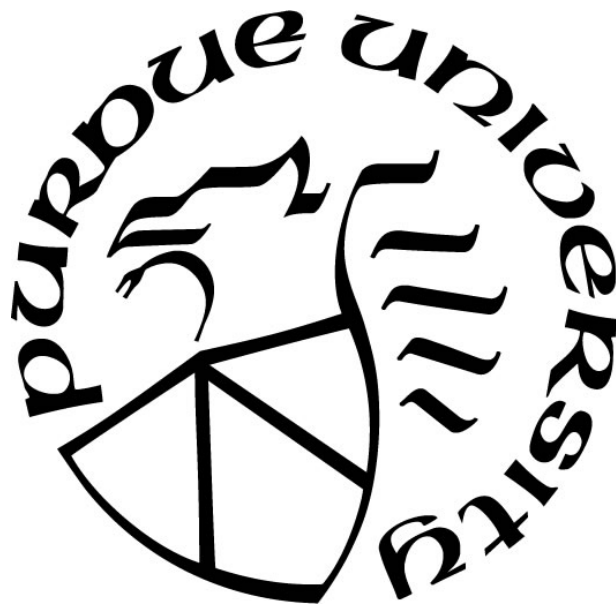# BIOMASS ALLOCATION VARIATION UNDER DIFFERENT NITROGEN AND WATER TREATMENTS IN WHEAT

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Agronomy

West Lafayette, Indiana

August 2019

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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ACKNOWLEDGMENTS

I greatly appreciate the time and effort my advisor, Mohsen Mohammadi, has put into making me a capable scientist. The patience, guidance, and support that he has given me throughout this work is very appreciated. I further am grateful for his edits and comments that have greatly improved the quality of this work.

I am further thankful for my committee members, Dr. Mitch Tuinstra and Dr. Jeff Volenec, for their time spent refining this thesis. Their thoughtful comments have been incredibly valuable.

The members of the Mohammadi lab throughout my short time at Purdue have been a tremendous resource, and I am very thankful for their knowledge that they have shared and for their friendship. Additionally, I would like to thank the remaining graduate students and staff for creating the positive culture that I associate with the Agronomy Department at Purdue.

I would like to thank my wife, Abby; my parents; and my family for their encouragement and love that has led me to this point.

Thank you for reading what I have spent the majority of my time working on these past two years.

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ABSTRACT

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Institution: Purdue University
Degree Received: August 2019
Title: Dynamic Response of Biomass Allocation to Nitrogen and Water in Wheat.
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Wheat is among the most important cereal crops in the world today with respect to the area harvested (219 million ha), production (772 million tonnes), and productivity (3.53 tons/ha). However, global wheat production goals for the coming decades are falling short of needed increases. Among the leading factors hindering yields is abiotic stress which is present in nearly 38% of wheat acres globally. Nevertheless, many standard wheat breeding programs focus on yield and yield related traits (i.e. grain yield, plant height, and test weight) in ideal environments rather than evaluating traits that could lead to enhanced abiotic stress tolerance. In this thesis, we explore the use of root and high-throughput phenotyping strategies to aid in further development of abiotic stress tolerant varieties.

In the first three experiments, root phenotypes were evaluated in two nitrogen (N) treatments. Over a series of seedling, adult, and multiple-growth-stage destructive plant biomass measurements, above-ground and below-ground traits were analyzed in seven geographically diverse wheat accessions. Root and shoot biomass allocation in fourteen-day-old seedlings were analyzed using paper-roll-supported hydroponic culture in two Hoagland solutions containing 0.5 (low) and 4.0 (high) mM of N. Root traits were digitized using a WINRhizo platform. For biomass analysis at maturity, plants were grown in 7.5-liter pots filled with soil mix using the same concentrations of N. Traits were measured as plants reached maturity. In the third N experiment, above- and below-ground traits were measured at four-leaf stage, stem elongation, heading, post-anthesis, and maturity. At maturity, there was a ~15-fold difference between lines

with the largest and smallest root dry matter. However, only ~5-fold difference was observed between genotypes for above-ground biomass. In the third experiment, root growth did not significantly change from stem elongation to maturity.

In the final experiment, two of these lines were selected for further evaluation under well-watered and drought treatments. This experiment was implemented in a completely randomized design in the Controlled Environment Phenotyping Facility (CEPF) at Purdue University. The differential water treatments were imposed at stem elongation and continued until post-anthesis, when all plants were destructively phenotyped. Image-based height and side-projected area were associated with height and shoot dry matter with correlations of r=1 and r=0.98, respectively. Additionally, 81% of the variation in tiller number was explained using convex hull and side-projected area. Image-based phenotypes were used to model crop growth temporally, through which one of the lines was identified as being relatively more drought tolerant. Finally, the use of the Munsell Color System was explored to investigate drought response.

These experiments illustrate the value of phenotyping and the use of novel phenotyping strategies in wheat breeding to increase adaptation and development of lines with enhanced abiotic tolerance.

CHAPTER 1: LITERATURE REVIEW

1.1 Global Importance of Wheat

For thousands of years, wheat has been among the most important grain crops around the world (Shewry, 2009). In 2017, wheat was planted on more hectares than any other crop, 219 million hectares, which produced 772 million tonnes of grain with an average grain yield of 3.53 tonnes/hectare (FAO, 2017). In addition, since it is easily digestible and contains large amounts of proteins and complex carbohydrates (Shewry, 2009), wheat is the staple food for more than 35% of the global population (FAO, 2013).

Bread wheat (*Triticum aestivum L.*) is a quantitative long-day, C3 species that is self-pollinating in nature (Vasil, 2007). While much of the wheat grown worldwide is allohexaploid bread wheat (2n = 6x = 42; AABBDD), differences in domestication events led to species with other ploidy levels. For example, tetraploid, durum wheat is commonly grown in both the Mediterranean climate and in North Dakota (Shewry, 2009).

A classification system is used to separate types of wheat based on three main characteristics: kernel hardness, kernel color, and growth habit (Finnie & Atwell, 2017). Kernel hardness (i.e. "hard" or "soft") refers to the force required to crush the grain. Kernel color (i.e. "red" or "white") describes the pigmentation of the grain. Growth habit refers to a plant's requirement for a vernalization period before flowering (i.e. "winter" or "spring"). Using these characteristics, wheat is classified as hard red winter, hard red spring, soft red winter, durum, hard white, and soft white. Hard red winter, hard red spring, and hard white types of wheat are primarily used to produce bread or dough-based products (Finnie & Atwell, 2017). Soft red winter and soft white wheats are grown for their use in batter-based products such as cakes and

cookies in addition to crackers and breakfast cereals. Durum wheat is primarily grown to produce pasta.

1.2 The History of Wheat Improvement

Through thousands of years of human selection, wheat became a non-shattering species. In non-shattering species, seed does not fall from the spike which allows for easier harvest and reduced loss of yield (Vasil, 2007). Fast-forward thousands of years to the first half of the 20th century, plant breeders were performing 'maintenance breeding' and were working to find resistant genes to overcome negative effects such as disease, lodging, and drought susceptibility (Hurd, 1971). Breeding for resistance/tolerance to these growth-limiting factors saw marginal gains of about 1% per year.

At this time, new varieties of staple crops in developing countries (wheat and rice) were no longer sustaining the rate of population growth and famine appeared inevitable (Vasil, 2007). In 1942, The risk of political uprise and war led the Rockefeller Foundation and the Mexican government to create the Cooperative Mexican Agricultural Program, currently known as the International Maize and Wheat Improvement Center (CIMMYT) (Schneider, 2014).

After World War II, factories that had previously produced nitrate for explosives transitioned into producing synthetic nitrogen (N) fertilizer for agriculture (Schneider, 2014). This addition of greater amounts of fertilizer made a huge impact toward increasing yields; however, greater N inputs increased plant lodging. In other words, the grain had become too heavy which caused the long stems to fall over or break.

Norman Borlaug developed semi-dwarf wheat by crossing tall, high-yielding, disease-resistant plants with a Norin 10, a dwarf variety from Japan. Through new plant breeding techniques, shuttle breeding and multi-location testing, semi-dwarf wheat was quickly tested. Shuttle breeding allowed multiple generations in one year by having a winter nursery in a

separate location to reduce the time to produce new cultivars, whereas multi-location testing allowed for more data to be collected per generation.

Borlaug tested these semi-dwarf varieties and found they had an increased harvest index of over 60% (2-3 times more grain with less above-ground biomass) (Schneider, 2014). In addition, these semi-dwarf plants were less prone to lodging in high-N environments. By 1963, 95% of the wheat grown in Mexico had adopted Borlaug's semi-dwarf wheat, and the wheat harvest was six times greater than the harvest nineteen years prior. Later, the germplasm was used to cut food-shortage and create self-sufficiency in India, Pakistan, and China (Vasil, 2007). In 1970, Borlaug was awarded the Nobel Peace Prize for his contribution in the Green Revolution that greatly increased wheat production worldwide (Schneider, 2014).

The Green Revolution was an essential time for wheat breeding and it allowed for greater nitrogen inputs, which are essential for high yields and high grain protein content (Barraclough et al., 2014). However, in the 1980s, global population growth once again exceeded cereal production. From 1985 to 2005, global crop production saw an increase of 28%; however, only ~20% of that increase was attributed to yield increases (Ray et al., 2012). The rest of the production increase was explained by environmentally concerning practices such as increased area harvested through deforestation (Neelin et al., 2006) and intensive agriculture through more frequent harvests (Montgomery & Matson, 2007). Both of these practices can have negative impacts on the environment and lead to an increased rate of climate change.

In addition to the bleak outlook that global supply might not be able to meet future global demand, from 1985 to 2005 yields stagnated on 38% of wheat acres (Ray et al., 2012) (Figure 1). Much work is going into increasing wheat production around the world in a sustainable manner without further increasing the area harvested. By the year 2050, world food demand will approximately double, and in order to meet the demands of this growing population, cereal

production must see a dramatic increase without increasing land, water, or nutrient requirements (Gilbert, 2010).
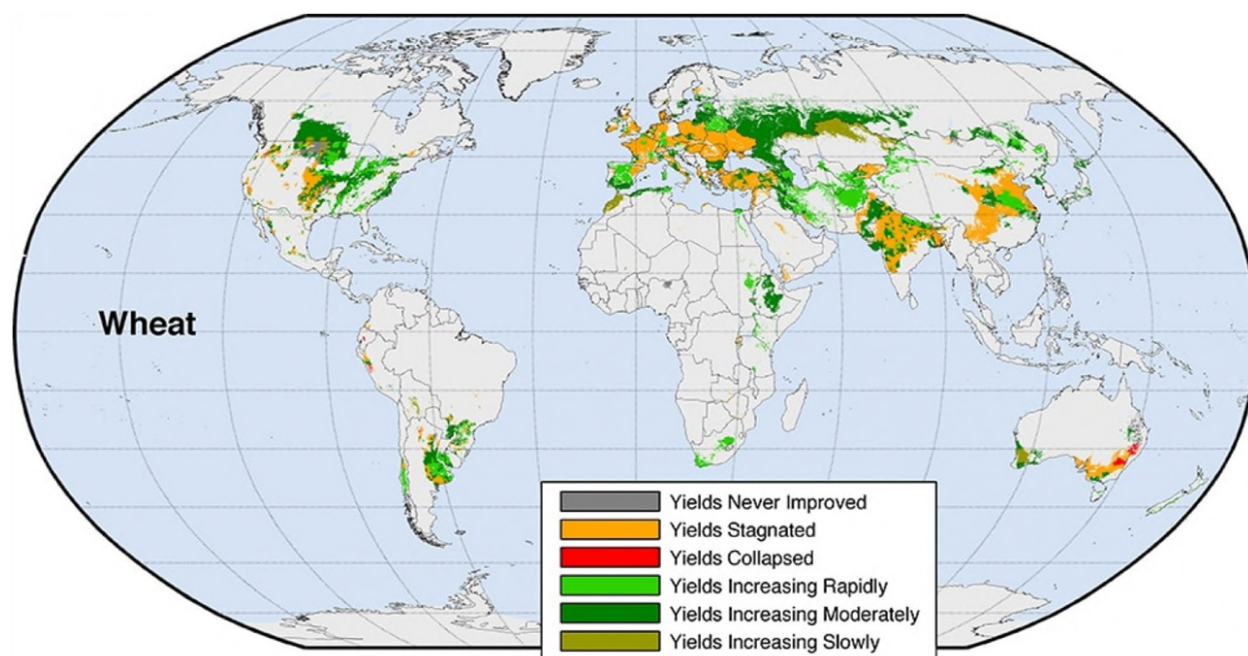
Figure 1. Ray et al. (2012). Global wheat yield trends from 1980 to 2012.

1.3 Factors Influencing Yield Potential

Yield potential is defined as the greatest yield attainable for a genotype grown in an environment in the absence of environmental stresses (Acquaah, 2012). However, realized yield potential is rarely observed due to presence of abiotic stresses, which account for the majority (~70%) of row-crop yield reductions. Two abiotic stresses that limit plant growth are nitrogen deficiency and drought (Acquaah, 2012). Crop breeding for tolerance to these abiotic stresses is of great importance for plant breeders of the 21st century.

To improve abiotic stress tolerance through selection, genetic variation is very important. Wheat is grown on nearly 18% of the arable land around the world (Vasil, 2007). The large number of acres used for wheat production can be attributed to its unrivaled adaptability and great genetic diversity, allowing it to be adaptable to many temperate regions (Acevedo, Silva, &

Silva, 2002; Feldman, 1995). As a testament to wheat's great adaptability, cultivars have been adapted to various regions so that wheat grows on six of the seven continents from southern Argentina to the Arctic Circle in Russia (Feldman, 1995).

This great genetic variability gives breeders hope that selection can be a viable strategy to improving yield and stress tolerance. Narayanan et al. (2014) evaluated 297 genotypes from various germplasm sources around the world in a greenhouse environment using a potted soilless medium searching for underlying genetic variability in many morphological shoot and root traits. They found significant (p value < 0.05) genetic variability for shoot traits (i.e. plant height, shoot dry weight, and tiller number per plant) and root traits (i.e. root dry weight, root:shoot ratio, root surface area, and root length). The range of shoot dry weight and root dry weight in their study ranged from 0.17 – 6.2 g and 0.23 – 7.6 g, respectively. While great genetic variation for root traits was described, this study was only performed in one environmental condition. As such, there is more work needed to evaluate root phenotypes in more environments.

1.4 Improving Performance and Stability of Wheat Yields in Various N Environments

Nitrogen is the most abundantly needed macronutrient for plant growth and the most expensive agriculture inputs. In higher pH, aerobic soils, it is most commonly applied as nitrate (NO_3^-) (Masclaux-Daubresse et al., 2010). Once N is taken up by plants, it is an essential constituent of amino acids, proteins, and nitrogenous compounds (Balotf et al., 2015). For these reasons, N has been found to be vitally important for plant growth, development, and yield (Good et al., 2004).

N is taken up from the soil primarily through mass flow and diffusion (Phillips et al., 1976). In optimal water conditions, nutrients are taken up mainly through mass flow as plants acquire water from the soil a part of the transpiration stream (Oyewole et al., 2014). In nutrient-poor, dry soils, the main process for nutrient uptake is diffusion (Comerford, 2005). Diffusion is

the process of the active uptake of N compounds in the soil because of a concentration gradient (Oyewole et al., 2014). A third way that plants acquire N is through direct contact between roots and nutrients; however, the amount of N acquired through this interaction is miniscule compared to the great influx from mass flow and diffusion.

Forms of N include nitrate (NO_3^-), ammonium (NH_4^+), and organic amino acid forms found naturally in soil (Sun et al., 2017). Since soil is negatively charged, ammonium (positively charged) is immobile while the negatively charged nitrate is mobile in the soil (Balotf et al., 2015). This mobility, in addition to soil type, N fertilization rate, and species (Masclaux-Daubresse et al., 2010), causes up to 35% of the nitrate applied to be lost to leaching and denitrification (Addiscott & Powlson, 1992). Raun & Johnson (1999) found that only 33% of the N applied on cereal crops are in the grains at harvest.

1.4.1 Implications of Various Nitrogen Environments

While so much of what is applied is lost, N fertilization increased 134% between 1961 – 2007 at an even greater rate than yields, which increased 120% (Conant et al., 2013). In order to meet expected agriculture needs in the future for food, feed, and fuel without increasing acreage or more excessive N use, nitrogen use efficiency (NUE) must be improved (Rasmussen et al., 2015). Genetic variation has been found in many species for traits believed to be important for NUE, and breeding efforts aimed at creating more nitrogen use efficient plants should be possible (Xu et al., 2012). While there are multiple ways that NUE is defined, one of the most common definitions is the amount of N in the grain per unit of N in the soil (mineral N and fertilized N) (Moll et al., 1982). When further broken up into components, NUE can be divided into nitrogen uptake efficiency and nitrogen utilization efficiency (Moll et al., 1982). Nitrogen uptake efficiency is the ratio of N acquired by the plant divided by the amount of N available in the soil (Moll et al., 1982). Previous studies focusing on wheat reported genetic variation for

nitrogen uptake efficiency in different N environments (Barraclough et al., 2014; Foulkes et al., 2009; Ortiz-Monasterio et al., 1997). Nitrogen utilization efficiency is defined as the ratio between yield and total N absorbed by the plant (Todeschini et al., 2016). Before maturity, N must be translocated from the shoots and roots to the grain (Le Gouis et al., 2000). Some lines that take up more N from the soil may not return a higher N content at harvest because they are less efficient with N translocation. During growth, N is stored in biomass or wasted as exudates (Rasmussen et al., 2015). In wheat, remobilization of N stored in biomass to grains before anthesis account for 60-95% of the grain N (Hirel et al., 2007). Improving the efficiency of N utilization is thus important. Breeding for nitrogen utilization efficiency is rarely practiced though as there is an inverse correlation between nitrogen utilization efficiency and grain yield.

However, a QTL on chromosome 6B, *Gpc-B1*, was found that is associated with an increase in grain protein content without negatively impacting yield. When introgressing *Gpc-B1* into an Argentinean germplasm, Tabbita et al. (2013) found an increase in grain protein content ranging from 3.6 to 9.9 g/kg without reducing yield. Additionally, in an Indian germplasm, introgression of *Gpc-B1* resulted in a significant increase in grain protein content without negatively impacting yield (Vishwakarma et al., 2014).

1.4.2 Using Root Traits to Improve Performance in Various Nitrogen Environments

Roots play an essential role in plant growth and development. Above-ground growth, especially yield, greatly depends on the ability of below-ground root biomass to absorb water and take up nutrients (Merrill et al., 2002). A greater understanding and manipulation of root architecture may be essential for a `second Green Revolution' (Lynch, 2007). In order to maximize nitrogen and water resources, incorporating root traits is increasingly important (Funk et al., 2007). However, the ideal root system architecture is not consistent across all environments (Ceccarelli, 1996; Hirel et al., 2007). Hirel et al. (2007) found that line

performance in high-N environments is not necessarily a good indicator for performance in low-N environments. Furthermore, lines selected to be high-yielding in favorable environments end up yielding less in unfavorable environments (Ceccarelli, 1996). As such, developing ideotypes dependent on the environment that the cultivar will be grown could provide greater productivity.

In high-N environments, important traits include large root biomass allocation (Ehdaie & Waines, 2008), root length density (Pierret et al., 2007), and early season root growth (Liao et al., 2004). Large root systems were found to be associated with more N take up from the soil, which resulted in increased yield, increased grain protein content, and reduced N pollution (Ehdaie & Waines, 2008). Root systems with greater root length density (root length per unit volume of soil) are capable of greater resource uptake (Pierret et al., 2007). While the increase in root length density does not necessarily increase the amount of nitrate absorbed, root proliferation in nitrate-rich environments greatly impacts the rate of nitrate capture allowing less to leak out of the root zone (Irving, 2015; Robinson, 1996). Additionally, Liao et al. (2004) found that root systems with quick development more than doubled the amount of N uptake 35 days after sowing over lines with slow root system development.

A limited amount of work has been performed to understand root traits that could add value in low-N environments. However, morphological traits such as smaller root dry matter (Gallais & Coque, 2005) and increased root:shoot ratio (Edwards et al., 2016) have increased yield performance in low-N environments. Gallais and Coque (2005) found that in maize, larger root systems were negatively correlated with yield in low-N environments. Similarly, Palta et al. (2011) found a similar result in a drought stressed environment. They proposed breeding for less root dry matter as a large root system does not add value necessarily when water is limiting. Edwards et al. (2016) concluded that the root:shoot ratio was negatively correlated to yield. The increase in the root:shoot biomass allocation did not increase water and/or nutrient uptake

enough to have a positive effect on the yield. The authors suggest that in stressful conditions, marginal increases to the root:shoot ratio may increase the chance of survival; however, increasing the ratio beyond the level needed for survival could decrease rather than increase yield.

Understanding the environment for which a breeder is adapting plants for is critical. This importance is further complicated in the fact that N and water are not the only limitations on plant growth and development. For example, when phosphorous is limiting, more roots should be grown in the first 30 cm of the soil; however, when N is limiting, less roots in the first 30 cm with more deep roots lead to greater productivity (Ho et al., 2004).

Previous Approaches to Phenotyping Root Traits

Bernardo describes an ideal trait for phenotyping as providing measurements that are accurate, precise, inexpensive, quick, and non-destructive (Bernardo, 2014). Unfortunately, studying root traits often is slow, lacks high-throughput capabilities, and accuracy of sampling is sometimes at question (Fitter, 2002). Furthermore, it is very difficult to compare results of different studies where different environments and species are used (Gratani, 2014).

Previous approaches to root phenotyping in the field include root digging (shovelomics) and soil boring (building an underground observatory) (Paez-Garcia et al., 2015). These methods are useful because they allow root analysis in a real-world environment; however, the heterogeneity of the field environment can produce variable data that can be hard to evaluate. Laboratory and greenhouse approaches to root phenotyping include agar-based systems, Growth and Luminescence Observatory for Roots (GLO-Roots), hydroponic procedures, and potted plant experiments (Paez-Garcia et al., 2015). In Figure 2, the environments where the researcher has the most control were the least correlated to commercial environments (Bruce et al., 2002). For example, in gel cultures, the Rht gene was strongly correlated to seedling root growth; however,

in soil, it was shown to have a strong, negative correlation (Wojciechowski et al., 2009). When the traits being studied deal with agronomic issues, soil-based methods are preferred for root analysis (Kuijken et al., 2015).
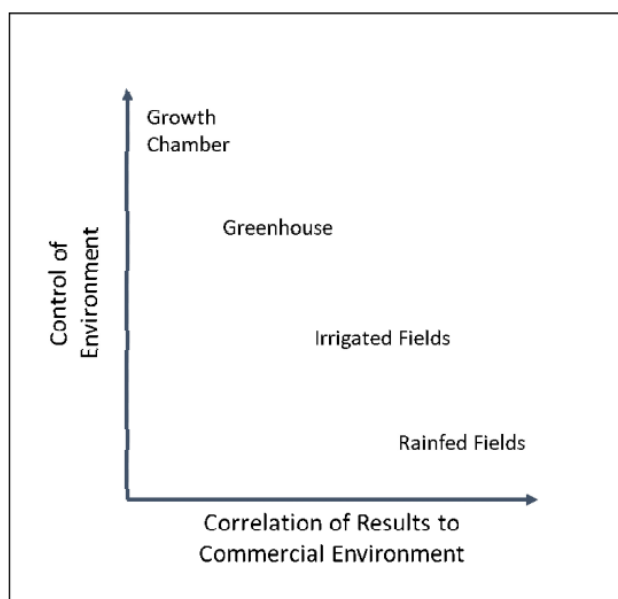
Figure 2. Adapted from Bruce et al. (2002). Represents the advantages and disadvantages of various studies.

1.5 Implications of Drought and Drought Tolerance on Yield Performance and Stability

Drought stress is one of the most severe abiotic stresses harming food security and socio-economics of many developing countries. In semi-arid regions, drought restricts yields in 37% of wheat growing areas (Rajaram, 2001). As the climate continues to change, projections forecast that in the decades to come, severe drying will cover most of Africa, southeast Asia, Australia, southern Europe, South America (especially Brazil and Chile), and the United States as a whole (Dai, 2011). With this severity in mind, a greater understanding of drought and crop adaptations to drought are becoming increasingly more important.

Characterization of drought can be split into three groups: meteorological drought, agricultural drought, and hydrological drought (Dai, 2011). Meteorological drought occurs as below-normal precipitation persists over a period of time (months to years) and is typically a

foreshadowing for the more severe forms of drought to come. Agricultural drought is the result of persistent meteorological drought where dry soils affect crop growth, development, and productivity. Finally, hydrological drought is the result of long-term depletion of water reserves (i.e. aquifers, lakes and reservoirs) where sources fall below their mean level. In addition to these characterization methods, drought is quantified based on its intensity, duration, and land-coverage of the event (Dai, 2011).

When designing an experiment related to drought tolerance, the severity of the drought is very important. For gene identification studies, a severe drought intensity, where plants cannot survive, could lead to the best results. For the identification of physiological and morphological traits of value, typically a less severe treatment similar to conditions that would be experienced in a farmer's field should be implemented (Ghanem et al., 2015).

Maqbool et al. (2015) performed a greenhouse study to evaluate three varieties experiencing drought at tillering, booting, anthesis, and grain filling. They found that water stress at any of these growth stages resulted in a decrease in plant height, fertile tiller number, shoot dry matter, grain yield, and 100-grain weight. Furthermore, their results indicate that drought stress at tillering produced the greatest decrease in fertile tiller number, drought stress at booting and anthesis produced the least spikelets/spike, and drought stress at grain filling resulted in the largest decrease in 100-grain weight. Thus, drought timing can have a large impact on morphological features and on the yield component that is most influenced. While the response to drought was thoroughly discussed in this experiment, genotypic variation of tolerance to drought was not.

Underlying the reduction in plant performance in drought is physiological changes (i.e. an increase in stomatal closure, an increase in oxidative stress, and a decrease in chloroplast activity) leading to a reduction in photosynthetic capabilities (Nezhadahmadi et al., 2013; Ogren

& Oquist, 1985). These reductions in photosynthetic capabilities reduced plant growth, biomass, and tillering potential (Akram, 2011).

Breeding for drought tolerance can take many forms as ideal drought responses can vary depending on the timing and intensity of the drought. Various forms of drought tolerance exist including escape, avoidance, tolerance, and rapid recovery, with the ideal strategy greatly dependent on the timing of the drought (Acquaah, 2012). Early in the growing season, during canopy development, plants adapted to drought stress will conserve water by producing smaller, more erectophile leaves with a reduced leaf area index (Araus et al., 2002). At the tillering stage, a suggested stress adaptation would be less wasteful tillers. On the other hand, to escape late season drought during the grain-filling period, shortening the crop-growing season is one way to mitigate the effects of the drought (Araus et al., 2002). Effective strategies for drought tolerance are greatly dependent on the timing of the stress in the plant's growth cycle and are largely site-specific.

In addition to morphologically important crop features, a number of physiological traits are important in the identification of drought tolerant varieties such as stay-green and relative water content. Stay-green is a physiological trait that has been identified as having the potential to improve drought tolerance (Lopes & Reynolds, 2012). Premature chlorophyll degradation is a common symptom of drought susceptibility affecting leaf area and photosynthesis (del Pozo et al., 2016). Lopes & Reynolds (2012) used the normalized difference vegetation index as an estimate for the stay-green phenotype in two populations. Broad-sense heritability estimates for stay-green were 0.60 and 0.13 for the two populations, respectively, with the correlation of r=0.32 and 0.23, respectively, between stay-green and yield in the two populations. While stay-green was not studied in this thesis because the destructive measurement was before senescence, it is a trait that must be studied in additional work.

Another physiological trait that is useful in the evaluation of drought stress is relative water content (RWC) (Turner, 1981). RWC is a measurement of the water status of a plant and is calculated in the following equation where the ratio of fresh weight (FW) minus dry weight (DW) is divided by turgid weight (TW) minus DW.

$$RWC = (FW - DW)/(TW - DW) *100$$

Ganji Arjenaki et al. (2012) found that RWC was significantly (p value <0.01) impacted by drought treatment and genotype and concluded that there was a positive correlation between yield and RWC in drought stress. In addition to all of these above-ground traits, below-ground root traits play a critical role in abiotic stress tolerance.

1.5.1 High-Throughput Phenotyping in Trait Identification

Phenotyping can be done in a variety of settings. For example, in many breeding programs, genotypes are evaluated in multi-environment, multi-location field trials with the goal of testing yield adaptability and stability across environments. While research trials performed in the field may be the gold-standard in terms of their correlation to a commercial environment, heterogeneity in the soil and control of the environment often make interpretation of data collected difficult (Bruce et al., 2002). In contrast to field trials, controlled-environment studies allow the researcher to control the external environmental variables, repeat in exact conditions (resulting in greater reproducibility), and have a quicker experimental turnover (Fahlgren et al., 2015).

Each of these techniques contains benefits and hindrances in their application; however, the best method for a given study depends on the main trait that is being evaluated. For example, while a field study should be performed to test productivity of commercial varieties ready to be released, greenhouse and controlled environments can help to distinguish the diversity of phenotypic responses to a given environment in a population (Ge et al., 2016).

In wheat, genetic gains for yield have stagnated in recent years, and low-throughput phenotyping (LTP) is suggested as a bottleneck for genetic advance (Araus et al., 2018). LTP is time-consuming and expensive which limits the collection of large-scale plant phenotypic datasets. However, large-scale, noninvasive, high-throughput phenotyping is now feasible through advancements in technology (i.e. image analysis and robotics) (Yang et al., 2014).

These new techniques, termed high-throughput phenotyping (HTP), use a variety of imaging techniques including, but not limited to, visible (RGB) imaging. RGB imaging platforms in crop breeding are used primarily to assess architectural traits and detect desirable morphological features, of a given genotype in various growth conditions (i.e. heat, drought, and disease stress). Accurate measurements of major traits using RGB images include seedling vigor, tiller count, biomass, height, leaf erectness and canopy as well as estimates for yield (Shakoor et al., 2017). Furthermore, since these practices are nondestructive to the plants, when utilized over a series of days and weeks assessment of growth rates, disease progression, and drought tolerance can be easily assessed (Shakoor et al., 2017). However, the same data collected from low-throughput means would require an exponential increase in the replication of the experiment (Fahlgren et al., 2015).

While still a relatively new science, studies utilizing HTP have been performed in maize (Ge et al., 2016), barley (Chen et al., 2014), and foxtail millet (Fahlgren et al., 2015). Ge et al. (2016) grew two maize inbreds, B73 and FFMM-A, in two water treatments with twenty replications per group. They performed analysis at two growth stages: from six to twenty-six days after sowing and then once again from twenty-six to forty-six days after sowing. At the first stage, leaf area, shoot fresh weight, and shoot dry weight were measured for each destructively sampled plant. They found that the RGB platform in a controlled environment had great potential for predicting leaf area ($r=0.99$), shoot fresh weight ($r=0.99$), and shoot dry weight ($r=0.98$).

In an experiment evaluating the drought tolerance of eighteen barley varieties, Chen et al. (2014) utilized visible, near-infrared, and fluorescence images to assess the implications of the drought on plant growth and development. They found a high correlation between an image-based trait, volume, and above-ground dry matter (r=0.95). In addition, they were able to distinguish morphological differences among genotypes in their response to the drought and their recovery after the drought had been lifted. They concluded that two traits used in their study, volume and compactness, serve as good estimates for biomass and tiller number, respectively.

Fahlgren et al. (2015) performed an experiment evaluating the drought tolerance of undomesticated *Setaria viridis* (green foxtail) in comparison to domesticated *Setaria italica* (foxtail millet). Interestingly, they reported that while these two species displayed similar biomass allocation patterns in water-limiting environments, in well-watered conditions, foxtail millet produced greater biomass through less efficient growth while green foxtail maintained the same level of water use efficiency. Furthermore, this study displayed the power of imaging techniques by detecting significant genotypic and environmental effects for height, biomass, water-use efficiency, color, and water status.

The additional phenotyping power from HTP could help to further the understanding, identification, and introgression of adaptations to drought tolerance into elite germplasm to support the development of yield stability (Berger et al., 2010).

1.5.2 Using Color to Explore Drought Response

In addition to reducing plant growth, biomass, and tillering potential (Akram, 2011), drought has also been shown to impact plant leaf color. Through visual assessment, extension specialists at Washington State University describe drought stress in alfalfa, maize, and small grains as having a more dull color compared to well-watered individuals (Ley, 2003). These subjective observations led to the exploration of the use of color characteristics to assess drought

tolerance. In a study using image-based technology, Fahlgren et al. (2015) found that principal component analysis of color traits visually separated the well-watered treatment from the drought treatment and explained 62% of the variation twenty-five days after planting in foxtail millet.

While there are multiple scales to assess color, one common scale is the Munsell Color System that measures hue, saturation, and intensity (Figure 3) (Bradley, 2013). On this scale, hue refers to the color that is displayed. Saturation refers to the purity of hue, the dullness or amount of gray in the color. Finally, intensity refers to the lightness of the hue which is a measurement of the amount of black/white in the color.
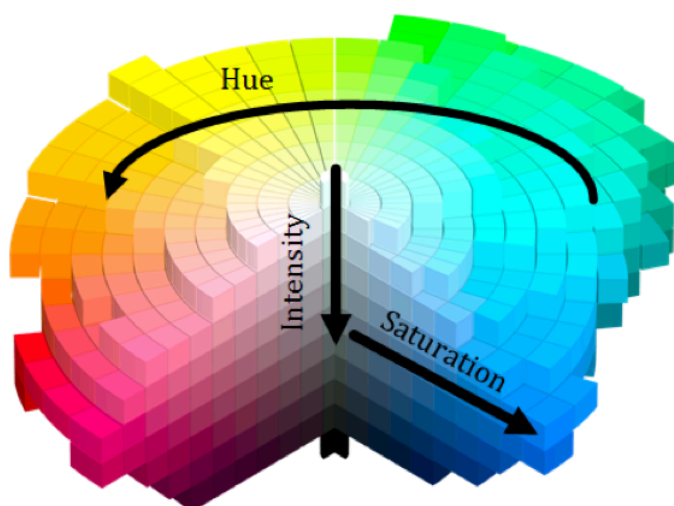
Figure 3. Cartoon description of the Munsell Color System.

1.6 Conclusion

Wheat is one of the most important cereal crops in the world today; however, ~38% of the acres around the world only marginally increased yields over the past thirty years (Ray et al., 2012). A large portion of the yield limitation in these wheat-growing regions is attributed to abiotic stresses. As such, systems for evaluation of germplasm for these environments will be increasingly important in the coming decades. While current breeding strategies primarily focus on selection of yield and yield-related traits, in this thesis a small germplasm collection with

diverse geographic origin was examined for important morphological and physiological traits attributed with heightened tolerance to nitrogen deficiency and drought stress.

In chapter two of this thesis, root traits of seven diverse lines in high- and low-N treatments were characterized to evaluate the genetic diversity of the root traits and associate these traits to N uptake and yield. Over the last century, progress has been made in agronomy and many studies report the importance of root architecture. However, relatively few studies have been performed to understand the genetic variability awaiting many of these traits especially in different N treatments. While many programs evaluate their current germplasm, a collection with great geographic diversity was assembled for this work.

In chapter three of this thesis, plant growth was modeled using HTP in differential water treatments. Currently, limitations in phenotyping hinder genetic gain, and HTP is an emerging technology with great potential. The advent of HTP is a strategy to produce large amounts of phenotypic data; however, studies must be performed to define the value of these systems in plant breeding.

The overall objective of this thesis was to phenotype a diverse set of spring wheat germplasm to gain a greater understanding of the genetic potential associated with important morphological and physiological traits in addition to the biomass allocation patterns associated with greater productivity in abiotic stress.

CHAPTER 2: DYNAMICS OF ROOT GROWTH AND ITS ASSOCIATION WITH WHEAT NITROGEN CONTENT AND GRAIN YIELD

A version of this chapter has been submitted to Agronomy Journal.

Data from the seedling experiment of this chapter comes from an unpublished study conducted by Mina Rostamza, a former postdoc in the wheat breeding program.

2.1 Introduction

During the Green Revolution from 1960 –1985, global wheat yields increased from 1088 kg/ha to 2172 kg/ha; however, from 1985 – 2010, global wheat yields only increased from 2172 kg/ha – 2972 kg/ha (FAO, 2017). Only 62% of wheat acres experienced yield increases from 1985-2010 (Ray et al., 2012). About 38% of global wheat acreage experienced no yield growth. To meet future demands, wheat production must be doubled by 2050 (FAO, 2009) without an increase in area harvested through deforestation (Neelin et al., 2006), harvest frequency (Montgomery & Matson, 2007), and water and nutrient demands (Gilbert, 2010). That requires increased yields of 2.4% per year (Ray et al., 2013), while from 1985 – 2017 yields only increased by 1.6% per year (FAO, 2017).

From 1961 to 2007, increased wheat yields were correlated to increased N inputs ($R^2 =$ 0.89) (Conant et al., 2013). While yields have increased, N loss has become a prevalent topic in the agriculture industry (Addiscott & Powlson, 1992; Rasmussen et al., 2015; Raun & Johnson, 1999). In high-N environments, Raun and Johnson (1999) found that only 33% of the applied N was taken up and remobilized into the grains of cereal crops. The remaining N was lost through denitrification, volatilization, and leaching or it remained in biomass at harvest and was not remobilized to the grain (Addiscott & Powlson, 1992). Rasmussen et al. (2015) studied wheat under different N treatments. They found that an increased N rate from 150 kg N ha^{-1} to 250 kg

N ha^{-1} led to an average of 36% of the supplemental N to remain in the soil where it was susceptible to leaching. When further increased from 250 kg N ha^{-1} to 350 kg N ha^{-1} almost 90% of the additional N remained in the soil and at risk of leaching.

On the other hand, many wheat growing regions with less resources for applying N fertilizer resulted in reduced leaf area index, above-ground biomass, tiller number, kernel number, and grain yield (Salvagiotti & Miralles, 2007). Kharel et al. (2011) found a decrease in yields of 28% in treatments with 0 N fertilization compared to 160 kg N ha^{-1}.

To increase yields in various N treatments, it was suggested by Lynch (2007) that a greater understanding and utilization of below-ground traits are key for a 'second Green Revolution'. Previous experiments identified important root traits in both high- and low-N treatments. However, root traits reported as beneficial in high-N treatments are not consistent with those found to be important in low-N treatments. In high-N treatments increasing root biomass (Ehdaie and Waines, 2008) and root length density (An et al., 2006; Pierret et al., 2007) were shown to be associated with greater N uptake and yield. Studies in low-N treatments report that increased `early vigor' (Liao et al., 2004), the root:shoot ratio (Edwards et al. 2016), and lower specific root length (root dry matter per unit length) (King et al., 2003) are associated with greater productivity.

To identify these traits and establish a vision for breeding programs, a diverse germplasm could be evaluated to draw preliminary conclusions about potentials of these phenotypes in the determination of grain yield. However, experiments involving root systems are difficult because roots are not easily observable (Fitter, 2002). The goals of the studies in this chapter were to examine the genetic variation in root dry matter under different N treatments and associate root dry matter to nitrogen uptake and yield. We aimed to characterize and demonstrate the relationship between below-ground and above-ground traits in seedling and maturity stages and

to examine the patterns of biomass allocation over multiple plant growth stages. In experiment one of this chapter, seven diverse lines were observed at the seedling stage in two N treatments to measure root traits at the seedling stage. Experiment two evaluated the same lines at maturity to assess root traits and grain yield in two N treatments. Finally, experiment three assessed four lines at multiple growth stages.

2.2 Materials and Methods

2.2.1 Plant Materials

Seven experimental breeding lines and landraces of wheat (*Triticum aestivum L.*) were used in this study. The germplasm were from various geographical origin or breeding geographical locations including: Opata originally from Mexico; PI 189823 from Santa Fe, Argentina; PI 245427 from Afghanistan; PI 519677 from Chile; PI 542457 from Nebraska, United States; PI 626655 from Iran; and PI 94379 from Armenia. From this point forward, the lines will be identified based on their geographic origin. Seven lines were studied at seedling stage and maturity stage experiments and four of them were studied for root analyses at multiple growth stages.

2.2.2 Seedling Experiment

Growth Conditions

In a growth chamber and paper-roll-supported hydroponic experiment, the biomass allocation patterns of these lines were examined under high- and low-N treatments at the seedling stage. Six uniform seeds from each line were grown in kraft paper (30 cm wide and 45 cm long) utilizing an experimental procedure explained by Rahnama et al. (2011). Before the seeds were 'planted', the paper was rolled by making a 1.5 cm crease for seed placement and a 3 cm crease along the side for further rolling. Seeds were treated with Maxim XL fungicide (100

uL/L) (active ingredients: fludioxonil and mefenoxam) and evenly placed in the kraft paper with the embryo facing the bottom of the paper. To ensure the seeds would remain in place, the paper was rolled tightly. Individually, the rolls were placed into a PVC tube (5.2 cm wide by 38.5 cm long) and filled with water. To reduce evaporation, the tubes were wrapped with parafilm. The growth chamber was set to 20˚C and a 12-hour photoperiod with a relative humidity of 50% for the duration of the experiment. The photosynthetic photon flux reached 300 umol m^{-2} s^{-1} at top of plant canopy. Five days after planting, the plants were exposed to a modified Hoagland solution. This solution contained: KH_2PO_4, 0.2 mM; $MgSO_4$, 0.5 mM; $CaCl_2$, 0.5 mM; H_3BO_3, $1x10^{-3}$ mM; $(NH_4)_6MO_7O_{24}$, $5x10^{-5}$ mM; $CuSO_4$, $5x10^{-4}$ mM; $ZnSO_4$, $1x10^{-3}$ mM; $MnSO_4$, $1x10^{-3}$ mM; Fe(III)–EDTA, 0.1 mM. The pH of the solution was adjusted to 6.2-6.3. The varying N levels, low (0.5 mM NO_3^-) and high (4.0 mM NO_3^-), were established by adding $Ca(NO_3)_2 4H_2O$ (Hoagland & Arnon, 1950; Zhu et al., 2014). To maintain the treatments, the nutrient solution was refreshed every two days.

Phenotyping

Two weeks after planting, shoot and root samples were collected. Roots were preserved in 50% ethanol. Roots and shoots were oven dried at 60˚C for two days and shoot dry matter (SDM) and root dry matter (RDM) were recorded. The ratio of root:shoot (RTS) was measured in each replicate by dividing RDM by SDM. Nitrogen concentration was assessed through dynamic flash combustion using a Thermo Scientific FlashEA 1112 Elemental Analyzer (CE Elantech, Lakewood, NJ). Briefly, in dynamic flash combustion, samples are heated to 900°C – 1000ºC, and the organic and inorganic material is converted into elemental gases once oxygen is added (Krotz & Giazzi, 2014). The N gases are carried to a reactor filled with copper, using helium, where the nitrogen oxides are reduced to elemental nitrogen. Through this reaction, the N passes through CO_2 and H_2O traps, and the shoot N concentration (SN) of the sample is sensed

by a Thermal Conductivity Detector. Shoot N content (SNC) was calculated by multiplying SN by SDM.

Design of Experiment and Statistical Analysis

This observation nursery was conducted in two side-by-side experiments. The six reps of a given line were in the same PVC tube, and therefore, this experiment did not allow for analysis of variance (ANOVA) to test for differences between lines, N treatments, or the line x N interaction. Instead, mean and standard errors of traits for each line in each treatment were calculated in R studio environment (R Core Team, 2014). To assess the relationship of RDM and SNC with regards to N treatment, a regression model [SNC = RDM + N + RDM x N interaction], was used to test the statistical difference between the slopes.

2.2.3 Maturity Experiment

Growth Conditions

A follow-up experiment was conducted on the same seven lines in a greenhouse, potted-soil environment to evaluate below-ground and above-ground traits and their relationship under high- and low-N treatments. The goal of this experiment was to examine genotypic differences in biomass allocation at maturity, and if these differences were dependent on N treatment. In addition, this experiment would validate whether roots measured at seedling stage in hydroponic growth environment correlate with those measured at maturity in soil.

Seeds were germinated in petri dishes and transplanted (one seedling per pot) into 7.5-liter pots containing a 1:1:1 mix of topsoil (Biotown Ag, Reynolds, IN), sand, and potting soil (Sungro Metro-mix 510). Plants were bottom-watered with tap water three times a week. The temperature was set to 23˚C and the photoperiod was 12 h for the duration of the experiment. Starting one week after transplanting, the same low (0.5 mM) and high N (4 mM) solutions

described in the seedling experiment were applied weekly. For the first 28 days, 125 mL of solution was applied to each pot every 7 days. After the 28th day, 250 mL of solution was applied to each pot every 7 days. In total, 2000 mL of Hoagland solution was added to each pot throughout the experiment.

Phenotyping

Above-ground traits measured included plant height (PLH), SDM, fertile tiller number (TN), kernel number (KN), and yield. A ruler was used to measure PLH, while TN and KN were counted by hand. Spikes were harvested and the above-ground tissue was cut at the crown level. Spikes and above-ground tissue were dried for two days at 60°C and dry matter was recorded. Spikes were then hand-threshed, and KN and yield were measured for each plant. For below-ground traits, roots where removed from the soil after the above-ground traits were collected. Roots and soil were submerged in water for ~30 minutes, then washed and rinsed with a medium-pressure hose several times. The clean roots were placed in 50% ethanol for future analysis, and later were dried at 60°C for two days and RDM was recorded.

Nitrogen concentration was measured in shoot (SN) and in grain samples (GN) through the same method used in the seedling experiment. SNC was the product of SN and SDM, whereas grain N content (GNC) was the product of GN and grain yield. For N measurement, plant material was ground through a 1 mm screen, and the method for analysis was described previously in the seedling experiment.

Design of Experiment and Statistical Analysis

This experiment was conducted in a split-plot design in eight replicates with N as the main-plot and line as the sub-plot (Table 1). ANOVA was performed in R environment (R Core Team, 2014). The N effect was tested against the main-plot error (N x Rep interaction) while line

and the line x N interaction were tested against the sub-plot error (residual MSE). For traits with a significant Line x N interaction, Tukey tests were performed on the interaction. For traits where the Line x N interaction was not significant, but line was significant, Tukey tests were performed to differentiate lines. Tukey tests were performed using agricolae::HSD.test. Since there were only two N treatments no further post-hoc tests were performed to separate N treatments when the difference was significant. Tukey tests distinguished large-, medium-, and small-RDM lines among the germplasm. Post-hoc ANOVA was performed to evaluate the difference in SNC, GNC, and yield in each N treatment between the various RDM classifications.

Table 1. Example ANOVA output for PLH in a split-plot experimental design as conducted in the maturity experiment. The Main-Plot Error is the N x Rep interaction, and the Sub-Plot Error is the Residual MSE with the Line x Rep interaction and the N x Line x Rep interaction pooled into the error.

Source of Variation	Df	MS	F value	Pr(>F)
N	1	126	1.05	ns
Rep	7	82	0.68	ns
Main-Plot Error	7	120		
Line	6	7395	80.54***	<0.001
Line x N	6	232	2.53*	<0.05
Sub-Plot Error	79	92		

P value Significance: *** < 0.001, ** 0.001 – 0.01; * 0.01 – 0.05; ns > 0.05

2.2.4 Dynamic Experiment

Growth Conditions

Two small RDM lines (Argentina and Chile) and two large RDM lines (USA and Iran) were selected for the growth stage experiment to evaluate below-ground traits and temporal patterns of biomass allocation. Single plant per 7.5-liter pots were grown in a 1:1:1 mix of topsoil (Biotown Ag Reynolds, IN), sand, and potting soil (Sungro: propagation mix). The

temperature of the greenhouse was set to 23˚C and the plants received a 12-hour photoperiod for the duration of the experiment.

The nutrient medium for this experiment was only the high-N (4 mM). Following emergence until heading, 125 mL of solution was applied to each pot; however, at heading, the amount applied per pot was doubled to 250 mL. In all, 2125 mL of Hoagland solution was applied to each pot throughout the experiment.

Phenotyping

Destructive sampling was performed at five growth stages: four-leaf stage, stem elongation, heading, post-anthesis, and maturity. Sampling began as each plant reached the desired growth stage and was recorded as days after planting. For each plant, phenotypes measured include PLH, TN, SDM, and RDM, similar to the maturity experiment. For destructive measurements at heading, post-anthesis, and maturity, emerged spikes were cut away from the above-ground biomass after PLH was measured.

Design of Experiment and Statistical Analysis

The experiment was completed in a randomized compete block design (RCBD) with four replicates at the four-leaf stage and five replicates in all other stages (Table 2). Lines, growth stage, and line x growth stage interaction were considered as fixed effects while replicates was considered as random effect. ANOVA was performed in R studio (R Core Team, 2014) using lmerTest::lmer. Type 3 SS was used to assess the significance of the fixed effects. When the line x growth stage interaction was significant for a given trait, Tukey tests were performed on the interaction using lsmeans::lsmeans and multcomp::cld. However, when the interaction of line x growth stage was not significant, significant differences between the levels of each line and growth stage were further investigated using Tukey tests.

Table 2. Example ANOVA output for PLH in a RCBD experimental design as conducted in the maturity experiment. Line, Growth Stage, and the Line x Growth Stage interaction were all tested using the Residual MSE (Error).

Source of Variation	DF	MS	F value	Pr(>F)
Line	3	5244	130***	<0.001
Growth Stage	4	10939	271***	<0.001
Line x Growth Stage	12	547	14***	<0.001
Error	72	40		

P value Significance: *** < 0.001; ** 0.001-0.01; * 0.01-0.05; ns >0.05

2.3 Results

2.3.1 Differential Nitrogen Treatments Resulted in Differences in Nitrogen Uptake

In the seedling experiment, SN of the high-N treatment was 43 mg/g while SN in the low-N treatment was 27 mg/g (Figure 4). At maturity, SN was significantly (p value <0.001) greater in the high-N treatment (4.54 mg/g) as compared to the low-N treatment (3.67 mg/g) (Figure 4). N treatments were also significantly (p value <0.05) different for GN as the high-N treatment was 27.8 mg/g and the low-N treatment was 23.3 mg/g (Figure 4).
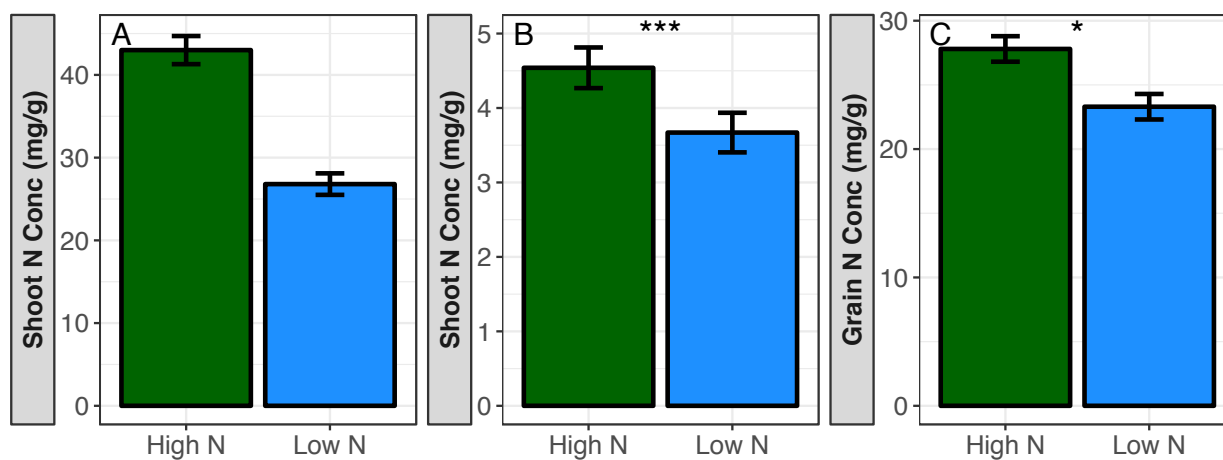
P value Significance: *** < 0.001; ** 0.001-0.01; * 0.01-0.05; ns >0.05

Figure 4. Means and standard errors of shoot N concentration (SN) under high- and low-N treatments at seedling (A). Means and standard errors of shoot N concentration (SN) for maturity experiment under high- and low-N treatments. Significant differences (p value <0.001) between N treatments for SN was provided at the top of the graph (B). Means and standard errors of grain N concentration (GN) for maturity experiment under high- and low-N treatments. Significant differences (p value <0.001) between N treatments for GN was provided at the top of the graph (C).

2.3.2 Greater Variation in Below-ground Traits Compared to Above-ground Traits

At maturity, the variation among the germplasm for RDM was far greater than the variation for SDM. Line and N treatment significantly impacted RDM; however, the line x N interaction was not significant. The effect of N treatment on RDM was significant (p value <0.05) (Table 3). On average, RDM in the high-N treatment was 0.78 g and that in low-N treatment was 0.68 g (Table 3). Significant differences (p value <0.001) for RDM were also found between lines (range: 0.12 g – 1.69 g) (Table 3; Figure 5). This difference corresponded to a ~15-fold difference in RDM, which was far greater than the ~5-fold difference in SDM.

The average RDM in high-N treatment was 15.3 mg (range: 10.4 mg — 20.6 mg) and the average RDM in low-N was 16.3 mg (range: 11.6 mg — 21.1 mg) (Table 3). Similar results were observed in the dynamic experiment where, at the four-leaf stage, the lines averaged 16.0 mg of RDM (range: 7.6 mg — 23.3 mg) (Table 3).

Interestingly, the effect of N was not significant on RTS at maturity. Which indicated that biomass allocation between RDM and SDM was not impacted by the N treatments. However, lines were significantly different (p value <0.001) in RTS (range: 0.06 g/g – 0.34 g/g). In the seedling experiment, the average RTS in low-N was 0.8 (range: 0.72 – 0.92 g/g), while the average RTS in high-N was 0.52, (range: 0.38 – 0.69 g/g) (Table 3).

Table 3. The means and standard errors of lines for root dry matter (RDM) and root:shoot ratio (RTS) of seedling and maturity experiments under low-N and high-N treatments. Since the line x N interaction was not significant in maturity experiment for both traits, the mean separation was performed only on lines. As the seedling experiment was an observation nursery ANOVA was not performed. For maturity experiment, since N was significant, the average RDM across lines was given for each N treatment. Since N was not significant for RTS, the overall average was presented. Similar letters indicate a non-significant difference based on Tukey test. For example, 'a' was greater than 'b'; however, 'ab' was not significantly different from 'a' or 'b'.

	Seedling				Maturity			
	RDM (mg)		RTS (g/g)		RDM (g)		RTS (g/g)	
ID	High N	Low N	High N	Low N	High N	Low N	High N	Low N
Mexico	14.4 ± 1.3	14.4 ± 1.7	0.68 ± 0.09	0.82 ± 0.11	0.21 ± 0.11 c		0.13 ± 0.02 cd	
Argentina	17.8 ± 1.6	21.1 ± 1.9	0.38 ± 0.11	0.72 ± 0.12	0.15 ± 0.11 c		0.06 ± 0.02 d	
Afghanistan	20.6 ± 3.2	18.8 ± 1.9	0.54 ± 0.23	0.92 ± 0.12	0.72 ± 0.11 b		0.3 ± 0.03 ab	
Chile	13.5 ± 1.4	19 ± 1.9	0.49 ± 0.1	0.89 ± 0.12	0.12 ± 0.11 c		0.11 ± 0.02 d	
USA	10.4 ± 1.4	11.8 ± 1.7	0.69 ± 0.1	0.77 ± 0.11	1.51 ± 0.11 a		0.34 ± 0.03 a	
Iran	10.7 ± 1.4	11.6 ± 1.9	0.47 ± 0.1	0.77 ± 0.12	1.69 ± 0.11 a		0.34 ± 0.02 a	
Armenia	19.5 ± 1.4	17.3 ± 1.7	0.38 ± 0.1	0.72 ± 0.11	0.84 ± 0.11 b		0.22 ± 0.02 bc	
Average	15.3 ± 1.7	16.3 ± 1.8	0.52 ± 0.12	0.8 ± 0.12	0.78 ± 0.1	0.68 ± 0.1	0.21 ± 0.02	
N Significance					*		ns	
Line Significance					***		***	
Line x N Significance					ns		ns	

P value Significance: *** < 0.001; ** 0.001-0.01; * 0.01-0.05; ns >0.05

Figure 5. Representative images of roots from each line in high- and low-N treatments at maturity.

2.3.3 Association of Root Dry Matter with Nitrogen Content and Yield

In the high-N treatment, RDM explained 96% of the variation of SNC; however, RDM explained 58% of the variation of SNC in the low-N treatment (Figure 6). Regression analysis indicated that an increase in SNC was more responsive to an increase in RDM in high-N compared with low-N treatment (Figure 6).
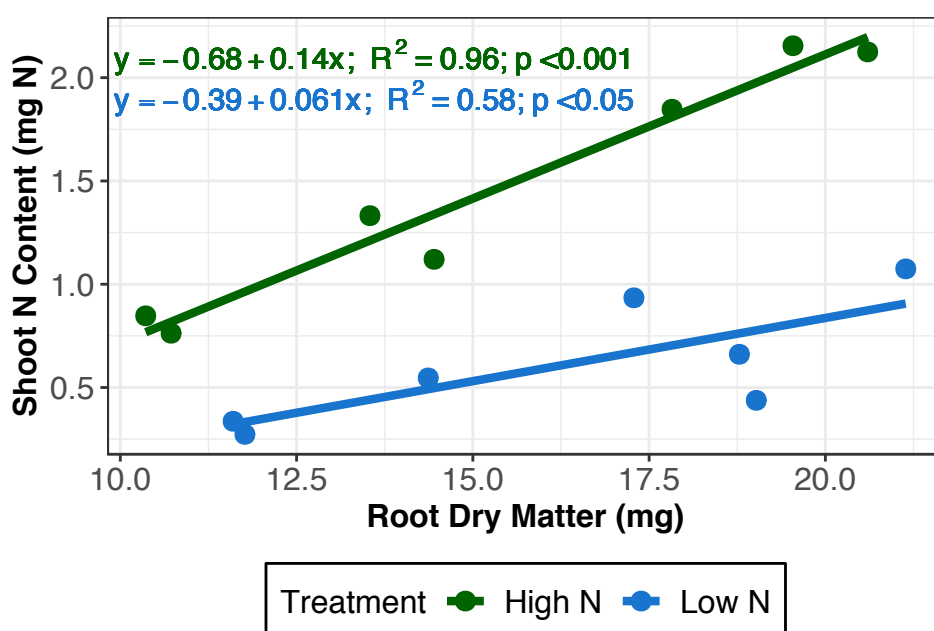
Figure 6. Relationship of Root Dry Matter (RDM) in mg on x-axis and Shoot N Content (SNC) in mg N on y-axis. Circles denote plants grown in the high-N treatment while triangles denote plants grown in the low-N treatment. Regression lines, R^2, and p values were given for each treatment.

Through a post-hoc Tukey test, three distinct groups were classified in this germplasm. Iran and USA had large RDM; Armenia and Afghanistan had medium RDM; and Mexico, Argentina, and Chile had small RDM (Table 3; Figure 5). In the high-N treatment, lines with large RDM, had significantly greater SNC than the medium- and small-RDM lines. (Figure 7). The GNC of all groups were not significantly different in the high-N treatment. Grain yield of the lines with large RDM was significantly greater than the lines with small RDM through a significant

increase in TN. However, the grain yield of medium RDM lines were not significantly different from grain yield of either large-RDM lines or the small-RDM lines (Figure 7). In the low-N treatment, SNC of the small-RDM lines was significant less than the SNC of the medium- and large-RDM lines. No significant differences were observed between RDM classifications for GNC or grain yield. In this experiment, large RDM was associated with increased SNC and yield in the high-N treatment but only with increased SNC in the low-N treatment (Figure 7).
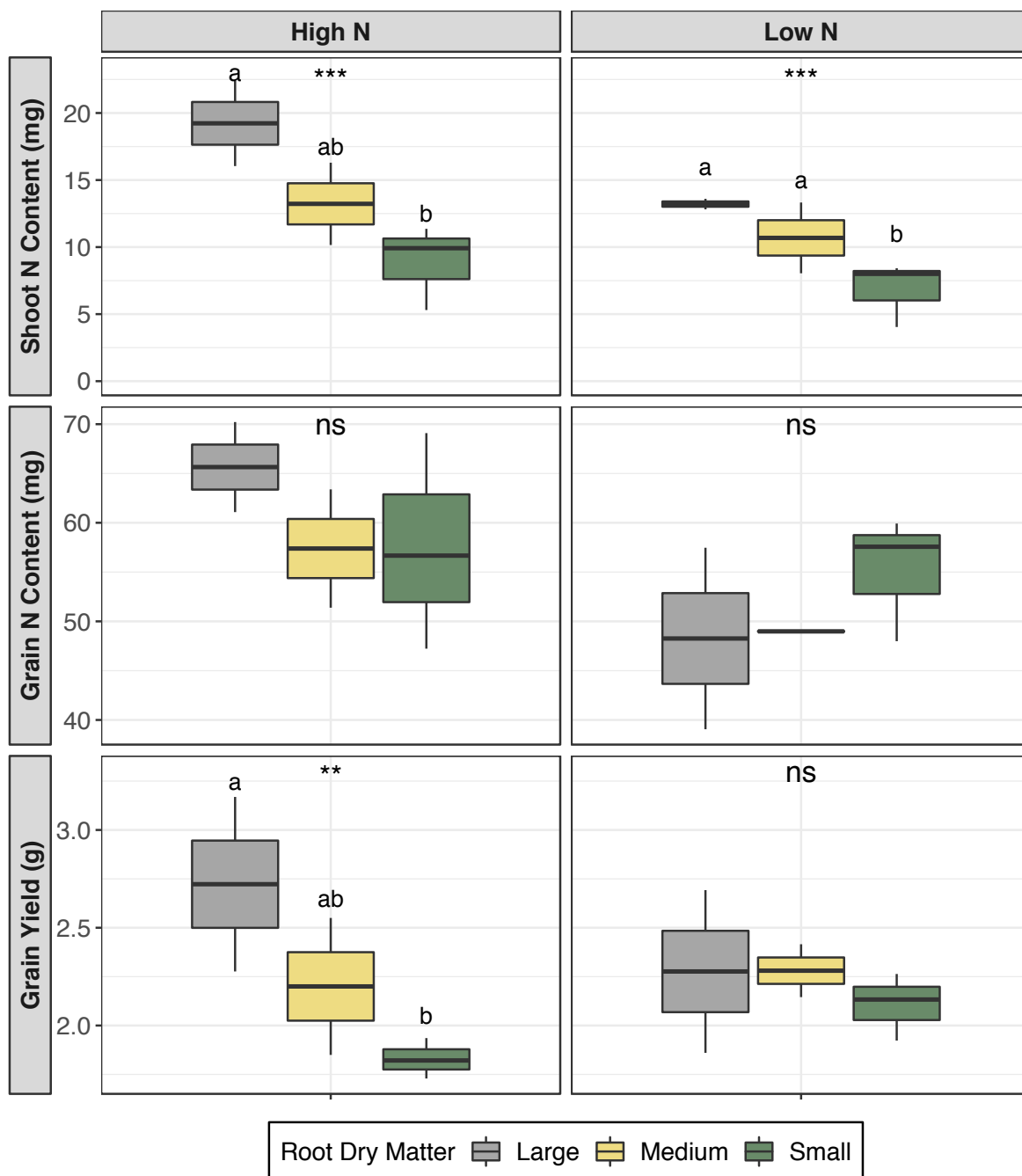
P value Significance: *** < 0.001; ** 0.001-0.01; * 0.01-0.05; ns >0.05
Figure 7. Representation of the impact of small, medium, and large RDM in high-N and low-N treatments for shoot N content (SNC), grain N content (GNC), and grain yield. Significant differences between RDM classifications were provided at the top of each graph. Where the RDM classifications were significantly different for a given trait in a given treatment, Tukey tests were performed, and letters above each boxplot describe significant differences between classifications. In these plots, 'a' was significantly different from 'b'; however, 'ab' was not significantly different from either 'a' or 'b'.

2.3.4 Root Dry Matter did not Significantly Change from Stem Elongation to Maturity

Data from seedling experiment and maturity experiment were used to calculate correlation of RDM between these two stages. The correlation of RDM between seedling and maturity experiment was r = -0.66 (Figure 8). Since this correlation was negative, we were interested to further characterize root growth patterns at more growth stages. However, only four of the seven lines were studied in this dynamic experiment.
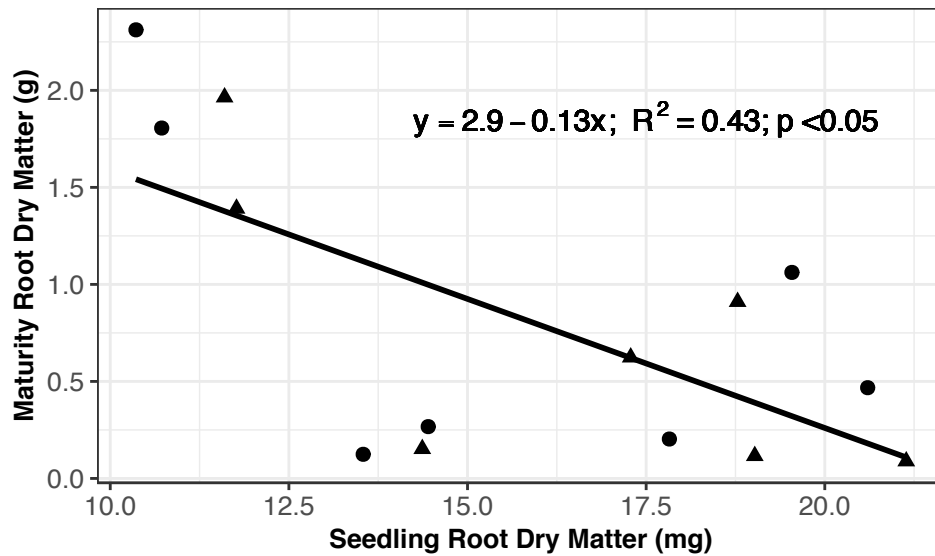
Figure 8. Depiction of the relationship between root dry matter (RDM) at seedling (Experiment 1) on x-axis and maturity (Experiment 2) on y-axis. Different colors represent the seven lines that were used in the experiment. Circles denote plants grown in the high-N treatment, while triangles denote plants grown in the low-N treatment.

In the dynamic experiment, the line x growth stage interaction was significant for RDM which indicated that lines had different patterns of biomass allocation. At the four-leaf stage, there was not a significant difference between the RDM of the four lines; however, by stem elongation, RDM of Iran was significantly greater than the other three lines. On the other hand, RDM of Chile had not significantly changed from the four-leaf stage to stem elongation. Which indicated that there was some variation in the timing of root growth. Invariably across all four lines in this experiment, RDM did not significantly change from stem elongation to maturity

(Figure 9). RDM of each line at stem elongation explained 99% of the variation in RDM at maturity. This correlation of RDM at these two growth stages indicated that root growth appeared to stagnate after stem elongation in our study regardless of the size of the root system.
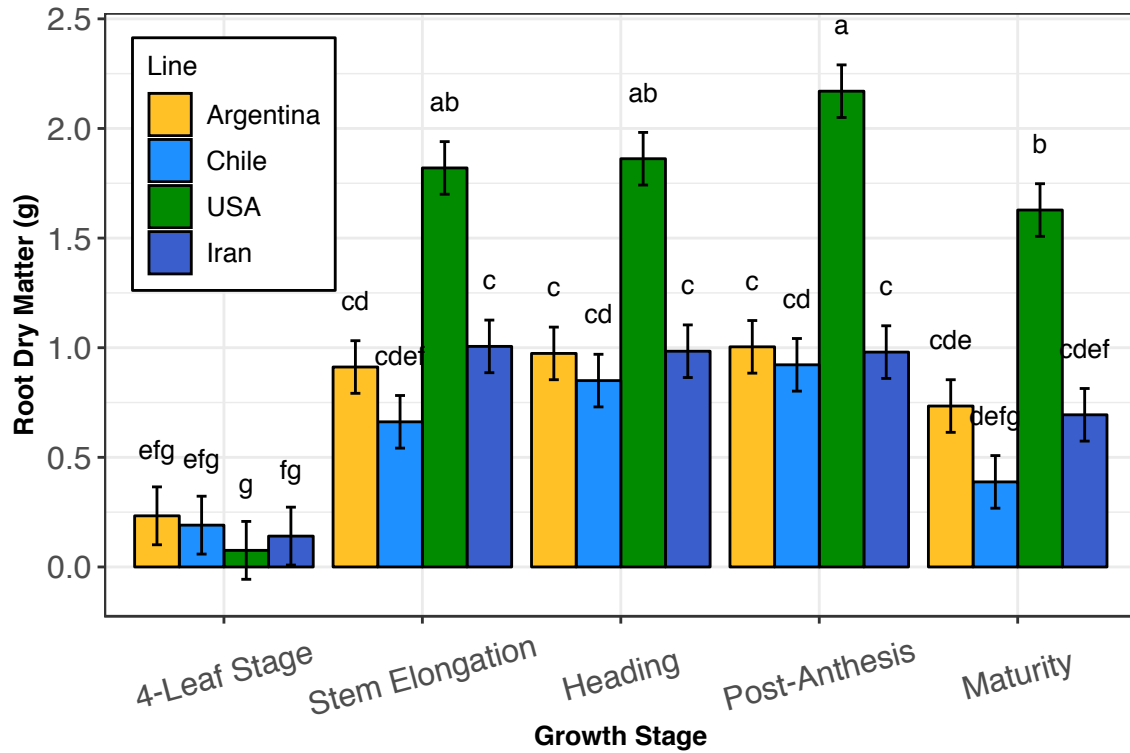
Figure 9. Illustration of RDM (y-axis) at the five growth stages sampled (x-axis). Growth stages sampled include: 4-leaf stage, stem elongation, heading, post-anthesis, and maturity. Error bars represent the standard error from the mean. Tukey tests were performed on the interaction of Line x Growth Stage. Letters above each bar classify significant differences between the groups. Letter 'a' was the largest group with 'g' being the smallest group. As an example, 'a' was significantly different from 'b', but 'ab' was not significantly different from either 'a' or 'b'.

2.4 Discussion

This study aimed to examine the genetic variation in RDM under different N treatments and associate RDM to nitrogen uptake and yield. Our objectives were to characterize and demonstrate the relationship between below-ground and above-ground traits in seedling and maturity stages and to examine the patterns of biomass allocation over multiple plant growth stages. This discussion was organized based on the observation of the relationship between root

traits to N uptake and yield and the potential importance of root traits with regards to different treatments. Finally, implications on breeding for root traits will be drawn from these studies for genetic variation and cycle length.

2.4.1 Relationship of Root Traits to N Uptake and Yield

Our data indicated the functionality of large roots to increase nitrogen uptake was dependent on the availability of N. In the high-N treatment, the large-RDM lines obtained a significantly greater SNC (p value <0.001) and grain yield (p value <0.01) than the small-RDM lines (Figure 7). In the low-N treatment, the large-RDM lines obtained significantly more SNC (p value <0.001) than the small-RDM lines; however, no significant differences were observed between the three RDM classifications for GNC and grain yield. Since the small-RDM lines had significantly less SNC in the low-N treatment, but GNC and grain yield were not significantly different from the medium- and large-RDM lines, the small-RDM lines were more efficient with the nitrogen taken up.

Similar results were observed by Ehdaie and Waines (2008). In a high-N treatment, they observed that large-root systems had the potential to increase both yield and grain protein content while reducing N pollution. In a field experiment, they found that where N inputs were readily available, especially when mobile in the soil, large-RDM lines had the potential to take up greater amounts of N from the soil.

In the low-N treatment, it appeared that small-RDM lines could be more efficient and allocating more resources to roots may not lead to greater N uptake or yield. This has previously been observed by (Lynch, 2007) in which large RDM was counterproductive in low-N treatments as the metabolic cost of maintaining the large root was a resource drain. Similarly, Kamiji et al. (2014) found that in low-N treatments, lines with reduced RDM were able to take

up more N per unit of RDM to meet shoot N demands. Edwards et al. (2016) concluded that in drought treatments, increases in root biomass allocation did not have a positive impact on yield. In drought treatments, they found that a marginal increase in RTS improved the chance for survival; however, increasing the ratio beyond the level needed for survival decreased rather than increased yield.

At seedling while only an observational study, lines with large-RDM also showed greater SNC in both high- ($R^2 = 0.96$; p value <0.001) and low-N ($R^2 = 0.58$; p value <0.05) treatments, while increased RDM had greater importance in the high-N treatments than the low-N treatment (Figure 6). Liao et al. (2004) found that 'early vigor' a trait termed for a quicker plant growth rate early in the growing season could be beneficial to increased N uptake. Their results indicated that vigorous lines with quicker early season root growth were able to increase N uptake in sandy soils across multiple N treatments.

The negative correlation (r = -0.66) between the seedling and maturity studies suggests that selection at the seedling stage is not an effective measure to improve RDM at maturity (Figure 8). While screening is commonly performed to evaluate root traits at the seedling stage, our data suggests that later growth stages might not be correlated with these early measurements. An additional source of variation between these experiments is the different growth conditions. While the seedling study was performed in a hydroponic growth condition, the maturity study was performed in potted-soil. The efficacy of hydroponic growth experiments should be further evaluated for their ability to correlate to field environments.

2.4.2 Great Genetic Variation in Root Dry Matter

Genetic variation is a fundamental prerequisite in breeding programs. At maturity, a ~15-fold difference was recorded in the RDM of this germplasm collection, which was far greater than the ~5-fold difference observed in SDM. This difference indicated a greater

variation in biomass allocation to below-ground organs than above-ground organs. Narayanan et al. (2014) evaluated a panel of 297 diverse spring wheat lines. Plants were grown in PVC tubes with diameter of 7.5 cm and height of 150 cm using Turface MVP (PROFILE Products LLC, Buffalo Grove, IL) as the soil media. They found that RDM ranged from 0.22 g to 7.6 g, a ~35-fold change. These results appear to verify the wide genetic diversity for RDM in spring wheat.

At seedling, RDM ranged from 10.5 mg – 19.0 mg in the high-N treatment and 11.7 mg – 20.2 mg in the low-N treatment. Beyer et al. (2018) sampled a historical panel of 215 lines in a paper-roll-supported hydroponic system and recorded a range of RDM measurements from 6 mg – 20 mg. While there was genetic variation at the seedling stages of these experiments, it was not close to the range seen at maturity, which suggested that plants did not have enough time to fully express the potential of root growth.

2.4.3 Reducing Cycle Time in Breeding for Root Dry Matter

Characterizing a plant phenotype early on during the plant growth and development is beneficial as breeders can make selections and allocate resources to selected genotypes. The dynamic experiment showed that RDM did not significantly increase or decrease from stem elongation to maturity and variation of RDM at stem elongation explained nearly 99% of variation at maturity stage. While this result was based on only four lines, it could cautiously be said that selecting RDM at stem elongation was as good as selection at maturity. Similar results were seen by Liu et al. (2018) where 71% of the variation in root weight density at maturity was explained in the variation in root weight density at jointing in winter wheat. Furthermore, they found that 82% of the variation in root weight density at maturity was explained in the root weight density at flowering.

2.4.4 Future Work

In these experiments, RDM was evaluated at multiple growth stages in a diverse panel of spring wheat. The goal of these projects was to evaluate the efficacy and feasibility of incorporating root traits (mainly RDM) into a breeding program. Especially, phenotyping RDM to assess beneficial traits in high-N and low-N treatments. From this work, a short-term goal of the wheat breeding program is to evaluate more lines with long-term goals of gene identification and field trials.

The short-term goal for the future work in this project is to evaluate more lines in more treatments. While the seven lines that were selected for this project were found to have a large-degree of genetic variation, screening a larger pool of germplasm would ideally uncover further variation in RDM.

Looking further out, in the future the wheat breeding program hopes to create a biparental mapping population between large-RDM and small-RDM lines to identify QTLs for root morphological traits. A similar phenotyping project within the wheat breeding program resulted in lines that were crossed for a future study. This approach will help to further the understanding of root trait inheritance, in addition to identifying important regions of the genome for root traits.

Many root studies, including those performed in these experiments, were performed in a greenhouse, controlled-environment setting. These studies are important as the heterogeneity of a field trial can lead to ambiguous results. However, with the knowledge of these studies, field trials must be performed to verify these results in a commercial environment with plant-plant interaction.

2.5 Conclusion

Experiments were performed to evaluate the performance and root characteristics of a diverse body of germplasm. A collection of germplasm from around the world was phenotyped

from which a ~15-fold difference was noted between lines for RDM whereas only a ~5-fold difference was measured between SDM. This diversity is an indication of the great potential that can be achieved if root traits are incorporated into breeding programs. Furthermore, RDM was identified across growth stages (i.e. seedling and maturity) from which selection could be beneficial as it was associated to greater N uptake and yield; however, selection at the seedling stage might not be an appropriate measure for RDM at maturity. Finally, varied biomass accumulation patterns above and below-ground were described on a single-plant basis from which it appears RDM remains consistent from stem elongation to maturity. From 1985 to 2005, wheat production stagnated on 38% of global wheat acres. While above-ground traits have commonly been used for selection in the past, incorporating root traits into breeding programs is an area from which there appears to be great, untapped potential. This potential could help to bridge the gap between current wheat production and the demands of the future.

CHAPTER 3: HIGH-THROUGHPUT PHENOTYPING IDENTIFIED PLANT GROWTH DIFFERENCES UNDER WELL-WATERED AND DROUGHT CONDITIONS USING A LIMITED NUMBER OF REPLICATES

A version of this chapter has been submitted to Journal of Integrative Agriculture.

3.1 Introduction

By the year 2050, global population is expected to increase by 1.4 billion people, primarily in developing countries, bringing the worldwide total to 9.1 billion people (FAO, 2009). To meet the growing food demand in the coming decades, annual cereal production must increase by almost one billion tons, which is equivalent to an annual wheat yield increase of 2.4% globally. However, wheat breeding efforts are only seeing sustained improvements of 1.6% growth per year (FAO, 2017). Furthermore, as climate change progresses, one such environment that will persist and likely expand, especially in developing countries, is drought. Annually, drought causes a loss of more than 20 million tons of grain in many semi-arid, wheat growing regions (CGIAR, 2018).

To address these challenges, researchers are approaching these problems through a variety of avenues. One of the main approaches to plant breeding for specific environments is to identify germplasm sources that are adapted to a given environment. Previous work on drought tolerance in wheat identified traits that could maintain productivity in drought conditions, including but not limited to, delayed leaf senescence in non-terminal drought (del Pozo et al., 2016), early vigorous root growth (Palta et al., 2011), and minimal reduction in above-ground growth rates (Maqbool et al., 2015). In addition, there are many morpho-physiological stress adaptive traits that are not utilized because there are no phenotyping techniques available to assess large populations (i.e. stay-green) (Lopes & Reynolds, 2012).

The International Wheat Genome Sequencing Consortium published the reference genome for hexaploid wheat providing information on more than 100,000 genes (Appels et al., 2018). While the entire wheat genome is a tremendous resource at researchers' disposal, genomic data is currently underutilized due to the lack of adequate phenotypic data. Manual phenotypic data collection on large germplasm panels is often time-consuming and laborious; however, current technological advancements enable the data collection of high-throughput phenotyping (HTP). HTP utilizes imaging and sensing technologies to avail surrogates for plant traits. HTP increases plant breeders' power to evaluate germplasm by the virtue of multiple, nondestructive measurements on larger populations (Ghanem et al., 2015). However, since HTP is the utilization of engineering, algorithmic, and computational techniques in agriculture, there is a need to determine if HTP-based surrogates can reliably measure traits of interests or characterize stress responses. For reliability, reference data must be collected to assess the accuracy of HTP predictions. Previous studies have used RGB imaging to measure plant characteristics such as biomass, shoot weight, and tiller number in rice (Yang et al., 2014) and leaf area and biomass in maize (Ge et al., 2016). A few projects provide evidence that HTP can be an important tool to assess drought tolerance temporally. Studies in maize (Ge et al., 2016), barley (Chen et al., 2014), and foxtail (Fahlgren et al., 2015) provided support for HTP in detecting drought and in assessing the ability of temporal models to distinguish drought tolerant lines throughout the growing season.

While HTP is gaining in popularity, more work is needed to verify the relationship between these image-based traits and the LTP. In this study, HTP was used to predict ground-truth measurements in wheat under two watering regimes. The objectives were as follows: (1) measure relative water content, shoot and root dry matter, and tiller number to assess drought tolerance,

(2) assess the ability of image-based HTP to predict LTP, and (3) evaluate the efficacy of image-based surrogates to model plant growth in well-watered and drought treatments.

3.2 Materials and Methods

3.2.1 Plant Materials and Growth Condition

In this experiment, phenotyping was performed in the Controlled Environment Phenotyping Facility (CEPF) at Purdue University. In the CEPF, the plants were watered daily using an automated irrigation system. For the duration of the experiment, these plants were grown in the CEPF at 26°C with a fourteen-hour photoperiod. Plant materials included two hexaploid wheat (*Triticum aestivum*) accessions PI 189823 from Argentina (referred to as Argentina) and PI 519677 from Chile (referred to as Chile). The seed was sourced from the National Small Grains Collection (NSGC, USDA-ARS) from Aberdeen, Idaho USA.

The potting medium was a mix of 1:1:1 of topsoil (Biotown Ag Reynolds, IN), sand, and propagation potting mix (Sungro: propagation mix). For measuring water-holding capacity, pots were filled with 5050g of sterilized potting medium, roughly 3/4 of the pot volume, and watered with reverse osmosis water to full saturation and allowed to drip dry until no water was coming out of the bottom of the pots. Using pressurized chambers, it was determined that the gravimetric water content of the soil used in this study at field capacity was 0.12 g of water/g of soil and the gravimetric water content at permanent wilting point was 0.03 g of water/g of soil. Seeds were germinated in petri-dishes and then transplanted in the pots (one seedling per pot). From the point when the plants were transplanted to pots (October 4th) until stem elongation (October 30th), all plants received similar water treatments of 0.12 g of water/g of soil associated with field capacity.

From stem elongation (twenty-six days after transplanting) to post anthesis, plants were divided into two treatment groups (i.e. well-watered and low-watered). The well-watered treatment was watered to near field capacity (0.12 g of water/g of soil) while the low-watered treatment was provided 0.03 g of water/g of soil, consistent with the permanent wilting point of the soil. Seven days after the drought was imposed, the low-watered treatment was further reduced to 0.015 g of water/g of soil. From this point forward, the low-watered treatment shall be called the drought treatment.

3.2.2 Low-Throughput Phenotyping

For LTP measurements, all plants were destructively phenotyped at the post-anthesis growth stage, which was twenty-two days after the imposition of differential water treatments. Traits collected at this time included plant height (PLH), shoot dry matter (SDM), root dry matter (RDM), spike dry weight (SDW), fertile tiller number (TN), and root:shoot ratio (RTS). PLH was measured by hand using a ruler and expressed in centimeter (cm). SDM, SDW, and RDM were measured after drying for two days at 60°C using a balance expressed in g. To extract roots from the soil, roots and soil was fully submerged in tap water for 30 minutes. After submersion, roots were loose from the soil and rinsed with a medium-pressure hose until clean.

3.2.3 High-Throughput Phenotyping

For HTP measurements, a custom-made RGB imager (Aris), with a standard 5 Megapixel RGB camera (Basler Ace) was used to acquire images. HTP traits included height (H), side projected area (SPA), and convex hull (CH) (Figure 10). HTP was conducted from stem elongation to post-anthesis. Three images per plant were taken from various angles. RGB images were analyzed by using a proprietary image analysis pipeline provide by Aris. Briefly, the image analysis pipeline conducted image segmentation using the chlorophyll fluorescence image of a

plant. The average from all three side-view images was used to represent the plant HTP traits.

Descriptions of each image-based trait were provided in Table 4. In addition, the Aris custom

RGB camera produced Munsell-based color characteristics (Figure 10). These are estimates of

hue (HUE), saturation (SAT), and intensity (INT). Professor Albert H. Munsell created the

Munsell color system which was adopted by the United States Department of Agriculture

(USDA) to evaluate soil color characteristics.
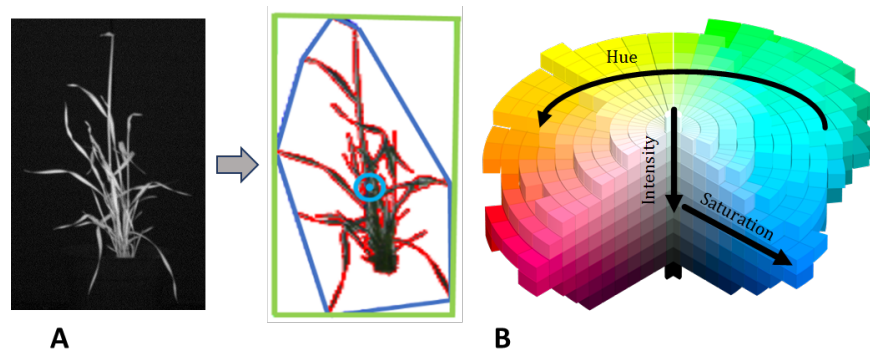
Figure 10. Depiction of the extraction of image-based traits from the RGB camera. The length of the green box was an estimate of height, the area of the blue polygon was convex hull, the red outline of the plant defines the area from which the Side Projected Area (SPA) was estimated (A). Cartoon description of the Munsell Color System characterized by color characteristics hue (HUE), saturation (SAT), and intensity (INT). In colorimetry, the Munsell color system is a color that specifies colors based on hue, saturation, and intensity. It was created by Professor Albert H. Munsell and is used as the official color system for soil research since the 1930s (B).

Table 4. Definition and abbreviation of each high-throughput trait.

Trait	Abbreviation	Definition
Height	H	Length from the lowest to the tallest point of the plant
Convex Hull	CH	Smallest polygon that contains all plant material
Side Projected Area	SPA	Total side-view projected area obtained by outlining all plant material
Hue	HUE	Average color of the plant material
Saturation	SAT	Attribute associated with the purity of the hue
Intensity	INT	Measurement of the amount of black/white in the hue

3.2.4 Experimental Design and Statistical Analysis

Through a power calculation (Table 5 using SDM data from chapter two of this thesis) it was determined this experiment would have sufficient power (0.96) and there would be little chance of a type II error when comparing the lines (Cohen, 1988). This power calculation does not give any information regarding the power when comparing the water treatments. Each treatment combination received eight replications totaling thirty-two experimental units in the study.

Table 5. Power calculation for Shoot Dry Matter (SDM) between lines as described by (Cohen, 1988) f describes the amount of separation between the lines (using data obtained in chapter two of this thesis), k is the number of treatments, n is the number of replications per treatment. Greater power is equivalent to a reduced risk of a type II statistical error.

Power Calculation for Shoot Dry Matter			
f	0.99	0.99	0.99
k	2	2	2
n	2	4	8
Power	0.21	0.65	0.96

This study was conducted in a factorial experiment with two factors, line and water treatment, in a completely randomized design with eight replications. Statistical analysis was completed in R environment (R Core Team, 2014) using `lm' command to perform ANOVA that

assessed differences between lines, water treatments, and among levels of line x water treatment interaction (Table 6). When the line x water treatment interaction was significant, a post-hoc Tukey test was performed. However, no further analysis was required when only the main effects were significant as line and water treatment each only had two levels. While the HTP traits were measured on 17 days, only the measurements drawn from the last day were used to assess the significance of the lines, water treatments, and the line x water treatment interaction. Prediction accuracy of HTP traits, derived from the images taken on the final day, to predict LTP counterparts was assessed and expressed by Pearson's coefficient of correlation.

The cor.test function in R environment (R Core Team, 2014) was used to determine the correlation and its significance between two traits. Within each line, Student's t-test was performed on SPA to test the significance of the water treatments on a given day. To model plant growth temporally, the growth rate of each plant was calculated as $(SPA_t - SPA_{t-1})/(day_t - day_{t-1})$. A three-day running average was used to reduce the day-to-day variation in growth rate.

Table 6. Example ANOVA table for plant height (PLH) in a completely randomized design with two factors. Line, Water Treatment, and the Line x Water Treatment (L x T) interaction were all tested using the Residual MSE (Error).

	d.f.	MS	F value	Pr(>F)
Line (L)	1	9453	563***	<0.001
Treatment (T)	1	561	33***	<0.001
L x T	1	72	4*	0.048
Error	28	17		

P value Significance: *** < 0.001, ** 0.001 – 0.01; * 0.01 – 0.05; ns > 0.05

3.3 Results

3.3.1 Line and Treatment Variation in Low-Throughput Traits

The effect of water stress was evaluated on two wheat accessions, Argentina and Chile, in a controlled environment. Plants grew in the controlled environment for forty-seven days and were

imaged with an RGB camera from three angles a total of seventeen of the final twenty-two days after the imposition of the drought treatment. In Figure 11, images for each treatment were shown at a variety of time points. In visual evaluation on Oct 30th, Argentina and Chile were easily distinguishable even though they are at the same growth stage, stem elongation. At this time, the drought was imposed. While minimal differences were visually seen on Nov 2nd, the effects of the drought were visible in Argentina on Nov 6th and continue to become more prevalent until the end of the experiment. While it is difficult to distinguish the treatments on Nov 6th in Chile, it appears that the drought effect was visually different from the well-watered treatment on Nov 9th and continues to worsen until the end of the experiment.
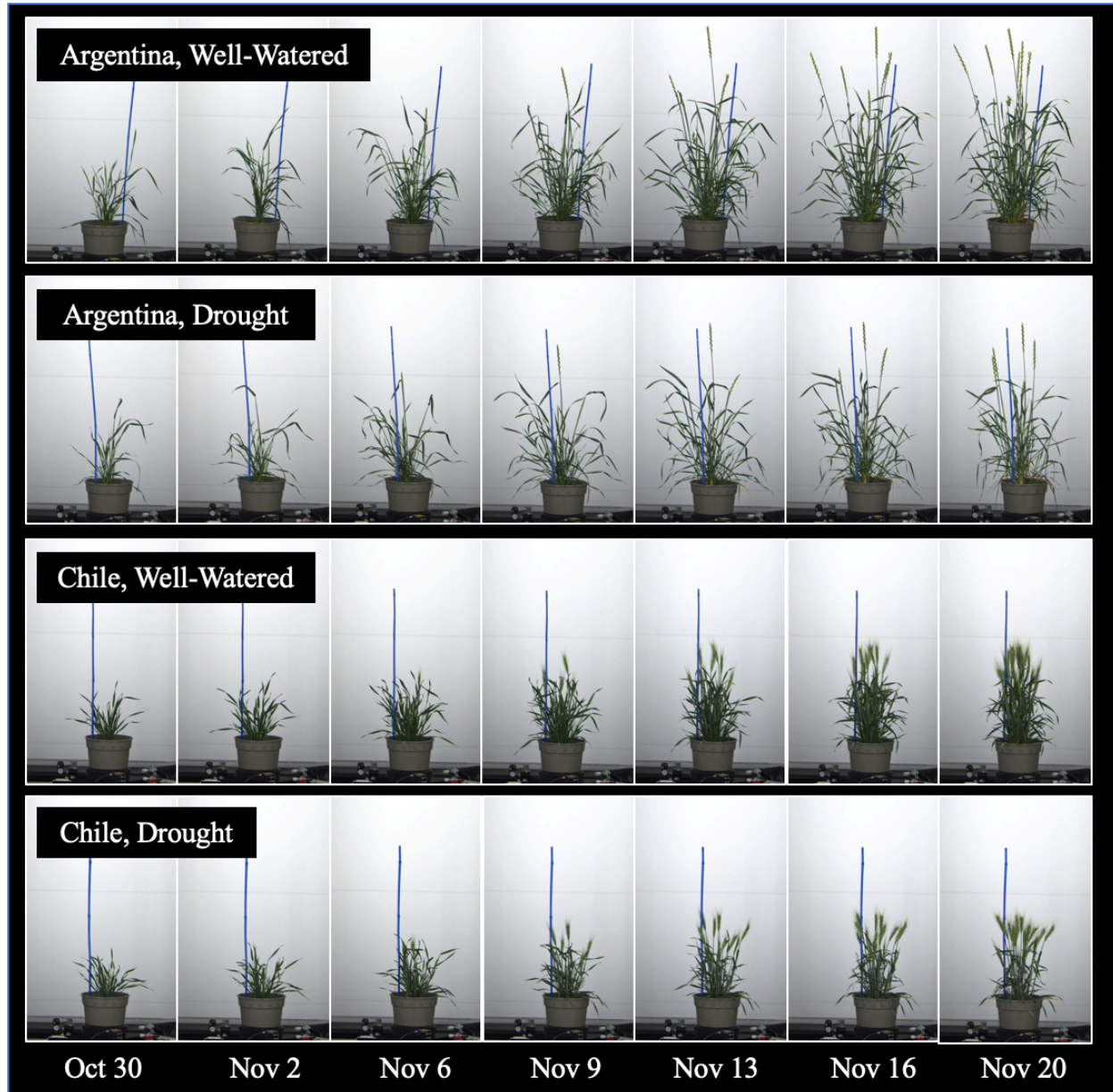
Figure 11. Temporal growth of the four treatment groups (i.e. Argentina, Well-Watered; Argentina, Drought; Chile, Well-Watered; and Chile, Drought). Progression is shown from the beginning of the differential water treatments, October 30th, to the destructive end of the experiment, November 20th.

Statistical analysis for testing significance of sources of variation was performed as mentioned in materials and methods. The line x treatment interaction effect was significant for PLH (Table 6; Figure 12). For Argentina, the PLH of well-watered (93.3 cm) plants was significantly different from PLH of drought stressed (82.0 cm) plants. However, the PLH of

Chile, well-watered (56.0 cm) plants was not significantly different from PLH of Chile, drought

stressed (50.6 cm) plants.

For all other LTP traits, line x treatment interaction was not significant. The effect of line
was significant for SDM, SDW, TN, and RDM but not RWC. The water treatment was
significant on SDM and RDM but not significant on RWC, SDW, and TN (

Table 7). On average, SDM was 33.9 g in well-watered condition compared to a

significantly smaller SDM of 21.8 g under drought condition. Furthermore, SDM of Argentina,

34.6 g, was significantly greater than SDM of Chile, 21.1 g. TN was significantly different

between the lines with 9.7 and 27.9 tillers for Argentina and Chile, respectively. A similar trend

was found for SDW where Argentina (5.9 g) was significantly less than that of Chile (12.9), but

the water treatment was not significant on SDW. Finally, line, treatment, and the line x treatment

interaction was not significant for RWC with a mean overall value of 73.2.
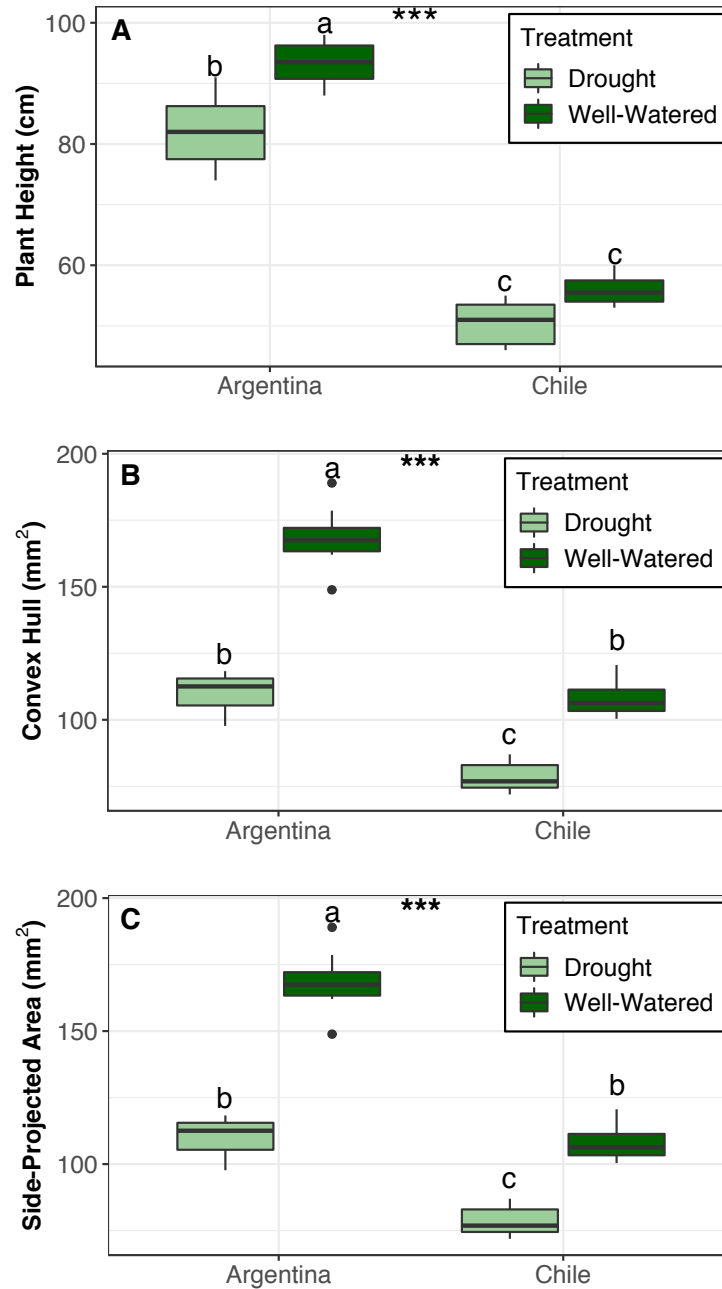
Figure 12. Boxplots of plant height (PLH) (A), convex hull (CH) (in thousands) (B), and side-projected area (SPA) (in thousands) (C). Lines in the center of each box correspond to the median of each treatment with the 1st and 3rd quartiles being represented by the bottom and top of the box, respectively. Whiskers represent 1.5 the inter-quartile range. The significance of the line x treatment interaction was presented at the top of the box-plot. Tukey tests were used to separate the treatment groups with different letters above the boxplots describing significant differences.

Table 7. Lsmeans and standard errors for lines and treatments for relative water content (RWC), shoot dry matter (SDM), spike dry weight (SDW), tiller number (TN), and root dry matter (RDM). For these traits, the Line x Treatment (L x T) was not significant. Significance of line and water treatment are to the right of each trait. No further post-hoc test was performed to separate treatments as line and treatment only had two levels.

	Line		Treatment		Line Significance	Treatment Significance
	Argentina	**Chile**	**Well-Watered**	**Drought**		
Relative Water Content		73.2 ± 2			**ns**	**ns**
Shoot Dry Matter (g)	34.6 ± 0.9	21.1 ± 0.9	33.9 ± 0.9	21.8 ± 0.9	*******	*******
Spike Dry Weight (g)	5.9 ± 0.7	12.9 ± 0.7	9.4 ± 0.8		*******	**ns**
Tiller Number (#)	9.7 ± 1.3	27.9 ± 1.3	18.8 ± 1.9		*******	**ns**
Root Dry Matter (g)	19.5 ± 1.1	14.3 ± 1.1	21.8 ± 1.1	12 ± 1.1	*******	*******

P value Significance: *** < 0.001, ** 0.001 – 0.01; * 0.01 – 0.05; ns > 0.05.

Line and treatment were both significant (*p* value <0.001) for RDM (

Table 7). For these measurements, water stress had a large impact on the overall size of the roots. RDM of Chile (14.3 g) was significantly smaller than that of Argentina (19.5 g). While these lines were significantly different for their RDM production, both lines appear to respond similarly to the drought stress through decreased biomass invested in the root systems. In the well-watered treatment, Argentina averaged 25.9 g of RDM which was significantly greater than the RDM observed under drought treatment, 13.1 g. This reduction translated to 49% less RDM under drought compared with well-watered treatment. A similar response was observed in Chile which was significantly smaller in the drought treatment (11.0 g) as opposed to the well-watered treatment (17.7 g), equivalent to a 38% smaller amount of RDM.

3.3.2 Line and Treatment Variation in High-Throughput Traits

Argentina CH was greater than Chile CH in both experimental conditions. The CH of the two lines also decreased from the well-watered to drought treatment. The line x treatment interaction was significant for CH. Tukey test significantly differentiated the four treatments groups (Figure 12). Convex Hull of Argentina, well-watered treatment (496,905 mm^2) was significantly greater than all other treatments. The drought treatment of Argentina (341,610 mm^2), while smaller than Argentina, well-watered by 30%, was significantly greater than both treatments of Chile. The well-watered treatment of Chile (196,446 mm^2) was significantly greater than the Chile, drought treatment (150,290 mm^2) – a 23% smaller CH in the drought treatment compared to the well-watered treatment.

Similar results were observed for SPA as the correlation between CH and SPA was 0.9. SPA of Argentina, well-watered (168,405 mm^2) was significantly greater than SPA of Argentina

drought treatment (109,983 mm^2) – a difference of 35%. SPA of Chile in well-watered treatment (107,970 mm^2) was significantly greater than SPA of Chile, drought (78,435 mm^2) – a difference of 27%. The Chile, drought treatment was significantly less than all other treatments (78,435 mm^2). However, the SPA of Argentina, drought and Chile, well-watered were not significantly different (Figure 12). Height was significantly greater in Argentina (1,044 mm) compared to Chile (737 mm). Additionally, H was significantly greater in well-watered treatment (936 mm) than in drought treatment (845 mm).

3.3.3 Prediction of Ground-Truth Traits Using High-Throughput Surrogates

High-throughput traits were associated with their counterpart low-throughput traits. The image-based H was able to predict plant height with an accuracy of $r = 1$ (Figure 13). Shoot dry matter was predicted using SPA and CH with coefficients of correlations of $r = 0.98$ (Figure 13) and $r = 0.87$ (Figure 13), respectively. CH was less predictive of SDM than SPA as tall plants led to a greater CH even though tall plants did not necessarily have more SDM. Tiller number showed coefficients of correlation of $r = -0.5$ and $r = -0.78$ with SPA and CH, respectively. Linear regression of TN using CH and SPA together (TN = 20 + -0.00014CH + 0.00033SPA) was able to explain 81% of the variation of TN.
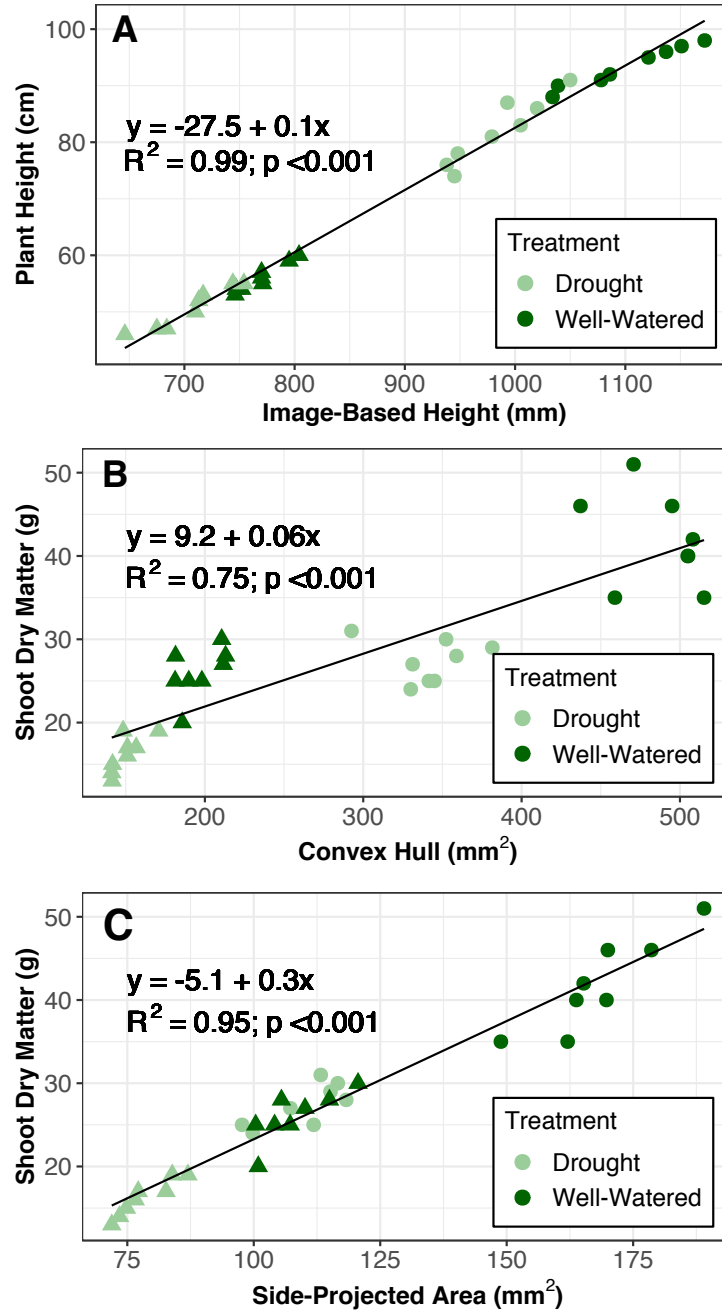
Figure 13. Pearson coefficient of determination between image-based height (H) (x-axis) and plant height (PLH) (y-axis), across plants grown under well-watered (dark green) and drought (pale green) treatments. The circles and triangles denote Argentina and Chile, respectively (A). Pearson coefficient of determination between side-projected area (SPA) (in Thousands) (x-axis) and shoot dry matter (SDM) (y-axis), across plants grown under well-watered (dark green) and drought (pale green) treatments. The circles and triangles denote Argentina and Chile, respectively (B). Pearson coefficient of determination between convex hull (CH) (in Thousands) (x-axis) and SDM (y-axis), across plants grown under well-watered (dark green) and drought (pale green) treatments. The circles and triangles denote Argentina and Chile, respectively (C).

3.3.4 Detecting onset of Drought Symptom and Visualizing Growth Rates Using High-Throughput Phenotyping

One advantage of HTP is the non-destructive nature of the image acquisition that allows for temporal analysis of plants during progression of growth. Because the correlation of SDM and SPA was r = 0.98, SPA was used to depict the temporal progression plant growth under the two water treatments for both lines (Figure 14). The t-tests between the water treatments for SPA revealed that the effects of the drought treatment were significant seven days from the start of the differential water treatments (*p* value <0.05) in Argentina; however, an additional three days were needed to find significance (*p* value < 0.05) between the SPA of the water treatments of Chile.

In addition to detecting onset of drought symptom, the repeated nature of image-based measurements on a near daily basis, allowed the growth rate to be further examined of each line under well-watered and drought conditions (Figure 14). To reduce the day-to-day variability in growth rate, a 3-day-running average was used to smooth the results. Growth rate of both lines in both treatments peaked 11 days after the imposition of the drought regardless of the treatment, which was 7973 $mm^2 day^{-1}$ of side projected area surrogate for Argentina and 5377 $mm^2 day^{-1}$ for Chile. This peak indicated a maximum growth rate of SPA between heading and anthesis, which coincided with the transition from vegetative to reproductive growth.
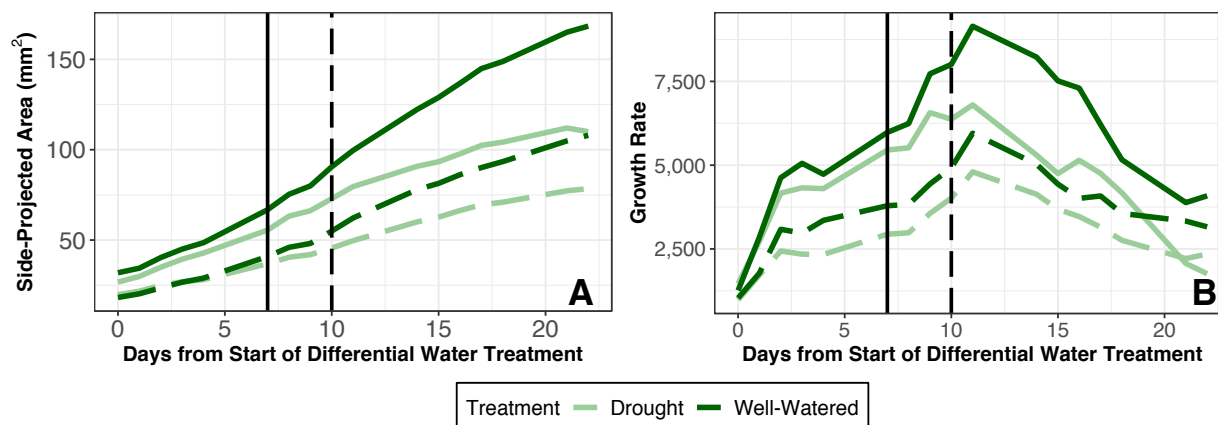
Figure 14. Temporal change in side-projected area (SPA). Argentina was denoted by a solid line whereas Chile was represented by the dashed line. Dark green lines indicate the well-watered treatment whereas pale green represented the drought treatment. The solid, black, vertical line displayed when the water treatment differences became significant in Argentina whereas the dashed, black, vertical line displayed when the water treatment differences became significant in Chile (A). Growth rate per 3-day running average for Argentina (solid line) and Chile (dashed line) under well-watered (dark green) and drought (pale green) treatments (B).

3.3.5 Exploring Applicability of the Munsell Color System in Plant Phenotyping

Color characteristics were explored in this experiment to see if color could be used to differentiate well-watered from drought stressed plants. Line and water treatment were significant for HUE, SAT, and INT. The line x water treatment interaction was significant for HUE but not for SAT and INT. The HUE observed between the well-watered (66.3) and drought (69.9) treatments of Argentina was significantly different; however, the water treatments of Chile were not significantly different. For SAT, the well-watered treatment (68.7) was significantly different from the drought treatment (60.8), and Chile (66.9) was significantly greater than Argentina (62.6). The behavior of INT was found to be more line-dependent. While lines behaved similarly in each treatment, both treatments of Argentina were found to be greater than both treatments of Chile. In the well-watered treatment, intensity of Argentina was 94.2 while in drought treatment it was 100. A similar significant difference was observed in Chile with an intensity of 83.1 in the well-watered treatment and 90.1 in the drought treatment. A 3D-plot was

built to visualize differences between lines and changes due to water treatments (Figure 15). The well-watered treatment was represented by the green dots and surface while the yellow dots and surface represent the drought treatment. In this experiment, SAT was best able to discriminate between the well-watered treatment compared to the drought treatment (Figure 15).
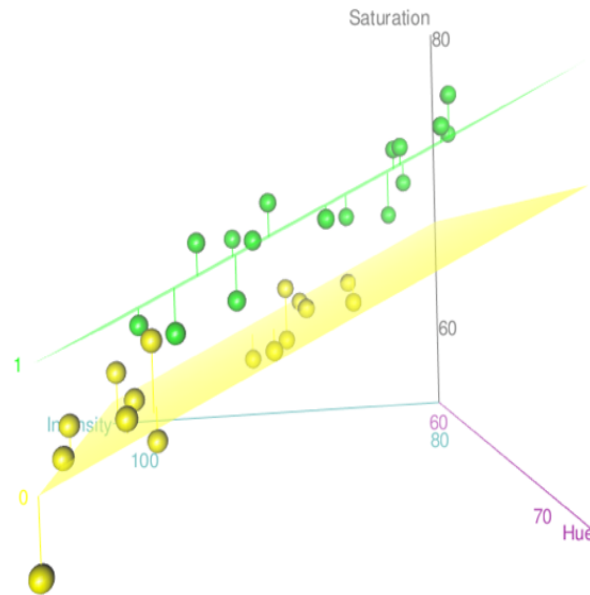
Figure 15. 3D plot graphing Hue (HUE), Saturation (SAT), and Intensity (INT) on the x, y, and z axes, respectively. Green dots represent plants in the well-watered treatment whereas yellow dots represent plants in the low-water treatment from the final day of imaging. Two surfaces illustrate the difference between these two treatments. The two figures were given to present this graph from two angles.

3.4 Discussion

Similar to previous work in barley (Chen et al., 2014), maize (Ge et al., 2016), and foxtail (Fahlgren et al., 2015), in this experiment, LTP and HTP techniques were used to assess plant growth in differential water treatments of two wheat lines. Furthermore, image-based phenotypes were used to visualize plant growth and growth rate. Finally, with this work we identified the SAT as a potential objective measurement for low-water detection with cautious. The findings of this study are discussed around (1) evaluation of HTP surrogates in modeling plant growth and

assessing the impact of water treatments and (2) correlation of HTP surrogates with ground-truth measurements.

3.4.1 Evaluation of High-Throughput Surrogates in Assessing the Impact of Water Stress Response

One goal of this study was to assess the applicability of HTP to characterize drought responses. Using SPA to assess plant growth in differential water treatments, Argentina was found to be more susceptible to drought than Chile. The effects of the drought significantly reduced above-ground biomass, inferred by reductions in SPA, of Argentina three days prior to Chile (Figure 14). The effects of the drought were observed quicker in Argentina than Chile since the bigger stature of Argentina used the supply of water faster than Chile. This genotypic difference would have easily been missed had there not been temporal analysis available through HTP.

Previous studies in corn (Ge et al., 2016) and foxtail (Fahlgren et al., 2015) have evaluated drought tolerance of their prospective species. Ge et al. (2016) found that the growth of plants in the drought treatment fell behind that of the well-watered treatment. Fahlgren et al. (2015) found significant differences between treatment groups (species and water treatment) in foxtail using imaged-based plant height and imaged-based biomass. Furthermore, using image-based estimates for biomass, they determined differences in absolute growth rates of individuals between species and water treatments that would not have been evident had the measurements only been destructively phenotyped at the end of the growing season.

Plant growth in the drought treatment fell behind that of plant growth in the well-watered treatment. Among LTP measurements, SDM, RDM, and PLH were all significantly affected by the water treatment with a decrease of ~37%, ~44%, and ~11%, respectively from the well-watered treatment to the drought treatment (

Table 7; Figure 12). RWC, which in many studies has been used to assess leaf water content (Turner, 1981), was not significantly impacted by line and treatment in this study. With regards to the significant decrease in SDM, RDM, and PLH from the well-water treatment to the drought treatment, it is likely that RWC was not significant due to an error in the phenotyping process. For example, Barrs and Weatherley (1962) studied RWC and found that the most likely causes of error were inconsistent dry weights of the samples and continued increase of water uptake after reaching full turgidity. Additionally, it has been hypothesized that removing the plants from the growth chamber, in the high-light environment, into the darker main area caused the stomata to close and the water content to be consistent across treatments.

Among other signs such as increased stomatal closure, reduced photosynthetic capacity, and reduced biomass (Nezhadahmadi et al., 2013; Ogren & Oquist, 1985), one sign of drought stress as described by (Lollato et al., 2013) is a change in leaf color. In drought treatments, they described a bluish tint to the leaf color. Extension from Washington State University described moisture stress in multiple crops including alfalfa, corn, and small grains as having a dull, green color (Ley, 2003). In this study, the application of HUE, SAT, and INT were explored. In examining each of these traits in this experiment, SAT appeared to have the greatest functionality in differentiating water treatments (Figure 15). SAT could be an objective measurement to describe the dull color of plants in a drought treatment.

Development of sensor-based techniques can speed up phenotyping and increase genetic gains by the virtue of increasing selection intensity. In addition, there are many stress-adaptive traits (i.e. stay-green and water use efficiency) that are not commonly used in wheat breeding as there are few phenotyping techniques available to assess a large population (Lopes & Reynolds,

2012). Therefore, many have proposed the use of HTP for future advancements in plant breeding (Araus et al., 2018; Chenu et al., 2017; Shakoor et al., 2017).

3.4.2 High-Throughput Surrogates Correlated with Ground-Truth Measurement

The use of HTP is an area of plant breeding with great potential; however, work remains to understand and utilize the potential of this new technology. In this controlled-environment study, the image-based traits accurately predicted PLH (r=1) and SDM (r=0.98) (Figure 12). Previous work has been performed to assess the performance of HTP in predicting reference measurements. Chen et al. (2014) analyzed the drought tolerance of eighteen barley varieties. Using a phenotyping platform in a controlled environment, they found a great correlation between `volume' and above-ground dry matter (r=0.95). Another study, performed by Ge et al. (2016) in a controlled environment, found RGB traits displayed strong prediction accuracies of r=0.993 and 0.98 in predicting shoot dry matter and leaf area, respectively, at early growth stages (<30 days after sowing). They further found that images were less predictive of these plant architecture traits at later growth stages.

In this experiment, TN = 20 + -0.00014CH + 0.00033SPA explained 81% of the variation within TN. Previously, Fahlgren et al. (2015) used the image-based trait height/width (HW) ratio alongside shoot fresh matter to model TN. They found that 64% of the variation in tiller number was explained by their model. Thus, it appears that in this experiment, SPA and CH might provide an increased prediction accuracy of TN.

3.4.3 Future Work

With this project, HTP was used to detect differential responses to various water treatments. While this project illuminated the use of the CEPF in evaluating stress tolerance, however, the limited number of lines limited the scope of this work. Nevertheless, this facility

has greater potential for future projects. To build upon this study, future projects should evaluate more lines in a variety of treatments. In addition, RWC should be measured again to ensure that these treatments are different.

While in this study destructive measurements were only performed at the end of the experiment, additional work should perform sampling at more growth stages. It is possible that image-based phenotypes were not able to predict SDM at previous growth stages. Sampling across multiple growth stages would be beneficial for the verification of image-based results.

3.5 Conclusion

In this experiment, the ability of HTP to predict reference low-throughput phenotypic measurements was assessed using two lines grown in two water treatments. Large prediction accuracies were recorded in estimating reference plant height ($r=1$) and SDM ($r=0.98$). Furthermore, image-based traits allowed for the assessment of temporal responses of each line to the water treatments. With an increased rate of sampling over LTP, this temporal analysis helped to reveal that Chile was less impacted by the drought than Argentina in that three additional days were needed to see an impact of the drought treatment on SPA. Argentina used the water faster as it was larger when the drought was imposed. Without HTP this line difference would have easily been missed. Finally, in analyzing color traits, it appears that saturation, which evaluates the purity of a color, could be an objective estimate of the 'dullness' of a plants color commonly experienced in drought. The results of this study showcase the ability of the high-throughput phenotyping in predicting reference measurements and modeling in plant growth.

CHAPTER 4: CONCLUSION

4.1 Thesis Summary

The overarching goal of this thesis was to find suitable phenotype a diverse wheat germplasm for tolerance to low nitrogen and drought. In chapter two of this thesis seven spring wheat lines were phenotyped to gain a greater understanding of root traits in high- and low-N treatments in hydroponic and potted-plants environments. In the first experiment, lines were evaluated at the seedling stage in a hydroponic solution. Lines with larger root dry matter were associated with a greater shoot nitrogen content in both high- and low-N treatments, but the added root dry matter appeared to have greater importance in the high-N treatment (Figure 6).

However, it was relatively unknown if changes to the root system at the seedling stage would be correlated with on root growth, nitrogen uptake, and yield at physiological maturity. In comparing the lsmeans for each line in each environment between the seedling and maturity studies, lines with larger RDM, at the seedling stage often resulted in smaller RDM at maturity (r=-0.66) (Figure 8). Due to this negative correlation, we conclude that selection of RDM at the seedling stage would not be an appropriate indirect measure for RDM at maturity.

At maturity, in the high-N treatment, grain yield and SNC of large-RDM lines were significantly greater than the small-RDM lines (Figure 7). However, the small-RDM lines in the low-N treatment were only significantly different from the large-RDM lines in terms of SNC (Figure 7). The reduction in SNC was due to N being remobilized to the grains in the small-RDM lines.

Due to the negative correlation of traits between the seedling and maturity growth stages the biomass allocation patterns of select lines were explored at more growth stages. In this study, small-rooted lines (Argentina and Chile) and large-rooted lines (USA and Iran) were analyzed at

five growth stages. From stem elongation to maturity, there was not a significant change in RDM. This indicated that sampling as early as stem elongation might be a suitable, time-saving approach to estimating RDM at maturity. However, it is possible that the roots were limited by the size of the pots in this experiment, so a field experiment must be done to verify these results. Measuring RDM at stem elongation would allow for the selection of lines before flowering which could help to save resources and years in breeding.

While chapter two of this thesis focused solely on low-throughput phenotyping of below-ground traits, chapter three focused on the use of high-throughput phenotyping to model plant growth and detect different line responses to well-water and drought treatments. In assessing Argentina and Chile, SPA was highly correlated to SDM (r=0.98). Furthermore, while LTP would have only been able to measure biomass at the one time per plant, HTP had the ability to separate different treatments with much greater specificity due to its nondestructive nature. Using the temporal, nondestructive nature of the image-based traits, Argentina used the water it was provided quicker, and the effects of the drought were seen in a shorter amount of time. The growth of plants in the drought treatment was significantly less than the well-watered treatment seven days after the imposition of the drought in Argentina. However, the water treatments were not significantly different until ten days after the imposition of the drought in Chile.

Finally, based on the results of this study, saturation was able to significantly differentiate the two lines and the two water treatments. Saturation should be tested further for its use in drought detection and drought tolerance in more lines across more drought treatments.

Technological advancements, like high-throughput phenotyping, should allow breeders to collect traits that are currently not feasible. For example, in this experiment a total of thirty-two plants where imaged on seventeen days. Instead of using HTP had destructive LTP been performed on all seventeen days, the experiment would have required 544 plants (32 plants x 17

sampling days). Additionally, once HTP grows past its infancy of using reference LTP measurements, this technology will allow traits that are currently not used in breeding programs, due to difficulty in sampling, large time investment, or low priority (i.e. root traits), to be further investigated and implemented.

In conclusion, the utilization of new traits and phenotyping strategies will be important to increase wheat yields in the coming decades. With this work, a diverse set of spring wheat germplasm was phenotyped, using a variety of methods, to gain a greater understanding of the genetic potential associated with important traits in addition to the biomass allocation patterns with greater productivity in nitrogen deficient and water stressed treatments.

4.2 Summary of Results

Chapter 2

- Great variation in root traits were observed among the germplasm screened in these experiments with an almost 15-fold difference among the root biomass production between lines.

- At the seedling stage, increased root dry matter was associated with an increase in shoot N content in high- and low-N treatments. However, shoot N content was more responsive to increases in root dry matter in the high-N compared with the low-N treatment.

- Root dry matter in the seedling study was negatively correlated to root dry matter at maturity, which suggested that selection of root dry matter at the seedling stage is not an appropriate indirect measure for root dry matter at maturity.

- Lines with large root dry matter had a significantly greater associated with a significant increase in shoot N content and grain yield in high-N treatments when compared to lines with small root dry matter. In low-N treatments, large root dry matter significantly increased shoot N content compared to lines with small root dry matter but not grain yield or grain N content.

- Root dry matter at stem elongation was not significantly different from root dry matter at maturity.

Chapter 3

- Nondestructive, temporal measurements were useful in monitoring the effects of the drought throughout the experiment.

- Plant height and shoot dry matter were accurately predicted ($r = 1$, $r = 0.95$) with high-throughput phenotyping. Furthermore, convex hull and side-projected area explained 81% of the variation of fertile tiller number.

- Saturation was significantly reduced in the drought treatment as opposed to the well-watered treatment. Lines differences were also significant for saturation.

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