CONSIDER THE ROOTS: EXAMINING BARLEY BELOWGROUND IN THE SEARCH FOR ADAPTATION

by

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ABSTRACT

Barley is important for global food security and sustaining the economically valuable malt industry. Montana is a top barley producer in the United States, but terminal drought stress poses a significant threat to this production by negatively impacting yield and quality. New crop varieties with enhanced environmental adaptation and soil resource extraction would help address this and other issues facing modern agriculture. Stay-green is a trait that extends grain fill in cereals and can stabilize yield and quality under drought. However, this trait and its benefits can be inconsistent across environments and its successful incorporation into breeding strategies would benefit from expanded understanding of stay-green. Roots may play a role in stay-green physiology. Roots provide plants with the water and nutrients needed for growth and are important for crop performance in their own right. Different root system architectures provide adaptation to different environmental stressors, but studying these belowground structures is challenging. Adaptation is also impacted by soil properties and microbial communities. For this dissertation, roots were examined in greenhouse seedling assays and field trials in Montana. Agronomic performance was also assessed for malt barley and spring wheat cultivars varying for stay-green, a biparental barley population segregating for stay-green, and a diverse mapping population of barley lines from different breeding programs. Stay-green cultivars had a greater percentage of deep roots, more prolonged root growth during grain fill, and greater yield. Analysis of the biparental population identified genetic co-segregations of stay-green, root phenotypes, grain quality, and quality stability between environments, further supporting the benefit of stay-green in dry environments and its connection to roots. The diverse mapping population was used to find associations between the relative abundances of bacteria in the rhizosphere and barley genetic loci, that were mostly environment specific. Rhizospheric bacteria may be involved in local adaptation of plants. Finding plant genetic signal for these microbial characteristics supports the idea that it may be possible to breed crops with enhanced ability to recruit beneficial microbiomes if environmental influence and agronomic impact can be understood. Root examination remains a challenging but worthwhile avenue to pursue in crop adaptation research.

INTRODUCTION

Roots tether plants to the earth and are conduits through which water and nutrients flow. These structures are the site of physically intimate and complex interactions between a plant and its environment. Roots move dendritically through soil encountering pores, blockages, resource gradients, and a staggering diversity of micro-, meso-, and macroscopic friends, foes, and neighbors. Roots make "decisions" (Hodge, 2009) in response to these encounters about where to go, what to do, and in the process transform the close dark world around them. Sometimes referred to as the "hidden half" of plants (Manske and Vlek, 2002), these belowground structures tend to be understudied as root researchers (myself included) are fond of stating in their introductions. It is difficult to investigate the structure, function, and environmental interactions of buried living tissue without destroying the very thing one wishes to observe. Yet, overcoming these challenges is worthwhile because of the myriad of critical roles roots play in plant fitness.

Roots present a deep reservoir of potential genetic improvement since they have historically been overlooked in plant breeding (Zeder, 2015). The development of crops and cropping systems since the Green Revolution of the 20th century has primarily focused on aerial traits to maximize yield, often paired with ample application of fertilizers, pesticides, and irrigation (Bishopp and Lynch, 2015). This has led to large production increases (Pinstrup-Andersen and Hazell, 1985), but key crops may be approaching a yield ceiling (Gerber et al., 2024), and such intensive management is not sustainable (Zhang et al., 2015). Resilient crops with enhanced ability to capture their own resources from the soil and use them efficiently could help reduce reliance on irrigation and chemical inputs, and would also be a boon to food production areas where access to water and inputs are limited (Bishopp and Lynch, 2015). As we

look for solutions to agriculture's most pressing problems, it would be unwise to not consider roots.

Roots are one of the common threads that run through the three research projects presented in this dissertation; the other is barley. Barley plays an important role in global food security partly as human food, and more so as feed and forage for livestock (Newton et al., 2011). It is also grown to be converted into malt, which is the primary end use for barley in the United States. Malt is used to brew the beloved beverage, beer among other products, and feeds a nearly four billion dollar industry (USDA NASS). Barley growers receive about 1.5 times the price for malt barley as for other end uses. In order for a barley crop to receive a malt designation, it needs to meet certain quality requirements, particularly high percent plump grain and low percent grain protein (AMBA, 2021).

Montana is a top producer of barley in the United States. Terminal drought stress occurring towards the end of the growing season is a recurring problem facing barley production areas in Montana and nearby regions (NOAA NCEI). This is predicted to worsen in coming years as precipitation patterns shift to concentrate further in the spring (Conant et al., 2018). Warmer, drier summers stress crops, and warmer drier winters reduce the mountain snowpack that supplies irrigation systems. The timing of terminal drought coincides with the grain filling period for barley. This impairs the plant's ability to pack seeds with starch resulting in reduced yield and quality (Gordon et al., 2020; Tarawneh et al., 2020). Malt barley production in Montana would greatly benefit from the development of varieties that are adapted to terminal drought and can acquire and use water resources more efficiently.

There are multiple avenues to explore when searching for plant adaptation enhancements. Stay-green is a trait that has been identified as a potential source of drought tolerance in cereals. Delayed onset or rate of senescence produces the functional stay-green phenotype (Thomas and Ougham, 2014), prolonging retention of photosynthetic leaf tissue and extending the length of the grain fill period. This can also be accompanied by an earlier start to the grain fill period which is beneficial under terminal drought stress patterns (Shavrukov et al., 2017). Stay-green plants often have more stable yield and or quality under drought and heat stress compared to plants without the trait (Christopher et al., 2008; Gous et al., 2013; Borrell et al., 2014; Trachsel et al., 2016; Shirdelmoghanloo et al., 2022). The physiological basis of stay-green is not well understood, especially in barley, and may differ between species. Genetic mapping studies in barley, wheat, and sorghum found that quantitative trait loci (QTLs) for stay-green co-located with QTLs for root traits measured in seedling assays (Mace et al., 2012; Borrell et al., 2014; Gous et al., 2016; Arifuzzaman et al., 2014). Roots systems are responsible for water uptake, so it is reasonable to suspect that these organs may be involved with stay-green and adaptation to drought.

Root system architecture (RSA) refers to the size, shape, and distribution of a root system within the soil. Traits that influence the RSA of cereals include the number of seminal, nodal, and lateral roots, root volume, weight, and surface area, root angle, and the distribution of root tissue between soil depths (Maqbool et al., 2022). Different RSA ideotypes are suited to different environments and conditions. In places with low intermittent rainfall resulting in shallow soil wetting, a widespread shallow root system might be more effective for moisture capture (Palta and Turner, 2019). Shallow roots are also useful for phosphorus uptake as this nutrient tends to

accumulate in upper soil layers (Lynch, 2019). On the other hand, deep root systems provide more nitrogen capture as this nutrient leaches to lower soil layers. Deep roots are also beneficial in cultivation systems that depend on water deposits persisting in the soil from precipitation earlier in the season. However, if deep rooting is accompanied by the rapid growth of a large plant above and belowground, water stores may become depleted prior to reproductive stages thus exacerbating terminal drought for the plant (Palta et al., 2011). Determining what root system ideotypes are beneficial for crop performance under different conditions requires examination of root phenotypes in relation to agronomics in a variety of realistic cultivation scenarios.

Creative and hardworking plant scientists have developed several methods over the years for characterizing root phenotypes. Extensive root research was done in the early twentieth century by J.E. Weaver (Weaver et al., 1922) and other researchers who carefully dug pits sometimes ten feet into the soil to expose complete root system profiles, then painstakingly drew the structures onto grids by hand. Digging into the ground is still one of the most effective methods we have for examining root systems and the space they occupy (Carter et al., 2019), but this is of course labor intensive. Other techniques such as shovelomics (Trachsel et al., 2011) or the pasta strainer method (El Hassouni et al., 2018), expose only the top portion of root systems then extrapolate predictions about the lower portions. Roots emerging from the base of the plant are roughly quantified and their angle is used as a proxy for rooting depth under the assumption that roots with a steeper angle will grow more directly down to deeper soil, while shallow angle roots will spread more laterally through upper layers. The soil core break method assesses rooting depth more directly (Wasson et al., 2014). All of these methods are destructive and thus

do not allow for repeated observations of the same root system as it develops. In 1937, inspired by rhizotron work where glass plates were affixed over the walls of soil pits to enable root observation, G.H. Bates developed a tool termed a minirhizotron (Bates, 1937). This was a three-foot-long glass cylinder with a grid etched into its surface, buried in a hole bored into the soil. Roots of surrounding plants would come into contact with the surface of the minirhizotron. A mirror and small lamp attached to a rod were lowered into the cylinder and these roots were then drawn onto a corresponding grid on paper by the researcher. Since this time, modern tools and materials have been developed for easier installation of more durable minirhizotrons, and cylindrical rotary scanners allow digital image capture of the roots and soil surrounding the tubes (Postic et al., 2019). These devices allow for repeated imaging of the same plant roots over the course of a growing season and quantification of roots at different depths. Still, the minirhizotron and other methods of root observation in the field are limited in their utility due to time and labor requirements.

Controlled environment assays often focusing on earlier developmental stages can be a higher throughput way to study roots. Methods have been developed in which plants are grown in transparent substrates (Maqbool et al., 2022) or clear pots (Robinson et al., 2016) such that roots can be directly observed as they grow. There are also methods that involve growing plants in devices or substrates from which roots can be easily removed for observation such as pouches or rolls of germination paper (Watt et al., 2013), or columns or pots filled with sand or calcined clay (Su et al., 2008). These techniques can facilitate the assessment of many genotypes. This is useful for genetic mapping which requires large plant populations such that there is sufficient diversity to detect association patterns between genotypes and phenotypes. Genetic mapping

results can be useful for marker assisted selection in plant breeding and to point towards genes and mechanisms behind the studied traits. Such understanding of young ex situ root phenotypes is useful for breeding if it can be reliably connected to field traits that are meaningful for crop performance. The challenge with this is that roots are highly plastic in their response to a plethora of environmental factors that they encounter in the soil, resulting in large impacts of genetic by environment interaction for root phenotypes (Schneider and Lynch, 2020). But, if interactions can be understood, such plasticity might be a resource to leverage for enhancing crop resilience and adaptation through breeding.

Soil properties of the area where a plant species evolved or a variety was developed have been said to influence the evolution or selection of that species or variety (Alegria et al., 2020, Rúa et al., 2016), which could impact its adaptation to different environmental conditions. A gram of soil may contain billions of microorganisms representing thousands of species (Torsvik and Øvreås, 2002). These microbial communities respond to climatic and edaphic properties of their habitat (Young and Ritz, 2005) and are an important component to the influence of soil on plants. As roots grow through soil they deposit a wide variety of compounds via secretion, excretion, and diffusion (Walk et al., 2003), accounting for 5% to 21% of the carbon fixed by photosynthesis in the plant aboveground (Marschner, 1995). This prompts the assembly of a dense, active, and distinct microbial community in this resource rich area immediately surrounding and impacted by the roots known as the rhizosphere. Here bacteria, fungi, archaea, protozoa, and microscopic animals may act as pathogens to the plant, simply coexist with their host, or even provide benefits to the plant (Bardgett and Van Der Putten, 2014). Rhizospheric microbes have been known to promote plant growth (Babalola et al., 2021), enhance plant

nutrient acquisition (Pii et al., 2015; Rosenblueth et al., 2018), protect against plant pathogens (Mendes et al., 2011), and assist with plant resilience or resistance to abiotic stress (Poudel et al., 2021). As such, intentional manipulation of this microbiome has been identified as another possible option to boost crop productivity without relying on chemical inputs (Gopal and Gupta, 2016). It has been demonstrated that differences between plant species and even genotypes within species can impact rhizosphere microbial community characteristics (Berendsen et al., 2012; Walters et al., 2018). Thus, it may be possible to select for the recruitment of beneficial microbiomes in plant breeding. This could be through microbe-mediated local adaptation to specific environments or microbe-mediated adaptive plasticity, which may be useful across a range of environments (Petipas et al., 2021). Understanding the mechanisms behind the plant, microbe, and environment interactions will be important for leveraging these adaptations appropriately in crop variety development.

This dissertation is comprised of three distinct research projects investigating root systems and environmental adaptation of malt barley. The first utilizes minirhizotrons and greenhouse assays to measure root traits in and out of the field, and draws correlations between these and the agronomic performance of stay-green and non-stay-green barley and spring wheat cultivars. The second conducts genetic linkage mapping of seedling root traits measured in one of the same greenhouse assays, and grain fill duration and grain quality traits measured at two field locations on a biparental barley population segregating for stay-green. The final project performs genome wide association analysis of the relative abundances of rhizospheric bacterial taxa and agronomic traits on a diverse barley population in two field locations. The projects add

to the body of knowledge on these topics and lay the groundwork for future investigations into remaining questions about the belowground world of barley.

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CHAPTER TWO

RELATIONSHIPS BETWEEN ROOTS, THE STAY-GREEN PHENOTYPE, AND AGRONOMIC PERFORMANCE IN BARLEY AND WHEAT GROWN IN SEMI-ARID CONDITIONS

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Core Ideas

1. In-field minirhizotrons revealed a connection between the stay-green phenotype and deep roots in barley.
2. Yield was most strongly positively correlated with deep root length in barley and total root length in wheat.
3. There were positive correlations with percent deep root length for both grain yield and protein in wheat.
4. Seminal root angle and deep rooting were negatively correlated in wheat but positively correlated in barley.

Abstract

Stay-green is a phenotype that crop breeders could use to improve drought adaptation. It increases the duration of grain fill in several species including barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*), maintaining yield in semi-arid conditions. Evidence from controlled environment experiments suggests a connection between stay-green and root systems. These belowground structures are understudied and thus represent opportunity for crop improvement if relationships to agronomics can be understood. Minirhizotrons facilitate study of these relationships by allowing repeated non-destructive root measurement in field conditions. However, this is time consuming and proxies would be useful for increasing throughput capacity of root research. Here we present results from field trials with minirhizotrons in a semi-arid environment, as well as greenhouse seedling assays conducted on stay-green and non-stay-green barley and wheat lines. In barley, stay-green and greater yield were primarily associated with

greater deep root length and delayed root senescence, while in wheat, yield was most strongly correlated with total root length, and root system differences for stay-green were not as apparent. We speculate that the physiology of stay-green is different between these two species, and that barley may employ a more efficient root system to withstand drought while wheat relies on a larger one. Several seedling traits related consistently to field root traits, but correlation directions were often opposite between barley and wheat. The connections between traits presented here could be useful for breeders seeking to improve crop adaptation to drought, but more genotypes and environments will need to be tested.

Introduction

Maintaining optimal crop performance in drought conditions is a major objective shared by researchers and breeders serving semi-arid areas around the world. Stay-green is a phenotype that has been targeted to provide drought adaptation and has been observed in many crop species including sorghum (*Sorghum bicolor* L. Moench) (Borrell et al., 2014), sunflower (*Helianthus annuus* L.) (Lisanti et al., 2013), rice (*Oryza sativa* L.) (Fu et al., 2011), maize (*Zea mays* L.) (Antonietta et al., 2016), wheat (*Triticum aestivum* L.) (Christopher et al., 2008), and barley (*Hordeum vulgare* L.) (Gous et al., 2016). Stay-green plants exhibit delayed senescence, retaining green leaf area until later in the season, increasing the duration of grain fill even in dry environments (Gregersen et al., 2013). A longer grain fill period extends photosynthesis and starch accumulation in barley and wheat, resulting in maintained yield and quality under drought conditions (Gous et al., 2013; Seiler et al., 2014; Christopher et al., 2016).

Though the physiology of the polygenic stay-green phenotype is not fully understood, there is evidence from studies in multiple species connecting aboveground stay-green

phenotypes with root traits that have been measured *ex situ*. In sorghum, multiple quantitative trait loci (QTL) for stay-green have co-located with QTL for nodal root angle (Mace et al., 2012; Borrell et al., 2014). Similarly in barley, Gous et al., (2016) mapped QTL for stay-green traits located near QTL associated with root length, root dry weight, and root to shoot ratio (Arifuzzaman et al., 2014). Further, in wheat, Christopher et al., (2018) found stay-green QTL that co-located with QTL identified for seminal root angle and seedling root number in a previous study (Christopher et al., 2013). Since mapping indicates genetic loci pleiotropically affecting root and aboveground stay-green traits in the greenhouse, and *in situ* examinations of the root systems of stay-green plants are lacking, field experiments are warranted.

Efforts to identify root phenotypes in the field associated with improved crop yield and drought adaptation have focused on deep rooting. Various measurements of root length deep in the soil profile in wheat have been connected to higher yield (Hayashi et al., 2013; Corneo et al., 2017; Uddin et al., 2018). However, results concerning relationships between root traits and associated aboveground characteristics are frequently inconsistent across locations, seasons, and management practices. Environment has a much greater effect on deep rooting than genotype (Hodgkinson et al 2017, Severini 2020). In their 2019 review, "Crop root system traits cannot be seen as a silver bullet delivering drought resistance," Palta and Turner describe how root phenotypes can be beneficial in some scenarios but provide no advantage in others. For example, wheat grain yield was strongly positively correlated to deep root length density in rainfed conditions but not under irrigation (Postic et al., 2019). Becker et al., (2016) also observed rooting depth plasticity in the greenhouse in response to drought stress. Ultimately there is likely no single root system trait or even set of traits that will confer drought adaptation across all

environments. The best mechanisms of crop adaptation in semi-arid environments are dependent upon the amount and timing of precipitation, temperature, soil type and depth, and other crop, environment, and management factors (Bodner et al., 2015). However, even though deep rooting does not consistently increase yield, it does not appear to negatively impact yield (Severini, 2020) and therefore remains a promising breeding target. More field studies examining roots *in situ* in a variety of locations and conditions are needed to understand the impact of deep roots on yield. Indeed, there is a lack of knowledge about root systems compared to the aboveground portions of plants, owing to the difficulty in observing them.

The high degree of root plasticity in response to environment also makes it difficult to correlate *ex situ* with *in situ* root traits. Nevertheless, the challenges involved in examining roots in the field makes *ex situ* root proxies an attractive research goal. Others have in fact uncovered correlations between root traits measured in controlled conditions at early growth stages and root traits measured in the field. Associations were found between seminal root length and weight measured in a hydroponic system, and yield in barley field trials (Bertholdsson et al., 2009). In wheat, Oyanagi et al., (1994) found that seminal root angle in pots was correlated with root depth index in the field. However, these relationships are inconsistent across environments and conditions. Watt et al., (2013) found that wheat seminal root length correlated well with maximum rooting depth in the field for vegetative but not reproductive stages. Rich et al., (2020) observed inconsistency in correlations between multiple controlled environment root screens and measurements of mature roots in the field in wheat. Still, heritability has been found for root traits in and out of the field (Atta et al., 2013; and Hohn and Bektas, 2020 respectively) so finding a proxy for roots could be possible with an improved understanding of genotype by

environment interactions. Thus, there is a need to compare more potential proxies to more field trials in different locations. Functional proxies should ideally have definite and reliable relationships to the traits they are proxies for. Such tools would greatly assist breeders in selecting for beneficial root phenotypes.

Here we present results from a field experiment in semi-arid northern Montana as well as two greenhouse experiments that examined the roots of stay-green and non-stay-green barley and wheat lines. The data are also used to investigate correlations between above- and belowground traits in this environment, as well as connections between roots in the field and seedling root traits in the greenhouse. Specifically, we ask: 1) Do these stay-green barley and wheat lines exhibit increased root length deeper in the soil profile, delayed root senescence, and or greater total root length? 2) Is greater total root length, greater deep root length, and or a change in root length during grain filling associated with enhanced crop yield and grain quality? 3) Does seminal root angle, or any root trait measured in our greenhouse assays, show potential as a proxy for deep root length or other root traits measured in our field study? 4) What differences might there be between barley and wheat for the root system characteristics investigated in this study?

Materials and Methods

Plant Material

Barley and wheat varieties and breeding lines that differ for the stay-green phenotype were used in this study (Table 2.1). For barley, the stay-green group of lines consisted of two breeding lines from North Dakota State University (NDSU), ND19119 and ND24260, known to exhibit a functional stay-green phenotype (Gous et al., 2013), as well as three Montana State

University (MSU) breeding lines that each have one of the NDSU lines as a parent and have exhibited stay-green characteristics in the breeding program. The other parents to the three MSU stay-green lines do not exhibit stay-green characteristics and thus formed the non-stay-green group along with a fourth non-stay-green line. This fourth line (MT124118) was added in 2019 so that it could be evaluated for its use in a genetic study of this trait (Williams et al., in preparation), and MT16M00803 was removed to accommodate this addition.

For wheat, only four lines were grown in 2018 due to resource constraints, but this was expanded to eight lines for 2019 and 2020. The stay-green wheat group consisted of Reeder, an NDSU line known to exhibit a functional stay-green phenotype (Naruoka et al., 2012), and two MSU lines, Dagmar and Vida, that have Reeder in their pedigrees and have shown stay-green characteristics. The non-stay-green wheat group consisted of lines that are commonly grown in Montana and or used in the MSU breeding program and do not exhibit delayed senescence.

Table 2.1. Plant Materials.

Species	Line	Stay-Green	Pedigree	Seasons Grown
Barley	Craft	non-SG	BETZES/DOMEN/BARONESE	2018, 2019, 2020
Barley	MT090190	non-SG	MT910189//*3Lk644/Eslick	2018, 2019, 2020
Barley	MT100120	non-SG	LK644/ESLICK//HOCKETT///HOCKETT	2018, 2019, 2020
Barley	MT124118	non-SG	HOCKETT/MT070174	2019, 2020
Barley	MT16M00803	SG	Craft/ND19119	2018
Barley	MT16M00503	SG	MT090190/ND19119	2018, 2019, 2020
Barley	MT16M01404	SG	MT100120/ND24260	2018, 2019, 2020
Barley	ND19119	SG	ND15403.3/ND15368//ND16453	2018, 2019, 2020
Barley	ND24260	SG	ND19869-1//ND17274/ND19119	2018, 2019, 2020
Wheat	Conan	non-SG	WESTBRED-RAMBO/WESTBRED-906-R	2018, 2019, 2020
Wheat	McNeal	non-SG	PI-125000/CENTANA//PK-176/FRONTEIRA(RS-6880)/3/GLENMAN	2019, 2020
Wheat	MTHW0202	non-SG	ID377s/MTHW9701	2018, 2019, 2020
Wheat	Spring Yellowstone	non-SG	CHOTEAU/6*YELLOWSTONE	2019, 2020
Wheat	Thatcher	non-SG	MARQUIS/(TR.DR)IUMILLO//(HN-3001)MARQUIS/KANRED	2019, 2020
Wheat	Dagmar[a]	SG	MT1133/MT1148	2019, 2020
Wheat	Reeder	SG	IAS-20*4/H-567.71//STOA/3/ND-674	2018, 2019, 2020
Wheat	Vida	SG	SCHOLAR/REEDER	2018, 2019, 2020

[a] A more detailed pedigree for Dagmar showing its relationship to Reeder can be found in Heo et al., 2020.

Experiment 1: Field Trials

Site Description Field trials were conducted in 2018, 2019, and 2020 at the Montana State University Northern Agricultural Research Center (NARC) (48.50°N, -109.80°W), 773 m above sea level, near the city of Havre, Montana, USA in the semi-arid Northern Great Plains region. The soils at this location are a mix of Joplin, Scobey, and Telstad clay loams officially classified by the USDA as well drained, very deep, fine to fine-loamy Aridic Arguistolls with slow to moderately slow permeability (USDA, 1998). The research field utilized for these trials

has been under no-till management for over twenty years, and each trial followed a fallow season to maximize stored soil moisture. Daily precipitation and temperature data for each growing season and historic averages were obtained from NOAA climatological summaries collected at NARC/Fort Assiniboine (National Weather Service, 2021) (Table 2.2). Below average growing season precipitation was received in 2018 and 2020, and below average precipitation during the grain fill period occurred in each season. Temperature, over the course of the study, did not differ greatly from one growing season to the next or from the historic averages.

Table 2.2. Growing season and historic weather conditions.

Weather Time Periods	**Trial Season**			
	2018	**2019**	**2020**	**Average (1916 – 2020)**
Precipitation			mm	
Planting to Flowering	84.3	134.1	112.0	111.5
Flowering to Harvest	13.2	27.2	15.2	65.8
Planting to Harvest (total)	97.5	161.3	127.3	141.2
Average Temperature			°C	
May	14.9	9.7	11.9	12.2
June	17.4	16.3	17.3	16.6
July	20.8	19.7	20.0	20.7
August	19.6	19.6	21.7	19.7

Experimental Design and Management A randomized complete block design with three blocks was used such that each line appeared in each block and was thus replicated three times in each season (with the exception of a few lines that were not planted every year, see Table 2.1 and Plant Materials). Plants were seeded during the last week of April each season, in five row plots with 0.3 m row spacing, that were 6.7 m long and trimmed to 5.2 m after emergence. Seed was treated before planting with CruiserMaxx Vibrance Cereals (Syngenta, Greensboro, NC, USA) seed treatment. The seeding rate was 10 g m^{-1} for barley and 7 g m^{-1} for wheat (approximately 60 seeds per m). Soil tests (Agvise Laboratories, Northwood, ND, USA) were conducted before

planting in 2019 and 2020 to give an idea of fertility (Table 2.3). In 2018, the soil tests were conducted the previous fall and an additional 32 g m^{-1} of 100-20-10-10, N-P-K-S, fertilizer was put down at the time of seeding. No fertilizer was added in 2019 and 2020. All field trials were rainfed and were maintained weed free.

Table 2.3. Fertility data from soil tests.

Trial Season	Depth	Nitrate	Phosphorus - Olsen	Potassium	pH	Organic Matter
	——cm——	——————————ppm——————————				———%———
2018	0 - 15	8.5	5	193	8.2	1.5
2018	15 - 61	14.0				
2019	0 - 15	4.0	15	218	7.7	1.2
2019	15 - 61	8.5			8.2	
2020	0 - 15	2.5	16	184	8.0	0.9
2020	15 - 61	12.0			8.3	

Aboveground Measurements Heading (Zadoks 59) and maturity (Zadoks 89) dates were assigned to each plot in 2020 according to the Zadoks decimal code for the growth stages of cereals (Zadoks et al., 1974). In 2019, Zadoks stage was assessed for each plot on three days around the time of heading as well as three days around the time of maturity, then plot heading and maturity dates were estimated based on these Zadoks scores. Planting date was subtracted from heading date and from maturity date to determine days to heading and maturity respectively. Heading date was subtracted from maturity date to calculate duration of grain fill. Plot heading and maturity dates were not recorded in 2018 but were monitored to inform the timing of data collection. Aboveground biomass samples were collected from each plot at the beginning of flowering (Zadoks 61), and after maturity (Zadoks 92), by cutting sections of plants at their base from two of the three central rows for an area of 0.27 m^2. At flowering, leaf area was measured using a leaf area meter (Li-Cor 3000, Lincoln, NE, USA), and the entire sample

was dried and weighed. At harvest, samples were already dry upon collection and were weighed. Seed heads were counted to determine productive tiller number, then threshed using a custom-built small capacity grain thresher. The grain was weighed to determine yield and harvest index (grain weight / total biomass). This grain was also used for near-infrared spectroscopy (NIR) measurement of grain protein content (Infratec NOVA, FOSS, Hillerod, Denmark) in 2019 and 2020.

Belowground Measurements The CI-600 In-Situ Root Imager minirhizotron system from CID Bio-Science (Camas, WA, USA) was used to collect root measurements in the field. Transparent acrylic root tubes, measuring 105 cm in length and 7 cm in diameter, were installed two days after planting in 2018 and 2020, and eight days after planting in 2019. A single tube was placed in every plot. Some tubes were initially placed in the wrong position in 2019, and needed to be reinstalled 14 days after planting, though this did not impact root measurements. Tubes were installed by first using a Giddings hydraulic probe to take soil cores with a diameter slightly larger than that of the tubes, at a 45º angle to the soil surface, directly in the middle row of each plot, then inserting the tubes into the resulting holes. If a tube was loose in a hole, a slurry of soil and water was poured into the gap around the tube to improve contact with the soil. Figure 2.1C in the publication by Postic et al., (2019) provides a clear diagram of this type of minirhizotron set-up.

Belowground images (Figure 2.1A) were captured with the CI-600 cylindrical rotary scanner at the same flowering and harvest collection time points that were used for biomass sampling (Zadoks 61 and 92). The scanner was lowered sequentially to capture 360º scans at four depths (~0-15, 15-30, 30-45 and 45-60 cm) below the soil surface. Bourgault et al. utilized

this same method in a previous study (2021) and estimated a 2-5 cm difference in actual imaging depth between tubes. CI-690 RootSnap! version 1.3.2.25, the companion program to the scanner, was used to trace over roots in the images (Figure 2.1B) to determine total root length at each depth. We acknowledge that the maximum rooting depth of spring wheat in field conditions usually extends beyond 60 cm (Thorup-Kristensen et al., (2009) found an average depth of 1.1 m for field grown spring wheat), and we wish to emphasize that our root length measurements are not an indication of maximum rooting depth achieved by the plants. However, for the sake of this study we refer to the top two scanning depths together (0-15 and 15-30 cm) as shallow, and the bottom two scanning depths together (30-45 and 45-60 cm) as deep. The percentage of the total root length that was deep was calculated as:

(Equation 1)

$$Percent\ Deep\ Root\ Length\ = \frac{root\ length\ 30\ to\ 60\ cm}{root\ length\ 0\ to\ 60\ cm}\ \text{x}\ 100$$

The percent difference in total root length between flowering and harvest was calculated as:

(Equation 2)

$$Percent\ Change\ in\ Root\ Length$$

$$= \frac{(harvest\ root\ length - flowering\ root\ length)}{flowering\ root\ length}\ \text{x}\ 100$$

Thus, a positive value indicates an increase in root length from flowering to harvest and a negative value indicates a decrease in root length.

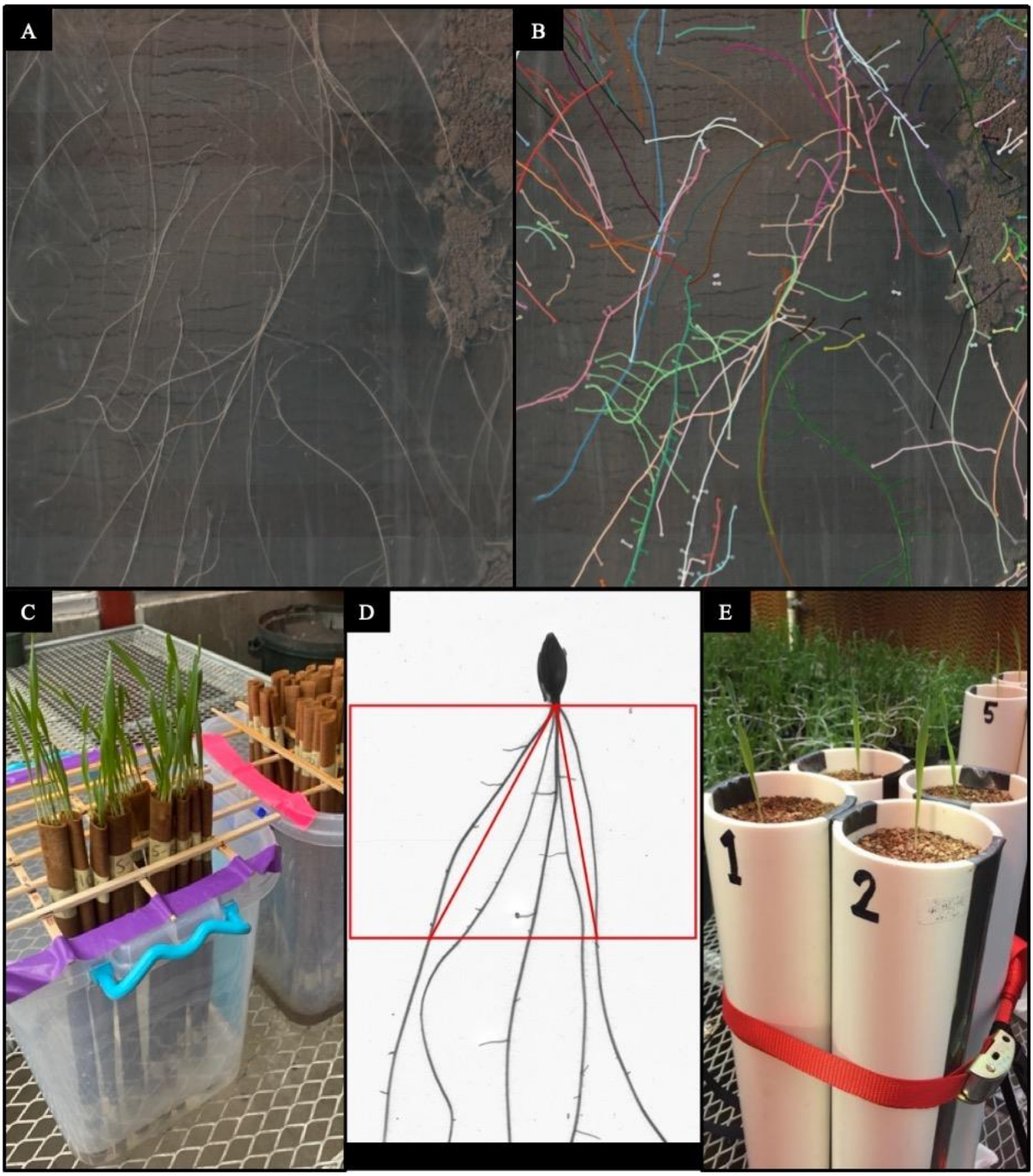

Figure 2.1. Images of root phenotyping methods. A) Minirhizotron scanned image of roots. B) Minirhizotron scanned image with roots traced in RootSnap! program. C) Set-up of the one-leaf seedling or root roll-up assay. D) Seminal root angle measurement in ImageJ on a scanned image from the root roll-up assay. E) Set-up of the four-leaf seedling or PVC pot assay.

Experiment 2: One-Leaf "Root Roll-Up" Seedling Assay

To observe root traits at the one-leaf stage (Zadoks 11) a method adapted from Watt et al. (2013) was used, hereafter referred to as the root roll-up assay. This was performed in the Montana State University Plant Growth Center greenhouse in Bozeman, Montana (45.67°N, -111.05°W). Temperature was maintained at 21°C during the day and 18°C at night. Artificial light supplemented natural light for a sixteen-hour photoperiod. A randomized complete block design was used in which all varieties and breeding lines listed in Table 2.1 appeared once in each of three blocks grown over time such that the experiment was replicated three times independently.

Seedling rolls were prepared using 25.4 cm by 38.1 cm sheets of germination paper (Anchor Paper Co., St. Paul, MN, USA) oriented with the shorter edge as the top. Seeds were weighed, then placed in a line parallel to and 1cm from the top of the paper with the embryos pointing down. The paper was then tightly rolled and secured with two small pieces of masking tape on the edge. Each roll contained four seeds of the same genotype with even spacing between each seed and the edges of the paper. These rolls were thoroughly wetted with water, then placed upright and close together in a 24x24x34 cm bucket with approximately 4 cm of water at the bottom. The rolls were held in place by a grid of chopsticks placed on top of the bucket and were kept moist by maintaining the water level at the bottom and misting the tops of the rolls daily. Set-up for the root roll-up assay is depicted in Figure 2.1C.

As seedlings began to grow, shoots emerged from the tops of the rolls as seminal roots grew down in between the layers of paper. The term seminal refers to roots that originate in the embryo within the seed (Manske and Vlek, 2002), and in this study we do not distinguish the primary seminal root (radicle) from other seminal roots. Growth was allowed to continue until

two out of the four seedlings in a roll (or one if there was a seed that failed to germinate) reached the point where the first leaf was fully emerged, and a second leaf tip was just barely visible. That roll was then removed from the bucket and either measured right away or stored in a plastic bag at 4°C for up to two days before data collection.

Shoots were removed from seeds and all shoots from one roll were placed in the same paper packet to be dried and weighed. Seminal roots, still attached to the seeds, were gently peeled off the paper. The number of seminal roots was counted as was the number of initiated lateral roots. The longest seminal root was measured with a ruler. Seedling root systems were placed one at a time in a transparent tray of shallow water on a flatbed scanner (Expression 12000XL Graphic Arts, Epson America Inc., Long Beach, CA, USA) and scanned at 400 dpi to produce 8-bit grayscale images. Before scanning, roots were gently separated to avoid crossover in the images, then left to settle in their most natural position to allow for subsequent measurement of the seminal root angle. After scanning, roots were removed from the seeds and all roots from one roll were placed in the same paper packet to be dried and weighed. The scanned images were analyzed using WinRHIZO software (Arsenault, 1995) to determine total root length and volume. The program ImageJ (Rasband, 1997) was used to measure the angle between the two outermost seminal roots at 3 cm below the seed (Figure 2.1D). The four seeds within one roll were considered pseudoreplications, and the measurements for each seedling were averaged to give the value for the roll.

Experiment 3: Four-Leaf "PVC Pot" Seedling Assay

A four-leaf seedling assay (Zadoks 14) adapted from a method used by Ali et al. (2016) and hereafter referred to as the PVC pot assay, was performed using the same growing conditions

and experimental design as Experiment 2. However, this experiment was replicated five times independently rather than three to account for a larger margin of error with this method.

Plants were grown in sections of PVC pipe that were 91 cm long, 10 cm in diameter, cut in half longitudinally, then taped back together. Porous rubber shelf liner material (Gorilla Grip, Westport, CT, USA) was used to create bottoms for the pots by taping it onto one end of the pipe. The pots were placed upright on a bench in the greenhouse, strapped to each other and the bench to secure them in place, and filled with Turface (Profile Products LLC, Buffalo Grove, IL, USA), a substrate made from baked calcined clay that is mechanically broken into approximately 4 mm diameter pieces. Set-up for the PVC pot assay is depicted in Figure 2.1E.

Two seeds of a genotype were weighed and planted in one pot in case one seed failed to germinate. Seeds were planted with the embryo pointing down, approximately 2.5 cm below the surface level of the Turface, which was approximately 5 cm below the top of the PVC pots. The seedling that emerged second was removed. Pots were watered daily and fertilized once per week with 20-20-20 general purpose fertilizer (Peter's Professional, Everris Na Inc., Geldermalsen, The Netherlands) at the manufacturers' recommended rate. Plants were allowed to grow until the first appearance of a fifth leaf tip with the fourth leaf fully emerged (Zadoks 14). The entire shoot of the plant was then carefully cut away from the seed and placed into a paper packet to be dried and weighed. The PVC pot was then laid on its side in a large bin and the tape was removed from the two pipe halves so they could be pulled apart. The root system was removed and dipped in water to wash off any Turface sticking to the roots. Turface was rinsed, autoclaved, and reused.

Root systems were either scanned right away or stored in jars of 50% ethanol at 4°C for up to two days. Roots were scanned in the same manner as the root roll-up seedlings, after spreading them out as much as possible in the water tray to avoid overlap. Then root systems were removed from the seed and placed into packets to be dried and weighed. Images were analyzed using WinRHIZO to measure total root length and volume.

Statistical Analysis

Statistical tests were performed in R (R Core Team, 2021). To compare stay-green and non-stay-green groups of lines, analysis of variance (ANOVA) was run for all traits measured in the greenhouse, and for all traits measured in the field by single season and across seasons. For greenhouse experiments and for single field seasons, a model was assessed for each trait with fixed effects stay-green (whether a line was designated as stay-green or non, Table 2.1), block, and their interaction as the only terms. For all field seasons together, each trait was analyzed using a model that included the fixed effects of stay-green, block, season, stay-green by block, and stay-green by season. In all models, if the interaction was associated with a p-value below 0.15, the interaction was included in the final model. Interactions were rare in the overall data set. Effects used in each model and their significance level in ANOVA ("car" package, Fox and Weisberg, 2019) are listed in Table A2.1.

To examine association between aboveground traits and root traits in the field, Spearman correlation tests were performed on values measured in the same individual plots using the “Hmisc” package (Frank et al., 2020). To examine association between root traits in the field and root traits in the greenhouse assays, Spearman correlation tests were performed using genotype

means of the traits, also with the "Hmisc" package. Correlations with greenhouse data were not done with the 2018 field season for wheat owing to the reduced number of lines grown that year.

Results

Comparison of Stay-Green and Non-Stay-Green Lines

Comparisons of agronomic traits of stay-green versus non-stay-green barley and wheat are presented in Table 2.4. Stay-green lines did exhibit longer duration of grain fill period than non-stay-green lines barley and in wheat, although this was not significant for wheat in 2019. The stay-green phenotype, indicated by longer duration of grain fill, was contributed to by delayed maturity and even more so by earlier heading. Stay-green did not have a significant impact on days to maturity wheat. There was also a pattern that was consistent though not always significant, of stay-green lines in both species having higher grain yield.

In barley, stay-green lines had less leaf area (though not in 2020), higher harvest index (again not in 2020), and tended to have higher productive tiller numbers than non-stay-green lines. There were no clear differences between stay-green and non-stay-green barley for aboveground biomass at flowering or percent grain protein.

In wheat, stay-green lines had more leaf area, higher productive tiller number, and greater percent grain protein than non-stay-green lines. There were no clear differences between stay-green and non-stay-green wheat for aboveground biomass at flowering, or harvest index.

Table 2.4. Agronomic trait means of non-stay-green (non-SG) and stay-green (SG) groups, with p-values from ANOVA for the fixed effect of stay-green for each species in single growing seasons and all seasons combined. Full ANOVA results are presented in Table A2.1. Sample sizes are listed in gray.

. $p < 0.1$ * $p < 0.5$ ** $p < 0.01$ *** $p < 0.001$

Agronomic Trait and Season	Barley					Wheat				
	non-SG		SG			non-SG		SG		
	Mean	n	Mean	n		Mean	n	Mean	n	
Aboveground Biomass at Flowering (g m^{-2})										
2018	270	9	270	15	.	181	6	167	6	
2019	541	12	563	11		530	15	537	8	
2020	407	12	407	12		359	15	322	9	
All	418	33	400	38		400	36	356	23	
Leaf Area at Flowering (cm^2)										
2018	1368	9	1181	15	***	976	6	1462	6	*
2019	1082	12	851	11	***	948	15	1018	8	
2020	842	12	891	12		836	15	887	9	
All	1073	33	994	38	*	906	36	1083	23	*
Productive Tiller Number										
2018	113	9	131	15	.	83	6	120	6	***
2019	188	12	219	11	.	151	15	176	8	.
2020	110	12	117	12		78	15	103	9	**
All	116	33	131	38		109	36	132	23	.
Days To Heading										
2019	63	12	60	11	****	58	15	57	8	
2020	64	12	63	12	.	61	15	58	9	.
All	63	24	61	23	***	59	30	58	17	*
Days To Maturity										
2019	90	12	92	11	*	92	15	92	8	
2020	93	12	94	12		93	15	94	9	
All	91	24	93	23	*	93	30	93	17	
Duration of Grain Fill (Days)										
2019	27	12	32	11	***	34	15	35	8	
2020	29	12	31	12	**	32	15	36	9	**
All	28	24	31	23	***	33	30	35	17	**
Harvest Index										
2018	0.42	9	0.44	14	.	0.42	6	0.44	6	.
2019	0.44	12	0.50	11	**	0.43	15	0.44	8	
2020	0.58	12	0.56	12	.	0.56	15	0.56	9	
All	0.49	33	0.50	37	*	0.49	36	0.49	23	
Grain Yield (g m^{-2})										
2018	232	9	233	15		197	6	228	6	.
2019	331	12	384	11	*	300	15	305	8	
2020	396	12	415	11		309	15	342	9	**
All	328	33	332	37	.	286	36	299	23	
Percent Grain Protein										
2019	12.2	12	12.1	11		14.1	15	14.7	8	
2020	10.4	12	10.9	12	.	12.6	15	13.4	9	.
All	11.3	24	11.5	23		13.4	30	14.0	17	*

In barley, the field root data suggested two patterns of differences between groups of lines that were consistent across all seasons and measurement time points, although stay-green did not always have a statistically significant effect in these ANOVA. Stay-green lines had a higher percentage of deep roots than non-stay-green lines at flowering (2019: $p<0.05$), and harvest (2018: $p<0.1$, 2019: $p<0.05$. combined seasons: $p<0.01$) (Figure 2.2C). Stay-green lines appeared to have less total root length than non-stay-green lines, but stay-green did not have a significant effect in these ANOVA (Figure 2.2A). With the exception of the 2018 season, stay-green barley exhibited an increase or lesser decrease in shallow and deep root length than non-stay-green, with a significant effect of stay-green in ANOVA for shallow root length in 2019, 2020, and combined seasons, and for deep root length in 2019 (Figure 2.2E). Corresponding positive correlations were observed between duration of grain fill (an indicator of the stay-green phenotype) and percent deep root length and percent change in root length as well as weakly negative correlations between duration of grain fill and total root length for all barley lines together (Table A2.3).

ANOVA revealed a significant effect of stay-green on multiple seedling traits in the greenhouse experiments (Table A2.4). Stay-green barley lines had a shorter longest root, wider seminal root angle, higher seminal root number, and lower lateral root number in the root roll-up assay, and less total root length in the PVC pot assay.

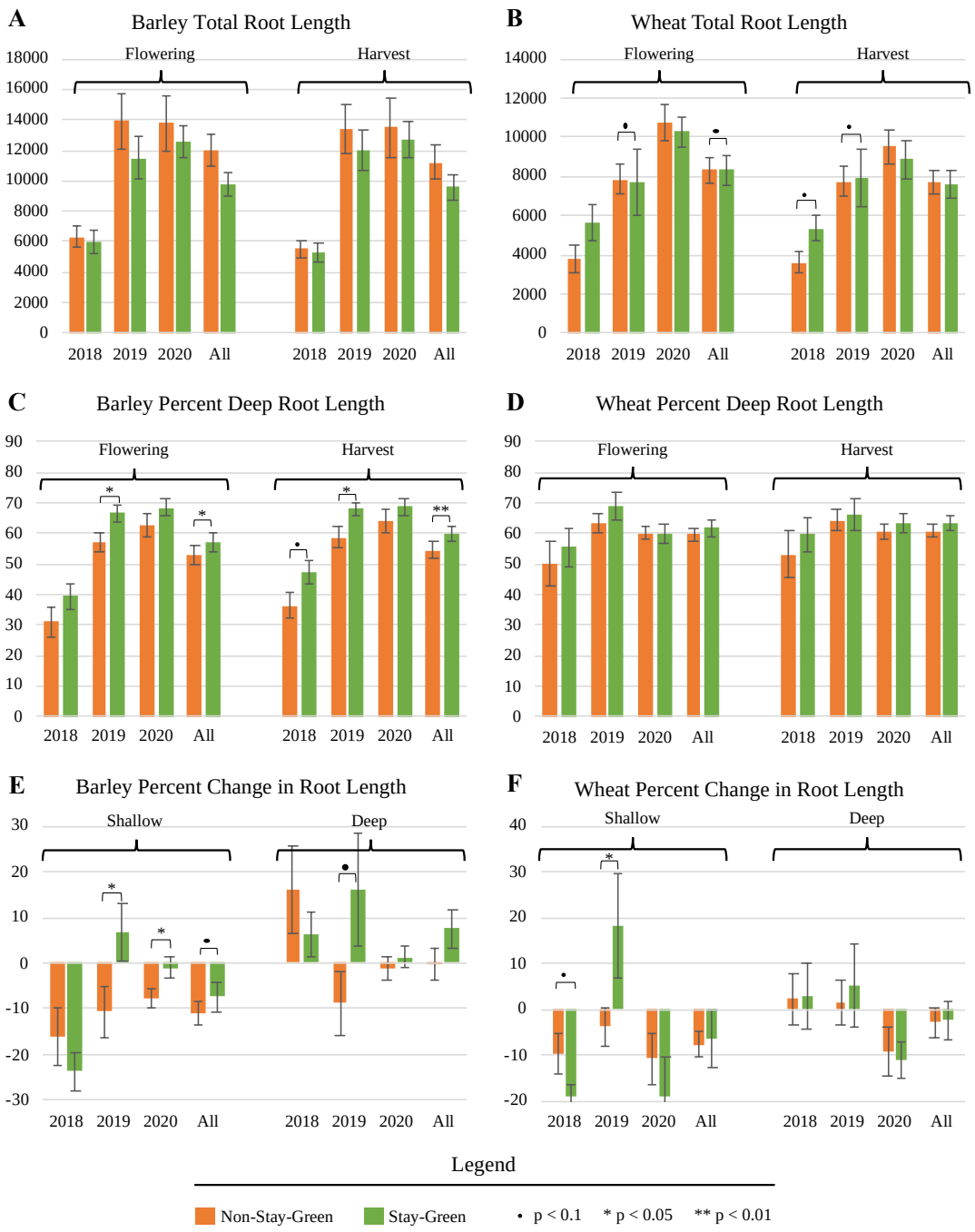

Figure 2.2. Means of non-stay-green and stay-green groups of lines for field root traits measured by minirhizotrons, with p-values from ANOVA for the fixed effect of stay-green. Sample sizes are listed in corresponding Table A2.2 A, C, E) Barley. B, D, F) Wheat. A, B) Total root length at flowering and harvest. C, D) Percent deep root length (Equation 1) at flowering and harvest. E, F) Percent change (Equation 2) in shallow and deep root length between flowering and harvest (during grain fill).

In wheat, the mean percent deep root length of stay-green lines was higher than that of non-stay-green lines at every instance of measurement, but the effect of stay-green was not significant in any of these ANOVA (Figure 2.2D). There were no consistent patterns of difference between the two groups for total root length or percent change in root length (Figure 2.2B and 2.2F). Duration of grain fill did not correlate with field root traits in wheat in the correlation analysis of all wheat lines together, however both days to heading and days to maturity had positive correlations with total root length and negative correlations with percent change in shallow and deep root length. (Table A2.3).

There were two seedling traits that were marginally impacted by stay-green in ANOVA (Table A2.4). Stay-green wheat lines had slightly less root volume and dry weight than non-stay-green lines the PVC pot assay.

Relationships of Field Root Traits to Agronomic Traits

In barley, grain yield was positively correlated with total root length at both flowering and harvest when data from all field seasons were analyzed together, but these correlations were weakly negative in 2018 and 2019 and weakly positive in 2020 (Figure 2.3A). Individual seasons showed consistent positive correlations of grain yield with percent deep root length at flowering and harvest (Figure 2.3C). Percent change in root length was also consistently positively correlated with grain yield, although this was much stronger for shallow than deep root length (Figure 2.3E). Spearman correlation coefficients for field root traits with grain yield and other agronomic traits for individual and combined seasons are listed in Table A2.3. While there were some instances of strong correlation with field root traits for aboveground biomass at flowering, leaf area at flowering, and harvest index, there was lack of consistency in the direction of these

correlations (positive or negative) across seasons (Table A2.3). Correlations with productive tiller number suggested a positive relationship to percent change in root length in barley but did not show a discernible relationship to total root length or percent deep root length. Percent grain protein in barley was negatively correlated with total root length in 2019, with weak negative correlations in 2020 and combined seasons (Figure 2.4A). Correlations between protein and percent deep root length were all negative but weak (Figure 2.4C). Correlation analysis did not demonstrate a connection between protein and percent change in root length in barley (Figure 2.4E).

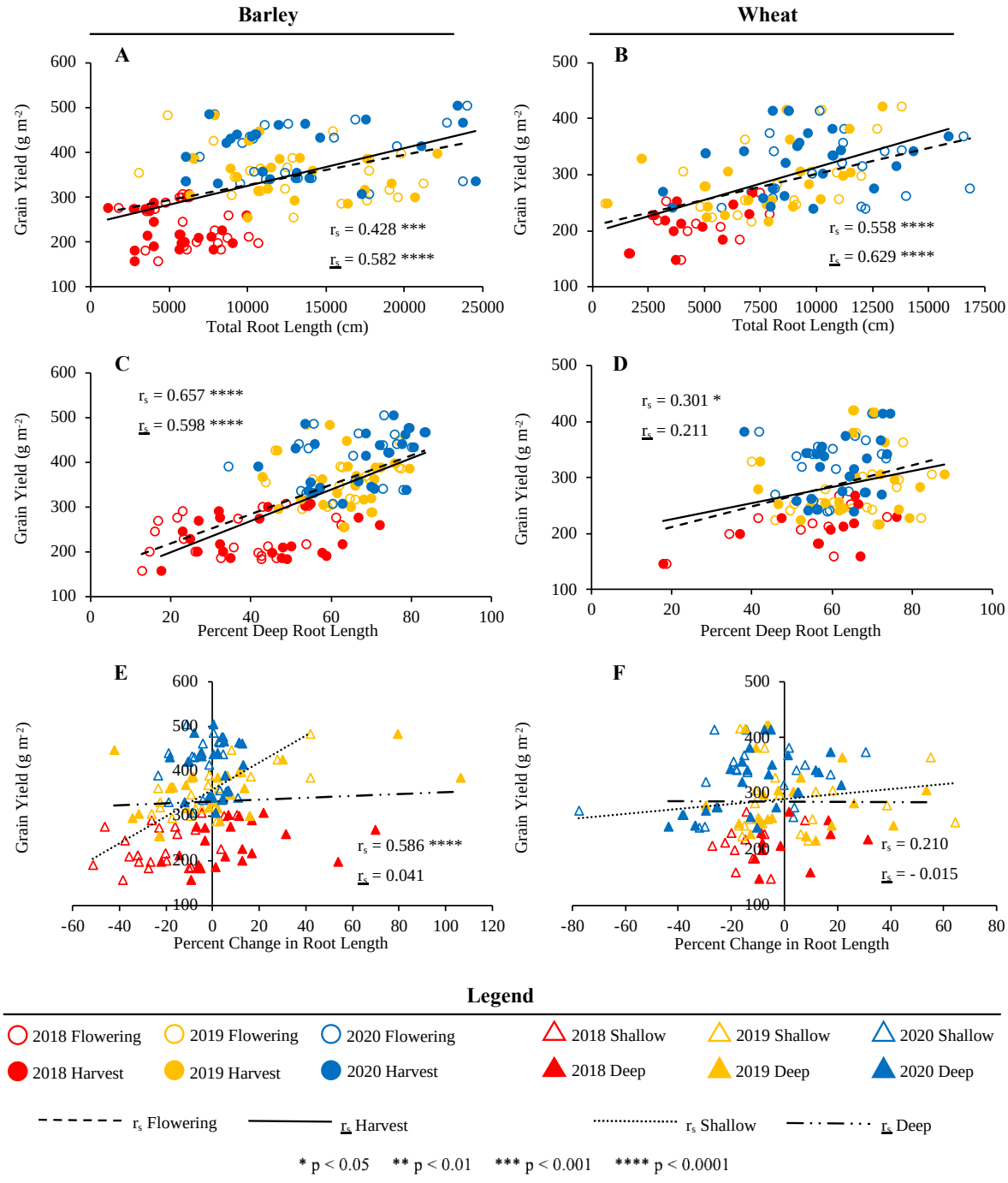

Figure 2.3. Correlations between grain yield and field root traits measured by minirhizotron. Points represent values measured on a single plot. Trendlines and Spearman correlation coefficients (r_s) are from all seasons combined. A, C, E) Barley. B, D, F) Wheat. A, B) Grain yield and total root length at flowering and harvest. C, D) Grain yield and percent deep root length (Equation 1) at flowering and harvest. E, F) Grain yield and percent change (Equation 2) in shallow and deep root length between flowering and harvest (during grain fill).

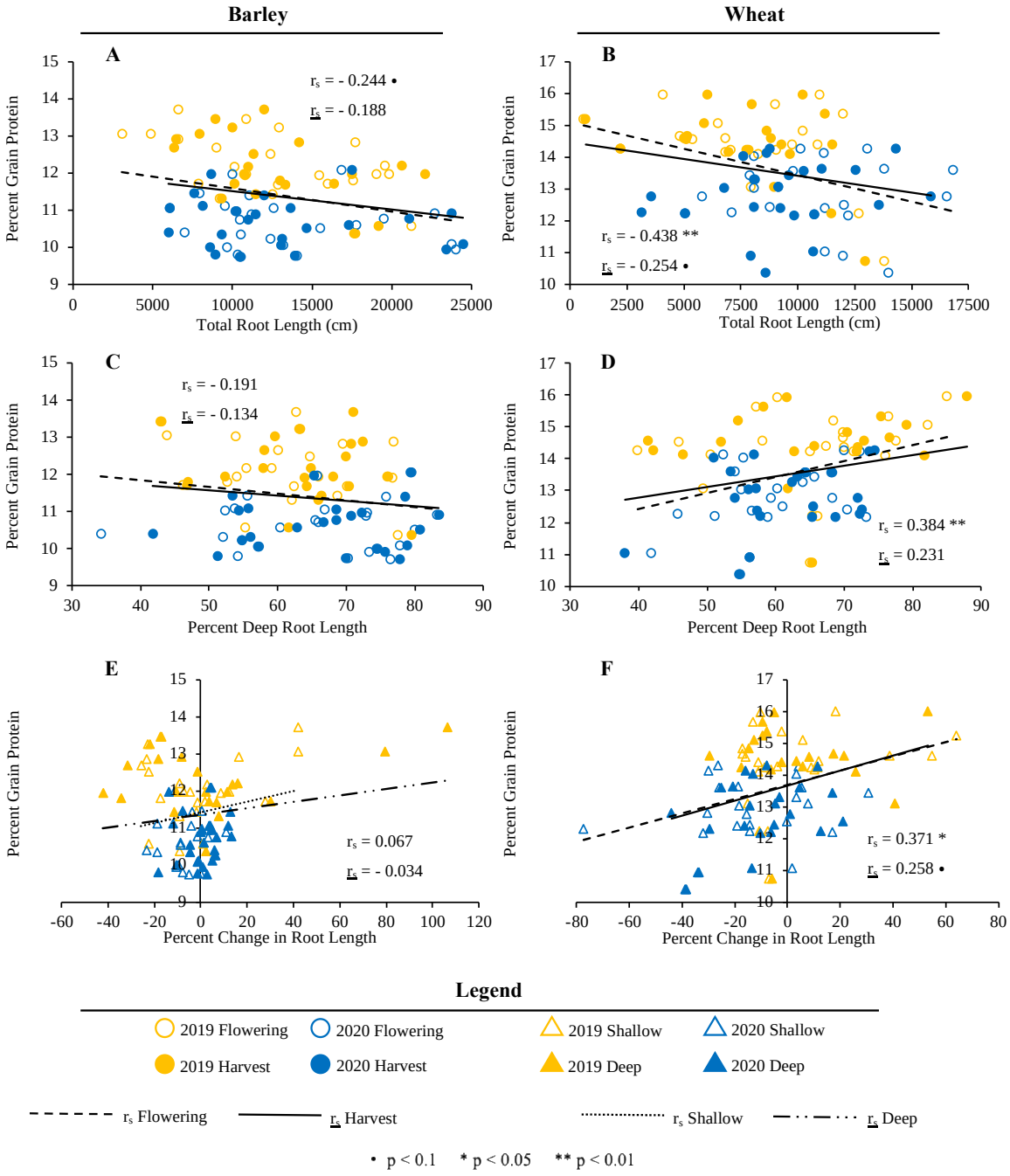

Figure 2.4. Correlations between percent grain protein and field root traits measured by minirhizotron. Points represent values measured on a single plot. Trendlines and Spearman correlation coefficients (r_s) are from all seasons combined. A, C, E) Barley. B, D, F) Wheat. A, B) Percent grain protein and total root length at flowering and harvest. C, D) Percent grain protein and percent deep root length (Equation 1) at flowering and harvest. E, F) Percent grain protein and percent change (Equation 2) in shallow and deep root length between flowering and harvest (during grain fill).

In wheat, correlation tests showed a positive relationship between grain yield and total root length, and suggested a positive relationship between yield and percent deep roots as well (Figure 2.3B, D). Yield was also positively correlated with percent change in root length in 2020, but this connection was weak in the other two seasons (Table A2.3). Correlations between other agronomic traits and root traits in wheat are presented in Table A2.3. There were positive correlations of aboveground biomass at flowering with percent deep root length at flowering and with percent change in shallow root length, except in 2018 where there was no correlation with either. A negative correlation with percent change in shallow root length in 2018 was the only strong relationship between a field root trait and leaf area in wheat. For productive tiller number, there were strong and moderate correlations with total root length in 2018 and 2019 respectively. There appeared to be a positive association of harvest index with total root length, but this did not carry through to 2018. While percent grain protein in wheat was negatively correlated with total root length when seasons were combined (Figure 2.4B), the correlation between these traits was weakly positive in 2020 and weakly negative in 2019 so did not match across seasons (Table A2.3). Positive but weak correlations with protein were observed for percent deep root length and percent change in root length (Figure 2.4D, F).

Seedling Traits as Proxies for Field Root Traits

In barley, correlations of total root length in the field with seminal root angle and seminal root number in the root roll-up assay were weak but were consistently negative for every instance of field measurement (Table 2.5 and Table A2.5). Percent deep root length at flowering and harvest in the field was consistently positively correlated with root volume, seminal root angle, and seminal root number, and consistently negatively correlated with length of longest

root, and lateral root number as measured by the root roll-up assay (Table 2.5 and Table A2.5). Percent deep root length was also negatively correlated with total root length and root dry weight as measured by the PVC pot assay, except for the 2020 field season where there was no correlation with either seedling trait (Table 2.5 and Table A2.6). Percent change in shallow and deep root length in the field in barley was positively correlated with seminal root angle (except for 2018 shallow roots: r_s: -0.262) and negatively correlated with length of longest root (except for 2020 deep roots: r_s: 0.119) in the root roll-up assay (Table 2.5 and Table A2.6).

Table 2.5. Lists of seedling assay traits with the most potential as proxies for field root traits based on strength of correlation and consistency of direction of correlation across seasons. Spearman correlation coefficients for all field versus greenhouse root comparisons can be found in Tables A2.5 and A2.6. Direction of correlation is indicated as positive (+) or negative (-). Method of proxy measurement is indicated as one-leaf root roll-up assay (RRU) or four-leaf PVC pot assay (PVC).

Barley		
Total Root Length	**Percent Deep Root Length**	**Percent Change in Root Length**
- seminal root angle RRU	+ root volume RRU	- length of longest root RRU
- seminal root number RRU	- length of longest root RRU	+ seminal root angle RRU
	+ seminal root angle RRU	- lateral root number RRU
	+ seminal root number RRU	
	- lateral root number RRU	
	- total root length PVC	
	- root dry weight PVC	
Wheat		
Total Root Length	**Percent Deep Root Length**	**Percent Change in Root Length**
+ length of longest root RRU	- root volume RRU	+ total root length RRU
- seminal root number RRU	- root to shoot ratio RRU	- root volume RRU
+ root to shoot ratio PVC	- seminal root angle RRU	- root to shoot ratio RRU
	- seminal root number RRU	

In wheat, correlations of total root length in the field with length of longest root in the root roll-up assay, and with root to shoot dry weight ratio in the PVC pot assay, were weak but consistently positive for every instance of field measurement (Table 2.5 and Tables A2.5 and

A2.6). Field total root length was negatively correlated with seminal root number in the root roll-up assay for the 2020 field season though there was no correlation in 2019 (Table 2.5 and Table A2.5). Percent deep root length in the field in wheat was consistently negatively correlated with root volume, root to shoot dry weight ratio, seminal root angle (except for 2020 harvest: r_s: 0.190), and seminal root number as measured by the root roll-up assay (Table 2.5 and Table A2.5). Percent change in shallow root length in the field was positively correlated with total root length in the root roll-up assay (Table 2.5), but there was no correlation with this trait for change in deep root length (Table A2.5). Change in shallow and deep root length in wheat was negatively correlated with root volume (except 2020 deep roots: r_s: 0.048) and root to shoot ratio as measured by the root roll-up assay (Table 2.5 and Table A2.5).

Discussion

Barley

Deep Roots There is a prevalent notion amongst crop scientists that deep rooting can help alleviate terminal drought stress (Bodner et al., 2015), as research has shown that enhanced root growth at depth does lead to more extraction of subsoil water when it is available (Pask and Reynolds, 2013; Vadez et al., 2013; Lilley and Kirkegaard, 2011). Our field trials were grown in deep soil and followed previous season fallow rotations, so deep water was most likely present. We observed that duration of grain fill (earlier heading and later maturity) was positively correlated with percent deep root length in barley (Table A2.3), and there was a trend in which stay-green barley lines had a higher percentage of their root length deeper in soil than non-stay green lines (Figure 2.2C). These results lend support to our hypothesis that increased root length at depth is related to the stay-green phenotype in barley. Deeper root growth has been linked to

earlier heading (and thus longer grain fill) in barley in a controlled environment (Voss-Fels et al., 2018). It is worth noting that in 2019 our field received above average precipitation before flowering and below average precipitation after flowering (Table 2.2), and this was also the year in which the dynamic of stay-green barley lines having more roots at depth and greater yield was most pronounced. We also saw evidence of a yield benefit associated with deep rooting when combining data for all barley lines (Figure 2.3C). These observations are consistent with the hypothesis that deep rooting is a useful adaptation to environments where precipitation is lacking but deep soil moisture is available.

Change in Root Length Our results imply that an increase (or smaller decrease) in shallow root length during the grain fill period might have been associated with stay-green lines (Figure 2.2E) and longer duration of grain fill (earlier heading and later maturity) (Table A2.3). This would suggest that delayed belowground senescence (although perhaps only at shallower depths) occurs along with the extended photosynthetic period observed aboveground in stay-green barley. Minirhizotrons have been used to document delayed root senescence associated with stay-green in sunflower (Lisanti et al., 2013). There was also a pattern of positive correlation between delayed root senescence and yield in barley when stay-green and non-stay-green lines were analyzed together (Figure 2.3E, Table A2.3). This is counter to the idea that continued root growth would negatively impact yield in water-limited environments as it would compete with grain for photosynthates (Yang and Zhang, 2006; Lynch, 2018). However, Lynch (2018) also points out that if roots die prematurely due to drought rather than programmed senescence, this could result in a loss of carbohydrates in the roots that would otherwise be reallocated to grain. Furthermore, Gous et al. (2013) showed that starch biosynthesis and

molecular structure in grain were unaffected under water stress for a stay-green barley line compared to a non-stay-green cultivar. Delayed root senescence could be part of a strategy for coping with terminal drought stress, but this might require a smaller more efficient root system so that its continued functioning would not detract too much energy from grain filling.

Total Root Length There was a consistent pattern where stay-green barley had less total root length than non-stay-green barley, even though the effect of stay-green was not significant in these ANOVA (Figure 2.2A). Furthermore, there were weak but consistent negative correlations of total root length with duration of grain fill and days to maturity (Table A2.3). These results fit with the idea that the root system of stay-green barley might be smaller than that of non-stay-green barley. However, correlations between total root length and grain yield were weakly negative in 2018 and 2019, but weakly positive in 2020 (Table A2.3), so the effect of this smaller root system might not always be beneficial and probably depends on environment.

Stay-Green Seminal Root Traits and Drought Adaptation The idea that the drought adaptation associated with the stay-green trait in barley might be due to a smaller deeper root system is further supported by some parallel relationships that we observed in our field and greenhouse experiments. Stay-green lines had a wider seminal root angle in the root roll-up assay (Table A2.4). This was unexpected because deep rooting is commonly thought to be associated with a narrower seminal root angle as has been shown in wheat (Oyanagi, 1994), sorghum (Singh et al., 2012), and maize (Lynch, 2013). Yet, a wider seminal root angle in our greenhouse experiment correlated with greater percent deep root length in our field experiment (strongly in 2019 though weakly in other seasons, Table A2.5). This difference between barley and other species with respect to root angle and deep rooting is not without precedent as Manschadi et al.,

(2006) found a stay-green wheat variety to have a narrow compact root system with more deep root activity than a non-stay-green wheat variety in a root observation chamber, while the root system of a drought tolerant barley variety had much more lateral spread at the top then narrowed with increasing depth. Robinson et al., (2016) also found a stay-green barley line to have a wider seminal root angle than a non-stay-green line. A shorter longest root, higher seminal root number, and lower lateral root number were also associated with more roots at depth (though not always strongly) and with stay-green lines (Table A2.5 and A2.4). As for a smaller root system size, there were weak but consistent patterns in which a wider seminal root angle and higher seminal root number were connected to less total root length and to our stay-green barley lines (Table A2.5 and A2.4).

Another stay-green characteristic for barley in our field study was that they had less leaf area at flowering and higher harvest index compared to non-stay-green (except in 2020, Table 2.4). This could be further indication of more efficient water use by drought adapted barley. Borrell et al., (2014) observed that in sorghum, stay-green varieties had less leaf area and the smaller vegetative canopy reduced water use prior to flowering, saving soil moisture for grain fill. It has been suggested that efficient water use is superior to more water use in high-input systems dependent on stored soil moisture (Passioura, 1983; Palta, 2011; Lynch, 2018).

Roots and Grain Protein Grain protein content did not appear to be influenced by root system characteristics for the barley lines in our study (Figure 2.4A, C, E). The relationship between roots and grain protein in barley was likely confounded by the fact that all but two of the barley lines (Craft and MT124118) carry the allele for *HvNAM1,* a NAC transcription factor gene, that reduces the percent grain protein (Alptekin et al., 2020). In the current study, both

stay-green and non-stay green lines carry the low protein allele, apparently reducing protein levels irrespective of the root architecture.

Wheat

Deep Roots While our stay-green wheat lines appeared to have greater means for percent deep root length than non-stay-green lines (Figure 2.2D), the effect of stay-green was not significant in ANOVA, and there were no strong correlations between deep rooting and duration of grain fill in wheat (Table A2.3). These results neither support nor refute the results of other studies. Manschadi et al., (2006) saw that a stay-green wheat line had more roots at depth and extracted more deep water than a non-stay-green line in a root observation chamber. In addition, higher normalized difference vegetation index (NDVI), which has been used as an indicator of the stay-green phenotype, has been correlated with more roots at depth and deep water use in the field (Li et al., 2019). Christopher et al., (2008) also observed the stay-green phenotype and yield advantage of a stay-green wheat line in multiple low rainfall environments but saw neither of these in a location where deep soil moisture was lacking. We did observe consistently positive though mostly weak correlations between percent deep roots and grain yield in our study (Figure 2.3D and Table A2.3). The environment for our field studies lacked late season precipitation (Table 2.2) but likely had stored soil moisture (Site Description). Thus, a positive association between yield and deep rooting in our results would be consistent with the results of Postic et al., (2019) where a correlation was observed between yield and deep roots in rainfed but not irrigated conditions in their minirhizotron field trials examining wheat.

Root Growth and Yield Positive correlations have been found between grain yield and root system size in wheat under water stress (Postic et al., 2019). Grain yield was positively

correlated with total root length at flowering and even more so at harvest in our study (Figure 2.3F). We also found positive correlations between yield and percent change in root length for wheat in 2020 and this pattern was seen in other seasons but was not significant (Table A2.3). Taken together these results imply that having a larger root system that persists for more time after flowering helps wheat withstand terminal drought stress.

Root Growth and Phenology Christopher et al., (2008) suspected that delayed water extraction from the soil after flowering played a role in producing the greater yield of their stay-green wheat line over a non-stay-green line. However, any connection of greater and or delayed root growth to the stay-green trait was unclear in our study. There were not consistent differences for change in root length or total root length between stay-green and non-stay-green groups of lines (Table A2.2). We did not observe a correlation between percent change in root length and duration of grain fill due to negative correlations of this root trait with both days to heading and days to maturity (Table A2.3). Similarly, there was no correlation between grain fill and total root length because total root length was positively correlated with both days to heading and days to maturity (Table A2.3). So, delayed root senescence appeared to be associated with earlier heading and earlier maturity, while larger root systems appeared to be associated with later heading and later maturity. Later heading was found to be associated with larger root systems in a minirhizotron greenhouse study by Ghimire et al., (2020), but many root studies in and out of the field do not measure roots beyond the timing of heading or flowering. Perhaps wheat yield is boosted by delayed heading coupled with delayed senescence and a root system that continues to function during grain filling. This would allow root systems to grow large prior to reproductive stages increasing the plant's capacity to capture resources, then maintain the plant's ability to

utilize those resources and fill seeds with more starch. The success of such a strategy would be dependent on environmental factors, likely requiring abundant stored soil moisture if late season precipitation was minimal.

Stay-Green Drought Adaptation Differences that we did observe between stay-green groups in wheat were greater leaf area (consistent though not significant in 2019 and 2020) and higher productive tiller number of stay-green lines compared to non-stay-green (Table 2.4). Christopher et al., (2008) also saw greater leaf area of a stay-green wheat line compared to a non-stay-green line in water-limited but not under non-limited conditions, and Pask and Reynolds (2013) observed that wheat lines that had the highest yield in dry environments were extracting more water from the soil due to greater stomatal conductance. The larger canopy associated with our stay-green lines suggests that the drought adaptation in stay-green wheat might have more to do with aerial physiology rather than root system architecture.

Roots and Grain Protein There was evidence of a connection between grain protein content and root system characteristics in wheat. Total root length was negatively correlated with percent protein for combined season (Figure 2.4B), although the correlation was weakly negative in 2019 and weakly positive in 2020 (Table A2.3). Grain yield was positively correlated with total root length, so an opposite negative correlation between total root length and protein would be consistent with expectations since yield and protein are well known to be negatively associated in wheat (Yu et al., 2018). Although interestingly, there were positive correlations for both protein and yield with percent deep root length and percent change in root length (Table A2.3). If this result were repeatable in more seasons and locations, it would be encouraging for wheat breeders as they could potentially focus on increasing deep root length and delaying root

senescence as a way to boost yield and percent protein together. A review on breaking the negative relationship between yield and protein in wheat, known as grain protein deviation (GPD), by Cormier et al. (2016) suggested that deep rooting could help maximize nitrogen uptake since nitrate leaches downward through soil.

Barley Versus Wheat In light of the differences observed in this study between barley and wheat, it seems that the best strategy for coping with drought depends on species. We speculate that while both species benefit from deep rooting and continued root functioning during grain fill, wheat relies in part on a larger root system to withstand drought, while barley employs a smaller more efficient one. Evidence of this was found in a study comparing barley and durum wheat, in which unit increases in root length density led to greater increases in water capture in barley than in durum (Ayad et al., 2010). Durum also increased its root length density and root to shoot ratio in response to decreased water availability compared to a smaller increase (Ayad et al., 2010) or even decrease (Carvalho et al., 2014) in barley. Of course, whether a larger or smaller root system is preferrable in a semi-arid growing environment likely also depends on the temporal drought stress pattern experienced by the crop (Lilley and Kirkegaard, 2011; Palta et al., 2011; Chenu et al., 2013.)

Proxy Considerations An ideal proxy trait can be measured quickly and easily and has a clear, consistent relationship to an important trait that is more difficult to measure (Reynolds et al., 2020). Between the methods used here, the root roll-up assay would be more practical for use in plant breeding as it was much faster and easier to perform than the PVC pot assay. There were also more correlations with field root traits coming out of the root roll-up assay. The root traits we measured in the field were influenced by seasonal variation (Table A2.1), but in instances

where correlations between the field and greenhouse were strong in one season, they were usually consistent in direction across seasons (Tables A2.5 and A2.6) indicating potential usefulness of these assay traits as proxies for field traits. The best proxy trait candidates are summarized in Table 2.5. Considering multiple of these traits in some sort of composite measurement may act as an improved proxy over examining any one trait in isolation.

While we tested our proxy traits against field data from three different seasons, all of these were in the same location. Robinson et al., (2018) found both positive and negative correlations between seminal root angle and yield when testing a barley breeding population in several locations. In our experiments, which assay traits were correlated with which field traits was not consistent between species (Table 2.5). Furthermore, in instances where field traits correlated with the same assay traits for both barley and wheat, the directions of those correlations were often opposite between the two species (Table A2.5, A2.6). It appears that proxies should be species specific as well as environment specific.

Conclusion

Minirhizotrons are useful tools that allow non-destructive repeatable imaging of roots growing in real field conditions. Barley and wheat roots have certainly been observed growing deeper (Thorup-Kristensen et al., 2009) than the range defined as deep in this study, and we wish to reiterate that we did not measure maximum depth reached by the roots, but rather the amount of root length present in fixed depth ranges captured by the minirhizotron imaging system (shallow: 0-30 cm, deep: 30-60 cm). Ours is the first study to use minirhizotrons to examine the same roots in the field at multiple time points for stay-green barley and wheat. This study also offers a first look at relationships of roots in the field with grain protein content in barley and

wheat. Our results suggested that: 1) Greater deep root length, delayed root senescence, and less total root length were associated with the stay-green phenotype in barley in this study, although in wheat, stay-green was associated with greater deep root length, but not related positively or negatively with either delayed root senescence or total root length. 2) Greater deep root length and delayed root senescence were associated with greater grain yield in barley, as was greater total root length but this relationship was inconsistent across seasons. In wheat, greater deep root length, delayed root senescence, and greater total root length were all associated with greater grain yield. 3) Several traits from the greenhouse assays showed potential as proxies for field root traits in our environment. 4) There were differences between barley and wheat with respect to how stay-green lines differed from non-stay-green lines, how field root traits related to agronomic traits, and the relationships between roots in the greenhouse and roots in the field. The patterns illustrated by this study were observed on a limited set of genotypes in a single location. It should not be assumed that what works for one species in one environment will work in a different environment, or for a different species in the same environment, even for two crops as similar as barley and wheat. In order to expand our understanding of and confidence in these associations, we would need to test them on more varieties in more locations. We are in the process of mapping the genetics of stay-green and root proxy traits in a biparental barley population. Once QTLs are identified, near isogenic lines (NILs) will be created for use in future minirhizotron experiments. Further characterization of root system physiology and its genetic controls could help barley and wheat breeders develop varieties that are better adapted to drought stress.

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Description of Appendix Material

Our appendix materials include tables showing the sample sizes, means, and p-values resulting from t-tests comparing stay-green and non-stay-green groups for root traits measured by field minirhizotrons and in greenhouse assays, as well as tables showing Spearman correlation coefficients for comparisons of field root traits with agronomic traits, and root assay traits in barley and wheat.

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CHAPTER THREE

QTL MAPPING REVEALS MALT BARLEY QUALITY IMPROVEMENT IN TWO DRYLAND ENVIRONMENTS ASSOCIATED WITH EXTENDED GRAIN FILL AND SEMINAL ROOT TRAITS

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Core Ideas

1. Extended grain fill improved and stabilized barley plump and protein quality for malting.
2. Grain quality was more affected by heading in the drier location and by maturity where there was more moisture.
3. Extended grain fill, early root development, and grain quality co-located at multiple QTLs.
4. Seminal root to shoot length ratio co-located with a gene (*HvNAM1*) that affects protein, plump, and maturity.

Abstract

To achieve malt grade and receive full price, barley (*Hordeum vulgare* L.) crops must meet standards for certain quality traits including percent plump and protein. Terminal drought stress reduces quality and is projected to worsen in barley cultivation areas, underscoring the need for varieties that maintain good malt production with unreliable precipitation. The stay-green trait extends the grain fill phase between heading and maturity and has been linked to stable quality under dry conditions. However, this relationship can be inconsistent and is not well understood. To effectively leverage a longer grain fill phenotype for drought adaptation, a better grasp of its genetics and environmental interaction is needed. Stay-green root system differences have been observed and could be at play. We performed correlation and quantitative trait locus (QTL) analysis on grain fill duration, grain quality, and seminal root traits using a recombinant inbred line (RIL) population segregating for stay-green. Agronomic data were collected in four field trials at two distinct semi-arid locations, and roots were measured in a greenhouse assay. Earlier heading and later maturity led to improved quality in both locations and more consistent

quality between locations. Earlier heading had a greater influence on quality in the drier environment while later maturity was more impactful in the less dry environment. We observed co-locations of seminal root trait QTLs with grain fill duration and grain quality. These QTLs lay the groundwork for further investigation into root phenotypes associated with stay-green and the deployment of these traits in breeding for drought adaptation.

Introduction

Barley (*Hordeum vulgare* L.) is an important cereal crop in the United States and globally. End use products for barley grain include animal feed, human food, and malt. Malt is used for brewing, distilling, and as a food additive and is usually the most profitable end use for a barley grower. From 2012 to 2022 in the United States, the price received for malt barley averaged 47% higher than the price for feed barley (USDA NASS). For a barley crop to be sold at this higher malt price, it must meet a series of strict quality requirements including low grain protein content (<13.0%) and high percent plump grain (>90% on a $^{6}/_{64}$ inch sieve) (AMBA, 2021). Thus, these crop traits are at least as important a consideration as yield in malt barley cultivation and breeding (McVay et al., 2017). In recent years, most barley in the United States has been grown in Montana, North Dakota, and Idaho (USDA NASS). A survey of Montana growers ranked malt quality as the second most important trait to consider when choosing a barley variety (USDA NASS, 2022). Drought tolerance was the highest ranked trait.

The states where most US barley is grown regularly experience terminal drought stress (NOAA NCEI), where a reduction in precipitation occurs during the latter phases of crop development. In barley, the grain fill phase when the plant is accumulating starch and protein in the seed endosperm, begins with heading and ends with maturity (Alqudah and Schnurbush,

2017). While barley has a reputation as a relatively drought tolerant crop, terminal drought conditions coinciding with grain fill negatively impact yield and malt quality parameters due to decreased starch accumulation, resulting in higher protein content and thinner grains (Gordon et al., 2020; Tarawneh et al., 2020). Many malt growers mitigate a lack of precipitation during grain fill with irrigation. However, the usefulness of irrigation as a risk management tool is likewise curtailed by limited availability of water and irrigable acres. Increasing temperatures and shifting precipitation patterns threaten the reliability of water supplies and are predicted to exacerbate the terminal drought hazard (Conant et al., 2018). Additionally, malting, brewing, and distilling businesses are increasingly concerned with sustainability. There is mounting pressure to conserve water and the drought adaptation of barley needs to be improved in order to maintain high quality malt production.

Stay-green is a trait that has been observed in several plant species (Thomas and Smart, 1993) including barley, and has received attention as an adaptation to drought. Functional stay-green is longer retention of photosynthetic leaf area due to delayed initiation of senescence and or a slower rate of senescence (Thomas and Ougham, 2014). This results in a prolonged grain fill period. We have also observed further extension of the grain fill period in stay-green barley lines via earlier heading in a previous study (Williams et al., 2022). Stay-green has been connected to yield stability in water limited environments in wheat (*Triticum aestivum* L.) (Christopher et al., 2008), sorghum (*Sorghum bicolor* L. Moench) (Borrell et al., 2014), and maize (*Zea mays* L.) (Trachsel et al., 2016). This trait has been studied less in barley than other cereals (Kamal et al., 2019), but has benefits for grain quality. A thorough examination of barley grain components in a controlled environment experiment by Gous et al. (2013) showed that a stay-green cultivar

maintained low protein content and stable starch structure under water stress, while these qualities were sensitive to water stress in a non-stay-green cultivar. Shirdelmoghanloo et al. (2022) observed instances of plumper barley grains correlating with longer green leaf area retention and grain fill duration in heat stressed field trials. However, expression of the stay-green phenotype and its agronomic advantages can be inconsistent across environments and genotypes, and the genetic and physiological basis of stay-green is not fully understood.

Stay-green is a complex trait that exhibits environmental plasticity, highlighting the need for further genetic dissection and field examination of the phenotype. In a glasshouse study of a doubled haploid (DH) barley population segregating for stay-green, different quantitative trait loci (QTL) were identified for measures of leaf greenness depending on whether the plants had been subjected to water stress or heat stress (Gous et al., 2016). Emebiri (2013) found different QTL for green color retention depending on the genetic source of stay-green in field trials of two different barley DH populations. Their measure of stay-green was also found to be either positively or negatively correlated with flowering time and grain plumpness depending on the population. In order to appropriately utilize a protracted grain fill period to enhance drought adaptation, the genetics of this phenotype and its relation to other agronomically significant traits need to be examined in the given genetic background. It is also important to test crop performance in the target production environment. A strategy that helps a plant maintain yield and quality in one drought stressed environment may not work in another (Palta and Turner, 2019). Yet, consistent expression of key phenotypes across locations is important to plant breeders striving to produce reliable varieties (Bernardo, 2010), so genotype by environment interactions need to be understood. In a multi-environment study of a stay-green wheat line,

Christopher et al., (2008) observed expression of the stay-green phenotype and its associated yield benefit in environments with deep soil moisture, but observed neither in an environment where deep moisture was lacking. Access to stored water during grain fill could be part of the mechanism behind the stay-green trait.

As the site of water acquisition, root systems are a logical focal point for investigations into the source of a putative drought tolerance trait. Indeed, multiple studies have uncovered evidence suggesting a relationship between roots and stay-green. Our previous study (Williams et al., 2022) examined roots in the field with the use of minirhizotrons and found stay-green barley lines with longer grain fill duration had a greater percentage of their roots deeper in the soil, and prolonged root growth compared to non-stay-green lines. Stay-green lines were also found to differ from non-stay-green in that study for traits measured in a greenhouse assay such as the length and number of seminal roots and the number of initiated lateral roots at the one-leaf stage. Furthermore, some of these seminal root phenotypes correlated with root traits measured in the field later in development. Other researchers have also drawn connections between stay-green and root attributes in controlled environment experiments. For example, a stay-green barley line was found to have a wider seminal root angle and lower root number than a non-stay-green line in a seedling assay (Robinson et al., 2016). Manschadi et al. (2006) found a stay-green wheat variety to have increased deep rooting compared to a non-stay-green variety in a root growth observation chamber. In QTL mapping studies, traits measured in root assays have co-located with stay-green QTL in barley (Gous et al., 2016), wheat (Christopher et al., 2018), and sorghum (Mace et al., 2012). Observation of root systems in the field poses many challenges, so *ex situ* root examinations are useful especially for large populations. Roots are also understudied

compared to more readily observed aboveground structures, and thus represent a largely untapped resource for potential crop improvement.

The goal of this study was to genetically dissect seminal root traits, the stay-green trait, and agronomic and grain quality traits in two distinct semi-arid environments with different precipitation patterns to discern the relationships between these phenotypes and their environmental interactions. We performed genetic linkage analysis on 168 recombinant inbred lines (RILs), which were derived from a cross between two-rowed barley lines; MT124118, a Montana State University experimental line, and ND19119, a line from North Dakota State University regarded as stay-green due to its longer grain fill duration, and known for large kernel size (Frankowiak et al., 2007, 2010). We performed QTL mapping for grain fill duration and grain quality parameters assessed in field trials, and seminal root traits measured in a greenhouse seedling assay. The locations used for our field trials allowed us to compare performance in environments differing for precipitation levels, soil nitrogen availability, and other factors. *In-situ* examination of mature root systems was not practical for a population of this size, but significant correlations were previously observed between seminal root traits measured by this greenhouse method and root traits measured in the field (Williams et al., 2022). Here we present genetic loci associated with length of grain fill, grain quality, and seminal root traits, as well as relationships between these phenotypes and with their environments.

Materials and Methods

Plant Material

To generate the biparental mapping population used in this study, female parent MT124118 (Hockett/MT070174) from Montana State University was crossed to pollen donor

ND19119 (ND15403.3/ND15368//ND16453) from North Dakota State University. Both are two-rowed barley lines developed for malt end use. ND19119 is characterized as having long grain fill and large kernels (Frankowiak et al., 2007), and a close descendant of this line has been shown to have prolonged retention of green leaf area under water stress (Gous et al., 2016). MT124118 exhibits comparatively shorter grain fill duration and lower percent plump (Williams et al., 2022). F_1 progeny resulting from this cross were advanced through single seed descent for six generations, to produce 168 sibling recombinant inbred lines (RILs) with approximately 98% homozygosity. This F_7 generation was grown in 0.3 m long single rows at the Post Agronomy Farm in 2018 to ensure that there was segregation within the population for grain fill duration. Seed from this preliminary field trial was used for multi-row field trials in 2019, which in turn provided seed for 2020 field trials. The two parent lines were used as checks in the experimental design, along with the common malt barley varieties Craft (BETZES/DOMEN/BARONESE) and Hockett (Bearpaw/ND7593).

Field Experiments

Site Descriptions A total of four field trials were conducted in 2019 and 2020 at two locations: the MSU Arthur H. Post Agronomy Farm (45.6°N, 111.2°W) in Bozeman, Montana, and the MSU Northern Agricultural Research Center (NARC) (48.5°N, 109.8°W) in Havre, Montana. Soil at the Bozeman location is an Amsterdam-Quagle silt loam, and at the Havre location it is a mix of Joplin, Scobey, and Telstad clay loams (USDA, 1998). Daily precipitation and temperature data were obtained from NOAA climatological summaries collected at the two study locations (Table 3.1). Both sites typically experience reduced precipitation during the

second half of the growing season, but this is usually more pronounced in Havre where there is also less precipitation during the winter between growing seasons.

Table 3.1. Environmental conditions for field trials conducted at the two locations. Total precipitation and mean temperature are given for each location-year for the previous September to planting date, planting date to mean heading date for the population, and from heading date to harvest date. Historic precipitation and temperature means are given for each site from 1966 to 2020, using mean dates from the current field trials to determine time periods. Yield goals and soil nitrogen levels for the top 24 in are also reported for each location-year.

Field Trial Condition	**Bozeman**			**Havre**		
	2019	2020	Historic Mean	2019	2020	Historic Mean
Total Precipitation (mm)						
Pre-Season	235	230	218	118	147	134
Planting to Heading	115	115	141	115	99	119
Heading to Harvest	110	31	67	48	28	62
Total	460	375	426	281	274	315
Mean Temperature (°C)						
Planting to Heading	13	13	13	12	15	14
Heading to Harvest	18	20	19	20	20	21
Fertility						
Yield Goal (lbs ac^{-1})	100	100	-	60	60	-
Total Available N (lbs ac^{-1})	145	148	-	29	34	-
Percent of Recommended N Available	121	123	-	40	47	-

Experimental Design and Management An augmented randomized complete block design (Federer, 1956) was used for the field study such that within each location-year trial, each RIL appeared once, and four replicated check lines (MT124118, ND19119, Craft, and Hockett) appeared at randomized positions in each of eight blocks. The replicated checks allowed for the calculation of best linear unbiased predictions (BLUPs) of RIL phenotypes to account for environmental variation within the field.

Field preparation differed between the two locations but was consistent with locations between seasons. In Bozeman, the soil was tilled and fertilized, while soil was not tilled or fertilized at the Havre location, which has been under no-till management for over twenty years in an effort to conserve soil moisture. Experience at these farms has shown that yields are

typically lower in Havre due to drier conditions and thus recommended fertility rates are lower. The nitrogen levels in soil tests for our field trials were below the recommended rate in Havre and above the recommended rate in Bozeman (Table 3.1).

Trials were seeded during the last week of April for each of the four location years except for Bozeman, 2019 which was seeded on May 8th due to weather constraints. Seed was treated prior to planting with CruiserMaxx Vibrance Cereals (Syngenta) to protect against early season pests and pathogens. Plots were three rows each with 0.3 m spacing and were 5.5 m long at the Bozeman location and 6.4 m long in Havre. All trials were rainfed and maintained weed free. Grain was harvested after drying to approximately 15% moisture content or less in the field.

Agronomic Data Collection Heading date, stage 59 on the Zadoks scale for the growth stages of cereals (Zadoks et al., 1974), was visually determined for each plot when 50% of the seed heads in the plot had emerged from the boot. Maturity date (Zadoks 89) was visually determined for each plot when 50% of the seed heads in the plot were no longer green. For the 2019 trial in Havre, Zadoks stage was assessed for each plot on three days around the time of heading and three days around the time of maturity, then heading and maturity dates were extrapolated from these observations. Heading and maturity dates were reported in Julian days, so using a continuous counting of days from the beginning of the respective year. Heading date was subtracted from maturity date to determine the duration of the grain fill period. Planting date was subtracted from maturity date to determine days to maturity. The Field Book app was used for data collection in the field (Rife and Poland, 2014).

After maturity, a measuring stick was used to find the heights of two plants per plot from the ground to the top of the seed head excluding awns, and the mean of these two measurements

was reported for plant height. Grain was harvested with a plot combine (Wintersteiger). Gross weight of harvested grain for each plot and measured individual plot lengths were used to calculate grain yield. Seed was threshed and cleaned after harvest. Test weight was measured using a Dickey-John Grain Analysis Computer 2500-UGMA (Corn Belt Testing Inc.). Grain protein content was measured by near-infrared spectroscopy (NIR) (Infratec NOVA, FOSS) following the protocol from the American Society of Brewing Chemists (ASBC, 1992). Percent plump grain was considered to be the percentage of a grain sample remaining on top of a $^{6}/_{64}$ by $^{3}/_{4}$ inch sieve after sieving as defined by the United States Standards for Barley (CFR, 2023).

Seedling Root Roll-Up Assay

A high throughput seedling assay called root roll-ups was conducted in a greenhouse at MSU, Bozeman to observe seminal root traits at the one-leaf stage (Zadoks 11). This method is described in Williams et al., (2022), and summarized here with modifications noted. Artificial light exposure and temperature were controlled in the greenhouse. The assay was conducted once running from December, 2019 to February, 2020, and again from June to August of 2020, for two replications with rolls grown in successive groups over each period. To be able to account for possible variation due to seasonal differences in natural daylength and climatic fluctuations within the greenhouse from week to week, the same augmented design and check varieties used in the field trials were used in this assay.

Four seeds of a single barley genotype were placed along the short edge of a 25.4 cm by 38.1 cm sheet of germination paper (Anchor Paper Co.) which was then rolled up from long edge to long edge. Rolls for the different barley lines were then wetted, placed together in a bucket with 2-3 cm of standing water, and held upright with the seeds at the tops of the rolls. Following

germination, the seminal roots (those originating in the seed embryo (Mankse and Vlek, 2002)), grew down into the rolls of paper while the shoots grew up out of the rolls. A roll was removed from the bucket when a second leaf tip was just visible in two out of its four seedlings.

Rolls were then unrolled for data collection. The number of seminal roots and initiated lateral roots were counted. The lengths of the longest and shortest seminal roots were measured with a ruler as was the length of the shoot. Root length range was determined by subtracting the length of the shortest root from the length of the longest root, then standardized by dividing that difference by the mean of those two root lengths. Root to shoot length ratio was defined as the length of the longest root divided by the length of the shoot. In this text, the term root length refers to the length of the longest root unless specified as referring to the length of the shortest root. The mean of the measurements for the four seedlings was reported as the value for a single roll.

Statistical Analysis

Field trials at the Bozeman and Havre farms were analyzed separately to facilitate comparison of the two distinct environments. Initial explorations of the data indicated that results were generally consistent between years within locations, so these were analyzed together to be concise. Trait ranges, means, and standard deviations were calculated for the RIL population and each parental line across seasons and within locations for the field trials, and across replications for the root roll-up assay. The two parental lines were compared by two sample un-paired t-test for each trait. Analyses were performed using R Statistical Software (R Core Team, 2022).

Environmental variation in the RIL data was accounted for as follows. Utilizing the augmented randomized complete block design, best linear unbiased predictions (BLUPs) were

calculated for each trait as described by Kehel et al. (2010). This was done using the "lmerTest" package (Kuznetsova et al., 2017) with restricted maximum likelihood (REML) estimation and the following mixed linear models.

$$Y_{ijkl} = \mu + Checks_i + Block_j + Year_k + Genotype_l + \varepsilon_{ijkl}$$

was used for field trial traits where $Checks_i$ was a fixed effect of the replicated check varieties, and $Block_j$, $Year_k$, and RIL $Genotype_l$ were random effects.

$$Y_{ijkl} = \mu + Checks_i + Block_j + Rep_k + Genotype_l + \varepsilon_{ijkl}$$

was used for root roll-up traits where $Checks_i$ was again fixed, and $Block_j$, Rep_k, and RIL $Genotype_l$ were random effects. BLUPs given by the genotype coefficient for each RIL were then centered around the raw population mean for each trait to provide RIL phenotypic values adjusted for spatial and temporal heterogeneity.

The same package and mixed linear models were used to calculate heritability for each trait as described by Holland et al. (2003). Variance due to RIL genotype was divided by the total variance due to all random effects and residuals.

In order to assess how consistent RILs were in their expression of phenotypes between the contrasting field environments, percent difference was calculated for yield and quality traits. For each RIL, the absolute difference between the Bozeman BLUP and the Havre BLUP was calculated. This difference was then divided by the mean of those BLUPs, so that the percent difference would be an indication of stability across locations regardless of whether the raw yield or quality measurements were high or low. The effect of location was also examined for each field trait via analysis of variance (ANOVA) on the complete dataset from all four field trials. For the RIL population, the same mixed linear models were used as for BLUPs and heritability with

the addition of location as a fixed effect. Parent lines were analyzed separately with location as a fixed effect, and year and block as random effects.

Pearson correlation tests using the R package "Hmisc" (Harrell, 2022) facilitated comparison of traits. BLUPs of heading date, days to maturity, and grain fill duration were tested for correlation with BLUPs of all other field trial traits within locations. The percent differences of traits that were calculated between locations were tested for correlation with heading, maturity, and grain fill means across locations for the RILs. Correlation analysis was also performed to compare all field trial traits with all root roll-up assay traits.

Figures were made using "R/QTL" (Broman et al., 2003), "ggplot2" (Wickham, 2016), and "ggpubr" (Kassambara, 2023).

Genotyping and Genetic Mapping

Genotyping of the RILs and two parental lines was done by the USDA North Central Small Grains Genotyping Lab (Fargo, ND, USA) using the barley 50K Illumina SNP microarray. Pervious work at MSU had indicated that the parent lines to the RIL population differed in allelic identity for the *HvNAM1* NAC transcription factor gene (Burcu Alptekin, personal communication, 2018), which significantly impacts protein content, percent plump grain, and time to maturity (Alptekin et al., 2021); traits of interest in the present study. Thus, it was decided to additionally genotype the population for this polymorphism. Genomic DNA extractions were performed as described in Alptekin et al., (2021). A competitive allele-specific PCR (KASP) assay was run at MSU, using primers designed to differentiate between the two parental alleles for *HvNAM1*. KASP primers (Table A3.1) were created by LGC, Biosearch Technologies' KASP on Demand system based on the *uhb6* primer sequences in Distelfeld et al.

(2008). The 61-55°C touchdown PCR protocol was run on 5 µl reaction volumes of DNA from the RILs and the parents.

For the construction of a genetic linkage map based on the genotyping data, markers were eliminated if the parents were not polymorphic, were heterozygous, if minor allele frequency in the population was less than 30%, or if more than 30% of the population was missing call data or heterozygous. The genotyping data were then imported into the program QTL IciMapping (Wang et al. 2019) to build a linkage map. The program's binning algorithm was used to remove redundant markers. Markers were then grouped by LOD score using a LOD threshold of 3. Ordering was done using k-Optimality by LOD. Rippling was done by LOD with a window size of 8. The map was further refined by dropping 23 markers that substantially increased the size of the map. The resulting map consisted of 1,316 markers covering 1,153 centimorgans as summarized in Table A3.2.

QTL mapping of the phenotype BLUPs was done using "R/QTL" (Broman et al., 2003). Composite interval mapping (CIM) was performed for each trait using the Haley-Knott regression method and an infinite window size. Logarithm of odds (LOD) score thresholds for QTL significance were determined for each trait by permutation test (n = 1,000). The genetic positions of QTLs identified by CIM with a LOD above the $\alpha = 0.2$ threshold were used to create an initial model. This model was used to check for interactions between QTLs. No QTL interactions above the $\alpha = 0.2$ LOD threshold were found in this study. Multiple interval mapping (MIM) was then performed to refine QTL positions, and search for additional QTLs. These were added to the model if their LOD scores were above the $\alpha = 0.2$ significance cutoff determined for the trait. This iterative process was repeated until no additional QTLs were

identified, thus arriving at the final model describing the positions, LOD scores, and percent of phenotypic variation explained for each QTL.

Results

Phenotypic Variation and Heritability

Transgressive segregation was observed for all traits in the field trials, as the distributions for the RIL population extended both above and below the ranges of the two parents (Table 3.2). This suggests that both positive and negative alleles were inherited from each parent. Consistent with expectations (Franckowiak et al., 2007, 2010; Williams et al., 2022), ND19119, the stay-green parent, displayed earlier heading and later maturity than MT124118 in both locations and thus a longer grain fill period (Table 3.2). ND19119 had greater percent plump grain and grain protein than MT124118 in both locations. The parent lines did not significantly differ for yield and test weight.

Table 3.2. Summary statistics for traits measured in the field trials and the root roll-up assay on the RIL population and each of the two parents. The two seasons are combined within locations for the field traits. Heritability estimates for the population are listed, as well as significance levels from t-tests comparing trait means of the parental lines:
$p \geq 0.1$ ns, $p < 0.1$. , $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***

Field Trait	**RIL Population**				**H^2**	**MT124118 Parent**				**Parent**	**ND19119 Parent**			
Location	Min	Mean	Max	SD		Min	Mean	Max	SD	**t-test**	Min	Mean	Max	SD
Heading Date (Julian)														
Bozeman	178	184.7	192	3.2	0.413	183	185.1	187	1.7	**	179	182.4	186	3.0
Havre	172	178.6	189	4.0	0.258	176	180.9	185	2.4	***	172	175.3	181	3.2
Grain Fill Duration (Days)														
Bozeman	30	40.2	52	4.5	0.246	33	37.1	41	3.0	***	39	43.8	48	2.7
Havre	18	30.5	40	4.5	0.247	21	25.3	30	2.6	***	28	33.7	40	4.4
Days to Maturity														
Bozeman	96	101.4	108	2.2	0.422	96	98.8	100	1.1	***	100	102.6	105	1.5
Havre	84	92.1	102	3.7	0.059	85	89.2	95	2.5	*	87	91.9	98	3.7
Plant Height (cm)														
Bozeman	73.0	89.2	101.5	5.4	0.693	79.0	84.1	89.5	2.6	***	86.0	94.5	102.5	4.0
Havre	51.7	70.0	86.0	6.7	0.264	61.0	65.9	73.5	3.8	**	60.8	71.6	80.5	5.9
Yield (kg ha^{-1})														
Bozeman	4000	6237	8255	746	0.076	5725	6840	7810	600	ns	5086	6507	7611	557
Havre	1552	3017	4397	520	0.198	2494	3248	4159	453	ns	2183	2974	3792	428
Test Weight (kg hl^{-1})														
Bozeman	62.0	69.0	75.8	3.7	0.026	66.5	70.0	74.1	3.1	ns	64.6	68.4	74.6	3.3
Havre	59.2	68.8	75.0	3.4	0.052	65.4	69.3	72.8	2.9	.	65.6	69.5	73.9	2.8
Percent Plump Grain														
Bozeman	88.9	97.6	99.3	1.4	0.331	90.4	94.5	97.3	2.0	***	98.3	99.0	99.3	0.2
Havre	74.0	94.4	99.2	3.6	0.349	80.8	86.1	91.6	3.0	***	93.5	97.2	99.2	1.4
Percent Grain Protein														
Bozeman	9.6	12.0	14.9	1.0	0.875	10.8	11.5	12.0	0.3	***	11.6	12.1	12.5	0.3
Havre	8.7	11.5	14.2	1.2	0.189	9.2	10.8	12.4	1.2	**	10.4	11.9	13.3	0.8
Root Roll-Up Trait														
Days to One-Leaf	9	12.6	18	1.8	0.430	9	11.6	15	1.8	**	11	13.7	16	1.5
Shoot Length (cm)	4.6	9.7	16.3	2.1	0.435	5.9	8.3	11.4	1.4	***	7.0	10.7	14.0	2.1
Longest Root Length (cm)	6.9	30.5	42.4	6.6	0.309	18.6	27.5	36.3	5.1	.	14.2	31.6	39.2	6.3
Root to Shoot Length Ratio	1.2	3.3	6.3	0.7	0.447	2.6	3.4	4.1	0.5	.	1.9	3.1	4.0	0.5
Shortest Root Length (cm)	0.8	9.1	18.4	3.4	0.072	2.7	8.8	14.6	3.4	ns	1.4	7.3	14.6	3.3
Root Length Range (cm)	4.7	20.5	34.2	5.7	0.346	8.3	16.4	27.7	5.4	***	12.8	24.2	30.5	4.8
Seminal Root Count	4	6.0	8	0.4	0.430	5	5.6	6	0.4	***	6	6.3	7	0.3
Lateral Root Count	0	24.2	189	30.2	0.176	0	18.7	75	22.9	ns	1	27.2	87	25.0

A wide range of heritability estimates was observed for field trial traits, and these varied between the two locations (Table 3.2). Heritability of heading date and days to maturity was about 0.4 in Bozeman but lower in Havre, particularly for days to maturity. Duration of grain fill was moderately heritable at about 0.2 in both locations. Grain plumpness was also moderately heritable (0.3), while grain protein had high heritability in Bozeman (0.872) but low heritability in Havre (0.185). Yield and test weight had low heritability in both locations.

Location had a large impact on all traits for the RILs and each parent as indicated in ANOVAs, excluding protein for ND19119, and test weight throughout (Table A3.3). Overall, grain fill was shorter and occurred earlier in the season in Havre (Table 3.2). Plant height, yield, and grain plumpness were higher in Bozeman. Protein content was slightly higher in Bozeman, and test weight barely differed between locations.

Transgressive segregation and a range of heritability estimates were also observed for all traits measured in the root roll-up seedling assay (Table 3.2). ND19119 had a longer shoot and root than MT124118 (Table 3.2). However, a smaller root to shoot length ratio indicated ND19119 had a shorter root relative to the shoot. This line also reached the one-leaf stage more slowly, had more seminal roots, and a larger root length range. The parents did not differ in t-tests for lateral root count or length of the shortest root.

Correlation of Traits

Pearson correlation tests revealed relationships between some traits. Longer grain fill duration was generally correlated with improved quality, although whether earlier heading or later maturity was more correlated varied between environments (Table 3.3). Correlations with grain quality were stronger with days to maturity than with heading date in Bozeman, and the

opposite was observed in Havre. Percent plump and test weight were both positively correlated with a longer grain fill duration in both locations. Conversely, lower percent protein was associated with a grain fill period that was shifted later in the season in both locations, as indicated by negative correlations with both heading and maturity. There were slight negative correlations between grain fill duration and grain yield in both locations.

Table 3.3. Pearson correlation coefficients for tests comparing RIL population BLUPs for heading date, grain fill duration, and days to maturity with agronomic traits within each location. The bottom four rows show correlations of mean RIL heading date, grain fill duration, and days to maturity for combined locations with the percent difference in agronomic traits between locations.
$p \geq 0.1$ ns, $p < 0.1$. , $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***

Agronomic Trait	**Heading Date**		**Grain Fill Duration**		**Days to Maturity**	
	Bozeman	Havre	Bozeman	Havre	Bozeman	Havre
Plant Height	0.361 ***	0.007 ns	-0.304 ***	-0.013 ns	0.012 ns	-0.011 ns
Yield	0.257 ***	0.015 ns	-0.174 **	-0.159 **	0.080 ns	-0.226 ***
Test Weight	-0.118 ns	-0.333 ***	0.272 ***	0.398 ***	0.284 ***	0.166 **
Plump	-0.040 ns	-0.323 ***	0.287 ***	0.510 ***	0.421 ***	0.355 ***
Protein	-0.192 **	-0.431 ***	-0.130 .	0.236 ***	-0.494 ***	-0.213 ***
Yield Percent Difference	0.004 ns		0.072 ns		0.092 ns	
Test Weight Percent Difference	0.072 ns		-0.068 ns		-0.004 ns	
Plump Percent Difference	0.340 ***		-0.480 ***		-0.288 ***	
Protein Percent Difference	0.022 ns		-0.256 ***		-0.384 ***	

Correlation analysis comparing heading, maturity, and grain fill BLUPs across locations to the percent difference in yield and quality traits between locations indicated association of more consistent percent plump with earlier heading and later maturity. Percent protein was more consistent between locations when maturity was later, but there was not a relationship with heading date. Consistency of test weight and yield between locations were not correlated with grain fill timing.

A pattern emerged when comparing the traits from the root roll-up seedling assay to the Bozeman and Havre field trial traits. A smaller root to shoot length ratio was correlated with

later maturity (r = -0.539*** in Bozeman, -0.369*** in Havre), greater test weight (r = -0.340*** in Bozeman, -0.343*** in Havre), and lower percent grain protein (r = 0.454*** in Bozeman, 0.359*** in Havre). The full set of correlations between field traits and seedling assay traits is presented in Table A3.4.

Identification of QTL

Genetic mapping revealed a total of 79 QTL associations from the field study and 32 from the root roll-up assay. These are listed in Table A3.5. There were six main loci displaying of co-segregation between grain fill timing and duration, grain quality, and seedling root traits (Table 3.4). In some cases, QTLs were observed in both environments, although with different effect sizes, but never opposite effects. In other cases, the association was only observed in one environment. On chromosome 2H, the allele from the ND19119 parent (ND allele) at *QGFhd-2H* was associated with earlier heading, higher grain protein, and shorter plants in both locations, and plumper grain in Havre. There were also associations with this locus for lateral root count and shortest root length in the root roll-up assay. Nearby, *QLN-2H* contained the highest number of QTLs for seminal root traits, with the ND allele extending the length of the longest and shortest roots and the root length range. Grain plumpness and plant height in Bozeman were the only traits from the field trials to map to this locus. *QDD-5H* contained smaller effect QTLs where the ND allele delayed development at the one-leaf stage, heading, and maturity, and increased longest root length, root length range, and shoot length. At *QGFmt-6H*, the ND allele was strongly associated with later maturity and lower protein in both field locations, as well as a smaller root to shoot length ratio in the root roll-ups. The *QGFmt-6H* ND allele was also associated with plumper grain and higher test weight in both locations. This locus overlapped the

position of the *HvNAM1* KASP marker (Table 3.4). At *QGFhd-7H*, it was the allele from the MT124118 parent (MT allele) that was associated with earlier heading and this was much stronger in Bozeman. The ND allele at *QGF-7H* was associated with longer grain fill in both locations, but this was stronger in Havre where it was also associated with greater percent plump. *QGF-7H* was the only locus where the grain fill period was extended on both ends by the same allele.

Table 3.4. Highlighted QTLs identified for traits from the field trials and the root roll-up assay. Chromosomes are indicated by the end of QTL names. LODs for all QTLs are above the $\alpha = 0.05$ LOD significance threshold determined for that trait. A BZ suffix in the trait name indicates a trait from the Bozeman field trials, HV for Havre, and RRU for the root roll-up assay.

QTL Trait	Position (cM)	LOD	Percent of Variance	Effect of ND Allele	Co-locating Trait and Reference
QGFhd-2H					
Longest Root Length, RRU	79.8	3.8	6.1	-	
Plump, HV	81.7	7.6	11.5	+	
Plump Percent Difference	81.7	7.9	14.4	-	Flowering time - Alqudah et al., 2014
Shortest Root Length, RRU	84.6	4.1	10.7	-	
Lateral Root Count, RRU	85.5	6.8	13.9	-	Flowering time - Dang et al., 2022
Heading Date, HV	85.5	22.4	40.3	-	
Heading Date, BZ	85.5	47.2	47.7	-	Multiple malt quality measures, plump, test weight, plant
Grain Fill, HV	85.8	18.9	25.9	+	height, heading date - Pauli et al., 2015
Plant Height, HV	85.8	8.7	12.7	-	
Protein, HV	85.8	10.7	13.3	+	Seedling root depth - Jia et al., 2019
Plant Height, BZ	86.0	15.8	15.5	-	
Protein, BZ	86.0	15.6	17.7	+	Multiple seedling root traits - Khodaeiaminjan et al., 2023
Grain Fill, BZ	86.8	26.4	22.0	+	
Days To One-Leaf, RRU	87.4	4.5	5.9	-	
QLN-2H					
Shortest Root Length, RRU	143.0	3.1	6.5	+	
Longest Root Length, RRU	145.6	12.3	22.6	+	
Shoot Length, RRU	145.6	11.1	13.7	+	Seedling root trait - Khodaeiaminjan et al., 2023
Plant Height, BZ	147.8	4.5	3.8	+	
Plump, BZ	147.8	5.8	6.3	+	Plump - Pauli et al., 2014
Days To One-Leaf, RRU	148.0	12.5	18.5	+	
Root Length Range, RRU	149.0	7.9	14.6	+	
QDD-5H					
Days To Maturity, BZ	174.3	5.4	7.8	+	
Heading Date, BZ	175.0	4.1	1.9	+	
Days To One-Leaf, RRU	178.7	6.1	8.2	+	Flowering time - Alqudah et al., 2014
Root Length Range, RRU	181.2	4.7	8.4	+	
Longest Root Length, RRU	182.2	3.9	6.4	+	Multiple malt quality measures - Pauli et al., 2015
Days To Maturity, HV	185.0	6.7	11.3	+	
Shoot Length, RRU	187.2	7.2	8.5	+	

Table 3.4 Continued.

QGFmt-6H					
Test Weight, HV	52.0	6.1	11.5	+	
Plump, BZ	54.0	5.5	6.0	+	
Plump, HV	58.8	6.2	9.3	+	
Plump Percent Difference	59.0	4.4	7.6	-	
Shoot Length, RRU	59.0	3.0	3.3	+	
Protein, HV	66.0	22.3	32.6	-	
Protein, BZ	66.0	34.4	51.9	-	Protein - See et al., 2002; Distelfeld et al., 2008
Grain Fill, BZ	66.8	21.0	16.1	+	
Longest Root : Shoot, RRU	67.0	14.1	29.3	-	Days to maturity, protein, plump, test weight - Alptekin et al., 2021
Protein Percent Difference	67.4	14.5	30.3	-	
Longest Root Length, RRU	68.4	4.6	7.5	-	
Test Weight, BZ	68.4	8.8	14.0	+	
Days To Maturity, BZ	69.0	18.5	32.2	+	
Grain Fill, HV	70.0	6.6	6.6	+	
Days To Maturity, HV	70.2	10.2	18.0	+	
Days To One-Leaf, RRU	72.4	3.0	3.9	-	
QGFhd-7H					
Protein Percent Difference	39.0	4.2	7.5	+	
Grain Fill, BZ	41.5	4.2	2.5	-	Flowering time - Alqudah et al., 2014
Heading Date, BZ	41.5	32.1	25.5	+	
Plant Height, BZ	41.5	6.1	5.2	+	Flowering time - Dang et al., 2022
Heading Date, HV	44.0	4.1	5.6	+	
Grain Fill, HV	46.0	4.4	4.4	-	Plant height - Pauli et al., 2014
QGF-7H					
Test Weight, BZ	72.9	4.0	5.9	+	
Heading Date, HV	76.8	5.0	7.0	-	
Plump, HV	76.8	8.0	12.2	+	Flowering time - Alqudah et al., 2014
Plump Percent Difference	76.8	6.0	10.6	-	
Days To Maturity, HV	78.0	3.3	5.2	+	Multiple seed size measures -
Days To One-Leaf, RRU	79.9	5.5	7.3	+	Gordon et al., 2020
Grain Fill, HV	80.8	10.7	10.7	+	
Heading Date, BZ	80.8	6.0	3.2	-	Heading date, plant height, test weight, plump -
Shoot Length, RRU	80.8	5.2	6.0	+	Pauli et al., 2015
Grain Fill, BZ	81.1	4.9	3.0	+	
Plant Height, HV	85.0	4.5	6.1	+	

0

The relationship between traits at one locus was not consistent across QTLs (Table 3.4). The timing of developmental stages (one-leaf, heading, and or maturity) co-segregated with the same direction of allelic effects at *QDD-5H* and *QGFhd-2H*, but there were opposite allelic effects on developmental timing at *QGFmt-6H*, and at *QGF-7H*, the ND allele was associated with earlier heading but later maturity and one-leaf stage. Likewise, there were opposite directions of allelic effects for grain fill duration and plant height at *QGFhd-2H* and *QGF-7H*, but effects for these traits were in the same direction at *QGF-7H*. The length of the shoot and the root in the root roll-up assay were both increased by the ND allele at *QLN-2H* and *QDD-5H*, but at *QGFmt-6H*, the shoot length was increased but the root length was decreased.

Several QTLs (*QGFhd-2H*, *QGFmt-6H*, *QGFhd-7H*, and *QGF-7H*) were associated with quality consistency, so the percent difference in plump, protein, or both between the Bozeman and Havre locations (Table 3.4). A smaller percent difference in quality indicates more stable trait expression between locations, or in other words more resistance to environmental heterogeneity. Figure 3.1 shows that the alleles associated with longer grain fill were also associated with smaller percent differences in plump and or protein between locations due to quality improvement with respect to requirements for malt.

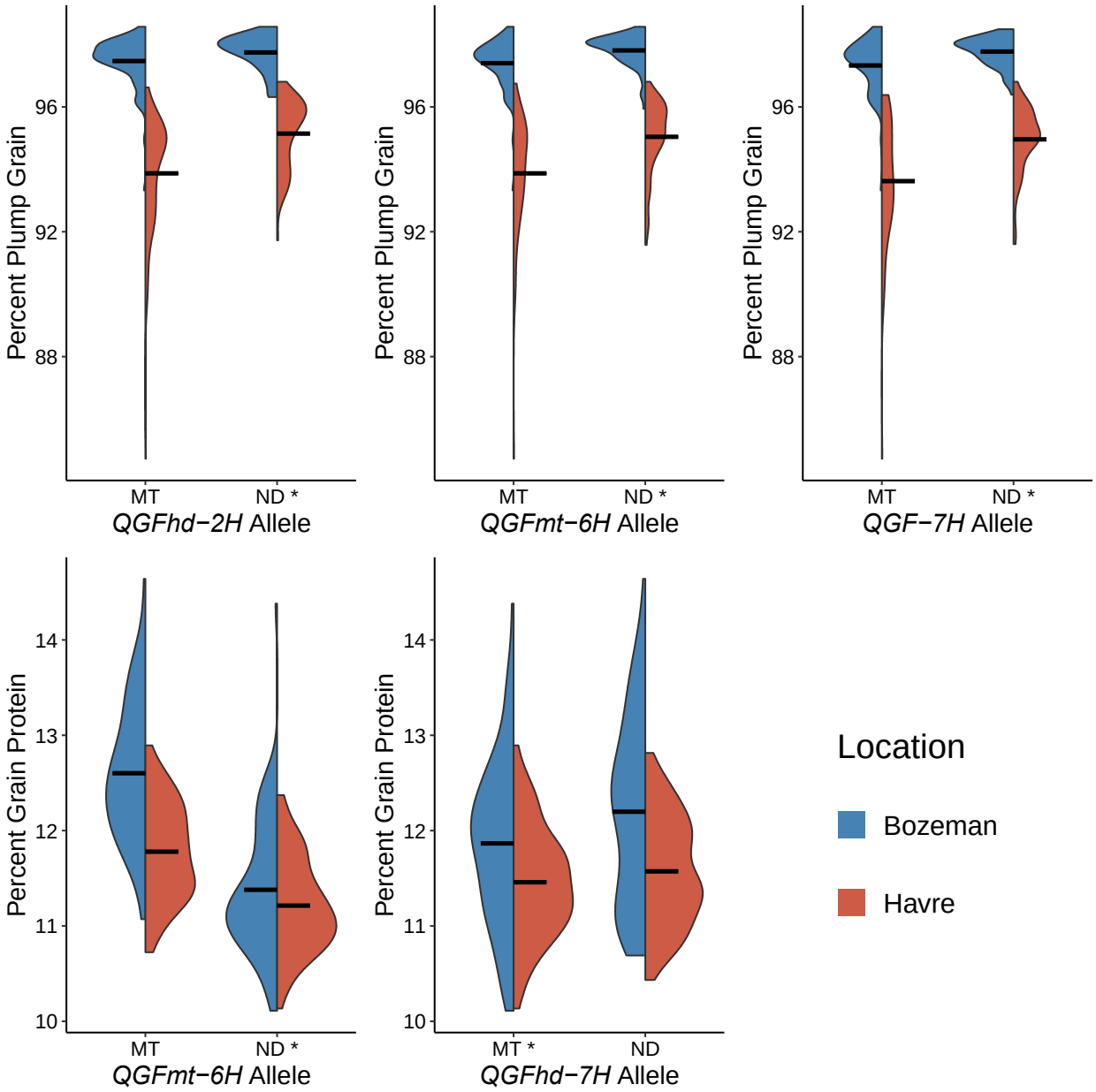

Figure 3.1. Split violin plots of percent plump grain and percent grain protein in Bozeman and Havre field trials at QTL positions for the percent differences of those traits between locations. The parental allele associated with a smaller difference or more consistent trait expression (as well as longer grain fill) is indicated with an asterisk. Horizontal bars represent trait means by location and allelic group.

Discussion

In the Western US, cultivating malt barley without irrigation is becoming increasingly risky for growers as lack of water during grain fill decreases plumps and increases protein (Tarawneh et al., 2020), negatively impacting quality such that grain is rejected for malting. In this study, through genetic dissection, we identified QTLs related to seminal root traits, plant development, and grain quality that may help to stabilize malt quality especially under dryland conditions. Our work supports and is supported by the findings of previous mapping analyses of these same and related traits in barley (Table 3.4).

Earlier Heading

Grain fill duration was impacted by heading date at *QGFhd-2H, QGFhd-7H,* and *QGF-7H*, and earlier heading appeared to benefit crop performance in our work. Negative correlations between plumpness and timing of development around heading have been documented before (Shirdelmoghanloo et al., 2022), and here we observed not only greater plumps with earlier heading (Table 3.3), but also more stable plumps due to plump improvement in drier environment at *QGFhd-2H* and *QGF-7H* (Figure 3.1). Dang et al. (2022) mapped flowering time in barley near *QGFhd-2H* and Alqudah et al., (2014) mapped flowering time near both *QGFhd-2H* and *QGF-7H*, as did Pauli et al. (2015) for heading date. The Pauli study also mapped plump and test weight near both loci, and multiple direct measures of malt quality such as beta-glucan content, free amino nitrogen, and malt extract near *QGFhd-2H*. Gordon et al., (2020) identified QTL near the *QGF-7H* locus as being associated with measures of barley seed size under terminal drought conditions. An early start to the grain fill period could be particularly beneficial for starch accumulation in environments like Havre that depend on finite stores of soil moisture.

The earlier heading *QGFhd-2H* ND allele was associated with greater plumps in Havre and higher protein levels in both locations (Table 3.4). This runs counter to the idea that grain size and protein content are inversely related, but research has shown that high soil nitrogen levels may be required for the inverse relationship (Magliano et al., 2014). Soil fertility at our Havre location was quite low (Table 3.1) so it is not entirely unexpected that both plump and protein were associated in the same direction with heading there. While lower percent protein content is generally better for malt quality, there is a lower bound to the ideal range of about 9.0% (Kumar et al., 2013). The protein minimums for the RIL population in both of our field locations were approaching that limit (Table 3.2). Earlier heading was correlated with greater percent protein and greater percent plump in Havre, but these correlations were weak or absent in Bozeman (Table 3.3) where soil nitrogen levels were much higher (Table 3.1). It could be that earlier heading enabled use of additional nitrogen for grain production relative to lines with later heading dates in Havre where nitrogen was more limited. It would be interesting to investigate *QGFhd-2H* further for potential improvement of nitrogen use efficiency. Grain quality was more affected by heading in the drier location and by maturity where there was more moisture.

Later Maturity

The timing of maturity also has important impacts on grain quality in different environments. Grain fill duration was affected by days to maturity at *QGFmt-6H and QGF-7H* in our study. Correlations and QTL co-segregations connected later maturity to better malt quality at both of our field study locations (Table 3.3, Table 3.4). Longer retention of green leaf area has benefitted grain plumpness in other studies, although this dynamic can change depending on plant genotype (Emebiri, 2013). More time to maturity was also correlated with more consistent

plump and protein between our locations (Table 3.3). The ND allele at *QGF-7H* was associated with later maturity and plumper grain in Havre, and a smaller percent difference in plump between locations due to this quality improvement in the drier environment (Table 3.4, Figure 3.1). It appears that a longer grain fill period may protect plumps from environmental variability.

Low heritability values for days to maturity and percent protein at our Havre location (Table 3.2) could call into question the QTL results for those traits. Heritability values are reduced by large environmental impact, which was likely pronounced in Havre with the low precipitation and fertility rates (Table 3.1, Table A3.3). Large population sizes with low replication of genotypes can also result in lower heritability values (Zila et al., 2014), and our model for heritability calculation depended on many unreplicated RIL genotypes in our augmented design. The model for QTL mapping however, considered alleles which are abundantly replicated amongst the RIL population. Other instances have been observed in RIL research where traits exhibit low heritability but robust QTL mapping results (Zhang et al., 2017). Furthermore, alignment of QTLs for maturity and protein in Havre with results from Bozeman where heritabilities for those traits were higher (Table 3.2), and the fact that other studies have also mapped maturity and protein to loci at or near *QGFmt-6H* and *QGF-7H* (Table 3.4) give us confidence in these QTLs.

The *QGFmt-6H* ND allele was even more strongly associated than *QGF-7H* with delayed maturity in both locations, and with greater test weight, plumper grain, lower protein, and smaller plump and protein differences between locations via quality improvement (Table 3.4, Figure 3.1). Our KASP assay placed the marker for the *HvNAM1* NAC transcription factor gene within this chromosome 6H locus (Table 3.4). Previous work (See et al., 2002; Distelfeld et al.,

2008; Alptekin et al., 2021) demonstrated that an allele for *HvNAM1* found in the barley variety Karl was associated with delayed senescence, lower grain protein content, and increased plumps and test weight compared to the allele found in most barley varieties. The ND19119 parent to our RIL population carries the Karl allele for *HvNAM1* and presents these corresponding phenotypes compared to the MT124118 parent which carries the more common allele (Table 3.2). NAC transcription factors are also involved in stress responses and senescence processes in several species (Nuruzzaman et al., 2013; Thomas and Ougham, 2014), so it is not surprising that one would be involved in the timing of maturity and grain quality in our dryland field trials.

Seedling Root Traits

Allocating energy to roots versus aerial structures is a balancing act for plants between capturing resources aboveground (sunlight) or belowground (water and nutrients). This balance can shift over the course of a plant's life in response to developmental and environmental cues especially abiotic stress (Koevoets et al., 2016). In a previous study (Williams et al., 2022), we used minirhizotrons to examine the root systems of barley lines varying for grain fill duration as they grew field trials at the same Havre location and performed the root roll-up assay on those same lines. Correlations between root roll-up traits, field root traits during grain fill, and agronomic traits, while circumstantial, motivated the genetic dissection of root traits in a population segregating for grain fill duration in the current study. However, resource limitation prohibited minirhizotron examination of the large mapping population, so root observations were limited to the root roll-up traits as tentative proxies. Multiple correlation and co-segregation relationships between seedling root traits and agronomic traits were observed in this study. QTLs are large genetic regions with respect to gene sequences and while co-segregation of traits may

indicate pleiotropic effects of individual genes, it is perhaps more likely that the relationship is due to linkage of different genetic elements. Correlation and co-segregation are not indicative of causation, but do suggest hypotheses to investigate with future work.

A shorter root length compared to shoot length in the root roll-ups correlated with deeper and more prolonged root proliferation during grain fill in the previous minirhizotron study. In the present study, this smaller root to shoot length ratio correlated with later maturity and better grain quality (Table A3.4) and strongly co-located with these phenotypes at the *QGFmt-6H*/*HvNAM1* locus (Table 3.4). Involvement in roots has been shown for other NAC transcription factors in barley (Christiansen et al., 2011), but this is the first suggestion of an association between *HvNAM1* and root growth, which we hope to investigate further. Slower belowground growth versus faster aboveground growth at early stages may facilitate accumulation of photosynthetically derived energy stores that could be used to support deeper root growth under dry conditions later in development. Along these lines, when comparing two spring wheat genotypes under drought conditions, Reynolds et al., (2007) found that the genotype with higher yield and biomass distributed more of its root system to deeper soil depths, but had less root mass overall and a smaller root to shoot ratio shortly after anthesis. If the dynamic of slower root growth compared to shoot growth at the one-leaf stage were to continue through later vegetative stages, it may help to conserve soil moisture for use later in the season by reducing uptake by the roots. Early vigor of young shoots in cereals can also prevent soil moisture loss by shading the soil surface and preventing weed competition (López-Castañeda et al., 1996), and connections have been made between early vigor and the stay-green trait under drought stress in maize (Trachsel et al., 2016). Considering how root and shoot growth rates compare to each other at

early developmental stages could be more informative for understanding subsequent root system architecture and crop performance than considering either in isolation.

Seedling root traits also mapped to *QGFhd-2H* and *QLN-2H* (Table 3.4). The involvement of these loci in root traits is supported by co-locations with other QTL studies. Jia et al. (2019) mapped barley seminal root system depth in rhizoboxes (similar to the seminal root length trait in our study) near *QGFhd-2H*. Khodaeiaminjan et al., (2023) examined the roots of three-week-old barley seedlings in paper pouches under osmotic stress and mapped root number and root system area near *QGFhd-2H*, and root system length near *QLN-2H*, further suggesting that these loci could be involved in root phenotypes contributing to drought adaptation. Of course, the one-leaf stage is distant from reproductive and maturity stages and connections drawn between seedling assay and field traits are speculative. However, correlations and co-locations between seminal root traits and agronomic traits have been presented before (Ali et al., 2015; Robinson et al., 2018) and have laid the groundwork for subsequent research that has identified causal genes behind the connections (Feng et al., 2022; Kirschner et al., 2021). More research is required to discover how seminal root trait loci might associate with root phenotypes throughout development in the field and how such phenotypes could impact crop performance and environmental adaptation.

Utility of Alleles in Different Environments

Although we did not observe any opposite effects of alleles in our study, there were differences in the presence and strength of effects, suggesting different alleles may be preferrable for breeding depending on the target environment. Shorter roots in our seedling assay were related to better quality in both locations at *QGFmt-6H* (Table 3.4), but at *QGFhd-2H* shorter

roots only associated with better quality at the Havre location, and at *QLN-2H* longer seedling root length was related to better quality in Bozeman. Havre was the drier of the two locations (Table 3.1), so having small roots early on may be more important in environments with less stored soil moisture. Havre was also the location where earlier heading had a more positive impact on grain quality, and it has been suggested (Voss-Fels et al., 2018) that when plants depend on water deep in the soil profile to withstand terminal drought, an early flowering time combined with deeper roots is an effective strategy for maintaining crop performance. Under such conditions, it could be beneficial to start putting water towards starch accumulation sooner, considering water extracted during grain fill is directed more towards grain production than water extracted during vegetative phases (Vadez, 2014). In support of this, Carter et al., (2019) found that barley varieties adapted to water stress flowered earlier, had more roots deeper in the soil at maturity, and used more water after rather than before anthesis in their field study. If water stores are sufficient and root systems can access them, then delayed maturity could further promote quality grain production. This may have been the case in the Bozeman location where precipitation levels are higher overall but still low during grain fill (Table 3.1), and better grain quality was more tightly correlated with later maturity than with heading (Table 3.3).

Grain plumpness was increased by ND alleles for *QGFhd-2H, QGFmt-6H* and *QGF-7H*, and the MT allele for *QGFhd-7H* in both environments. However, the utility of the same QTLs to improve protein depends on the end use and soil fertility conditions. For example, the ND allele at *QGFmt-6H* decreases grain protein, perhaps even below acceptable levels in low nitrogen environments, but could potentially be deployed with higher fertility to maintain low protein for malt while yields are increased. Conversely, the ND allele for *QGFhd-2H* increases

protein and so could be deployed in low nitrogen environments and to increase protein levels for feed, forage, and food barley. Of course, to more fully understand how these alleles function and might behave in a wider variety of environments and genetic backgrounds, more research is needed.

Overall longer grain fill led to better more consistent crop performance with respect to malt quality indicator traits in our study, however the effect on yield was neutral. While the stay-green trait can provide higher yield under dry conditions for sorghum (Borrell et al., 2014), maize (Trachsel et al., 2016), and wheat (Christopher et al., 2008), mixed or neutral relationships between yield and stay-green indicators have been observed in barley (Emebiri, 2013) and rice (*Oryza sativa* L.) (Kamal et al., 2019), so our yield results are not without precedent. Yield is a highly complex trait. Although the parent varieties in this study differed for development and grain quality, they did not differ phenotypically for grain yield. They thus may not have differed for yield genetically, resulting in low heritability of this trait (Table 3.2) and lack of correlation and QTL results for yield in our work. However, since a much higher price is paid for a barley crop if it receives the malt designation (USDA NASS) which is largely based on plump and protein (AMBA, 2021), a neutral or even slightly negative impact on yield would be acceptable if these malt parameters were reliably and sufficiently improved.

Conclusion

Here we have demonstrated a relationship between extended grain fill duration and improved and consistent grain quality for malting, and have identified associated QTLs. The relationships between traits observed in our correlation and QTL analyses in connection with the results from our field study of roots (Williams et al., 2022) suggest a plant strategy for producing

high quality barley grain under terminal drought conditions. Slower growth belowground compared to faster growth aboveground early in the season could gather energy to support the growth of a deeper mature root system and or save soil moisture to be available for filling grain. An earlier start to grain fill and deeper root system would allow more resources to be put towards seeds than other plant structures. Delayed senescence aboveground and belowground could further enhance quality grain production if water stores lasted.

Understanding the genetic component in these interactions is important for breeding malt barley varieties suited to different cultivation areas and conditions. The present study lays the groundwork for this but has limitations. QTLs are large genetic regions with respect to actual genes, and the relationships between traits and with genetic loci presented in this study are not causative or fully elucidated. Similarly, a greenhouse seedling assay is far removed in developmental time and environmental condition from field trials on farms, and the connections we have drawn between traits from these different experiments are speculative. We plan to address these remaining knowledge gaps in future work with near isogenic lines (NILs) we have developed for the key QTLs identified here. These will allow us to confirm QTLs, narrow their genetic regions with fine mapping, and identify gene candidates. We also plan to directly examine the impact of these alleles on root systems in the field throughout development with the use of these NILs and minirhizotrons. We hope this work will be helpful to scientists developing the barley varieties of the future.

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Description of Appendix Material

Table A3.1 provides the DNA sequence used as the basis for the KASP primers for *HvNAM1* genotyping. Information about marker numbers and distances used in the linkage map are presented in Table A3.2. Table A3.3 shows the significance levels for the effect of location on field trial traits in ANOVAs. Results from correlation tests comparing field traits and root roll-up traits are presented in Table A3.4. Table A3.5 is a complete list of QTLs identified in this study and shows references to co-locations between our main QTLs and QTLs from other mapping studies.

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CHAPTER FOUR

BARLEY GENETICS INTERACTING WITH CONTRASTING ENVIRONMENTS INFLUENCE RHIZOSPHERIC BACTERIAL COMMUNITY COMPOSITION AND AGRONOMICS

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Abstract

Microorganisms assembled into the plant rhizosphere from the surrounding soil can benefit the fitness of their host. Previous research has linked variations in plant genetics to variations in rhizospheric microbial community composition. An attractive goal is to boost crop production and or reduce reliance on synthetic agricultural inputs by using plant breeding to enhance recruitment of beneficial microbiomes. However, what impact the abiotic environment has on connections between microbes and host genetics, and whether those connections in turn impact crop performance in realistic agricultural scenarios is still unclear. We assessed agronomic performance and 16S sequence-based rhizosphere bacterial community composition on a large diverse barley population grown at two semi-arid field locations in a major barley production area. Genome wide association analysis comparing these characteristics to barley genetic loci revealed a few instances of co-localization between agronomic traits and the relative abundances of specific microbial taxa. There were also some loci that were linked to microbial traits in both field locations, but most associations were location specific. Here we place these preliminary results in the context of this rapidly growing research field, and lay out ideas for additional analysis in this ongoing research project.

Introduction

Utilizing plant-beneficial microorganisms to reduce dependence on agrochemicals is an attractive idea against the backdrop of high agrochemical costs, global population growth, climate change, and calls for sustainable agriculture (Lau et al., 2022; Maja and Ayano, 2021). Soil-borne microbes can benefit plant health and function by facilitating the acquisition of

nitrogen, phosphorus, and other nutrients (Rosenblueth et al., 2018; Pii et al., 2015), stimulating hormonal pathways to promote plant growth (Babalola et al., 2021), and protecting against pathogens (Mendes et al., 2011) and abiotic stressors such as drought (Poudel et al., 2021). The development of microbial inoculants as a biological solution to some of agriculture's environmental degradation issues is an active area of research producing mixed results (Alori et al., 2017). Large scale production, distribution, application, and establishment of these products pose hurdles to their efficacy (Kaminsky et al., 2019). A different tactic that began receiving attention more recently is the idea of breeding crop varieties that have an enhanced ability to use microbes to their advantage (Gopal and Gupta, 2016; Contreras-Liza, 2021; Clouse and Wagner, 2021). To do this, a detailed understanding of the impacts that plants and microbiomes have on each other and how this is altered by varying environmental conditions is needed. Thus, large knowledge gaps remain to be filled before microbial communities can be predictably manipulated via host genetic selection to benefit crop performance.

The term microorganism or microbe simply refers to an organism that is microscopic in size. A microbiome is a community of microbes cohabitating in a given environment. In soil habitats this includes bacteria, fungi, viruses, archaea, protozoa, and microscopic animals such as nematodes. However, most studies researching plant-associated microbes use the term to refer only to bacteria and to a lesser extent fungi, as these organisms have abundant, diverse, and impactful relationships with plants (Bardgett and Van Der Putten, 2014). The present paper will focus on bacteria. Different microbiomes exist within different compartments of plants (Gopal and Gupta, 2016). The term rhizosphere was first introduced by Lorenz Hiltner in 1904 to describe the area of soil immediately surrounding and impacted by plant roots. It is the site of

nutrient and water acquisition for plants and a hot spot of microbial activity (McNear, 2013). While soil microbial communities are driven by edaphic factors, the rhizosphere harbors a unique microbiome that is assembled from the soil microbiome and driven by plant traits (Bulgarelli et al., 2013). The characteristics of microbial communities living in close association with a plant can be regarded as extended phenotypes of the host (Dawkins, 1982; Whitham et al., 2003).

Only traits under the influence of plant genetics can be targeted by selection in plant breeding. The fact that rhizosphere microbial communities differ from those in bulk soils (Bulgarelli et al., 2015; Alegria et al., 2020) demonstrates on a basic level that plants do influence these assemblages. Contrasts between rhizosphere microbiomes of different plant species (Berendsen et al., 2012) take that demonstration a step further. Studies have also been able to attribute some rhizosphere microbiome variation to plant genotype which shows heritability of these extended phenotypes (Peiffer et al., 2013; Walters et al., 2018), suggesting it may be possible to select for them. But heritability alone is not sufficient for directed breeding efforts. Knowledge of the specific genetic loci involved is needed to lead to discovery of the plant genes and mechanisms involved in microbe interactions, and provide the necessary information for allelic selection. Recent research has begun to uncover some of these loci through genetic mapping of root and rhizosphere microbiome extended phenotypes in Arabidopsis (Bergelson et al., 2019), sorghum (Deng et al., 2021), switchgrass (Sutherland et al., 2022), tomato (Oyserman et al., 2022), barley (Escudero-Martinez et al., 2022), and foxtail millet (Wang et al., 2022). These studies have minimized variation in the plants' abiotic and biotic environment by providing a uniform starting soil microbial community, growing in a controlled

environment, and or examining a single field location and time point. This may aid in detecting the effects of plant genetics, but overlooks the effects of important genetic by environment interactions. Some studies have begun to incorporate these interactions into plant microbiome research. A study by Meier et al., (2022) performed host genetic mapping of the maize rhizosphere microbiome under different nitrogen treatments, and a study by Edwards et al., (2023) mapped the root microbiome of switchgrass varieties with contrasting evolutionary histories in different corresponding environments. Microbial communities are sensitive to a plethora of environmental factors such as water and nutrient availability, so the success of manipulating the rhizosphere microbiome for agricultural systems through crop breeding depends on incorporating a deep comprehension of plant-microbe-environment interactions (Bano et al., 2021).

It has been postulated that plant interactions with soil microorganisms in their center of domestication impact plant fitness and therefore have implications for their selection history (Alegria et al., 2020), although conclusions on this subject are not always straightforward. The Edwards switchgrass study (2023) found evidence of an affinity between host subpopulations and the local microbiota of their native geographic range, with some exceptions. A predisposition for interaction between plants and microbes native to the same region may contribute to local adaptation or home field advantage where a cultivar performs exceptionally well in a specific location (Ewing et al., 2024). Understanding these dynamics could be critical for effectively breeding for beneficial rhizospheric microbial interactions, and there is more work to be done in this arena.

As a self-pollinating diploid, barley lends itself to genetic study more readily than most cereals and grasses. Barley is also an important cereal crop globally and within the United States (Newton et al., 2011). Montana is one of the primary growing areas for barley and drought poses the most significant challenge to its growth there (USDA NASS, 2022). Breeding cultivars with heightened ability to reap benefits from rhizospheric microbes, especially drought protection, would be a boon to the Montana barley industry. Escudero-Martinez et al., (2022) identified barley genetic loci and candidate genes associated with the rhizosphere microbiome in a controlled environment experiment using linkage mapping of a biparental population, but examination of the rhizosphere microbiome in the context of expanded environmental and genetic diversity is needed.

Using a diverse barley population compiled from several different breeding programs (Spring Two-Row Multi-Environment Trial (S2MET) panel; *Hordeum vulgare* L.; Neyhart et al., 2019), we performed field trials in multiple locations within as well as far outside barley's growing region to investigate connections between barley genetics, rhizosphere bacterial and fungal community characteristics, and adaptation to environments indicated by agronomic performance. This is an ongoing study and here I will present some preliminary results on bacteria and agronomics from the first year of field trials at two locations in Montana. I will also set the stage for subsequent analysis and experimentation and review other relevant research. Our project specifically seeks to address the following research questions: 1) Are compositional variations in barley rhizospheric microbial communities heritable? 2) Can these variations be associated with specific barley genetic loci? 3) Are associations consistent across environments (could be broadly deployed in plant breeding) or environment specific (could be involved in

local adaptation)? 4) Is there co-localization of loci associated with microbial traits and with agronomic traits?

Methods

Description of Field Trials

Field trials were conducted in 2021 in Bozeman, MT at the Montana State University (MSU) Arthur H. Post Agronomy Farm (45.6°N, 111.2°W) and in Moccasin, MT at the MSU Central Agricultural Research Center (47.1°N, 109.0°W). Soil at the Bozeman location is an Amsterdam-Quagle silt loam, while at the Moccasin location there is a thin layer of fine Judith clay loam over a coarser layer with gravel and cobbles (USDA, 1998).

This study utilized 232 malt barley lines out of the Spring Two-Row Multi-Environment Trial (S2MET) population (Neyhart et al., 2019) from breeding programs at USDA-ARS Aberdeen in ID, University of Minnesota, Montana State University, North Dakota State University, and Washington State University. An augmented randomized complete block design (Federer, 1956) was used for the field trials in which each of the 232 experimental lines appeared once in the trial at each location, and four barley check varieties (Craft, Hockett, Lavina, and Merit 57) appeared at random positions in each of twelve blocks. This design is useful for large populations and multi-environment trials where replication of experimental lines is not practical, because it allows for estimation of within-environment variation via the replicated checks (Bernardo, 2010).

The Bozeman trial was planted on April 30th, and the Moccasin trial on May 6th. CruiserMaxx Vibrance Cereals (Syngenta) was used to treat seed prior to planting for broad range pest and pathogen protection. In Bozeman, 90 lbs ac^{-1} of 46-0-0 fertilizer was applied prior

to planting. In Moccasin, 50 lbs ac^{-1} of 20-30-20-10 fertilizer was applied at planting and an additional 100 lbs ac^{-1} of urea after planting. Composite soil samples from the top six inches of the check plots were collected for each block in each trial at the time of rhizosphere sampling using hand soil core samplers, and were sent for chemical analysis (Table 4.1). Soil was tilled prior to planting in Bozeman as is common practice on that farm, whereas the Moccasin farm has been under no-till management for over 15 years. Alleys between ranges were planted with fill barley in Moccasin and left bare in Bozeman. Both trials were rainfed and there was a severe drought throughout the state during the 2021 growing season. Precipitation data were collected on both research farms and pulled from NOAA climatological summaries (NOAA NCEI) (Table 4.2).

Table 4.1. Means and standards deviations across blocks for soil chemistry.

Soil Property	**Bozeman**	**Moccasin**
Soil pH	6.0 $\pm$ 0.1	7.4 $\pm$ 0.2
Nitrate (ppm)	7.7 $\pm$ 4.5	3.0 $\pm$ 0.6
Phosphorus (ppm)	54.4 $\pm$ 5.6	49.1 $\pm$ 8.6
Potassium (ppm)	287.3 $\pm$ 22.5	280.8 $\pm$ 29.4
Sulfate (ppm)	5.2 $\pm$ 0.6	13.1 $\pm$ 2.0
Organic Matter (%)	3.3 $\pm$ 0.1	4.9 $\pm$ 0.2

Table 4.2. Weather data for the two field trials. Precipitation totals are shown for the period of time from the end of the previous growing season to the start of the field trial season, and for each month during the field trials from planting (April, 30th for Bozeman and May, 6th for Moccasin) to mean maturity (July 30th for Bozeman and August, 1st for Moccasin). Mean temperature is shown for each month during the growing season.

Time Period	**Precipitation (in.)**		**Mean Temperature (°F)**	
	Bozeman	Moccasin	Bozeman	Moccasin
September 2020 to April 2021	5.82	4.24	-	-
May 2021	3.1	2.68	50.2	47.8
June 2021	0.8	0.6	65.5	64.4
July 2021	0.83	1.06	72.5	72.3
August 2021	-	-	65	64.7
Total in-season	4.73	4.34	-	-
Total	10.55	8.58	-	-

Agronomic Data Collection

The barley population was assessed for various agronomic traits in the field trials. Heading and maturity dates (stages 59 and 89 on the Zadoks et al. (1974) cereal growth scale) were noted when seed heads were visible for half of the plants in a plot and when half of the seed heads had lost green color respectively. Duration of the grain fill period was calculated by subtracting heading date from maturity date. Plant height was measured after maturity from the base of the plant to the top of the seed head. After the plants had dried down, before harvest, three 6 in. sections were cut from each plot by hand and were weighed for total biomass. Seed heads from these samples were counted to measure productive tiller number and were removed and threshed. The weight of this seed was divided by the total biomass to calculate harvest index. Full plots were harvested by combine after grain ripening, and seed was weighed for gross yield calculations. Test weight, percent plump grain, and percent grain protein were measured on cleaned grain samples from each plot.

Rhizosphere Sample Collection,
Processing, and Sequencing

Root samples from field plots were collected between late elongation (Zadoks 37) or early booting (Zadoks 43) phase depending on the line. This occurred on June 16th in Bozeman and June 24th in Moccasin. Trowels were used to dig up six plants from throughout each plot. The goal was to extract the top six inches of the root systems out of the soil, but this was not always achieved. Shoots were removed from roots and loose soil was shaken off, retaining soil that was adhered to the roots. All roots from an individual plot were put into a ziplock bag together and these composite samples were kept on ice in coolers until being brought back to the lab. There samples were stored at 4°C while awaiting processing over the next few days.

To extract rhizosphere samples from the root system samples, roots with adhered soil were placed in 50 mL conical tubes with 30 mL of 0.9% NaCl and sonicated in a sonicating water bath for one minute. The contents were then poured through a mesh strainer into a small cup and the flow through was returned to the conical tube. A fresh cup was used for each sample and the strainer was cleaned and sanitized between samples. Rhizosphere soil in the conical tubes was then pelleted by centrifuging at 2000 x g for 5 minutes. Supernatant was discarded, pellets were resuspended in 1 mL sterile water and transferred to 1.5 mL microcentrifuge tubes which were centrifuged, then supernatant was again discarded and pelleted rhizosphere samples were stored at -80°C. The bulk soil samples that were collected for chemical analysis as described above were also run over a sieve and a subsample was stored in a 1.5 mL tube at -80°C. Genomic DNA was extracted from these samples using the DNeasy Power Soil Pro kit from QIAGEN following the manufacturer's instructions.

DNA samples were submitted to Integrated Microbiome Resource (Dalhousie University, Halifax, NS) for amplicon sequencing of the bacterial 16S V6-V8 region. Sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a 2x350 PE kit. This is a highly conserved rRNA gene region that allows reliable amplification of intervening hypervariable regions that when sequenced indicate the presence of different operational taxonomic units (OTUs) or amplicon sequencing variants (ASVs) within a genomic sample. After sequencing, reads were filtered for quality and given taxonomic assignments down to the genus level using the "dada2" pipeline (Callahan et al., 2023) in R (R Core Team, 2022). Phyloseq objects were created for downstream processing and analysis of the Bozeman and Moccasin microbial datasets in the "phyloseq" package (McMurdie and Holmes, 2013).

Analyses

Phenotypic Data Preparation The rhizospheric microbial extended phenotypes examined were relative abundances of different bacterial phyla and genera determined for each plot. It is important to note that 16S sequencing datasets are compositional in nature and indicate relative abundances of taxa rather than absolute quantities (Gloor et al., 2017). The approach determines the number of times an ASV appears in a sample, but there is a limit to the total number of sequence calls that are made during amplicon sequencing. This results in the potential for abundant taxa to be overrepresented and rare taxa can be underrepresented or missing, thus, these datasets must be processed and interpreted carefully. Nevertheless, this method is high-throughput and remains a powerful tool for microbial community research.

To prepare these relative abundance "traits" for analysis, ASV read counts were first agglomerated by either the phylum or genus level. Counts of 0 were replaced with the small value of 0.000001 prior to performing centered log ratio (CLR) transformations (Aitchison, 1982) on all ASV counts using the "compositions" package (Van den Boogaart et al., 2024). This transforms ASV counts into relative abundance representations that are comparable between samples.

While rare taxa can be interesting and informative for microbial community analysis (Lynch and Neufeld, 2015), traits that have a missing or null value for many entries in a GWAS panel do not lend themselves to genetic mapping. So, the prevalence of each phylum and genus was determined (i.e. the percent of barley population rhizosphere samples that had a count of at least one for that taxon). Phyla and genera with prevalence values of at least 95% in either location were retained in the datasets of both locations for further analysis.

For agronomic traits, ranges, means, and standard deviations were first calculated for the entire population in each field trial using raw values. Then best linear unbiased predictions (BLUPs) were calculated for each line to account for within-field variation utilizing the augmented design (Kehel et al., 2010). For each trait a mixed linear model:

$$Y_{ijk} = \mu + Checks_i + Block_j + Genotype_k + \varepsilon_{ijk}$$

was created using the "lmerTest" package (Kuznetsova et al., 2017) where $Checks_i$ was a fixed effect representing the replicated check varieties, $Block_j$ was also a fixed effect, and $Genotype_k$ was a random effect for the experimental barley lines from the S2MET panel. Coefficients of the Genotype factor corresponding to each line were the BLUPs used for GWAS analysis.

Heritability The linear models that were created to determine BLUPs of agronomic traits were also used to calculate heritability of those traits as discussed by Holland et al., (2003). For each phenotype, variance due to genotype was divided by the sum of the variance due to all random effects plus residual variance. This same method utilizing the BLUPs was also used to calculate the heritability of relative abundances of bacterial taxa.

GWAS The S2MET panel that was the source for lines used in this study was previously genotyped by sequencing as described in (Neyhart et al., 2019), and this genotyping data is publicly available (https://doi.org/10.13020/cp4r-0v95, Neyhart and Smith, 2019). After matching the genotyping data to the subset of S2MET lines that were included in the current experiments, markers with a minor allele frequency (MAF) less than 5% were removed from the dataset. This left 4,787 single nucleotide polymorphism (SNP) markers that were used in the genome-wide association study (GWAS).

Genetic mapping was carried out in the program GAPIT using the FarmCPU model (Wang and Zhang, 2021). Three principal components were used to account for population structure in addition to adjustments using the kinship matrix produced by GAPIT, and these parameters appeared to yield appropriately fit models for traits when examining QQ plots. Significance thresholds for trait marker associations were based on a value of $\alpha = 0.2$.

A window of 1 Mb was used to conduct an initial search for co-localizations of trait associated loci. These were also examined for overlap with quantitative trait loci (QTLs) from two different barley linkage mapping studies; one by Escudero-Martinez et al., (2022) that mapped barley rhizosphere bacterial communities in a greenhouse, and the Williams et al., (2024) study that mapped barley seminal root traits and agronomics. Positions for markers corresponding to the QTLs in these studies were located on the Morex 2 reference genome to compare to the marker positions in the current study which also utilize Morex 2.

Preliminary Results and Discussion

Contrasting Locations and Agronomic Performance

Differences in agronomic performance between environments generally followed expectations given that prior experience with breeding trials in these locations has shown Bozeman to be the wetter more productive environment. During the 2021 drought year, in-season precipitation was only slightly higher in Bozeman than Moccasin (Table 4.2), but the pre-season precipitation was substantially higher, and Bozeman soils likely have greater water holding capacity compared to the shallow rocky soils in Moccasin. Soil nitrate levels were also higher but more variable in Bozeman (Table 4.1). The barley population exhibited a shorter grain filling period in Moccasin mostly due to a later heading date (Table 4.3). Plants were larger overall in

Bozeman with greater mean plant height, biomass, and productive tiller number, although harvest index and test weight were slightly higher in Moccasin. Bozeman also had plumper grain and lower grain protein content, which are often inversely related (Magliano et al., 2014), and important for malt quality with high plump and low protein being more desirable (AMBA, 2021). Bozeman was the higher yielding location.

Table 4.3. Ranges, means, standard deviations, and heritability of agronomic traits for the population within each location.

Trait	**Bozeman**					**Moccasin**				
	Min	Max	Mean	SD	H^2	Min	Max	Mean	SD	H^2
Heading Date (Julian)	173	188	179.2	2.8	0.902	178	193	188.9	1.8	0.000
Maturity Date (Julian)	207	218	211.7	2.6	0.649	207	216	212.6	2.3	0.070
Grain Fill Duration (Days)	24	40	32.5	3.3	0.781	19	37	23.7	2.7	0.000
Plant Height (cm)	42.0	67.0	57.5	4.3	0.338	15.2	50.8	41.1	5.4	0.481
Biomass (g m^{-2})	829	1734	1221	154	0.005	137	528	327	68	0.096
Productive Tiller Number	352	962	615	100	0.150	39	488	310	61	0.054
Harvest Index	0.232	0.619	0.410	0.062	0.000	0.322	0.619	0.522	0.060	0.271
Yield (kg ha^{-1})	2271	5497	4289	511	0.709	341	2091	1057	320	0.286
Test Weight (kg hl^{-1})	61.9	69.8	66.1	1.4	0.559	48.3	74.5	69.5	3.4	0.156
Plump (%)	83.9	99.8	96.0	2.9	0.303	22.8	96.7	77.0	15.5	0.636
Grain Protein (%)	9.6	15.4	12.1	0.9	0.797	10.0	18.8	13.9	1.6	0.642

Relationships Between Barley Genotypes and Microbial Taxa Differ by Environment

Preliminary results from our field trials found heritability and associations with barley genetic loci for the relative abundances of several bacterial phyla and genera in the rhizosphere (Table 4.4). These relationships differed between our two environments. We speculated that the relationships between host genotypes and the microbiome would be more apparent at the Moccasin location. The no-till and planted alley management practices over the years there might have cultivated higher levels of soil organic matter (SOM) along with a more diverse resident soil microbial community. Positive relationships have been demonstrated between these

factors in semi-arid environments (Murphy et al., 2011; Sokol et al., 2019) and we did observe greater % SOM in Moccasin compared to Bozeman in our soil tests (Table 4.1). It could be that plants had more opportunities to form relationships with a more diverse soil microbial community, and were under more pressure to do so in Moccasin where there was less soil nitrogen and moisture (Table 4.1, Table 4.2). Meier et al., (2022) observed that heritability of the rhizobiome was higher under a low nitrogen treatment compared to high nitrogen in maize. Our heritability observations ran counter to this as the mean value in Bozeman ($H^2 = 0.281$) was higher than in Moccasin ($H^2 = 0.118$), with several more taxa having a heritability of 0 in Moccasin (Table 4.4). However, there were far more bacterial taxa over the 95% prevalence threshold in only Moccasin than in only Bozeman (Figure 4.1). There were also a greater number of associations in the GWAS for the relative abundances of taxa from the Moccasin trial than from the Bozeman trial (Table 4.4). Thus, our hypothesis that interactions between microbes and plant genotypes are more prolific in the Moccasin environment remains to be tested further.

Table 4.4. Prevalence, heritability, and number of significant GWAS associations by location for microbial taxa appearing in at least 95% of samples in either Bozeman or Moccasin.

Taxonomic Level	**Taxon**	**Prevalence**		**Heritability**		**Number of GWAS Associations**	
		Bozeman	Moccasin	Bozeman	Moccasin	Bozeman	Moccasin
Genus	Acidibacter	70.22	97.02	0.000	0.000	0	0
Genus	Arthrobacter	97.06	97.45	0.933	0.000	0	2
Genus	Belnapia	99.26	98.72	0.000	0.000	0	8
Genus	Blastococcus	79.78	99.15	0.000	0.000	0	2
Genus	Bradyrhizobium	95.59	98.72	0.965	0.918	0	6
Genus	Bryobacter	95.59	98.30	0.974	0.000	0	0
Genus	Candidatus.Alysiosphaera	62.50	99.57	0.003	0.000	0	0
Genus	Gaiella	87.50	98.72	0.455	0.000	0	7
Genus	Gemmatimonas	97.43	95.74	0.945	0.030	0	1
Genus	Geodermatophilus	55.51	99.15	0.000	0.000	0	0
Genus	Haliangium	92.65	99.57	0.405	0.837	0	2
Genus	Jatrophihabitans	71.69	99.15	0.000	0.000	0	3
Genus	Methylobacterium-Methylorubrum	86.03	95.74	0.087	0.000	0	1
Genus	Microlunatus	88.97	100.00	0.000	0.000	0	0
Genus	Microvirga	74.26	100.00	0.000	0.000	0	0
Genus	Mycobacterium	88.24	100.00	0.515	0.000	1	0
Genus	Neo-b11	9.56	96.60	0.000	0.416	0	0
Genus	Nitrospira	73.53	98.72	0.075	0.000	0	0
Genus	Nocardioides	95.96	98.72	0.000	0.000	1	3
Genus	Pedobacter	90.07	95.32	0.151	0.000	0	0
Genus	Pseudarthrobacter	99.26	99.57	0.806	0.815	5	0
Genus	Pseudomonas	99.26	89.36	0.722	0.611	0	0
Genus	Pseudonocardia	98.53	100.00	0.000	0.000	1	0
Genus	Psychroglaciecola	24.26	99.15	0.238	0.000	1	10
Genus	RB41	61.40	100.00	0.059	0.000	0	0
Genus	Reyranella	37.87	98.30	0.000	0.000	0	7
Genus	Rhodococcus	99.26	87.23	0.791	0.062	0	1
Genus	Rubellimicrobium	14.71	99.57	0.098	0.000	0	0
Genus	Rubrobacter	98.16	100.00	0.895	0.000	2	0
Genus	Segetibacter	100.00	100.00	0.000	0.000	0	1
Genus	Skermanella	99.63	99.57	0.657	0.834	1	7
Genus	Solirubrobacter	89.71	99.15	0.199	0.861	0	8
Genus	Sphingomonas	100.00	97.87	0.000	0.034	0	5
Genus	Streptomyces	81.25	95.74	0.000	0.000	0	0
Phylum	Acidobacteriota	100.00	100.00	0.000	0.000	0	0
Phylum	Actinobacteriota	100.00	100.00	0.000	0.000	0	0
Phylum	Bacteroidota	100.00	100.00	0.038	0.000	0	0
Phylum	Bdellovibrionota	74.63	96.17	0.000	0.000	0	2
Phylum	Chloroflexi	100.00	100.00	0.015	0.000	0	0
Phylum	Firmicutes	94.85	99.57	0.448	0.000	0	0
Phylum	Gemmatimonadota	99.63	99.57	0.428	0.000	6	0
Phylum	Myxococcota	98.90	100.00	0.747	0.000	8	0
Phylum	Nitrospirota	73.53	98.72	0.021	0.000	0	0
Phylum	Planctomycetota	93.01	100.00	0.325	0.000	0	0
Phylum	Proteobacteria	100.00	100.00	0.057	0.000	0	0
Phylum	Verrucomicrobiota	97.06	100.00	0.892	0.000	0	0
Mean		83.62	98.39	0.281	0.118	0.57	1.65

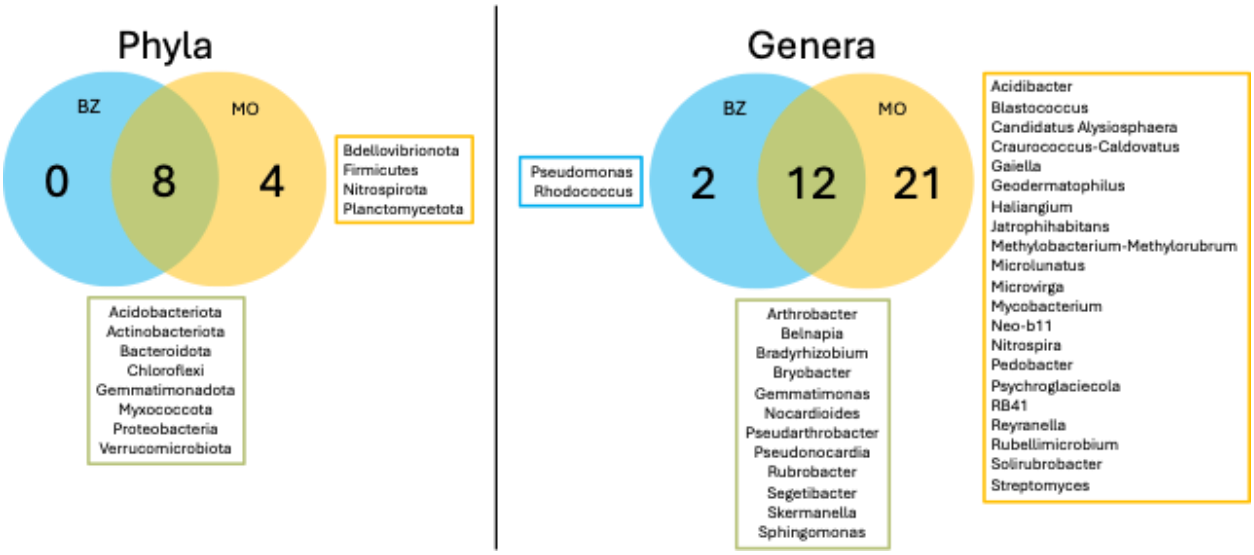

Figure 4.1. Venn diagrams of prevalent bacterial taxa at each field trial location. Phyla and genera that appeared at least once in at least 95% of samples in the Bozeman (BZ) dataset, Moccasin (MO) dataset, or both are listed.

We may paint a different picture of barley-microbe and genetic by environment interactions as we incorporate data from additional seasons and locations and refine our analysis in this ongoing project. The 95% prevalence criterion we used to select taxa for heritability and GWA analyses may have been overly stringent. Edwards et al., (2023) and Walters et al., (2018) defined the core microbiome as taxa that appeared in at least 80% of samples. In a multi-environment study this results in location specific cores as well as a study-wide core. It would be interesting to examine whether cultivars with a greater proportion of local core microbiota compared to study-wide core taxa in their rhizospheres also specifically perform better at that location. Our heritability estimates were calculated for the two locations separately using the replicated check varieties to compensate for the lack of replication of experimental lines, and ranged from a minimum of 0 to a maximum of 0.974 (Table 4.4). This may be unusually high for such traits, as ranges of 0 to ~0.650 have been reported previously by similar studies (Deng et al., 2021; Meier et al., 2022). The compositional nature and interconnectedness of 16S datasets

can lead to issues when calculating heritability such as over- and imprecise estimation, particularly for dominant taxa (Bruijning et al., 2023), and additional or different data analysis may be necessary. There were also instances where a microbial trait had very low heritability but still had significant marker associations in the GWAS, or vice versa (Table 4.4). Some marker associations for traits with low heritability are probably due to low MAF of the SNP leading to overestimation of the percent of variance explained (Table A4.1). Yet the 5% MAF threshold we used is common for GWAS (Bergelson et al., 2019; Edwards et al., 2023) and many studies use a lower 1% threshold (Deng et al., 2021; Meier et al., 2022; Wang et al., 2022). Disconnect between heritability and GWAS results in our study could also be due to the fact that the model for heritability analysis considers unreplicated genotypes or barley lines in our augmented design, while the GWAS model considers replicated alleles throughout the population. This idea is also suggested by Edwards et al., (2022) in their paper. It may also explain some of the low heritability values obtained for agronomic traits (Table 4.3). Further exploration of the data and analysis techniques may resolve some of these issues.

Identifying loci that associate with bacterial taxa across study environments would encourage the idea that such loci could be broadly deployed in breeding for multiple locations, while environment specific associations could be implicated in local adaptation. We found some potential instances of the same barley genetic regions associating with different microbial taxa between Bozeman and Moccasin in our study thus far (Figure 4.2, Table A4.1). However, most loci were location specific which could support the idea that the soil microbiome contributes to home field advantage, helping plants to perform best in certain places (Petipas et al., 2021; Revillini et al., 2016). Meier et al., (2022) also observed environmental specificity in their

mapping study as none of the maize genetic loci they found associating with microbial traits were shared between their high and low nitrogen field treatments. When mapping the switchgrass root microbiome in three different locations, Edwards et al., (2023) also produced different results for each location. But, they were able to detect more consistent association between bacteria and host genetics across environments by combining p-values from the three location-specific GWAS using Fischer's method, and this process even revealed some associations that did not appear in any of the three locations alone. This suggests that host genetic loci involved in belowground microbial relationships regardless of surrounding soil community do exist, but may require more in-depth analysis to discover due to large environmental impact on these processes. Both broadly associated and locally associated host genetic loci could be useful in crop breeding.

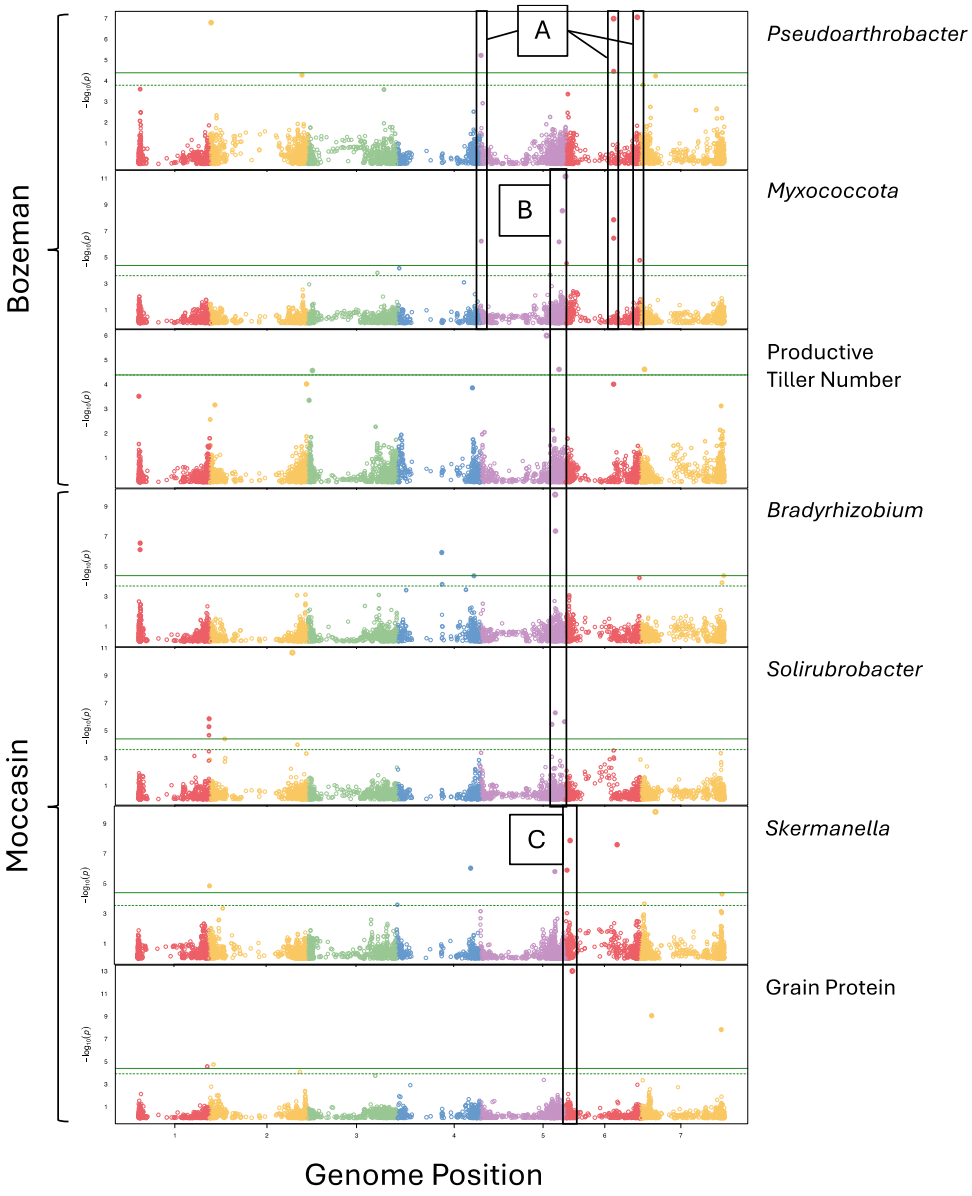

Figure 4.2. Manhattan plots showing selected GWAS results. Dashed horizontal green lines indicate an $\alpha = 0.2$ significance threshold and the solid horizontal green lines indicates an $\alpha = 0.05$ significance threshold. The top three plots are from the Bozeman field trial and the bottom four are from the Moccasin field trial. The boxes labeled A) highlight a repeating pattern of loci associating with relative abundances of the bacterial genus, *Pseudoarthrobacter* and the phylum, *Myxococcota* in Bozeman. The B) box indicates the approximate location of the overlapping regions of a QTL on chromosome 5H from Escudero-Martinez et al., (2022) and *QDD-5H* from Williams et al., (2023), and contains co-localizations between *Myxococcota* and productive tiller number in Bozeman, and the genera *Bradyrhizobium* and *Solirubrobacter* in Moccasin. The C) box shows loci associated with the genus *Skermanella* and grain protein content in Moccasin that are within the approximate location of *QGFmt-6H* from the Williams study. Grain protein in Bozeman mapped to this region as well.

Connections Between Microbial Traits, Host Genetic Loci, and Agronomic Traits Are Complex

Co-localization of loci associated with microbial traits and with agronomic traits could suggest a link between rhizospheric bacterial community composition and crop performance. Such co-localizations could be spurious or due to genetic linkage or pleiotropy. A rhizosphere microbial community characteristic occurring during early host development might prime the plant for success later on, or a phenotype that impacts the relative abundance of a certain microbial taxon may share genetic basis with a gene product such as a transcription factor that also contributes to plant fitness. We only observed one instance in which an agronomic associated SNP was within 1Mb of a bacterial associated SNP from the same field location. This was for productive tiller number and *Myxococcota* in Bozeman (Figure 4.2). However, there were additional examples where significant SNPs for agronomic traits and for microbial traits from different field locations were within 1Mb of each other, or where such trait combinations from Bozeman and or Moccasin mapped within putative regions of QTLs from other studies (Figure 4.2, Table A4.1). Such alignments should be interpreted with caution as this is not an indication of causation. It is also worth noting that our agronomic and rhizosphere data were collected at different time points during host development which has been shown to have an impact on microbial community composition (Chaparro et al., 2014). Additional research will be required to confirm observed marker-trait associations and co-localizations and determine the mechanisms behind them.

Linking agronomic and rhizosphere microbial traits to the same genetic loci has been a challenge in this research field. Escudero-Martinez et al. (2022) examined nearly identical sibling barley lines differing only for a QTL they had demonstrated to have a significant impact

on the relative abundance of several different bacterial taxa in the rhizosphere. They observed no differences between the lines for root system architecture traits or seed yield measured in a greenhouse. Maize leaf traits, particularly canopy coverage as measured by UAV, were found to correlate either positively or negatively with 62 different microbial groups that had been mapped in a GWAS, but shared loci between these traits were not reported (Meier et al., 2022). A study by Wang et al., (2022) took an interesting three-pronged approach in a rather large investigation of foxtail millet. They conducted a GWAS of agronomic traits, and of rhizoplane microbial traits (mGWAS), and they also performed an MWAS where bacterial OTUs essentially took the place of host genetic data in a GWAS against which to map the agronomic phenotypes. Many of the OTUs that correlated with agronomic traits in the MWAS also associated with SNP markers in the mGWAS, supporting the involvement of certain bacteria in crop performance, but only 4 specific SNPs were implicated in both the mGWAS and the agronomic GWAS out of 2,194 total SNPs with significant associations in either analysis. Shared loci may be difficult to discover owing to the complexity of interacting host, microbe, and environment factors.

Conclusion and Future Directions

The research questions for this project aim to increase understanding of the influence of barley genetics on the rhizosphere microbiome, whether those relationships in turn have an impact on agronomic performance, and the role that environment plays in those interactions. The relative abundances of some bacterial phyla and genera were found to be heritable in our study (Table 4.4). We were also able to link these microbial traits to specific loci in our GWAS. Co-localization of microbial traits within our GWAS as well as with QTLs from the barley rhizosphere genetic linkage study by Escudero-Martinez et al., (2022) lend confidence to these

results (Figure 4.2, Table A4.1). While there was some consistency of loci appearing in the GWAS results from each of our locations, most marker associations were environment specific. Some agronomic traits co-localized with microbial traits but not many. Such alignments have also been elusive in other similar studies, though correlations have been demonstrated in other ways (Meier et al, 2022; Wang et al., 2022). Microbial community characteristics are very sensitive to environmental factors and highly quantitative in their response to host genetics making reliable signals challenging to detect and validate (Bergelson et al., 2021). Further analysis and research are required before directed plant breeding for these extended phenotypes will be effective.

Additional data and analysis for this project are forthcoming. We repeated the field trials for another season in Bozeman and Moccasin, and conducted the same experiments for two seasons in South Dakota and one season in Hawaii for a total of seven location-years. Examining such a suite of field trials has not been done previously in rhizosphere microbiome genetic mapping research and will allow us to dive deeply into examining the effects of environment and genetic by environment interaction. It will be interesting to see if there are consistent GWAS results not only across locations, but across seasons within locations as we continue to explore the question of broad versus local adaptation. Furthermore, these trials will allow us to study consistency of agronomic performance and how host genetics and the microbiome might play a role in crop robustness. We will also be analyzing microbial sequences from bulk soil samples corresponding to each field trial. These could be useful for examining within field variation as we so far have not adjusted for that in our 16S datasets. Characterizing the microbial communities in the bulk soil will also provide a baseline against which to compare the

communities around the barley roots, allowing us to see which taxa are enriched or depleted in the rhizosphere as done by Edwards et al., (2023). These bulk samples will also help to determine whether differences in rhizosphere community assembly between locations are due to differences in starting material of the surrounding soil communities. Another question we will be able to address is whether an impact of breeding history is detectable in rhizosphere microbiome composition since the S2MET population used in this project contains lines from multiple different breeding programs. Similar ideas were addressed in other studies by looking for an impact of barley ecotype (Alegria et al., 2020) or switchgrass subpopulation (Edwards et al., 2023) on rhizosphere or root microbiomes. Finally, and perhaps most exciting, ITS sequencing has been done on all of our samples to allow characterization of fungal communities alongside their bacterial counterparts. Fungi are often overlooked in microbiome research. All of the rhizosphere genetic mapping studies that have been referenced in this paper focused solely on bacteria except for Bergelson et al., (2019) which analyzed both kingdoms. They found that fungi were more sensitive to *Arabidopsis* genetics than bacteria, and argue the importance of considering both these types of microbes in the rhizosphere. We will perform heritability analysis and GWAS on the ITS data as with the 16S and compare fungi and bacteria in the context of our research questions. Once we focus in on important barley genetic loci, we will begin to search for gene candidates and clues pointing to possible mechanisms behind the trait associations.

There are multiple possible approaches for validation and exploration of GWAS results. Genetic mapping requires rather large populations, but curating a much smaller collection of barley lines containing specific known genetic differences facilitates closer examination of the varying regions. In a previous study (Williams et al., 2024), members of our group identified

QTLs associated with seedling root architectural traits in barley. We have generated near isogenic lines (NILs) that are genetically identical except at the positions of these QTLs. The lines could possibly conveniently be used to investigate microbial associated loci identified in this project as there is some co-localization between the results of these two mapping studies (Figure 4.2, Table A4.1). There is mounting evidence to suggest that root morphology could impact microbial assembly as well (Galindo-Castañeda et al., 2022). A bulked segregant analysis (BSA) would be another option for targeted observation of microbe associated host genetic loci. This would involve selecting a subset of lines from the S2MET population with contrasting allelic identities at the loci of interest, and ideally as much genetic similarity as possible throughout the rest of the genome. Deng et al., (2021) used a BSA approach to validate a locus from their sorghum rhizosphere GWAS and were able to demonstrate differential enrichment for diderm versus monoderm bacteria between major and minor allele genotypes. Using NILs or bulked segregants could also allow for more in-depth experiments such as imposing different levels of drought stress in a greenhouse, or tracking microbiome compositions over multiple host developmental time points, that would further characterize the focal loci.

While ITS and 16S sequencing are powerful tools for gaining insight into microbial communities from large numbers of samples collected under a variety of circumstances, it is important to be aware of their shortcomings when conducting such research. ITS and 16S sequencing are powerful tools for giving insight into microbial communities from large numbers of samples collected under all sorts of circumstances, but they also have their shortcomings. Different taxa can have different numbers of the 16S gene (Kembel et al., 2012), and using primer pairs targeting different variable regions can produce somewhat different results (Peiffer

et al., 2013). Relic DNA in soil samples from dead or dormant microbes can also contribute misrepresentation of the community (Blagodatskaya et al., 2013). The rhizosphere provides an ideal microhabitat for accelerated bacterial growth, changes in community structure, and horizontal gene transfer which has led to parallel evolution and blurred lines between soil-born taxa (Maheshwari et al., 2017). Bacterial taxa with identical or highly similar 16S sequences can have distinct genomes and ecological functions (Jaspers and Overmann, 2004), and dissimilar heritability values in host genetic studies (Meier et al., 2022). Also, genera from different families or phyla can share the same functions. Thus, functional groupings can be very different from taxonomic relationships. This presents a potential problem for basing microbial extended phenotypes solely on taxonomic classifications.

Leveraging different omics techniques to look beyond taxonomy and describe rhizosphere communities in terms of microbial functions could be a powerful way to identify beneficial extended phenotypes to select for in plant breeding (Zancarini et al., 2021), as well as understand the host side of interaction mechanisms. A GWAS using shotgun metagenomic sequencing of tomato rhizospheres mapped bacterial functional potentials based on predicted proteins that were related to root colonization, import of certain root exudate compounds, and siderophores that had been previously shown to benefit disease suppression in tomato (Oyserman et al., 2022). Transcriptomic analysis of a barley rhizosphere QTL implicated host genes involved in pathogen protection and cell wall structure (Escudero-Martinez et al., 2022). Several rhizosphere mapping studies have identified host genes having to do with these functions as candidates for their loci based on gene ontology (Bergelson et al., 2019; Sutherland et al., 2022; Oyserman et al., 2022; Edwards et al., 2023) and the Escudero-Martinez finding provides some

experimental support for the suggestion. This is still far from demonstrating causative connections between plant and microbe genotypes and phenotypes and delineating mechanisms behind quantitative genetic associations.

To purposefully target the rhizosphere microbiome in plant breeding, connections need to be made from plant genotype to plant phenotype to microbial extended phenotype and back to plant performance, all with consideration of the impact of environment on these connections. Many unknowns and challenges remain, and replacing agrochemicals with microbes whilst maintaining necessary food production levels remains an unrealized aspiration. But, there is evidence of microorganisms providing benefits for plants, and separately, evidence of connection between plant genetics and microbial community characteristics. If the gap between these two lines of evidence can be bridged, then microbial plant breeding goals may be achieved.

Description of Appendix Material

Table A4.1. List of significant marker trait associations with co-localizations within 1Mb indicated by numbered groups. Co-localizations are also noted with QTLs from the Escudero-Martinez et al., (2022) linkage mapping study of the barley rhizosphere microbiome, and the Williams et al., (2024) study of barley seminal root traits, phenology and grain quality.

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CHAPTER FIVE

CONCLUSION AND FUTURE DIRECTIONS

This dissertation is the culmination of extensive field- and greenhouse-based investigation into barley's agronomic performance around Montana and its "hidden half". The findings support the notion that root systems play an important role in crop adaptation, add to the collective knowledge about the stay-green trait in barley, and show that plant breeding for microbial extended phenotypes remains an idea worth pursuing. These results contain the seeds of future projects and will hopefully be helpful to other barley breeders and researchers.

Chapters 2 and 3 both examined crop performance and stay-green or increased grain fill duration in barley. Chapter 2 did this using a group of four stay-green and four non-stay-green lines, while Chapter 3 utilized a biparental population of 168 sibling recombinant inbred lines (RILs) varying for the trait. In Chapter 2 there was a correlation between the stay-green trait and higher grain yield, but the relationship between these was neutral in the RIL study. While stay-green has been repeatedly linked to greater yield in other species, mixed results with respect to yield for stay-green barley are not unusual (Kamal et al., 2019). The RIL study revealed a connection between the stay-green trait and plumper grain and lower grain protein content, both of which are highly desirable quality characteristics for malt barley (AMBA, 2021). Grain plumpness was not measured on the smaller set of lines in Chapter 2, and no impact of stay-green on grain protein was detected. This was likely due to the fact that most of the lines in that study, with and without the stay-green trait, contained an allele for the *HvNAM1* NAC transcription factor gene that confers low grain protein (Distelfeld et al., 2008). The parents of the RIL population differed for this gene and its locus was identified as a major quantitative trait

locus (QTL) in that work for grain protein as well as time to maturity which it is also known to affect (Alptekin et al., 2021). The *HvNAM1* locus was also a QTL for a trait that was important in both the Chapter 2 and Chapter 3 studies; root to shoot length ratio.

Two of the field trials in Chapter 2 were conducted in the same location and seasons as two of the trials in Chapter 3, and the lines from both studies were examined using the same seedling root assay. A smaller root to shoot length ratio correlated with delayed maturity and lower grain protein content in addition to co-locating with these traits to the *HvNAM1* locus. in the RIL study. This ratio was not presented in Chapter 2, but its components, the length of the longest seedling root and the length of the seedling shoot, were measured and presented. When the ratio of these traits was calculated and compared to root traits measured on mature plants in the field by minirhizotron, a smaller ratio correlated strongly with a greater percentage of deep roots (r_s = -0.929), and an increase in total root length over the course of grain fill (r_s = -0.762). Deeper and more prolonged root growth were also characteristic of the stay-green lines in that Chapter 2 study. These correlations and co-locations between root to shoot length ratio, deeper more prolonged field root growth, extended grain fill, and lower protein certainly do not demonstrate causation, and finding reliable proxies for in situ root phenotypes is challenging due to root plasticity in response to dynamic environmental factors. However, since there was shared environment between the two studies it may be reasonable to speculate that there is a genetic connection between these above and belowground traits. The *HvNAM1* transcription factor could pleiotropically affect early and mature root development in addition to delaying maturity and lowering grain protein, or these root traits may be influenced by linked genes located near *HvNAM1*. Near isogenic lines (NILs) have been created for this QTL and further examination of

these phenotypes using these genetic tools could shed light on the nature of the relationship between these traits. This was one of multiple instances of co-location in the RIL study between seedling root traits and agronomic traits including the consistency of crop performance between the two field trial environments.

An important consideration for plant breeders is the range of geographic and environmental conditions that their varieties are adapted to. Adaptation to a broad spectrum of conditions is efficient from a variety development standpoint, but might miss out on the superior performance that some genotypes display only in specific locations. It may also be possible to breed varieties that demonstrate good broad performance and exceptional local performance (Ewing et al., 2024). The QTL mapping study in Chapter 3 found that alleles which were associated with extending grain fill duration were also associated with more consistent grain quality between the two study environments due to quality improvement in the lower performing location. Thus, the stay-green trait, which is thought to confer drought and heat tolerance (Kamal et al., 2019), may be beneficial for broad adaptation without reducing high local performance. The QTLs in that study were impactful in both environments, though earlier heading was more important in the drier location and later maturity made a bigger impact in the less dry environment. This further suggests the broad adaptability and environmental plasticity of the trait. Future in-field examination of roots using NILs may help to answer whether part of this adaptability has a belowground basis.

The study in Chapter 4 also conducted genetic mapping in two different Montana environments but for very different traits compared to Chapter 3. In this chapter, a genome wide association study (GWAS) was conducted on a large diverse barley population using the relative

abundance of different microbial taxa in the rhizosphere surrounding the roots as the mapped traits or extended phenotypes. The abiotic and biotic aspects of soil are thought to play an important role in the optimal functioning of plants in their native environment, sometimes referred to as the "home field advantage" (Rúa et al., 2016). Unlike the agronomic trait QTLs that consistently produced genetic signal between the two field environments in the Chapter 3 study, the loci identified for microbial traits in the Chapter 4 GWAS were mostly location specific, which could support the idea of bacterial involvement in local adaptation.

Finding any GWAS associations between rhizospheric microbial taxa and plant genetics is an exciting result as this has only been demonstrated by a handful of other recent studies so far (Bergelson et al., 2019; Deng et al., 2021; Sutherland et al., 2022; Oyserman et al., 2022; Escudero-Martinez et al., 2022; Wang et al., 2022; Meier et al., 2022; Edwards et al., 2023). This is one step towards the goal of crop breeding for rhizosphere microbiomes that benefit plant fitness. More work will need to be done to validate loci and understand their impact on crop performance in different environments. Some connection has been demonstrated between agronomics and rhizosphere microbial traits (Meier et al., 2022; Wang et al., 2022), but only a few instances of co-localization were identified in the Chapter 4 GWAS and in one other study (Wang et al., 2022). Mapping bacterial functions may be more informative in future analysis as there is a high degree of functional redundancy amongst soil microbial taxa (Maheshwari et al., 2017). It will also be important to understand the plant functioning that is involved in microbial interactions.

Root architecture and anatomy create distinct niches for root-associated microbial communities to occupy, so it makes sense to consider root phenotypes when investigating these

microbiomes (Galindo-Castañeda et al., 2022). Both impact plant fitness and these effects could be synergistic. A strong effect of depth has been demonstrated on bulk soil microbial communities (Eilers et al., 2012; Hao et al., 2021) with abundance and diversity decreasing with increasing depth, but it is much less clear what if any effect depth might have on the microbial community within the rhizosphere. One hypothesis is that the presence of plant roots and the resources they provide would attenuate the impact of edaphic factors (Naylor et al., 2023) including depth. Depth was not found to be a determinant of wheatgrass rhizosphere bacterial community composition overall by Naylor et al. (2023), but there did appear to be an interactive impact of depth and irrigation level. Bacterial community composition was also to found to differ by rhizosphere depth for three grass species in an arid grassland (Kuske et al., 2002). Additionally, microbial communities have been found to vary along the length of a developing root from the nascent tip through the subsequent zones, with the community becoming more organized along older sections of tissue (Rüger et al., 2021). For roots that are growing generally downward through the soil, this could contribute to a dynamic depth effect over the course of plant growth. So, depth may indeed have an effect on the rhizosphere microbial community. However, the studies that have examined this in the field have been limited to 30 cm deep or shallower, while the rooting depth of annual cereal crops can be in the 1 to 2 m range (Hoad et al., 2001). Deep rooting has been identified as an important trait for adaptation to certain low precipitation environments (Lynch, 2013), so the development of drought tolerant varieties may benefit from an understanding of the deep rhizosphere microbiome. The challenge of accessing the rhizosphere at such depths, particularly in dry soils, poses a significant hurdle to this research.

The world beneath our feet is a complex one. This dissertation adds to a body of work that describes plant phenotypes belowground, interactions with their biotic and abiotic environment, and the genetics underpinning these characteristics. Many challenging questions remain to be answered on these topics, but they are questions worth pursuing. As efficient acquisition and use of water and nutrients becomes ever more critical to modern agriculture, crop breeders would do well to take roots into consideration.

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APPENDICES

APPENDIX A

ADDITIONAL TABLES FOR CHAPTER TWO

Table A2.1. Effects used in linear models for comparing stay-green and non-stay-green groups, and significance levels of F-values in ANOVA. A "-" indicates the effect was not included in the model.
p > 0.15 ns, p < 0.15 . , p < 0.05 *, p < 0.01 **, p < 0.001 ***

Barley							
Experiment	**Trait and Season**		**Effects**				
			Stay-Green	Block	Season	Stay-Green x Block	Stay-Green x Season
Field Trial, Aboveground	Aboveground Biomass at Flowering (g m^{-2})	2018	.	***	-	*	-
		2019	ns	ns	-	-	-
		2020	ns	ns	-	-	-
		All	ns	.	***	-	-
	Leaf Area at Flowering (cm^2)	2018	***	.	-	.	-
		2019	***	ns	-	-	-
		2020	ns	ns	-	-	-
		All	*	ns	***	-	*
	Productive Tiller Number	2018	.	.	-	-	-
		2019	.	ns	-	-	-
		2020	ns	ns	-	-	-
		All	ns	ns	ns	-	-
	Days To Heading	2019	***	ns	-	-	-
		2020	.	ns	-	-	-
		All	***	ns	***	-	-
	Days To Maturity	2019	*	ns	-	-	-
		2020	ns	ns	-	-	-
		All	*	ns	ns	-	-
	Duration of Grain Fill (Days)	2019	***	ns	-	-	-
		2020	**	ns	-	-	-
		All	***	ns	***	-	-
	Harvest Index	2018	.	ns	-	-	-
		2019	**	ns	-	-	-
		2020	.	ns	-	-	-
		All	*	ns	***	-	*
	Grain Yield (g m^{-2})	2018	ns	**	-	-	-
		2019	*	ns	-	-	-
		2020	ns	.	-	-	-
		All	.	*	***	-	-
	Percent Grain Protein	2019	ns	**	-	-	-
		2020	.	ns	-	-	-
		All	ns	.	***	-	-

Table A2.1 Continued.

Field Trial, Belowground	Total Root Length at Flowering (cm)	2018	ns	ns	-	-	-
		2019	ns	.	-	-	-
		2020	ns	ns	-	-	-
		All	ns	ns	***	-	-
	Total Root Length at Harvest (cm)	2018	ns	ns	-	-	-
		2019	ns	.	-	-	-
		2020	ns	ns	-	-	-
		All	ns	ns	***	-	-
	Percent Deep Root Length at Flowering	2018	ns	ns	-	-	-
		2019	*	ns	-	-	-
		2020	ns	ns	-	-	-
		All	*	ns	***	-	-
	Percent Deep Root Length at Harvest	2018	.	ns	-	-	-
		2019	*	ns	-	-	-
		2020	ns	ns	-	-	-
		All	**	ns	***	-	-
	Percent Change in Shallow Root Length	2018	ns	.	-	-	-
		2019	*	ns	-	-	-
		2020	*	*	-	-	-
		All	.	ns	ns	-	.
	Percent Change in Deep Root Length	2018	ns	ns	-	-	-
		2019	.	ns	-	-	-
		2020	ns	**	-	-	-
		All	ns	ns	ns	-	-
Root Roll-Up Assay	Total Root Length (cm)		ns	*	-	.	-
	Root Volume (cm^3)		ns	.	-	*	-
	Root to Shoot Weight Ratio		ns	***	-	-	-
	Length of Longest Root (cm)		.	ns	-	-	-
	Seminal Root Angle (°)		***	.	-	*	-
	Seminal Root Number		**	ns	-	-	-
	Lateral Root Number		**	**	-	-	-
PVC Pot Assay	Total Root Length (cm)		*	***	-	-	-
	Root Volume (cm^3)		ns	.	-	*	-
	Root Dry Weight (g)		ns	.	-	-	-
	Root to Shoot Weight Ratio		ns	***	-	-	-

Table A2.1 Continued.

Wheat							
Experiment	**Trait and Season**		**Effects**				
			Stay-Green	Block	Season	Stay-Green x Block	Stay-Green x Season
Field Trial, Aboveground	Aboveground Biomass at Flowering (g m^{-2})	2018	ns	*	-	.	-
		2019	ns	**	-	-	-
		2020	ns	*	-	-	-
		All	ns	*	.	-	-
	Leaf Area at Flowering (cm^2)	2018	*	ns	-	-	-
		2019	ns	ns	-	-	-
		2020	ns	ns	-	-	-
		All	*	ns	ns	-	*
	Productive Tiller Number	2018	***	ns	-	-	-
		2019	.	ns	-	-	-
		2020	**	ns	-	-	-
		All	.	ns	*	-	-
	Days To Heading	2019	ns	ns	-	-	-
		2020	.	ns	-	-	-
		All	*	ns	*	-	-
	Days To Maturity	2019	ns	ns	-	-	-
		2020	ns	ns	-	-	-
		All	ns	ns	.	-	-
	Duration of Grain Fill (Days)	2019	ns	ns	-	-	-
		2020	**	ns	-	-	-
		All	**	ns	ns	-	-
	Harvest Index	2018	.	ns	-	-	-
		2019	ns	ns	-	-	-
		2020	ns	ns	-	-	-
		All	ns	***	-	-	-
	Grain Yield (g m^{-2})	2018	.	.	-	-	-
		2019	ns	ns	-	-	-
		2020	**	**	-	.	-
		All	ns	*	***	-	-
	Percent Grain Protein	2019	ns	ns	-	-	-
		2020	.	ns	-	-	-
		All	*	ns	***	-	-

Table A2.1 Continued.

Field Trial, Belowground	Total Root Length at Flowering (cm)	2018	ns	ns	-	-	-
		2019	.	ns	-	.	-
		2020	ns	ns	-	-	-
		All	.	ns	***	.	-
	Total Root Length at Harvest (cm)	2018	.	ns	-	-	-
		2019	.	ns	-	.	-
		2020	ns	ns	-	-	-
		All	ns	.	***	-	-
	Percent Deep Root Length at Flowering	2018	ns	ns	-	-	-
		2019	ns	ns	-	-	-
		2020	ns	ns	-	*	-
		All	ns	.	ns	-	-
	Percent Deep Root Length at Harvest	2018	ns	ns	-	-	-
		2019	ns	ns	-	-	-
		2020	ns	ns	-	.	-
		All	ns	.	ns	-	-
	Percent Change in Shallow Root Length	2018	.	.	-	-	-
		2019	*	.	-	-	-
		2020	ns	ns	-	-	-
		All	ns	*	ns	-	-
	Percent Change in Deep Root Length	2018	ns	*	-	-	-
		2019	ns	ns	-	-	-
		2020	ns	.	-	-	-
		All	ns	.	*	-	-
Root Roll-Up Assay	Total Root Length (cm)		ns	**	-	-	-
	Root Volume (cm^3)		ns	*	-	-	-
	Root to Shoot Weight Ratio		ns	ns	-	.	-
	Length of Longest Root (cm)		ns	ns	-	-	-
	Seminal Root Angle (°)		ns	ns	-	-	-
	Seminal Root Number		ns	ns	-	-	-
	Lateral Root Number		ns	***	-	-	-
PVC Pot Assay	Total Root Length (cm)		ns	**	-	-	-
	Root Volume (cm^3)		.	ns	-	*	-
	Root Dry Weight (g)		.	***	-	-	-
	Root to Shoot Weight Ratio		ns	ns	-	-	-

Table A2.2. Field root trait means of non-stay-green and stay-green groups, with p-values from ANOVA for the fixed effect of stay-green for each species in single growing seasons and all seasons combined.
. p < 0.1 * p < 0.5 ** p < 0.01

Barley						
Field Root Trait	**Season**	**Group Means**		**p**	**Sample Size (n)**	
		Non-Stay-Green	Stay-Green		Non-Stay-Green	Stay-Green
Total Root Length at Flowering (cm)	2018	6341	5998		8	14
	2019	13920	11490		9	11
	2020	13809	12540		12	12
	All	11983	9753		32	37
Total Root Length at Harvest (cm)	2018	5506	5322		9	15
	2019	13406	12030		8	11
	2020	13492	12686		12	12
	All	11216	9589		32	38
Percent Deep Root Length at Flowering	2018	31	39		8	14
	2019	57	67	*	9	11
	2020	63	68		12	12
	All	53	57	*	32	37
Percent Deep Root Length at Harvest	2018	36	47	.	9	15
	2019	59	68	*	8	11
	2020	64	69		12	12
	All	54	60	**	32	38
Percent Change in Shallow Root Length	2018	-16	-24		8	14
	2019	-11	7	*	8	11
	2020	-8	-1	*	12	12
	All	-11	-7	.	31	37
Percent Change in Deep Root Length	2018	16	6		8	14
	2019	-9	16	.	8	11
	2020	-1	1		12	12
	All	0	8		31	37

Table A2.2 Continued.

Wheat						
Field Root Trait	**Season**	**Group Means**		**p**	**Sample Size (n)**	
		Non-Stay-Green	Stay-Green		Non-Stay-Green	Stay-Green
Total Root Length at Flowering (cm)	2018	3803	5658		6	5
	2019	7860	7719	.	14	7
	2020	10738	10265		14	9
	All	8329	8319	.	34	21
Total Root Length at Harvest (cm)	2018	3608	5367	.	6	6
	2019	7719	7914	.	14	7
	2020	9516	8890		14	9
	All	7733	7619		34	22
Percent Deep Root Length at Flowering	2018	50	55		6	5
	2019	63	69		14	7
	2020	60	60		14	9
	All	60	62		34	21
Percent Deep Root Length at Harvest	2018	53	60		6	6
	2019	64	66		14	7
	2020	60	63		14	9
	All	61	63		34	22
Percent Change in Shallow Root Length	2018	-10	-19	.	6	5
	2019	-4	18	*	14	7
	2020	-11	-19		14	9
	All	-8	-7		34	21
Percent Change in Deep Root Length	2018	2	3		6	5
	2019	2	5		14	7
	2020	-9	-11		14	9
	All	-3	-2		34	21

Table A2.3. Spearman correlation coefficients comparing field root traits and aboveground traits using individual plot values.

Legend							
$r_s < -0.5$		$-0.5 < r_s < -0.1$		$0.1 < r_s < 0.5$		$r_s > 0.5$	
$p < 0.05$		$0.05 < p < 0.1$		$0.1 < p$			
Barley							
Aboveground Trait	**Season**	**Field Root Trait**					
		Total Root Length		Percent Deep Root Length		Percent Change in Root Length	
		Flowering	Harvest	Flowering	Harvest	Shallow	Deep
Aboveground Biomass at Flowering	2018	-0.037	0.022	-0.246	0.158	0.069	0.013
	2019	-0.239	-0.269	-0.010	-0.162	0.130	0.063
	2020	0.401	**0.447**	**0.467**	**0.484**	0.206	0.354
	All	**0.485**	**0.605**	**0.524**	**0.492**	**0.371**	-0.043
Leaf Area at Flowering	2018	0.027	0.177	-0.068	-0.165	0.254	**0.504**
	2019	0.033	0.113	-0.363	-0.304	-0.240	-0.173
	2020	-0.170	-0.161	0.184	0.181	-0.223	-0.061
	All	**-0.487**	**-0.511**	**-0.585**	**-0.534**	**-0.390**	0.117
Productive Tiller Number	2018	-0.256	-0.027	0.080	-0.038	0.297	0.085
	2019	-0.101	0.053	0.041	-0.010	**0.490**	**0.479**
	2020	0.246	0.247	0.346	0.267	0.249	0.057
	All	-0.073	0.066	0.014	0.013	0.198	**0.253**
Days To Heading	2019	0.110	**0.073**	-0.480	**-0.509**	-0.400	-0.321
	2020	0.061	0.008	-0.139	-0.184	-0.193	-0.082
	All	0.064	0.027	-0.205	-0.217	-0.196	-0.163
Days To Maturity	2019	-0.102	0.194	0.034	0.170	**0.550**	0.396
	2020	-0.210	-0.236	0.107	0.052	0.004	**-0.414**
	All	-0.136	-0.059	0.208	0.221	0.265	0.056
Duration of Grain Fill	2019	-0.130	0.079	0.252	0.380	**0.459**	0.407
	2020	-0.161	-0.127	0.329	0.333	0.231	-0.217
	All	-0.167	-0.048	**0.356**	**0.386**	**0.429**	0.191
Harvest Index	2018	-0.182	-0.089	0.134	0.259	-0.071	0.093
	2019	**-0.397**	-0.207	0.374	**0.462**	**0.473**	0.255
	2020	-0.172	-0.208	-0.301	-0.270	-0.121	-0.087
	All	**0.239**	**0.339**	**0.515**	**0.506**	**0.390**	-0.028
Grain Yield	2018	-0.336	0.003	0.254	0.083	**0.439**	0.395
	2019	-0.304	-0.136	0.131	0.125	**0.669**	0.350
	2020	0.131	0.137	0.397	0.356	0.077	0.074
	All	**0.428**	**0.582**	**0.657**	**0.598**	**0.586**	0.041
Percent Grain Protein	2019	**-0.462**	**-0.430**	-0.227	-0.073	0.031	-0.038
	2020	-0.172	-0.208	-0.301	-0.270	-0.121	-0.087
	All	-0.244	-0.188	-0.191	-0.134	0.067	-0.034

Table A2.3 Continued.

Wheat							
Aboveground Trait	**Season**	**Field Root Trait**					
		Total Root Length		Percent Deep Root Length		Percent Change in Root Length	
		Flowering	Harvest	Flowering	Harvest	Shallow	Deep
Aboveground Biomass at Flowering	2018	0.027	-0.091	0.000	0.042	-0.036	0.200
	2019	-0.004	0.062	0.237	0.046	**0.580**	-0.068
	2020	-0.102	0.234	0.409	0.220	0.400	0.362
	All	0.119	**0.285**	**0.440**	0.214	**0.515**	0.089
Leaf Area at Flowering	2018	0.018	-0.042	0.027	0.140	**-0.709**	0.318
	2019	0.351	0.234	0.095	0.001	-0.143	-0.152
	2020	0.067	-0.017	-0.104	0.166	-0.077	-0.062
	All	-0.107	-0.183	0.002	0.125	-0.217	0.062
Productive Tiller Number	2018	0.579	**0.585**	0.187	0.091	-0.260	0.096
	2019	0.411	0.397	-0.079	-0.094	-0.007	-0.062
	2020	0.058	0.279	0.313	0.371	0.221	0.264
	All	0.091	0.172	0.184	0.185	0.110	0.151
Days To Heading	2019	0.250	0.119	-0.094	-0.099	-0.114	-0.278
	2020	**0.511**	0.223	-0.079	-0.104	-0.122	-0.181
	All	**0.478**	0.237	-0.155	-0.101	-0.272	-0.284
Days To Maturity	2019	0.292	0.149	-0.286	-0.300	-0.092	-0.371
	2020	0.323	-0.005	-0.209	-0.024	**-0.468**	**-0.487**
	All	**0.435**	0.172	-0.218	-0.125	**-0.437**	**-0.485**
Duration of Grain Fill	2019	0.026	0.015	-0.067	-0.089	0.017	-0.080
	2020	-0.387	-0.219	0.051	0.190	-0.081	-0.043
	All	0.058	0.045	-0.138	-0.035	-0.218	-0.137
Harvest Index	2018	-0.264	-0.070	-0.027	0.000	-0.218	**0.709**
	2019	0.238	0.300	0.240	0.194	0.151	-0.019
	2020	0.122	0.239	-0.008	0.033	0.265	0.383
	All	**0.520**	**0.433**	-0.003	0.011	-0.135	-0.164
Grain Yield	2018	0.273	0.497	0.600	0.399	0.082	0.455
	2019	0.392	**0.462**	0.171	0.131	0.134	-0.069
	2020	0.011	0.335	0.264	0.256	**0.427**	**0.428**
	All	**0.558**	**0.629**	**0.301**	0.211	0.210	-0.015
Percent Grain Protein	2019	-0.275	-0.305	0.318	0.160	0.135	-0.111
	2020	0.122	0.239	-0.008	0.033	0.265	0.383
	All	**-0.438**	-0.254	**0.384**	0.231	**0.371**	0.258

Table A2.4. Seedling assay trait means of non-stay-green and stay-green groups, with p-values from ANOVA for the fixed effect of stay-green for each species.
. p < 0.1 * p < 0.5 ** p < 0.01 *** p < 0.001

Barley						
Seedling Assay	**Trait**	**Group Means**		**p**	**Sample Size (n)**	
		Non-Stay-Green	Stay-Green		Non-Stay-Green	Stay-Green
One-Leaf Root Roll-Ups	Total Root Length (cm)	137.3	140.8		12	15
	Root Volume (cm^3)	0.18	0.18		12	15
	Root to Shoot Weight Ratio	2.60	2.54		11	12
	Length of Longest Root (cm)	34.9	32.9	.	12	15
	Seminal Root Angle (°)	28.2	34.0	***	12	15
	Seminal Root Number	5.6	6.0	**	12	15
	Lateral Root Number	19.9	9.1	**	12	15
Four-Leaf PVC Pots	Total Root Length (cm)	1790.5	1524.9	*	20	25
	Root Volume (cm^3)	1.12	1.10		20	25
	Root Dry Weight (g)	0.08	0.07		20	25
	Root to Shoot Weight Ratio	1.17	1.30		20	25
Wheat						
Seedling Assay	**Trait**	**Group Means**		**p**	**Sample Size (n)**	
		Non-Stay-Green	Stay-Green		Non-Stay-Green	Stay-Green
One-Leaf Root Roll-Ups	Total Root Length (cm)	82.0	83.1		15	9
	Root Volume (cm^3)	0.14	0.14		15	9
	Root to Shoot Weight Ratio	1.12	1.25		11	7
	Length of Longest Root (cm)	27.2	27.0		15	9
	Seminal Root Angle (°)	19.5	21.9		15	9
	Seminal Root Number	3.9	4.1		15	9
	Lateral Root Number	8.8	11.5		15	9
Four-Leaf PVC Pots	Total Root Length (cm)	1200.2	1098.2		25	15
	Root Volume (cm^3)	0.91	0.83	.	25	15
	Root Dry Weight (g)	0.06	0.05	.	25	15
	Root to Shoot Weight Ratio	1.29	1.28		25	15

Table A2.5. Correlations between field root traits and one-leaf seedling assay or root roll-up traits using genotype means.

Legend							
$r_s < -0.5$		$-0.5 < r_s < -0.1$		$0.1 < r_s < 0.5$		$r_s > 0.5$	
$p < 0.05$		$0.05 < p < 0.1$		$0.1 < p$			
Barley							
Root Roll-Up Seedling Assay Traits	**Season**	**Field Root Trait**					
		Total Root Length		Percent Deep Root Length		Percent Change in Root Length	
		Flowering	Harvest	Flowering	Harvest	Shallow	Deep
Total Root Length	2018	0.119	-0.095	0.190	0.381	0.095	-0.095
	2019	-0.190	-0.452	-0.024	-0.238	-0.095	-0.048
	2020	0.071	0.214	0.167	0.000	0.071	0.190
	All	-0.317	-0.367	-0.417	-0.350	-0.450	-0.317
Root Volume	2018	0.071	0.667	**1.000**	**0.929**	0.524	0.048
	2019	0.071	0.429	0.619	0.119	0.571	0.452
	2020	0.048	-0.024	0.143	0.167	-0.286	0.190
	All	0.167	0.233	0.650	0.600	0.417	0.067
Root to Shoot Weight Ratio	2018	0.143	0.071	0.286	0.286	0.238	-0.548
	2019	-0.119	0.095	-0.190	-0.548	0.024	-0.071
	2020	0.333	0.381	0.167	0.071	-0.524	-0.024
	All	0.117	0.050	-0.167	-0.267	-0.150	-0.583
Length of Longest Root	2018	0.143	-0.381	-0.667	-0.595	-0.143	0.048
	2019	0.429	-0.286	-0.571	-0.595	**-0.976**	**-0.929**
	2020	0.024	0.048	-0.571	-0.500	-0.524	0.119
	All	0.083	-0.033	-0.617	-0.533	**-0.800**	**-0.733**
Seminal Root Angle	2018	-0.381	-0.167	0.024	0.024	-0.262	0.214
	2019	-0.190	-0.048	0.667	**0.857**	0.500	0.571
	2020	-0.524	-0.500	0.119	0.048	**0.786**	0.095
	All	-0.383	-0.300	0.367	0.400	0.367	**0.700**
Seminal Root Number	2018	-0.193	-0.181	0.422	0.578	-0.133	-0.349
	2019	-0.407	-0.216	**0.719**	0.707	0.503	0.587
	2020	-0.180	-0.036	0.204	0.084	0.635	0.419
	All	-0.370	-0.311	0.269	0.269	-0.017	0.210
Lateral Root Number	2018	0.143	-0.190	-0.405	-0.333	0.167	-0.048
	2019	-0.048	-0.262	**-0.786**	**-0.810**	-0.524	-0.524
	2020	0.429	0.476	-0.048	-0.071	-0.500	-0.214
	All	0.467	0.350	-0.433	-0.417	-0.217	-0.517

Table A2.5 Continued.

Wheat							
Root Roll-Up Seedling Assay Traits	**Season**	**Field Root Trait**					
		Total Root Length		Percent Deep Root Length		Percent Change in Root Length	
		Flowering	Harvest	Flowering	Harvest	Shallow	Deep
Total Root Length	2019	-0.238	-0.119	0.548	0.690	0.667	0.071
	2020	0.190	0.286	0.333	0.000	0.333	0.024
	All	-0.405	-0.214	0.238	-0.119	**0.714**	0.119
Root Volume	2019	0.310	0.190	-0.143	-0.095	-0.619	**-0.762**
	2020	-0.595	**-0.738**	**-0.762**	-0.429	-0.167	0.048
	All	-0.310	-0.524	-0.690	-0.690	-0.548	-0.667
Root to Shoot Weight Ratio	2019	0.595	0.524	-0.310	-0.143	-0.595	-0.071
	2020	-0.310	-0.524	-0.690	-0.452	-0.619	-0.524
	All	0.476	0.262	-0.476	-0.357	**-0.714**	-0.214
Length of Longest Root	2019	0.190	0.310	0.381	**0.833**	0.071	0.119
	2020	0.381	0.619	0.119	-0.167	0.238	-0.167
	All	0.405	0.571	0.238	-0.214	0.286	0.071
Seminal Root Angle	2019	0.452	0.429	-0.381	-0.333	-0.190	-0.071
	2020	**-0.786**	-0.476	-0.190	0.190	0.238	0.381
	All	-0.190	-0.190	-0.667	-0.405	-0.286	0.167
Seminal Root Number	2019	0.071	0.000	-0.429	-0.333	0.048	0.048
	2020	**-0.881**	-0.690	-0.190	-0.095	0.405	0.571
	All	-0.643	-0.524	-0.595	-0.500	0.024	0.238
Lateral Root Number	2019	-0.524	-0.452	0.429	0.405	0.381	0.214
	2020	0.310	0.214	-0.048	-0.476	-0.024	-0.167
	All	0.333	0.310	0.667	0.214	0.429	-0.048

Table A2.6. Correlations between field root traits and four-leaf seedling assay or PVC pot traits using genotype means.

Legend							
$r_s < -0.5$		$-0.5 < r_s < -0.1$		$0.1 < r_s < 0.5$		$r_s > 0.5$	
$p < 0.05$		$0.05 < p < 0.1$		$0.1 < p$			
Barley							
PVC Pot Seedling Assay Traits	**Season**	**Field Root Trait**					
		Total Root Length		Percent Deep Root Length		Percent Change in Root Length	
		Flowering	Harvest	Flowering	Harvest	Shallow	Deep
Total Root Length	2018	-0.190	-0.452	**-0.810**	**-0.810**	-0.048	0.357
	2019	0.381	0.024	**-0.738**	-0.452	-0.667	-0.690
	2020	0.167	0.119	-0.024	0.048	-0.095	-0.143
	All	0.333	0.250	-0.367	-0.283	-0.150	-0.017
Root Volume	2018	-0.524	-0.524	-0.595	-0.667	-0.024	0.381
	2019	0.452	0.238	-0.143	0.095	-0.286	-0.333
	2020	-0.262	-0.333	0.071	0.095	0.286	0.000
	All	-0.133	-0.167	-0.167	-0.067	-0.067	0.333
Root Dry Weight	2018	0.190	-0.143	**-0.714**	-0.667	-0.048	0.476
	2019	0.143	-0.190	**-0.762**	-0.452	-0.476	-0.452
	2020	0.190	0.143	0.048	0.095	0.000	-0.357
	All	0.017	-0.033	-0.617	-0.517	-0.183	0.100
Root to Shoot Weight Ratio	2018	0.381	0.119	-0.167	-0.143	**-0.714**	-0.500
	2019	-0.310	0.000	0.095	0.429	0.095	0.190
	2020	0.214	0.238	-0.310	-0.143	0.048	0.095
	All	0.317	0.400	0.383	0.267	0.117	0.067

Table A2.6 Continued.

Wheat							
PVC Pot Seedling Assay Traits	**Season**	**Field Root Trait**					
		Total Root Length		Percent Deep Root Length		Percent Change in Root Length	
		Flowering	Harvest	Flowering	Harvest	Shallow	Deep
Total Root Length	2019	0.000	-0.048	-0.262	-0.357	-0.476	0.000
	2020	0.190	0.238	-0.048	0.071	-0.071	0.071
	All	0.571	0.476	0.190	0.310	-0.381	-0.071
Root Volume	2019	0.048	0.024	-0.333	-0.310	-0.381	0.119
	2020	-0.024	0.262	0.048	0.143	0.262	0.357
	All	0.476	0.500	0.095	0.167	-0.238	0.143
Root Dry Weight	2019	-0.048	-0.095	-0.405	-0.262	-0.381	0.143
	2020	-0.190	0.143	-0.048	-0.071	0.452	0.548
	All	0.333	0.429	0.048	-0.024	-0.143	0.167
Root to Shoot Weight Ratio	2019	0.405	0.500	0.238	0.690	-0.024	0.214
	2020	0.119	0.214	-0.214	-0.405	-0.119	-0.452
	All	0.571	0.619	0.024	-0.333	0.024	0.071

APPENDIX B

ADDITIONAL TABLES FOR CHAPTER THREE

Table A3.1. HvNAM1 sequence for KASP assay PCR primer.

FAM Allele	HEX Allele	Sequence
C	G	ACGAGGAGCTGGTCGTGCACTACCTCAAGAAGAAGGCCGCCAAGGCGCCGCTCCCC GTCACCATCATCGCCGAGGTGGACCTCTACAAGTTCGACCCATG[C/G]GAGCTCCCCG GTATGTACTACTAGTTAGTACTATGTCTATCCCTATCTCGTCGATCGTGCTTGCTTGCTC TATCAAGCGCCGTAATTTCCCGGTGCAATT

Table A3.2. Significance levels for the effect of location on field trial traits in ANOVAs. p > 0.1 ns, p < 0.1 . , p < 0.05 *, p < 0.01 **, p < 0.001 ***

Trait	**Effect of Location**		
	RILs	MT124118	ND19119
Heading Date	***	***	***
Days To Maturity	***	***	***
Grain Fill	***	***	***
Plant Height	***	***	***
Yield	***	***	***
Test Weight	ns	*	.
Plump	***	***	***
Protein	**	**	.

Table A3.3. Pearson correlation coefficients for tests comparing traits from the field trials to traits from the root roll-up assay using BLUPs for the RIL population.

$p \geq 0.1$ ns, $p < 0.1$. , $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***

Field Trait	**Location**	**Root Roll-Up Trait**							
		Days to One-Leaf	Shoot Length	Longest Root Length	Longest Root : Shoot	Shortest Root Length	Root Length Range	Seminal Root Count	Lateral Root Count
Heading Date	Bozeman	0.001 ns	-0.112 ns	-0.023 ns	0.065 ns	0.112 ns	-0.114 ns	-0.020 ns	0.107 ns
	Havre	-0.029 ns	-0.059 ns	-0.029 ns	0.016 ns	0.112 ns	-0.111 ns	-0.037 ns	0.065 ns
Grain Fill	Bozeman	-0.047 ns	0.256 ***	-0.071 ns	-0.379 ***	-0.136 *	0.014 ns	0.009 ns	-0.092 ns
	Havre	0.057 ns	0.191 **	-0.030 ns	-0.249 ***	-0.096 ns	0.033 ns	0.087 ns	-0.023 ns
Days To Maturity	Bozeman	-0.076 ns	0.266 ***	-0.152 **	-0.539 ***	-0.065 ns	-0.140 *	-0.014 ns	0.000 ns
	Havre	0.038 ns	0.220 ***	-0.086 ns	-0.369 ***	-0.004 ns	-0.097 ns	0.099 ns	0.055 ns
Plant Height	Bozeman	0.217 ***	0.168 **	0.232 ***	0.101 ns	0.118 ns	0.180 **	0.102 ns	0.155 **
	Havre	0.211 ***	0.124 ns	0.195 **	0.100 ns	0.063 ns	0.173 **	0.127 ns	0.160 **
Yield	Bozeman	-0.045 ns	-0.083 ns	-0.004 ns	0.087 ns	0.045 ns	-0.035 ns	-0.032 ns	0.010 ns
	Havre	-0.160 **	-0.095 ns	-0.053 ns	0.026 ns	-0.018 ns	-0.052 ns	-0.019 ns	0.027 ns
Test Weight	Bozeman	0.006 ns	0.295 ***	0.015 ns	-0.340 ***	-0.014 ns	0.023 ns	-0.027 ns	0.166 **
	Havre	-0.031 ns	0.228 ***	-0.039 ns	-0.343 ***	-0.049 ns	-0.008 ns	-0.093 ns	0.058 ns
Plump	Bozeman	0.104 ns	0.161 **	0.033 ns	-0.111 ns	-0.001 ns	0.034 ns	0.067 ns	-0.129 *
	Havre	0.063 ns	0.180 **	0.033 ns	-0.149 *	0.009 ns	0.036 ns	0.086 ns	-0.111 ns
Protein	Bozeman	0.003 ns	-0.259 ***	0.073 ns	0.454 ***	-0.065 ns	0.127 ns	-0.019 ns	-0.194 **
	Havre	0.012 ns	-0.221 ***	0.033 ns	0.359 ***	-0.156 **	0.138 *	0.017 ns	-0.179 **
Yield Absolute Difference		0.153 **	0.065 ns	0.056 ns	0.012 ns	0.040 ns	0.040 ns	0.004 ns	-0.025 ns
Test Weight Absolute Difference		0.123 ns	0.017 ns	0.060 ns	0.061 ns	-0.017 ns	0.081 ns	0.009 ns	-0.008 ns
Plump Absolute Difference		-0.029 ns	-0.147 *	-0.025 ns	0.130 *	-0.014 ns	-0.028 ns	-0.074 ns	0.079 ns
Protein Absolute Difference		-0.006 ns	-0.186 **	0.098 ns	0.382 ***	0.068 ns	0.068 ns	-0.040 ns	-0.127 ns

Table A3.4. Complete list of QTLs found in this study. A BZ suffix in the trait name indicates a trait from the Bozeman field trials, HV for Havre, and RRU for the root roll-up assay. All QTL are above the α = 0.1 LOD significance threshold determined for the trait.

Highlighted QTL	Trait	Chromosome	Position (cM)	LOD	Percent of Variance	Effect of ND allele	Upstream Marker	Closest Marker	Downstream Marker
	Plump, BZ	1H	74.09	3.85	4.10	-	BOPA1_6142-1544	JHI-Hv50k-2016-37341	JHI-Hv50k-2016-37374
	Grain Fill, BZ	1H	80.13	3.55	2.11	-	BOPA1_713-972	JHI-Hv50k-2016-37604	JHI-Hv50k-2016-37631
	Plump, HV	1H	138.68	3.50	5.03	+	JHI-Hv50k-2016-52646	JHI-Hv50k-2016-53147	JHI-Hv50k-2016-53246
	Heading Date, HV	1H	149.98	3.30	4.49	-	JHI-Hv50k-2016-55341	JHI-Hv50k-2016-55555	SCRI_RS_141322
	Heading Date, BZ	1H	152.17	11.41	6.61	-	SCRI_RS_153896	JHI-Hv50k-2016-56943	JHI-Hv50k-2016-56402
	Plump, BZ	1H	152.17	3.16	3.33	+	SCRI_RS_153896	JHI-Hv50k-2016-56943	JHI-Hv50k-2016-56402
	Lateral Root Count, RRU	2H	16.00	3.71	7.32	-	JHI-Hv50k-2016-66640	SCRI_RS_231057	JHI-Hv50k-2016-66810
	Seminal Root Count, RRU	2H	17.05	3.38	7.14	+	JHI-Hv50k-2016-66640	SCRI_RS_231057	JHI-Hv50k-2016-66810
	Root Length Range, RRU	2H	18.91	3.39	5.91	-	JHI-Hv50k-2016-66810	JHI-Hv50k-2016-67558	JHI-Hv50k-2016-67865
	Days To One-Leaf, RRU	2H	19.21	3.30	4.27	-	JHI-Hv50k-2016-67558	JHI-Hv50k-2016-67865	JHI-Hv50k-2016-68257
	Grain Fill, BZ	2H	51.87	7.69	4.86	+	JHI-Hv50k-2016-75973	JHI-Hv50k-2016-76228	JHI-Hv50k-2016-76189
	Yield, HV	2H	58.62	3.35	7.93	-	JHI-Hv50k-2016-77454	JHI-Hv50k-2016-77472	JHI-Hv50k-2016-77668
	Protein, HV	2H	59.55	5.36	6.12	+	BOPA1_1862-765	JHI-Hv50k-2016-77657	JHI-Hv50k-2016-77766
QGFhd-2H	Longest Root Length, RRU	2H	79.85	3.78	6.15	-	JHI-Hv50k-2016-89276	JHI-Hv50k-2016-89584	BOPA1_4630-1036
	Plump, HV	2H	81.74	7.58	11.54	+	JHI-Hv50k-2016-90347	JHI-Hv50k-2016-91422	SCRI_RS_130072
	Plump Percent Difference	2H	81.74	7.89	14.41	-	JHI-Hv50k-2016-90347	JHI-Hv50k-2016-91422	SCRI_RS_130072
	Shortest Root Length, RRU	2H	84.58	4.15	10.74	-	JHI-Hv50k-2016-91961	JHI-Hv50k-2016-93366	JHI-Hv50k-2016-93148
	Lateral Root Count, RRU	2H	85.51	6.75	13.89	-	JHI-Hv50k-2016-94875	BOPA2_12_10035	BOPA1_2634-2228
	Heading Date, HV	2H	85.51	22.42	40.28	-	JHI-Hv50k-2016-94875	BOPA2_12_10035	BOPA1_2634-2228
	Heading Date, BZ	2H	85.51	47.21	47.68	-	JHI-Hv50k-2016-94875	BOPA2_12_10035	BOPA1_2634-2228
	Grain Fill, HV	2H	85.82	18.94	25.87	+	BOPA2_12_10035	BOPA1_2634-2228	JHI-Hv50k-2016-97346
	Plant Height, HV	2H	85.82	8.71	12.74	-	BOPA2_12_10035	BOPA1_2634-2228	JHI-Hv50k-2016-97346
	Protein, HV	2H	85.82	10.75	13.28	+	BOPA2_12_10035	BOPA1_2634-2228	JHI-Hv50k-2016-97346
	Plant Height, BZ	2H	86.00	15.77	15.52	-	BOPA1_2634-2228	JHI-Hv50k-2016-97346	SCRI_RS_12492
	Protein, BZ	2H	86.00	15.56	17.66	+	BOPA1_2634-2228	JHI-Hv50k-2016-97346	SCRI_RS_12492
	Grain Fill, BZ	2H	86.75	26.41	22.00	+	JHI-Hv50k-2016-97819	JHI-Hv50k-2016-98104	JHI-Hv50k-2016-98482
	Days To One-Leaf, RRU	2H	87.38	4.45	5.85	-	JHI-Hv50k-2016-98104	JHI-Hv50k-2016-98482	JHI-Hv50k-2016-98582
QLN-2H	Shortest Root Length, RRU	2H	142.98	3.08	6.51	+	JHI-Hv50k-2016-114052	JHI-Hv50k-2016-114164	JHI-Hv50k-2016-115620
	Longest Root Length, RRU	2H	145.62	12.30	22.57	+	JHI-Hv50k-2016-114164	JHI-Hv50k-2016-115620	SCRI_RS_128484
	Shoot Length, RRU	2H	145.62	11.11	13.74	+	JHI-Hv50k-2016-114164	JHI-Hv50k-2016-115620	SCRI_RS_128484
	Plant Height, BZ	2H	147.83	4.54	3.80	+	SCRI_RS_227965	BOPA1_NCC05640-1-1-248	JHI-Hv50k-2016-116864
	Plump, BZ	2H	147.83	5.77	6.31	+	SCRI_RS_227965	BOPA1_NCC05640-1-1-248	JHI-Hv50k-2016-116864
	Days To One-Leaf, RRU	2H	148.00	12.51	18.45	+	BOPA1_NCC05640-1-1-248	JHI-Hv50k-2016-116864	SCRI_RS_151556
	Root Length Range, RRU	2H	149.00	7.86	14.60	+	BOPA1_NCC05640-1-1-248	JHI-Hv50k-2016-116864	SCRI_RS_151556
	Plump, HV	3H	32.86	2.98	4.25	-	SCRI_RS_144410	JHI-Hv50k-2016-161228	SCRI_RS_230486

Table A3.4 Continued.

	Plant Height, HV	3H	39.84	2.99	4.04	-	JHI-Hv50k-2016-163538	JHI-Hv50k-2016-163892	JHI-Hv50k-2016-164079
	Plump, BZ	3H	51.79	6.51	7.20	-	JHI-Hv50k-2016-178277	SCRI_RS_181360	JHI-Hv50k-2016-180310
	Yield, BZ	3H	53.04	4.20	8.77	+	JHI-Hv50k-2016-181260	BOPA1_2067-775	JHI-Hv50k-2016-181307
	Lateral Root Count, RRU	3H	74.00	4.27	8.49	+	BOPA1_11116-257	JHI-Hv50k-2016-194817	JHI-Hv50k-2016-195060
	Yield, HV	3H	114.68	4.12	9.86	-	JHI-Hv50k-2016-198689	JHI-Hv50k-2016-198862	JHI-Hv50k-2016-199520
	Yield Percent Difference	3H	114.70	3.79	9.87	+	JHI-Hv50k-2016-198689	JHI-Hv50k-2016-198862	JHI-Hv50k-2016-199520
	Shoot Length, RRU	3H	116.94	3.28	3.63	+	JHI-Hv50k-2016-198862	JHI-Hv50k-2016-199520	JHI-Hv50k-2016-199950
	Seminal Root Count, RRU	4H	38.99	2.99	6.29	+	JHI-Hv50k-2016-233133	SCRI_RS_220122	JHI-Hv50k-2016-233609
	Shoot Length, RRU	4H	42.00	5.71	6.55	-	JHI-Hv50k-2016-234288	JHI-Hv50k-2016-234379	JHI-Hv50k-2016-235181
	Test Weight, BZ	4H	44.11	7.72	12.07	-	JHI-Hv50k-2016-234379	JHI-Hv50k-2016-235181	SCRI_RS_144322
	Heading Date, BZ	4H	56.10	4.21	2.20	+	SCRI_RS_136648	JHI-Hv50k-2016-253123	JHI-Hv50k-2016-254192
	Yield, BZ	4H	68.00	3.68	7.63	+	JHI-Hv50k-2016-258005	JHI-Hv50k-2016-258419	JHI-Hv50k-2016-258481
	Days To One-Leaf, RRU	4H	114.00	4.97	6.59	+	JHI-Hv50k-2016-270367	BOPA2_12_10032	JHI-Hv50k-2016-270513
	Root Length Range, RRU	4H	116.00	3.23	5.62	+	JHI-Hv50k-2016-270367	BOPA2_12_10032	JHI-Hv50k-2016-270513
	Plant Height, BZ	4H	125.63	6.84	5.91	+	JHI-Hv50k-2016-276166	SCRI_RS_119390	JHI-Hv50k-2016-276557
	Heading Date, BZ	5H	36.72	3.19	1.65	+	JHI-Hv50k-2016-284294	JHI-Hv50k-2016-284434	SCRI_RS_205508
	Plant Height, BZ	5H	51.00	15.73	15.46	+	BOPA2_12_31155	JHI-Hv50k-2016-308617	JHI-Hv50k-2016-308711
	Plant Height, HV	5H	71.00	4.30	5.91	+	JHI-Hv50k-2016-311439	JHI-Hv50k-2016-313234	JHI-Hv50k-2016-314454
	Test Weight, BZ	5H	74.00	3.26	4.80	-	JHI-Hv50k-2016-311439	JHI-Hv50k-2016-313234	JHI-Hv50k-2016-314454
	Plant Height, BZ	5H	111.78	10.19	9.25	+	JHI-Hv50k-2016-327706	SCRI_RS_195813	JHI-Hv50k-2016-328604
	Root Length Range, RRU	5H	150.00	3.13	5.45	-	JHI-Hv50k-2016-342507	JHI-Hv50k-2016-342820	JHI-Hv50k-2016-354835
QDD-5H	Days To Maturity, BZ	5H	174.28	5.41	7.78	+	SCRI_RS_12757	JHI-Hv50k-2016-354890	JHI-Hv50k-2016-355060
	Heading Date, BZ	5H	175.00	4.13	1.94	+	JHI-Hv50k-2016-355060	SCRI_RS_186008	JHI-Hv50k-2016-355514
	Days To One-Leaf, RRU	5H	178.74	6.10	8.22	+	JHI-Hv50k-2016-357373	JHI-Hv50k-2016-357516	JHI-Hv50k-2016-357952
	Root Length Range, RRU	5H	181.22	4.73	8.42	+	SCRI_RS_210006	JHI-Hv50k-2016-359326	JHI-Hv50k-2016-359869
	Longest Root Length, RRU	5H	182.16	3.92	6.38	+	JHI-Hv50k-2016-359869	JHI-Hv50k-2016-360315	JHI-Hv50k-2016-360588
	Days To Maturity, HV	5H	185.00	6.73	11.28	+	SCRI_RS_190416	JHI-Hv50k-2016-362259	JHI-Hv50k-2016-362422
	Shoot Length, RRU	5H	187.20	7.23	8.46	+	SCRI_RS_141226	JHI-Hv50k-2016-365259	JHI-Hv50k-2016-365534
	Test Weight, BZ	6H	0.00	5.43	8.22	-		JHI-Hv50k-2016-369992	BOPA1_1603-1165
	Plump, BZ	6H	3.56	5.39	5.86	+	JHI-Hv50k-2016-370838	BOPA1_5148-1414	JHI-Hv50k-2016-370664
	Test Weight, HV	6H	3.56	6.02	11.40	-	JHI-Hv50k-2016-370838	BOPA1_5148-1414	JHI-Hv50k-2016-370664

Table A3.4 Continued.

QGFmt-6H	Test Weight, HV	6H	52.00	6.05	11.47	+	JHI-Hv50k-2016-380543	JHI-Hv50k-2016-380886	JHI-Hv50k-2016-381001
	Plump, BZ	6H	54.00	5.48	5.97	+	JHI-Hv50k-2016-380948	JHI-Hv50k-2016-381068	BOPA2_12_30361
	Plump, HV	6H	58.77	6.24	9.32	+	JHI-Hv50k-2016-382955	JHI-Hv50k-2016-382870	JHI-Hv50k-2016-382628
	Plump Percent Difference	6H	59.00	4.39	7.63	-	JHI-Hv50k-2016-382955	JHI-Hv50k-2016-382870	JHI-Hv50k-2016-382628
	Shoot Length, RRU	6H	59.00	2.97	3.27	+	JHI-Hv50k-2016-382955	JHI-Hv50k-2016-382870	JHI-Hv50k-2016-382628
	Protein, HV	6H	66.00	22.26	32.59	-	BOPA1_2176-891	JHI-Hv50k-2016-385462	JHI-Hv50k-2016-385540
	Protein, BZ	6H	66.00	34.36	51.93	-	BOPA1_2176-891	JHI-Hv50k-2016-385462	JHI-Hv50k-2016-385540
	Grain Fill, BZ	6H	66.79	20.96	16.07	+	BOPA1_2176-891	JHI-Hv50k-2016-385462	JHI-Hv50k-2016-385540
	Longest Root : Shoot, RRU	6H	67.00	14.12	29.34	-	BOPA1_2176-891	JHI-Hv50k-2016-385462	JHI-Hv50k-2016-385540
	Protein Percent Difference	6H	67.40	14.53	30.34	-	JHI-Hv50k-2016-385540	SCRI_RS_143259	JHI-Hv50k-2016-385944
	Longest Root Length, RRU	6H	68.35	4.58	7.53	-	JHI-Hv50k-2016-387063	JHI-Hv50k-2016-388223	BOPA1_2041-1317
	Test Weight, BZ	6H	68.35	8.79	13.96	+	JHI-Hv50k-2016-387063	JHI-Hv50k-2016-388223	BOPA1_2041-1317
	Days To Maturity, BZ	6H	69.00	18.52	32.16	+	BOPA1_2041-1317	JHI-Hv50k-2016-387236	HvNAM1
	Grain Fill, HV	6H	70.00	6.55	6.55	+	JHI-Hv50k-2016-387236	HvNAM1	JHI-Hv50k-2016-389670
	Days To Maturity, HV	6H	70.17	10.21	17.98	+	JHI-Hv50k-2016-387236	HvNAM1	JHI-Hv50k-2016-389670
	Days To One-Leaf, RRU	6H	72.42	3.02	3.90	-	SCRI_RS_2834	JHI-Hv50k-2016-391034	SCRI_RS_150504
	Yield, BZ	6H	100.06	3.89	8.09	-	SCRI_RS_137215	SCRI_RS_153227	JHI-Hv50k-2016-413558
	Lateral Root Count, RRU	6H	105.55	4.07	8.07	+	JHI-Hv50k-2016-414074	SCRI_RS_164341	JHI-Hv50k-2016-414438
	Plump, BZ	6H	111.35	8.41	9.55	+	SCRI_RS_12874	JHI-Hv50k-2016-415471	JHI-Hv50k-2016-415739
	Test Weight, BZ	6H	122.11	5.29	8.00	-	JHI-Hv50k-2016-417327	JHI-Hv50k-2016-418570	JHI-Hv50k-2016-418731
	Test Weight Percent Difference	7H	18.69	3.42	8.39	-	JHI-Hv50k-2016-451447	JHI-Hv50k-2016-451426	JHI-Hv50k-2016-451629
	Protein, BZ	7H	23.08	3.23	3.07	+	JHI-Hv50k-2016-452700	JHI-Hv50k-2016-452747	JHI-Hv50k-2016-452912
	Shoot Length, RRU	7H	23.08	3.48	3.86	-	JHI-Hv50k-2016-452700	JHI-Hv50k-2016-452747	JHI-Hv50k-2016-452912
	Grain Fill, BZ	7H	25.61	4.41	2.66	-	JHI-Hv50k-2016-452912	JHI-Hv50k-2016-453316	SCRI_RS_162631
	Plump, BZ	7H	27.86	7.09	7.90	+	JHI-Hv50k-2016-453455	JHI-Hv50k-2016-453831	JHI-Hv50k-2016-453618

Table A3.4 Continued.

QGFhd-7H	Protein Percent Difference	7H	39.00	4.16	7.49	+	JHI-Hv50k-2016-458499	SCRI_RS_138457	JHI-Hv50k-2016-459853
	Grain Fill, BZ	7H	41.47	4.19	2.52	-	JHI-Hv50k-2016-459853	BK_05	JHI-Hv50k-2016-460074
	Heading Date, BZ	7H	41.47	32.15	25.46	+	JHI-Hv50k-2016-459853	BK_05	JHI-Hv50k-2016-460074
	Plant Height, BZ	7H	41.47	6.06	5.18	+	JHI-Hv50k-2016-459853	BK_05	JHI-Hv50k-2016-460074
	Heading Date, HV	7H	44.00	4.06	5.59	+	BK_05	JHI-Hv50k-2016-460074	BOPA2_12_10368
	Grain Fill, HV	7H	46.00	4.39	4.39	-	BK_05	JHI-Hv50k-2016-460074	BOPA2_12_10368
QGF-7H	Test Weight, BZ	7H	72.86	4.00	5.93	+	JHI-Hv50k-2016-473042	JHI-Hv50k-2016-472911	JHI-Hv50k-2016-472745
	Heading Date, HV	7H	76.76	5.04	7.04	-	JHI-Hv50k-2016-481205	SCRI_RS_140553	JHI-Hv50k-2016-474421
	Plump, HV	7H	76.76	7.99	12.23	+	JHI-Hv50k-2016-481205	SCRI_RS_140553	JHI-Hv50k-2016-474421
	Plump Percent Difference	7H	76.76	5.98	10.61	-	JHI-Hv50k-2016-481205	SCRI_RS_140553	JHI-Hv50k-2016-474421
	Days To Maturity, HV	7H	77.99	3.25	5.20	+	JHI-Hv50k-2016-475000	JHI-Hv50k-2016-475638	JHI-Hv50k-2016-475672
	Days To One-Leaf, RRU	7H	79.85	5.50	7.35	+	JHI-Hv50k-2016-475672	JHI-Hv50k-2016-475698	BOPA2_12_30492
	Grain Fill, HV	7H	80.78	10.70	10.70	+	JHI-Hv50k-2016-485419	BOPA1_8491-1138	JHI-Hv50k-2016-487580
	Heading Date, BZ	7H	80.78	6.05	3.25	-	JHI-Hv50k-2016-485419	BOPA1_8491-1138	JHI-Hv50k-2016-487580
	Shoot Length, RRU	7H	80.78	5.23	5.95	+	JHI-Hv50k-2016-485419	BOPA1_8491-1138	JHI-Hv50k-2016-487580
	Grain Fill, BZ	7H	81.09	4.92	2.99	+	BOPA1_8491-1138	JHI-Hv50k-2016-487580	BOPA1_1578-552
	Plant Height, HV	7H	85.00	4.46	6.15	+	JHI-Hv50k-2016-489580	SCRI_RS_122512	JHI-Hv50k-2016-490074
	Plant Height, BZ	7H	101.00	8.69	7.71	+	JHI-Hv50k-2016-492409	JHI-Hv50k-2016-494771	SCRI_RS_175893
	Plant Height, HV	7H	106.03	4.30	5.90	+	JHI-Hv50k-2016-494771	SCRI_RS_175893	JHI-Hv50k-2016-495129
	Plump, BZ	7H	146.68	5.10	5.52	+	JHI-Hv50k-2016-512813	JHI-Hv50k-2016-513238	BOPA1_1590-544

APPENDIX C

ADDITIONAL TABLE FOR CHAPTER FOUR

Table A4.1. All marker-trait associations from the GWAS for agronomic and microbial traits, and co-localizations with QTLs from Escudero-Martinez et al., (2022) and Williams et al., (2024).

Chromosome	Position (bp)	SNP	Location	Trait Type	Trait	-log10(p)	Percent of Variance	Effect of Minor Allele	Minor Allele Frequency	Co-localizations Within 1Mb	Co-localizations From Other Studies
1	2739186	S1_2739186	Bozeman	Agronomic	Grain Yield	5.055	1.776	102.400	0.208		
1	10258898	S1_10258898	Moccasin	Genus	Bradyrhizobium	6.106	54.752	13.330	0.081	1.1	
1	10961647	S1_10961647	Moccasin	Genus	Bradyrhizobium	6.541	0.000	-0.634	0.081	1.1	
1	10965736	S1_10965736	Bozeman	Agronomic	Percent Grain Protein	6.797	1.902	-0.350	0.072	1.1	
1	21498804	S1_21498804	Bozeman	Phylum	Gemmatimonadota	4.616	2.688	0.561	0.123		
1	53877355	S1_53877355	Moccasin	Genus	Gaiella	4.540	0.000	-1.716	0.102		
1	58732002	S1_58732002	Moccasin	Genus	Gaiella	4.470	0.000	-1.749	0.094		
1	346195874	S1_346195874	Bozeman	Agronomic	Maturity Date	5.506	4.098	0.406	0.294		
1	379634641	S1_379634641	Moccasin	Genus	Gaiella	4.730	4.942	-1.781	0.096		
1	411156124	S1_411156124	Moccasin	Phylum	Bdellovibrionota	7.039	1.078	-1.573	0.114		
1	413955188	S1_413955188	Moccasin	Genus	Gaiella	8.786	13.179	1.186	0.125		
1	528013882	S1_528013882	Moccasin	Genus	Gaiella	5.571	2.842	0.864	0.132		
1	535416475	S1_535416475	Moccasin	Agronomic	Percent Grain Protein	4.557	2.049	0.217	0.262		
1	548442398	S1_548442398	Moccasin	Genus	Solirubrobacter	5.275	0.000	0.854	0.132	1.2	
1	549126160	S1_549126160	Moccasin	Genus	Solirubrobacter	4.652	0.000	-1.237	0.419	1.2	
1	550242008	S1_550242008	Moccasin	Genus	Solirubrobacter	5.849	0.000	-0.325	0.474		
1	554061215	S1_554061215	Bozeman	Agronomic	Grain Yield	4.877	1.507	-106.220	0.335		
2	2829368	S2_2829368	Moccasin	Genus	Sphingomonas	7.695	3.125	-2.139	0.116	2.1	
2	3238548	S2_3238548	Moccasin	Genus	Skermanella	4.838	3.066	0.512	0.081	2.1	
2	3238548	S2_3238548	Moccasin	Genus	Sphingomonas	7.227	10.439	2.100	0.081	2.1	
2	8691157	S2_8691157	Bozeman	Genus	Pseudoarthrobacter	6.788	69.267	-1.150	0.071		
2	21194828	S2_21194828	Moccasin	Genus	Gaiella	5.520	0.645	-0.672	0.387		
2	27377110	S2_27377110	Moccasin	Agronomic	Percent Grain Protein	4.734	0.099	-0.202	0.465		
2	57273147	S2_57273147	Moccasin	Agronomic	Plant Height	6.809	3.278	0.408	0.273		
2	102399312	S2_102399312	Moccasin	Genus	Haliangium	4.402	0.000	-1.119	0.039	2.2	
2	102399312	S2_102399312	Moccasin	Genus	Nocardioides	4.475	0.000	-1.181	0.039	2.2	
2	106853205	S2_106853205	Moccasin	Genus	Haliangium	4.442	21.295	-1.137	0.045	2.3	
2	106853205	S2_106853205	Moccasin	Genus	Nocardioides	4.612	10.698	-1.213	0.045	2.3	
2	115721968	S2_115721968	Moccasin	Genus	Solirubrobacter	4.383	0.023	0.550	0.175		
2	199847612	S2_199847612	Moccasin	Genus	Arthrobacter	6.152	3.287	1.273	0.322		*QGFhd-2H* : seminal root traits, heading date, grain quality (Williams et al., 2024)
2	644152401	S2_644152401	Moccasin	Genus	Solirubrobacter	10.647	0.023	1.561	0.133		2.1 QTL: rhizosphere bacterial genus QTL (Escudero-Martinez et al., 2022); QLN-2H: seminal root traits (Williams et al., 2024)
2	652035186	S2_652035186	Bozeman	Agronomic	Percent Plump Grain	7.291	7.480	0.339	0.140		
2	670213296	S2_670213296	Moccasin	Genus	Blastococcus	4.397	9.607	0.944	0.073		
2	689755797	S2_689755797	Bozeman	Agronomic	Grain Yield	5.209	4.460	-126.213	0.240		
2	702515240	S2_702515240	Moccasin	Genus	Belnapia	6.510	5.065	-1.120	0.069		2.1 QTL: rhizosphere bacterial genus QTL (Escudero-Martinez et al., 2022)
2	745314952	S2_745314952	Bozeman	Phylum	Gemmatimonadota	4.385	1.869	0.585	0.431		
2	750395721	S2_750395721	Bozeman	Phylum	Gemmatimonadota	5.153	0.486	0.487	0.397		

Table A4.1 Continued.

2	752927131	S2_752927131	Moccasin	Agronomic	Harvest Index	6.639	0.914	0.009	0.145		
3	8187279	S3_8187279	Bozeman	Agronomic	Percent Plump Grain	4.561	6.989	-0.312	0.105		
3	17464358	S3_17464358	Moccasin	Phylum	Bdellovibrionota	4.504	45.423	-0.864	0.320		
3	19191454	S3_19191454	Bozeman	Genus	Rubrobacter	5.893	8.273	1.401	0.103	3.1	
3	19847104	S3_19847104	Bozeman	Phylum	Gemmatimonadota	6.500	3.823	0.736	0.086	3.1	
3	23418538	S3_23418538	Bozeman	Agronomic	Grain Yield	5.875	8.426	155.553	0.086		
3	33266982	S3_33266982	Bozeman	Genus	Nocardioides	4.475	11.181	-1.501	0.419		
3	34960422	S3_34960422	Bozeman	Agronomic	Productive Tiller Number	4.563	3.524	4.742	0.373		
3	51808788	S3_51808788	Moccasin	Agronomic	Harvest Index	5.410	3.281	0.005	0.421		
3	101733471	S3_101733471	Moccasin	Agronomic	Percent Plump Grain	4.448	25.991	-2.376	0.451		
3	493728860	S3_493728860	Moccasin	Agronomic	Harvest Index	4.605	1.141	0.007	0.164		
3	504134127	S3_504134127	Moccasin	Genus	Reyranella	5.056	0.016	0.464	0.381		
3	528973679	S3_528973679	Bozeman	Phylum	Gemmatimonadota	5.059	3.972	0.775	0.092		
3	542015340	S3_542015340	Moccasin	Agronomic	Plant Height	4.812	5.327	-0.385	0.112		
3	545127707	S3_545127707	Moccasin	Genus	Reyranella	9.119	44.122	-0.696	0.106		
3	547843435	S3_547843435	Moccasin	Genus	Reyranella	6.932	54.273	2.641	0.084		
3	594146568	S3_594146568	Bozeman	Genus	Skermanella	4.491	27.987	0.947	0.056		
3	664821347	S3_664821347	Bozeman	Genus	Mycobacterium	4.603	9.285	-2.170	0.236		
3	671890267	S3_671890267	Moccasin	Genus	Psychroglaciecola	31.259	54.565	-0.418	0.137	3.2	
3	671893297	S3_671893297	Moccasin	Genus	Psychroglaciecola	29.062	42.264	6.246	0.134	3.2	
3	680223770	S3_680223770	Bozeman	Genus	Psychroglaciecola	4.536	38.112	-3.821	0.104		
3	688562174	S3_688562174	Moccasin	Genus	Belnapia	5.610	3.782	0.962	0.079		
4	35550931	S4_35550931	Moccasin	Genus	Belnapia	7.882	16.922	-1.292	0.079	4.1	
4	36211080	S4_36211080	Moccasin	Genus	Psychroglaciecola	5.240	0.000	-0.560	0.085	4.1	
4	60957465	S4_60957465	Moccasin	Agronomic	Percent Plump Grain	5.156	4.368	3.219	0.124	4.2	
4	60957465	S4_60957465	Bozeman	Agronomic	Percent Plump Grain	5.781	2.010	0.275	0.123	4.2	
4	346970208	S4_346970208	Moccasin	Genus	Bradyrhizobium	5.916	0.000	-1.025	0.110		
4	465989932	S4_465989932	Moccasin	Agronomic	Percent Plump Grain	5.327	1.704	-2.087	0.294		
4	576137567	S4_576137567	Moccasin	Genus	Skermanella	6.016	11.793	0.717	0.075		4.1 QTL: rhizosphere bacterial ASV
4	581252202	S4_581252202	Bozeman	Agronomic	Maturity Date	5.999	10.483	-0.672	0.114		(Escudero-Martinez et al., 2022)
4	598068305	S4_598068305	Moccasin	Genus	Reyranella	4.716	0.000	-0.857	0.064		
4	620873337	S4_620873337	Moccasin	Agronomic	Percent Plump Grain	8.474	2.248	-3.676	0.427		
4	623321296	S4_623321296	Moccasin	Genus	Jatrophihabitans	4.554	0.590	0.217	0.335	4.3	
4	623334098	S4_623334098	Moccasin	Genus	Jatrophihabitans	4.830	1.865	0.225	0.310	4.3	
4	623334098	S4_623334098	Moccasin	Genus	Segetibacter	4.607	12.474	0.239	0.310	4.3	
4	623708635	S4_623708635	Moccasin	Genus	Jatrophihabitans	4.535	0.493	0.214	0.351	4.3	
5	6863100	S5_6863100	Moccasin	Genus	Sphingomonas	7.022	4.611	-1.664	0.254	5.1	
5	7400480	S5_7400480	Moccasin	Genus	Psychroglaciecola	7.566	0.000	0.836	0.096	5.1	
5	7478887	S5_7478887	Bozeman	Genus	Pseudoarthrobacter	5.215	0.209	0.696	0.155	5.1	
5	9490806	S5_9490806	Bozeman	Phylum	Myxococcota	6.225	10.583	1.838	0.063		
5	97248843	S5_97248843	Bozeman	Agronomic	Percent Grain Protein	4.742	9.243	0.211	0.330		

Table A4.1 Continued.

5	520669130	S5_520669130	Bozeman	Agronomic	Productive Tiller Number	5.991	1.717	5.048	0.194		
5	556799189	S5_556799189	Bozeman	Agronomic	Percent Grain Protein	5.643	4.363	0.273	0.140		5.2 QTL: rhizosphere bacterial genus (Escudero-Martinez et al., 2022)
5	559348031	S5_559348031	Moccasin	Agronomic	Harvest Index	4.447	1.636	-0.004	0.305		
5	562310313	S5_562310313	Moccasin	Genus	Solirubrobacter	5.440	0.000	1.106	0.052		
5	564846721	S5_564846721	Moccasin	Genus	Belnapia	5.227	3.115	-0.751	0.160		
5	587058397	S5_587058397	Moccasin	Genus	Bradyrhizobium	9.771	0.284	1.189	0.121	5.2	
5	587058397	S5_587058397	Moccasin	Genus	Skermanella	5.793	2.631	0.656	0.121	5.2	
5	587764196	S5_587764196	Moccasin	Genus	Solirubrobacter	6.280	52.160	-0.751	0.209	5.2	
5	589739761	S5_589739761	Moccasin	Genus	Bradyrhizobium	7.346	0.000	-2.369	0.165		5.3 QTL: rhizosphere bacterial family (Escudero-Martinez et al., 2022); *QDD-5H* : seminal root traits, delayed heading and maturity (Williams et al., 2024)
5	594829374	S5_594829374	Moccasin	Genus	Reyranella	13.761	0.009	-2.074	0.120		
5	607073518	S5_607073518	Moccasin	Genus	Reyranella	20.392	0.000	2.391	0.097		
5	617105739	S5_617105739	Bozeman	Agronomic	Productive Tiller Number	4.608	6.806	4.823	0.189	5.3	
5	617193546	S5_617193546	Bozeman	Phylum	Myxococcota	6.171	0.262	-0.895	0.139	5.3	
5	644002020	S5_644002020	Bozeman	Phylum	Myxococcota	8.528	3.204	-1.643	0.100		
5	659541058	S5_659541058	Moccasin	Genus	Solirubrobacter	5.631	42.620	1.140	0.068		*QDD-5H* : seminal root traits, delayed heading and maturity (Williams et al., 2024)
5	668265933	S5_668265933	Bozeman	Phylum	Myxococcota	11.138	4.521	1.992	0.075	5.4	
5	668937320	S5_668937320	Moccasin	Genus	Belnapia	5.291	4.161	0.866	0.120	5.4	
6	6977154	S6_6977154	Moccasin	Genus	Gemmatimonas	4.640	39.869	1.894	0.195	6.1	
6	6977154	S6_6977154	Bozeman	Phylum	Myxococcota	4.538	0.727	0.630	0.203	6.1	
6	7681197	S6_7681197	Moccasin	Genus	Sphingomonas	7.473	5.752	-1.129	0.153	6.1	
6	12287518	S6_12287518	Moccasin	Genus	Skermanella	5.885	1.183	0.486	0.227		
6	16206598	S6_16206598	Moccasin	Agronomic	Plant Height	6.109	5.615	-0.499	0.172		
6	18483107	S6_18483107	Bozeman	Agronomic	Duration of Grain Fill	7.951	12.270	-1.158	0.080		
6	36341900	S6_36341900	Moccasin	Genus	Psychroglaciecola	7.239	0.052	-0.767	0.091	6.2	
6	36341900	S6_36341900	Moccasin	Genus	Skermanella	7.857	3.236	-0.796	0.091	6.2	*QGFmt-6H* : seminal root traits, delayed maturity, low protein (Williams et al., 2024)
6	51815946	S6_51815946	Moccasin	Agronomic	Percent Grain Protein	13.003	8.621	0.523	0.400		
6	55770233	S6_55770233	Bozeman	Agronomic	Percent Grain Protein	14.128	19.975	0.420	0.397		
6	368527525	S6_368527525	Bozeman	Agronomic	Maturity Date	12.035	6.220	-1.044	0.153		
6	374871945	S6_374871945	Bozeman	Genus	Pseudoarthrobacter	6.981	0.402	0.770	0.171	6.3	
6	374871945	S6_374871945	Bozeman	Phylum	Myxococcota	6.443	4.959	2.882	0.171	6.3	
6	374871948	S6_374871948	Bozeman	Genus	Pseudoarthrobacter	4.451	0.000	-2.263	0.161	6.3	
6	374871948	S6_374871948	Bozeman	Phylum	Myxococcota	7.845	4.845	-0.542	0.161	6.3	
6	403258165	S6_403258165	Moccasin	Genus	Psychroglaciecola	8.701	0.000	-0.660	0.243	6.4	
6	403258165	S6_403258165	Moccasin	Genus	Skermanella	7.583	1.632	-0.742	0.243	6.4	
6	547835533	S6_547835533	Moccasin	Genus	Methylobacterium-Methylorubrum	4.510	23.462	1.983	0.073	6.5	
6	547837831	S6_547837831	Moccasin	Genus	Gaiella	6.046	4.801	1.029	0.082	6.5	
6	552075138	S6_552075138	Bozeman	Genus	Pseudonocardia	5.255	6.235	-0.982	0.154		
6	559847890	S6_559847890	Bozeman	Genus	Pseudoarthrobacter	7.046	0.522	-0.750	0.159		
6	567938334	S6_567938334	Moccasin	Genus	Sphingomonas	5.290	1.565	0.757	0.482		
6	576416464	S6_576416464	Moccasin	Genus	Blastococcus	4.446	14.407	1.065	0.050		
6	578867407	S6_578867407	Bozeman	Phylum	Myxococcota	4.771	0.557	0.594	0.251		

Table A4.1 Continued.

6	582790283	S6_582790283	Moccasin	Genus	Psychroglaciecola	6.121	0.041	-0.404	0.421	6.6	
6	583094559	S6_583094559	Bozeman	Genus	Rubrobacter	4.618	8.132	1.339	0.067	6.6	
6	583094559	S6_583094559	Bozeman	Phylum	Gemmatimonadota	9.272	10.046	1.165	0.067	6.6	
7	13265841	S7_13265841	Moccasin	Genus	Reyranella	5.041	0.000	0.718	0.106		
7	18484768	S7_18484768	Bozeman	Agronomic	Percent Plump Grain	4.719	3.719	0.200	0.375		
7	32643252	S7_32643252	Bozeman	Agronomic	Productive Tiller Number	4.611	1.116	-3.465	0.399	7.1	
7	32727474	S7_32727474	Moccasin	Genus	Nocardioides	4.721	7.718	1.003	0.075	7.1	
7	40360840	S7_40360840	Bozeman	Agronomic	Duration of Grain Fill	4.802	6.245	-0.694	0.158		*QGFhd-7H.1*: heading date, protein stability
7	41744480	S7_41744480	Bozeman	Agronomic	Grain Yield	5.213	4.271	-146.782	0.154		(Williams et al., 2024)
7	51968281	S7_51968281	Moccasin	Genus	Psychroglaciecola	6.350	0.020	0.522	0.408		
7	75212014	S7_75212014	Moccasin	Genus	Belnapia	6.374	1.783	0.834	0.397		
7	88663228	S7_88663228	Moccasin	Agronomic	Percent Grain Protein	9.051	2.603	0.349	0.368		
7	119396882	S7_119396882	Moccasin	Genus	Psychroglaciecola	12.347	0.000	-1.179	0.052	7.2	
7	119396882	S7_119396882	Moccasin	Genus	Skermanella	9.776	15.107	-1.096	0.052	7.2	
7	632732436	S7_632732436	Moccasin	Agronomic	Percent Grain Protein	7.818	3.447	0.454	0.097		
7	636567177	S7_636567177	Moccasin	Genus	Belnapia	7.116	2.173	-0.957	0.151		
7	638708843	S7_638708843	Moccasin	Genus	Arthrobacter	4.789	11.064	0.974	0.095	7.3	
7	638708843	S7_638708843	Moccasin	Genus	Psychroglaciecola	5.744	0.035	0.489	0.095	7.3	
7	643864910	S7_643864910	Moccasin	Genus	Belnapia	7.274	1.313	0.895	0.171		
7	652403921	S7_652403921	Moccasin	Genus	Bradyrhizobium	4.383	0.841	1.093	0.089	7.4	7.1 QTL: rhizosphere bacterial genus and family
7	652403921	S7_652403921	Moccasin	Genus	Rhodococcus	4.925	23.520	3.423	0.089	7.4	(Escudero-Martinez et al., 2022)