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From Forage to Grain: Structural and Functional Changes Occurred During 10 Generations of Transition of Intermediate Wheatgrass (*Thinopyrum intermedium*) to Kernza®

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Abstract

Thinopyrum intermedium (c.n. intermediate wheatgrass), marketed under the trade name Kernza, is a promising species for perennial grain production based on seed size, ease of threshing, resistance to shattering, and grain quality. Although numerous generations of breeding for seed yield have been completed, the impact of selection on non-target traits is unknown. Here, we evaluated structural and functional changes brought about by selection for seed yield over a sequence of nine selection cycles (C0 to C9). In two experiments under semi-controlled environmental conditions, we compared gas exchange (A, E, gs, and A/Ci curves), leaf and root morphology, and the structure of seedlings from 10 generations. We found that the selection for yield throughout cycles indirectly changed the leaf structure (leaf size, leaf thickness, and leaf anatomy) and physiology (carbon acquisition and transpiration per unit area), with later cycles showing larger leaves with higher rates of CO_2 assimilation and transpiration. Changes in root structure followed similar trends: selection resulted in longer, more branched, and finer roots. These changes in non-target traits are linked to resource-use strategies and to ecosystem services provided by Kernza. Understanding how the domestication of perennial grains impacts non-target traits will aid in the design of integrated breeding programs for Kernza and other perennial grain crops.

Keywords: domestication; gas exchange; leaf anatomy; root morphology

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1. Introduction

The domestication of grasses has historically favored annual species due to their rapid growth and suitability for crop rotation. Breeding over the last century has focused on yield increases and disease resistance, resulting in crops with a domestic phenotype (bigger seed size, loss of seed dormancy, improved flavor, loss of shattering or seed dispersal, lack of branching, etc. [1]), but it also resulted in reduced fitness in the wild and a reliance on intensive chemical management [2,3]. The environmental effects of annual cropping underscore the urgent need for a shift toward more sustainable practices [4].

Intermediate wheatgrass (IWG, *Thinopyrum intermedium* [Host] Barkworth & D.R. Dewey) is a perennial grass native to Eurasia that has been used extensively as forage in the USA and Canada [5], and the seeds can also be used in baked products or beverages to augment or replace grains such as wheat, rye, or barley [6]. This grass has a large perennial root system that can provide several ecosystem services such as increased soil

carbon, efficient nutrient cycling, and reduced nutrient leaching relative to annual grain crops [7–10]. Intermediate wheatgrass was selected for domestication from an evaluation of over 100 perennial grasses in the 1980s by work at the Rodale Institute, Kutztown, PA [11]. Evaluation of germplasm started in the late 1980s at the Rodale Research Center [5] and was followed by a breeding program at the USDA Plant Materials Center in Big Flats, NY, in 1990 [12]. A breeding program was started at The Land Institute in 2001 [13]. After six generations of phenotypic selection (Cycle 6), the breeding program transitioned to genomic selection [14]. The Land Institute provides licenses to sell the grain as "Kernza", but its lower grain yield compared to annual grain crops is the primary reason for the lack of full adoption by farmers [13]. Hence, the added economic value of this perennial crop, such as ecological benefits or the dual-purpose for food and fodder, are critical for its adoption [15].

Most domestication programs are primarily focused on breeding and selection using yield and yield-related characters, as these traits are undoubtedly the most important to reach economic yields and to allow for the cultivation and commercialization of the crop. Modern breeding programs for perennial crops face the challenge to achieve in a few years what thousands of years of informal selection and 100 years of formal breeding have achieved in the annual counterparts [16,17]. Because of this, other traits related to perenniality and the provision of ecosystem services tend to not be included in the breeding programs of new crops.

During the process of domestication, plants were moved from relatively low resource environments to higher resource agricultural ecosystems, where competition and herbivory pressure were reduced by human intervention [18]. The release of natural stressful conditions has resulted, in many crops, in a shift in resource acquisition strategy from conservative to more acquisitive [19–21], although some authors claim that this change may not be widespread among annual crops [16].

For perennial new crops, there is evidence that selection using yield characters can inadvertently change key traits involved in resource acquisition, use, and recycling at the leaf [22,23] and root levels [24,25], plant defenses [26], relationships with pollinators, and diversity and activity of soil microorganisms such as pathogens and arbuscular mycorrhiza fungi [27], among others. Some of these changes may be inconsequential for the plant's performance as a crop and the services it provides, although there is some evidence of lost resilience (i.e., reduced drought tolerance [28] and lower resistance to pests and pathogens [29]). In any case, the understanding of unintended changes that go unnoticed and might deeply impact crop performance is crucial to guide breeding and to design appropriate agronomic technologies.

Our objective was to evaluate non-targeted structural and functional changes brought about by selection, using yield and harvestability as the main selection traits, in a sequence of nine breeding cycles (C0 to C9) of IWG. We evaluated a set of root, leaf, and whole-plant traits associated with resource-use strategies within the theoretical frame of trait-based ecology [30]. Considering that the breeding program of IWG was performed under high resource availability (wide plant spacing allowed for greater access to water, nutrients, and light), we assessed whether plants shifted towards resource acquisitive strategies throughout the breeding cycles.

We discuss our results in the context of multifunctionality of perennial species as a grain and service crop, which would help plant breeders to select for crop traits that support these functions in addition to yield.

2. Materials and Methods

2.1. General

In order to assess unintended and indirect changes related to breeding IWG for seed-yield related traits, we compared structural and functional characters of plants from 9 selection cycles, starting from cycle 0 (established from selections by the Big Flats Plant Materials Center) to C9. Plants were grown in semi-controlled field environments. The number of the cycle increases with each successive selection for desirable yield traits in IWG at The Land Institute, USA [13].

The phenotypic breeding program spans 14 years and six cycles of selection. Selection was focused primarily on improving grain yield, seed mass, free-threshing ability, and shatter resistance. The first six selection cycles relied on traditional breeding approaches: thousands of individual plants were evaluated at non-competitive spacings, from which the best plants were selected and intermated to initiate the next generation. Using this approach, each breeding cycle required 2–3 years to complete. Details on the breeding program are available in [13]. Compared with the initial population, grain yield and free-threshing ability increased by 144 and 129%, respectively. The seed size, a trait with strong impact on the properties of processed grains and flour, has evolved more slowly, increasing by just 52% over five generations, indicating that greater progress for this trait may require adjusted breeding priorities or a shift in methodology.

From the seventh selection cycle to the ninth cycle, breeding shifted entirely to genomic selection, reducing the time required for each generation to one year. Genomic selection was performed using young seedlings with emphasis on free threshing, shattering, spike yield, and seed mass for selecting the parental plants [14]. The selected parents were randomly intermated in a greenhouse crossing block to form the subsequent cycle [31]. Grain yield increased 205% during the entire selection process (from C0 to C9). The grain yield improvement per cycle of selection followed a linear trend from the initial population (C0) to the ninth cycle (C9), with a rate of 58.5 kg grain per ha^{-1} per cycle [31].

2.2. Experimental Setup

Both experiments were located at The Land Institute near Salina, KS (38°46′14″ N; 97°35′30″ W). The location is 370 m above sea level on the western fringe of the tallgrass prairie region. Mean annual precipitation in the area is 737 mm, with approximately one-tenth of that precipitation in the form of snow. Rainfall is concentrated in spring and fall, and extended droughts are common in summer. The mean daily high temperature in July is 34 °C, the mean nightly low in January is −7 °C, and rapid, wide swings in temperature are common in all seasons.

Experiment 1: Greenhouse. Plants of 7 selection cycles were compared (C0, C1, C2, C4, C6, C7, and C8) under semi-controlled conditions (greenhouse). Seeds (ten seedlings per selection cycle) were placed in 4 l containers filled with a commercial mix (ProMIx, PremireTech.com, Delson, QC, Canada). The experimental design was completely randomized; containers were randomly distributed in the greenhouse. Seedlings were allowed to grow in a greenhouse for 3 weeks under irrigation. Greenhouse conditions were as follows: temperature 30 °C/20 °C (day/night), and radiation was approximately 1400 µmol m^{-2} s^{-1}.

Leaf traits were measured 21 days after germination (10 July). Gas exchange under well-watered conditions was measured 2 times 4 weeks after germination (17 and 18 July) on 6 plants per each selection cycle (n = 6).

Experiment 2: Rain-out shelter. Plants of 10 selection cycles (C0–C9) were grown under a rain-out shelter to control water availability. Seeds (ten seedlings per selection cycle) were placed in 10 L containers filled with ProMix (Premier Tech Horticulture, Delson, QC, Canada). The experimental design was completely randomized; containers were randomly

distributed in the rain-out shelter. Temperature and humidity conditions followed weather conditions in Salina, Kansas for May (13 °C, 18 °C, and 24 °C mean minimum, mean, and maximum mean temperatures, respectively) and June (19 °C, 24 °C, and 31 °C, mean minimum, mean, and maximum mean temperatures, respectively), and PPFD was reduced by 30% of ambient radiation (@ 2300 μmol m^{-2} s^{-1}) by the rain-out. Plants were allowed to grow in a greenhouse for 40 days under an irrigation frequency to maintain pots at field capacity.

Gas exchange was measured several times during the experiment (38 days after seed germination), A/Ci curves were performed, and leaf samples were taken for anatomy. The experiment was terminated on 14 June, plants were uprooted, and above-ground biomass was determined. Roots were washed, immediately sealed in plastic bags, and transported in ice-cooled boxes to the laboratory for morphological trait measurements (Section 2.3).

2.3. Structural and Functional Traits

2.3.1. Early Vigor: Seed Size and Seedling Biomass

Seed weight was calculated for each selection cycle, with 3 replicates of 100 seeds each. For seedling weight, 10 seedlings of each selection cycle were uprooted and the soil washed from the roots. Fresh weight was recorded, and seedlings were then placed in an oven at 60 °C for drying for 48 h. Dry weight was then recorded.

2.3.2. Anatomical and Morphological Leaf Traits

The following morpho-anatomical data were assessed: (i) leaf area (cm^2); (ii) leaf thickness (excluding the main vein; mm); (iii) leaf weight (g); (iv) specific leaf area (SLA; cm^2 g^{-1}) and epidermis thickness (μm); (v) cuticle thickness (μm); (vi) stomatal density (number of stomata per lineal unit); (vii) vein density (number of vascular bundles per lineal area); (viii) vein size (cross-sectional length; μm); (ix) xylem/phloem proportion (%); and (x) metaxylem vessel diameter (μm).

Individual leaves (3 subsamples per plant) of six plants per selection cycle were harvested and immediately transported to the lab in sealed plastic bags (21 and 40 days after germination in experiments 1 and 2, respectively). Fresh leaf thickness was measured using a Mitutoyo Digital Thickness Gage 547–301 (Mitutoyo America Corp., Aurora, IL 60502, USA when leaves were harvested. Leaves were immediately sealed in plastic bags and transported in ice-cooled boxes and stored in the laboratory at 4 °C prior to analysis. Leaf area was determined by scanning the harvested leaves and processing the images using UTHSCSA Image Tool for Windows, Version 2.0 [32]. After scanning, leaves were dried at 60 °C for 48 h and weighed. SLA was calculated as the ratio of leaf area to dry weight, as described by [33]. Individual leaf values of thickness, leaf area, leaf weight, and SLA were averaged (3 reps per plant; 3 plants per selection cycle).

Anatomical features were compared by digitally measuring traits from scans of transverse sections. Young, fully expanded leaves were fixed in FAA (formaldehyde: 96% ethyl alcohol/glacial acetic acid/water, 2:10:1:7, by vol.) and sent to the Kansas State Veterinary Diagnostic Laboratory, Manhattan, KS, to be prepared, sectioned, and stained. Digital slides were stored at Aperio management system (Aperio eSlide Manager Version 12.5.1.5997eSlideManager Hub: 12.5.1.5997).

2.3.3. Physiological Leaf Traits: Gas Exchange

Gas exchange was measured using a LICOR LI-6800 Portable Photosynthesis system (LI-COR Enviromental, Lincon, NE 68504, USA). For three individual plants per selection cycle, gas exchange variables were measured on 3 leaves per plant (averaged for a single value per plant): A: CO_2 uptake; E: transpiration, Ci internal CO_2 concentration; and Gs: stomatal conductance. Measurements were taken around noon (11:00 to 14:30) on fully

expanded leaves and exposed to full sun and on two consecutive dates (to accommodate all measurements). For narrow leaves, the method for estimating leaf area when the leaf does not fill the chamber of the LI-Cor 6800 is to use the leaf area calculator and enter the leaf width, which computes the area based on a known or measured length. Leaf width of our plants ranged from 0.7 to 0.99 cm from C0 to C9.

To quantify biochemical processes limiting photosynthesis, A/Ci response curves were fitted using the Farquhar's model for C_3 photosynthesis through non-linear regression analysis [34]. We measured A at external CO_2 concentrations (Ca) = 400, 300, 200, 100, 50, 10, 400, 400, 600, 800, 1000, and 1200, ppm [35]. Three individual plants of each selection cycle were chosen. On each plant, curves were generated using the average instantaneous gas exchange of 3 leaves per plant. Measurements were performed under controlled chamber conditions of 2000 μmol m^{-2} s^{-1} PPFD and 50% humidity. Air and leaf chamber temperature were 27 °C, between 10:30 and 15:00.

2.3.4. Morphological Root Traits

Root traits were obtained by measuring features from root images obtained after scanning one harvested tiller per plant (resolution 2000 dpi) of three plants per selection cycle (40 days after germination in experiment 2, Figure 1). We used the program Rhizovision Explorer [36], which calculated metrics such as root length, diameter, area, and volume. Specific root length (SRL, cm g^{-1}) was calculated for the entire tiller root system as the ratio of the total root length and the dry root biomass; root tissue density (RTD, g cm^{-3}) was calculated by dividing the dry weight of a root intersection by its volume (calculated assuming the cylindrical shape of roots [37]).

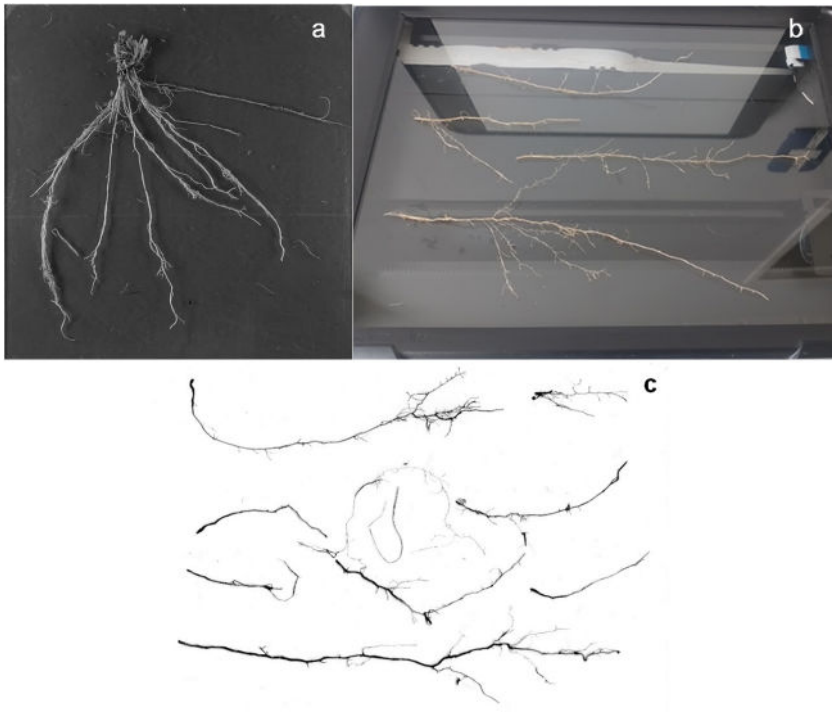

Figure 1. Root processing and digitalization methodology showing (**a**) a clean harvested root tiller-system; (**b**) root scanning to generate high resolution images at 2000 dpi; and (**c**) digital images were then analyzed using RhizoVision [36].

2.4. Statistical Analysis

To evaluate the trend of changes in the leaf (gas exchange, morphology, and anatomy) and root traits as a result of the breeding history in IWG, we performed a linear regression analysis using the selection cycle (from C0 to C9) as the independent continuous variable,

representing the intensity of phenotypic or genetic change. Each selection cycle involves a series of actions such as parent selection, crossbreeding, and offspring evaluation, which have a cumulative effect on the genetic structure of the population. Linear models had a good fit for all dependent variables (R^2 showed in the figures). The residuals vs. fitted values plot showed a random dispersion, suggesting linearity.

To compare differences between plants of different selection cycles (C0 to C9), we also used a one-way analysis of variance. To check for data normality, we used Shapiro–Wilk's test and Levene's test for homogeneity of variance. When these assumptions were violated, data were transformed using logarithm or root square transformation as appropriate. Statistical analyses were performed using InfoStat Software, version 2014, Universidad Nacional de Córdoba, Córdoba, Argentina.

3. Results

3.1. Experiment 1 (Greenhouse)

Leaf morphology. Starting with C0, the leaf width increased at a rate of 0.034 cm/cycle, following selection for seed yield (Figure 2a). The individual leaf area also increased at a rate of 3.38 cm^2/cycle (Figure 2b).

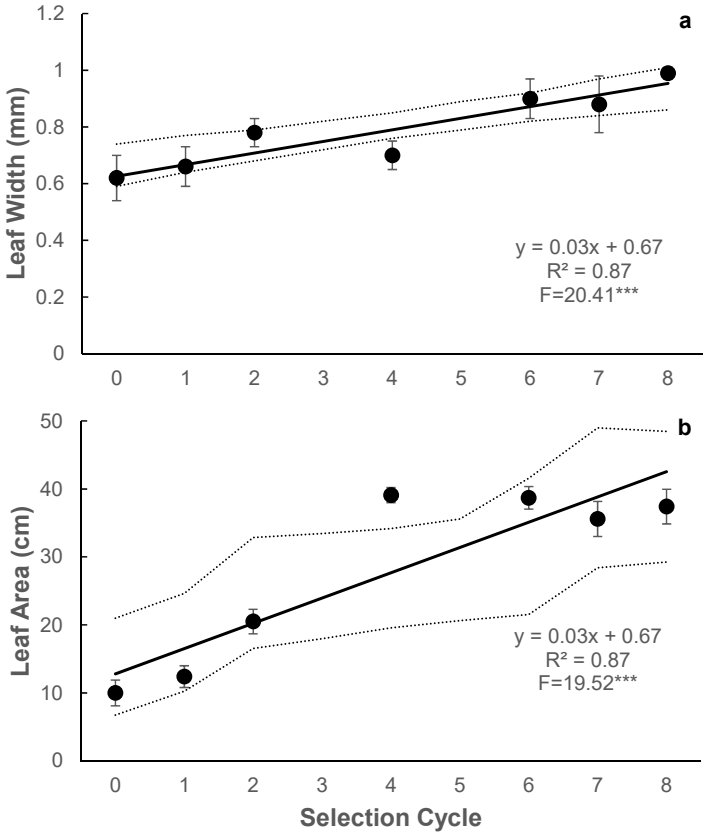

Figure 2. Leaf width (**a**) and individual leaf area (**b**) of 7 generations of *T. intermedium* selected for seed yield. Leaf traits were measured on six plants per selection cycle (three leaves per plant). Predicted lines (black line), confidence intervals (95%. Dashed lines) R^2, F-test, and significance (*** $p \leq 0.0001$) are from linear regression models.

Gas Exchange. Net CO_2 uptake (A; μmol m^{-2} s^{-1}; Figure 3a) and transpiration (E; mmol m^{-2} s^{-1}; Figure 3c) also increased with selection for seed yield, following a steady increase in stomatal conductance (gs, μmol m^2 s^{-1}; Figure 3b). The internal CO_2 concentration (Ci, ppmCO_2; Figure 3d) also increased with the selection cycle.

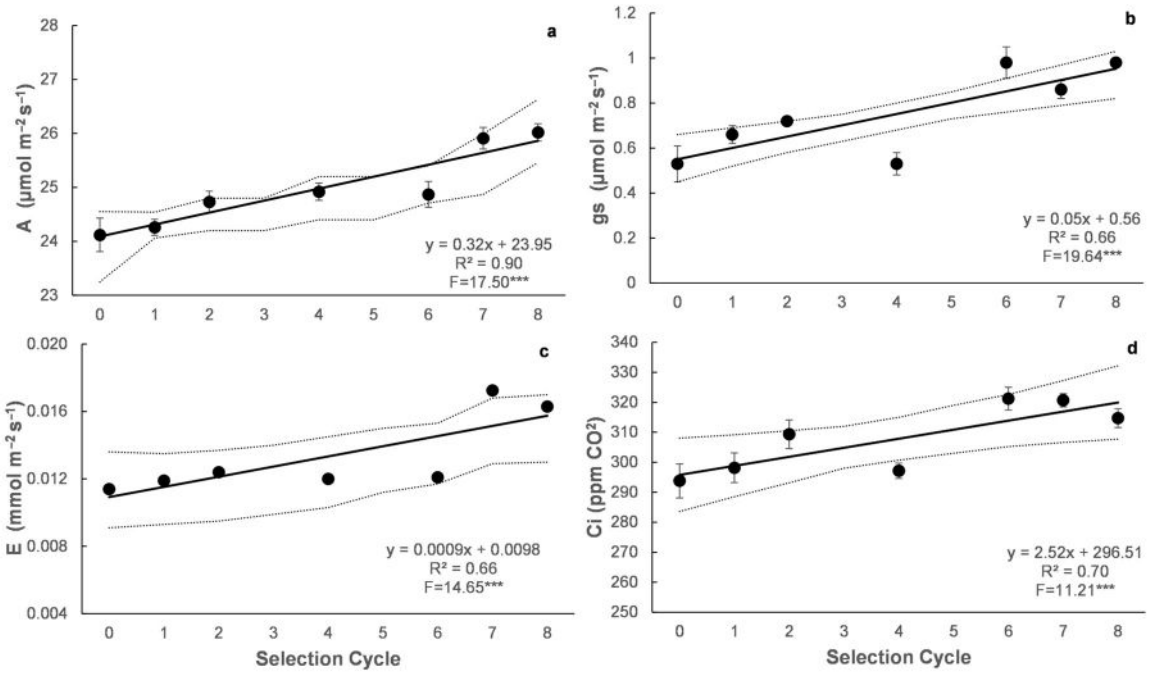

Figure 3. Net CO_2 uptake (µmol m^{-2} s^{-1}, A; (**a**)); stomatal conductance (gs; (**b**)); transpiration (mmol m^{-2} s^{-1}, E; (**c**)); internal CO_2 concentration (Ci; and (**d**)) 7 generations of *T. intermedium* selected for seed yield measured on three plants per selection cycle (3 leaves per plant). Predicted lines (black lines), confidence intervals (95%, dashed lines) R^2, F-test, and significance (*** $p \leq 0.0001$) are from linear regression models.

3.2. Experiment 2 (Rain-Out Shelter)

Early Vigor. Selection for seed yield produced a strong change in seed weight and initial seedling growth. After an initial drop in seed size from C0 to C1, the seed weight increased from 5.4 to 14 g/1000 seeds (average C8–C9), with an overall rate of 1.1 g/1000 seeds with each selection cycle (Figure 4). Seedling size had a similar change from 0.02 g (total dry weight) to 0.09 g (Figure 4). A significant change in the rate of seed and seedling weight was found after C6, when genomics selection started.

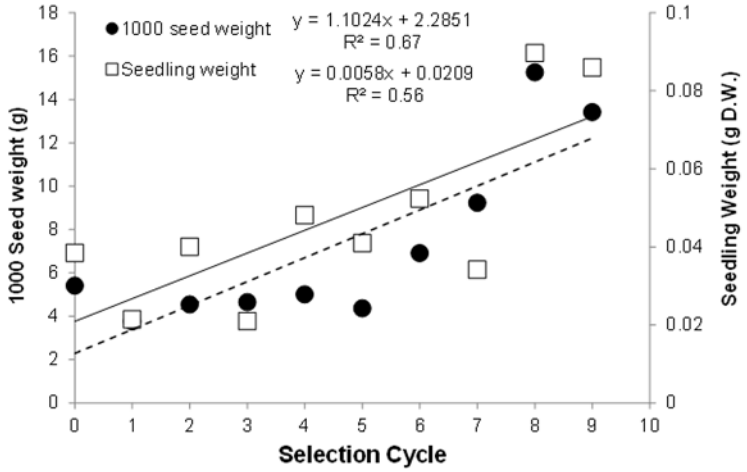

Figure 4. Seed weight and seedling weight (1 true leaf) of 10 cycles (C0 to C9) of selection of *T. intermedium*, grown in the greenhouse. Seed weight was measured in three replicates of 100 seeds per selection cycle, while seedling weight was measured for ten plants. Predicted lines (black for seed weight and dashed for seedling weight), equations and R^2 are from linear regression models.

Leaf Morphology and Anatomy. The leaf area increased with selection (Figure 5a). Leaves of C8 and C9 plants were significantly bigger than those of the previous cycles ($p < 0.001$; F = 4.48). The size increased as much as 80% from C0 to C9. The thickness of the lamina also increased (Figure 5b). Leaves of C8 and C9 plants were significantly thicker than those of the previous cycles ($p < 0.001$; F = 23.36). The thickness increased by 60% from C0 to C9. Despite changes in size and thickness, early selections (C0–C1–C2) showed a similar specific leaf area (SLA) than late selections (C8–C9), which implies that there are changes in leaf anatomy.

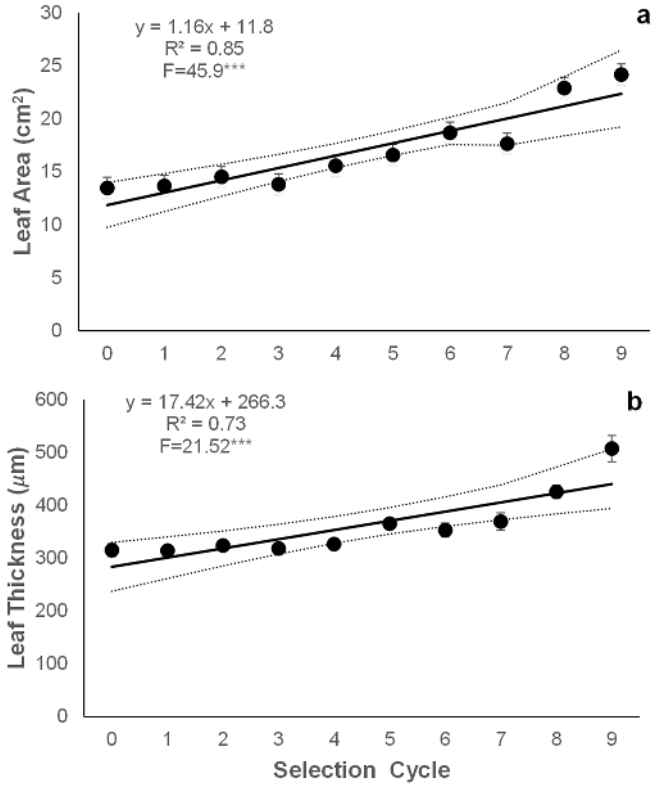

Figure 5. Leaf area of individual leaves (**a**) and leaf thickness (**b**) of plants of 10 generations (C0 to C9) of selection of *T. intermedium*. Predicted lines (black lines), confidence intervals (95%, dashed lines) R^2, F-test, and significance (*** $p \leq 0.0001$) are from linear regression models. Leaf traits were measured on six plants per selection cycle (three leaves per plant).

Selection affected the size and distribution of vascular bundles (Figure 6). The veins grew smaller, from C0–C2 to C8–C9, but also closer together. The distance between bundles (a proxy trait to the bundle's density; number of bundles per lineal μm) in C8–C9 was 26% lower than in C0–C2 (1 bundle per 232 μm vs. 294 μm, respectively; F = 5.29; $p < 0.0001$; Figure 7). The main vascular bundle's cross-section reduced its length by 15% in C8–C9 compared to that from C0–C2 (132 vs. 167 μm; $p = 0.01$; F = 14.59; Figure 6). The relative proportion of xylem and phloem was unaffected by the main vein's reduction ($p > 0.05$). Both groups' metaxylem vessel diameters were similar ($p > 0.05$; F = 0.09; Figure 6). As vascular bundles grew closer, so did the bulliform cells, increasing their density per lineal unit. The size of bulliform cells was not significantly affected by selection (43.6 ± 1.32 μm; $p > 0.05$). The width of the epidermis (24.98 ± 0.57 μm) and cuticle (3.31 ± 0.11 μm) and the number of stomata per lineal unit (3.47 per lineal 1000 μm of leaf blade) also remained similar across cycles ($p > 0.05$).

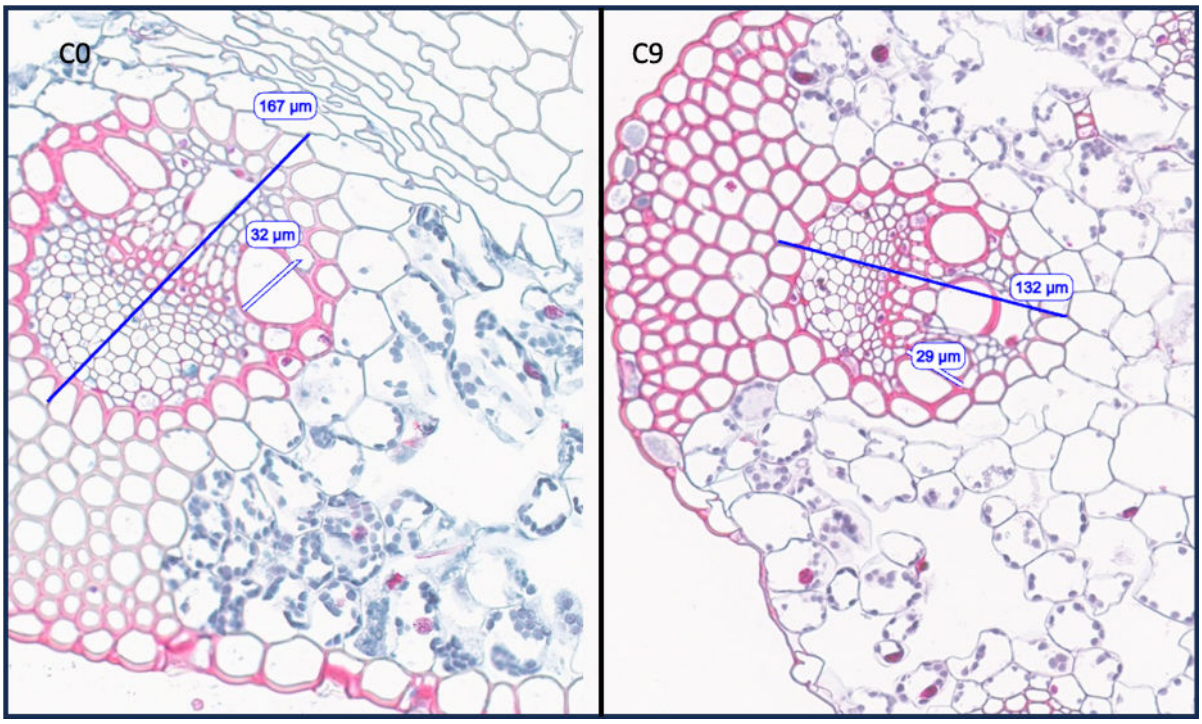

Figure 6. Leaf cross-section of *T. intermedium* showing main vascular bundle. On the left, C0. On the right, C9. The tissues conforming to the vascular bundle are marked. In both pictures, the length of the cross-section of the main bundle and the diameter of a metaxylem vessel are shown. Pictures were taken in Aperio eSlide Manager at 40× magnification.

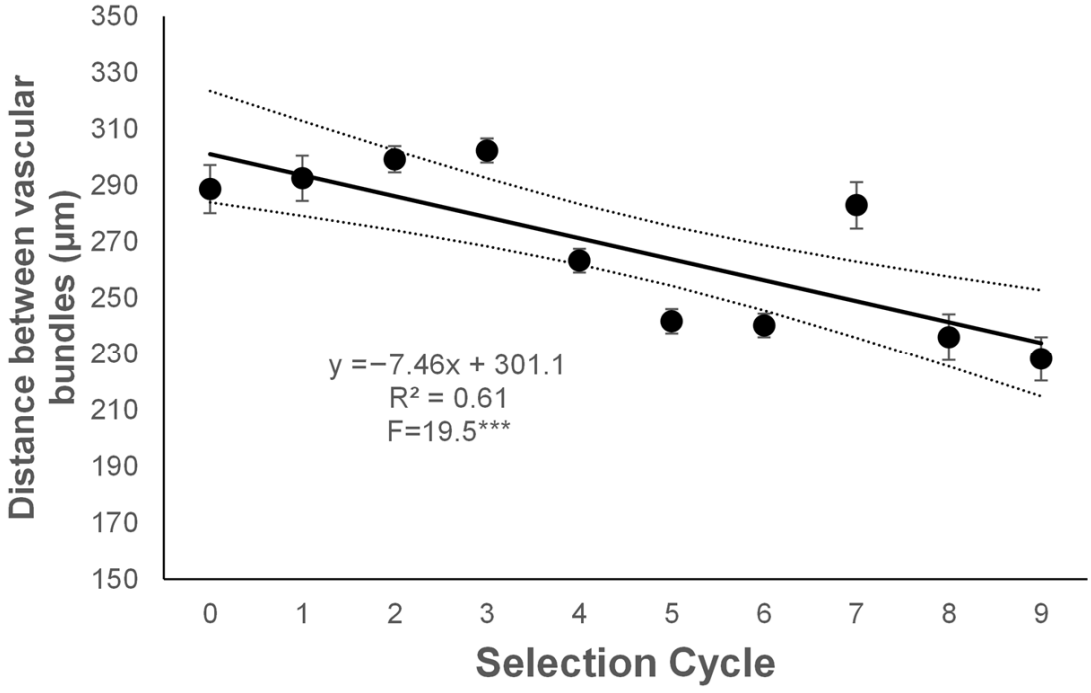

Figure 7. Distance between vascular bundles located in the leaf blade, from the central vein to the edge of the leaf of the leaf, for plants with 10 cycles (C0 to C9) of selection of *T. intermedium*. The distance between transversal veins was measured in µm using Aperio Manager for e-slides. Predicted lines, confidence intervals (95%) R^2, F-test, and significance (*** $p \leq 0.0001$) are from linear regression models. Anatomical traits were measured on three plants per selection cycle (three leaves per plant).

Gas Exchange. A/Ci curves were pulled into three groups (C7–C8–C9; C3–C4–C6; and C0–C2) to more clearly show differences between cycles (Figure 8). Above 100 ppm, external CO_2 concentration cycles C7–C8–C9 had a higher Net CO_2 uptake than both of the other two groups (C0–C2 and C3–C4–C6), indicating a higher biochemical capacity for carbon fixation. The difference (increase) in Net CO_2 with the selection cycle was more noticeable at an ambient CO_2 concentration (400 ppm) than at a saturating concentration (1500 ppm; Figure 8).

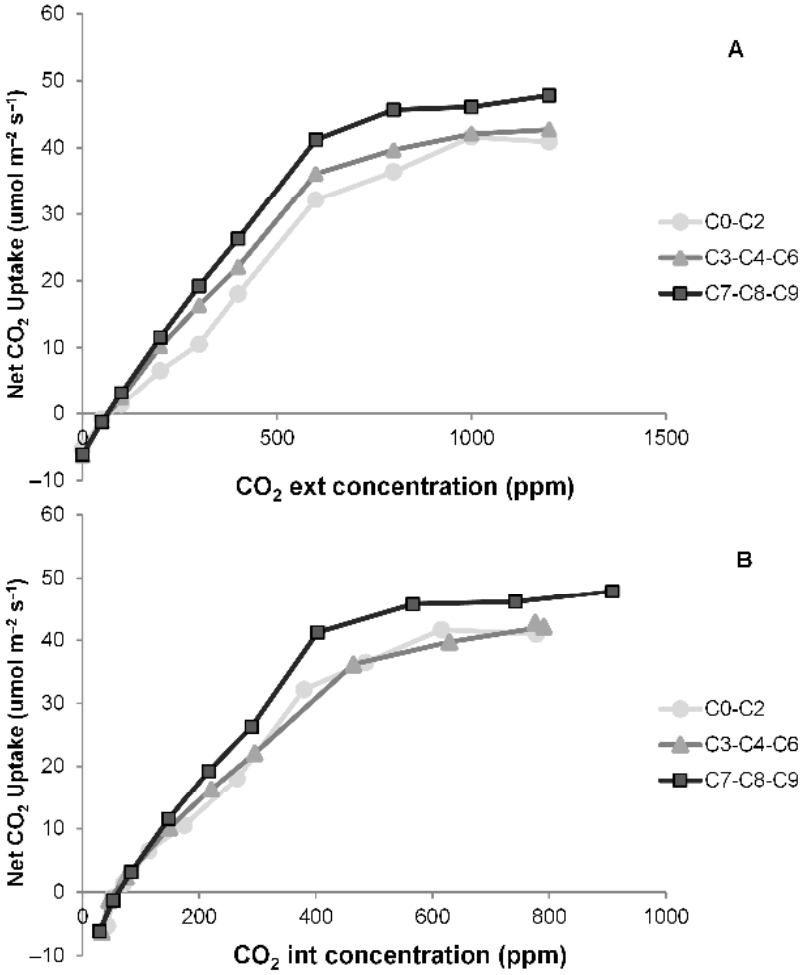

Figure 8. A/Ci curves for 8 cycles of *T. intermedium*. Data was grouped for C0–C2, C3–C4–C6, and C7–C8–C9. For each selection cycle, 3 plants were measured and Net CO_2 uptake data for each external concentration was averaged before averaging with the corresponding pooled cycles. Measurements were obtained using external CO_2 concentrations (**A**) = 400, 300, 200, 100, 50, 10, 400, 400, 600, 800, 1000, 1200, and 1500 ppm, and internal CO_2 concentration (**B**).

Root Morphology. Root traits were measured in five selection cycles: C0, C2, C4, C8, and C9. Selection affected the root morphology starting from the fourth selection cycle in a linear trend along the cycles from C4 to C9. Roots became less deep but more branched, resulting in a greater total length per tiller (Figure 9a–c,h). More improved accessions (C8 and C9) produced more acquisitive roots: thinner with a higher SLR but also a higher RDT compared to C0 and C2 (Figure 9d,e,g).

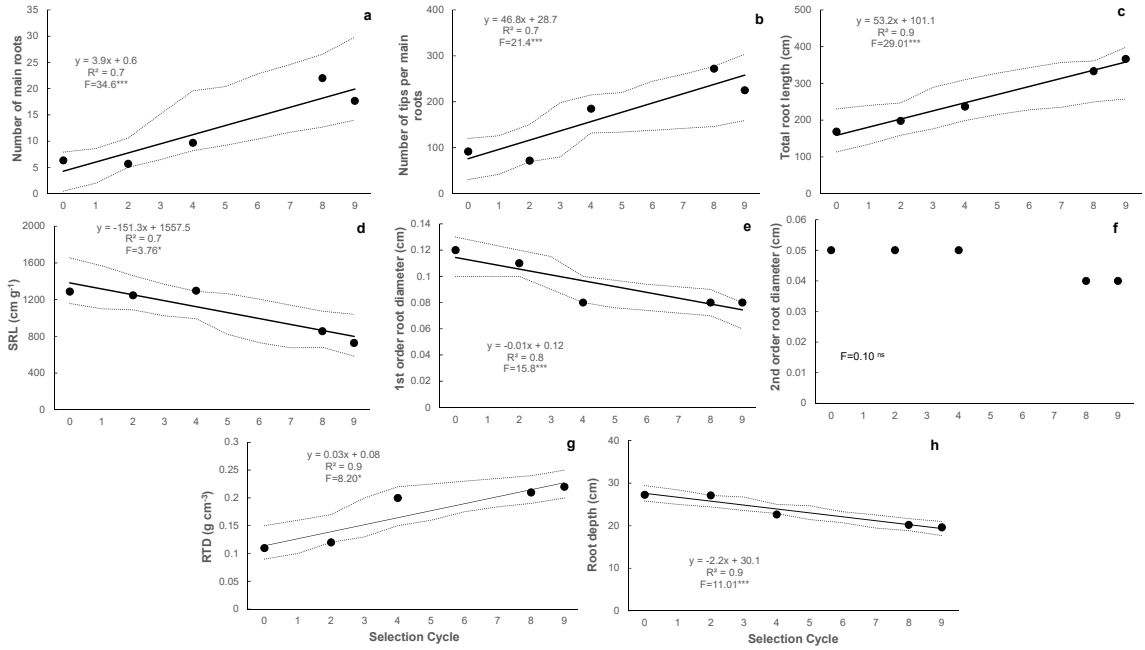

Figure 9. Root functional traits: number of main roots (**a**), number of tips per main root (**b**), total root length (**c**), specific root length-SRL (**d**), 1st order root diameter (**e**), 2nd order root diameter (**f**), root tissue density-RTD (**g**), and root depth (**h**) in five selection cycles (C0, C2, C4, C8, and C9) of *T. intermedium*. Different letters indicate a significant difference among cycles. Predicted lines (black lines), confidence intervals (95%, dashed lines) R^2, F-test, and significance (* $p \leq 0.05$; *** $p \leq 0.0001$) are from linear regression models. ns = non-significant. Root traits were measured on three plants per selection cycle (the roots of three tillers per plant).

Against expectations, these findings do not follow the typical trade-off between an acquisitive versus conservative strategy. SRL was positively related to RTD but was negatively related to root diameter. Also, the root diameter was negatively related to RTD. All together this means there was a high SRL and thinner roots were denser (Figure 10).

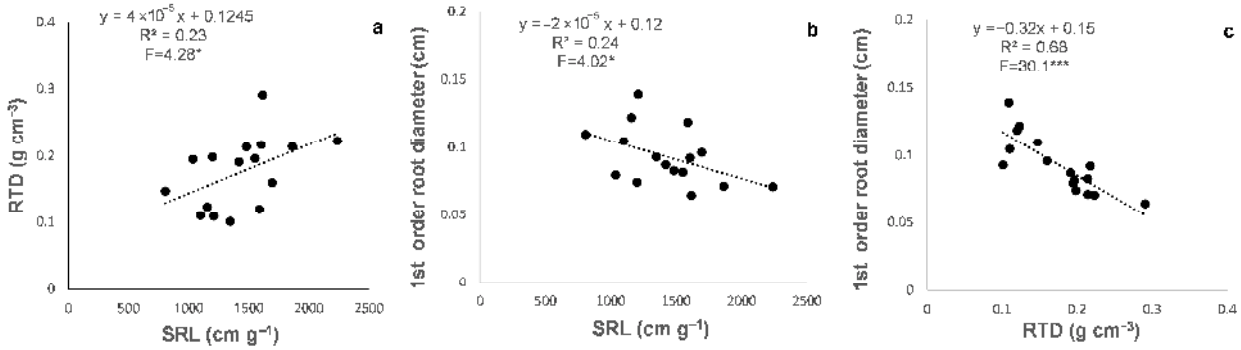

Figure 10. Relationship between specific root length (SRL) and (**a**) root tissue density (RTD) and (**b**) 1st order root diameter, and relationship between RTD and 1st order root diameter (**c**). Regressions were analyzed using five selection cycles (0, 2, 4, 8, and 9) of *T. intermedium* together. Regression equations, F-tests, and *p*-values (* $p \leq 0.05$; *** $p \leq 0.0001$) are presented in the panels. Dashed lines are the predicted regression lines.

4. Discussion

In our experiments to understand pathways of domestication in intermediate wheatgrass, selection following a grain yield increase had profound effects on the plant's structural and functional traits. Both phenotypic and genomic selection resulted in significant changes at the leaf and root levels, with consequences in the acquisition and use of resources

at the plant level. These linear changes in structural and functional traits across breeding cycles aligned with the previously reported rate of grain yield increase (58.4 Kg ha^{-1} per cycle) under both phenotypic and genomic selection using these lines [31]. Changes in many traits occurred both unconsciously at early stages of plant domestication [38] and later with conscious, directed selection and breeding focused on a few beneficial traits. Early changes occurred when plants were moved from wild habitats to cultivated land, where the availability of resources was more predictable [16]. In these cultivated environments, direct selection for yield favored acquisitive traits [19,21,23,39]. Similar changes have been described in other crops after domestication [38,40], although these modifications were mostly the consequences of many years of unconscious and conscious selection.

Increased seed size in IWG resulted in early vigor, and these two traits had a significant increase after genomic selection was introduced as the breeding method in cycle 7 (C7). Leaves became larger as they grew wider as selection proceeded. Increased seedling size and leaf size are common domestication traits found in all crops when compared to their wild relatives [41].

Contrary to predictions, leaf thickness increased with the selection cycle, although the specific leaf area (SLA) did not change (i.e., leaf density). Despite the idea that SLA is dependent on leaf size variation [42] and that larger leaves have a lower SLA [43], we did not find significant changes as leaves grew larger with selection. Specific leaf area is one of the main parameters used to predict the response of species to environmental factors, because it determines how much new leaf area to deploy for each unit of biomass produced. Still, leaves sharing similar SLA can show widely different photosynthetic rates [44]. On the other hand, leaf thickness can have important consequences for leaf function. For example, in *Silphium integrifolium*, a perennial new crop, an increased leaf thickness was associated with anatomical changes, while the SLA remained unchanged [23]. In this species, mesophyll cells became larger and the intercellular open space increased, which explained the higher Net CO_2 exchange rates found in semi-domesticated species compared to their wild predecessors. In our current experiment, we did not find strong anatomical differences linked to leaf thickness, either in mesophyll cell size or intercellular air space.

Leaf functions such as photosynthesis, transpiration, and respiration are closely related to leaf shape or three-dimensional architecture [45], which can be dynamically modified by leaf structures, such as bulliform cells. Under stress, bulliform cells generally assist in rolling leaves to avoid water loss during transpiration, but it has been shown in rice that changes in bulliform cell number, size, or localization can result in adaxial or abaxial leaf rolling, which contributes to the improvement of photosynthetic efficiency and grain yields by maintaining relatively erect plant architecture and minimizing shadowing between leaves [46,47]. Yuan [48] proposed that the long, rolled, and thick last three leaves were the primary characteristics of super-high-yield hybrid rice. The identification of genes controlling moderate leaf rolling through the controls of size and number of bulliform cells has been targeted for the improvement of grain yields in rice [47]. In our experiment, we documented an increase in the density of bulliform cells; as vascular bundles grew closer, so did the bulliform cells, increasing their density per lineal unit, while the size of these cells was not significantly affected by selection. Leaf vein density has been suggested to be the most important leaf trait determining leaf hydraulic conductance [49], because maintaining a high transpiration rate requires a vascular system that minimizes transport in the photosynthetic tissue by delivering water directly from the vein close to the stomata where gas exchange takes place [50]. Huang [49] suggests that the manipulation of vascular bundles is a promising approach to improve photosynthesis in rice plants.

As predicted, we found clear evidence of the links between structure and function, both at the leaf and root level, linked to the increase in grain yield from lines C0 to C9. The increased closeness of vascular bundles found in C8 and C9 is in line with their higher stomatal conductance and gas exchange found in both experiments under non-limiting water conditions. Later cycles had more stomatal conductance that allowed for more Net CO_2 uptake and also more transpiration. These findings show the integration of structural and functional changes along with yield when selection for yield is the chosen breeding trait. Similar changes have been found in other perennial crops during domestication [23].

While our experiments were not designed to test responses to water deficits, these findings suggest that higher selection cycles would be more susceptible to low water availability. The physiological model that links gas exchange to the distance between veins and stomata [50] has been verified across a wide phylogenetic sample that extends from mosses to angiosperms, and, therefore, we have considerable confidence in using relationships between the vein architecture and gas exchange as an a priori index of leaf function [51]. On the other hand, the denser bulliform cells found in C8 and C9 could enhance stress tolerance. These two seemingly contrasting changes in leaf anatomy and their linked mechanisms for stress tolerance need to be further evaluated.

As expected, the root system of *T. intermedium* became more acquisitive across the selection cycles, increasing the total root length and SRL and decreasing the diameter. This trend throughout the selection cycles is in accordance with the change in the resource-use strategy from conservative to acquisitive reported for above-ground traits in other annual and perennial crops [19–21,23]. The unexpected increase in RTD linked to the production of thinner roots in higher selection cycles could be explained by changes in the root anatomy, as reported in other studies. The negative correlation between the RTD and root diameter may be related to the root cortex being less dense than root stele and because, in thicker roots, a larger proportion of the root cross-sectional area is accounted for by the cortex [52,53]. On one hand, if the stele tissue occupies most of the diameter of thin roots, the mycorrhizal habitat would be highly reduced and roots would be more self-sufficient in resource acquisition [54,55]. However, McKenna [27] reported that selection for agronomic traits in IWG increases responsiveness to arbuscular mycorrhizal fungi and root colonization, contradicting our hypothesis about the selection of a root system less dependent of AMF and relying on their own roots for soil exploration. Our experiments were not designed to test responses to AMF because plants grew on sterile soil, and the relationship between root morphology and AMF colonization capacity should be further evaluated. On the other hand, thinner and denser roots with a high proportion of stele can be beneficial for drought tolerance, as it may enhance water and nutrient uptake and transport efficiency [56].

The longer roots found in higher selection cycles were the result of shallow but more branched roots; while unselected accessions had 30 cm root depths, after nine cycles of selection the root depth decreased 30% in C8 and C9 and the number of lateral roots or root tips increased. Other studies have reported that most of the below-ground biomass (79% of root biomass and 95% of rhizome biomass) of IWG occurs within the first 20 cm of the soil [57].

The development of perennial grain crops has been proposed as a way for agriculture to provide an increased production of food and materials in a more sustainable manner by requiring less land, reducing soil erosion, and increasing carbon capture [58]. Coupled with less-favorable climates, these immense pressures will require extraordinary efforts to develop sustainable and resilient cropping systems to meet future demands. Perennial crops could provide the ecosystem benefits of reduced soil erosion by limiting the annual disruption of the soil [59], reduced nitrate leaching [9,60], and increased carbon capture [10].

During the domestication process, strong forces modify structural and functional traits to capture and mobilize the resources needed by the plant to sustain the yield increase, which is needed to develop a successful crop that can compete with its annual counterparts. Some of these changes can go unnoticed as selection proceeds, although they should be considered by breeders and physiologists, as they may hinder resource-use efficiency as the yield keeps increasing. What we present here is a series of initial studies working with plants from seedling to the start of booting. To be able to take detailed measurements of some of the traits, the experiments needed to be performed on first-year plants and under controlled conditions. These experiments also provided strong data on which traits should be followed in longer term experiments. Also, detecting early changes in perennial crops, particularly during the seedling and young plant stages, is crucial for accelerating the domestication process and improving breeding efficiency. By identifying and selecting for desirable traits early in development, breeders can significantly reduce the time required to achieve improvements in mature plants or to avoid undesirable changes. Additionally, early screening allows for the identification of key structural and functional traits that will influence the plant's long-term performance (such as seedling vigor, gas exchange traits, and root traits), including its ability to grow, produce grain, and persist over multiple seasons. These early indicators can help predict how the mature plant will behave under field conditions.

Our findings show that selection for grain yield in intermediate wheatgrass (IWG) drives substantial and coordinated changes in plant structure and function, both above- and below-ground. Resource-use-related traits change with a linear trend across both the phenotypic and genotypic selection cycles, matching the pattern reported for grain yield. Seedlings grew larger, and leaves became larger, thicker, and more productive. The shift in root traits was coordinated toward a more acquisitive strategy: high SRL, thinner, and more branched roots as selection enhanced the seed yield. While these changes enhance resource capture, biomass production, and grain yield under favorable conditions, they may also reduce resilience under stress, particularly in water-limited environments. Anatomical traits such as the increased bulliform cell and vascular bundle density suggest potential adaptative benefits, although they may have long-term impacts on stress tolerance, and they require further investigation. As domestication progresses, it is critical to consider these functional shifts, not only for maximizing yield but also for ensuring the sustainability and resilience of perennial cropping systems.

Finally, while our study provides valuable insights into the effects of selection on structural and functional traits in IWG, it is important to acknowledge some limitations that may affect the broader applicability of our conclusions. First, the controlled environment in which the experiments were conducted may not fully capture the complexity of field conditions, where factors such as soil type, environmental stress, and biotic interactions could influence the traits examined. Second, the focus on young plants means that we did not observe the ontogenetic changes that might occur with aging and neither did we assess the relationships of these traits with productivity. These factors may affect the interpretation of the results, and future studies with plants grown in field conditions for several seasons are undergoing to validate and extend our findings to agricultural scenarios.

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Abbreviations

The following abbreviations are used in this manuscript:

IWG	Intermediate wheatgrass
SLA	Specific leaf area
A	Net CO_2 uptake
E	Transpiration rate
Ci	Internal CO_2 concentration
Gs	Stomatal conductance
SRL	Specific root length
RTD	Root tissue density

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