Evaluation of Root System Architecture (RSA) As A New Breeding Target for Climate-Resilient Soft Red Winter Wheat (*Tritium aestivum* L.)

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Crop and Soil Environmental Science

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August 30th, 2024
Blacksburg, VA

Keywords: Root System Architecture (RSA), Climate Resiliency, Winter Wheat (*Tritium aestivum L.*), Accumulation, Remobilization

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The Evaluation of Root System Architecture (RSA) As A New Breeding Target for Climate-Resilient Winter Wheat (*Tritium aestivum* L.)

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ABSTRACT

Crop yields are expected to face more threatening circumstances due to ongoing climatic and environmental change. The continued sustainability of crop production will depend on the genetic capacity of crops to adapt to increased biotic and abiotic barriers induced by climate change. Historically, shoot-based traits were breeding targets for overcoming yield gaps between developed and undeveloped nations. However, the rate of genetic gain has stabilized with conventional breeding targets for indirect yield improvement. As the availability of mineral fertilizers is steadily declining and the occurrence of low-fertility soils has increased, reoccurring yield disparities worldwide are propelling us to evaluate new breeding targets. There is potential for plant breeders to shift their focus to the root system architecture (RSA) as a new target for indirect selection, enabled by the phenotypic plasticity of winter wheat (*Triticum* sp.), one of the main staple agronomic crops. Our current limited understanding of the dynamic nature of the root system architecture is due to the difficulties associated with *in situ* phenotyping and characterization of anatomical traits. The objectives of this thesis were to 1) review advancements in root phenotyping methodologies and past, present, and future predictions; 2) evaluate differences in root biomass accumulation and remobilization among 22 Virginia Tech-developed elite germplasm; 3) evaluate potential genetic variability for root biomass accumulation across breeding lines. Minimal genetic variation was observed for root biomass accumulation through time. Soil coring proved

not to be a very effective method for capturing genetic variability of root biomass accumulation from a soil depth of 10 cm. However, a low genetic signal was also observed for shoot biomass, even though the entire field plot for each genotype was sampled. Yet, a considerably higher genetic signal was observed for plant height. These results suggest that both root and shoot biomass are complex, polygenic traits that require significantly more attention to evaluate genetic differences.

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GENERAL AUDIENCE ABSTRACT

Climate change induces numerous abiotic and biotic barriers to our global cropping systems. Mineral fertilizer reserves are expected to deplete within the next 80 years while our agricultural lands continue losing fertility. This translates into increased yield discrepancies among the most prominent staple agronomic crops. Historically, crop improvement has been performed through indirect selection upon shoot-based traits for yield improvement. However, the capacity of genetic gain from these conventional selection criteria is projected to stabilize. Therefore, it would be beneficial for future global crop production if the initiative was taken to identify a new breeding target that can ensure climate resiliency in staple crops, such as winter wheat (*Triticum*). Root system architecture (RSA) is defined as the spatial distribution of embryonic and post-embryonic roots throughout a growth medium. This has the potential to become a new breeding target. However, there are numerous difficulties to overcome when evaluating roots *in situ*. In addition, there is no standardized root phenotyping method that can be implemented nationwide due to the variability in phenotypic response in various growing environments. The objectives of this thesis are to 1) reveal the advancements in root phenotyping and its legitimacy for standardization, 2) explore the genetic architecture of root system architecture, and 3) evaluate the genetic variability of root biomass accumulation for climate resiliency. Minimal genetic variation was observed for root

biomass accumulation through time. Soil coring proved not to be a very effective method for capturing genetic variability of root biomass accumulation from a soil depth of 10 cm. However, a low genetic signal was also observed for shoot biomass, even though the entire field plot for each genotype was sampled. Yet, a considerably higher genetic signal was observed for plant height. These results suggest that both root and shoot biomass are complex, polygenic traits that require significantly more attention to evaluate genetic differences.

DEDICATION

I want to dedicate this thesis to my family, who has provided constant love and support throughout my academic journey and beyond.

ACKNOWLEDGEMENTS

I want to express my gratitude to my research committee: Dr. Nicholas Santantonio (Committee Chair), Dr. Bastiaan Bargmann, and Dr. Angela Possinger, who have provided me with support, guidance, and valuable insights and facilitated a learning environment that challenges me to become a better researcher.

Additionally, I would like to thank Dr. Felipe Sabadin for agreeing to combine research focuses and for providing considerable assistance when preparing my thesis. He has contributed significantly to planting germplasm, data collection, and data analysis, and his aid has always been greatly appreciated.

I would also like to extend my sincere gratitude to the Virginia Tech Small Grains Breeding program and laboratory, who provided all the germplasm used in my field trial, assisted with planting and data collection, and provided emotional support to help me fulfill my responsibilities and research objectives.

I want to thank the George Washington Carver Scholars Program for funding my graduate degree, facilitating a safe space and community for BIPOC (Black, Indigenous, People of Color) graduate students to congregate, strengthening my professional development skills, and providing me the opportunity to present my research at a conference hosted by an internationally accredited organization.

Lastly, I thank my family and friends for their unwavering physical, emotional, and moral support throughout my academic journey.

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EMI - Electromagnetic Inductance

ERT - Electrical Resistance Tomography

EV - Early Vegetative

GF – Grain Fill

GWAS - Genome-Wide Association Study

HD – Heading

LV – Late Vegetative

M – Maturity

MAS - Marker-Assisted Selection

MRI - Magnetic Resonance Imaging

NUE - Nutrient Use Efficiency

NAE - Nutrient Acquisition Efficiency

NAM - Nested Association Mapping

PET - Positron Emission Tomography

QTL - Quantitative Trait Loci

R:S - Root to Shoot Ratio

RCA - Root Cortical Aerenchyma

RSA - Root System Architecture

X-ray CT - X-ray Computed Tomography

Chapter 1. General Introduction and Literature Review

1.1 Introduction: Winter wheat (*Triticum*) and Its Impact on Global Food Security

As climatic conditions intensify, wheat-growing regions increasingly face abiotic stresses like heat waves, droughts, and flooding, which negatively impact yields during the grain-filling stage. Consequently, abiotic stress is becoming the primary yield-limiting factor in winter wheat production (Colombo et al. 2022). Additionally, yields of cereal crops, including wheat, have plateaued as they approach their potential limits (Khan et al. 2016). To address the yield gap and feed the growing global population, plant breeders must integrate new technologies and methods to enhance the genetic gain of staple crops such as winter wheat (*Triticum aestivum*).

Root system architecture (RSA)—the spatial distribution and morphological characteristics of roots—plays a crucial role in plant survival (Ober et al. 2021). RSA is influenced by genetic factors, environmental conditions (soil type, moisture content, pH), and biotic interactions (Paez-Garcia et al. 2015). Historically, breeding efforts have focused on shoot traits, which has led to yield plateaus. As a result, RSA has emerged as a promising target for improving yield and global food security (Pflugfelder et al. 2021).

Developing standardized methods for phenotyping RSA traits has proven challenging compared to above-ground characteristics due to the need for specialized equipment for in-situ imaging and visualization. RSA growth variation among genotypes and environmental conditions further complicates this process (Paez-Garcia et al. 2015). Nevertheless, a standardized root phenotyping method could significantly advance breeding efforts by selecting RSA traits that enhance agricultural production.

Environmental factors such as climate, precipitation, photoperiod, humidity, and soil properties critically impact plant development. It is essential to consider how these factors influence RSA to effectively breed climate-resilient winter wheat. For instance, drought stress triggers root physiological responses, with changes in RSA often indicating plant response to water shortages (Li et al. 2021). Without adequate genetic resilience, wheat is vulnerable to disease and pest pressure, which can severely reduce yield under changing environmental conditions. Understanding RSA's response to soil environment and stress factors is vital for developing more resilient cultivars (Colombo et al. 2022).

A shallow RSA may suffice under optimal conditions, but drought stress compels more profound root growth to access water and nutrients, making rooting depth an essential trait for drought tolerance. Deep-rooted wheat genotypes have been shown to yield 35-38% more under drought compared to shallow-rooted ones (Li et al. 2021). Additionally, soil physical properties like compaction and penetration resistance affect RSA and should be evaluated in breeding programs (Colombo et al. 2022).

Efforts to enhance RSA must integrate genetic factors with environmental variables. This review aims to: (1) examine above-ground traits and developmental stages of winter wheat and their impact on RSA; (2) explore the phenotypic and genetic architecture of RSA; (3) assess the advantages and limitations of various root phenotyping methods; (4) discuss genetic approaches for validating field evaluations; (5) analyze RSA plasticity; and (6) consider future directions for using innovative technology to develop wheat germplasm with highly adaptive RSA.

1.2 Winter Wheat (*Triticum*) Life Cycle and Above-Ground Biomass Growth Rate

Leaf & Tiller Development

Winter wheat undergoes eleven developmental stages throughout its life cycle. The cycle starts with [1] germination, where the coleoptile and radicle emerge from the seed under favorable moisture and temperature conditions. Seedling vigor can be enhanced by applying fertilizer before or at planting. Early planting at uniform rates is beneficial, as it increases the likelihood of more head-bearing tillers per unit area, leading to higher grain yields (Bruns, Croy 1983). Full tillering begins only after three leaves have developed. During this stage, the root system expands and forms the root crown structure at the base of the main stem (Broeske et al. 2022). Adequate phosphorus application during stages 1-3 is crucial for effective root initiation and tillering (Broeske et al. 2022).

The [2] tillering stage features rapid expansion of lateral meristems, making tillers more noticeable. In winter wheat, tillers formed in the fall contribute more to yield than those formed in the spring. Drought stress during this stage can significantly restrict tiller growth, negatively impacting yield depending on stress duration and intensity (Broeske et al. 2022). The crown roots continue to develop during this period.

During the [3] primary tiller stage, tillers will grow from the first four leaves, with the first three contributing up to half the total yield (Broeske et al. 2022). Secondary tillers emerge from the base of primary tillers. The crown root system becomes well-established. Monitoring for weeds and insects and applying herbicides as needed is advisable. A nitrogen application of 20-30 lbs./acre in the fall helps ensure the survival of

fall tillers. However, excessive nitrogen can lead to excessive vegetative growth, making the plant more vulnerable to fungal diseases, insect damage, and winter kill (Broeske et al. 2022). The plant experiences 3-8 weeks of cold weather-induced vernalization during this time. The [4] pseudo-stem (the secondary leaf sheath surrounding the primary leaf) will become erect, and the leaf stem will elongate. Spring green-up begins in winter wheat, marking an optimal period for spring nitrogen applications (Broeske et al. 2022).

Stem Elongation

The pseudo-stem should be erect at the [5] pseudo-stem stage, and the leaf sheaths elongated (Broeske et al., 2022). During this phase, the developing head pushes into the pseudo-stem, and the potential number of spikelets is established. Extreme stress at this stage can reduce kernel production. The plant absorbs about 0.1 inches of water daily, while tillers formed after this stage do not contribute to grain yield (Broeske et al. 2022). Stem elongation, driven by increased photoperiod and temperature in late winter and early spring, is a vital feature of this period (Broeske et al. 2022).

Rapid stem elongation occurs in the [6] jointing stage, making the first stem node visible due to internode stretching (Broeske et al. 2022). The plant becomes more sensitive to temperature drops, and the developing head moves up the elongating stem. During this stage, each plant absorbs about 0.25 inches of water daily (Broeske et al. 2022). If disease pressure is observed, applying a fungicide is advisable.

The second node and second-to-last leaf should be visible during the [7] rapid vegetative growth stage. Water and nutrient demands increase, and temperatures below 24°F can negatively impact productivity (Broeske et al. 2022). The [8] flag leaf, the last

growing leaf, should emerge above the third and fourth nodes. This leaf is crucial for yield, contributing 50-75% of the photosynthetic activity for grain development (Broeske et al. 2022). The [9] flag leaf should fully emerge from the main stem.

The plant's energy shifts to producing reproductive tissues and filling grain. To protect the flag leaf from foliar diseases, applying fungicides is recommended. Additional nitrogen may improve grain protein quality but will likely have only a minor effect on yield (Broeske et al. 2022). The [10] boot stage occurs just before heading when the head is encased in the flag leaf sheath, causing the plant to appear swollen. The flag leaf sheath and peduncle (stem supporting the head) continue to grow, leading to the head emerging from the flag leaf sheath (Broeske et al. 2022).

Heading and Flowering

The heading stage follows the boot stage, with the developing head emerging from the flag leaf sheath and anthesis (flowering) imminent. The number of kernels per head depends on the number of flowers pollinated, and wheat is sensitive to temperature during this stage (Broeske et al. 2022). About ten days after flowering, the kernels become fully established and initially appear watery-ripe, with clear liquid being squeezable from them (Broeske et al. 2022).

Grain Filling

During this stage, 15-18 days after flowering, [11] kernels transition from watery to milky ripe, with solid contents in the endosperm increasing (Broeske et al. 2022). The milk-like substance within the kernels gradually converts to a dough-like starch. The

plant's green color begins to fade, and kernel moisture decreases to 30-40%, leading to kernel hardening (Broeske et al. 2022). Monitoring moisture content is crucial as the wheat nears physiological maturity (Broeske et al. 2022).

Ripening

In this final stage, kernels are fully ripe, with moisture content reduced from 30% to 15%, which is optimal for harvest. Further moisture loss could jeopardize grain quality (Broeske et al. 2022). The green biomass has dried completely and turned into straw.

1.2 Fibrous Root System of Winter Wheat (*Triticum*)

Winter wheat (*T. aestivum*) is a monocot with a fibrous root system, including both embryonic (coarse, seminal roots from the radicle) and post-embryonic roots (fine, lateral roots arising from seminal roots) that can penetrate deep into the soil under optimal conditions. Seminal roots are the primary roots responsible for axial transport from the root system architecture (RSA) to the shoots. In contrast, fine roots, including lateral roots and root hairs, are crucial for water and nutrient uptake (Placido et al. 2020). A key trait of fine roots is a high surface area, which enhances water and nutrient absorption, especially in drought-prone environments (Ghimire et al. 2020). Lateral roots and root hairs are critical for a highly adaptive RSA due to their sensitivity to changes in the root microenvironment, affecting root length, formation, and orientation (Placido et al. 2020).

Winter wheat exhibits genetic variation in various traits as a hexaploidy species with six sets of chromosomes from A, B, and D diploid genomes. For example, the D genome influences the number of lateral roots (Placido et al. 2020). Understanding the genetic architecture of winter wheat is crucial for practical breeding. Breeding for RSA traits should account for specific temperature and moisture conditions to accurately address genotype-by-environment interactions in statistical analyses (Placido et al. 2020).

1.4 Root System Architecture (RSA) Response to Edaphic Factors

Soil Structure

Soils consist of gas, liquid water, and solid particles, with solid material making up about 50% of the total soil volume. Soil texture, determined by sand, silt, and clay proportions, influences aggregate size and distribution within the soil profile (Rich, Watt 2013). This texture affects soil structure, impacting bulk density and ped strength. Soil moisture content is a crucial edaphic factor influencing soil strength and plant development. Edaphic factors directly affect the phenotypic expression of root system architecture (RSA) traits.

Due to increased mechanical impedance, seminal roots growing in hardened soils experience reduced elongation rates. Rich and Watt (2013) found that primary roots in hardened soils showed stunted growth, limited surface area, and reduced dry matter compared to roots in softer soils, although they had a larger diameter. Lateral root initiation also varies with edaphic factors such as soil texture, pore size, and clod density (Rich, Watt 2013).

Root-limiting Features

Understanding soil characteristics is essential for predicting potential physical barriers affecting yield. Mechanical impedance and soil strength can vary across a given environment due to differences in soil structure, texture, and compaction (Rich, Watt 2013). Hardpans, such as fragipans and duripans, found in the subsoil, can restrict rooting depth but may not significantly impact total root mass, as roots can still explore above these layers (Rich, Watt 2013). Uneven soil compaction, often caused by heavy machinery during planting, can result in less dense root growth in compacted areas (Rich, Watt 2013). Additionally, roots will avoid expending energy to penetrate dense soil aggregates if more accessible routes, such as pore spaces or channels from previous crops, are available.

Subsoil Porosity

Root exploration into soil cracks and pores increases the likelihood of accessing essential water and nutrients, which supports early tiller development and disease resistance. Rich and Watt (2013) found that maize planted after alfalfa had 41% of its roots in alfalfa root channels. Similarly, wheat roots that extended deeper than 40 cm exhibited clustered growth in cracks and pores of reddish-brown clay soil. A fundamental mechanism of root extension involves the accumulation of turgor pressure within cells in the elongation zone, which helps overcome the constraints of the cell wall and surrounding soil (Rich, Watt 2013). Roots must exert significant energy to penetrate soil aggregates and facilitate elongation (Rich, Watt 2013).

Future plant breeding research could focus on developing genotypes with desirable root traits, such as rapid elongation rates, to improve performance in dense, resistant, and compacted soils. However, variability in subsoil porosity must be considered, as roots in large pore spaces may show poorer shoot biomass growth than those in more compacted soils (Rich, Watt 2013). The reasons behind the reduced shoot biomass in plants with roots in large pore spaces remain unclear (Rich, Watt 2013).

Soil Water Content

Water is the most critical factor limiting plant growth, influencing the physical and chemical properties of the rhizosphere. Water-stress conditions occur when water potential within plant biomass exceeds that in the soil, leading to dehydration, reduced hydrostatic pressure, and stunted root growth (Rich, Watt 2013). Water-stressed plants can mobilize nutrients from the shoots to the roots, enabling more expansive root growth to access water (Rich, Watt 2013).

Wheat growers could benefit from research on diverse root length responses under varying water availability. Such research could lead to crop varieties with enhanced water-stress tolerance. Rich and Watt (2013) found that in dry conditions, parts of the root system may extend deeper into the soil for moisture, with approximately 50% of water for this root exploration sourced from the phloem and the remaining 50% from soil aggregates. Manipulating shoot-based traits like phloem efficiency could improve water-use efficiency and support root elongation as subsoil moisture declines (Rich, Watt 2013).

Anoxia and Hypoxia

Prolonged soil inundation leads to hypoxia (low oxygen) or anoxia (no oxygen) in the rhizosphere, which impairs primary seminal root elongation and increases susceptibility to root rot. Chronic inundation can significantly reduce agronomic crop growth and yield potential (Rich, Watt 2013). Root decay from extended waterlogging alters the root-to-shoot ratio (R:S), with flood-tolerant paddy rice showing a lower R:S of 0.12-0.23 compared to 0.4-0.55 in wheat grown in well-drained soils. R:S is crucial for understanding plant responses to environmental stress and merits further study (Rich, Watt 2013).

Excessive anoxic conditions inhibit both primary root elongation and lateral root initiation. In wheat seedlings, waterlogging for 10-14 days, followed by a return to aerated conditions, often results in the seminal roots not recovering or continuing elongation, showing severe damage or death (Rich, Watt 2013). Shorter waterlogging periods may terminate seminal root apices but can still stimulate lateral root emergence from basal zones. Similar effects are observed in maize, where waterlogging for 12-13 days significantly slows or halts seminal root elongation (Rich, Watt 2013). Nodal roots, part of the crown structure, can vary in resilience among species and significantly affect overall root development (Rich, Watt 2013).

Nutrients

Root System Architecture (RSA) shape and orientation, including elongation, curving, and branching, critically influence a plant's ability to access nutrients in the soil.

For example, a higher horizontal root angle in the topsoil in beans allows the root system to exploit areas with higher phosphorus concentrations, as phosphorus is immobile in soil and crucial for plant growth (Rich, Watt 2013). Increased root proliferation often correlates with soil fertility; however, specific nutrients, such as nitrate, can inhibit root elongation in specific maize genotypes at high concentrations (Rich, Watt 2013). This highlights significant variability in how different nutrients affect root growth across plant species.

Future plant breeding efforts should improve RSA plasticity to better adapt to fluctuating nutrient concentrations and soil conditions. Evaluating RSA plasticity will enhance climate resilience and ensure consistent crop productivity and high yields despite variable nutrient distribution and environmental conditions (Rich, Watt 2013). Extreme nutrient deficiencies severely hinder root growth and increase disease susceptibility, while moderate deficiencies often lead to carbon being reallocated from shoot to root biomass to sustain root elongation (Rich, Watt 2013).

Temperature

Soil temperature significantly impacts root morphological mechanisms, such as elongation, branching, respiration, and metabolism, affecting above-ground biomass development. Unlike atmospheric temperature, soil temperature fluctuates daily, seasonally, and with soil depth, leading to variable root responses (Rich, Watt 2013). Research shows that root elongation generally increases with rising temperatures up to an optimal point, after which growth rates decline (Rich, Watt 2013). For cereals like wheat, barley, and oats, root elongation rates increase linearly from 5°C to 25°C, with peak

growth rates around or above 25°C, though optimal temperatures can vary with experimental conditions (Rich, Watt 2013). Among cereals, maize roots grow fastest at 30°C, while oat roots peak at 5°C (Rich, Watt 2013).

Previous studies on temperature effects on root growth may not have adequately controlled temperature, leading to variations in optimal growth rates based on root age (Rich, Watt 2013). While there is a general correlation between temperature and root diameter, variability in root thickness responses can arise from factors such as experimental design, methodology, root type, age, or branching order (Rich, Watt 2013). Higher temperatures typically enhance root water and nutrient uptake, boosting photosynthesis and vegetative growth. Conversely, cooler soil temperatures may negatively impact shoot growth. Recent research has improved our understanding of root-to-shoot signaling mechanisms and how soil temperature influences plant productivity (Rich, Watt 2013).

1.5 Molecular Mechanisms of Trophic Responses

Hydrotropism

Hydrotropism is the ability of roots to sense water gradients and grow toward areas with higher water potential. This phenomenon was first documented in 1887, distinguishing it from gravitropism in the gravitropic mutant (*ageotropum*) of pea (*Pisum sativum*) (Dalal et al. 2023). The study of hydrotropic responses remained largely inactive until research was revived by discovering hydrotropic mutants in Arabidopsis: no hydrotropic response1 (nhr1), mizu-kussei1 (miz1), mizu-kussei2 (miz2), and altered

hydrotropic response 1 (ahr1) (Dalal et al. 2023). In Arabidopsis, MIZ1 regulates hydrotropic responses in both primary and lateral roots, and overexpression of MIZ1 (MIZ1OE) enhances these responses, aiding plant survival under drought stress (Dalal et al. 2023).

Hydrotropic responses have been observed in various agronomic and ornamental crops, including cucumber, maize, rice, wheat, and lotus (Dalal et al. 2023). However, except for Arabidopsis, hydrotropic response genes have not been identified due to significant variations in molecular mechanisms across plant species. For instance, the site of perception can be the root cap in maize and peas or the transition/elongation zone in rice and Arabidopsis (Dalal et al. 2023). Cucumbers' root tips inhibit hydrotropic responses, whereas de-tipped roots may show higher hydrotropic responses when temporarily immobile (Dalal et al. 2023). Additionally, the hydrotropic response in peas, cucumbers, and rice is regulated by auxin (a plant growth regulator). At the same time, lotus roots do not rely on polar auxin transport for this response (Dalal et al. 2023).

In Arabidopsis, the plant hormone cytokinin, which promotes cell division, is found at higher concentrations in areas of the rhizosphere with lower water potential. This higher cytokinin concentration increases the regulation of ARR16 and ARR17 (Arabidopsis Response Regulators), promoting cell division in the meristematic zone and root bending toward high-water potential areas (Dalal et al. 2023). Due to varying levels of degradation or biosynthesis at the root tip, the asymmetric cytokinin distribution leads to root elongation on the water-deficit side, enhancing resource acquisition (Dalal et al. 2023). MIZ1 is crucial for hydrotropic response in Arabidopsis, but other plant species likely have unique molecular mechanisms for hydrotropism. Phytochemicals such as

auxin, ABA, and cytokinin also play distinct and independent roles in hydrotropic responses (Dalal et al. 2023).

Xerotropism

Lateral roots naturally exhibit plagiotropism, meaning they grow at an angle relative to the plant's vertical axis. Xerotropism refers to the ability of lateral roots to alter their growth angle and direction in response to water-deficit conditions in the soil. In Arabidopsis, hydrotropic and xerotropic responses are independent of one another. For example, the hydrotropic mutant MIZ1 can demonstrate both hydrotropic and xerotropic responses, while a xerotropic mutant is limited to only displaying xerotropic responses (Dalal et al. 2023).

Xerotropism in Arabidopsis is regulated by auxin, with the TIR1/AFB-dependent auxin signaling pathway playing a central role. This signaling pathway modulates several PIN proteins, including PIN4, PIN7, and PIN3, which are crucial for root angle regulation (Dalal et al. 2023). The genetic mechanisms underlying root angle adjustments are complex and involve various auxin-regulated genes, which will be discussed in more detail in subsequent sections.

Hydropatterning & Xerobranching

As roots grow and navigate a diverse soil environment, they may encounter various micro- or macro-pores filled with either water or air. Xerobranching occurs when root branching is inhibited due to encountering air-filled pores or limited contact with soil aggregates. This situation typically triggers a localized ABA (abscisic acid) signaling

pathway. The accumulation of ABA within the roots suppresses lateral root initiation and auxin responses. However, lateral root formation can resume once the root tip penetrates saturated soil aggregates (Dalal et al. 2023).

Roots are capable of detecting microscale variations in water availability within their surroundings. Hydropatterning is a developmental response that enables roots to grow towards regions of higher water availability. In this process, lateral root initiation occurs in the direction of saturated soil aggregates, while root hairs, aerenchyma, and anthocyanins develop in areas with lower water potential (Dalal et al. 2023).

Research on hydropatterning in maize, rice, and Arabidopsis has identified critical regulatory mechanisms. This response is controlled by auxin and involves the expression of the auxin biosynthesis gene TAA1 (L-tryptophan pyruvate aminotransferase), the PIN3 protein transporters, and the regulating gene ARF7 (Auxin Response Factor 7). ARF7, which influences lateral root formation through auxin-dependent transcriptional activity mediated by LBD (LATERAL ORGAN BOUNDARIES-DOMAIN) genes, activates LBD16 in areas of the root with higher water availability. In contrast, ARF7 undergoes SUMOylation (Small Ubiquitin-like Modifier proteins) in air-exposed tissues, which recruits IAA3 (Indole-3-Acetic Acid 3) to inhibit ARF7's DNA-binding activity. This inhibition negatively impacts the auxin response related to lateral root initiation (Dalal et al. 2023). Consequently, both hydropatterning and xerobranching involve similar environmental triggers and root responses to pockets of dry air, particularly under water-deficit conditions.

Halotropism

Halotropism is defined as the growth and bending of roots away from areas with high salt concentrations. This negative response to salinity helps roots avoid potentially damaging saline environments. In Arabidopsis, primary roots have been observed to counteract gravitropic responses, redirecting their growth to evade highly saline conditions. This phenomenon is not unique to Arabidopsis; halotropic responses have also been identified in the primary roots of other plants, such as tomato and sorghum, indicating that halotropism is a widespread adaptive strategy across various plant species (Dalal et al., 2023).

1.6 Root System Architecture (RSA) Traits

Plant breeders are focused on developing winter wheat lines with high tolerance to abiotic stresses such as drought and flooding. The traditional breeding process for winter wheat, as practiced by Virginia Tech's Small Grains Breeding Program, spans approximately 8-13 years from initial development to commercial release. This lengthy timeline necessitates incorporating advanced root phenotyping methods early in the breeding pipeline to effectively identify and select genotypes with desirable RSA traits in real time.

Since the performance of a genotype can vary significantly across different environments, understanding root morphology is crucial. This understanding allows breeders to develop RSA ideotypes—ideal root structures tailored to perform optimally in diverse cropping systems, soil types, and temperature regimes. By educating farmers and providing them with a range of RSA ideotypes, they can make informed decisions about

which breeding lines will be most effective in their specific environmental conditions (Ober et al. 2021). Successful integration of this research into winter wheat breeding pipelines can lead to the development of lines with enhanced climate resilience, meaning they possess traits that allow them to withstand and thrive under variable weather patterns (Ober et al. 2021).

RSA is defined as the spatial and vertical distribution of both embryonic (seminal roots from the radicle) and post-embryonic (crown and lateral roots) roots throughout the soil profile or growth medium. RSA encompasses distinct morphological characteristics critical for plant survival (Dalal et al. 2023; Paez-Garcia et al. 2015). RSA's three main developmental components are root number, elongation, and angle (Colombo et al. 2022). In water-scarce environments, root distribution—including root diameter, length, and length density at various soil depths—plays a significant role in water uptake, water-use efficiency, and crop yield (Colombo et al. 2022).

Rooting Depth

Rooting depth is a critical trait in plant breeding because a deep root system enhances a plant's ability to access water and nutrients, directly influencing its resilience (Paez-Garcia et al. 2015). Several factors impact rooting depth, including soil's physical and chemical properties. Soil compaction, often caused by heavy machinery, and low soil moisture can hinder root penetration. Chemical factors, such as nutrient availability and soil pH, also play a role. For example, nitrogen (N) leaches to deeper soil layers and is only accessible if roots can grow deeply. Conversely, phosphorus (P) is immobile, making lateral root distribution crucial for its uptake (Paez-Garcia et al. 2015).

Recent research has highlighted the importance of anatomical traits in rooting depth. For instance, maize genotypes with fewer but larger cortical cells exhibit deeper roots under water stress, likely due to lower metabolic costs associated with soil exploration (Paez-Garcia et al. 2015). Root cortical aerenchyma (RCA), which forms in response to hypoxia or other stressors like drought and nutrient deficiencies, can enhance root performance. Maize with high RCA formation shows significantly higher biomass and yield than genotypes with low RCA (Postma, Lynch 2011).

Gravitropism, the root's response to gravity, also affects rooting depth by guiding root growth direction (Paez-Garcia et al. 2015). Osmotic stress influences root development by affecting the apical meristem, primary root length, and lateral root initiation. Effective regulation of stem cell division and differentiation is crucial for maintaining root growth during drought stress (Li et al. 2021). The auxin biosynthetic gene TaTAR2.1 in wheat enhances lateral root number and length, improving grain yield. The auxin response factor TaARF4 regulates primary root length and plant height through auxin signaling (Li et al. 2021). In rice, the quantitative trait locus DEEP ROOTING 1 (DRO1) is associated with deeper rooting and increased drought tolerance, impacting yield positively (Paez-Garcia et al., 2015; Li et al. 2021).

Root Number

Root number is a quantitative trait that describes the number of the primary, distinct root types, such as seminal, lateral, and crown roots (Li et al. 2021). The root number trait is expressed differently among plant species, and variability may exist among contrasting environments. An example is that rice may only develop one seminal

root, while wheat may produce three to six times as many seminal roots (Li et al. 2021). Seminal root initiation stems from root primordia from the embryo and emerges before the crown roots, fulfilling their role of absorbing water and nutrients throughout the plant growth cycle (Li et al. 2021). It has been reported that the number of seminal roots is associated with the branching attribute, yield potential, and adaption to water deficits of mature plants (Li et al. 2021). Several quantitative trait loci (QTL) controlling seminal root number have been discovered, but essential genes remain unknown (Li et al. 2021). As for crown root development, auxin is also responsible for regulating its development, and several known genes play a role in the process. Crown root primordium is derived from the innermost ground meristem cells near the peripheral cylinder of vascular bundles within the stem (Li et al. 2021). In rice, auxin-induced Crl1 inhibits the initiation of crown roots and is directly regulated by an auxin response factor, OsARF1 (Li et al. 2021).

Lateral root development occurs during the post-embryonic stage, which is after the formation of seminal and crown roots; the combination of lateral root emergence and patterning reflects the adaptive responses to water availability in heterogeneous soil, further described as hydropatterning (root branching in contact with water) (Li et al. 2021). While initiation begins from the semi-meristematic xylem-pole pericycle cells, lateral root development consists of four stages: positioning, initiating, outgrowth, and emergence (Li et al. 2021). The pre-branch sites upon a given (seminal) root are determined by the oscillations in gene expression by the periodic expression of *AtARF7* (protein); *AtMAKR4* (MEMBRANE-ASSOCIATED KINASE REGULATOR 4) (Li et al 2021). Since researchers first uncovered the importance that auxin plays in lateral root

development, auxin transporter genes (*AUXs* and *PINs*), signaling genes (*ARFs*), and *AUX/IAA* have also been identified to engage in the development of lateral roots (Li et al. 2021). The function of SOLITARY-ROOT (SLR), an auxin signaling module, has been well studied in lateral root initiation; auxin triggers the degradation of AtSLR, which subsequently releases AtARF7 and AtARF19 to regulate downstream gene expression (Li et al. 2021).

Root Hairs

Root hairs, unicellular tubular outgrowths from root epidermal cells, are crucial for plant water absorption, contributing up to 50% of total water uptake (Paez-Garcia et al. 2015; Li et al. 2021). In barley (Hordeum vulgare), root hairs facilitate the penetration of hard, compacted soils, enhancing soil exploration and resource acquisition. This trait is essential for plant establishment and the uptake of water and nutrients (Paez-Garcia et al. 2015). Similarly, in common bean (Phaseolus vulgaris), large root hairs combined with shallow basal roots improve phosphorus (P) acquisition, leading to a 300% increase in total biomass (Paez-Garcia et al. 2015). In wheat (Triticum aestivum), root hair formation influences aluminum tolerance and soil aggregates' adhesion to the root system (Paez-Garcia et al. 2015).

Root hair development varies among species and is classified into three types based on epidermal cell arrangements. Type I species, such as rice, have indistinguishable hair and non-hair cells before hair development and exhibit variable environmental responses. Type II species, like *Brachypodium distachyon*, feature smaller trichoblasts and more prominent atrichoblasts arranged asymmetrically. Type III species,

such as Arabidopsis thaliana, have vertically aligned hair and non-hair cells (Li et al. 2021).

Transcription factors play a key role in regulating root hair development. For instance, AtRHD6 and its homologs are crucial for root hair growth, positioning, and identity. Positive regulators such as AtRSL2 and AtRSL4 enhance root hair development, with AtRSL4 showing significant effects when overexpressed. Conversely, GT2-LIKE1 (AtGTL1) and OBF BINDING PROTEIN 4 (AtOBP4) negatively regulate root hair elongation by suppressing AtRSL4 and AtRSL2, respectively (Li et al., 2021). RSL genes in species like Brachypodium and rice positively impact root growth, while wheat orthologs TaRSL2 and TaRSL4 enhance root hair length and nutrient uptake (Li et al., 2021).

Root Xylem

The root xylem is crucial in influencing yield and drought tolerance. The tracheary elements within the root xylem, which feature secondary cell walls, are essential for effective water transport from roots to shoots (Li et al., 2021). The formation and differentiation of xylem tissue are regulated by various phytohormones, including ABA, auxin, cytokinin, brassinosteroids (BRs), and jasmonic acid (JA). Among these, cytokinin is a negative regulator of xylem differentiation (Li et al., 2021).

Root Branching

Root branching, or lateral root formation, is a key component of root system architecture (RSA) and is crucial for improving nutrient and water uptake efficiency.

Breeders target RSA to enhance the uptake of essential nutrients like phosphorus (P) and nitrogen (N) and to boost overall crop performance (Paez-Garcia et al. 2015). For example, in maize grown in nitrogen-limiting soils, genotypes with fewer but longer lateral roots yielded 30% more than those with many shorter lateral roots. This is because fewer, longer lateral roots allowed more energy to be directed towards elongating the primary root, improving access to nitrogen at greater soil depths.

Conversely, crops with abundant lateral roots are better adapted to low-phosphorus (P) soils. Recent research, including transgenic studies, has identified genes that enhance RSA and phosphorus efficiency. For instance, the overexpression of the β-expansin gene in soybean and the TaEXPB23 expansin gene in tobacco have led to increased lateral root formation and improved phosphorus uptake (Paez-Garcia et al. 2015).

Despite their distinct types, different crops, such as wheat and maize, exhibit similar RSA features, like cluster or crown root structures. These structures enable the development of deep primary and shallow adventitious roots. This dual root system allows crops to explore the soil environment more effectively, adapting to varying environmental and soil conditions (Paez-Garcia et al., 2015).

1.7 Root Phenotyping Methodology Based on Environment Type

Breeders typically require three fundamental inputs to improve traits in newly developed varieties: [1] adequate germplasm that contains favorable alleles for the intended target trait, [2] an established selection methodology to effectively identify the expressed target trait, and [3] the necessary (capital and human) resources to carry out the breeding pipeline (Ober et al. 2021).

Lab-based Root Phenotyping

Lab-based root phenotyping methods, such as using germination paper or agar plates, are convenient due to their simplicity and the ability to screen hundreds of genotypes under controlled conditions (Ober et al. 2021). These methods involve germinating seeds in soil-free media, which minimizes resistance and allows for the observation of RSA traits at the seedling stage. However, the genetic variability observed in seedlings may not accurately predict traits in mature plants.

While lab-based phenotyping is useful for initial evaluations, it lacks the predictive power for root biomass growth and development under field conditions. Therefore, although these methods are valuable for pre-breeding selection, field validation is essential to ensure that selected traits perform well in realistic growing environments.

Greenhouse-based Root Phenotyping

Greenhouse-based root phenotyping methods offer a middle ground between lab and field approaches, providing moderately controlled conditions that can introduce variability affecting plant growth. Managing disease and pest pressures in greenhouses is crucial to avoid skewed results (Paez-Garcia et al. 2015).

The development of rhizotrons—glass-walled containers for real-time root observation—has advanced root phenotyping since their inception in early 20th-century Germany (Bohm, 1979). Rhizoboxes, rectangular containers often used in larger setups known as rhizotrons, can observe root growth up to 2 or even 4 meters deep (Ober et al. 2021). These setups are more space-intensive and may limit the number of genotypes that

can be tested simultaneously. Rhizotrons are more physiologically relevant than lab-based methods, as they use soil, but they still may not fully replicate natural conditions if the soil doesn't match the target environment. Soil moisture and texture in rhizotrons can affect root growth differently than in natural settings, and physical space constraints can limit root development.

Studies comparing different methods found that the clear pot method, which allows for real-time observation of germplasm growth, is cost-effective and requires less labor and space than growth pouch methods (Richard et al. 2015). However, the root development in clear pots is restricted by container size, making it less representative of field conditions. Overall, controlled environment phenotyping methods allow for efficient and reproducible evaluation of young plants, but they may not fully predict performance in mature plants or field conditions. Various factors, including genetic background, phenotyping methodology, and environmental conditions, influence how well seedling traits predict mature plant traits (Ober et al. 2021).

Field-based Root Phenotyping

Field-based root phenotyping methods are the most physiologically relevant for studying root growth in cropping systems. The earliest approach, developed by American scientist King in 1892, involved using a vertically long soil monolith with metal wires to expose root structures after soil washing (Bohm, 1979). By 1926, J.E. Weaver introduced trenching, which involved digging a soil pit to observe root expansion. Though initially used for trees, trenching was later applied to annual crops, but its labor-intensive nature makes it impractical for large-scale experiments.

The development of soil coring methods improved root phenotyping by allowing the extraction and examination of soil cores, first with hand augers in 1951 and later with hydraulic corers in 1963 (Bohm, 1979). Soil coring, however, captures only a small cylindrical sample of roots, which may not fully represent root spatial distribution. To obtain a comprehensive view, multiple cores need to be extracted, making this method challenging for large-scale experiments and modern breeding pipelines.

The mini-rhizotron, first introduced in 1937 by G.H. Bates, is a less invasive field-based technique. Mini-rhizotrons involve inserting clear tubes into the soil at a 45-degree angle before planting. Roots grow around these tubes, allowing for real-time imaging of root growth without removing the soil. Although mini-rhizotrons provide valuable in situ observations, they only capture 2-D images, necessitating estimates to assess the full spatial distribution of roots (McGrail et al. 2020).

Since 2011, shovelomics has emerged as a popular root phenotyping method, particularly for maize. This technique involves digging out crown roots to assess traits such as root angle and number (Takahashi, Pradal 2021). While shovelomics is suitable for large-scale experiments, it is labor-intensive and can damage roots, leaving finer roots unaccounted for. The method’s effectiveness varies with soil type, as sandy soils are more porous and easier to work with than clayey soils, which can be harder and more prone to changes in moisture. While field-based methods provide high physiological relevance, they each have limitations regarding root data's practicality, scale, and completeness.

Image-based Approaches for Indirect Root Phenotyping

Researchers have identified the advantages and limitations of various root phenotyping methodologies across controlled, semi-controlled, and field environments. For example, combining the GLO-Roots System with rhizotron technology enables high-throughput root system architecture (RSA) phenotyping. The GLO-Roots System uses luminescence-based imaging to produce high-resolution, 2-D root images. In contrast, sophisticated image processing software consolidates images from both sides of the rhizotron to analyze gene expression patterns (Atkinson et al. 2019). Despite these advancements, 2-D technologies struggle to capture the complex, 3-D nature of root systems fully (Atkinson et al. 2019).

Shovelomics has improved throughput with automatic imaging software like DIRT and REST, though manual excavation remains labor-intensive (Atkinson et al. 2019). Soil coring has also advanced with UV illumination and fluorescence spectroscopy, enhancing image capture of core break faces and improving soil-root contrast (Atkinson et al. 2019). However, non-destructive 3-D imaging technologies, such as X-ray computed tomography (X-ray CT), magnetic resonance imaging (MRI), and positron emission tomography (PET), offer promising alternatives.

X-ray CT, first applied to plant roots over 30 years ago, provides 3-D visualization based on differential X-ray attenuation. Recent improvements in scan time, image resolution, and segmentation software have made X-ray CT viable for root phenotyping, including cultivar-specific studies (Atkinson et al. 2019). MRI, which uses radio frequency waves and magnetic fields to map hydrogen and oxygen atoms, has been used to image root systems of various crops. MRI's effectiveness can be affected by

substrate type and soil moisture levels (Pflugfelder et al. 2017; Atkins et al. 2018). X-ray CT is more widely adopted due to its cost-effectiveness and adaptability, though X-ray CT and MRI struggle with inundated soil conditions (Atkinson et al. 2019).

PET visualizes radioactive tracers but has a relatively coarse resolution (~1.4 mm) and is best used with X-ray CT and MRI for comprehensive root system analysis (Atkinson et al. 2019). The DEEPER project, led by Penn State University, is working to address the challenges of field-based root phenotyping by developing mobile X-ray CT and MRI platforms for non-destructive, in-field 3-D RSA imaging (Atkinson et al. 2019).

Geophysical-based Approaches for Root Phenotyping

Recent advances in non-destructive geophysical technology offer new ways to quantify root traits. Electronic resistance tomography (ERT) measures soil water content and relates it to root growth, providing a non-destructive method with higher throughput than soil coring. However, ERT has the lowest throughput among geophysical techniques due to the need for numerous probes to be inserted into the soil, and its application has been primarily limited to tree root traits (Atkinson et al. 2019). Electromagnetic inductance (EMI) technology, which does not require direct soil contact, offers significantly higher throughput than ERT. EMI has recently been used to assess root activity in wheat (Atkinson et al. 2019).

Ground penetrating radar (GPR) uses high-frequency radio waves to detect subsurface objects and material boundaries. GPR has a similar throughput to EMI but faces limitations in detecting roots smaller than 2 mm in diameter. While GPR has been used to measure bulk root biomass in crops like wheat and sugar cane, it cannot

independently distinguish phenotypic differences among genotypes (Atkinson et al. 2019). Combining 2-D imaging methods with these non-destructive 3-D techniques could enhance root phenotyping by providing a more comprehensive understanding of root traits and facilitating the selection of genotypes with improved climate resilience.

1.8 Plasticity

Root System Architecture (RSA) Plasticity

In root system architecture (RSA), phenotypic plasticity is the ability of roots to adjust their growth and development in response to environmental stimuli (Dalal et al. 2023). It reflects how a genotype can produce different phenotypes depending on environmental conditions, adapting to abiotic stress. Plasticity can be observed at various levels: morphological (i.e**.,** root length, lateral roots, root hair development, anatomical (i.e., changes in vasculature, cell wall modifications), and physiological (i.e., adjustments in water uptake, metabolic reprogramming).

These adaptations can be short-term (e.g., metabolic changes) or long-term (e.g., changes in height or biomass) (Dalal et al. 2023). Root plasticity allows roots to adjust their orientation and function in response to uneven nutrient distribution and varying water availability. Historically, the study of RSA plasticity was less prioritized than above-ground traits, partly due to its complexity. Recent advancements indicate that phenotypic plasticity is genetically controlled and heritable, linked with developmental processes (Dalal et al. 2023). This plasticity is often specific to traits and environments, potentially enhancing crop fitness, yield, biomass, and disease resistance.

Developing root ideotypes—optimized root structures for resource-limited conditions—has been suggested to boost crop productivity. Identifying the genes and molecular mechanisms underlying these traits is crucial for designing effective RSA for specific environments (Dalal et al. 2023). Future research should focus on understanding root phenotypic plasticity to improve climate resilience in agronomic crops.

1.9 Genetic Approaches for Root System Architecture (RSA) Evaluation

Quantitative Trait Loci (QTL) Controlling Root System Architecture (RSA)

Quantitative Trait Loci (QTL) analysis is a powerful tool for exploring genetic variation, developing molecular markers, and identifying candidate genes to enhance crop traits (Li et al. 2021). Numerous QTLs related to root system architecture (RSA) have been identified in wheat, which can potentially improve yield and drought tolerance by targeting these QTLs under drought conditions (Li et al. 2021).

Population structure significantly affects stress response variability. Various genetic populations, including doubled haploids (DHs), recombinant inbred lines (RILs), and introgression lines (ILs), are used to assess root traits under different water regimes to uncover associated QTLs (Li et al. 2021). Given the low throughput of field-based phenotyping methods and the critical role of seminal roots in determining adult rooting depth and water extraction, seminal root traits are particularly important in wheat RSA studies (Li et al. 2021). For instance, twenty-three QTLs linked to six seminal root traits

under water stress have been identified in a DH population from a cross between a high-yielding and drought-tolerant variety (Li et al. 2021).

Further, QTLs associated with adult root traits under drought stress have been mapped, including a significant region on chromosome 4B that influences root biomass and harvest index during drought conditions (Li et al. 2021). In contrast, thirty-two QTLs for seminal root traits have been identified under adequate and limited water conditions using a backcrossed population derived from crossing a cultivar with synthetic hexaploidy wheat (Li et al. 2021). Notably, a translocation wheat line with introgression of chromosome segment 7DL from *Agropyron elongatum* showed increased root biomass under drought stress (Li et al. 2021). The most impactful QTLs are those considered to have large effects on the target trait(s), which significantly influence phenotypes and are ideal for developing molecular markers, genetic dissection, and candidate gene discovery (Li et al. 2021).

Genetic Control of Root System Architecture (RSA) in Wheat

Despite identifying hundreds of QTLs associated with RSA, only two genes have been linked to RSA regulation in wheat (*Triticum*): VERNALIZATION 1 (VRN1) and EGT2. VRN1 influences root growth angle throughout the wheat growth cycle. At the same time, EGT2 encodes a domain-containing protein that narrows the seminal root growth angle, suggesting its potential as a target for improving root traits in cereal crops (Ober et al. 2021). Model plants, such as Arabidopsis, have facilitated the discovery of additional genes in wheat, such as Ta ARF4 and TaLAMP1, that control primary root morphology and growth (Ober et al. 2021). Techniques like genome-wide association

studies (GWAS), quantitative trait loci (QTL) mapping, and marker-assisted selection (MAS) have enhanced precision in allele identification and selection. These methods allow for comprehensive genome-wide assessments of phenotypic data across diverse populations (Ober et al. 2021).

GWAS identified a significant genetic locus on chromosome 6A of durum wheat that controls root angle. This finding was validated through field studies of elite germplasm (Ober et al. 2021). Further validation using Ethiopian durum accessions, and a durum NAM population confirmed the effects of this QTL on root angle and agronomic performance (Ober et al. 2021). Additionally, GWAS of RSA traits in bread wheat revealed two epistatic loci on chromosome 5B controlling root biomass. The high-biomass allele combination was notably absent in elite European germplasm (Ober et al. 2021).

Challenges in QTL discovery include accurately quantifying specific root traits amidst confounding above-ground traits, as populations often segregate for both root and shoot characteristics (Ober et al., 2021). Experiments typically require large populations, often in the thousands, to reliably map QTLs to increase the likelihood of identifying large-effect QTLs. However, this requirement for large test populations can also limit the production of reliable results (Ober et al., 2021).

Genetic Engineering and Gene Editing Advancements of Root System Architecture (RSA) in Elite Wheat Germplasm

Current genetic engineering advancements are enhancing RSA traits in wheat. For example, the increased development of root hairs in wheat is achieved through the expression of TaRSL4-A. Additionally, the VRNI gene, known as a flowering regulator, has been validated for modulating RSA in wheat and barley (Ober et al. 2021).

Site-specific nucleases, such as zinc finger nucleases, TALENs, and CRISPR/Cas9, enable precise modifications of DNA sequences in elite wheat germplasm. These nucleases can target specific gene families or individual genes to alter or terminate gene function (Ober et al. 2021). The use of ribonucleoprotein (RNP) complexes, which facilitate haploid induction, eliminates the need for foreign T-DNA, potentially reducing regulatory hurdles for genetically modified (GM) products (Ober et al. 2021).

Despite these advancements, many countries still regulate gene-edited crops as GM events, even if the foreign T-DNA has been removed. The application of CRISPR/Cas9 RNP-edited wheat could challenge existing global GM regulations and drive further innovations in wheat breeding (Ober et al. 2021).

1.10 Conclusion and Future Perspectives

The reliance on cereals like wheat (Triticum) for global nutrition has been longstanding. As the world population grows, so does the demand for nutritious grain. However, increasing temperatures, erratic weather, and abiotic stresses pose significant threats to crop resilience, disease tolerance, and yield stability. Historically, wheat

breeding has focused on above-ground traits to improve yield and disease resistance, often neglecting root system architecture (RSA). This oversight limits genetic gains and the genotype's adaptability to environmental changes.

Recent shifts in breeding focus aim to incorporate RSA traits to enhance crop resilience. Despite significant variability in root growth due to soil temperature and moisture, and challenges in observing root development in natural environments, RSA traits are crucial for plant survival. RSA encompasses various morphological traits like rooting depth, root number, root hairs, and branching, each with distinct molecular pathways.

Phenotypic plasticity, or a genotype’s ability to display different phenotypes in varying environments, adds complexity to RSA phenotyping. Methods for root phenotyping can be categorized as lab-based (e.g., germination paper), greenhouse-based (e.g., rhizotrons), field-based (e.g., shovelomics), or image-based (e.g., UAV imagery). While field-based methods provide high physiological relevance, they generally offer low throughput, which can slow breeding progress. In contrast, image-based approaches, such as UAV hyperspectral imaging, provide high-throughput phenotyping but require sophisticated technology and significant processing time.

Genome-based approaches like genome-wide association studies (GWAS) and nested association mapping (NAM) identify allele variations linked to RSA traits under drought stress. Recent discoveries include large-effect QTLs that significantly impact phenotype and gene editing technologies using ribonucleoprotein (RNP) complexes, which reduce the risk of off-target mutations.

Future efforts should focus on (1) integrating high-throughput, high-precision field-based phenotyping methods, (2) developing molecular tools to track beneficial alleles for adaptive RSA, and (3) understanding RSA morphology to select ideal RSA traits for specific environments. Establishing standardized root phenotyping methods and integrating them into genomic prediction models will enable precise parent selection, allelic combination calculations, and kinship matrices for breeding lines. Ensuring wheat yields meet global demand will require continued exploration of RSA traits and innovation in RSA phenotyping procedures.

Chapter 2. Evaluating Genetic Variability of Root Biomass Accumulation and Remobilization in Soft Red Winter Wheat (*Triticum aestivum* L.)

ABSTRACT

Climate change threatens crop yields through increased temperatures and more extreme stress events. To address this, plant breeders need to enhance genetic resilience against biotic and abiotic stresses. Traditionally, breeding has focused on shoot traits for crop productivity in various environments due to their significance and ease of measurement. However, as many resilience traits, such as drought tolerance, are linked to belowground structures, there is an opportunity to shift focus to root system architecture (RSA) for developing resilient staple crops like soft red winter wheat (Triticum aestivum). Understanding RSA is challenging due to difficulties in in situ phenotyping of below-ground traits. This research examines the genetic variability of root growth and nutrient remobilization in historic and modern soft winter wheat germplasm.Twenty-two wheat lines representing Virginia germplasm and other important varieties released between 1967 and 2024 were planted in a randomized block design with five replicates at two Virginia locations. Roots were sampled from plots using a soil core, isolated, and quantified during the five time points corresponding to early vegetative, late vegetative, heading, grain fill, and maturity. Mean root growth and remobilization patterns differed across environments, demonstrating that roots responded to environmental stimuli. Minimal genetic variation for root biomass was only detected at grain fill and maturation time points, suggesting little standing variation for the selection of root growth but the potential for selection to increase the remobilization of nutrients from roots to the developing grain.

2.1 Introduction

In high-production cropping systems, yields are sustained through intensive fertilizer use and, in arid regions, irrigation. However, excessive fertilizer application can lead to environmental issues such as nutrient run-off, which contributes to anoxic zones and disrupts aquatic ecosystems, particularly near large bodies of water like the Mississippi Delta (Charles et al. 2005). Similarly, extensive irrigation can deplete surface water resources and spark disputes over water rights (Robinson and Kenney 2012).

The Green Revolution introduced modern mechanized farming and synthetic fertilizers to developing nations, significantly enhancing crop yields. Yet, volatile fertilizer prices and the anticipated depletion of non-renewable resources like phosphorus within 80 to 100 years (McGrail et al. 2020) present growing challenges. This situation underscores the need for more sustainable practices, including smarter fertilizer use, adaptive management to conserve soil fertility, and germplasm development suited to low-fertility soils and minimal inputs (McGrail et al. 2020).

Conventional breeding methods have achieved annual wheat yield gains of 0.9% (Wasson et al. 2014), attributed to the focus on shoot-based traits as proxies for yield improvement. As climate change intensifies, relying solely on fertilizer additions and expanding farmland is no longer viable. The next wave of crop production innovation must integrate technological advancements and develop elite germplasm capable of thriving under increased abiotic stress.

To address these challenges, plant breeders should focus on improving root system architecture (RSA). Enhanced RSA can contribute to better nutrient and water acquisition, increased yield stability, and greater climate resilience. This approach

represents a crucial step toward achieving competitive yields and sustainable crop production in the face of global climate change.

2.1.1 Root System Architecture (RSA) and Its Importance

Root System Architecture (RSA) encompasses the spatial distribution of both embryonic roots (from the radicle) and post-embryonic roots (from existing roots or non-root tissues) throughout a growth medium. This complex organ is crucial for anchoring plants, acquiring nutrients and water, and reallocating resources to support seed production. RSA is dynamic, with growth patterns heavily influenced by climate and soil conditions. Extensive RSA allows plants to access more moisture and nutrients, potentially enhancing survival, growth, and yield in nutrient- or moisture-limited environments. However, in conditions where water and nutrients are abundant, the benefits of an extensive RSA are less clear beyond providing structural support.

The challenge of observing root system development over time has limited research on RSA traits. Breeding efforts have primarily focused on indirect selection if environments conducive to advantageous RSA traits—such as those with limited water or nutrients—will improve performance. While shoot-based traits are more straightforward to observe and manipulate for yield improvement, RSA traits are less directly measurable. For example, harvest index has been used to develop drought-adapted wheat varieties, assuming that nutrient partitioning to grain is more critical than total nutrient accumulation.

Roots are crucial for plant stress responses. They support the plant's ability to manage water stress by accessing deeper soil moisture and regulating water loss through stomatal closure. However, the direct impact of RSA traits on final yield under stress

remains unclear. While deeper roots and efficient water regulation are beneficial, their effects on harvested material under stress are not always straightforward (Ober et al. 2021; Condon et al. 2002; Santantonio et al. 2018).

Breeding trials in fertile soils, whether naturally occurring or enhanced by fertilizers, typically focus on shoot traits like yield and lodging. These trials often overlook root traits for water and nutrient acquisition efficiency (NAE), potentially leading to poor root performance (McGrail et al. 2020). Incorporating RSA traits into breeding programs could enhance plant resilience to water and nutrient stress, crucial for maintaining crop production under changing climate conditions.

Molecular mechanisms for root stress responses have been studied in Arabidopsis, revealing species-specific defense mechanisms like hydrotropism and xerobranching (Dalal et al. 2023; Waadt et al. 2022). However, similar insights for agronomic crops are limited due to the difficulties in evaluating root development in field conditions. Roots also sense nutrient gradients, with RSA reflecting nutrient and water availability. In low-fertility soils, roots exhibit adaptive responses like elongating seminal roots toward nitrogen gradients and expanding lateral roots in search of phosphorus (Rich & Watt, 2013). While an extensive RSA can be advantageous under stress, its impact on yields depends on the severity and duration of resource limitations (Broeske et al., 2022).

2.1.2 Quantitative Root System Architecture (RSA) Traits

Nutrient Use Efficiency (NUE) is a multifaceted quantitative trait representing the ratio of produced outputs relative to nutrient inputs. It incorporates several fundamental components that define its effectiveness (McGrail et al. 2020). As a target for crop

improvement, NUE holds promise for enhancing productivity in low-input cropping systems. However, implementing NUE screening in breeding schemes is challenging due to micro-environmental variability across fields. This variability can result in neighboring plants exhibiting similar phenotypic performance due to shared environmental conditions, complicating the assessment and improvement of NUE (Guo et al. 2020).

Nutrient Acquisition Efficiency (NAE) refers to roots' ability to access and absorb nutrients from the soil profile. It includes roots' proximity to target resources and their ability to translocate these nutrients throughout the plant (McGrail et al. 2020). Despite their significance, NAE-related root traits are seldom used in conventional breeding pipelines due to the difficulties in developing and standardizing effective screening methods (McGrail et al. 2020).

Shoot-based traits are influenced by their growing environment; the same is true for root system development. RSA is particularly sensitive to changes in the soil environment, as it detects initial stress signals and manages resource disparities (Placido et al. 2020). Soil typically presents a heterogeneous distribution of water and nutrients, affecting root spatial and lateral distribution. The primary morphological characteristics of RSA include crown/adventitious roots, lateral roots, seminal roots, root hairs, and root caps (Ober et al., 2021).

Root Morphological Characteristics:

1. **Crown/Adventitious Roots**: These roots anchor the plant in the soil profile and provide support. They also help with nutrient uptake from the topsoil layers.
2. **Lateral Roots**: Emerging from crown roots, lateral roots are vital for acquiring immobile nutrients such as phosphorus from the topsoil.

3. **Seminal Roots**: Serving as the primary root system, seminal roots are crucial for navigating soil pore spaces to access nutrients and water. Their role is especially important during critical growth stages, such as the grain-filling period in wheat, where they must source adequate resources for grain development. Poor soil conditions during this stage, such as low moisture and nutrient availability, can significantly reduce grain yield.
4. **Root Hairs**: These tubular extensions from the root epidermis enhance the root surface area, improving nutrient and water absorption. Root hairs are crucial for penetrating hard or drying soils and are responsible for a significant portion of phosphorus acquisition (Paez-Garcia et al. 2015).
5. **Root Tips**: Root tips are foundational for root exploration. Wider root tips can improve root penetration into hard or drying soils, facilitating better resource acquisition (Paez-Garcia et al. 2015).

Understanding and improving RSA traits can be critical for enhancing crop resilience and productivity, particularly under stress conditions. Advancing screening methods for root traits and integrating them into breeding programs can contribute to the development of crops better adapted to varying soil and climatic conditions.

2.1.3 Advancements in Root Phenotyping

Integrating root traits into modern breeding pipelines could greatly enhance crop resilience and productivity. Key traits to focus on include rooting depth, root angle, root number, root diameter, and branching degree. Reliable methods for screening these traits

across various environments are essential for connecting RSA traits to above-ground characteristics like yield and plant height and developing climate-resilient germplasm.

Historically, breeding efforts have focused on shoot traits due to their ease of observation and direct correlation with yield. However, the study of root traits has evolved significantly. The earliest documented root phenotyping, as noted by Bohm (1979), dates back to 1873, when Sachs used glass windows to observe root systems. Since then, a variety of root phenotyping methods have been developed and categorized by their environment and platform.

Lab-based approaches include methods like germination paper and agar plates, which offer precise but controlled conditions that may not fully replicate field scenarios. Greenhouse-based approaches such as rhizotrons and clear pots provide semi-controlled environments, allowing for stress simulations while controlling some variables. Field-based approaches like shovelomics, minirhizotrons, and soil-coring offer realistic conditions but are labor-intensive and variable. Image-based approaches, including UAV High-Throughput Phenotyping (HTPP) and GLO-Roots, use advanced technology for high-throughput analysis but require sophisticated equipment and data processing. Geophysical-based approaches such as Ground-Penetrating Radar (GPR) and Electrical Resistivity Tomography (ERT) offer non-invasive insights but can be complex and costly.

Each method has its strengths and weaknesses, and the choice of approach depends on the specific needs of the breeding program. Balancing precision, scalability, and realism in root phenotyping can improve breeding pipelines, leading to elite

germplasm capable of thriving in diverse conditions and ultimately enhancing global food security.

Uncontrolled (Field-based) Environment

Despite their high physiological relevance, field-based root phenotyping methods are often the most invasive and labor-intensive techniques. They involve significant soil disturbance, which can be impractical for large-scale studies. Trenching, an early method, involves digging deep pits to visually assess root systems (Bohm, 1979). It was initially used for tree roots before being adapted for annual crops. While informative, trenching is unsuitable for extensive experiments due to its labor and time demands.

Mini-rhizotrons, a more modern and less invasive technique, involve inserting transparent tubes into the soil at a 45-degree angle before planting. Roots grow around these tubes, allowing imaging devices to capture root growth *in situ*. While mini-rhizotrons enable detailed observation without disturbing the soil, the 2-D images only represent a limited portion of the root system, necessitating complex estimations to assess the total spatial growth.

Soil coring, introduced in 1963, involves extracting soil cores with a hydraulic corer and examining the roots within (Bohm, 1979). This method provides valuable data but produces small, cylindrical samples that may not capture the full extent of the root system. Soil coring's limitations include its inability to reveal the entire shape and orientation of roots and the need for numerous samples to ensure representativeness, making it challenging for large-scale experiments.

Shovelomics, designed for maize, manually digs out crown roots and measures root angle and number (Takahashi, Pradal 2021). While it can be applied to large-scale

studies, this method can be time-consuming and may damage finer roots in deeper soil layers. Additionally, the effectiveness of shovelomics for other crops and its variability with different soil types remain concerns (Takahashi, Pradal 2021). Overall, while each field-based method has its strengths and limitations, combining these techniques may be necessary to achieve comprehensive and accurate root phenotyping for modern breeding pipelines.

Semi-controlled (Greenhouse-based) Environment

The development of rhizotrons marked a significant advancement in semi-controlled root phenotyping. Rhizotrons are glass-walled containers that allow real-time observation of root growth in a soil medium, providing high physiological relevance compared to water-based systems like rhizoponics. They are designed to accommodate root growth up to 1 meter, with some models extending to 3 meters (Ober et al. 2021). However, their glass walls eventually restrict root development, limiting the growth of both lateral and seminal roots. Additionally, the need for sufficient space to house multiple rhizotrons can be a significant constraint (Ober et al. 2021).

In contrast, mesh bags and clear pots offer practical alternatives when space is limited. These methods facilitate the screening of numerous experimental units within a single experiment. However, they also restrict natural root growth, leading to results with lower physiological relevance compared to field conditions. Given these limitations, root length is not an ideal trait to assess using these methods. Instead, evaluating root branching and angle, along with genetic variability in germination rates and seedling root initiation, provides more meaningful data in semi-controlled environments.

Controlled (Lab-based) Environment

Lab-based root phenotyping methods, such as those using germination paper or agar plates, offer convenience due to their simplicity and ability to screen hundreds of genotypes under controlled conditions (Paez-Garcia et al. 2015). These methods facilitate the observation of root system architecture (RSA) at the seedling stage, with minimal resistance from the growth medium. However, lab-based approaches are limited to evaluating RSA traits during early development and may not accurately reflect genetic variability observed in mature plants (Ober et al. 2021).

While lab-based methods are effective for initial screening, they lack the statistical power to predict root biomass growth and development in natural field conditions. The highly controlled environment of these methods results in plant growth patterns that differ significantly from those in the field, limiting their utility for standard breeding practices. Consequently, lab-based phenotyping should be complemented by field-based validation to ensure that selection criteria are applicable to real-world conditions where crops are ultimately grown and harvested.

Image-based (High-Throughput Phenotyping) Approaches

The Growth and Luminescence Observatory for Roots (GLO-Roots) integrates luminescence-based reporters with imaging technologies to bridge the gap between imaging capability and physiological relevance in soil-grown roots (Rellán-Álvarez et al. 2015). This sophisticated system, often used in conjunction with rhizotrons, provides detailed 2-D root images.

X-ray computed tomography (CT) has traditionally been used for imaging in controlled environments and holds the potential for high-throughput root phenotyping. However, its use is limited by the time required for scanning and image reconstruction, though it shows promise for revealing RSA plasticity (Teramoto et al. 2020). Magnetic resonance imaging (MRI) and positron emission tomography (PET) also offer imaging capabilities. MRI's efficacy is influenced by soil moisture, which can confound results, while PET, with a resolution of ~1.4 mm, struggles with imaging fine roots (Atkinson et al. 2019). Additionally, image-based approaches often face challenges related to the time and staffing needed for processing, which can hinder efficient breeding pipelines.

Remote sensing via uncrewed aerial vehicles (UAVs) has become increasingly prevalent when evaluating cropping systems. This method enables high-throughput phenotyping by assessing above-ground traits as proxies for below-ground characteristics. For instance, UAV-based remote sensing has linked wheat root mass with canopy temperature under drought and arid conditions, validating these findings through subsequent root excavation (Ober et al. 2021). Moreover, integrating soil coring with UV illumination and fluorescence spectroscopy enhances high-resolution imaging of root systems, offering precise root phenotyping (Atkinson et al. 2019).

Geophysical-based Approaches

Recent advancements in non-destructive geophysical technologies have enhanced root trait quantification. Electronic resistance tomography (ERT) measures soil water content and its relationship with root growth. While non-destructive and has a higher

throughput than soil coring, ERT requires multiple probes for accurate readings and has predominantly been used to quantify tree roots (Atkinson et al. 2019).

In contrast, electromagnetic inductance (EMI) offers higher throughput by avoiding direct soil contact and has recently been used to measure root activity in wheat (Atkinson et al. 2019). Ground-penetrating radar (GPR) uses high-frequency radio waves to detect subsurface objects and boundaries based on their permittivity. GPR's throughput is comparable to EMI, but it currently lacks the resolution to detect roots smaller than 2 mm in diameter. Despite this limitation, GPR has been applied to quantify bulk root biomass in wheat and sugarcane, though it struggles with detecting phenotypic differences among genotypes (Atkinson et al. 2019). Integrating 2-D and 3-D non-destructive root phenotyping methods could provide a comprehensive approach to selecting competitive genotypes based on root traits, enhancing climate resilience.

2.2 Research Hypotheses

This purpose of this study is to evaluate the capacity of the soil coring method to detect genetic variability of root biomass accumulation while revealing differences in remobilization among 22 soft red winter wheat (Triticum aestivum) genotypes developed by the Virginia Tech Small Grains Breeding Program.

This study tested the following hypotheses:

1. Significant genetic variation for root biomass accumulation exists in each test environment.
2. Breeding lines will exhibit differential rates of root biomass accumulation and remobilization through time.
3. Root biomass can be used as a proxy to predict shoot/above-ground biomass growth among breeding lines.

2.3 Research Objectives

1. To evaluate the genetic variation and heritability of root biomass accumulation.
2. Evaluate changes in below-ground/root biomass accumulation and remobilization through time.

2.4 Materials and Methods

2.4.1 Plant Genetic Materials

A sample of 22 soft red winter wheat (*Tritium aestivum* L.) accessions representing historic and elite wheat germplasm were used in this study. These lines were selected for another experiment intended to estimate growth and development curves, and these lines were chosen to be replicated for biomass sampling to serve as a ground truth for estimates of biomass obtained from multispectral aerial imaging. Ten lines, 'Massey' (1984), 'Roane' (1998), 'McCormick' (2002), 'Tribute' (2002), 'Jamestown' (2007), 'Shirley' (2008), 'Hilliard' (2015), 'USG 3118' (2017), 'Liberty 5658' (2019), and 13VTK429-3 (2020; marketed as 'SH 7222'), represent historic and modern varieties adapted to the mid-Atlantic region released by Virginia Tech. Five lines, 16VDH-SRW03-018, 15VTK-1-101, DH15SRW67-151, VA18W-71 and 15VDH-FHB-MAS22-14, were elite experimental breeding lines in the Virginia Tech breeding program at the time the panel was selected in fall of 2020. Line 15VTK-1-101 has since been released in 2024 but has yet to be given a variety name. Two lines, 'Blueboy' (1967), and 'Coker 9835' (1990), were included in the panel for their historical importance as parents. For example, 'Massey' was derived from the cross 'Blueboy' / 'Knox 62'. Five private company lines still under patent or PVP, 'L11541' (Limagrain), '26R59' and '26R46' (Pioneer), 'SY Viper' (Syngenta), and 'USG 3316' (Uni-South Genetics), were included in the study to serve as agronomic checks.

Of these twenty-two lines, seventeen were either developed by Virginia Tech or had expired PVP and could be genotyped. Genotype-by-sequencing (GBS; Elshire et al. 2011) is used routinely in the Virginia Tech small grains breeding program to obtain

genome-wide genetic marker data. GBS calls of these 17 lines were obtained from the much larger set of 5,532 individuals genotyped within the program in 2023. Briefly, GBS library construction was conducted at the USDA-ARS Wheat Genotyping Lab in Raleigh NC with restriction enzymes, Pst1 and Msp1, used for genome reduction. Libraries were sequenced on a paired-end Illumina platform. Variant discovery and calling were completed using Tassel (Bradbury et al., 2007) and bwa (Li and Durbin, 2010), with sequences aligned to the Ref Seq v2.1 wheat genome (Zhu et al. 2021). Missing marker data was imputed with Beagle (version 5.4; Browning et al. 2018).

2.4.2 Experimental Design

The panel of 22 soft red winter wheat lines was planted in a replicated complete block design with five replications (blocks) within each test location, Blacksburg, VA and Warsaw, VA in the fall of 2022 (Table 1). Test plots were planted as seven rows spaced 15.2 cm apart, 2.74 m long by 1.22 m wide, with 30.5 cm margins established between each pass (column) of plots. Each replicate served as a time of sampling, structured to coincide with the five major winter wheat developmental stages: early vegetative (EV), late vegetative (LV), heading (H), grain-filling (GF), and maturity (M) (Table 2). At each time point, a scheduled UAV flight captured multispectral aerial images of both experiments in the field immediately prior to above-ground biomass collection, followed by below-ground biomass sampling.

The above-ground biomass collection procedure involved first measuring the plant height (PH) of each plot, before each plot was cut with a rice knife at ground level and all above-ground biomass placed in a bin. Fresh weight was collected in the field by

placing bins onto a scale. Bin weights were measured and subtracted from the total weight. A representative subsample of above-ground biomass weighing approximately 0.5 kg to 1 measured, and subsequently dried in a dryer at 65 °C for 48 hours. Dry weight was then collected and used to correct the total plot fresh weight to a total plot dry shoot biomass weight (SB). This process was repeated for each of the first four time points. For the final time point at grain maturity (M) when plants had fully senesced, the same process was repeated, but the remaining total biomass was fed through a research plot combine to separate the grain from the straw and chaff. The fresh weight sample from the fifth time point had the grain extracted in a threshing machine after the sample was dried and weighed. Total grain weight was calculated by summing the grain weight from the sample, and the remaining from the plot. Grain weight was then used to estimate end-use trait values, including grain yield (GY; g/plot), test weight (TW; kg/l), and harvest index (HI; GY/SB).

The below-ground root sampling was conducted using a custom-built soil coring tool constructed to sample root systems in coniferous forests at a depth of 1 m with a core diameter of 5 cm (Santantonio and Hermann 1985). Two intra-plot soil cores (pseudo-replicates) were collected from each plot (experimental unit), from the third and fifth crop row to a depth of 30 cm at each time point and test location. A polyethylene sleeve was inserted into the coring cylinder to maintain the structure and integrity of each soil core sample. Once extracted from the soil, the core was divided into 10 cm increments to create three sub-strata per soil core. Samples were dried for 48 hours at 65 °C.

The processing of soil cores was done in sequential order of each time point within location. Each sub-sample was crushed by a weight to free roots from the soil-

Table 1. Soil classifications and characteristics from both test environments. Soil data had been retrieved from the NCRS Soil Web Survey and National Cooperative Survey (2007). The Montgomery County (Blacksburg) test location encompassed two soil types, asymmetrically spanning across all five blocks.

Location (reference coordinates)	**Map Unit Symbol**	**Map Unit Name**	**Taxonomic Class**	**Taxo-nomic Order**	**Soil Moisture Class**	**Soil Temperature Regime**	**Particle Size Class**	**Percent of AOI**
Montgomery County, Virginia (37.1982366, -80.57492917)	25	McGary and Purdy soils	Fine, mixed, active, mesic Aeric Epiaqualfs	Alfisols	Udic	Mesic	Fine	79.2%
Montgomery County, Virginia (37.1982366, -80.57492917)	33	Weaver soils	Fine-loamy, mixed, active, mesic Fluvaquentic Eutrudepts	Inceptisols	Udic	Mesic	Fine-loamy	20.8%
Westmoreland County, Virginia (38.01365596, -76.72310621)	21B	Suffolk sandy loam, 2 to 6 percent slopes	Fine-loamy, siliceous, semiactive, thermic Typic Hapludults	Ultisols	Udic	Thermic	Fine-loamy	100.0%

matrix, with any remaining shoot biomass still attached to the crown root structure removed prior to further processing. Root tissue was separated from the soil by dry sieving (2 and 5 mm), with remaining soil aggregates washed off through wet sieving. The resulting isolated root biomass samples were then placed in a dryer for 48 hours at 65 °C. The mass of root biomass accumulation (g; RB) was measured on a balance.

2.4.3 Statistical Analysis

Statistical analysis was conducted in R. An Analysis of Variance (ANOVA) was conducted to determine the relative variance explained by each of the independent variables in the experimental design: test location (l), genotype (g) and the experimental unit from which subsamples were taken, plot (p, sub-sampling variation) in relation-

Table 2. Dates of soil core sampling throughout each of the five major winter wheat developmental stages: early vegetative (EV), late vegetative (LV), heading (H), grain-filling (GF), and maturity (M), per location.

Location	EV	LV	H	GF	M
Blacksburg, VA	March 29th	April 19th	May 6th	June 2nd	June 25th
Warsaw, VA	March 22nd	April 13th	May 10th	May 23rd	June 19th

to the dependent variable, root biomass accumulation, under the following model,

$$y_{ijkl} = l_i + g_j + p_{ijk} + e_{ijkl}, \quad \textit{Eq. 1}$$

where *e* is the error due to subsampling (Table 3). The mean square for subsampling was used as the denominator to produce the F test statistic for the plot term. In contrast, the mean square for the plot term was used as the denominator to perform the F test statistics for the location and genotype terms.

Total root biomass per experimental plot was estimated by correcting the average sample weight per plot by the ratio of the plot area to the core surface area (3.34 m^2 / 0.00196 m^2 = 1703.4). However, since samples were not collected between crop rows, this estimate is likely biased high; the 'ggplot2' function in R was used to plot the distribution of dry, shoot, and root biomass over time, as well as the mean shoot and root biomass accumulation during each time point based on location. Traits only collected at the fifth time point were combined to visualize relationships between biomass accumulation and end-use traits (GY, TW, HI). Best Linear Unbiased Estimators (BLUEs) for genotypes were fit for each trait, RB, SB, GY, TW, and HI, using the same linear model as the abovementioned ANOVA (Eq. 1). Location was established as a

fixed effect variable, and line (accession) was established as the random effect variable. Pairwise Pearson correlations were calculated to create a phenotypic correlation matrix of all phenotypic traits and visualized using the 'ggcorrplot' package in R.

A Genomic Best Linear Unbiased Predictor (GBLUP; Van Raden, 2008) was fit to predict genotype effects for each trait in each time point across test locations using the 'rrBLUP' package in R. The same linear model as used to calculate BLUEs was used to calculate GBLUPs, and only the genetic effect was instead considered random and normally distributed, with an additive genetic covariance, **G**, estimated from genome-wide markers as

$$\boldsymbol{G} = c^{-1}\boldsymbol{WW} \qquad \textit{Eq. 2}$$

where the constant $c = 2\boldsymbol{p}'(1-\boldsymbol{p})$, with **p** representing the vector of allele frequencies at each locus. Variance components for genetic effects (V_A), plot variability (V_E), and the subsampling error (V_S) were estimated in the model fit and used to calculate a narrow sense heritability for each trait as

$$h^2 = \frac{V_A}{V_P}, \qquad \textit{Eq. 3}$$

where V_A is the additive variance, and $V_P = V_A + V_E$ is the phenotypic variance. The subsampling variance, V_S, was not included in the phenotypic variance.

The 'ggplot2' package was used to plot the changes in random effects and narrow sense heritability through time. A heatmap and hierarchical clustering of lines was made using the 'heatmap' function in R.

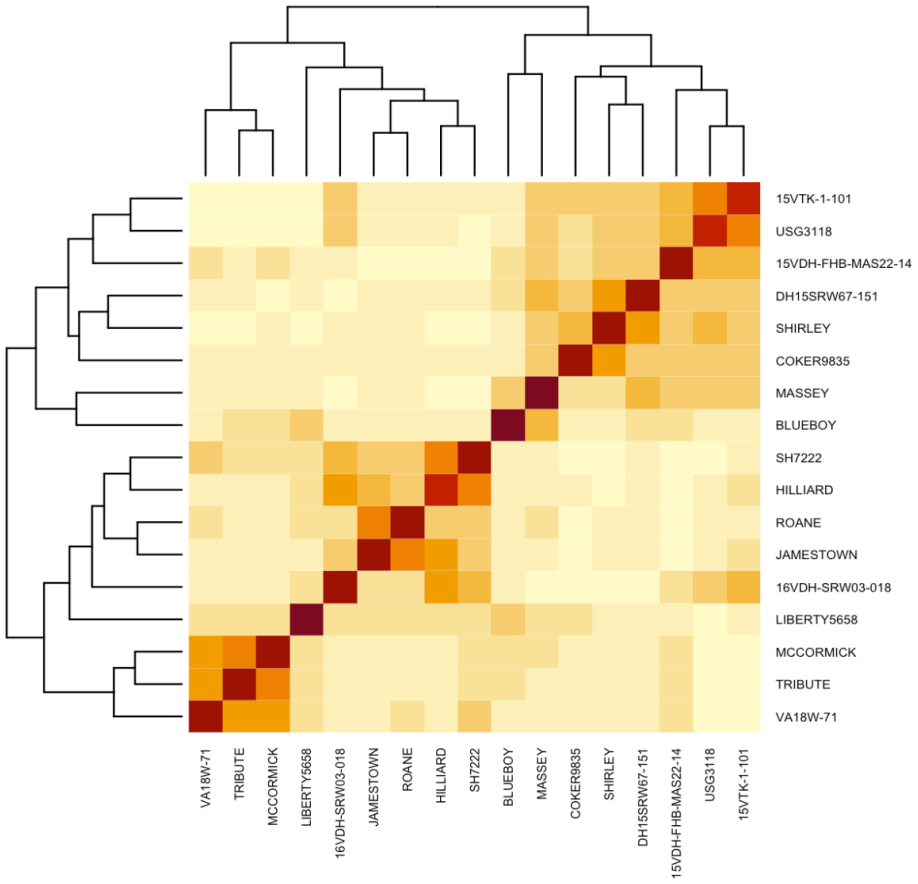

Figure 1. A heat map visualization of a genomic relationship matrix encompassing 17 elite genotypes used in this field trial with available genetic marker information reveals their degree of relatedness to one another. Three significant blocks of relatedness exist among this test population, indicating the development of three separate families within the Virginia Tech Small Grains Breeding Program.

2.5 Results

Table 3. ANOVA tables of total Root Biomass Accumulation at each time point, early vegetative (EV), late vegetative (LV), heading (H), grain-filling (GF), and maturity (M).

Time Point 1	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Location	1	3.72	3.72	11.04	0.003
Genotype	21	5.44	0.26	0.77	0.724
Plot	21	7.08	0.38	2.40	0.007
Subsampling	44	6.19	0.14		

Time Point 2	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Location	1	0.51	0.51	1.94	0.178
Genotype	21	6.06	0.29	1.01	0.417
Plot	21	5.53	0.26	1.53	0.117
Subsampling	44	7.58	0.17		

Time Point 3	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Location	1	0.14	0.14	0.12	0.733
Genotype	21	10.99	0.52	0.44	0.967
Plot	21	25.01	1.19	1.34	0.206
Subsampling	43	38.30	0.89		

Time Point 4	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Location	1	71.46	71.46	16.08	6.4×10^{-4}
Genotype	21	87.86	4.18	0.94	0.555
Plot	21	93.35	4.44	4.36	1.9×10^{-5}
Subsampling	44	44.89	1.02		

Time Point 5	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Location	1	1.38	1.38	8.46	0.008
Genotype	21	4.55	0.21	1.33	0.258
Plot	21	3.41	0.16	1.24	0.263
Subsampling	44	5.74			

Table 4. Mean values of various phenotypic traits based on location. Mean values were calculated from raw phenotypic data measured during plant maturity (5th time point).

Location	Mean HI (GY/SB)	Mean GY (g/plot)	Mean RB (g/plot)	Mean SB (g/plot)
Blacksburg, VA	0.44	2930	1550	3745
Warsaw, VA	0.55	2538	1124	2100

Analysis of variance (ANOVA) across locations for each time point showed significant plot effects ($P < 0.05$; i.e. not all plot values are the same; Table 3) for EV and GF time points. Location effects were significant ($P < 0.05$) for EV, GF, and M time points. The effect of genotype was not significant at any time point. The estimates of genetic variance were not significantly greater than zero based on a likelihood ratio test.

Means for shoot, root, and grain yield were higher in Blacksburg. However, the linear increase in shoot biomass (78%) did not translate to a linear increase in root biomass (38%) or grain yield (15%) from the Warsaw to Blacksburg environments (Table 4). The harvest index demonstrated a higher efficiency of resources in producing the final harvested product and grain yield in the Warsaw location than in the Blacksburg location.

Average root biomass accumulation over time, as mean root biomass values were evaluated per location is shown in Figure 2. An average increase in root biomass from the 1st to 2nd time point was not observed in Blacksburg. Generally, roots increased in biomass through HD and then decreased either after HD (Blacksburg) or after GF (Warsaw). The Warsaw environment reflected the highest root biomass accumulation during the GF (grain filling) stage of the winter wheat (*Triticum*) development cycle. In contrast, the Blacksburg environment had the highest root biomass accumulation during the H (heading) stage during the winter wheat (*Triticum*) development cycle.

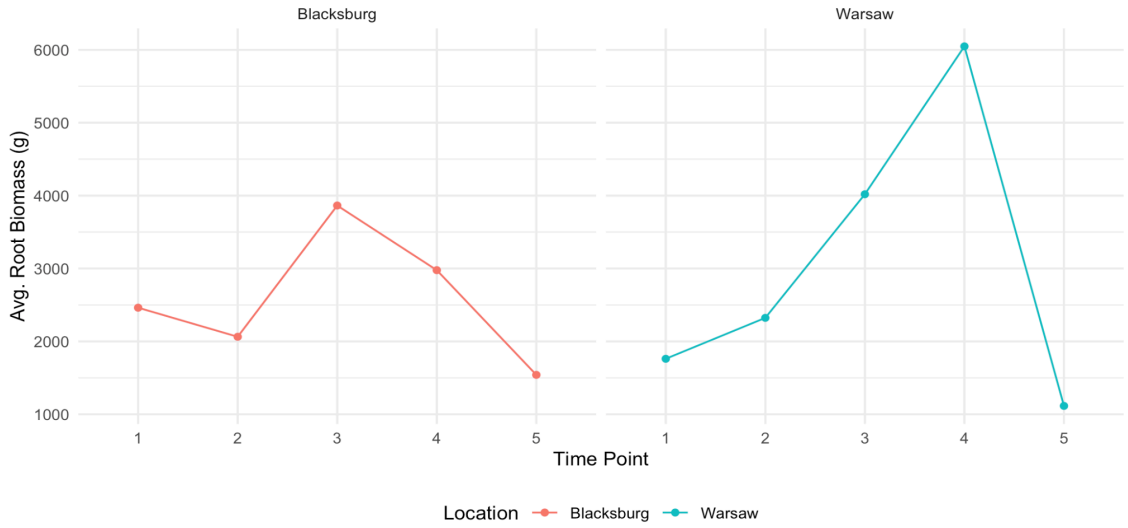

Figure 2. Distribution of mean dry root biomass accumulation through time; the distribution mean values of each replicate were plotted per location. Peak root biomass accumulation is acquired at differing points along the winter wheat (*Triticum*) development cycle.

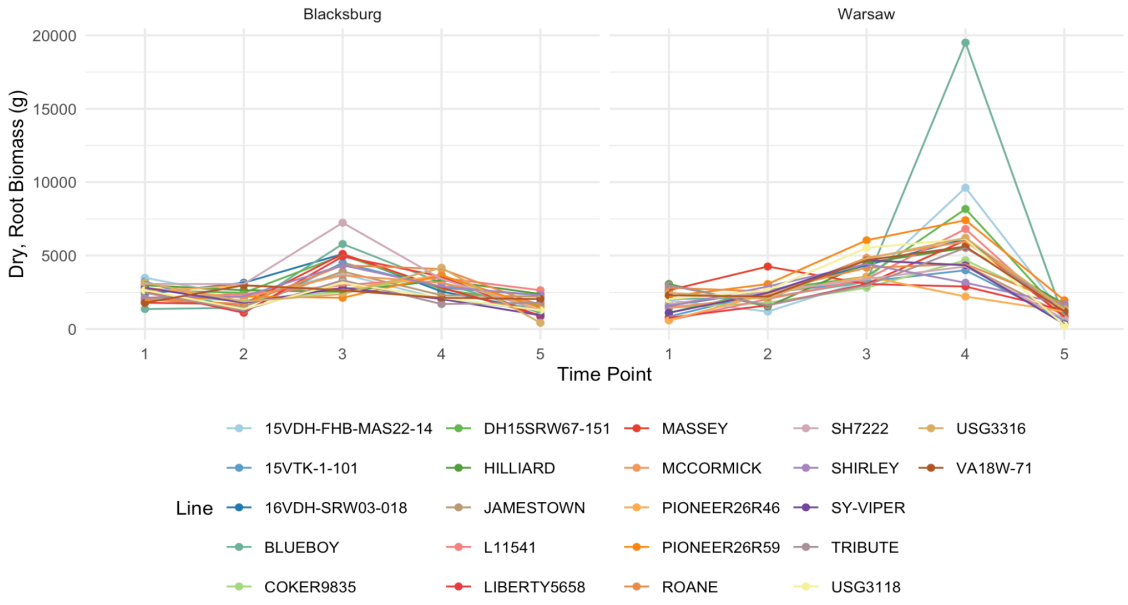

Figure 3. Distribution of dry root biomass accumulation phenotypes through time for each of the 22 winter wheat breeding lines.

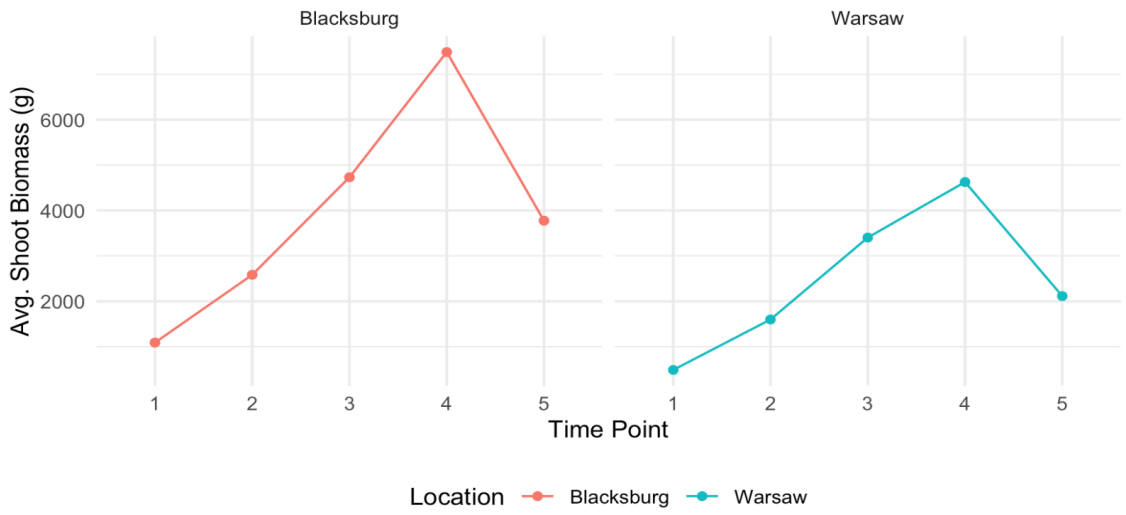

Figure 4. Distribution of the mean dry shoot biomass accumulation through time; a mean value from each replicated was plotted per location. The highest shoot biomass accumulation occurred in both locations during the 4th time point or GF stage. Genotypes grown in Blacksburg expressed an overall higher level of shoot biomass accumulation.

Across the 22 elite genotypes and among each location, some variability in the rate of root biomass accumulation through time was observed (Figure 3). 'Blue Boy' expressed the highest phenotypic response in Warsaw, while 'SH 7222' had the highest phenotypic response in Blacksburg. An additional example of phenotypic variation was observed in Warsaw, where 'Massey' expressed its peak root biomass accumulation during EV. Both locations had peak shoot biomass accumulation during the GF time point, and Blacksburg expressed a higher overall growth trend than Warsaw (Figure 4). These results suggest that the environmental type significantly affects biomass growth (Figure 4; Table 3).

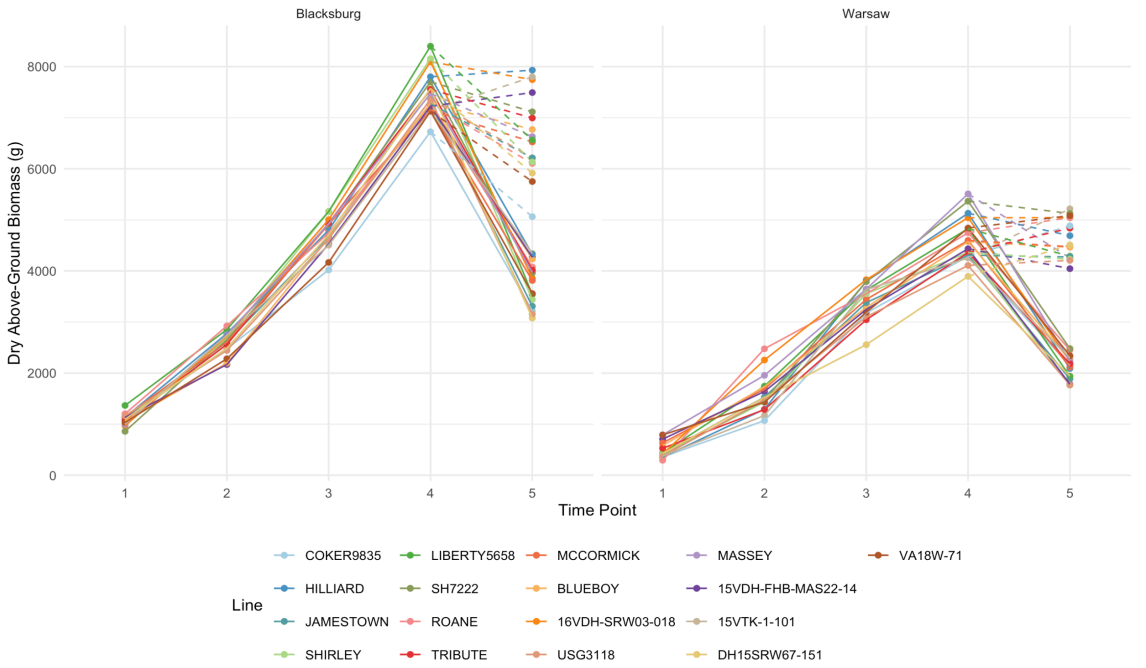

Figure 5. Distribution of dry, shoot biomass accumulation through time for each genotype. The dashed lines of each genotype reflected the total above-ground biomass accumulated during maturity per location. The solid lines from grain filling to maturity reflected the difference in shoot biomass and grain yield. This aims to visualize how much biomass is remobilized for grain production in both locations.

Shoot biomass continually increased at both locations through GF and remained steady (Warsaw) or decreased slightly (Blacksburg; Figure 5). The dashed lines represent the accumulation of total biomass during maturity, and higher variability of this accumulation was observed in Blacksburg than in Warsaw. In Blacksburg, ‘Liberty 5658’ obtained the highest measurement of shoot biomass accumulation. In Warsaw, ‘Massey’ obtained the highest shoot biomass accumulation. Overall, the Blacksburg environment achieved higher above-ground biomass than the Warsaw environment. A linear increase

Table 5. Pearson correlations between phenotypes of grain yield and root biomass, as well as grain yield and shoot biomass at maturity for each location.

Location	Cor(GY, RB)	Cor(GY, SB)
Blacksburg, VA	-0.008	0.317
Warsaw, VA	0.146	0.259

Table 6. Pearson correlations between root and shoot biomass phenotypes during each sampling period per location.

Location	EV	LV	H	GF	M
Blacksburg, VA	0.008	-0.109	0.487	0.075	0.075
Warsaw, VA	0.212	0.342	0.448	-0.102	0.067

in shoot biomass accumulation was observed in both locations through GF (Figure 5). Blacksburg achieved a comparable mean shoot biomass by HD than the highest observed during GF in Warsaw.

Phenotypic correlations between root and shoot biomass were nearly zero for most time points. The exception was during HD, when phenotypic correlations were moderate, with values of 0.49 and 0.45 for Blacksburg and Warsaw, respectively (Table 6). Correlations at EV and LV were moderate to low in Warsaw, ranging from 0.21 to 0.32. At maturity, grain yield and root biomass had low to no correlation to grain yield in Blacksburg ($r = -0.01$) and Warsaw ($r = 0.15$; Table 5). The correlations between shoot biomass and grain yield were higher but still moderate in Blacksburg ($r = 0.32$) and Warsaw ($r = 0.26$).

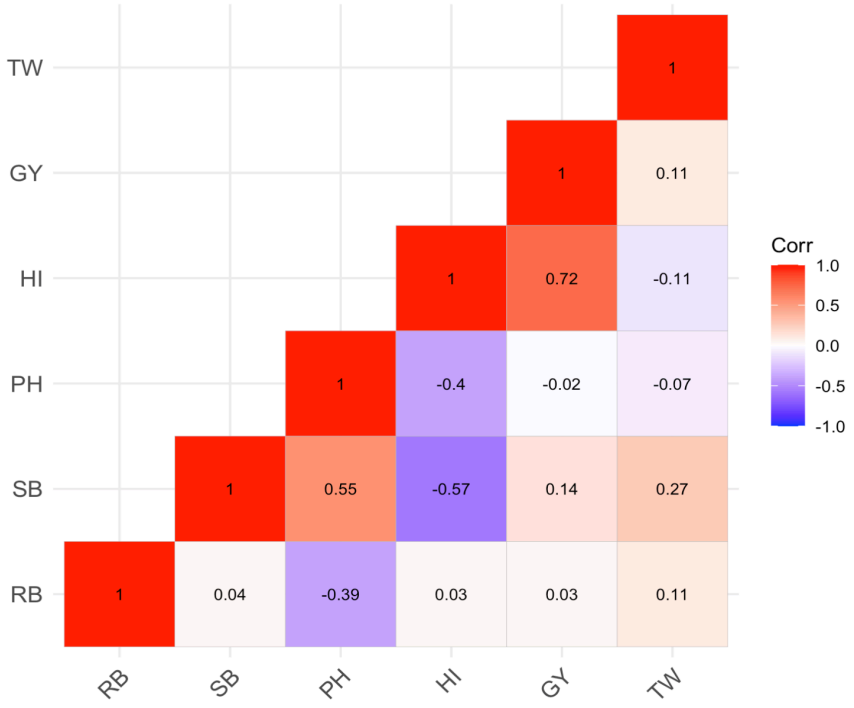

Figure 6. Phenotypic correlation matrix generated via BLUEs model. The BLUEs values for each trait were calculated from a subset of phenotypic data from the 5th time point.

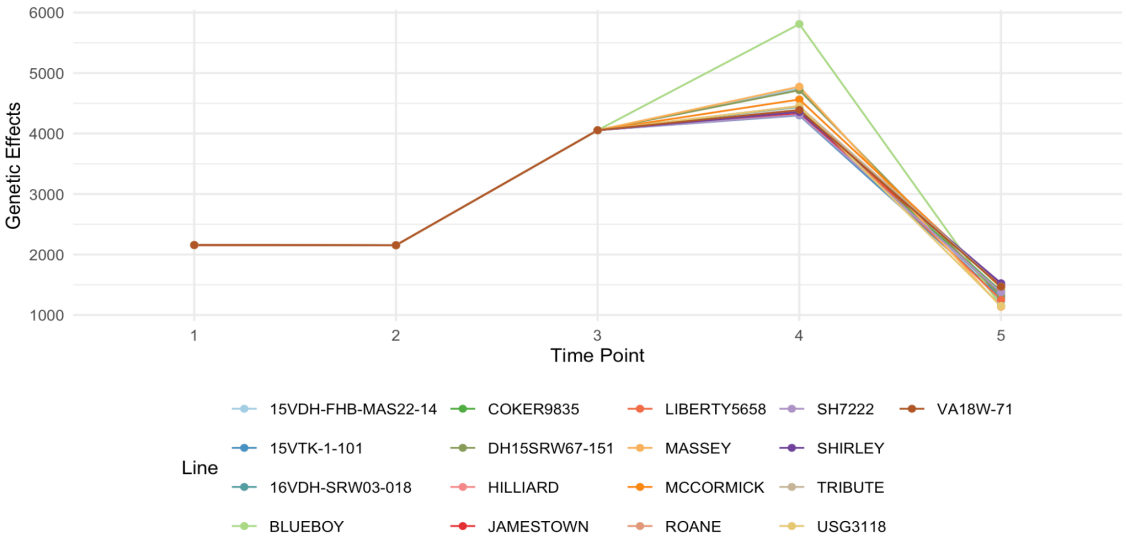

Figure 7. Distribution of genetic effects of root biomass accumulation among breeding lines through time, via GBLUPs model. The range of plot-level genetic effects of root biomass was moderate, ranging from 1050 g to 5811 g.

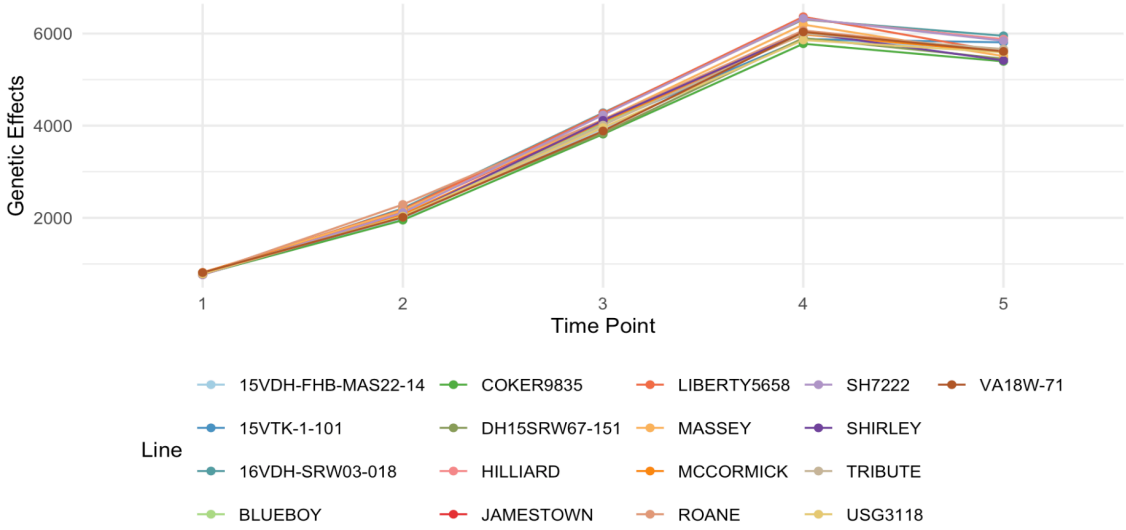

Figure 8. Distribution of genetic effects of shoot biomass accumulation among breeding lines through time, via GBLUPs model. The range of plot-level genetic effects of shoot biomass through time was substantial, ranging from 765 g to 6365 g.

Genetic correlations were calculated for all pairwise traits using BLUEs for the final time point, M (Figure 6). Root biomass (RB) did not correlate highly with any other end-use trait. Similarly, shoot biomass also did not demonstrate high correlations with end-use traits.

The estimate of genetic variance for root biomass was zero for the first three time points but was non-zero during GF, and M, resulting in non-zero genetic effects for those latter time points (Figure 7). The range of genetic effects of shoot biomass (Figure 8) through time was considerable and consistent with its corresponding raw, phenotypic values across breeding lines. However, no significant differences in the genetic effects of shoot biomass across breeding lines were observed over time. Root biomass narrow-sense heritability at HD and GF was near zero at 0.06 and 0.17, respectively, but was considerably higher at M, with a value of 0.72 (Figure 9). The heritability of shoot biomass was calculated to compare the effect of subsampling relative to the collection of all biomasses. Heritability estimates for shoot biomass ranged from 0.05 to 0.27, with all stages having non-zero estimates. In contrast, heritability estimates for plant height, a known high heritability trait, were all above 0.7, except for the early vegetative time point with no genetic signal.

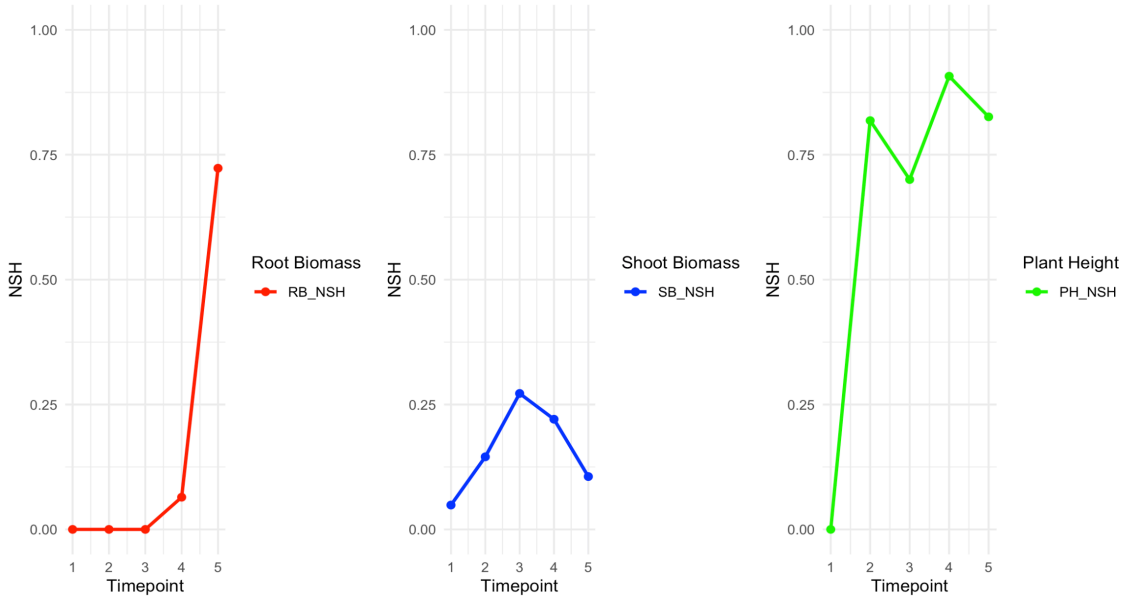

Figure 9. Comparing the distribution of narrow sense heritability (NSH) among root biomass, shoot biomass, and plant height through time. Above-ground and below-ground traits vary considerably in their level of narrow sense heritability through time.

2.6 Discussion

Across all environments, genotypes demonstrated low root biomass during early seedling stages, higher root biomass during early reproductive stages, and subsequent decline at maturity (Figures 2 and 3), following the expected pattern of accumulation during growth and remobilization of resources during maturation. However, timing of peak root biomass varied across the two locations. Peak root biomass occurred during the heading stage in Blacksburg (2.27 g), but not occur until the grain fill stage in Warsaw (3.55 g; Figure 2). The higher total shoot biomass accumulated in Blacksburg coupled with the lower total root biomass at that location indicates that Blacksburg was a lower stress environment than Warsaw.

Plants in Warsaw invested more resources into below ground structures (73% of Blacksburg root biomass) proportional to their above ground structures (56% of Blacksburg shoot biomass), and obtained lower, but comparable, grain yields to Blacksburg (87% of Blacksburg grain yield). Genotypes in Warsaw also demonstrated more efficient resource remobilization for grain development, with a harvest index of 0.55 compared to 0.44 in Blacksburg (Table 4). This suggests that Warsaw had higher abiotic stress, reflected in steep root biomass accumulation through grain fill (Figure 2). Grain yield, shoot biomass, and root biomass were not highly correlated (Figure 6; Table 6), indicating that these traits do not increase proportionally across environments.

The higher rate of root biomass remobilization in Warsaw did not result in a higher yield compared to Blacksburg (Figure 4 and Table 5). Shoot biomass peaked at the GF stage and was significantly higher in Blacksburg (3,745 g) than in Warsaw (2,100 g), where both environments had similar grain production. These findings support the

ANOVA results, showing location (soil and weather) as a major factor influencing biomass growth (Table 3). Figures 6 and 7 indicate that shoot and root biomass have a weak phenotypic correlation, contradicting the hypothesis that root biomass could predict shoot biomass.

The research aimed to assess differences in root biomass accumulation and remobilization across breeding lines and evaluate the efficacy of soil coring as a phenotyping method. The ANOVA analysis assuming genotype independence (Table 3) showed significant plot effects only during the EV and GF stages, with location effects being significant at the EV, GF, and M stages. Genotypic effects were not found to be significant at any time point. Including genomic information in a linear mixed model did result in non-zero estimates of genetic variance for the last three time points between heading and maturity. However, these estimates of genetic variance were not significantly greater than zero based on a likelihood ratio test.

The GBLUP model results (Figure 7) showed minimal genetic effects on root biomass, ranging from 0.35 g to -0.10 g. Genetic variation for root biomass was not significant early on, but some evidence (i.e. non-zero variance parameter estimates) at maturity suggests some genetic variation exists for remobilization. The limited genetic variability detected may be due to the constraints of the soil coring method. However, biomass traits are expected to be complex, polygenic traits governed by many genes throughout the genome. Shoot biomass, which was collected in full, did not show strikingly higher heritability than root biomass despite root biomass being estimated from a relatively small sample from the plot. These results suggest that even extraction of all the roots in the plot would not have resulted in significantly increased estimates of root

biomass heritability. The general small diameter of winter wheat root systems makes it challenging to screen and accurately quantify the RSA. Future studies with advanced phenotyping technologies that can better characterize and measure roots in situ may better capture genetic variability in root biomass.

2.6.1 Limitations of Experimental Design

The soil coring method did not yield a high narrow-sense heritability for root biomass accumulation, underscoring this trait's complexity and polygenic nature. The conventional soil coring approach was insufficient for detecting microscale, but crucial, differences in root anatomical traits—such as root angle, diameter, branching, length, and hairs—contributing significantly to root biomass spatial distribution (RSA). As such, the current study was limited in its ability to characterize the RSA.

The test population's size may have impacted the ability to detect genetic differences and estimate genetic variance. Including a relationship matrix estimated from markers allowed information sharing across relatives and resulted in non-zero estimates of genetic variance, suggesting that a wider or large population of wheat lines may have produced higher estimates of genetic variance. While replication of plots at each time point (i.e. 10 plots per genotype per location) would have allowed for testing of genotype effects directly within each location without the need for genetic information, this would have doubled the time and labor involved in sampling. If twice as many plots were to have been planted and sampled, it is likely doubling the population size would have resulted in better ability to separate genetic variability from error if genetic information were available for the additional lines.

2.6.2 Limitations of Soil Coring

A significant limitation of this experiment was the lack of adequate equipment for repeated rapid root sampling. The small grains breeding program at Virginia Tech, which had not previously focused on root phenotyping, lacked the necessary tools for extracting and analyzing root biomass. Recent root phenotyping methods often involve high equipment and operational costs or extensive construction and labor. Consequently, an inexpensive manual steel soil-root coring apparatus, previously used for analyzing fine roots of Douglas-fir trees (Santantonio and Hermann 1985), was chosen for sample collection.

Field-based methods like soil coring offer moderate throughput but are time-consuming, with manual extraction in this study taking approximately 2 hours per replication with a team of 3 to 5 people. Hydraulic operated coring equipment mounted on a tractor could potentially decrease the time needed to sample and allow enough plots to be sampled to make the effort worthwhile for routine breeding efforts. Although this method has the potential for higher throughput, it has the same sample size limitations and does not necessarily allow for root phenotypes other than biomass to be measured. Achieving this could require a larger research team and additional financial resources for advanced imaging technologies, such as UV illumination and fluorescence spectroscopy. These technologies could enhance root imaging accuracy when combined with soil coring.

Routine breeding program operations generally do not allow large amounts of time or resources to be spent to quantify a single individual or plot. Thus, the soil coring method was chosen as a compromise between sampling and processing time, and the

information that could be obtained from the sample, which in this case was limited to total biomass of the sample core. Were a small grain breeding program to begin measuring root biomass as a routine operation, the time constraint of sampling and sample processing would be a substantial consideration. As such, we do not believe this method is suitable for routine implementation in a small grain breeding program, as soil coring was still rather labor and time intensive and did not yield highly heritable phenotypes.

2.7 Conclusion

Agricultural operations globally face declining fertilizer availability and increasing instances of low-fertility soils. This situation compels plant breeders and researchers to seek sustainable solutions for crop improvement. One potential approach is to shift focus from shoot-based to root-based traits, which could help close yield gaps between developed and developing nations and enhance plant resilience to environmental changes. Root system architecture (RSA) is crucial in acquiring and transporting water and nutrients essential for high-yielding crops. Variability in crop yields, often linked to climate-induced soil changes, highlights the need for breeding strategies that emphasize RSA traits.

Root phenotyping is labor-intensive and challenging. This experiment employed the soil coring method to assess genetic variability in root biomass accumulation among a small sample of elite lines from Virginia Tech's small grains breeding program. The results revealed minimal genetic variability and narrow sense heritability for root biomass. However, notable differences in root biomass accumulation and remobilization

rates were observed across locations. The experiment's limited statistical power likely stemmed from the constraints of the soil coring method (core size), the small test population, limited number of replicated field plots, and most importantly, the complex polygenic nature of root biomass traits. There was some evidence of genetic differences at later time points, suggesting genetic variability may be higher for remobilization of nutrients from the roots, rather than root biomass accumulation alone. Large numbers of genotypes, broader and more diverse testing could potentially provide more insight into the potential for breeders to change RSA traits.

2.8 Future Directions

Breeding programs nationwide should prioritize evaluating root system architecture (RSA) traits for enhancing vegetative growth and yield stability. This experiment used soil coring, a field-based method, due to its relative speed and convenience for root sampling. However, this approach is destructive (although with a limited impact on the total plot area), labor-intensive, and time-consuming, which may limit its overall efficacy, and the value of the data obtained.

Future root phenotyping research should focus on more automated, non-destructive methods. For instance, Wasson et al. (2015) used the ‘BlueBox’ fluorescence imaging system to create high-contrast soil core-break images. Additionally, researchers at Penn State University are developing portable X-ray CT and MRI devices for non-destructive, 4-D root phenotyping (Atkinson et al. 2019).

Given that the rhizosphere significantly impacts root development, further investigation into abiotic and biotic interactions within this soil zone could enhance our

understanding. The rhizosphere, where soil chemistry and microorganism populations directly affect root growth and nutrient uptake, plays a crucial role. Increasing our knowledge of these interactions could improve nutrient availability and disease control while reducing reliance on synthetic agrochemicals. The heterogeneity of natural soil affects root penetration, elongation, branching, and resource acquisition. Understanding root plasticity in response to these abiotic factors is essential for translating phenotypic responses from controlled environments to field conditions (Downie et al. 2014). High-resolution imaging technologies offer promise for characterizing RSA traits more effectively and accelerating crop improvement.

Developing elite germplasm with improved climate resilience is just one step. To achieve competitive yields globally, there must be a balanced focus on genotypes, environment, and agronomic management (GEM) (Mahmood et al. 2022). Establishing a standardized, non-destructive, high-throughput root phenotyping methodology will enable efficient screening of RSA traits across various environments. Accumulating root phenotypic and genetic data will support accurate genomic selection models linked to crop yields. Integrating data from diverse environments may help identify genotypes adapted to drought or low-fertility conditions. Enhancing agronomic management through precision agriculture technologies may reduce fertilizer waste, optimize pesticide application, and improve disease detection. Improving soil health may also contribute to better water and nutrient retention. Successful implementation of a GEM model requires harmonizing these factors to achieve optimal gains (Mahmood et al. 2022).

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