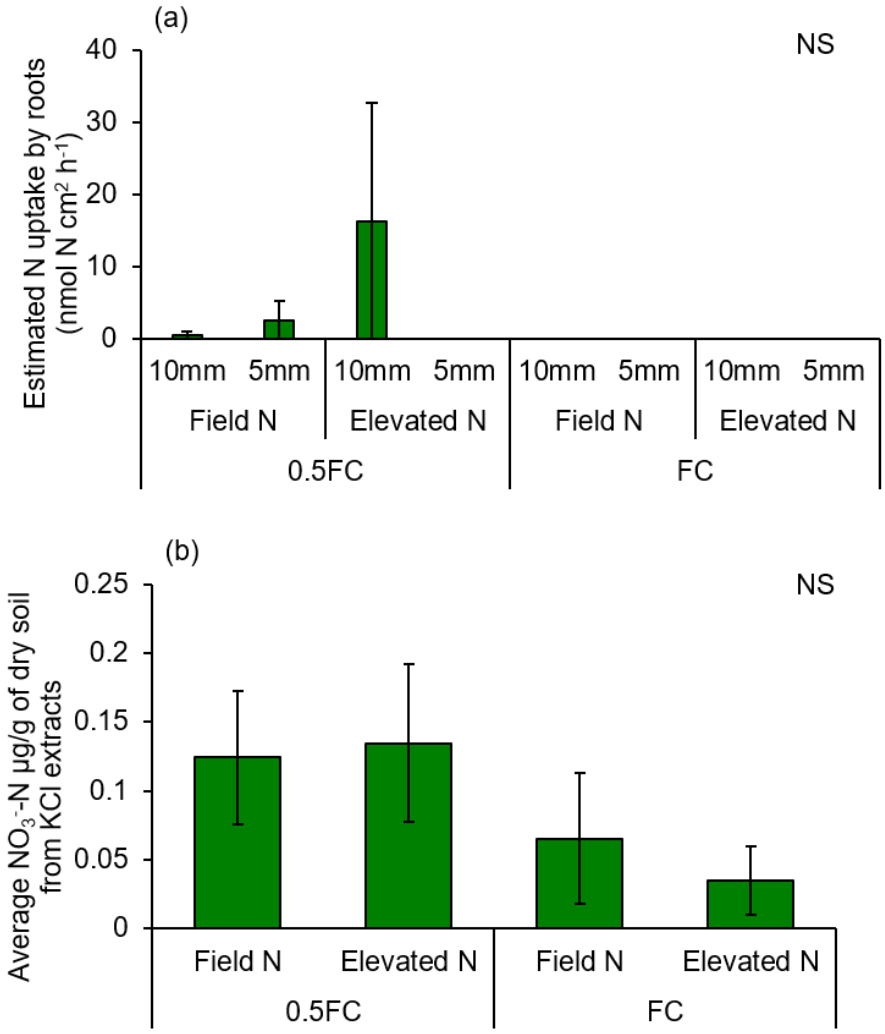

Figure 5.12. Estimated N uptake from the soil and average NO_3^--N concentrations from KCl extractions on soils treated with two water treatments (FC; 0.5FC) and two N treatments (Field N, 29.6 mg/kg of dry soil; Elevated N, 120 mg of N/kg). For microdialysis, values are presented as mean values ± SE n=8, except n=7 for treatments 0.5FC Field N 10 mm, FC Elevated N <5 mm; n=5 for 0.5FC Elevated N <5 mm; FC Field N 10 mm; n=4 for 0.5FC Elevated N 10 mm; n=3 for FC Field N <5 mm, FC Elevated N 10 mm. For KCl extracts, values are shown as mean values ± SE n=6, except for n=5 for treatment FC Field N. Using ANOVA and LSD of means 5% level, N uptake by microdialysis shows no significance (NS), and NO_3^--N soil concentrations show no significance (NS).

5.3.5.2. Nitrogen uptake (Experiment B)

Uptake of ^{15}N for Kukri grown in rhizoboxes is shown in Figure 5.13. The average of ^{15}N uptake was highest in plants treated with 0.5FC and Field N, and the lowest uptake was in 0.5FC x Elevated N (Figure 5.13a). However, when ^{15}N uptake is sub-divided into the different forms of N, NH_4-^{15}N, NO_3-^{15}N, glycine-^{15}N, the highest uptake of ^{15}N observed was via nitrate-^{15}N, regardless of treatment, with the lowest being via glycine-^{15}N (Figure 5.13b). However, under FC and Elevated N, uptake of ^{15}N was similar for both ammonium-^{15}N and glycine-15N.

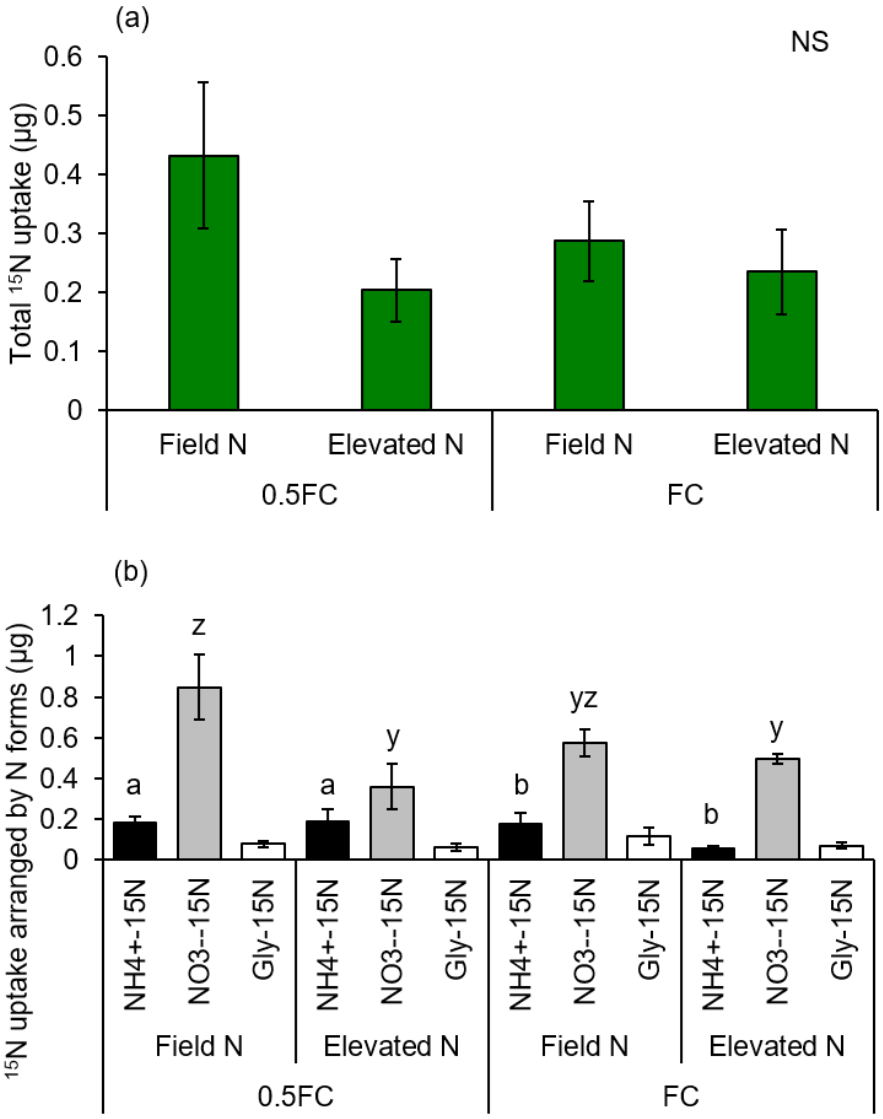

Figure 5.13. Uptake of ^{15}N in three different forms of N (NH_4-^{15}N, NO_3-^{15}N, glycine-^{15}N) for plants grown under two water treatments (0.5FC, FC) and two N treatments (Field N, 29.6 mg of N/kg of soil; and Elevated N, 120 mg of N/kg of soil). Values are presented as mean values ± SE n=12, except n=10 for treatments FC/Elevated N, 0.5FC/Field N. Using ANOVA and LSD of means 5% level, means with different letters are shown to be significantly different ($p<0.05$). Total ^{15}N uptake (a) shows no significance (NS). For individual forms of ^{15}N uptake, NH_4-^{15}N (b) shows main effect of water, p=0.047; NO_3-^{15}N shows main effect of N, p=0.024; glycine-^{15}N shows no significance (NS).

5.4. Discussion

This section summarises the results from two experiments, one which used microCT to investigate root architecture in response to water and N treatments (Experiment A), and another using rhizoboxes which allowed a longer growing period to observe root growth over time and sampling of roots and N at specific locations (Experiment B). Since the water and N treatments were as similar as possible the results will be discussed together.

5.4.1. Experimental comparison of soil N and water conditions

The soil textures of both soils used in Experiments A and B were very similar and matched that of a sandy loam texture. A sandy loam has quite a fine texture, so this may have affected the way in which the soil dries or re-wets (Fierer and Schimel, 2002, Padilla *et al.*, 2009). Additionally, although the water treatments were similar in both experiments (100% of FC and 50% of Field capacity), the slight differences in percentages of sand:silt:clay would also slightly change the water holding capacity of the soils (Austin *et al.*, 2004). Ultimately, as soil texture affects the way water moves through the soil it influences N availability. High soil moisture would increase N mobility and encourage mineralisation of NH_4^+ NO_3^- to NH_4^+-N and nitrification of NH_4^+-N to NO_3^--N (Austin *et al.*, 2004). Since both the soil columns and rhizoboxes were watered every other day (approximately every 48h interval), this would most likely have induced some drought-like symptoms within the soil profile via a wetting-drying mechanism (Padilla *et al.*, 2009), especially in the rhizoboxes, due to the increased soil volume. The 48h watering intervals is standard procedure for many glasshouse experiments, but still can be equated to pulsed water supply, which does still cause some level of drought stress, although not as severe as after a pulsed water supply (Chapter 4, Cousins *et al.*, 2020, Padilla *et al.*, 2013).

In both experiments, the constantly changing soil moisture might have affected N supply. The water pulses of both 0.5FC and FC impacted N mineralisation, due to a significant decrease in Elevated N. A higher concentration of mineral N in the Field N treatment compared to the Elevated N treatment could be because 0.5FC still provided enough water to flush the soil system every watering interval (48h). Also, it is possible that the FC treatment added more water than the soil could hold, with possible leaching of N through the base of the soil column (which was meshed to facilitate drainage but not held under suction). Upon scaling up Experiment A to the rhizoboxes (Experiment B), the soil volume, water supply, and soil N increased substantially, but NO_3^--N concentrations at harvest were considerably lower in the rhizoboxes compared to the CT soil columns. Under the FC treatment, water did leach out of the bottom of the rhizoboxes, therefore N would have leached out too, shown by the decrease in NO_3^--N under FC compared 0.5FC (Bijay *et al.*, 1995, Carstensen *et al.*, 2014). Additionally, water stress can increase soil NO_3^--N under low N but decrease it under higher N levels (Zhong *et al.*, 2017). The size of the rhizobox means that there was a high chance of N microsites developing. As a result, these microsites could encourage root proliferation in that zone, thus increasing ion uptake (Grossman and Rice, 2012, Hodge, 2004).

The use of microdialysis in Experiment B to determine the available N at a specific location is not novel (Brackin *et al.*, 2017, Brackin *et al.*, 2015), but it allowed me to identify hotspots of N within a soil profile. The lack of NO_3^--N observed under the FC treatments, does seem to confirm that high water availability increased flow of N through the soil system and subsequently increased root access to NO_3^--N. Having a low water supply resulted in some NO_3^--N still available in the soil at harvest and is complementary to results found in Cousins *et al.* (2020) and Chapter 2, where higher mineral N (NH_4^+-N and NO_3^--N) concentrations were found under low water or variable (wet/dry cycle) water. While the small size of microdialysis probes means that a smaller volume of soil can be sampled, relative to that sampled via soil extracts, it also allows the study of N dynamics in soil microsites.

Another difference between the two experiments is the duration of growth. Growing wheat in the rhizoboxes (Experiment B) meant the plants could be grown for longer, and allowed observations of root growth over time as well as easier sampling of N from soil close to the roots and other known locations. In Experiment A, wheat was restricted to two weeks' growth, due to the size of the columns and the restrictions on scanning resolution. As the wheat plants were more established in the rhizoboxes, it was possible to measure photosynthetic capacity and plant stress with photosynQ.

5.4.2. Plant biomass allocation

Since Kukri was grown until two different growth stages, the results will be discussed accordingly. Findings from both experiments show that neither water nor N stress affected Kukri root to shoot ratios, with root and shoot biomass similar to each other. This contradicts research showing increased root growth relative to shoots under water stress (Eghball and Maranville, 1993, Palta and Gregory, 1997). The lack of treatment effect on the root to shoot ratio in both experiments can be explained by Kukri having a high N use efficiency, thus root growth did not differ significantly between treatments because to Kukri, N was not a limiting factor (Mahjourimajd *et al.*, 2016). Also another possible explanation is that having a smaller root to shoot ratio could mean it is less expensive for the plant to maintain (Withington *et al.*, 2006), because having a greater root to shoot ratio means that there is more non-photosynthetic tissue (roots) for each unit of leaf area to sustain (Lynch *et al.*, 2014).

The difference in growing period affected shoot and root biomass 40-fold. Kukri at five weeks had a longer period where it was not reliant on the seed reserves. Therefore, differences in biomass at five weeks could also be attributed to the effect of the seed reserves becoming negligible by two weeks. However, the seed reserve can be depleted more quickly when soil moisture is high, as a high soil moisture would increase seedling growth, exacerbating seed reserve use (Bouaziz and Hicks, 1990). Rate of seedling establishment relative to water supply was not measured, but is something worth considering in light of the results.

The increase in root to shoot ratios for Gladius under Elevated N could be explained by several factors. The addition of high soil N increases the overall amount of N potentially available to roots, meaning

plants would not be pressured into producing longer roots to access available N. Since Gladius was grown in small soil columns, this would have contained the concentration of N in a much smaller volume, so the proximity of roots to available N patches is increased.

5.4.3. Plant photosynthetic capacity an indicator of plant stress

As soil texture impacts on soil moisture and movement of water and N, the distribution of water and N also plays a role in affecting the efficiency of photosynthesis in Kukri grown in rhizoboxes (Experiment B). It is well-known that water and N variability can affect shoot biomass, and this can be partially explained by photosynthetic capacity.

FvP/FmP is defined as the maximum quantum yield of chlorophyll fluorescence (the ratio between the minimal amount of fluorescence from the leaf to the maximum fluorescence) in the dark-adapted state of Photosystem II (Maxwell and Johnson, 2000, Sheng *et al.*, 2008). The amount of chlorophyll fluorescence (as part of the maximum photochemical efficiency) detected is a good indicator of plant stress (Sharma *et al.*, 2015). Other research has demonstrated that having a low FvP/FmP ratio coincides with a form of stress, because low FvP/FmP values are related to photoinhibition and low stomatal conductance (Prieto *et al.*, 2009). This photoinhibition is usually reflected in reduced photosynthesis and subsequently plant growth (Farquhar *et al.*, 1989, Prieto *et al.*, 2009).The low FvP/FmP ratio observed under Elevated N for both water treatments in the rhizoboxes could be explained by two possible reasons. It is possible that because of the soil texture and quantity of water added, a lot of the available N under the Elevated N treatment was in fact lost through leaching, thus the Elevated N treatment became a 'stressed' treatment. However, this does not explain the high FvP/FmP ratio under the Field N treatment. A possible explanation for this increase in chlorophyll fluorescence is that it is a possible strategy the plant utilises to combat stress, because under stress, wheat cultivars that have higher FvP/FmP ratios are better able to maintain high photosynthesis, total chlorophyll, stomatal conductance, transpiration and dry matter (Sharma *et al.*, 2015).

Another predictor of plant stress is Non-photochemical Quenching (NPQt) which is when light energy absorbed by the leaf is dissipated as heat (Sarlikioti *et al.*, 2010). The dissipation of heat from the plant is one way in which the plant can protect the photosystems from oxidation. As a result, there is an increase in NPQt. Again, the increase in NPQt for Kukri (Experiment B) in FC x Elevated N could be due to an imposed N stress due to excess water leaching nutrients through the soil. It is also possible that with an increase in N (Elevated N), plant water uptake could be increased due to increased root activity (Xu *et al.*, 2015), thus decreasing the amount of soil moisture available, as research has shown that a decrease in soil moisture results in an increase in NPQt (Hazrati *et al.*, 2016). However, plant antioxidant capacity as a stress tolerance mechanism is dependent on N availability. Thus having a higher level of available N can improve the stress tolerance of plants by increasing antioxidant producing ability and inhibiting relative oxygen species and oxidative stress (Fu and Huang, 2003,

Zhong *et al.*, 2017). As a result, this can increase the effective dissipation of energy, demonstrating high NPQt levels (Zhong *et al.*, 2017).

PhiNO is the proportion released as free energy (energy that can potentially cause damage to photosystems) (Maxwell and Johnson, 2000). The difference of phiNO levels under Field N x Elevated N can possibly be attributed to the fact that low soil N drives root activity, with low N possibly decreasing root thickness but/and increasing root length. The increase in specific root length can be a stress response mechanism, thus increasing the ratio of incoming light that is lost via non-regulated processes (phiNO) (Maxwell and Johnson, 2000). Alternatively, the decrease in phi2 (photosynthetic efficiency) (Kramer *et al.*, 2004) under Field N x FC could be due to high levels of soil moisture leaching out some of the available N. Because the majority of assimilated N is invested in photosynthetic machinery (Nunes-Nesi *et al.*, 2010), it is perhaps not surprising that the photosynthetic efficiency of the plant would naturally decrease under low N availability. Finally, the high ratio of phiNPQ (ratio of incoming light that goes towards NPQt (Maxwell and Johnson 2000)) under FC x Elevated N is comparable to the high NPQt level observed under the same treatments.

Finally, chlorophyll content is one of the major factors affecting plant photosynthetic capacity (Arjenaki *et al.*, 2011), but changes in chlorophyll affecting photosynthetic capacity depends on the level of stress and its duration. Relative chlorophyll (level of greenness) did not significantly decrease under water or N stress, which has also been observed in other studies, where drought stress did not impact chlorophyll content, but instead light reflection from the leaf was increased (Medina *et al.*, 2016, Schlemmer *et al.*, 2005). In addition, Kukri maintained a steady relative chlorophyll level under all treatments combinations could suggest that the water treatments did not cause enough stress.

5.4.4. Root architectural response to soil water and N

In the CT images (Experiment A), the high total root volume for Kukri under 0.5FC x Elevated N could suggest that although water is limiting, it did not affect growth because N is not limiting. Since N is important for healthy plant growth, it is not surprising that N addition helps mitigate the negative influence of water stress on plant growth (Saud *et al.*, 2017, Thakur *et al.*, 2012). This is the opposite effect to Gladius, where the lowest root volume occurred under 0.5FC x Field N. This is most likely due to Gladius having a lower N use efficiency than Kukri (Mahjourimajd *et al.*, 2016). Also, under low soil water, NH_4^+-N concentrations increase and NO_3^--N decreases (Huang *et al.*, 2018). This, also considering NO_3^--N is more mobile than NH_4^+-N (Liu *et al.*, 2017) could impact root C allocation in terms of root length, diameter and specific root length. Postma and Lynch (2011) showed in their model SimRoot that under severe N, maize growth was drastically reduced due to root maintenance costs, i.e. root loss, thinner roots, longer roots, fewer roots.

An increase in seminal number in Kukri (Experiment A) with an increase in soil water content could be explained by the increase in soil N availability as a result of water flushes with the 48h watering. This

result also suggests that for Kukri, N is more important for root plasticity than water. A different pattern was observed in Gladius, (highest seminal number under FC x Field N) suggesting the potential for Gladius to be better adapted to water stress. Alternatively, an increase in root tip number with Field N x 0.5FC suggests limiting resources encourages root growth, in order to maintain or increase shoot biomass. Under FC x Elevated N, where neither variable is technically limiting, there is an increase in root tip number. This increase in root tips under high N and/or water means the plant may not be stressed and so roots are potentially thicker, so C allocation is not as regulated (Postma and Lynch, 2011, Withington *et al.*, 2006).

The increase in root length under 0.5FC x Field N is comparable to other research, where longer roots under limited water or nutrients are able to access deeper soil layers (Paez-Garcia *et al.*, 2015, Postma and Lynch, 2011). Also, Grossman and Rice (2012) showed that barley plants invested more into the roots when grown in low-nutrient soils, and that root proliferation increased in nutrient hotspots. The lack of significance in specific root length for Kukri could be due to a higher N use efficiency, which changes the allocation of N within the plant, allowing more N to be allocated for photosynthesis, thus not allowing photosynthetic capacity to be negatively affected (DaMatta *et al.*, 2002). If photosynthesis is not negatively affected, the plant does not feel stressed and so there is little need to change the C allocation in the roots, i.e. C is distributed more evenly in the roots (lower specific root length). This can also be observed in the increased root diameter under Elevated N. However, an increase in specific root length in Gladius (Experiment A) under Field N and Elevated N for both water treatments could be explained by the change in soil moisture. By limiting both water and N, this restrains C allocation to the roots, reducing root diameter. Having longer thinner roots increases the root surface (Fitter, 2002) which helps to maximise water and N uptake. By increasing specific root length, it becomes less expensive for the plant to maintain (Withington *et al.*, 2006).

Although root volume values for Kukri and Gladius were not statistically significant from WinRhizo and CT analysis, some interesting patterns can be observed and discussed. Again, a higher root volume under limited water (0.5FC) and limited N (Field N) is not surprising, with root proliferation particularly observed under reduced N supply (López-Bucio *et al.*, 2003, Manschadi *et al.*, 2006). It is important to note the differences of volume observed using WinRhizo and CT, with WinRhizo analysis resulting in maximum of ~20 mm^3 and CT presenting volumes up to 700 mm^3. This can be attributed to the sensitivity of CT to capture roots at very small resolutions, i.e. 61 µm, as well as capturing the root architecture in real-time. Howeve, one disavdantage of using X-ray CT at a resolution of 61 µm was that it was unable to capture finer (usually lateral) roots (Tracy *et al.*, 2012a). WinRhizo is a destructive procedure and therefore only able to capture the roots which were harvested, and subsequently there is a high chance of root breakage and loss of root material. Kukri was able to produce a high root volume under Elevated N x 0.5FC; although the limited water supply would have undoubtedly affected N

movement (even if N is not limiting), it is possible that Kukri had to utilise its high N use efficiency to maintain root and shoot biomass.

As wheat was only grown for two weeks in Experiment A, it was not possible to determine rate of root growth over time. However, by conducting the second experiment in rhizoboxes (Experiment B), this allowed root growth of Kukri to be observed and the rate over time to be calculated. High N use efficiency observed in Kukri can be used to explain Field N encouraging quicker root growth than 0.5FC (Mahjourimajd *et al.*, 2016).

5.4.5. Plant N uptake dynamics

Although average ^{15}N uptake was not significantly affected by the treatment combinations, there was an increase under low N compared to high N. Although the roots used for ^{15}N uptake were excised, it is possible to conclude that a high NO_3^--N uptake under 0.5FC x Field N could be the roots may have increased the number of N transporters to try and maintain N influx (Lee and Drew, 1989) meaning plants that have been N-limited end up with an enhanced capacity to absorb N when that limitation is removed, i.e. when roots are submerged in ^{15}N solution (Lee and Rudge, 1986). This could help explain the minimal growth response observed in Kukri, in that Kukri potentially has a higher capacity for N uptake, due to its high N use efficiency. This in return compensates for low N levels.

Plant N preference can depend on whether the roots are excised or remain intact. In Experiment B, the roots were excised, so it is possible that the uptake of ions that are influenced by mass flow, i.e. NO_3^--N, may be underestimated or overestimated (Brackin *et al.*, 2015).

Another interesting result is the differentiation between N form taken up by the roots, with roots preferring to take up NO_3^--N over any other form of N, followed by ammonium then glycine. Wheat preference for NO_3^--N over NH_4^+-N or glycine could be because wheat shows sensitivity to soil NH_4^+-N concentrations (Cramer and Lewis, 1993, Liu *et al.*, 2017, Thornton and Robinson, 2005); this could explain biomass accumulation lower under NH_4^+-N than NO_3^--N (Cramer and Lewis, 1993). The highest uptake of NO_3^--N occurred under 0.5FC x Field N. These results show that wheat can take up different forms of N, and this is also affected by the soil water and N supply.

5.4.6. Conclusions

This chapter highlights the impact of water and N variability on biomass allocation to shoot and roots and soil and root N dynamics. Root architecture and root plasticity were quantified using X-ray CT to visualise and measure root architecture in 3-D in plants grown in soil. Additionally, N uptake under water and N stress was measured using ^{15}N isotopes and microdialysis.

Overall, N played a bigger role in differentiating root and shoot biomass, with Kukri performing better under either low water or low N, proving the first hypothesis. In terms of root architecture, Field N x 0.5FC water encouraged longer roots as well as more root tips, whereas Elevated N x FC water resulted in larger root diameter, proving the second hypothesis. Interestingly, under high water, the number of

seminals increased, although number of root tips was lowest, suggesting that under 0.5FC water, there was an increased number of laterals from fewer seminals. Not surprisingly, the highest volume of roots was under high N but low water, demonstrating that low water was the driver for increased root growth. Looking at the photosynthetic measurements, we conclude that the plants may not have been as stressed as initially thought, therefore not agreeing with the third hypothesis, with NPQt levels lowest under Field N, FvP/FmP ratio highest under Field N, and relative chlorophyll levels not differing between treatments. The decrease in N levels under both water treatments for Elevated N could suggest that the roots were able to access most of the available N, and water helped in encouraging NO_3^--N uptake in Kukri grown in the rhizoboxes. The ^{15}N isotopic work was crucial in helping to identify differences in uptake between NH_4-^{15}N, NO_3-^{15}N and glycine-^{15}N, distinguishing root preference for N forms. Irrespective of N or water treatment, roots preferred to take up NO_3^--N over the other two forms of N, disproving our fourth hypothesis. By analysing this data, it is possible to understand root response to its environment. There is an increasing pressure to learn how plants adapt to their environment and identify traits responsible for improving plant plasticity (whether roots or shoots) under water and N stresses. As rainfall becomes more erratic, this will impact N fertiliser efficiency and plant N use efficiency or water use efficiency, thus posing more agricultural challenges around the world. Plant production needs to be optimised, no longer just under optimal conditions, but under sub-optimal conditions, even extreme drought or nutrient deficit. This research has reinforced the idea that water and N are co-dependent in terms of plant plasticity and soil N management. By thoroughly understanding plant plasticity to soil water and N content, it will narrow down traits and response mechanisms that would greatly enhance plant survival under moderate to extreme resource limitations. Ultimately, this knowledge can be utilised in two different ways: changing the way we manage our crops, purposefully creating resource deficits in order to improve crop growth or implement the best growth response traits into breeding programs. This will aid in maximising crop yields with minimum resource allocation.

5.5. Supplementary Material

Table S5.1. Statistical analysis sorted by variable.

Experiment	Genotype	Variable	Data transformation
A	Gladius	Shoot dry weight	Log_e
		Root dry weight	Log_e
		Root:shoot ratio	Original data
		Total root length	Original data
		Specific root length	Original data
		Root volume (from WinRhizo)	Square root
		Total root tip number	Original data
		Root diameter	Original data
		Seminal number	Square root
		Root volume (from X-ray CT)	Original data
		Total mineral N (NH_4^+-N and NO_3^--N)	Original data
	Kukri	Shoot dry weight	Original data
		Root dry weight	Original data
		Root:shoot ratio	Original data
		Total root length	Square root
		Specific root length	Original data
		Root volume (from WinRhizo)	Original data
		Total root tip number	Square root
		Root diameter	Log_e
		Seminal number	Original data
		Root volume (from X-ray CT)	Original data
		Total mineral N (NH_4^+-N and NO_3^--N)	Log_e
B	Kukri	Shoot dry weight	Log_e
		Root dry weight	Log_e
		Root:shoot ratio	Log_e

Rate of root growth	
Leaf temperature differential	Original data
FvP/FmP (maximum quantum yield of chlorophyll fluorescence)	Original data
NPQt (Non-photochemical quenching, energy absorbed by the leaf and dissapated as heat)	Log_e
Linear electron flow (number of electrons flowing out of Photosystem II)	Original data
Phi2 (energy used for sugara production)	Log_e
PhiNO (energy released as free energy)	Log_e
PhiNPQ (energy quenched in the leaf)	Original data
Relative chlorophyll (level of greenness)	Original data
Total NO_3^--N concentration (from microdialysis	
Total NO_3^--N (from KCl soil extracts)	Square root
Total ^{15}N uptake	Log_e
Uptake of NH_4-^{15}N	Log_e
Uptake of NO_3-^{15}N	Original data
Uptake of glycine-^{15}N	Square root

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Chapter 6

Summary, conclusions and future work

6.1. Conclusions

The work presented in this thesis provides new insights into the interactive effects of water and N supply on important soil properties and the growth and physiology of two wheat varieties. Water and N supply affected root plasticity, shoot growth, soil N dynamics and microbial biomass C. Soil mineral N availability was strongly influenced by soil moisture, with the availability of NH_4^+-N and NO_3^--N decreasing with low soil moisture. Changes to soil physiochemical properties were associated with changes in root architecture, C allocation to roots and shoots, and aboveground physiology (including photosynthetic efficiency, see Chapter 5). Moreover, the differing physiological responses of wheat varieties Kukri and Gladius to variable water and N supply have provided insights into the phenotypic responses that could potentially aid in enhancing water productivity, nutrient use efficiency and yields.

The main findings of the study were:

- Chapter 2
    - The use of a gravimetric platform to set up and maintain specific water contents.
    - The confirmation of the co-dependency of water and N for overall plant growth, soil nutrition and microbial activity.
    - Reduced water supply encouraged root growth when combined with medium and high N.
    - Plant growth was more responsive to N than water treatments.
    - Variable water treatment resulted in less biomass overall, suggesting wheat prefers consistency.
- Chapter 3
    - The determination of soil water and legacy effect on subsequent crop growth.
    - Increase in biomass with increased N.
    - Variable water encouraged mineralisation, producing largest biomass under high N for second crop growth.
    - Mineral N decreased between 2 seasons of crop growth.
- Chapter 4
    - Kukri is better adapted to water variability due to its high N use efficiency.
    - Less frequent watering had more negative effect on plant growth compared to decreased quantity of water.
    - The water regimes imposed affected N availability.
    - Differences in C allocation because of the water variability, with changes to root thickness even when root biomass remained same across N treatments.
    - Differences in biomass with constant watering and 48 h watering intervals show that trying to maintain water levels manually (watering to weight 3x per week) maybe

compromise on plant physiology, soil water physics and nutrient cycling, which all aid with biomass allocation.

- Chapter 5
    - Kukri performed better under either low water content or low N.
    - Low water content and low N encouraged longer roots and more root tips, but high water and high N produced larger root diameter.
    - Highest volume of roots found under high N but a low water content, demonstrating that low water was the driver for increased root growth.
    - More phiNO (potentially dangerous energy) released under low N, more phiNPQ quenched under high water (safety mechanism), NPQt levels lowest under low N, FvP/FmP ratio highest under low N, and relative chlorophyll levels not differing between treatments.
    - Identified root uptake preference between NH_4-^{15}N, NO_3-^{15}N and glycine-^{15}N, with roots preferring NO_3 N uptake.

Taken together, the research discussed in this thesis demonstrates the importance of understanding the co-dependency of water and N in relation to plant growth and soil N dynamics. From this, it is possible to identify plant response mechanisms to varying levels of water and N stress, to subsequently select levels of water and N that would maximise crop growth even under sub-optimal conditions. In this final chapter, some emerging themes that can be drawn from across all chapters are highlighted along with identification of new areas for potential future research.

6.2. Biomass allocation impacted by water and N variability

As rainfall variability becomes increasingly common, it will impact soil nutrient dynamics, and hence, the ways in which farmers utilise their fertilisers. A decrease in soil moisture levels will inevitably result in plant death if more optimal moisture levels are not achieved. In addition, the more efficient use of N fertiliser is becoming more important as climate change impacts increase, with deficit soil moisture decreasing soil N mobility, patches of N unavailable to plants can develop (Chapter 5). Alternatively, high soil moisture or soil saturation can create conditions conducive to soil N loss as N_2 and N_2O emissions (Butterbach-Bahl *et al.*, 2013), as well as leaching of N fertilisers and surface run-off (Bijay *et al.*, 1995, Carstensen *et al.*, 2014). These losses can result in annual N losses of up to 160 kg per hectare in some systems (Herzog *et al.*, 2008), with only ~50% of N captured by crops or remaining in the soil (Brackin *et al.*, 2015). This inefficiency of N use can result in more fertiliser being applied to compensate, but this exacerbates the problem. Managing water and N resources more efficiently will a) optimise agricultural water productivity and b) decrease N fertiliser waste. By controlling water and N

resources during vegetative growth stages, i.e. tillering, and the pre-cropping season will aid in improving crop resource use efficiency.

6.2.1. Limiting water supply

By limiting water supply, i.e. reduced water (Chapter 2, 3), 0.5FC (Chapter 4, 5), the results showed significant changes to shoot and root biomass. In Chapter 2, under variable water (wet/dry cycle) shoot dry weights were lowest, compared to well-watered treatments, even under high N. Additionally, at a later growth stages as revealed in a second harvest, roots were most responsive to N under reduced water, with the highest root biomass found under high N (Cousins *et al.*, 2020; Chapter 2).

The reduction in overall plant growth (seen in Chapter 2, 3 and 5) mirrors other studies where a reduction in the amount of water can be detrimental to plant growth, with a 50% reduction in rainfall amount resulting in smaller total biomass and having a negative effect on plant physiology, creating shallower rooting systems (Gibson-Forty *et al.*, 2016). Padilla *et al.* (2013) demonstrated that frequent watering at a reduced quantity resulted in water deficit over the short period of time between watering but increased plant water use efficiency (Lambers *et al.*, 2008, Larcher, 2003). In contrast, plants subjected to wetting and drying cycles or partial root-zone irrigation showed an increase in root growth, also reflected in increased shoot growth (Kang and Zhang, 2004, Zhang *et al.*, 2009). This can be seen with the increased roots and shoots found under Wet/Dry compared to the dry treatments 0.5FC and 0.5FC 48h (Chapter 4).

Furthermore, the work presented in Chapter 2 (Cousins *et al.*, 2020) suggested an increase in shoot and root growth, as well as water use efficiency from Harvest 1 and Harvest 2, meaning wheat was able to produce more biomass with less water. The increased root growth helped to maintain shoot growth (Araus *et al.*, 2013, Ayad *et al.*, 2010, Passioura, 2002). Increased total root length or biomass in general, coupled with increased root density at deeper soil layers, would aid in accessing deep soil moisture and N (Tardieu, 2011). This increase in root biomass was reported in previous chapters, particularly with Kukri, with the highest root biomass and length corresponding to 0.5FC x field N (Chapter 5); subsequently, shoot growth was highest under these same treatments. The opposite effect was found in Chapter 4, where shoot and root biomass were severely reduced under 0.5FC, 0.5FC 48h and Wet/Dry. Studies have shown that excess irrigation or rainfall can be detrimental to plant growth and yield. Although it is hard to conclude whether high water supply was detrimental to overall plant growth in this research, under reduced water (0.5FC) shoot and root biomass was highest, especially when coupled with low N (Field N) (Chapter 5). The application of excess water will result in water loss through unproductive soil evaporation and could cause yields to decrease due to waterlogging and nutrient leaching (Cabello *et al.*, 2009, Sun *et al.*, 2006).

There are several studies that have demonstrated better crop growth with co-limitation of resources (i.e. both water and N limited). This can have the benefit of increasing water use efficiency and nitrogen use

efficiency as well as grain yields (Cossani *et al.*, 2010). Root uptake of N can be significantly increased as a result of just one simulated rainfall event (Ivans *et al.*, 2003). In the work presented in Chapter 2 (Cousins *et al*., 2020), root biomass increased the most under reduced water when combined with high N. As water was limiting, the level of soil N was able to help the plant to positively respond to stress and maintain shoot and root growth. Perhaps having the highest level of N is not necessary for efficient plant growth, because when combining reduced water with medium soil N levels, overall plant growth was maintained, but root biomass particularly increased (Chapter 4). Under water stress conditions (namely 0.5FC and Wet/Dry treatments in Chapter 4), specific root length increased, meaning roots were becoming longer and thinner. Not only did water supply affect specific root length, but N supply also. For example, under Wet/Dry treatment, root biomass was the same across N treatments, however specific root length decreased with increasing N (Chapter 4). An increase in specific root length generally means C allocation to roots is restrained which increased access to both water and N pools further down the pot (Chapter 4). The reduction in root diameter but increase in length as a result of lower water or N levels, creates a larger root surface (Fitter, 2002), thus enabling the plant to maximise water and N uptake. Ultimately, having thinner and longer roots is less expensive for the plant to maintain (Withington *et al.*, 2006). This may be one adaptation to combat against drier or more variable climate conditions, and a possible trait to be considered in the breeding of drought resistant crops.

6.2.2. Changing frequency of water

Not only does the quantity of water affect crop growth, the frequency of water can change the plastic response of a plant both positively and negatively. In Chapter 4, under 0.5FC 48h, the root to shoot ratio of Kukri was significantly reduced compared to 0.5FC. These plants had smaller biomass (both shoots and roots) than plants subjected to either the Wet/Dry treatment or 0.5FC treatment. The difference between the 0.5FC 48h and Wet/Dry treatment is the timing between watering. The dry-down period of the wet/dry cycle is considerably longer than 48h, forcing the plants into a form of drought. During the wet period of the wet/dry cycle, the soil water supply could have encouraged root proliferation, especially when combined with low N. This increased root system could have helped root N uptake and maintained plant growth under stress.

The ability of plants to adapt to variable watering frequency can also be attributed to plasticity in different root types. Sebastian *et al.* (2016) found some grasses suppress shoot-borne roots (otherwise known as crown roots or aerial roots) in order to conserve water during water deficits. The crown roots of some grasses, like maize, are important for water and nutrient uptake and drought stress signalling. After imposing a wet/dry cycle on the grasses, it was found that crown root development was affected. Under deficit water, crown root growth was suppressed, but when plants were re-watered, the crown roots were revived and development resumed. Moreover, the crown roots were able to identify where the water supply was coming from and respond accordingly. Only if the plants were watered from the top was crown root growth reactivated. If plants were watered from the base of the pot, the plant was

only able to recover water status and maintain root growth (Sebastian *et al.*, 2016). In addition, increasing the number of crown roots has been found to enhance topsoil foraging for both water and nutrients (Sun *et al.*, 2018).

In Chapter 5, the use of X-ray CT allowed the root system to be imaged and analysed in 3-D, non-destructively. Having a higher number of seminal roots under 0.5FC (which is comparative to the 0.5FC 48h watering treatment established in Chapter 4), suggests that this may have aided the plant to adapt to the reduced water supply and variability of watering. Regardless of N treatment, variable watering i.e. 48h watering or wet/dry cycles, will impact N mineralisation, ammonification, nitrification, and subsequently uptake and N use efficiency. The use of the DroughtSpotter in Chapters 2 and 4 is an effective approach to understand how even small changes in watering (either hourly or daily changes) can affect biomass accumulation. However, it does not necessarily create a perfect comparison to field conditions, since in the field, soil moisture is not confined by a pot and roots are thus able to access deeper soil moisture layers than would otherwise be found in a pot.

6.2.3. Limiting soil N supply

Limiting soil N supply was also found to affect plant biomass, particularly root growth (Chapter 2, 4, 5). As N mobility and availability is dependent on soil water availability, N uptake is subsequently impacted. Although N is important for plant growth, having a lower soil N availability has been found to encourage more root biomass in wheat (López-Bellido *et al.*, 2005). This was observed in Chapters 2 and 5. Even under medium N levels, if there is high water supply, this encourages more root production (Cousins *et al*., 2020; Chapter 2). In Chapter 5, Field N (low N) produced higher root biomass with 0.5FC (low water), but when paired with FC (high water), root growth decreased. The reduced root length, root tip number, and seminal number as a result of increasing N and water (Chapter 5), has also been previously observed (Comfort et al 1988), and demonstrates a reduction in root surface area and possible inefficiency in nutrient and water uptake.

Optimal partitioning theory, which suggests resources are allocated to the plant organ that is experiencing resource limitation, has been tested and discussed in Chapters 2, 4, and 5. In Chapter 2, it was demonstrated that when plants were subjected to less N and reduced water, they produced larger root systems, compared to plants under higher N and higher water supply. Having a larger root system could boost N uptake and water uptake, especially at earlier growth stages. As the shoots are necessary for photosynthesis, under N stress having a high FvP/FmP (Chapter 5) could be beneficial to aid stress relief, through a better maintenance of photosynthesis, total chlorophyll, stomatal conductance, transpiration and biomass (Sharm et al 2014). However, plant response to co-limitation or resource deficiency can be genotype dependent. For example, root and shoot growth in Kukri increased under medium N as opposed to high N like Gladius (Cousins *et al.*, 2020; Chapter 2). This was most likely due to Kukri having a higher NUE, which allows it to adapt under lower N levels than normal and still

produce more roots and shoots. In agreement with this, in Chapters 4 and 5, it was shown that Kukri produced more overall shoot and root biomass than Gladius (Chapter 5), and the effect of water and N was more pronounced in Gladius (Chapter 5).

By reducing N supply, root proliferation is promoted (López-Bucio *et al.*, 2003, Manschadi *et al.*, 2006). This increased allocation of biomass below-ground typically comes at the expense of shoot growth; which is reflected in a higher root to shoot ratio, and improved N assimilation (Evans *et al.*, 1975, Sims *et al.*, 2012). An example of root proliferation at the expense of shoot growth was observed in Chapter 4. In Kukri, an increase in root biomass was observed under medium N and high water, whereas the opposite trend was observed under 0.5FC (low water) which showed a decrease in root biomass and overall shoot biomass but there was a lower root to shoot ratio. Another trait of root systems under low N supply is a higher specific root length compared to root systems under high N supply (Fitter, 2002). This has been observed in Chapter 5, with the highest specific root length found under Field N and 0.5FC in Gladius. Kukri also showed the highest specific root length under medium N, even though root biomass remained the same across all N treatments under Wet/Dry cycles (Chapter 4). A higher specific root length, resulting in thinner and longer roots, has the potential to conserve more energy; instead of producing thicker roots, which use more C (and thus more photosynthetic energy).The plant can channel resources (namely C) to produce deeper roots that are able to access nutrients and/or water at deeper levels (Lynch *et al.*, 2014, Postma and Lynch, 2011). In contrast, increasing N supply can encourage larger root biomass. In Chapter 4, Gladius root biomass increased with increasing N. Although a larger root biomass can be beneficial for overall plant health, it is more expensive for the plant to maintain (Withington *et al.*, 2006), and is also dependent on water supply (and other environmental factors). Instead, plants may develop more root branching, which results in an increase in thinner roots with a higher specific root length (Elazab *et al.*, 2016, Herrera *et al.*, 2007). This trait was observed in Chapter 5, with Field N x 0.5FC producing the highest seminal number in Gladius and number of root tips in Kukri. Although root branching was not directly measured, it can be inferred by both seminal number and root tip number, by looking at the X-ray CT images from Chapter 5. With both Gladius and Kukri, there appeared to be a lot of roots in the lower part of the soil column, which shows extensive lateral root growth (root branching). This could be a result from growing wheat in pots, however, the root architectural response was seen multiple times in all experiments.

6.3. Soil N dynamics impacted by variable water and N

The co-dependency of water and N means that combining water pulses or partial irrigation with low N supply encourages the breakdown of N forms locked up in soil organic matter, increasing N availability (López-Bucio *et al.*, 2003, Schwinning and Sala, 2004, Wang *et al.*, 2015). This concept was explored in Chapters 2 and 4. In Chapter 4, mineral N concentrations were highest under high N for both varieties,

with Kukri marginally better at N uptake (shown by slightly lower mineral N values from soil extracts of Kukri). However, mineral N was highly variable, and this can be attributed to the different watering treatments; the dry water treatments (0.5FC, 0.5FC 48h, Wet/Dry) had slightly higher soil mineral N. Lower levels of mineral N under 0.5FC 48h and Wet/Dry with low and medium N levels, suggest that the water pulses received (either every 48h or after a dry-down) flushed the soil system and unlocked potential N from dry microsites. The high levels of mineral N under high N with reduced or variable water in Chapter 2 also backed up the argument that without adequate water supply, N efficiency is lowered due to the inability of N to move, be mineralised, nitrified or accessed by plant roots.

6.3.1. Impact of variable watering on soil N

By modifying the frequency and/or quantity of water supply, the soil drying dynamics change. The DroughtSpotter platform used in Chapter 2 and 4 not only permitted high precision watering of plants, but also the monitoring of soil moisture over the entire growth cycle of the plant. The way a soil dries or re-wets can differ depending on quantity and frequency of water, i.e. wet/dry cycles or pulsed water supply (Fierer and Schimel, 2002, Padilla *et al.*, 2009). This is partly due to soil type. Austin *et al.* (2004) established that soils with a fine texture naturally have a greater water holding capacity, resulting in a greater flush of mineralised N upon watering. Whereas, soils with a higher organic matter content have more pools of potentially available nutrients. Any form of water supply has the chance to unlock these nutrient pools by encouraging microbial activity and mineralisation or denitrification of N substrates (Cui and Caldwell, 1997, Gordon *et al.*, 2008). In line with this, both NH_4^+-N and NO_3^--N concentrations were highly variable in the soil in all experiments (Chapters 2, 3, 4 and 5). However, the importance of water variability on N supply is clearly seen in Chapter 3, where high levels of mineral N are observed under variable water after one crop season. During a dry-down, overall soil moisture decreases, but there may still be differences in soil moisture throughout the soil. Any N available within the wetter soil patches will most likely have been used up by plant roots sooner, leaving any N residing in drier patches to remain either bound up in forms of N plants cannot take up or simply inaccessible due to decreased mobility. Upon re-watering, N previously locked up in the drier soil patches will be accessible once again.

6.3.2. Impact of variable watering on N uptake

The use of ^{15}N stable isotopes allowed N uptake to be measured in Kukri (Chapter 5). An increase in ^{15}N uptake was observed under field N (low N) compared to elevated N. This could possibly be due to the roots under the field N treatment were N-stressed or N-starved. As nutrient uptake is facilitated by transporters in the root (Steffens and Rasmussen, 2016), nutrient deficiency has been shown to increase expression of transporter genes. In return, this helps to improve nutrient uptake capacity (Steffens and Rasmussen, 2016). Variable water and N can also affect formation of different root types. Although the differentiation of crown roots from seminals was not directly measured, X-ray CT imaging allowed the visualisation of the root crown and root architecture traits (seminal number, root volume) (Chapter 5).

The number of seminals, root tips and total root volume will impact N uptake and could either increase or decrease N use efficiency. Under N stress, plants show an enhanced capacity to absorb N when the N limitation is removed (Lee and Drew, 1989); in this case, the N limitation was removed upon submersion of the excised roots in the different ^{15}N solutions.

6.4. Implications of legacy effect on crops

Not only can soil water supply during a cropping season greatly affect plant growth, but soil moisture conditions prior to planting (hereby known as legacy effect) can also have a profound impact on plant plasticity and nutritional status. The concept of a legacy effect was explored in Chapter 3, where a set of wheat plants were subjected to three different water and N treatments, harvested, and then a second crop was grown in the same soil as the previous crop. Both plant growth and soil N dynamics were affected by a previous set of soil water and N treatments and crop. The idea of legacy effect has been explored previously in the literature. Cavagnaro (2016) identified that NH_4^+-N and NO_3^--N levels in the starting soil were higher compared to those at planting. Wet/dry cycles resulted in the smallest decrease in NH_4^+-N levels, suggesting that levels of nitrification or NH_4^+-N were lower and higher, respectively. This reflects previous findings which show that wet/dry cycles can lower the rate of nitrification (Xiang *et al.*, 2008). Alternatively, NO_3^--N concentrations under wet/dry cycles were exceptionally high pre-planting. This can be explained by increased mineralisation and nitrification as a result of the water pulses after each dry-down (Austin *et al.*, 2004). This is mirrored in Chapter 3, where the variable (wet/dry cycle) watering in the first crop most likely created N-rich and N-poor zones microsites (Cousins *et al.*, 2020, Cui and Caldwell, 1997, Harrison-Kirk *et al.*, 2013). For the second crop, there is a possibility that the increase in soil moisture (Well-watered treatment) would have flushed out any remaining NH_4^+-N or NO_3^--N (Cui and Caldwell, 1997). In the context of a changing climate, the knowledge of previous rainfall or irrigation and/or soil N supply is something that can be utilised to improve both water and N use efficiency. Results from this thesis have shown that crop growth efficiency is variable (Chapters 2 and 4). It would be interesting to further explore water and N legacy effects under similar watering treatments as those established in Chapter 4 and 5. The frequency of watering would undoubtedly affect soil N acquisition, based on preliminary results in Chapter 3.

6.5. General conclusions

In summary, reduced water supply resulted in higher root biomass (Chapter 2, 3), with the addition of 48 h watering intervals increasing root, especially when paired with medium N levels (Chapter 4). Root biomass was also encouraged when both water and N levels were low, but total root volume was lowest (Chapter 5). Specific root length was affected by water quantity and frequency, but reductions in specific root length were driven by increasing soil N changes particularly under variable water (wet/dry

cycling) (Chapter 4). The variability of water and N also impacted average ^{15}N uptake, as well as demonstrating plant preference to nitrate-^{15}N over ammonium-^{15}N and glycine-^{15}N. These overall findings have been discussed in the context of a changing climate, with an increase in erratic rainfall and limited N resources, and can be used to develop knowledge surrounding crop management and breeding to improve overall plant resource use efficiency and yields.

6.6. Future Work

This thesis generated new data in an important area as well identifying several potential gaps in the literature. In order to develop and improve crop management strategies under variable water and N supply, further research is highly recommended in the following areas.

1. Include more wheat varieties

This study focused on the phenotypic behaviour of two Australian wheat varieties but in two hemispheres. In order to consider the effects of a changing climate across the globe, it would be valuable to include more wheat varieties grown under different environmental conditions. It would be helpful to identify varieties that are drought-tolerant, drought-susceptible, and with higher and lower N use efficiencies to be able to target phenotypic traits.

2. Use different soils

The effect of variable water and N was only studied in sandy-loam textured soils. Soil texture affects the soil drying characteristics, therefore in order to improve crop water productivity and N use efficiency, it is necessary to first identify what properties change within a soil profile dependent on its particle size distribution, and then investigate root (direct effect) and shoot (indirect effect) phenotypic traits as a result of the soil profile.

3. Explore the role of growth stage on plant response

One aspect that would need to be investigated further is the impact of growth stage on plant response to water variability. In maize, the highest water productivity was observed when deficit irrigation was applied at the early vegetative stages. Any later, and this caused a significant reduction in yield (Pandey *et al.*, 2000a, Pandey *et al.*, 2000b). Alternatively, additional irrigation or a wet seasonal start can strengthen crop establishment and improve grain quality, especially when occurring during sensitive growth stages such as tillering, booting or heading (the emergence of extra shoots, flag leaf, and grain head, respectively) (Poole *et al.*, 2015, Salter and Goode, 1967)).

4. Explore timing of N application

Frequency of water played a major role in both root and shoot growth, but the influence of timing of N fertiliser application on root and shoot physiology is not very well known or understood. By comparing different N application timings, alongside variable watering (either by quantity or frequency), it would be possible to explore different plant physiological responses. From this, we could tailor water and N management for a specific crop and/or environment (e.g. future climate change scenarios) to stabilise or even maximise plant growth and yields.

5. Explore localised root proliferation phenomena

As N mobility is affected by soil moisture, identifying N-rich and N-poor microsites and the forms of N that reside in these microsites, would be beneficial to understanding root proliferation and movement of N through the soil and into the root, and how this can affect C allocation.

6. Conduct field trials

Clear effects of water and N were demonstrated throughout this thesis via glasshouse experiments. In order to compare these results to more realistic scenarios, it would be important and useful to conduct field trials for different wheat varieties (with differing water and N use efficiencies or root traits) under similar watering and N treatments. By translating watering treatments from the glasshouse to the field would require identifying irrigation schedules that would be both environmentally friendly and cost-effective (minimising water loss and cost). In a field environment, other variables may play a part in water and N uptake. In addition, water movement through a soil profile would be more varied due to soil structure and soil physiochemical properties. This in turn would affect N movement and root proliferation.

7. Measure all N pathways

Although the concept of N leaching and movement of N is discussed in this thesis, N loss pathways, such as denitrification, or NO_3^--N leaching were not measured. Measurements of these losses would allow more accurate N budgets to be calculated, and thus help to improve N use efficiency in the field from both a physiological and economical viewpoint.

8. Measure microbial activity

Microbial activity was touched upon in Chapter 2, with microbial biomass C measured; however, to identify different microbial communities and activity of these communities, further research would be necessary. Measurements such as microbial biomass C, microbial biomass N, microbial community genetic screening would add to the understanding of microbial activity in relation to variable water and N. By identifying microbial communities and activity, it may be possible to identify plant growth promoting bacteria that could aid water and/or N uptake, thus improving water and N use efficiencies.

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