SPECIAL SECTION: BELOWGROUND PHENOTYPING

Relationships between roots, the stay-green phenotype, and agronomic performance in barley and wheat grown in semi-arid conditions

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Abstract

Stay-green is a phenotype that crop breeders could use to improve drought adaptation. It increases the duration of grain fill in several species including barley (Hordeum vulgare L.) and wheat (Triticum aestivum L.), maintaining yield in semi-arid conditions. Evidence from controlled environment experiments suggests a connection between stay-green and root systems. These belowground structures are understudied and thus represent opportunity for crop improvement if relationships to agronomics can be understood. Minirhizotrons facilitate study of these relationships by allowing repeated nondestructive root measurements in field conditions. However, this is time-consuming, and proxies would be useful for increasing throughput capacity of root research. Here we present results from field trials with minirhizotrons in a semi-arid environment, as well as greenhouse seedling assays conducted on stay-green and non-stay-green barley and wheat lines. In barley, stay-green and greater yield were primarily associated with greater deep root length and delayed root senescence, whereas in wheat, yield was most strongly correlated with total root length, and root system differences for stay-green were not as apparent. We speculate that the physiology of stay-green is different between these two species, and that barley may use a more efficient root system to withstand drought whereas wheat relies on a larger one. Several seedling traits related consistently to field root traits, but correlation directions were often opposite between barley and wheat. The connections between traits presented here could be useful for breeders seeking to improve crop adaptation to drought, but more genotypes and environments will need to be tested.

INTRODUCTION

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

Though the physiology of the polygenic stay-green phenotype is not fully understood, there is evidence from studies in multiple species connecting aboveground stay-green phenotypes with root traits that have been measured ex situ. In sorghum, multiple quantitative trait loci (QTL) for stay-green have co-located with QTL for nodal root angle (Borrell et al., 2014; Mace et al., 2012). Similarly in barley, Gous et al. (2016) mapped QTL for stay-green traits located near QTL associated with root length, root dry weight, and root-to-shoot ratio (Arifuzzaman et al., 2014). Further, in wheat, Christopher et al. (2018) found stay-green QTL that co-located with QTL identified for seminal root angle and seedling root number in a previous study (Christopher et al., 2013). Because mapping indicates genetic loci pleiotropically affecting root and aboveground stay-green traits in the greenhouse, and in situ examinations of the root systems of stay-green plants are lacking, field experiments are warranted. Efforts to identify root phenotypes in the field associated with improved crop yield and drought adaptation have focused on deep rooting. Various measurements of root length deep in the soil profile in wheat have been connected to higher yield (Corneo et al., 2017; Hayashi et al., 2013; Uddin et al., 2018). However, results concerning relationships between root traits and associated aboveground characteristics are frequently inconsistent across locations, seasons, and management practices. Environment has a much greater effect on deep rooting than genotype (Hodgkinson et al., 2017; Severini et al., 2020). In Palta and Turner’s (2019) review, “Crop Root System Traits Cannot Be Seen as a Silver Bullet Delivering Drought Resistance,” they describe how root phenotypes can be beneficial in some scenarios but provide no advantage in others. For example, wheat grain yield was strongly positively correlated to deep root length density in rainfed conditions but not under irrigation (Postic et al., 2019). Becker et al. (2016) also observed rooting depth plasticity in the greenhouse in response to drought stress. Ultimately there is likely no single root system trait or even set of traits that will confer drought adaptation across all environments. The best mechanisms of crop adaptation in semi-arid environments are dependent upon the amount and timing of precipitation, temperature, soil type, soil depth, and other crop, environment, and management factors (Bodner et al., 2015). However, even though deep rooting does not consistently increase yield, it does not appear to negatively impact yield (Severini et al., 2020) and therefore remains a promising breeding target. More field studies examining roots in situ in a variety of locations and conditions are needed to understand the impact of deep roots on yield. Indeed, there is a lack of knowledge about root systems compared with the aboveground portions of plants, owing to the difficulty in observing them.

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

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### Core Ideas

- In-field minirhizotrons revealed a connection between the stay-green phenotype and deep roots in barley.
- Yield was most strongly positively correlated with deep root length in barley and total root length in wheat.
- There were positive correlations with percent deep root length for both grain yield and grain protein in wheat.
- Seminal root angle and deep rooting were negatively correlated in wheat but positively correlated in barley.
locations. Functional proxies should ideally have definite and reliable relationships to the traits they are proxies for. Such tools would greatly assist breeders in selecting for beneficial root phenotypes.

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

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material

Barley and wheat cultivars and breeding lines that differ for the stay-green phenotype were used in this study (Table 1). For barley, the stay-green group of lines consisted of two breeding lines from North Dakota State University (NDSU), ND19119 and ND24260, known to exhibit a functional stay-green phenotype (Gous et al., 2013), as well as three Montana State University (MSU) breeding lines that each have one of the NDSU lines as a parent and have exhibited stay-green characteristics in the breeding program. The other parents to the three MSU stay-green lines do not exhibit stay-green characteristics and thus formed the non-stay-green group along with a fourth non-stay-green line. This fourth line (MT124118) was added in 2019 so that it could be evaluated for its use in a genetic study of this trait, and MT16M00803 was removed to accommodate this addition.

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

### 2.2 | Experiment 1: Field trials

#### 2.2.1 | Site description

Field trials were conducted in 2018, 2019, and 2020 at the MSU Northern Agricultural Research Center (48.50˚ N lat, −109.80˚ W long), 773 m asl, near the city of Havre, MT in the semi-arid Northern Great Plains region. The soils at this location are a mix of Joplin, Scobey, and Telstad clay loams officially classified by the USDA as well-drained, very deep, fine to fine-loamy Aridic Argustolls with slow to moderately slow permeability (USDA, 1998). The research field utilized for these trials has been under no-till management for over 20 yr, and each trial followed a fallow season to maximize stored soil moisture. Daily precipitation and temperature data for each growing season and historic averages were obtained from NOAA climatological summaries collected at the Northern Agricultural Research Center/Fort Assiniboine (National Weather Service, 2021) (Table 2). Below average growing season precipitation was received in 2018 and 2020, and below average precipitation during the grain fill period occurred in each season. Temperature, over the course of the study, did not differ greatly from one growing season to the next or from the historic averages.

#### 2.2.2 | Experimental design and management

A randomized complete block design with three blocks was used such that each line appeared in each block and was thus replicated three times in each season (with the exception of a few lines that were not planted every year, see Table 1). Plants were seeded during the last week of April each season, in five row plots with 0.3 m row spacing that were 6.7 m long and trimmed to 5.2 m after emergence. Seed was treated before planting with CruiserMaxx Vibrance Cereals (Syngenta) seed treatment. The seeding rate was 10 g m⁻¹ for barley and 7 g m⁻¹ for wheat (approximately 60 seeds m⁻¹). Soil tests (Agvise Laboratories) were conducted before planting in 2019 and 2020 to give an idea of fertility (Table 3). In 2018, the soil tests were conducted the previous fall and an additional 32 g m⁻¹ of 100-20-10-10, N-P-K-S, fertilizer was put down at the time of seeding. No fertilizer was added in 2019 and 2020. All field trials were rainfed and were maintained weed free.

#### 2.2.3 | Aboveground measurements

Heading (Zadoks 59) and maturity (Zadoks 89) dates were assigned to each plot in 2020 according to the Zadoks decimal code for the growth stages of cereals (Zadoks et al., 1974). In 2019, Zadoks stage was assessed for each plot on three days
### TABLE 1  Plant materials

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

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

### TABLE 2  Growing season and historic weather conditions

| Weather time periods | Trial season | 2018  | 2019  | 2020  | Average (1916–2020) |
|----------------------|--------------|-------|-------|-------|---------------------|
|                      |              |       |       |       |                     |
| Precipitation        |              |       |       |       |                     |
| Planting to flowering|              | 84.3  | 134.1 | 112.0 | 111.5               |
| Flowering to harvest |              | 13.2  | 27.2  | 15.2  | 65.8                |
| Planting to harvest (total) | | 97.5  | 161.3 | 127.3 | 141.2               |
| Average temperature  |              |       |       |       |                     |
| May                  |              | 14.9  | 9.7   | 11.9  | 12.2                |
| June                 |              | 17.4  | 16.3  | 17.3  | 16.6                |
| July                 |              | 20.8  | 19.7  | 20.0  | 20.7                |
| August               |              | 19.6  | 19.6  | 21.7  | 19.7                |

### TABLE 3  Fertility data from soil tests

| Trial season | Depth (cm) | Nitrate (ppm) | Phosphorus - olsen % Olsen Potassium (ppm) | Organic matter (%) |
|--------------|------------|---------------|--------------------------------------------|--------------------|
| 2018         | 0–15       | 8.5           | 5                                          | 193                | 8.2 | 1.5        |
| 2018         | 15–61      | 14.0          |                                            |                    |     |            |
| 2019         | 0–15       | 4.0           | 15                                         | 218                | 7.7 | 1.2        |
| 2019         | 15–61      | 8.5           |                                            |                    |     |            |
| 2020         | 0–15       | 2.5           | 16                                         | 184                | 8.0 | 0.9        |
| 2020         | 15–61      | 12.0          |                                            |                    |     |            |
around the time of heading as well as three days around the
time of maturity, then plot heading and maturity dates were
estimated based on these Zadoks scores. Planting date was
subtracted from heading date and from maturity date to deter-
mine days to heading and maturity, respectively. Heading date
was subtracted from maturity date to calculate duration of
grain fill. Plot heading and maturity dates were not recorded
in 2018 but were monitored to inform the timing of data col-
lection. Aboveground biomass samples were collected from
each plot at the beginning of flowering (Zadoks 61), and after
maturity (Zadoks 92), by cutting sections of plants at their
base from two of the three central rows for an area of 0.27 m².
At flowering, leaf area was measured using a leaf area meter
(Li-Cor 3000), and the entire sample was dried and weighed.
At harvest, samples were already dry upon collection and were
weighed. Seed heads were counted to determine productive
tiller number, then threshed using a custom-built small capac-
ity grain thresher. The grain was weighed to determine yield
and harvest index (grain weight/total biomass). This grain was
also used for near-infrared spectroscopy (NIR) measurement
of grain protein content (Infratec NOVA, FOSS) in 2019 and
2020.

2.2.4 Belowground measurements

The CI-600 In-Situ Root Imager minirhizotron system from
CID Bio-Science was used to collect root measurements in
the field. Transparent acrylic root tubes, measuring 105 cm in
length and 7 cm in diameter, were installed 2 d after planting
in 2018 and 2020, and 8 d after planting in 2019. A single tube
was placed in every plot. Some tubes were initially placed in
the wrong position in 2019 and needed to be reinstalled 14 d
after planting, though this did not impact root measurements.
Tubes were installed by first using a Giddings hydraulic probe
to take soil cores with a diameter slightly larger than that of
the tubes, at a 45˚ angle to the soil surface, directly in the mid-
dle row of each plot, then inserting the tubes into the resulting
holes. If a tube was loose in a hole, a slurry of soil and water
was poured into the gap around the tube to improve contact
with the soil. Figure 1C in the publication by Postic et al.
(2019) provides a clear diagram of this type of minirhizotron
set-up.

Belowground images (Figure 1A) were captured with the
CI-600 cylindrical rotary scanner at the same flowering and
harvest collection time points that were used for biomass
sampling (Zadoks 61 and 92). The scanner was lowered
sequentially to capture 360˚ scans at four depths (~0—15,
15—30, 30—45 and 45—60 cm) below the soil surface.
Bourgault et al. (2021) used this same method in a previous
study and estimated a 2—5 cm difference in actual imag-
ing depth between tubes. CI-690 RootSnap! Version 1.3.2.25,
the companion program to the scanner, was used to trace
over roots in the images (Figure 1B) to determine total root
length at each depth. We acknowledge that the maximum root-
ing depth of spring wheat in field conditions usually extends
beyond 60 cm (Thorup-Kristensen et al., 2009, found an aver-
age depth of 1.1 m for field grown spring wheat), and we wish
to emphasize that our root length measurements are not an
indication of maximum rooting depth achieved by the plants.
However, for the sake of this study we refer to the top two
scanning depths together (0—15 and 15—30 cm) as shallow,
and the bottom two scanning depths together (30—45 and
45—60 cm) as deep. The percentage of the total root length
that was deep was calculated as:

\[
\text{Percent deep root length} = \frac{\text{root length 30 to 60 cm}}{\text{root length 0 to 60 cm}} \times 100
\]  

(1)

The percent difference in total root length between flower-
ing and harvest was calculated as:

\[
\text{Percent change in root length} = \frac{(\text{harvest root length} - \text{flowering root length})}{\text{flowering root length}} \times 100
\]  

(2)

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

2.3 Experiment 2: One-leaf root roll-up
seedling assay

To observe root traits at the one-leaf stage (Zadoks 11), a
method adapted from Watt et al. (2013) was used, hereafter
referred to as the root roll-up assay. This was performed in
the MSU Plant Growth Center greenhouse in Bozeman, MT
(45.67˚ N lat, −111.05˚ W long). Temperature was maintained
at 21 °C during the day and 18 °C at night. Artificial light
supplemented natural light for a 16-h photoperiod. A random-
ized complete block design was used in which all cultivars
and breeding lines listed in Table 1 appeared once in each of
three blocks grown over time such that the experiment was
replicated three times independently.

Seedling rolls were prepared using 25.4 cm \( \times \) 38.1 cm
sheets of germination paper (Anchor Paper Co.) oriented with
the shorter edge as the top. Seeds were weighed, then placed
in a line parallel to and 1 cm from the top of the paper with the
embryos pointing down. The paper was then tightly rolled and
secured with two small pieces of masking tape on the edge.
Each roll contained four seeds of the same genotype with even
spacing between each seed and the edges of the paper. These
rolls were thoroughly wetted with water, then placed upright
and close together in a 24 × 24 × 34 cm bucket with approximately 4 cm of water at the bottom. The rolls were held in place by a grid of chopsticks placed on top of the bucket and were kept moist by maintaining the water level at the bottom and misting the tops of the rolls daily. Set-up for the root roll-up assay is depicted in Figure 1c.

As seedlings began to grow, shoots emerged from the tops of the rolls as seminal roots grew down between the layers of paper. The term seminal refers to roots that originate in the embryo within the seed (Manske & Vlek, 2002), and in this study we do not distinguish the primary seminal root (radicle) from other seminal roots. Growth was allowed to continue until two out of the four seedlings in a roll (or one if there was a seed that failed to germinate) reached the point where the first leaf was fully emerged, and a second leaf tip was just barely visible. That roll was then removed from the bucket and either measured right away or stored in a plastic bag at 4°C for up to 2 d before data collection.

Shoots were removed from seeds and all shoots from one roll were placed in the same paper packet to be dried and weighed. Seminal roots, still attached to the seeds, were gently peeled off the paper. The number of seminal roots was counted as was the number of initiated lateral roots. The longest seminal root was measured with a ruler. Seedling root systems were placed one at a time in a transparent tray of shallow water on a flatbed scanner (Expression 12000XL Graphic Arts, Epson America Inc.) and scanned at 400 dpi to produce 8-bit grayscale images. Before scanning, roots were gently separated to avoid crossover in the images, then left to settle in their most natural position to allow for subsequent measurement of the seminal root angle. After scanning, roots were removed from the seeds and all roots from one roll were placed
in the same paper packet to be dried and weighed. The scanned images were analyzed using WinRHIZO software (Arsenault et al., 1995) to determine total root length and volume. The program ImageJ (Rasband, 1997) was used to measure the angle between the two outermost seminal roots at 3 cm below the seed (Figure 1d). The four seeds within one roll were considered pseudoreplications, and the measurements for each seedling were averaged to give the value for the roll.

2.4 | Experiment 3: Four-leaf PVC pot seedling assay

A four-leaf seedling assay (Zadoks 14) adapted from a method used by Ali et al. (2016) and hereafter referred to as the polyvinyl chloride (PVC) pot assay, was performed using the same growing conditions and experimental design as Experiment 2. However, this experiment was replicated five times independently rather than three to account for a larger margin of error with this method.

Plants were grown in sections of PVC pipe that were 91 cm long, 10 cm in diameter, cut in half longitudinally, then duct taped back together. Porous rubber shelf liner material (Gorilla Grip) was used to create bottoms for the pots by taping it onto one end of the pipe. The pots were placed upright on a bench in the greenhouse, strapped to each other and the bench to secure them in place, and filled with Turface (Profile Products LLC), a substrate made from baked calcined clay that is mechanically broken into approximately 4 mm diameter pieces. Set-up for the PVC pot assay is depicted in Figure 1e.

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

Root systems were either scanned right away or stored in jars of 50% ethanol at 4 °C for up to 2 d. Roots were scanned in the same manner as the root roll-up seedlings, after spreading them out as much as possible in the water tray to avoid overlap. Then root systems were removed from the seed and placed into packets to be dried and weighed. Images were analyzed using WinRHIZO to measure total root length and volume.

2.5 | Statistical analysis

Statistical tests were performed in R (R Core Team, 2021). To compare stay-green and non-stay-green groups of lines, analysis of variance (ANOVA) was run for all traits measured in the greenhouse, and for all traits measured in the field by single season and across seasons. For greenhouse experiments and for single field seasons, a model was assessed for each trait with fixed effects stay-green (whether a line was designated as stay-green or non), Table 1, block, and their interaction as the only terms. For all field seasons together, each trait was analyzed using a model that included the fixed effects of stay-green, block, season, stay-green by block, and stay-green by season. In all models, if an interaction was associated with a p value above .15, the interaction was not included in the final model. Interactions were rare in the overall data set. Effects used in each model and their significance level in ANOVA (“car” package, Fox & Weisberg, 2019) are listed in Supplemental Table S1.

To examine association between aboveground traits and root traits in the field, Spearman correlation tests were performed on values measured in the same individual plots using the “Hmisc” package (Frank et al., 2020). To examine association between root traits in the field and root traits in the greenhouse assays, Spearman correlation tests were performed using genotype means of the traits, also with the “Hmisc” package. Correlations with greenhouse data were not done with the 2018 field season for wheat owing to the reduced number of lines grown that year.

3 | RESULTS

3.1 | Comparison of stay-green and non-stay-green lines

Comparisons of agronomic traits of stay-green vs. non-stay-green barley and wheat are presented in Table 4. Stay-green lines did exhibit longer duration of grain fill period than non-stay-green lines in barley and in wheat, although this was not significant for wheat in 2019. The stay-green phenotype, indicated by longer duration of grain fill, was contributed to by delayed maturity and even more so by earlier heading. Stay-green did not have a significant impact on days to maturity wheat. There was also a pattern that was consistent though not always significant, of stay-green lines in both species having higher grain yield.

In barley, stay-green lines had less leaf area (though not in 2020), higher harvest index (again not in 2020), and tended
TABLE 4  Agronomic trait means of non-stay-green (non-SG) and stay-green (SG) groups, with p values from ANOVA for the fixed effect of stay-green for each species in single growing seasons and all seasons combined. Full ANOVA results are presented in Supplemental Table S1. Sample sizes are listed in gray.

| Agronomic trait and season          | Barley                  | Wheat                   |
|-------------------------------------|-------------------------|-------------------------|
|                                     | Non-SG      | SG       | Non-SG     | SG       | Non-SG     | SG       |
|                                     | Mean | n | Mean | n | Mean | n | Mean | n | Mean | n | Mean | n |
| Aboveground biomass at flowering (g m⁻²) |             |             |             |             |             |             |             |             |             |             |             |             |
| 2018                                | 270     | 9 | 270   | 15       | 181       | 6 | 167       | 6 |
| 2019                                | 541     | 12 | 563   | 11       | 530       | 15 | 537       | 8 |
| 2020                                | 407     | 12 | 407   | 12       | 359       | 15 | 322       | 9 |
| All                                 | 418     | 33 | 400   | 38       | 400       | 36 | 356       | 23 |
| Leaf area at flowering (cm²)        |             |             |             |             |             |             |             |             |             |             |             |             |
| 2018                                | 1368     | 9 | 1,181  | 15       | 976       | 6 | 1,462     | 6 |
| 2019                                | 1082     | 12 | 851   | 11       | 948       | 15 | 1,018     | 8 |
| 2020                                | 842      | 12 | 891   | 12       | 836       | 15 | 887       | 9 |
| All                                 | 1073     | 33 | 994   | 38       | 906       | 36 | 1,083     | 23 |
| Productive tiller number            |             |             |             |             |             |             |             |             |             |             |             |             |
| 2018                                | 113      | 9 | 131   | 15       | 83        | 6 | 120       | 6 |
| 2019                                | 188      | 12 | 219   | 11       | 151       | 15 | 176       | 8 |
| 2020                                | 110      | 12 | 117   | 12       | 78        | 15 | 103       | 9 |
| All                                 | 116      | 33 | 131   | 38       | 109       | 36 | 132       | 23 |
| Days to heading                     |             |             |             |             |             |             |             |             |             |             |             |             |
| 2019                                | 63       | 12 | 60    | 11       | 58        | 15 | 57        | 8 |
| 2020                                | 64       | 12 | 63    | 12       | 61        | 15 | 58        | 9 |
| All                                 | 63       | 24 | 61    | 23       | 59        | 30 | 58        | 17 |
| Days to maturity                    |             |             |             |             |             |             |             |             |             |             |             |             |
| 2019                                | 90       | 12 | 92    | 11       | 92        | 15 | 92        | 8 |
| 2020                                | 93       | 12 | 94    | 12       | 93        | 15 | 94        | 9 |
| All                                 | 91       | 24 | 93    | 23       | 93        | 30 | 93        | 17 |
| Duration of grain fill (d)          |             |             |             |             |             |             |             |             |             |             |             |             |
| 2019                                | 27       | 12 | 32    | 11       | 34        | 15 | 35        | 8 |
| 2020                                | 29       | 12 | 31    | 12       | 32        | 15 | 36        | 9 |
| All                                 | 28       | 24 | 31    | 23       | 33        | 30 | 35        | 17 |
| Harvest index                       |             |             |             |             |             |             |             |             |             |             |             |             |
| 2018                                | 0.42     | 9 | 0.44  | 14       | 0.42      | 6 | 0.44      | 6 |
| 2019                                | 0.44     | 12 | 0.50  | 11       | 0.43      | 15 | 0.44      | 8 |
| 2020                                | 0.58     | 12 | 0.56  | 12       | 0.56      | 15 | 0.56      | 9 |
| All                                 | 0.49     | 33 | 0.50  | 37       | 0.49      | 36 | 0.49      | 23 |
| Grain yield (g m⁻²)                 |             |             |             |             |             |             |             |             |             |             |             |             |
| 2018                                | 232      | 9 | 233   | 15       | 197       | 6 | 228       | 6 |
| 2019                                | 331      | 12 | 384   | 11       | 300       | 15 | 305       | 8 |
| 2020                                | 396      | 12 | 415   | 11       | 309       | 15 | 342       | 9 |
| All                                 | 328      | 33 | 332   | 37       | 286       | 36 | 299       | 23 |
| Percent grain protein               |             |             |             |             |             |             |             |             |             |             |             |             |
| 2019                                | 12.2     | 12 | 12.1  | 11       | 14.1      | 15 | 14.7      | 8 |
| 2020                                | 10.4     | 12 | 10.9  | 12       | 12.6      | 15 | 13.4      | 9 |
| All                                 | 11.3     | 24 | 11.5  | 23       | 13.4      | 30 | 14.0      | 17 |

*p < .1, *p < .05, **p < .01, *** p < .001.
to have higher productive tiller numbers than non-stay-green lines. There were no clear differences between stay-green and non-stay-green barley for aboveground biomass at flowering or percent grain protein.

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

In barley, the field root data suggested two patterns of differences between stay-green and non-stay-green groups of lines that were consistent across all seasons and measurement time points, although stay-green did not always have a statistically significant effect in these ANOVA. Stay-green lines had a higher percentage of deep roots than non-stay-green lines at flowering (2019: \( p < .05 \)), and harvest (2018: \( p < .1 \); 2019: \( p < .05 \); combined seasons: \( p < .01 \)) (Figure 2c). Stay-green lines appeared to have less total root length than non-stay-green lines, but stay-green did not have a significant effect in these ANOVA (Figure 2a). With the exception of the 2018 season, stay-green barley exhibited an increase or lesser decrease in shallow and deep root length than non-stay-green, with a significant effect of stay-green in ANOVA for shallow root length in 2019, 2020, and combined seasons, and for deep root length in 2019 (Figure 2e). Corresponding positive correlations were observed between duration of grain fill (an indicator of the stay-green phenotype) and percent deep root length and percent change in root length as well as weakly negative correlations between duration of grain fill and total root length for all barley lines together (Supplemental Table S3).

ANOVA revealed a significant effect of stay-green on multiple seedling traits in the greenhouse experiments for barley (Supplemental Table S4). Stay-green barley lines had a shorter longest root, wider seminal root angle, higher seminal root number, and lower lateral root number in the root roll-up assay, and less total root length in the PVC pot assay.

In wheat, the mean percent deep root length of stay-green lines was higher than that of non-stay-green lines at every instance of measurement, but the effect of stay-green was not significant in any of these ANOVA (Figure 2D). There were no consistent patterns of difference between the two groups for total root length or percent change in root length (Figure 2B and F). Duration of grain fill did not correlate with field root traits in wheat in the correlation analysis of all wheat lines together, since both days to heading and days to maturity had positive correlations with total root length and negative correlations with percent change in shallow and deep root length. (Supplemental Table S3).

There were two seedling traits that were marginally impacted by stay-green in ANOVA for wheat (Supplemental Table S4). Stay-green wheat lines had slightly less root vol-

### 3.2 Relationships of field root traits to agronomic traits

In barley, grain yield was positively correlated with total root length at both flowering and harvest when data from all field seasons were analyzed together, but these correlations were weakly negative in 2018 and 2019 and weakly positive in 2020 (Figure 3A). Individual seasons showed consistent positive correlations of grain yield with percent deep root length at flowering and harvest (Figure 3C). Percent change in root length was also consistently positively correlated with grain yield, although this was much stronger for shallow than deep root length (Figure 3E). Spearman correlation coefficients for field root traits with grain yield and other agronomic traits for individual and combined seasons are listed in Supplemental Table S3. Although there were some instances of strong correlation with field root traits for aboveground biomass at flowering, leaf area at flowering, and harvest index, there was lack of consistency in the direction of these correlations (positive or negative) across seasons (Supplemental Table S3). Correlations with productive tiller number suggested a positive relationship to percent change in root length in barley but did not show a discernible relationship to total root length or percent deep root length. Percent grain protein in barley was negatively correlated with total root length in 2019, with weak negative correlations in 2020 and combined seasons (Figure 4A). Correlations between protein and percent deep root length were all negative but weak (Figure 4C). Correlation analysis did not demonstrate a connection between protein and percent change in root length in barley (Figure 4E).

In wheat, correlation tests showed a positive relationship between grain yield and total root length and suggested a positive relationship between yield and percent deep roots as well (Figure 3B, D). Yield was also positively correlated with percent change in root length in 2020, but this connection was weak in the other two seasons (Supplemental Table S3). Correlations between other agronomic traits and root traits in wheat are presented in Supplemental Table S3. There were positive correlations of aboveground biomass at flowering with percent deep root length at flowering and with percent change in shallow root length, except in 2018 where there was no correlation with either. A negative correlation with percent change in shallow root length in 2018 was the only strong relationship between a field root trait and leaf area in wheat. For productive tiller number, there were strong and moderate correlations with total root length in 2018 and 2019, respectively. There appeared to be a positive association of harvest index
FIGURE 2 Means of non-stay-green and stay-green groups of lines, with p values from ANOVA for the fixed effect of stay-green for field root traits measured by minirhizotrons. Sample sizes are listed in corresponding Supplemental Table S2. A, C, E: Barley. B, D, F: Wheat. A, B: Total root length at flowering and harvest. C, D: Percent deep root length (Equation 1) at flowering and harvest. E, F: Percent change (Equation 2) in shallow and deep root length between flowering and harvest (during grain fill).

3.3 Seedling traits as proxies for field root traits

In barley, correlations of total root length in the field with seminal root angle and seminal root number in the root roll-up assay were weak but were consistently negative for every instance of field measurement (Table 5 and Supplemental Table S5). Percent deep root length at flowering and harvest with total root length, but this did not carry through to 2018. Although percent grain protein in wheat was negatively correlated with total root length when seasons were combined (Figure 4B), the correlation between these traits was weakly positive in 2020 and weakly negative in 2019 so did not match across seasons (Supplemental Table S3). Positive but weak correlations with protein were observed for percent deep root length and percent change in root length (Figure 4D and F).
in the field was consistently positively correlated with root volume, seminal root angle, and seminal root number, and consistently negatively correlated with length of longest root, and lateral root number as measured by the root roll-up assay (Table 5 and Supplemental Table S5). Percent deep root length was also negatively correlated with total root length and root dry weight as measured by the PVC pot assay, except for the 2020 field season where there was no correlation with either seedling trait (Table 5 and Supplemental Table S6).

Percent change in shallow and deep root length in the field in barley was positively correlated with seminal root angle (except for 2018 shallow roots: \( r_s = -0.262 \)) and negatively correlated with length of longest root (except for 2020 deep roots: \( r_s = -0.119 \)) in the root roll-up assay (Table 5 and Supplemental Table S6).

In wheat, correlations of total root length in the field with length of longest root in the root roll-up assay, and with root to shoot dry weight ratio in the PVC pot assay, were weak.
FIGURE 4  Correlations between percent grain protein and field root traits measured by minirhizotron. Points represent values measured on a single plot. Trendlines and Spearman correlation coefficients ($r_s$) are from all seasons combined. A, C, E: Barley. B, D, F: Wheat. A, B: Percent grain protein and total root length at flowering and harvest. C, D: Percent grain protein and percent deep root length (Equation 1) at flowering and harvest. E, F: Percent grain protein and percent change (Equation 2) in shallow and deep root length between flowering and harvest (during grain fill).

but consistently positive for every instance of field measurement (Table 5 and Supplemental Tables S5 and S6). Field total root length was negatively correlated with seminal root number in the root roll-up assay for the 2020 field season though there was no correlation in 2019 (Table 5 and Supplemental Table S5). Percent deep root length in the field in wheat was consistently negatively correlated with root volume, root to shoot dry weight ratio, seminal root angle (except for 2020 harvest: $r_s = .190$), and seminal root number as measured by the root roll-up assay (Table 5 and Supplemental Table S5). Percent change in shallow root length in the field was positively correlated with total root length in the root roll-up assay (Table 5), but there was no correlation with this trait for change in deep root length (Supplemental Table S5). Change in shallow and deep root length in wheat was negatively correlated with root volume (except 2020 deep roots: $r_s = .048$).
TABLE 5  Lists of seedling assay traits with the most potential as proxies for field root traits based on strength of correlation and consistency of direction of correlation across seasons

| Total root length | Percent deep root length | Percent change in root length |
|-------------------|--------------------------|------------------------------|
| **Barley**        |                          |                              |
| - seminal root angle RRU | + root volume RRU         | - length of longest root PVC  |
| - seminal root number RRU | - length of longest root RRU | + seminal root angle RRU    |
|                    | + seminal root angle RRU  | - lateral root number RRU    |
|                    | + seminal root number RRU |                              |
|                    | - lateral root number RRU |                              |
|                    | - total root length PVC   |                              |
|                    | - root dry weight PVC     |                              |
| **Wheat**         |                          |                              |
| + length of longest root RRU | - root volume RRU         | + total root length RRU      |
| - seminal root number RRU | - root to shoot ratio RRU |                              |
| + root to shoot ratio PVC | - seminal root angle RRU  |                              |
|                    | - seminal root number RRU |                              |

Note. Spearman correlation coefficients for all field vs. greenhouse root comparisons can be found in Supplemental Tables S5 and S6. Direction of correlation is indicated as positive (+) or negative (-). Method of proxy measurement is indicated as one-leaf root roll-up assay (RRU) or four-leaf PVC pot assay (PVC).

and root to shoot ratio as measured by the root roll-up assay (Table 5 and Supplemental Table S5).

4 | DISCUSSION

4.1 | Barley

4.1.1 | Deep roots

There is a prevalent notion amongst crop scientists that deep rooting can help alleviate terminal drought stress (Bodner et al., 2015), as research has shown that enhanced root growth at depth does lead to more extraction of subsoil water when it is available (Lilley & Kirkegaard, 2011; Pask & Reynolds, 2013; Vadez et al., 2013). Our field trials were grown in deep soil and followed previous season fallow rotations, so deep water was most likely present. We observed that duration of grain fill (earlier heading and later maturity) was positively correlated with percent deep root length in barley (Supplemental Table S3), and there was a trend in which stay-green barley lines had a higher percentage of their root length deeper in soil than non-stay-green lines (Figure 2C). These results lend support to our hypothesis that increased root length at depth is related to the stay-green phenotype in barley. Deeper root growth has been linked to earlier heading (and thus longer grain fill) in barley in a controlled environment (Voss-Fels et al., 2018). It is worth noting that in 2019 our field received above average precipitation before flowering and below average precipitation after flowering (Table 2), and this was also the year in which the dynamic of stay-green barley lines having more roots at depth and greater yield was most pronounced. We also saw evidence of a yield benefit associated with deep rooting when combining data for all barley lines (Figure 3C). These observations are consistent with the hypothesis that deep rooting is a useful adaptation to environments where precipitation is lacking but deep soil moisture is available.

4.1.2 | Change in root length

Our results imply that an increase (or smaller decrease) in shallow root length during the grain fill period might have been associated with stay-green lines (Figure 2E) and longer duration of grain fill (earlier heading and later maturity) (Supplemental Table S3). This would suggest that delayed belowground senescence (although perhaps only at shallower depths) occurs along with the extended photosynthetic period observed aboveground in stay-green barley. Minirhizotrons have been used to document delayed root senescence associated with stay-green in sunflower (Lisanti et al., 2013). There was also a pattern of positive correlation between delayed root senescence and yield in barley when stay-green and non-stay-green lines were analyzed together (Figure 3E, Supplemental Table S3). This is counter to the idea that continued root growth would negatively impact yield in water-limited environments as it would compete with grain for photosynthates (Lynch, 2018; Yang & Zhang, 2006). However, Lynch (2018) also points out that if roots die prematurely due to drought rather than programmed senescence, this could result in a loss of carbohydrates in the roots that would otherwise be reallocated to grain. Furthermore, Gous et al. (2013) showed that starch biosynthesis and molecular structure in grain were
unaffected under water stress for a stay-green barley line compared with a non-stay-green cultivar. Delayed root senescence could be part of a strategy for coping with terminal drought stress, but this might require a smaller more efficient root system so that its continued functioning would not detract too much energy from grain filling.

4.1.3 Total root length

There was a consistent pattern where stay-green barley had less total root length than non-stay-green barley, even though the effect of stay-green was not significant in these ANOVAs (Figure 2A). Furthermore, there were weak but consistent negative correlations of total root length with duration of grain fill and days to maturity (Supplemental Table S3). These results fit with the idea that the root system of stay-green barley might be smaller than that of non-stay-green barley. However, correlations between total root length and grain yield were weakly negative in 2018 and 2019, but weakly positive in 2020 (Supplemental Table S3), so the effect of this smaller root system might not always be beneficial and probably depends on environment.

4.1.4 Stay-green seminal root traits and drought adaptation

The idea that the drought adaptation associated with the stay-green trait in barley might be due to a smaller deeper root system is further supported by some parallel relationships that we observed in our field and greenhouse experiments. Stay-green lines had a wider seminal root angle in the root roll-up assay (Supplemental Table S4). This was unexpected because deep rooting is commonly thought to be associated with a narrower seminal root angle as has been shown in wheat (Oyanagi, 1994), sorghum (Singh et al., 2012), and maize (Lynch, 2013). Yet, a wider seminal root angle in our greenhouse experiment correlated with greater percent deep root length in our field experiment (strongly in 2019 though weakly in other seasons, Supplemental Table S5). This difference between barley and other species with respect to root angle and deep rooting is not without precedent, as Manschadi et al. (2006) found a stay-green wheat cultivar to have a narrow compact root system with more deep root activity than a non-stay-green wheat cultivar in a root observation chamber, whereas the root system of a drought-tolerant barley cultivar had much more lateral spread at the top then narrowed with increasing depth. Robinson et al. (2016) also found a stay-green barley line to have a wider seminal root angle than a non-stay-green line. A shorter longest root, higher seminal root number, and lower lateral root number were also associated with more roots at depth (though not always strongly) and with stay-green lines (Supplemental Tables S5 and S4). As for a smaller root system size, there were weak but consistent patterns in which a wider seminal root angle and higher seminal root number were connected to less total root length and to our stay-green barley lines (Supplemental Table S5 and S4).

Another stay-green characteristic for barley in our field study was that they had less leaf area at flowering and higher harvest index compared to non-stay-green (except in 2020, Table 4). This could be further indication of more efficient water use by drought adapted barley. Borrell et al. (2014) observed that in sorghum, stay-green cultivars had less leaf area and the smaller vegetative canopy reduced water use prior to flowering, saving soil moisture for grain fill. It has been suggested that efficient water use is superior to more water use in high-input systems dependent on stored soil moisture (Passioura, 1983; Palta et al., 2011; Lynch, 2018).

4.1.5 Roots and grain protein

Grain protein content did not appear to be influenced by root system characteristics for the barley lines in our study (Figure 4A, C, E). The relationship between roots and grain protein in barley was likely confounded by the fact that all but two of the barley lines (Craft and MT124118) carry the allele for HvNAM1, a NAC transcription factor gene, that reduces the percent grain protein (Alptekin et al., 2021). In the current study, both stay-green and non-stay-green lines carry the low protein allele, apparently reducing protein levels irrespective of the root architecture.

4.2 Wheat

4.2.1 Deep roots

Although our stay-green wheat lines appeared to have greater means for percent deep root length than non-stay-green lines (Figure 2D), the effect of stay-green was not significant in ANOVA, and there were no strong correlations between deep rooting and duration of grain fill in wheat (Supplemental Table S3). These results neither support nor refute the results of other studies. Manschadi et al. (2006) saw that a stay-green wheat line had more roots at depth and extracted more deep water than a non-stay-green line in a root observation chamber. In addition, higher normalized difference vegetation index (NDVI), which has been used as an indicator of the stay-green phenotype, has been correlated with more roots at depth and deep water use in the field (Li et al., 2019). Christopher et al. (2008) also observed the stay-green phenotype and yield advantage of a stay-green wheat line in multiple low rainfall environments but saw neither of these in a location where deep soil moisture was lacking. We did observe consistently positive though mostly weak correlations between percent deep roots and grain yield in our study (Figure 3d and
Supplemental Table S3). The environment for our field studies lacked late season precipitation (Table 2) but likely had stored soil moisture (see Section 2.2.1). Thus, a positive association between yield and deep rooting in our results would be consistent with the results of Postic et al. (2019), where a correlation was observed between yield and deep roots in rainfed but not irrigated conditions in their minirhizotron field trials examining wheat.

4.2.2 Root growth and yield

Positive correlations have been found between grain yield and root system size in wheat under water stress (Postic et al., 2019). Grain yield was positively correlated with total root length at flowering and even more so at harvest in our study (Figure 3F). We also found positive correlations between yield and percent change in root length for wheat in 2020 and this pattern was seen in other seasons but was not significant (Supplemental Table S3). Taken together these results imply that having a larger root system that persists for more time after flowering helps wheat withstand terminal drought stress.

4.2.3 Root growth and phenology

Christopher et al. (2008) suspected that delayed water extraction from the soil after flowering played a role in producing the greater yield of their stay-green wheat line over a non-stay-green line. However, any connection of greater and or delayed root growth to the stay-green trait was unclear in our study. There were not consistent differences for change in root length or total root length between stay-green and non-stay-green groups of lines (Supplemental Table S2). We did not observe a correlation between percent change in root length and duration of grain fill due to negative correlations of this root trait with both days to heading and days to maturity (Supplemental Table S3). Similarly, there was no correlation between grain fill and total root length because total root length was positively correlated with both days to heading and days to maturity (Supplemental Table S3). So, delayed root senescence appeared to be associated with earlier heading and earlier maturity, whereas larger root systems appeared to be associated with later heading and later maturity. Later heading was found to be associated with larger root systems in a minirhizotron greenhouse study by Ghimire et al. (2020), but many root studies in and out of the field do not measure roots beyond the timing of heading or flowering. Perhaps wheat yield is boosted by delayed heading coupled with delayed senescence and a root system that continues to function during grain filling. This would allow root systems to grow large prior to reproductive stages increasing the plant’s capacity to capture resources, then maintain the plant’s ability to utilize those resources and fill seeds with more starch. The success of such a strategy would be dependent on environmental factors, likely requiring abundant stored soil moisture if late season precipitation was minimal.

4.2.4 Stay-green drought adaptation

Differences that we did observe between stay-green groups in wheat were greater leaf area (consistent though not significant in 2019 and 2020) and higher productive tiller number of stay-green lines compared to non-stay-green (Table 4). Christopher et al. (2008) also saw greater leaf area of a stay-green wheat line compared to a non-stay-green line in water-limited but not under nonlimited conditions, and Pask and Reynolds (2013) observed that wheat lines that had the highest yield in dry environments were extracting more water from the soil due to greater stomatal conductance. The larger canopy associated with our stay-green lines suggests that the drought adaptation in stay-green wheat might have more to do with aerial physiology rather than root system architecture.

4.2.5 Roots and grain protein

There was evidence of a connection between grain protein content and root system characteristics in wheat. Total root length was negatively correlated with percent protein for combined seasons (Figure 4B), although the correlation was weakly negative in 2019 and weakly positive in 2020 (Supplemental Table S3). Grain yield was positively correlated with total root length, so an opposite negative correlation between total root length and protein would be consistent with expectations because yield and protein are well known to be negatively associated in wheat (Yu et al., 2018). Although interestingly there were positive correlations for both protein and yield with percent deep root length and percent change in root length (Supplemental Table S3). If this result was repeatable in more seasons and locations, it would be encouraging for wheat breeders as they could potentially focus on increasing deep root length and delaying root senescence as a way to boost yield and percent protein together. A review on breaking the negative relationship between yield and protein in wheat, known as grain protein deviation, by Cormier et al. (2016) suggested that deep rooting could help maximize nitrogen uptake because nitrate leaches downward through soil.

4.3 Barley vs. wheat

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

4.4 | Proxy considerations

An ideal proxy trait can be measured quickly and easily and has a clear, consistent relationship to an important trait that is more difficult to measure (Reynolds et al., 2020). Between the methods used here, the root roll-up assay would be more practical for use in plant breeding as it was much faster and easier to perform than the PVC pot assay. There were also more correlations with field root traits coming out of the root roll-up assay. The root traits we measured in the field were influenced by seasonal variation (Supplemental Table S1), but in instances where correlations between the field and greenhouse were strong in one season, they were usually consistent in direction across seasons (Supplemental Tables S5 and S6) indicating potential usefulness of these assay traits as proxies for field traits. The best proxy trait candidates are summarized in Table 5. Considering multiple of these traits in some sort of composite measurement may act as an improved proxy over examining any one trait in isolation.

Although we tested our proxy traits against field data from three different seasons, all of these were in the same location. Robinson et al. (2018) found both positive and negative correlations between seminal root angle and yield when testing a barley breeding population in several locations. In our experiments, which assay traits were correlated with which field traits was not consistent between species (Table 5). Furthermore, in instances where field traits correlated with the same assay traits for both barley and wheat, the directions of those correlations were often opposite between the two species (Supplemental Table S5, S6). It appears that proxies should be species specific as well as environment specific.

5 | CONCLUSION

Minirhizotrons are useful tools that allow non-destructive repeatable imaging of roots growing in real field conditions. Barley and wheat roots have certainly been observed growing deeper (Thorup-Kristensen et al., 2009) than the range defined as deep in this study, and we wish to reiterate that we did not measure maximum depth reached by the roots, but rather the amount of root length present in fixed depth ranges captured by the minirhizotron imaging system (shallow: 0–30 cm, deep: 30–60 cm). Ours is the first study to use minirhizotrons to examine the same roots in the field at multiple time points for stay-green barley and wheat. This study also offers a first look at relationships of roots in the field with grain protein content in barley and wheat. Our results suggested that (a) greater deep root length, delayed root senescence, and less total root length were associated with the stay-green phenotype in barley in this study, although in wheat, stay-green was associated with greater deep root length, but not related positively or negatively with either delayed root senescence or total root length. (b) Greater deep root length and delayed root senescence were associated with greater grain yield in barley, as was greater total root length but this relationship was inconsistent across seasons. In wheat, greater deep root length, delayed root senescence, and greater total root length were all associated with greater grain yield. (c) Several traits from the greenhouse assays showed potential as proxies for field root traits in our environment. (d) There were differences between barley and wheat with respect to how stay-green lines differed from non-stay-green lines, how field root traits related to agronomic traits, and the relationships between roots in the greenhouse and roots in the field. The patterns illustrated by this study were observed on a limited set of genotypes in a single location. It should not be assumed that what works for one species in one environment will work in a different environment, or for a different species in the same environment, even for two crops as similar as barley and wheat. In order to expand our understanding of and confidence in these associations, we would need to test them on more varieties in more locations. We are in the process of mapping the genetics of stay-green and root proxy traits in a biparental barley population. Once QTLs are identified, near isogenic lines will be created for use in future minirhizotron experiments. Further characterization of root system physiology and its genetic controls could help barley and wheat breeders develop cultivars that are better adapted to drought stress.

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**AUTHOR CONTRIBUTIONS**

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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