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# Drought-Tolerant Barley: II. Root Tip characteristics in Emerging Roots

**Andrea Y. Carter [1], Michael J. Ottman [1], Gilberto Curlango-Rivera [1], David A. Huskey [2], Brooke A. D'Agostini [3] and Martha C. Hawes [2,*]**

1 School of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA; aycarter@email.arizona.edu (A.Y.C.); mottman@ag.arizona.edu (M.J.O.); curlango@email.arizona.edu (G.C.-R.)
2 Soil, Water, and Environmental Sciences, University of Arizona, Tucson, AZ 85721, USA; dahuskey@email.arizona.edu
3 Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721, USA; bdagostini@email.arizona.edu
* Correspondence: mhawes@email.arizona.edu; Tel.: +520-621-5490

Received: 11 February 2019; Accepted: 28 April 2019; Published: 30 April 2019


**Abstract:** Reduced water resources are of increasingly urgent global concern. One potential strategy to address the crisis is the use of drought tolerant crops in agriculture. Barley varieties developed for reduced irrigation ("Solum" and "Solar") use significantly less water than conventional varieties ("Cochise" and "Kopious"). The underlying mechanism of this drought tolerance is unknown but root structure and function play a key role in plant water uptake. In this study, an empirical survey compared early root development between drought tolerant and conventional varieties. Traits associated with root meristem-regulated cell division including rate of seed germination, border cell number and root cap mucilage production, and root hair emergence were quantified during root emergence. Preliminary results revealed that drought tolerant varieties exhibited faster seed germination and root hair production than conventional varieties. Border cell number and mucilage production in the drought tolerant varieties also were higher than in the conventional variety "Kopious," but lower than in "Cochise". Each trait, if found to be linked to the observed drought tolerance, could yield a simple, rapid, and inexpensive tool to screen for new crop varieties. Further detailed studies are needed.

**Keywords:** drought tolerance; root traits; border cells; mucilage; barley

## 1. Introduction

Characterization of the physiological aspects of crops grown under drought conditions is a well-recognized methodology for the identification of important secondary traits when breeding for water-limited environments [1–3]. Barley is characterized by fast-pre-anthesis growth and high tiller output, traits that are used to explain why barley is well adapted to dry areas [4]. Described as fast development of leaf area and/or shoot biomass, early seedling vigor is attributed to advantageous growth of barley over wheat in Mediterranean-type environments [5,6]. Vigorous early growth and canopy shading serve to reduce evaporation from the soil surface thereby increasing available water for crop transpiration [3].

Vigorous root systems are another effective means by which cereals can maximize soil moisture use, particularly if able to access subsoil moisture during reproductive growth when additional water is used for grain development and filling [7]. Root vigor describes early and fast root extension and proliferation, greater root biomass, and greater root length and density [8]. A study of wheat roots demonstrated an additional 10 mm of water accessed by roots from the subsoil late in the season (post-anthesis) increased grain yield by over 0.50 tons per hectare [9].

This study focused on four varieties of commercially available six-row spring barley varieties with varied adaptation to drought stress [10,11]. "Kopious" and "Cochise" are characterized as semi-dwarf, high-input, high-yielding conventional varieties bred for high irrigation conditions, with 5 to 7 flood irrigations of 150 mm per irrigation each season. Both conventional varieties were developed from the same germplasm Composite Cross XXXII [12]. "Solar" and its predecessor "Solum" are characterized as low-input varieties that were bred for adaptation to water limited environments, receiving a single irrigation of 150 to 200 mm of water applied immediately after planting. Both drought tolerant varieties were derived from the Composite Cross XXXIX germplasm [13,14].

Two years of replicated field trials have found the drought resistant barley varieties "Solum" and "Solar" to exhibit distinct rooting and water use patterns compared to the conventional varieties "Cochise" and "Kopious" [15]. Observed traits associated with improved performance of the drought tolerant varieties included greater average root growth and greater water extraction post-anthesis [15]. The goal of this study was to determine if variation in root traits can be detected during early development of these varieties whose divergence in drought tolerance has been established in field trials.

## 2. Materials and Methods

### *2.1. Plant Material*

Seeds of barley (*Hordeum vulgare*) "Solar", "Solum," "Kopious," and "Cochise" were surface sterilized with 95% ethanol for five minutes, followed by a treatment with 0.8% sodium hypochlorite solution for five minutes. Seeds were rinsed five times with sterile deionized water ($sdH_2O$), 17.3 MΩ-cm e-Pure (Barnstead/Thermolyne. Dubuque, IA, USA). After one hour imbibition in $sdH_2O$, seeds were placed onto 1% agar (Sigma-Aldrich. St. Louis, MO, USA) overlaid with sterile germination paper (Anchor Paper. St. Paul, MN, USA) and incubated for 24 or 48 h at 25 °C.

### *2.2. Root Border Cell Number*

Germinating seeds of all barley varieties under study were collected 24 h after incubation and then transferred into CYG seed germination pouches (Mega International. St. Paul, MN, USA) and kept at bench laboratory conditions at room temperature (~25 °C). Radicles reaching a length of at least 25 mm were selected and single root tips were immersed in 100 µL of $sdH_2O$ for inspection under low magnification with a Zeiss SV8 stereo microscope (Carl Zeiss. Oberkochen, Germany) to ensure proper root tip and border cell formation. Root border cells, which dissociate from the root tip upon contact with water, were separated and collected from the root tip by micropipetting [16–24]. Border cell numbers were counted from 10 µL aliquot samples of the border cell-$sdH_2O$ suspension using an Olympus BX60F5 compound microscope. One replicate consisted of one border cell sample collected from one radicle from one seedling for each barley variety grown in a seed germination pouch. Each experiment included at least three replicate samples for each barley variety and each experiment was independently repeated three times. Shapiro-Wilk normality tests were performed for each barley variety under study to determine if assumptions of normal distribution were met for analysis of variance (ANOVA). Pairwise comparison analyses of border cell numbers between barley varieties were performed using Welch Two Sample *t*-tests.

### *2.3. Root Tip Mucilage Dimensions*

Healthy barley seedlings were collected 48 h after incubation and single root tips were then immersed in 100 µL $sdH_2O$ for inspection under low magnification. Mucilage dimensions were analyzed by India ink negative stain. One µL of India ink solution, which cannot penetrate the boundaries of the mucilage layer, was added to a single root tip immersed in 100 µL of $sdH_2O$ and visualized using a Zeiss SV8 stereo microscope, as described in previous studies [16–20,24]. Images of the root tip mucilage were captured using a Leica DFC290 HD digital camera (Leica Camera. Wetzlar,

Germany) with Leica LAS software V 4.0.0 (Leica Microsystems. Heerbrugg, Switzerland). Area of the mucilage surrounding the root tip was measured from image files using ImageJ 1.x image processing program [16]. One replicate consisted of one root tip sample from one radicle from one seedling for each barley variety germinated for 48 h. Each experiment included at least four replicate samples for each barley variety and each experiment was independently repeated three times. Pairwise comparison analyses of measured mucilage areas between barley varieties were performed using Welch Two Sample *t*-tests.

### *2.4. Measurement of Root Hairs in Presence of Water*

Germinating seeds of all barley varieties under study were collected 24 h after incubation and then transferred into sterile Falcon MULTIWELL 6 well tissue culture plates (Becton Dickinson. Franklin Lakes, NJ, USA). Under aseptic conditions, germinating seeds were placed individually in each well containing one mL of $sdH_2O$ and kept at bench laboratory conditions for two days at room temperature (~25 °C). Root hair development of each radicle for each seedling was analyzed using a Zeiss SV8 stereo microscope, and radicles with presence of normal or extra production of root hairs were quantified. Each replicate consisted of one radicle from one seedling for each barley variety grown in 6 well tissue culture plates. Each experiment included at least seven replicate samples for each plant variety and each experiment was independently repeated at least three times. Fisher's exact tests were performed to analyze contingency tables with numbers of radicles with normal or extra production of root hairs, and for pairwise comparison analyses between barley varieties.

### *2.5. Root Tip and Root Elongation Zone Dimensions*

Healthy barley seedlings with a radicle length of at least 25 mm were collected 48 h after incubation and radicles were then individually placed onto microscope slides and covered with $sdH_2O$ to be visualized using an Olympus BX60F5 compound microscope (Olympus Optical. Tokyo, Japan). After inspection for proper root tip and border cell formation, the emergence of the youngest root hair was visualized in order to identify the elongation zone. The longitudinal distance between the youngest emergent root hair and the root tip was measured with an eyepiece reticle under 100x magnification. Each replicate consisted of one root tip sample collected from one radicle from one seedling for each barley variety incubated for 48 h. Each experiment included at least three replicates samples for each barley variety and each experiment was independently repeated three times. Shapiro-Wilk normality tests were performed for each barley variety under study. After ANOVA, pairwise comparison analyses between barley varieties were performed using Welch Two Sample *t*-tests.

### *2.6. Rate of Germination and Root Growth*

After sterilization and transfer of seeds to agar plates, the germination rate (number of seeds with at least one emerging root >1 mm in length) was recorded at 24, 48, 72 and 96 h of incubation. Seeds with an emerging root tip were transferred into seed germination pouches, and root length of all emerging roots was measured daily to assess rate of root growth.

### *2.7. Statistical Analysis*

Statistical analyses of assays were performed using R version 3.4.3 [17].

## 3. Results

### *3.1. Increased Mucilage on "Solar" Root Tips*

A divergence in the size of the mucilage layer on "Solar" and "Kopious" was evident without magnification, so microscopic analyses were carried out on replicate samples. Significant variation in the size of the mucilage layer was confirmed by direct measurement (Table 1). When immersed into

India ink solution, microscopic visualization revealed an obvious difference in mucilage size between the two species (Figure 1).

**Table 1.** Increased root tip mucilage area in drought tolerant [z] barley varieties.

| Plant Variety | Root Tip Mucilage Area ($mm^2$) |
|---|---|
| "Kopious" | 0.74 ± 0.28 [a] |
| "Cochise" | 0.80 ± 0.31 [a] |
| "Solar" [z] | 1.14 ± 0.46 [b] |
| "Solum" [z] | 0.92 ± 0.26 [a] |

Values represent the mean ± standard deviation of at least four replicates for each plant variety in at least three independent experiments ($n \geq 12$). Mucilage area with the same letter are not significantly different according to Welch Two Sample *t*-test ($p < 0.05$).

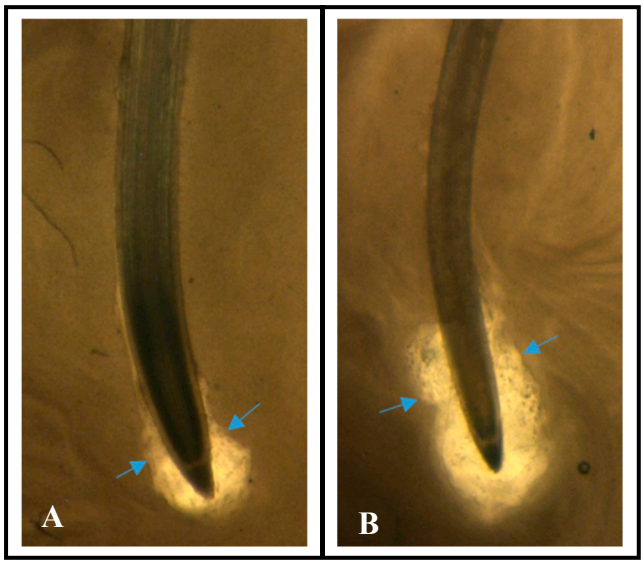


**Figure 1.** Increased mucilage production in "Solar". The size of the mucilage layer (clear area denoted by blue arrows) from root tips of (**A**) conventional barley "Kopious" and (**B**) drought tolerant "Solar" is visualized using India ink, which cannot penetrate the mucilage surrounding border cells.

### *3.2. Increased Border Cell Production on "Solar" Root Tips*

The root cap mucilage layer is exported from root border cells [18–24]. Experiments therefore were carried out to determine if the observed difference in mucilage layer stems from altered border cell production. The results revealed that border cell number from the drought tolerant varieties under study are significantly higher than "Kopious," but significantly lower than "Cochise" (Table 2).

**Table 2.** Number of border cells produced by conventional [y] and drought tolerant [z] barley varieties.

| Plant Variety | Root Border Cell Number |
|---|---|
| "Kopious" [y] | 289 ± 28 [a] |
| "Cochise" [y] | 634 ± 183 [b] |
| "Solar" [z] | 472 ± 52 [c] |
| "Solum" [z] | 460 ± 95 [c] |

Values represent the mean ± standard deviation from at least three replicate samples in at least three independent experiments ($n \geq 9$). Cell numbers with the same letter are not significantly different according to Welch Two Sample *t*-test ($p < 0.01$).

### 3.3. Altered Root Hair Development

In the process of evaluating aspects of root tip mucilage and border cells, a previously unseen phenotype was evident in barley roots as they developed while immersed in water (Figure 2). In the first set of experiments, root hair emergence from "Solum" and conventional varieties under study was consistent with that seen in previous studies with roots from many plant species (Figure 2A). The region of elongation between the root tip and emerging root hairs was evident (arrows). In contrast, prolific root hairs developed on "Solar" roots within 2–3 mm behind the root tip (arrow), and the root hairs were significantly longer and more abundant (Figure 2B).

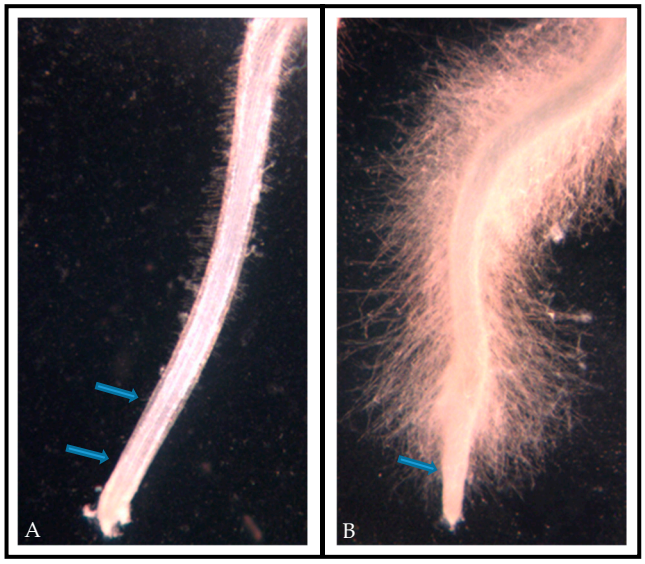


**Figure 2.** Divergent hairy root development in conventional variety "Kopious" (**A**) and drought-tolerant variety "Solar" (**B**) when the roots emerged from seedlings immersed in water.

This 'hairy' phenotype was found to occur in "Solar" and conventional varieties under study in additional experiments, but the percentage of roots with the increased root hairs was significantly smaller than the percentage of "Solar" roots exhibiting this phenotype (Table 3).

**Table 3.** Measurement of root hairs in conventional [y] and drought tolerant [z] barley varieties.

| Plant Variety | Number of Roots with Normal Root Hairs | Number of Roots with Extra Root Hairs | *n* | % Hairy Phenotype |
|---|---|---|---|---|
| "Kopious" [y] | 28 | 5 | 33 | 15 [a] |
| "Cochise" [y] | 44 | 8 | 52 | 15 [a] |
| "Solar" [z] | 18 | 19 | 37 | 51 [b] |
| "Solum" [z] | 48 | 6 | 54 | 11 [a] |

Values reflect results from at least seven replicates for each plant variety in at least three independent experiments ($n \geq 21$). Percentages of roots with hairy phenotype with the same letter are not significantly different according to Fisher's Exact test ($p < 0.01$).

The altered root hair emergence was confirmed by direct measurement of the site of the first emerging root hairs at the start of the region of elongation (Table 4).

**Table 4.** Decreased root elongation zone in drought tolerant [z] barley varieties.

| Plant Variety | Root Tip + Elongation Zone (um) |
|---|---|
| "Kopious" | 2155.6 ± 199.3 [a] |
| "Cochise" | 2067.8 ± 317.8 [a] |
| "Solar" [z] | 1765.0 ± 228.4 [b] |
| "Solum" [z] | 1786.7 ± 314.9 [b] |

Values reflect the mean ± standard deviation from at least three replicates for each plant variety in at least three independent experiments ($n \geq 9$). Root tip and elongation zone distances with the same letter are not significantly different according to Welch Two Sample *t*-test ($p < 0.01$).

### *3.4. Altered Rate of Seed Germination in Drought Tolerant and Conventional Barley*

Throughout experimentation, "Solar" and "Solum" seeds repeatedly demonstrated an increased rate of germination compared to "Kopious" and "Cochise." Many environmental factors (weather, soil, seed contamination, harvest, etc.) can influence germination, root development and seedling vigor in the field. In order to estimate the genotype contribution to observed germination rate differences, seed lots harvested from "Solar," "Solum," "Kopious" and "Cochise" were compared. To control for environmental factors, all seeds were grown during 2018, in the same field, and same time of year. The results revealed a 24-h delay in initial root emergence in "Cochise" and "Kopius" vs "Solar" and "Solum" (Table 5). The overall germination rate over time was similar for all four varieties, with 95–100% after three days for "Solar" and "Solum," and 90–100% for "Cochise" and "Kopious" after four days.

**Table 5.** More rapid seed germination in drought tolerant "Solar" than conventional Kopious."

| Plant Variety | 24 h | 48 h | 72 h | 96 h | Total |
|---|---|---|---|---|---|
| "Solar" | 26/60 (43%) | 24/60 (40%) | 10/60 (17%) | | 60/60 (100%) |
| "Solum" | 27/60 (45%) | 19/60 (32%) | 13/60 (22%) | | 59/60 (98%) |
| "Cochise" | 0/60 | 16/60 (27%) | 22/60 (37%) | 19/60 (32%) | 57/60 (95%) |
| "Kopious" | 0/60 | 19/60 (32%) | 19/60 (32%) | 19/60 (32%) | 57/60 (95%) |

### *3.5. Rate of Root Growth*

To assess potential variation in rate of root growth, healthy, germinated seeds of all four varieties were transferred to seed germination pouches after 24 h of incubation. Root length was measured every 24 h over the course of seven days (data not shown). Rate of root growth at 25 ± 2 mm per day was the same for all four varieties, and consistent with values obtained with other species under the same conditions (Figure 3).

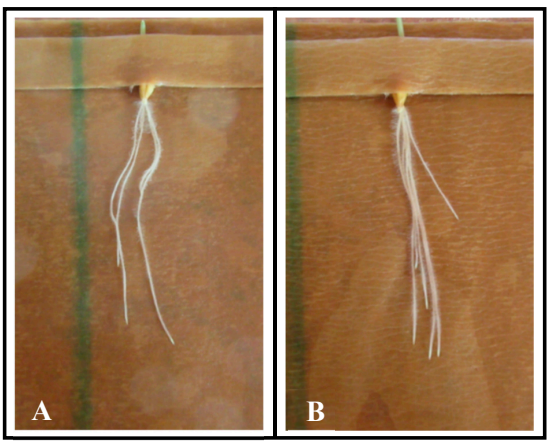


**Figure 3.** Similar root morphology and growth of "Kopious" (**A**) and "Solar" (**B**) barley roots 4 days after transfer from petri plates to growth pouches.

## 4. Discussion

The increasingly urgent need for strategies to cope with drought is well recognized [25]. Although the impact of developing drought tolerant crops is a key strategy, the underlying mechanisms that can guide breeding efforts remain elusive [26,27]. Root architecture including root hair development plays a critical role in overall water uptake; thus, possible correlation between variation in root hair emergence between drought tolerant ("Solar" and "Solum") and conventional ("Kopious" and "Cochise") varieties necessitates of future assessment [28–31]. The correlation of the higher grain yield of "Solar" under low-irrigation conditions and the hairy root phenotype observed in this study suggests root hairs may play a role in drought tolerance. In addition, although the hairy root phenotype was rarely observed in "Solum," both drought tolerant genotypes did exhibit shorter elongation zones than the conventional varieties, suggesting genotypic similarities in root hair morphology.

Further exploration between water uptake and border cell number will also be of interest. Border cells are specialized to protect vulnerable root tips from pathogenic infection and damage from toxic metals, and to stimulate associations with beneficial organisms [18,19]. They are produced by a dedicated meristem programmed to deliver a specific number of viable cells with gene expression and protein secretion patterns that are distinct from progenitor cells in the root cap [32–34]. Border cells produce signals needed to facilitate associations with microbes such as mycorrhizal fungi which confer increased plant water uptake [35]. Even a difference of a few border cells has been shown to create a measurable difference in the rate of mycorrhizal associations [36]. Species in the Brassicaceae family which do not produce border cells are not susceptible to infection by these beneficial fungi [37].

Stability of genotype in regard to border cell numbers was found for the drought tolerant varieties but not for the conventional varieties, for instance, "Kopious" showed the lowest border cell production and "Cochise" the highest (as well as the highest standard deviation in border cell number of all varieties). The observed differences in border cell production between the varieties surveyed in this study highlight the need for further investigation into the causes and impacts of border cell variation.

The mucilage secreted by border cells has been shown to underlie production of the 'rhizosheath' which surrounds the root and facilitates root growth and penetration [38–41]. As such, the correlation between increased border cell number and mucilage production with improved soil penetration by "Solar" roots is an interesting parallel. For instance, it was found a 12-fold increase in the number of root border cells of maize as a result of soil compaction [42]. Lubrication provided by border cells may explain how "Solar" roots were able to penetrate through a Caliche (soil calcium carbonate deposits) layer at 70 cm depth [15].

Faster germination may be part of an early vigor and/or drought escape mechanism employed by "Solar" and "Solum," which reach physiological maturity six to nine days earlier than "Cochise" and "Kopious" under low irrigation conditions [15]. Speed of germination as an indicator of yield has been previously reported in crops under both optimal and stress conditions and may be a valuable screening trait, particularly for short-duration varieties such as the spring-barley varieties included in this study [43–46].

Variation in germination rate also suggests potential hormonal differences between barley varieties. Gibberellins, in particular, warrant further study due to gibberellin-regulated reduced biosynthesis in semi-dwarf crops, promotion of seed germination, and potential modulation of stress response [47–49]. Variation in root hair phenotypes may also be attributed to hormonal differences between the varieties under study. Expression of root hair-promoting hormones, such as auxin and ethylene, should be further explored as well as their interaction with root exudates such as strigolactones [50,51].

## 5. Conclusions

Future work to understand the evolution of the evaluated phenotypes in the development of the drought tolerant varieties would be worthwhile. Review of these traits in the breeding population and progenitor lines would establish if any traits tested in this study were indirectly selected for in the course of breeding barley varieties under drought conditions. The results of this study highlight the

dynamic nature of root tip properties, such that all or one of the observed traits could have a significant impact on drought tolerance. A causative relation between border cell number, mucilage production, or root hairs, and soil penetration or water uptake may provide a rapid and cost-effective tool to screen for drought tolerance in crop plants.

**Author Contributions:** All authors contributed to conceptualization, methodology, validation, writing, and investigation.

**Funding:** This research was funded by AZ Grain Research & Promotion Council, #4240140.

**Conflicts of Interest:** The authors declare no conflict of interest.

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