DISSERTATION

PHENOTYPIC PLASTICITY IN THE RESPONSE OF SORGHUM TO WATER STRESS AND RECOVERY INDICATES PRE-FLOWERING DROUGHT TOLERANCE

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2018

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ABSTRACT

PHENOTYPIC PLASTICITY IN THE RESPONSE OF SORGHUM TO WATER STRESS AND RECOVERY INDICATES PRE-FLOWERING DROUGHT TOLERANCE

Drought stress is a major limiting factor to agricultural production worldwide that is predicted to become more frequent and intense under climate change. With a limited water supply, it is crucial that we identify phenotypic traits in plants that resist drought stress and can be utilized in breeding to improve crop drought tolerance. Sorghum [*Sorghum bicolor* (L.) Moench] is a crop species noted for its adaptation to drought stress, however significant variation for drought tolerance exists within the species. Therefore, this study was conducted to evaluate phenotypic traits in sorghum that conferred pre-flowering drought tolerance. The objectives were to 1) determine similarities and differences in morphological and physiological traits among sorghum lines varying in drought tolerance in response to water stress, 2) evaluate a method to assess root exudation of sorghum lines grown in substrates that differed in physicochemical properties and 3) implement the aforementioned method to assess quantitative and qualitative similarities and differences in root exudation among a subset of sorghum lines in response to water stress.

In chapter one, I aimed to determine morphophysiological traits that indicated pre-flowering drought tolerance and were universal among tolerant lines, therefore potentially serving as early drought tolerance screens for variety selection. Nine sorghum lines varying in pre-flowering drought tolerance were evaluated for their adjustments in water economy-related morphological and physiological characteristics under water stress. I focused on analyzing changes in leaf area, total root length and carbon assimilation rate among lines that varied in pre-flowering drought tolerance and at different time points reflecting water stress and subsequent recovery. This chapter specifically sought to determine if timing and magnitude of changes (plasticity) in these traits could predict pre-flowering

drought tolerance. Phenotypic plasticity refers to the range in phenotypic values expressed in response to a changing environment (well-watered versus water-stressed conditions) and is under genetic control and therefore may be useful in variety selection. In this study, we found pre-flowering drought tolerant lines maintained morphological stability while moderately changing carbon assimilation during water stress. Upon the readdition of water, pre-flowering drought tolerant lines displayed greater abilities to recover development to that of their well-watered counterparts. Therefore, phenotypic plasticity might serve as a valuable tool for plant breeders to identify pre-flowering drought tolerance in sorghum early in the season, saving time and money in plant breeding programs.

In chapter two, my goal was to establish a method to evaluate sorghum root exudation in substrates that differed in physicochemical properties and could be utilized in the future to determine sorghum root exudation under water stress. Root exudates are molecules released by roots into the adjacent soil that respond to the physical, chemical and biological environment to aid in overall plant health. Current methods determine root exudation utilizing artificial systems (*i.e.* hydroponics, sterile media), but there is a major gap in our knowledge translating to crops grown in more realistic conditions. To this end, I utilized a non-targeted metabolomics approach using both gas chromatography- and liquid chromatography-mass spectrometry to survey the rhizosphere-associated exudate composition of two sorghum lines grown in three substrates that represent different soil types. Here, I found that exudation varied largely by substrate. Furthermore, two types of changes were characterized within each substrate between each plant treatment (genotype) and respective no-plant control: rhizosphere-enhanced metabolites (REMs) and rhizosphere-abated metabolites (RAMs). Many more REMs were detected in the sand and clay substrates than the soil substrate. However, this was likely due to several factors including plant-to-plant variability and edaphic factors that may impact the detection and extraction of metabolites. Additionally, two sorghum genotypes exuded metabolites at different magnitudes, yet many exudates of interest could not be identified, reflecting that metabolite

annotation remains a major bottleneck in non-targeted metabolite profiling. Nevertheless, this method can be utilized in future studies to determine genotypic variation in root exudation in response to environmental changes. As root exudation is likely under genetic control, these adjustments in exudation may serve a role in conventional or molecular plant breeding.

In chapter three, I utilized the method that I established in chapter two to evaluate sorghum root exudation under water stress and upon the readdition of water. Drought stress elicits a combination of abiotic stressors including nutrient deficiency and soil mechanical impedance on root growth. Root exudation serves to buffer these environmental changes such as through the release of chelators to acquire nutrients. Thus, it is likely that root exudation plays a role in mitigating the multi-dimensional effects of drought stress. To assess this hypothesis, I characterized root exudate adjustments of sorghum lines varying in pre-flowering drought tolerance under water stress. In this study, I utilized non-targeted metabolite detection and gas chromatography-mass spectrometry to characterize root exudate adjustments between drought tolerant and susceptible lines. Additionally, I extracted exudates at two time points: four days after a dry down commenced at 21 days after sowing and 24 h after the readdition of water. Here, I found that drought tolerant lines displayed more exudate adjustments in response to water stress that likely serve in defense against the multidimensional effects of water deficit. Additionally, tolerant lines also displayed fewer exudate adjustments in response to rewatering, which may indicate the return to normal development. However, the composition of exudate adjustments varied between the tolerant lines, indicating that mechanisms underlying drought tolerance likely differ. For instance, in one of the tolerant lines, there was a much larger adjustment in the content of citric acid, an exudate that aids in nutrient acquisition. The results of this study indicate that exudate adjustments may be specific for tolerant lines and thus the functional roles of these exudates should be explored in future research for potential use in plant breeding.

Overall, the results of this dissertation indicate that an effective combination of morphological, physiological and biochemical mechanisms are likely required to appropriately acquire and distribute limited resources under water stress that confer drought tolerance. In these studies, I determined phenotypes that indicate pre-flowering drought tolerance in sorghum. My goal was to characterize phenotypes that can be utilized in both conventional and molecular plant breeding programs to promote crops that use their water wisely in the face of our declining water supply. Future studies should continue to determine the functional roles of these drought tolerance mechanisms. In particular, future research should evaluate root exudation and its interaction with the environment to promote sustainable agricultural practices that protect our limited resources and support an increasing global population.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 2016-67-11-25103. Additional funding provided by Colorado State University Agricultural Experiment Station, the Energy Institute at Colorado State University and the Sustainable Bioenergy Development Center at Colorado State University. Funding bodies did not participate in the design, collection, analysis and interpretation of data or in preparation of the dissertation. I would also like to sincerely thank the village it took to complete this dissertation, comprised of professional acquaintances and dear friends and family.

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CHAPTER 1: TIMING AND MAGNITUDE OF PLASTICITY IN RESPONSE TO WATER LIMITATION AND RECOVERY INDICATE PRE-FLOWERING DROUGHT TOLERANCE IN SORGHUM

SUMMARY

Drought stress is a major limiting factor of agricultural production and will likely become more frequent and severe under predicted future climate conditions. Under such conditions, the discovery of early-season phenotypes to identify plants that are drought tolerant and maintain yield across environments is critical for shortening the breeding cycle. Here, our goals were to determine individual traits and combinations thereof associated with pre-flowering drought tolerance. In addition, we evaluated the timing and magnitude of plasticity in these traits. Nine sorghum lines differing in drought tolerance were subjected to pre-flowering water stress. Multiple morphological and physiological traits were evaluated at four time points during water stress and recovery. We evaluated whether certain traits, or plasticity in those traits, were linked to drought tolerance. We found that individual trait values were not predictive of pre-flowering drought tolerance in sorghum but that the degree of phenotypic plasticity in certain traits was linked to drought tolerance for this species. Most notably, tolerant lines exhibited low morphological plasticity (homeostasis) and moderate physiological plasticity in response to water stress as well as high plasticity in leaf area after rewatering. In addition, the ability of tolerant lines to recover leaf area after rewatering was associated with recovery of normal development. The plastic responses observed in this study, particularly the index of recovery for leaf area, can be important selection criteria in breeding for drought tolerance in sorghum. Overall, we found that specific combinations of the timing and degree of morphological and physiological responses are valuable indicators of pre-flowering drought tolerance.

INTRODUCTION

Drought is a major limiting factor in global agricultural production and is predicted to increase in frequency and severity as a result of climate change (Dai 2011). As water supply becomes more limited,

a premium will be placed on varieties that conserve moisture and maintain yield under reduced water availability (Cattivelli et al. 2008). To develop such varieties across crops, it is crucial to first identify the types of strategies drought tolerant plants employ. The study of drought tolerance is challenging, as it is multidimensional and the product of many physiological, morphological, biochemical and molecular plant responses (Mitra 2001). Strategies to tolerate drought stress can also vary across species and genotypes, and include both constitutive and induced traits that together provide different types of drought tolerance such as escape, avoidance, or phenotypic flexibility (plasticity) (Farooq et al. 2009). In addition, these various strategies are differentially affected by factors such as duration or intensity of drought stress, plant adaption, and plant developmental stage (Blum 2011, Basu et al. 2016).

Drought stress can occur at several critical developmental stages of plant growth that ultimately limit yield, including germination, seedling establishment, vegetative, and reproductive stages (Blum 1996, Tuinstra et al. 1997). In particular, germination and seedling establishment are critical stages that are highly sensitive to drought stress (Blum 1996). Stress at these early stages often leads to a failure of plant establishment and therefore directly limits yield (Baalbaki et al. 1999). The impact of drought on seedling establishment is also of concern because early, rapid development of above ground biomass, known as early vigor, contributes to later success as it allows for greater light interception, increased productivity, and reduced evaporation from the soil (Richards 2000, Richards et al. 2002). Therefore, investigation of phenotypic responses to water stress that maintain seedling development is essential to better breeding for drought tolerance.

Many morphological and physiological traits reduce the effects of drought stress (as reviewed by Farooq et al. 2009, Simova-Stoilova, Vassileva and Feller 2016, Comas et al. 2013, Amelework et al. 2015). These traits increase survival, conserve resources, or aid in rapid recovery after drought (Vassileva et al. 2011). Often, one of the first plant responses to water deficit is closure of stomata, which manages water loss and risk of xylem embolism, but also reduces intake of CO_2 (Jones and

Sutherland 1991, Alscher and Cumming 1990, Chaves, Maroco and Pereira 2003) and thereby reduces assimilation rate (Cornic 2000). It is important to note, however, that morphological and physiological responses are not mutually exclusive, and reductions in assimilation may occur not only because of decreased stomatal conductance, but also because of reduced leaf area and impaired photosynthetic machinery (Chaves et al. 2003, Basu et al. 2016, Bradshaw 1965). Other notable responses to drought include reduced plant growth and allocation of resources to the root system for continued extraction of soil moisture (Farooq et al. 2009). Taken together, under stress, these responses increase the root: shoot ratio and improve water use efficiency at the whole plant level (Ludlow and Muchow 1990). In addition, the way in which plants allocate resources upon the reintroduction of water is an important mechanism driving tolerance. Therefore, it is an effective combination of responses under both water stress and recovery that lead to increased plant survival (Metlen, Aschehoug and Callaway 2009, Vassileva et al. 2011, Blum 2011). Because of this, morphological and physiological phenotyping should occur not only at various points during water stress, but also during recovery (Simova-Stoilova et al. 2016) and new approaches that go beyond comparing commonly measured traits are required.

One such approach may be to examine phenotypic plasticity of relevant morphological and physiological traits under water-stressed and well-watered conditions. Plants are sessile organisms and their survival and fitness depend on the ability to adjust to their environment. Phenotypic plasticity refers to the range of expressed trait values of a genotype in response to different environments (e.g., with adequate versus inadequate rainfall) (Valladares, Sanchez-Gomez and Zavala 2006). Plasticity is under genetic control and is observed in both physiological and morphological traits (Schlichting and Pigliucci 1993, Bradshaw 2006, Bradshaw 1965). The timing of measurements to determine plasticity is critical, as physiological and morphological responses differ temporally and the benefit of plastic responses is influenced by the timing of response (Bradshaw 1965, DeWitt, Sih and Wilson 1998, Sultan 2000). Although the ability to respond to and buffer environmental conditions can be advantageous,

extreme shifts in phenotype in response to an environment may not always be favorable (Nicotra, 2010). For instance, plastic changes may result in a loss of fitness due to the resources required for sensing and responding to environmental cues (DeWitt et al. 1998). Additionally, an inappropriate plastic response to an environment can result in a costly phenotype-environment mismatch (DeWitt et al. 1998, Valladares, Gianoli and Gomez 2007, De Jong and Leyser 2012). Therefore, it may sometimes benefit a plant to remain stable across environments (a phenomenon termed homeostasis), particularly in non-terminal drought stress conditions often found in agriculture (Bradshaw 2006, Aspinwall et al. 2015, Metlen et al. 2009). However, morphological homeostasis is likely the product of other altered intrinsic mechanisms such as physiological or molecular plasticity (Bradshaw 2006, Forsman 2014, Hua et al. 2001).

Sorghum (*Sorghum bicolor* (L.) Moench) is a globally important, C4 model crop used for food, forage and fuel, and known for its tolerance to drought stress. It is especially useful in the study of drought as it exhibits different types of tolerance depending on whether the stress occurs before the reproductive stage (pre-flowering drought tolerance) or during grain fill (post-flowering drought tolerance). Pre-flowering drought tolerance is a particularly critical area of study as early season stress can have impacts on plant establishment as well as grain yield (Kebede et al. 2001). Furthermore, although several notable sorghum breeding lines have been identified as either pre-flowering or post-flowering drought tolerant, there is little evidence that a genotype can display both types of tolerance (Rosenow et al. 1983). It is likely that the mechanisms employed to combat drought stress differ among these lines due to domestication across a wide range of environments (Kimber, Dahlberg and Kresovich 2013).

Here, we characterize the timing and degree of change in morphological and physiological responses to pre-flowering water stress across a diverse selection of sorghum lines that vary for pre-flowering drought tolerance. Our goal was to identify traits common to all the lines, and traits found in

individual lines that contribute to pre-flowering drought tolerance. Specifically, we wanted to 1) identify morphological and physiological traits that were predictive of pre-flowering drought tolerance, 2) evaluate whether combinations of traits were effective at predicting pre-flowering drought tolerance, and 3) understand whether the timing and magnitude of changes (plasticity) in traits during water stress and recovery could predict pre-flowering drought tolerance. We compared physiological and morphological traits of well-watered and water-stressed plants and calculated plasticity and recovery indices in response to water stress and rewatering. We found that pre-flowering drought tolerant lines exhibit more moderate morphological responses and only subtle changes in physiology during water stress, but greater abilities to recover normal development after water stress.

MATERIALS AND METHODS

Plant materials and growth conditions

Nine sorghum lines were selected based on their importance in molecular plant breeding (e.g., recombinant inbred line (RIL) parents, sequenced lines, transformable lines) and diverse range of drought tolerances (Table 1.1). Seeds were germinated on filter paper in Petri dishes with fungicide solution (Maxim XL, Syngenta, Greensboro, NC, USA) at 27°C for four days (two days dark, two days light). Seedlings were then transplanted into 1.4 liter pots with fritted clay (Field & Fairway, Profile Products LLC, Buffalo Grove, IL, USA) and grown in the greenhouse for 14 days. Daily temperatures ranged between 20°C and 30°C during a 16-h photoperiod with supplemental lighting and relative humidity averaged 55%. Pots were soaked in water until saturated, then removed and weighed to determine 100% field capacity (FC). Prior to treatment, each plant was watered every other day to maintain 100% FC and fertilized weekly by watering with 6.3 grams/liter of Miracle Gro® (24:8:16) (The Scotts Company LLC, Marysville, OH, USA). At 15 days after sowing, plants were transferred to a walk-in growth chamber (mean 30°C day; 23°C night; 50% relative humidity; 16-h photoperiod; photosynthetic

photon flux density of 300 μmol m^{-2} s^{-1} for lighting) where they were acclimated before the treatment began at 21 days after sowing.

Experimental design

Three replicates of each of nine lines were subjected to each of two treatments which began at 21 days after sowing: (1) a water-stressed treatment where water was withheld for six days, then returned to 100% field capacity (severe drought stress and recovery), and (2) a well-watered treatment, which was consistently maintained at 100% field capacity. Gradual soil dry-down over six days in the water-stressed treatment using fritted clay allowed for slow development of stress similar to that which occurs in agricultural and natural systems and at a uniform rate across lines (Des Marais et al. 2012). At the point of most severe water stress, percent field capacity was reduced to 40% field capacity. A total of 297 plants were grown in a randomized complete block design. As morphological evaluations required destructive sampling, individual replicates were required for each time point. Five replicates per line were utilized for the first time point evaluating traits prior to treatment (5 plants x 9 lines = 45 plants). Three replicates per genotype and treatment were utilized for the next three time points evaluating morphological and physiological traits during water stress and recovery (3 time points x 3 plants x 9 lines x 2 treatments = 162 plants). For the last time points that measure development and biomass/grain yields, the same five replicates were utilized for both time points per line and treatment (5 plants x 9 lines x 2 treatments = 90 plants).

Traits were evaluated at each of six time points (Figure 1.1): (1) pre-stress, (2) 4 days of withholding water (limited water), (3) 24 h after rewatering (24-h recovery), (4) nine days after rewatering (nine-day recovery), (5) 70 days after sowing just prior to full maturity and (6) at maturity. A subset of five replicates were evaluated at the last two time points. These plants were transplanted from fritted clay into 11.4 L pots with potting soil (Pro-Mix BX General Purpose, Premiere Tech

Horticulture, Quakertown, PA, USA) after the nine-day recovery time point and grown in the greenhouse. An additional 13.0 grams of fertilizer (19:6:12) (Osmocote, The Scotts Company LLC, Marysville, OH, USA) was applied at 70 days after sowing.

Assessing morphological & physiological traits

Green leaf area was determined using the LICOR LI-3100C leaf area meter (LI-COR, Inc., Lincoln, NE, USA). Belowground traits were measured with WinRHIZO root scanning equipment (Epson Expression 1100 XL, Epson America, Inc., Long Beach, CA, USA) and software (Regent Instruments, Inc. Quebec, QC, CA). Root: shoot ratios were calculated as total root length/leaf area. At 70 days after sowing, leaf stage was assessed by the number of leaves on the primary shoot with visible leaf collars. To determine impacts of water stress on development and yield, days to flowering as well as biomass and grain characteristics were measured (Table 1.2). Dry weights were determined by drying samples at 34°C for one week. Additionally, several other morphological traits were measured during water stress and recovery including: plant height, fresh and dry root and shoot weights, root surface area, average root diameter, fine root length, ratio of root length <0.04 mm diameter: root length >0.04 mm diameter, and number of nodal roots (Table 1.S1).

Physiological measurements were taken on the youngest fully expanded leaf of each plant inside the growth chamber during a four-h midday period using the LI-6400XT and leaf chamber fluorometer attachment (LI-COR, Inc., Lincoln, NE, USA). Measurements were made under the following conditions: block temperature (28°C), photosynthetically active radiation (400 µmol $m^{-2}s^{-1}$), CO_2 (400 µmol mol^{-1}), and relative humidity (ambient). Physiological traits assessed included: carbon assimilation rate, stomatal conductance, intracellular CO_2, ratio of internal to atmospheric CO_2 and intrinsic water use efficiency (Table 1.1, Table 1.S1). Fluorescence measurements were also taken and included quantum yield of PSII (Φpsii), photochemical quenching (Qp) and electron transport rate (ETR) (Table 1.S1).

Plasticity was calculated using indices of plasticity (IP) and indices of recovery (IR) to standardize impacts of drought and recovery on morphological and physiological traits among lines. Within one time point and for each line, trait means of plants under well-watered and water-stressed conditions were used in the following formula, where M_{max} is the treatment with the highest mean and M_{min} is the treatment with the lowest mean as calculated in Valladares et al. (2000) and Couso and Fernández (2012):

IP or IR = $(M_{max} - M_{min})/M_{max}$

An initial survey searched for trends in plasticity values to narrow focus to traits with the most significant patterns. IP values for assimilation rate, leaf area and total root length at the limited water time point and for leaf area and total root length at the 24-h recovery time point are discussed here (see Figure 1.1 for indices and time points at which they were calculated).

IP indicates the magnitude of impact of drought stress on each trait for each line, with values ranging between 0 and 1. Higher values indicate greater differences between well-watered and stressed phenotypes, and therefore, more extreme responses to drought (higher plasticity). At 24-h recovery time point, index values for morphology were termed IP instead of IR, since we assumed morphology at this time point would not yet reflect a response to rewatering, but still be a response to water stress. The same formula was used to calculate values for physiology at 24-h recovery; however, we termed these indices of recovery (IR) because we assumed assimilation would have a more immediate response to rewatering, unlike morphological responses which can take days to recover (DeWitt et al. 1998). In addition, IR was calculated for both morphological and physiological traits at the nine-day recovery time point to characterize to what degree water-stressed lines recovered phenotypes of their well-watered counterparts. Again, higher values indicate greater differences between well-watered and stressed lines, and therefore, longer lasting impacts of water stress on recovery.

Statistical analysis

All traits within a time point were analyzed in JMP Pro 12 (SAS Institute) using analysis of variance (ANOVA) with treatment, line and their interaction term included as fixed effects. The analysis was then sliced by line to determine between-treatment differences within lines. Data that did not pass the Shapiro-Wilk Normality Test were Box-Cox transformed prior to analysis. Statistical significance was determined by $P < 0.05$ unless otherwise stated. Correlations between Ips, Irs, total seed weight and days to flowering were determined using Spearman's rank correlations.

Classification of pre-flowering drought tolerances

To examine whether certain morphological and physiological traits were common to tolerant and susceptible groups, previously unclassified lines were categorized as either pre-flowering drought tolerant or susceptible based on yield and flowering data from this study. Susceptibility was determined by a reduction in grain, except for lines in which a delay in development was used as in Ejeta et al. (2000) (Table 1.2, Figure 1.2)). Susceptible lines included Rio, SC170, and IS3620C. No IS3620C plants set seed in our greenhouse conditions, but we classified it as susceptible according to other trends it displayed during development. M35-1 had no significant reduction in grain or delay in flowering under water stress and was classified as tolerant. Previously categorized lines were BTx623, RTx430 and Tx7000 (pre-flowering drought tolerant) (Rosenow et al. 1983) and BTx642 and SC56 (pre-flowering drought susceptible) (Tuinstra et al. 1996, Kebede et al. 2001). Our data and characterization scheme support these designations.

RESULTS

A total of 22 physiological and morphological traits were evaluated during the first four time points: (1) pre-stress, (2) limited water, (3) 24-h recovery and (4) nine-day recovery (Figure 1.1, Table 1.2). In addition, developmental and yield traits were assessed at (5) 70 days after sowing and at (6)

maturity. A subset of trait values that were most relevant in addressing our goals is discussed below (Table 1.2).

Physiological traits do not show a relationship with drought tolerance

Significant variation in assimilation rates existed among lines at pre-stress and as well as among lines and between treatments at all other time points (Tables 1.2 and 1.3). Before onset of water stress, assimilation ranged from 6.14 to 16.34 mol CO_2 m^{-2} s^{-1} (Table 1.2). At limited water time point, well-watered lines ranged from 4.70 to 13.52 mol CO_2 m^{-2} s^{-1} and water-stressed lines ranged from -0.90 to 15.16 mol CO_2 m^{-2} s^{-1}. At 24-h recovery time point, well-watered lines ranged from 2.14 to 10.32 mol CO_2 m^{-2} s^{-1} and water-stressed lines from 2.34 to 10.79 mol CO_2 m^{-2} s^{-1}. At nine-day recovery time point, well-watered lines ranged from 0.76 to 11.64 mol CO_2 m^{-2} s^{-1} and water-stressed lines from 1.02 to 7.14 mol CO_2 m^{-2} s^{-1}. At pre-stress time point, all tolerant (T) lines and two susceptible (S) lines (IS3620C, Rio) had significantly higher assimilation than the three other susceptible lines (Tables 1.2 and 1.3). At limited water, susceptible lines BTx642, IS3620C and SC170 as well as tolerant lines M35-1 and Tx7000 were stable, with water-stressed lines not differing from well-watered lines. However, water-stressed Rio (S), SC56 (S), as well as BTx623 (T) and RTx430 (T) had decreased assimilation. At 24-h recovery, most water-stressed lines were no different from well-watered controls except for susceptible lines BTx642 (lower), and IS3620C (higher). At nine-day recovery, only water-stressed M35-1 (T) and Rio (S) had significantly lower assimilation than well-watered controls. Overall, there was not a consistent pattern in assimilation rates between well-watered and water-stressed conditions to predict pre-flowering drought tolerance in sorghum.

Stomatal conductance rates among lines at pre-stress ranged from 0.042 to 0.083 mol H_2O m^{-2} s^{-1} (Table 1.2). At limited water, well-watered lines ranged from 0.023 to 0.069 mol H_2O m^{-2} s^{-1} and water-stressed lines from 0.007 to 0.082 mol H_2O m^{-2} s^{-1}. At 24-h recovery, well-watered lines ranged from

0.018 to 0.062 mol H_2O m^{-2} s^{-1} and water-stressed lines from 0.014 to 0.058 mol H_2O m^{-2} s^{-1}. At nine-day recovery, well-watered lines ranged from 0.006 to 0.0635 mol H_2O m^{-2} s^{-1} and water-stressed from 0.012 to 0.048 mol H_2O m^{-2} s^{-1}. In summary, assimilation and stomatal conductance did not predict pre-flowering drought tolerance/susceptibility in sorghum.

Alone, neither root length nor leaf area show relationships with drought tolerance

Significant variation in total root length and leaf area existed among lines at pre-stress as well as among lines and between treatments at other time points (Tables 1.2 and 1.3). Root length among lines at pre-stress ranged from 301.66 to 1101.26 cm (Table 1.2). At limited water, well-watered lines ranged from 274.54 to 1689.82 cm and water-stressed from 580.45 to 1906.98 cm. At 24-h recovery, well-watered lines ranged from 473.85 to 2071.47 cm and water-stressed from 663.22 to 1832.16 cm. At nine-day recovery, well-watered lines ranged from 1009.10 to 3168.03 cm and water-stressed from 637.89 to 2129.97 cm. At pre-stress, root lengths of Tx7000 (T) and IS3620C (S) were the smallest (Tables 1.2 and 1.3). At limited water, root length did not differ between treatments. At 24-h recovery, only water-stressed BTx623 (T) and Rio (S) had significantly reduced root lengths compared to well-watered controls. At nine-day recovery, water-stressed BTx623 (T) continued to have a marginally reduced root length ($P < 0.07$). Water-stressed lines M35-1 (T), BTx642(S) and SC56 (S) also had smaller root lengths at this time.

Leaf areas among lines at pre-stress ranged from 47.16 to 169.06 cm^2 (Table 1.2). At limited water, well-watered lines ranged from 53.39 to 305.39 cm^2 and water-stressed from 67.35 to 176.90 cm^2. At 24-h recovery, well-watered lines ranged from 83.84 to 376.41 cm^2 and water-stressed from 93.31 to 203.34 cm^2. At nine-day recovery, well-watered lines ranged from 212.81 to 926.82 cm^2 and water-stressed from 93.64 to 515.01 cm^2. Leaf areas varied significantly among lines prior to treatment (Tables 1.2 and 1.3). At limited water, water-stressed BTx623 (T), RTx430 (T), Tx7000 (T) and SC170 (S)

had significantly reduced leaf areas compared to well-watered counterparts. At 24-h recovery, all water-stressed lines had reduced leaf areas compared to well-watered controls except the two smallest lines in each of the tolerant and susceptible categories, Tx7000 (T), and IS3620C (S). At nine-day recovery, only water-stressed Tx7000 (T), RTx430 (T), and SC170 (S) did not differ from well-watered 51controls. Overall, neither root length nor leaf area, when considered individually, displayed a relationship with pre-flowering drought tolerance.

Root: shoot ratios increase in response to water stress

We calculated root: shoot ratios (root length: leaf area) to better observe relationships of water-economy related traits with water stress. Root: shoot ratios at pre-stress ranged from 0.79 to 3.12 (Table 1.2). At limited water, well-watered lines ranged from 4.88 to 7.06 and water-stressed from 5.68 to 11.56. At 24-h recovery, well-watered lines ranged from 5.17 to 7.43 and water-stressed from 6.37 to 11.35. At nine-day recovery, well-watered lines ranged from 3.40 to 4.62 and water-stressed from 3.54 to 7.26. At pre-stress, only IS3620C (S) had a smaller root: shoot ratio than other lines (Tables 1.2 and 1.3). At limited water, all water-stressed lines had increased root: shoot ratios compared to well-watered lines ($P < 0.06$), except for M35-1 (T). At 24-h recovery, all water-stressed lines retained at least marginally increased root: shoot ratios ($P < 0.07$). At nine-day recovery, most lines exhibited no significant difference between treatments except water-stressed IS3620C (S) and Rio (S), which still had higher root: shoot ratios.

Plasticity differentiates between tolerant and susceptible lines

IP values were calculated to examine the magnitude of trait differences between well-watered and water-stressed treatments at limited water and 24-h recovery time points. Index of plasticity ranges from 0 to 1, with 1 indicating the greatest change in response to water stress and 0 indicating the

smallest change in response to water stress. At limited water, assimilation rates of susceptible lines ranged from 0.03 to 1.00 and were either the most or least plastic in response to water stress, except for IS3620C (S), while tolerant lines were moderately plastic (Figure 1.3a). Morphological traits were less plastic than assimilation rate (i.e., effects of water stress were more substantial on physiology than morphology) (Figure 1.3). At the limited water time point, IP for leaf area ranged from 0.08 to 0.44; M35-1 (T) was least plastic in response to water stress and SC170 (S) was most plastic (Figure 1.3b). IP for root length at limited water ranged from 0.13 to 0.53; M35-1 (T) was least plastic and IS3620C (S) was most plastic (Figure 1.3c).

IP were also calculated for morphological traits at the 24-h recovery time point (Figures 1.4b and 1.4c). For morphology, we assumed that 24-h recovery still reflected response to water stress because morphological changes occur more slowly than physiological changes. At 24-h recovery, IP for leaf area ranged from 0.02 to 0.64; Tx7000 (T) was least plastic and SC56 (S) was most plastic. IP for root length ranged from 0.09 to 0.49; M35-1 (T) was least plastic and IS3620C (S) was most plastic. Overall, at 24-h recovery, susceptible lines (except BTx642), showed greatest morphological change due to water stress while tolerant lines were most stable.

Moderate changes in carbon assimilation differentiate tolerant lines at short-term recovery

IR was calculated for assimilation at 24-h and nine-day recovery to determine short and longer term physiological responses to rewatering after water limitation (Figure 1.3a). Higher IR values indicate larger differences between well-watered and water-stressed treatments (water-stressed exhibits lesser recovery of well-watered phenotypic value) and lower values indicate smaller differences (water-stressed exhibits greater recovery of well-watered phenotypic value). Unlike morphological traits, we assumed that assimilation at 24-h recovery would reflect response to rewatering. IR for assimilation at 24-h recovery ranged from 0.08 to 0.72; SC56 (S) had the lowest value and BTx642 (S) had the highest

(i.e., water-stressed SC56 resumed normal assimilation after rewatering; BTx642 did not.) In general, at 24-h recovery, tolerant lines made more moderate adjustments to assimilation rate while susceptible lines made either extreme or very small adjustments. At nine-day recovery, IR for assimilation ranged from 0.09 to 0.91; IS3620C (S) had the lowest value and RTx430 (T) had the highest value (Figure 1.3a). There was no apparent relationship with assimilation IR and pre-flowering drought tolerance at the longer-term nine-day recovery.

Morphological index of recovery indicates the impact of water stress

IR was calculated for morphological traits at nine-day recovery to determine the impact of water stress on recovery (Figures 1.3b and 1.3c). Higher values indicate that drought continued to have larger impacts on morphology; lower values indicate smaller impacts. Leaf area IR ranged from 0.10 to 0.57 and root length IR ranged from 0.02 to 0.59. Leaf areas of SC56 (S), Rio (S) and IS3620C (S) were impacted most while leaf areas of RTx430 (T) and SC170 (S) were impacted least (Figure 1.3). Root lengths of SC56 (S) and BTx642 (S) were impacted most while RTx430 (T) and SC170 (S) exhibited diminished impacts of drought stress. In general, susceptible lines showed the greatest differences between treatments, particularly for leaf area, indicating greater lasting effects of water stress on morphological recovery. Tolerant lines demonstrated smaller effects of water stress on recovery.

Leaf area recovery is correlated with later developmental milestones

At 70 days after sowing, all water-stressed susceptible lines displayed at least a marginally reduced leaf stage ($P < 0.08$) relative to well-watered controls, indicating compromised development (Tables 1.2 and 1.3). All water-stressed susceptible lines took longer to flower, except for IS3620C (photoperiod sensitive) (Figure 1.2). Dry matter was decreased for water-stressed SC170 (S) and SC56 (S) and seed weights were reduced for water-stressed BTx642 (S) and SC170 (S). There were no significant

differences between well-watered susceptible and tolerant lines in either days to flowering or seed weight (Figure 1.2). Days to flowering and seed weight for water-stressed lines were correlated with morphological IP and IR values (Table 1.S2). Leaf area IR was positively correlated with days to flowering (Spearman's $\rho = 0.795$, $P < 0.05$). Days to flowering was negatively correlated to seed weight (Spearman's $\rho = -0.762$, $P < 0.05$).

DISCUSSION

Knowledge of characteristics that differentiate pre-flowering drought tolerance early in the breeding cycle has potential to advance sorghum improvement programs. Targeting early-season tolerance traits that are linked to yield performance at end of the season saves time and money. Historically, emphasis has been on the selection of genotypes able to maintain yield stability across environments; however, high-yielding crop varieties have typically been selected for local, relatively consistent environments. Although the word "stability" implies a lack of change, it is in fact phenotypic flexibility that enables stable yields through compensating plastic responses (De Jong and Leyser 2012, Bradshaw 1965, Bradshaw 2006). Therefore, under the uncertainty of climate change, selecting for phenotypic plasticity in response to adverse conditions may be more valuable to plant breeding (De Jong and Leyser 2012, Nicotra et al. 2010).

Breeding for drought tolerance is notoriously difficult because tolerance can involve many different strategies due to differences in plant evolutionary history (Blum and Sullivan 1986, Amelework et al. 2015). Although phenotypic flexibility has been noted as an important part of drought response, the study of plasticity for specific traits other than yield is only recently receiving attention in agriculture (as reviewed by Aspinwall et al. 2015, Nicotra et al. 2010, Bloomfield, Rose and King 2014). The magnitude of plasticity in response to drought differs across environments, species and genotypes and varies with duration, severity and timing of water stress (Aspinwall et al. 2015, Sanad, Campbell and Gill 2016). Here, we demonstrate that the timing and magnitude of physiological and morphological

adjustments to water stress and resource reintroduction are most important in differentiating tolerant and susceptible lines.

Physiological and morphological trait values do not predict pre-flowering drought tolerance

No individual characteristics that distinguished pre-flowering drought tolerant lines were found when we compared responses of physiological and morphological traits under water stress and short-term recovery (Table 1.2). However, we did identify differences in long-term traits, such as flowering time and yield (Figure 1.2). Yield is a complex trait defined by many other characteristics such as flowering time, biomass, tillering, root architecture, and resistance to biotic and abiotic stresses (as reviewed by Shi et al. 2009). Therefore, looking at any one trait individually may not be the most effective strategy in breeding for drought tolerance. For instance, although smaller varieties are often considered more drought adapted as they have a smaller leaf area to maintain (Blum and Sullivan 1986), we measured no effect of plant size on pre-flowering drought tolerance (Table 1.2). In this study, many morphological responses to drought stress such as maintenance of the root system or reduction of leaf area were common to both tolerant and susceptible lines. However, differentiating patterns emerged upon examination of the timing and magnitude (plasticity) of responses.

Indices of plasticity and recovery distinguish pre-flowering drought tolerance

When studying drought stress, the ability to effectively utilize resources may occur either 1) under water stress and/or 2) upon rewatering (Vassileva et al. 2011). An inappropriate response to the environment resulting in a phenotype-environment mismatch can compromise development, particularly for morphological traits that are not flexible on a short time-scale or are irreversible (DeWitt et al. 1998). If resources are restored in the environment, inability to reverse the phenotype is detrimental for recovery (De Jong and Leyser 2012, Metlen et al. 2009). We observed that drought

tolerant lines responded to pre-flowering water stress in the following ways: 1) low morphological plasticity (leaf area and root length) under water stress, 2) moderate assimilation rate plasticity under water stress and 3) greater ability to recover morphologically after rewatering (Figure 1.3). Similarly, the maintenance of green leaf area and appropriate physiological adjustments under water stress have been used to identify drought tolerance in plants including sorghum and Arabidopsis (Farooq et al. 2008; Blum & Sullivan 1986; Xiong et al. 2006). We found that susceptible sorghum lines were unable to recover leaf area to the same degree as tolerant lines, indicating an inability to respond to water stress and/or rewatering in an appropriate time frame. Also in sorghum, there is evidence that a drought tolerant genotype within the durra race was better able to maintain morphological homeostasis under stress and recover leaf area at six days after rewatering, compared to a susceptible durra genotype (Fracasso, Trindade and Amaducci 2016). In our study, susceptibility also did not depend on race, though it is a typical indicator of geographical origin and adaptation to drought stress (Kimber et al. 2013, Amelework et al. 2015).

Other species demonstrate both commonalities and differences in response to drought and rewatering when compared to sorghum, implying a need for case by case studies. In tolerant cowpea lines, patterns of leaf area recovery after mid-season water stress were similar to tolerant sorghum lines in our study (Anyia and Herzog 2004). However, during mid-season water stress, some tolerant cowpea lines displayed greater reductions in leaf area compared to well-watered controls than susceptible lines. In contrast, rice lines that were able to continue leaf expansion under early drought stress were better able to recover dry matter production after rewatering (Siopongco et al. 2006). Therefore, a greater degree of plasticity in leaf area under water stress may be advantageous for cowpea, but not for rice or sorghum. Although phenotypic plasticity in plants is valued as a mechanism to buffer changes in the environment {Nicotra et al. 2010}, the advantage of plasticity is, in fact, dependent on species, context, trait, timing, and magnitude.

Leaf area recovery is associated with normal reproductive development in sorghum

The ability to recover after water stress has become increasingly recognized as an important drought tolerance mechanism that helps re-establish development (Blum 2011, Xu, Zhou and Shimizu 2010). Overall, we found that water-stressed tolerant lines could recover normal leaf area by nine days after rewatering, whereas susceptible lines could not (Figure 1.3b). A rapid leaf area response to rewatering (lower IR) was particularly important for pre-flowering drought tolerance in sorghum as it was associated with the maintenance of flowering time (Table 1.S2). Flowering time of water-stressed tolerant lines was not significantly different from respective well-watered tolerant lines, demonstrating sorghum lines that capitalize on the reintroduction of resources after stress, can recover normal development (Figure 1.2). Reduced leaf expansion under drought may be associated with a delay in development (Simane, Peacock and Struik 1993) and the importance of maintaining green leaf area during post-flowering drought stress is widely documented in sorghum as it supports photosynthetic activity during grain-filling (Borrell, Hammer and Douglas 2000, Kebede et al. 2001). However, to our knowledge, this is the first time a link has been established between recovering leaf area after pre-flowering water stress and maintaining flowering time.

Delays in development allow for longer vegetative periods, however, delays in flowering time without delays in maturity shorten the grain-filling stage and impact yield (Jung and Muller 2009). For instance, wheat lines that preserved anthesis time across low and high yielding environments produced higher yields at low-yielding sites (Sadras et al. 2009). Wheat lines with highly plastic durations of the post-anthesis interval (lines that shortened this interval) had lower yields at these sites. Therefore, although developmental plasticity is often recognized as a drought tolerance mechanism, it can also have negative impacts on yield. In this study, tolerant sorghum recovered leaf area before susceptible lines without a subsequent delay in flowering or yield (Figures 1.2 and 1.3). It follows that leaf area IR is

an early season phenotype that can predict pre-flowering drought tolerance and help decrease the length of the breeding cycle.

Stability in one trait is likely due to the compensating flexibility in another trait

Studying multiple factors in concert (e.g., morphological, physiological, biochemical) provides a better understanding of plasticity and identifies the most effective responses to environmental conditions (Kurimoto et al. 2004, Aspinwall et al. 2015, Nicotra et al. 2010). Because of the close association of these factors, maintenance of stability in one trait is often associated with a compensating mechanism and flexibility in another (Pigliucci 2003, Forsman 2014). In our study, tolerant lines were morphologically stable under limited water and made moderate changes in assimilation. These controlled physiological adjustments are likely due to compensating reductions in stomatal aperture, changes in metabolism, or production of osmolytes, hormones, and reactive oxygen species (Pinheiro and Chaves 2011). In winter wheat, the maintenance and subsequent recovery of respiration under water stress was associated with the maintenance of cytochrome pathway activity and mitochondrial energy production (Vassileva et al. 2009, Vassileva et al. 2011). In *Arabidopsis*, physiological homeostasis under water stress was likely the product of several changes in gene expression (Juenger et al. 2010). These genes were found to be involved in pathways including abscisic acid and cytokinin biosynthesis, two hormones involved in drought response that modulate plant growth (Farooq et al. 2009). It is likely that moderate changes in assimilation rates seen here in tolerant sorghum lines, (unlike the shut down or lack of adjustment seen in susceptible lines), are at least partly responsible for their morphological stability (Figure 1.3a). Furthermore, the stability in less flexible morphological traits under water stress appears to contribute to rapid morphological recovery. As there are many costs of and limits to plasticity, the right degree of response is essential for controlled functioning of metabolism under water stress, particularly when environmental conditions are short-

term. Further exploration into the underlying biochemistry and genetics of physiological processes occurring under water stress would help establish the internal mechanisms behind morphological and physiological plasticity. Although there is some debate about the extent to which phenotypic plasticity is under genetic control, there is also evidence, our own data included, which suggests plasticity can be exploited for agricultural use (Schlichting and Pigliucci 1993, De Jong and Leyser 2012, Aspinwall et al. 2015). Recent research also suggests various epigenetic processes are behind plasticity and could provide beneficial targets for plant breeding (Metlen et al. 2009, Bloomfield et al. 2014).

In conclusion, our study shows the benefit of plastic responses varies by trait, timing and magnitude. In sorghum, we found morphological stability and moderate physiological plasticity to be most effective in combatting pre-flowering drought stress. However, as maintenance of plant tissues is likely produced through changes in biochemistry, further research should determine coincident metabolic changes during water stress. Our findings also suggest additional focus on the recovery period is needed to better understand how tolerant plants allocate resources upon rewatering. Furthermore, index of recovery for leaf area is useful for early identification of pre-flowering drought tolerance in sorghum. Our study supports that for sorghum, specific combinations of timing and degree of morphological and physiological responses likely enable pre-flowering drought tolerance. Therefore, future research should explore use of phenotypic plasticity as a tool for breeding drought resilient crops.

Table 1.1. Lines in this study and relevant information.

Line	*Type*	*Characterized Pre-flowering Drought Tolerance*	*Characteristics*	*Race*	*Publications*
BTx623	Grain	Tolerant	Sequenced	Caudatum	(Paterson et al. 2009)
RTx430	Grain	Tolerant	Transformable	Unknown	(Liu and Godwin 2012)
Tx7000	Grain	Tolerant	Sequenced	Kafir	(Evans et al. 2013, Kebede et al. 2001)
BTx642	Grain	Susceptible	Sequenced; stay green	Durra	(Evans et al. 2013, Subudhi, Rosenow and Nguyen 2000)
SC56	Grain	Susceptible	Stay green	Caudatum	(Kebede et al. 2001)
SC170	Grain	Unknown (S)[a]	Parent to BTx623 and RTx430	Caudatum	(Evans et al. 2013, Miller 1984)
IS3620C	Grain	Unknown (S)	Converted inbred line	Guinea	(Brown et al. 2006, Hart et al. 2001)
M35-1	Biomass	Unknown (T)	RIL[b] parent for agronomically important traits	Durra	(Reddy et al. 2013)
Rio	Sweet	Unknown (S)	RIL parent for energy-related traits	Unknown	(Murray et al. 2008)

[a] unknown drought tolerance lines characterized in this study are indicated in brackets: Susceptible (S) and Tolerant (T); [b] recombinant inbred line

Table 1.2. Least-square means and standard errors for morphological, physiological and developmental traits measured at various time points for **(a)** tolerant lines and **(b)** susceptible lines.

Figure 3.. *Tolerant Lines*

	Line	BTx623		M35-1		RTx430		Tx7000	
		Well-Watered	Water-Stressed	Well-Watered	Water-Stressed	Well-Watered	Water-Stressed	Well-Watered	Water-Stressed
	Trait								
Pre-Stress	**A[b]**	13.88 ± 0.54 (AB)	N/A	15.53 ± 1.58 (A)	N/A	16.34 ± 0.59 (A)	N/A	13.36 ± 0.88 (ABC)	N/A
	gsw[c]	0.073 ± 0.005 (A)	N/A	0.075 ± 0.01 (A)	N/A	0.083 ± 0.003 (A)	N/A	0.076 ± 0.004 (A)	N/A
	LA[d]	147.99 ± 12.33 (ABC)	N/A	126.24 ± 11.61 (CD)	N/A	88.26 ± 17.49 (EF)	N/A	72.13 ± 6.59 (FG)	N/A
	TRL[e]	793.12 ± 155.08 (AB)	N/A	811.81 ± 208.65 (AB)	N/A	548.38 ± 130.35 (BC)	N/A	364.55 ± 37.22 (C)	N/A
	R:S[f]	3.12 ± 0.70 (A)	N/A	1.99 ± 0.47 (AB)	N/A	1.67 ± 0.39 (AB)	N/A	1.94 ± 0.97 (AB)	N/A
Limited Water	**A**	11.59 ± 1.56	3.31 ± 2.96**	11.03 ± 1.2	8.14 ± 4.13	13.52 ± 1.45	4.42 ± 3.3**	11.2 ± 2.34	9.72 ± 4.49
	gsw	0.061 ± 0.009	0.021 ± 0.016**	0.051 ± 0.004	0.042 ± 0.021	0.069 ± 0.006	0.023 ± 0.015**	0.061 ± 0.012	0.05 ± 0.021
	LA	229.66 ± 30.06	152.52 ± 6.29*	181.93 ± 21.62	166.61 ± 37.11	142.45 ± 16.54	104.45 ± 11.99*	107.8 ± 7.44	69.92 ± 2.19**
	TRL	1207.74 ± 166.64	1381.53 ± 195.1	1071.2 ± 181.56	918.2 ± 165.78	811.28 ± 132.18	1087.7 ± 123.39	793.36 ± 63.45	684.6 ± 77.59
	R:S	5.18 ± 0.22	9.53 ± 0.99**	5.81 ± 0.41	5.68 ± 0.81	4.88 ± 0.52	9.57 ± 1.33**	7.06 ± 0.4	9.76 ± 0.75*
24-H Recovery	**A**	5.55 ± 1.31	7.28 ± 1.21	8.31 ± 2.29	10.34 ± 2.92	9.39 ± 2.25	6.83 ± 0.32	10.32 ± 4.27	8.77 ± 0.41
	gsw	0.061 ± 0.009	0.021 ± 0.016	0.051 ± 0.004	0.042 ± 0.021	0.069 ± 0.006	0.023 ± 0.015	0.061 ± 0.012	0.05 ± 0.021
	LA	345.97 ± 27.68	179.2 ± 41.83**	245.23 ± 47.62	180.7 ± 15.98*	195.56 ± 57.03	105.79 ± 4.95**	101.52 ± 11.65	104.12 ± 2.21
	TRL	1946.78 ± 78.96	1214.73 ± 266.00*	1503.72 ± 266.16	1650.71 ± 598.23	1064.9 ± 263.93	843.62 ± 145.56	473.85 ± 98.43	663.22 ± 58.61
	R:S	5.89 ± 0.23	7.20 ± 0.39**	6.18 ± 0.12	8.91 ± 2.52*	5.56 ± 0.21	8.01 ± 0.62**	5.17 ± 0.74	6.37 ± 0.3**
Nine-Day Recovery	**A**	4.17 ± 1.26	7.14 ± 0.48	5.23 ± 1.28	1.68 ± 1.05*	11.67 ± 3.12	6.94 ± 1.51	1.49 ± 1.14	1.32 ± 0.73
	gsw	0.017 ± 0.005	0.048 ± 0.006**	0.029 ± 0.006	0.017 ± 0.005	0.064 ± 0.017	0.039 ± 0.004	0.016 ± 0.007	0.012 ± 0.006
	LA	737.13 ± 86.60	468.58 ± 107.93*	622.05 ± 18.59	325.14 ± 42.49**	350.04 ± 70.79	316.67 ± 70.76	323.46 ± 38.4	215.42 ± 17
	TRL	3168.03 ± 671.98	1823.79 ± 538.16*	2650.79 ± 86.02	1355.19 ± 328.7**	1411.07 ± 344.8	1443.71 ± 385.45	1100.24 ± 166.83	830.95 ± 78.18
	R:S	4.22 ± 0.44	3.75 ± 0.38	4.26 ± 0.06	4.06 ± 0.45	3.94 ± 0.33	4.43 ± 0.36	3.4 ± 0.37	3.85 ± 0.09
70 DAS[a]	**LS[g]**	14.33 ± 0.33	14.33 ± 0.33	12.40 ± 0.51	12.17 ± 0.48	13.75 ± 1.11	14.00 ± N/A	14.75 ± 0.25	13.33 ± 0.88
Maturity	**FD[h]**	72.00 ± 0.41	73.33 ± 0.88	79.00 ± 0.63	79.83 ± 0.95	75.25 ± 0.63	74.00 ± N/A	72.75 ± 0.85	73.67 ± 0.67
	SW[i]	41.98 ± 3.58	35.90 ± 6.35	34.64 ± 2.61	30.35 ± 2.86	24.92 ± 1.29	36.63 ± N/A	35.46 ± 3.80	37.26 ± 6.09
	DM[j]	91.92 ± 9.91	75.33 ± 14.05	143.64 ± 17.77	143.67 ± 7.50	50.48 ± 4.05	62.47 ± N/A	83.72 ± 5.74	85.31 ± 9.37
	DW[k]	133.90 ± 13.27	111.23 ± 20.18	178.28 ± 20.12	174.02 ± 9.6	75.40 ± 3.74	99.10 ± N/A	119.18 ± 9.4	122.57 ± 15.45

[a] days after sowing; [b] assimilation rate (mol CO_2 m-2 s-1); [d] stomatal conductance (mol H_2O m-2 s-1); [d] leaf area (cm^2); [e] total root length (cm); [f] leaf area / total root length; [g] leaf stage; [h] days to flowering; [i] seed weight (g); [j] dry matter (g); [k] dry weight (g)

Uppercase letters indicate significant differences (Student's t) among lines at pre-stress time point. Level of significance (* P<0.08 and ** P<0.05) between treatments within genotypes are shown.

(b) Susceptible Lines

	Line	BTx642		IS3620C		Rio		SC170		SC56	
		Well-Watered	**Water-Stressed**	**Well-Watered**	**Water-Stressed**	**Well-Watered**	**Water-Stressed**	**Well-Watered**	**Water-Stressed**	**Well-Watered**	**Water-Stressed**
	Trait										
Pre-Stress	**A**[b]	6.14 ± 1.6 (E)	N/A	11.57 ± 0.75 (BC)	N/A	14.41 ± 0.57 (AB)	N/A	7.9 ± 0.7 (DE)	N/A	10.52 ± 1.74 (CD)	N/A
	gsw[c]	0.063 ± 0.022 (AB)	N/A	0.063 ± 0.006 (AB)	N/A	0.073 ± 0.003 (A)	N/A	0.042 ± 0.004 (B)	N/A	0.068 ± 0.01 (A)	N/A
	LA[d]	141.18 ± 5.74 (BC)	N/A	47.16 ± 4.30 (G)	N/A	164.94 ± 4.10 (AB)	N/A	169.06 ± 8.03 (A)	N/A	108.97 ± 7.81 (DE)	N/A
	TRL[e]	872.27 ± 19.30 (AB)	N/A	301.66 ± 21.81 (C)	N/A	1101.26 ± 162.37 (A)	N/A	1034.34 ± 47.98 (A)	N/A	945.33 ± 70.61 (A)	N/A
	R:S[f]	2.20 ± 0.15 (AB)	N/A	0.79 ± 0.06 (B)	N/A	2.87 ± 0.33 (A)	N/A	2.42 ± 0.32 (AB)	N/A	2.00 ± 0.14 (AB)	N/A
Limited Water	**A**	6.49 ± 1.47	6.29 ± 2.08	9.5 ± 1.72	15.16 ± 1.62	13.45 ± 1.77	0.59 ± 1.16**	4.7 ± 1.34	4.38 ± 2.73	8.34 ± 1.01	-0.90 ± 0.18**
	gsw	0.037 ± 0.006	0.032 ± 0.009	0.054 ± 0.012	0.082 ± 0.01	0.067 ± 0.008	0.008 ± 0.005**	0.023 ± 0.006	0.021 ± 0.012	0.051 ± 0.008	0.007 ± 0.004**
	LA	142.13 ± 14.05	176.9 ± 29.23	53.39 ± 0.76	67.35 ± 6.42	237.58 ± 55.47	163.82 ± 3.19	305.39 ± 33.49	172.27 ± 29.42**	160.31 ± 4.31	108.93 ± 17.72
	TRL	854.52 ± 170.23	1137.89 ± 205.29	274.54 ± 19.86	580.45 ± 79.17	1557.94 ± 260.23	1906.98 ± 180.95	1689.82 ± 326.16	1289.09 ± 61.06	854.06 ± 46.93	1355.97 ± 8.56
	R:S	5.32 ± 0.51	9.47 ± 1.51**	5.89 ± 0.5	8.46 ± 0.92*	6.71 ± 1.16	10.3 ± 1.17**	5.58 ± 0.42	8.51 ± 0.87**	5.85 ± 0.36	11.56 ± 2.05**
24-H Recovery	**A**	9.11 ± 1.72	2.51 ± 2.86**	4.88 ± 1.00	10.79 ± 1.64**	6.78 ± 2.68	2.95 ± 0.34	2.14 ± 1.88	2.34 ± 1.19	8.9 ± 1.43	9.71 ± 0.07
	gsw	0.037 ± 0.006	0.032 ± 0.009**	0.054 ± 0.012	0.082 ± 0.01**	0.067 ± 0.008	0.008 ± 0.005	0.023 ± 0.006	0.021 ± 0.012	0.051 ± 0.008	0.007 ± 0.004
	LA	263.99 ± 39.37	172.36 ± 2.86*	83.84 ± 14.86	96.72 ± 12.31	476.06 ± 40.26	203.34 ± 8.54**	376.41 ± 20.82	172.87 ± 23.19**	260.09 ± 42.28	93.31 ± 26.38**
	TRL	1682.35 ± 260.22	1332.33 ± 166.38	547.44 ± 129.22	1070.03 ± 171.18*	3330.38 ± 475.61	1832.16 ± 411.3**	2061.21 ± 70.5	1329.05 ± 233.19	2071.47 ± 319.68	1307.57 ± 458.23
	R:S	6.38 ± 0.39	7.68 ± 0.45*	6.78 ± 0.33	9.68 ± 0.78**	6.77 ± 0.37	8.33 ± 0.81*	5.45 ± 0.34	7.53 ± 0.54**	7.43 ± 0.42	11.35 ± 1.06**
Nine-Day Recovery	**A**	0.76 ± 0.50	1.33 ± 0.21	6.83 ± 0.15	6.19 ± 2.59	5.34 ± 1.98	1.02 ± 1.25**	5.02 ± 1.08	6.36 ± 1.11	8.09 ± 0.68	4.58 ± 1.01
	gsw	0.008 ± 0.001	0.029 ± 0.014**	0.034 ± 0.001	0.034 ± 0.014	0.027 ± 0.010	0.016 ± 0.006	0.031 ± 0.004	0.039 ± 0.002	0.049 ± 0.008	0.028 ± 0.003*
	LA	593.94 ± 93.74	283.98 ± 44.13**	212.81 ± 57.57	93.64 ± 15.91**	926.82 ± 132.01	404.02 ± 46.99**	651.11 ± 91.32	515.01 ± 134.92	424.54 ± 114.74	182.73 ± 18.16**
	TRL	2571.96 ± 475.18	1050.23 ± 230.07**	1009.1 ± 333.08	637.89 ± 27.07	3482.43 ± 746.71	2129.97 ± 69.94	2239.41 ± 336.75	1799.75 ± 460.41	2093.84 ± 789.78	918.98 ± 108.56**
	R:S	4.29 ± 0.15	3.64 ± 0.27	4.62 ± 0.38	7.26 ± 1.38**	3.68 ± 0.27	5.38 ± 0.48**	3.43 ± 0.04	3.54 ± 0.18	4.61 ± 0.59	5.02 ± 0.29
70 DAS[a]	**LS**[g]	14.75 ± 0.48	12.50 ± 0.50**	11.00 ± 0.58	9.33 ± 0.33*	13.50 ± 0.65	10.75 ± 0.48**	14.00 ± 0.00	11.67 ± 0.67**	13.00 ± 0.58	11.00 ± 0.41**
Maturity	**FD**[h]	77.20 ± 0.20	82.67 ± 0.33**	98.00 ± 4.58	93.77 ± 3.28	76.75 ± 1.31	82.50 ± 0.96**	74.75 ± 0.48	77.33 ± 0.67**	78.67 ± 0.67	84.25 ± 0.95**
	SW[i]	30.60 ± 1.79	21.70 ± 1.82**	N/A	N/A	38.97 ± 3.93	31.19 ± 2.81	28.96 ± 2.23	15.92 ± 2.85**	16.07 ± 1.88	12.44 ± 0.97
	DM[j]	93.94 ± 2.27	69.87 ± 6.11	37.97 ± 5.90	35.5 ± 4.44	122.41 ± 19.35	116.82 ± 19.75	93.79 ± 17.14	52.02 ± 4.2**	57.00 ± 1.45	34.87 ± 4.25**
	DW[k]	124.54 ± 3.37	91.57 ± 4.29*	37.97 ± 5.90	35.5 ± 4.44	161.38 ± 19.35	148.00 ± 21.08	122.75 ± 16.38	67.93 ± 6.7**	73.07 ± 2.41	47.30 ± 4.64**

[a] days after sowing; [b] assimilation rate (mol CO_2 m-2 s-1); [d] stomatal conductance (mol H_2O m-2 s-1); [d] leaf area (cm^2); [e] total root length (cm); [f] leaf area / total root length; [g] leaf stage; [h] days to flowering; [i] seed weight (g); [j] dry matter (g); [k] dry weight (g)
Uppercase letters indicate significant differences (Student's t) among lines at pre-stress time point. Level of significance (* P<0.08 and ** P<0.05) between treatments within genotypes are shown.

Table 1.3 Analyses of variance (ANOVA) for selected morphological and physiological traits.

	Trait	F	R Squared	Line	Treatment	Line x Treatment
Pre-Stress	A[b]	8.57	0.70	<.0001	NS[l]	NS
	gsw[c]	1.78	0.33	0.120	NS	NS
	LA[d]	21.86	0.85	<.0001	NS	NS
	TRL[e]	5.92	0.62	0.0002	NS	NS
	R:S[f]	1.38	0.28	0.247	NS	NS
Limited Water	A	3.38	0.70	0.0738	<.0001	0.0253
	gsw	3.27	0.63	0.0339	0.0009	0.0291
	LA	10.86	0.85	<.0001	0.001	0.0702
	TRL	5.55	0.74	<.0001	0.1029	0.2108
	R:S	5.35	0.54	0.1458	<.0001	0.3028
24-H Recovery	A	2.55	0.59	0.0064	0.6237	0.0866
	gsw	3.54	0.67	0.0007	0.469	0.0250
	LA	11.10	0.86	<.0001	<.0001	0.0021
	TRL	5.90	0.77	<.0001	0.0615	0.0514
	R:S	7.19	0.62	<.0001	<.0001	0.8853
Nine-Day Recovery	A	4.18	0.7	<.0001	0.0571	0.0876
	gsw	3.91	0.69	0.0002	0.3993	0.0182
	LA	9.56	0.82	<.0001	<.0001	0.4908
	TRL	4.36	0.67	<.0001	0.0001	0.6433
	R:S	3.60	0.63	0.0004	0.0662	0.0424
70 DAS[a]	LS[g]	6.70	0.72	<.0001	<.0001	0.1587
Development & Maturity	FD[h]	20.93	0.88	<.0001	<.0001	0.0008
	SW[i]	11.34	0.79	<.0001	0.0053	0.0244
	DM[j]	13.64	0.82	<.0001	0.0164	0.1333
	DW[k]	20.96	0.88	<.0001	0.0078	0.0537

[a] days after sowing; [b] assimilation rate (mol CO_2 m-2 s-1); [c] stomatal conductance (mol H_2O m-2 s-1); [d] leaf area (cm^2); [e] total root length (cm); [f] leaf area / total root length; [g] leaf stage; [h] days to flowering; [i] seed weight (g); [j] dry matter (g); [k] dry weight total above ground biomass (g); [l] not significant

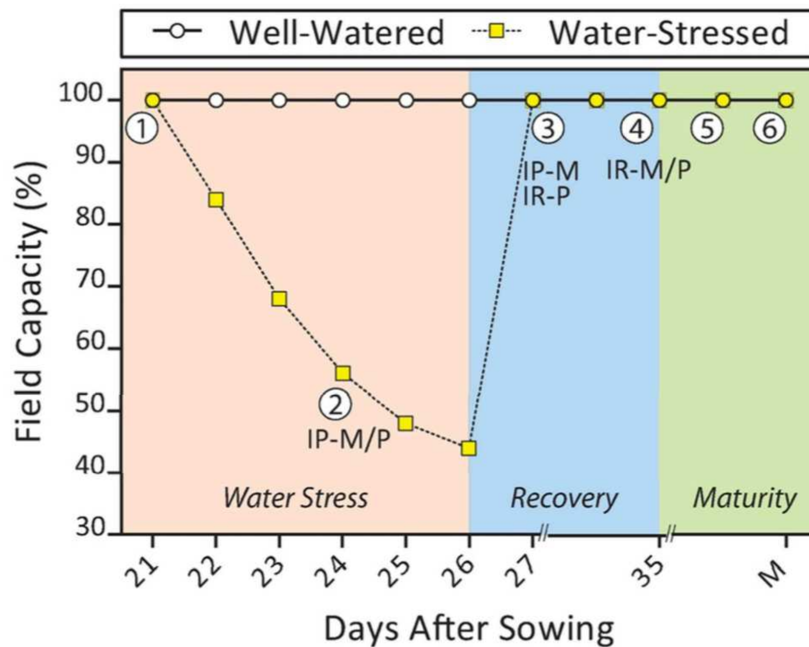

Figure 1.1. Marked time points marked (white numbered circles) demonstrate when morphological and physiological data were collected. Indices of plasticity (IP) and indices of recovery (IR) were calculated for morphological (M) and physiological (P) traits at specific time points as indicated on the figure. Time points include: (1) Pre-stress, (2) Limited Water (IP), (3) 24-H Recovery (IP, IR) and (4) Nine-Day Recovery (IR). Time points at which developmental data were collected (5) 70 Days After Sowing and (6) Maturity. A dotted line with yellow squares and a solid line with white circles represent average field capacity of water-stressed treated plants and well-watered treated plants, respectively.

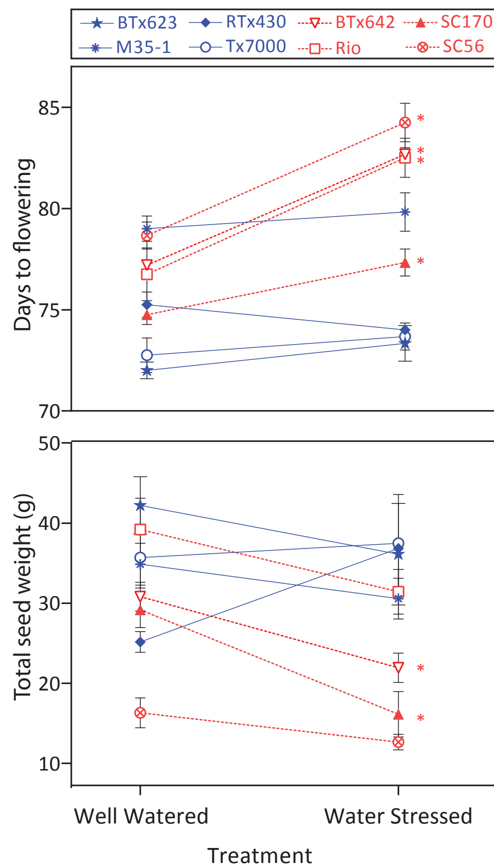

Figure 1.2. Reaction norms for **(a)** days to flowering and **(b)** total dry seed weights (g) of tolerant (blue) and susceptible (red) lines for well-watered and water-stressed treatments. Asterisks indicate significant differences ($P < 0.05$) between the two treatments within each genotype and vertical bars indicate standard errors of means.

a) Assimilation Rate

	Genotype	IP LW	IR 24-H R	IR 9-D R
Tolerant	*BTx623*	0.71	0.24	0.71
	M35-1	0.26	0.20	0.68
	RTx430	0.67	0.27	0.91
	Tx7000	0.13	0.15	0.11
Susceptible	*BTx642*	0.03	0.72	0.43
	IS3620C	0.37	0.55	0.09
	Rio	0.96	0.56	0.81
	SC170	0.07	0.09	0.21
	SC56	1.00	0.08	0.43

b) Leaf Area

	Genotype	IP LW	IP 24-H R	IR 9-D R
Tolerant	*BTx623*	0.34	0.48	0.36
	M35-1	0.08	0.26	0.48
	RTx430	0.27	0.46	0.10
	Tx7000	0.35	0.02	0.33
Susceptible	*BTx642*	0.20	0.35	0.52
	IS3620C	0.21	0.13	0.56
	Rio	0.31	0.57	0.56
	SC170	0.44	0.54	0.21
	SC56	0.32	0.64	0.57

c) Root Length

	Genotype	IP LW	IP 24-H R	IR 9-D R
Tolerant	*BTx623*	0.13	0.38	0.42
	M35-1	0.14	0.09	0.49
	RTx430	0.25	0.21	0.02
	Tx7000	0.14	0.29	0.24
Susceptible	*BTx642*	0.25	0.21	0.59
	IS3620C	0.53	0.49	0.37
	Rio	0.18	0.45	0.39
	SC170	0.24	0.36	0.20
	SC56	0.37	0.37	0.56

Figure 1.3. Index of plasticity (IP) and index of recovery (IR) for **(a)** assimilation rate, **(b)** leaf area, and **(c)** root length. Values indicate the magnitude of difference between well-watered and water-stressed treatments. Shading within columns ranges from white (smallest difference) to dark blue (largest difference) at limited water (LW), 24-H recovery (24-H R) and nine-day recovery (9-D R).

Supplementary Table 1.1.

See Miller Dissertation Supplementary Tables & Figures.xlsx

Supplementary Table 1.2

See Miller Dissertation Supplementary Tables & Figures.xlsx

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CHAPTER 2: SORGHUM RHIZOSPHERE-ENHANCED METABOLITES ARE INFLUENCED BY THE BELOWGROUND INTERACTION OF SUBSTRATE AND GENOTYPE

SUMMARY

Root exudation is an important plant process by which roots release small molecules into the rhizosphere. There is a major gap translating knowledge of root exudation in artificial systems (*i.e.* hydroponics, sterile media) to crops, specifically when grown in soils under field conditions. Sorghum (*Sorghum bicolor* L. Moench) root exudation was determined using non-targeted metabolomics and both ultra performance liquid chromatography (UPLC-) and gas chromatography (GC-) mass spectrometry (MS) to evaluate variation in exudate composition of two sorghum genotypes among three substrates (sand, clay, and soil). Above and below ground plant traits were measured to determine the interaction between sorghum genotype and belowground substrate. Plant growth and quantitative exudate composition varied largely by substrate. Two types of changes to rhizosphere metabolites were observed: rhizosphere-enhanced metabolites (REMs) and rhizosphere-abated metabolites (RAMs). There were more REMs detected in sand and clay substrates than the soil substrate. RAMs were detected at higher levels in the no-plant controls than in respective plant treatments. This study demonstrated that belowground substrate influences root exudation in sorghum, and the magnitude of exuded metabolites varied by sorghum genotype. However, metabolite identification remains a major bottleneck in non-targeted metabolite profiling of the rhizosphere.

INTRODUCTION

Plant root systems are plastic, allowing them to respond to physical, chemical and biological changes in their belowground environment (Bengough et al. 2006; Bertin et al. 2003). Root exudates, chemical compounds released from the roots into the adjacent soil (the rhizosphere), are a critical component of this plastic response (Badri & Vivanco 2009). These versatile exudates serve many purposes, including facilitating water and nutrient acquisition, mediating positive and negative microbial

symbioses, and functioning as natural pesticides and herbicides (Bais et al. 2006; Berendsen et al. 2012). The composition of these root exudates is highly variable, varying both quantitatively and qualitatively to changes in the environment as well as varying among plant species, genotypes, and even plant developmental stages (Badri & Vivanco 2009; Chaparro et al. 2013; Jones et al. 2004). The potential to utilize this variation to promote agricultural sustainability and improve crop yields is receiving attention as a promising tool in both plant breeding and agronomic practices (Bakker et al. 2012; Berendsen et al. 2012; Chaparro et al. 2012; Kuijken et al. 2015). Root exudate variation represents an opportunity to harness a plant's natural ability to respond to its environment without applying costly chemical inputs such as fertilizers, herbicides and pesticides.

Root exudation can either directly or indirectly improve plant fitness to help mitigate stressful conditions (Jones et al. 2004). For instance, when soils become compacted or dry, roots can secrete viscous mucilage to promote root growth, which increases the plant's ability to search for water and nutrients (Bengough et al. 2006). Plants can also improve fitness indirectly by exuding metabolites that recruit specific plant growth-promoting microorganisms (hereafter PGPM) in the rhizosphere that are most beneficial in the given environment (Berg & Smalla 2009; Kumar 2016). These PGPM can help buffer against extreme conditions by acquiring deficient nutrients, regulating hormone production, or by acting as biological controls to defend against pathogens (Glick 2012). Exploring how the plant interacts with its physical, chemical and biological environment can therefore help to understand the specific roles of root exudates. Furthermore, this knowledge can potentially be implemented in sustainable agricultural practices through plant variety selection, crop rotation, or biochemical soil inoculations.

Many studies have observed root exudation by evaluating plants or individual plant-microbe interactions in artificial conditions (*e.g.* hydroponic systems or sterile media), providing a baseline of knowledge. (Neumann & Romheld 2007). However, belowground interactions between the plant and its abiotic and biotic environment are much more dynamic in agricultural settings (Chaparro et al. 2012).

For example, the amount of root exudation is influenced by the presence of microorganisms and inherent soil properties (Jones et al 2004).

Several physical and chemical characteristics of the soil such as structure, Ph and previous plant cultivation greatly influence the amount of nutrient availability and impact root growth, exudation and microbial presence (Berg & Smalla 2009; Jones et al. 2004; Neumann et al. 2014; Pierret et al. 2007). Autoclaving the substrate alters these inherent soil properties including macronutrient availability, soil aggregation and organic matter structure, thus influencing patterns of root exudation (Berns et al. 2008; Liegel 1986). For instance, sterilizing soils can increase nutrient adsorption, increasing the exudation of chemical compounds such as chelators to bind nutrients (Chairidchai and Ritchie 1993). Additionally, root exudation increases in the presence of microorganisms due to microbial consumption and turnover. Therefore, sterile systems using artificial media or autoclaved soils likely underestimate the rate of root exudation in comparison to natural systems (Jones et al. 2004; Vranova et al. 2013). Therefore, by utilizing and characterizing substrates that represent soil ecosystems, we can determine the ecological significance of root exudation to improve crop production.

Root exudation differs in quantitative and qualitative composition of several different classes of metabolites, small molecules formed from plant metabolism, including carbohydrates, amino acids, organic acids, vitamins, secondary metabolites and high molecular weight compounds such as mucilage (Badri & Vivanco 2009; Dakora & Philips 2003). It is estimated that 200 plant-biosynthesized compounds can be released as root exudates (Curl & Truelove 1986). However, root exudate studies often target single metabolites or groups of metabolites, such as the case with the root exudate sorgoleone produced by the crop species, sorghum (*Sorghum bicolor* L. Moench). Sorgoleone is an allelopathic root exudate that has been studied in sorghum for its genotypic variation and its mechanism for weed suppression (Czarnota et al. 2001; Dayan et al. 2009; Netzley & Butler 1986). Yet, sorghum is a crop species that is noted for its adaption to drought and heat and it is unknown if root exudation of this

species contributes towards these tolerances. Therefore, evaluation of the broad spectrum of exudates produced in response to environmental conditions and that may aid in the plant's success is required.

Most root exudates are low molecular weight compounds that are products of both primary or specialized plant metabolism (Walker et al. 2003). Therefore, metabolomics is an attractive method to characterize how genetic and environmental factors influence root exudation. Plant metabolomics is often performed using gas chromatography-mass spectrometry (GC-MS) and/or ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) (Chaparro et al. 2013; Heuberger et al. 2014; Turner et al. 2016), with each of these platforms having their own strengths and limitations (Zhang et al. 2012). Although the progression of the metabolomics field to identify and quantify compounds is rapidly occurring with an increasing number of standards and improving methodologies (Hong et al. 2016, van Dam & Bouwmeester 2016), metabolite annotation remains a major bottleneck in non-targeted metabolomics (Dunn et al. 2012). Nevertheless, the use of non-targeted metabolomics in plant biology to understand genotypic effects on metabolite variation is becoming more common, ranging from applications in stress physiology to food quality (Hardy & Hall 2012). The use of non-targeted metabolomics across multiple platforms will identify a broad range of metabolites in the rhizosphere to determine the root exudate profile.

Here, we characterized variation in plant growth and root exudation between sorghum genotypes grown in substrates differing in physico-chemical properties. We utilized non-targeted metabolomics and both GC- and UPLC-MS platforms to ascertain the ability of each platform to extract metabolites from the rhizosphere. Furthermore, we evaluate the viable microbial presence in the rhizosphere of each genotype in each substrate to further assess the exudate profile. Taken together, we describe a robust method to evaluate genotypic exudate variation in response to various environmental conditions.

MATERIALS AND METHODS

Plant Cultivation

Two grain sorghum genotypes were utilized for this study due to their importance in breeding programs. BTx623 is a sequenced genotype that is pre-flowering drought tolerant (Paterson et al. 2009; Smith et al. 1985), whereas SC56 is a post-flowering drought tolerant genotype (Kebede 2001). After seed germination on filter paper with fungicide solution (Maxim XL, Syngenta, Greensboro, NC, USA) contained within Petri dishes, seedlings were transplanted into 1.4 liter pots containing one of three different substrates and grown in a greenhouse experiment (30°C day/ 23°C night; 50% relative humidity; 12-h photoperiod with supplemental lighting). Substrates included an all-purpose potting mix (Fafard® 4P, Sun Gro Horticulutre, Agawam, MA, USA), fritted clay (Field & Fairway™, Profile Products LLC, Buffalo Grove, IL, USA), or sand (Quikrete®, The Quikrete Companies, Atlanta, GA, USA), hereafter referred to soil, clay and sand respectively. Each pot was lined with muslin cloth, filled with substrate, soaked in water overnight, drained for one h and weighed previous to seedling transplanting to determine 100% field capacity (FC). All pots were watered every other day to 100% FC and fertilized weekly by watering with 75% Hoagland's solution to 100% FC which consisted of: KH_2PO_4; KNO_3; $Ca(NO_3)_2$; $MgSO_4$; H_3BO_3; $MgCl_2$-$4H_2O$; $ZnSO_4$-$7H_2O$; $CuSO_4$-$5H_2O$; MoO_3-H_2O; and Sequestrene 138 iron chelate.

Experimental Design

Five replicates for each genotype within a substrate were grown for 21 days after sowing (DAS) that represent the plant treatments. In addition, five replicates of bulk substrate containing no plant (no-plant control) for each of the substrates were maintained during that period by watering and fertilizing the same as the plant treatments and serving as no-plant controls. Plants were grown in a

randomized complete block design and morphological and physiological traits were assessed in addition to root exudation.

Characterization of Soil Properties & Quantitative Estimation of Viable Soil Microorganisms

In a separate experiment following the same experimental design, 50-gram substrate samples from the bulk substrates were mixed and sent to Ward Laboratories, Inc. (Kearney, NE, USA) to determine soil properties (Table 2.1). To estimate the viable microbial presence, five-gram substrate samples from the rhizosphere of each replicate containing a plant or the bulk soil of the no-plant control were taken and placed into 45 Ml of 0.85% sterile saline solution. Samples were mixed for one minute and the solution was allowed to settle. Serial dilutions were completed and transferred to 10% tryptic soy broth plus 1.5% agar plates. Plates were incubated at 28°C and colony forming units (CFU) were counted daily. Counts were then calculated by multiplying CFU by the dilution factor and soil moisture to obtain the total number of bacteria/g of dry soil.

Assessment of Morphological & Physiological Plant Traits

Green leaf area was evaluated using the LICOR LI-3100C leaf area meter (LI-COR, Inc., Lincoln, NE, USA). To assess root morphological traits, roots were extracted from the substrates and scanned using the WinRHIZO root-scanning equipment (Epson Expression 1100 XL, Epson America, Inc., Long Beach, CA, USA) and software (Regent Instruments, Inc. Quebec, QC, CA).

Metabolite Extraction

In this study, we applied a modified method from Lundberg et al. (2012). Briefly, samples were extracted from soil, clay, and sand on 21-day old sorghum plants by cutting the plant at the substrate line (if plant was present), removing the roots with rhizosphere soil attached, and placing roots into 10

Ml of 70% methanol or high performance liquid chromatography (HPLC) grade water contained within a 50 Ml conical tube. The tube was shaken for ten seconds by hand and the roots were extracted and placed into a one gallon bag with water for storage for root morphological analysis. The remaining bulk substrate from the plant treatment was then placed into a sanitized food processor and mixed for ten seconds on pulse. A five-gram subsample of the substrate was taken and placed into the respective 50 Ml conical tube, that previously contained roots. The same process to collect a five-gram subsample of substrate was completed for bulk substrates from no-plant controls. Tubes were placed on a shaker on the tube's side for two h at 24 °C and centrifuged at 23 °C, 4750xg for seven min. A two-Ml sample of the liquid portion was placed into a microcentrifuge tube and the extract was evaporated using Thermo Savant™ AES 2010 Speedvac® system (Thermo Fisher Scientific, Waltham, MA, USA). Afterwards, the extract was resuspended by adding 100 µL of 70% methanol and briefly vortexed. The samples were divided for GC- and UPLC-MS analyses, with 50 µL transferred into respective microcentrifuge tubes for GC-MS, and the other 50 µL transferred into glass inserts in autosampler vials for UPLC-MS.

Metabolite Detection by Gas Chromatography – Mass Spectrometry

To prepare samples for GC-MS analysis, 50 Ml of extract was dried using a speedvac, resuspended in 50 Ml of pyridine containing 50 mg/Ml of methoxyamine hydrochloride, incubated at 60 °C for 45 min, sonicated for 10 min, and incubated for an additional 45 min at 60 °C. Next, 25 Ml of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS, Thermo Scientific) was added and samples were incubated at 60°C for 30 min, centrifuged at 3000xg for 5 min, cooled to room temperature, and 80 Ml of the supernatant was transferred to a 150 Ml glass insert in a GC-MS autosampler vial. Metabolites were detected using a Trace GC Ultra coupled to a Thermo ISQ mass spectrometer (Thermo Scientific). Samples were injected in a 1:10 split ratio twice in discrete randomized blocks. Separation occurred using a 30 m TG-5MS column (Thermo Scientific, 0.25 mm i.d.,

0.25 µm film thickness) with a 1.2 Ml/min helium gas flow rate, and the program consisted of 80°C for 30 seconds, a ramp of 15°C per minute to 330°C, and an 8 min hold. Masses between 50-650 m/z were scanned at 5 scans/sec after electron impact ionization.

Metabolite Detection by Ultra Performance Liquid Chromatography – Mass Spectrometry

For UPLC-MS analysis, 50 Ul of extract was dried under nitrogen and resuspended in 100 Ml of methanol. Then, 5 Ml of extract was injected twice (n=2 replicates) onto a Waters Acquity UPLC system in discrete, randomized blocks, and separated using a Waters Acquity UPLC HSS T3 column (1.8 µM, 1.0 x 100 mm), using a gradient from solvent A (water, 0.1% formic acid) to solvent B (Acetonitrile, 0.1% formic acid). Injections were made in 100% A, held at 100% A for 1 min, ramped to 98% B over 12 min, held at 98% B for 3 min, and then returned to starting conditions over 0.05 min and allowed to re-equilibrate for 3.95 min, with a 200 µL/min constant flow rate. The column and samples were held at 50 °C and 5 °C, respectively. The column eluent was infused into a Waters Xevo G2 Q-TOF-MS with an electrospray source in positive mode, scanning 50-1200 m/z at 0.2 sec per scan, alternating between MS (6 V collision energy) and MSE mode (15-30 V ramp). Calibration was performed using sodium formate with 1 ppm mass accuracy. The capillary voltage was held at 2200 V, source temperature at 150 °C, and nitrogen desolvation temperature at 350 °C with a flow rate of 800 L/hr.

Metabolomics Data Analysis

For each sample, raw data files were converted to .cdf format, and matrix of molecular features as defined by retention time and mass (m/z) was generated using XCMS software in R (Smith et al. 2006) for feature detection and alignment. Raw peak areas were normalized to total ion signal in R, outlier injections were detected based on total signal and PC1 of principle component analysis, and the mean area of the chromatographic peak was calculated among replicate injections (n=2). Molecular features

were clustered using RAMClustR (Broeckling et al. 2014), which groups features into spectra based on coelution and covariance across the full dataset, whereby spectra are used to determine the identity of observed compounds in the experiment (*i.e.* spectral clusters approximate individual compounds). The peak areas for each feature in a spectrum were condensed via the weighted mean of all features in a spectrum into a single value for each compound. Metabolites were annotated by searching against in-house and external metabolite databases including NIST v12, Massbank, Golm, and Metlin. Annotated compounds were grouped into the following chemical classes: carbohydrates, amino acids, organic acids, vitamins and other (Badri & Vivanco 2009; Dakora & Philips 2003).

Statistical Analysis

Morphological traits were statistically analyzed by using an Analysis of Variance (ANOVA) for genotype, treatment and their interaction using JMP Pro 11 (SAS Institute, Cary, NC, USA), followed by the Student's t-test. Data were box-cox transformed prior to analysis in order to improve normality. Statistics assessing microbial presence were completed in JMP Pro 11 using ANOVA and a student's t-test was computed to determine statistical significance among genotypes and substrates. Data were log transformed prior to analysis.

For metabolite statistical analysis, GC- and UPLC-MS data were combined and a principle components analysis (PCA) was performed using SIMCA v14.0 (Umetrics, Umea, Sweden) with unit variance (UV) scaling. Within each substrate, ANOVAs were performed by using the aov function in R (R Development Core Team, 2012). A false discovery rate (FDR) adjustment was used on the p-values using p.adjust function (Benjamini and Hochberg 1995). Log_2 fold changes (FC) were calculated by: log_2 (plant treatment mean trait value / control mean trait value). Rhizosphere-enhanced metbolites (REMs) were those that were significant ($p < 0.05$) after applying the FDR adjustment and had a log_2 FC of greater

than one. Rhizosphere-abated metabolites (RAMs) were those that were significant after applying the FDR adjustment and had a log_2 FC of less than negative one.

RESULTS

Three substrates (clay, sand and soil) differing in physico-chemical properties were utilized to compare plant growth and exudate production in sorghum (see Table 2.

2.1 for soil properties). Two sorghum genotypes were evaluated within each substrate for their above and belowground morphological characteristics to determine how substrate affects plant growth. To assess metabolites enriched by the plant's rhizosphere, controls within each substrate did not contain a plant (no-plant controls) and were designed to distinguish metabolites that were characteristic of the bulk substrate, and therefore determine which metabolites were rhizosphere-associated. We termed exudates as rhizosphere-associated as they may encompass both plant and microbial exudates. Additionally, we evaluated two extraction buffers, 70% methanol and 100% HPLC grade water, to determine which extract buffer results in metabolite data that is consistent and encompasses a wide range of biochemical compounds from plant rhizospheres among substrates.

Variation in Plant Morphology is Largely Influenced by Substrate

An experiment was conducted to understand how substrates influence sorghum's allocation of resources to above and below ground traits. Sorghum plants were grown in three substrates for 21 days, after which leaf areas and several root traits were measured (Figure 2.1). Leaf areas ($p < 0.0001$) were smaller for plants grown in sand and clay than plants grown in soil, and there were no differences between sorghum genotypes (Figure 2.1A). Substrate also affected root morphology (Figures 2.1B and 2.1C). Plants grown in sand had the shortest total root lengths ($p < 0.0001$) and largest average root diameters ($p < 0.0001$), and this effect was comparable across genotypes. Total root lengths and average root diameters were more similar between plants grown in clay and soil in comparison to those grown in

sand. However, genotype BTx623 had longer total root lengths than SC56 in soil, while genotype SC56 had larger average root diameters than those of BTx623 in both clay and soil substrates. Overall, plants grown in sand had smaller above and below ground biomass investments than plants grown in clay or soil.

Non-Targeted Metabolomics Detected Rhizosphere-Associated or –Abated Exudates

We detected metabolites using a non-targeted metabolomics approach and two extraction buffers: 70% methanol and 100% HPLC water. The water extraction resulted in overall very low metabolite diversity and signal intensity and was determined to be insufficient for metabolomics analysis (data not shown). Thus, we focused on utilizing samples extracted with 70% methanol. The GC- and UPLC-MS analyses resulted in 34,718 and 2,929 molecular features (Smith et al. 2006) that were deconvoluted into an estimated 829 and 475 compounds, respectively (Broeckling et al. 2014).

The metabolomics data was evaluated to compare trends in the root-exuded metabolite profiles using principal component analysis (PCA) on the total 1,304 compounds. Four principle components (PC) explained 64% of the variation. PC1 (28.1%) and PC3 (10.6%) explained variation associated with substrate and plant treatment (Figure 2.2A), respectively. The PCs separated by substrate (PC1, soil and clay/sand) and plant treatment (PC3, BTx623/SC56 and Control). PC4 also displayed variation attributed to substrate (7.5%) (clay and soil/sand) (Figure 2.2B). PC2 (17.8%) was variation not attributed to sorghum genotype or substrate, for example potentially due to variation by plant replicates (Figure 2.2B). The PCA supports that overall variation in metabolites (*i.e.* the type of metabolites, and the abundance of the metabolite) is influenced by both substrate and plant treatment.

Individual metabolites that varied due to genotype and substrate were determined by an ANOVA, as well as an ANOVA conducted within each substrate (FDR adjusted $p < 0.05$). Additionally, each plant treatment (BTx623 and SC56) was evaluated for metabolites that increased or decreased

compared to the no-plant control within each substrate. Metabolites that changed by ±2-fold (plant treatment / no-plant control) were considered changing within the system. Changes that were 2-fold or greater were considered *rhizosphere-enhanced metabolites* (REMs), as it cannot be determined if they were produced from the plant or by microorganisms within each substrate. Additionally, metabolites of – 2-fold or less were considered diminished, and are termed *rhizosphere-abated metabolites* (RAMs). ANOVA p-values and $\log_2$ fold changes (FC) between each genotype and control for all detected metabolites are displayed as volcano plots (Figure 2.S1). Hereafter, we will describe metabolites using the term $\log_2$ FC to indicate the relative amounts detected between plant treatments and no-plant controls and compare across substrates.

Using p-values (FDR adjusted $p < 0.05$) from ANOVAs conducted within each substrate and fold change criteria ($\log_2 FC > 1.0$) for both sorghum genotypes, a total of 219 compounds varied. It was found that 73 REMs varied in clay (5.6% of the detected metabolites), 105 varied in sand (8.1%), and 11 REMs varied in soil (0.8%) (Table 2.2). Of the REMs, only eight were common to all three substrates (Figure 2.3A). Clay and sand had the most shared compounds (49 compounds) and sand had the most substrate specific compounds (47 compounds). For rhizosphere-abated metabolites, 62 RAMs varied in clay (4.8%), 57 RAMs varied in sand (4.4%), and 2 RAMs varied in soil (0.2%) (Table 2.2). Sand and clay shared the highest number of RAMs with 25 compounds (Figure 2.3B). Clay had the largest number of substrate specific RAMs (37 compounds).

Annotated Metabolites Represent Known Root Exudates

Of the 1,304 detected compounds, only 42 metabolites could be annotated based on matching retention time and mass spectra to in-house, external, and theoretical metabolite databases including 30 metabolites from GC-MS and 14 metabolites from UPLC-MS dataset (Table 2.3). These metabolites include carbohydrates (18), amino acids (15), organic acids (5), vitamins (1) and other metabolites (3)

that are known to be root exudates. A list of annotated metabolites can be found in Table 2.3. It should be noted that the annotated metabolites represent a small portion of the varying metabolites within each substrate, and not all of the annotated metabolites demonstrated signficant differences when comparing each plant treatment to respective no-plant controls (Table 2.3). There were many other significantly represented, unidentifiable metabolites that displayed consistent trends across the substrates. We present a subset of annotated metabolites that were rhizosphere-enhanced metabolites to include two sugars (sucrose, trehalose), an amino acid (tryptophan), and organic acids (quinic acid, malic acid) (Figure 2.4). In addition, we provide an example of a metabolite that was a rhizosphere-abated metabolite (glycerol).

Within each of the clay, sand, and soil substrates, sucrose was detected at the lowest levels in no-plant controls compared to plant treatments (Figure 2.4A). In both BTx623 and SC56 plant treatments, sucrose was detected at significantly higher levels in clay and trended to higher levels for both plant treatments in sand compared to respective no-plant controls (Figure 2.4A; Table 2.3). In clay, sucrose was found to have the highest $\log_2$ FCs for each plant treatment compared to those in other substrates (Table 2.3). Additionally, sucrose had the highest $\log_2$ FC compared to all other metabolites detected within the clay substrate.

Tryptophan was detected at low levels in each of the substrate's no-plant controls (Figure 2.4B). In both clay and sand, tryptophan was detected in both plant treatments at significantly higher levels than their respective no-plant controls. Tryptophan was detected at the highest level in the plant treatments of the sand substrate, followed by the clay and soil substrates. The organic acid quinic acid was detected at significantly higher levels in each of the plant treatments within all of the substrates (Figure 2.4C). Malic acid in both plant treatments was detected at higher levels in clay (Figure 2.4D). Although not significant, malic acid was detected with the highest $\log_2$ FC in sand (Table 2.3; Figure 2.4D).

Across no-plant controls, trehalose varied in abundance, with its lowest detected presence in the sand no-plant control (Figure 2.4E). Trehalose was detected with the highest $\log_2$ FC in sand and was significantly different in the SC56 plant treatment although it also trended higher in BTx623 within this substrate. One annotated metabolite, glycerol, was detected at significantly higher levels in the no-plant controls than both plant treatments grown in sand or clay (Figure 2.4F).

Viable Microbial Presence Indicates Varying Levels of Detected Microorganisms

When comparing the no-plant controls of the three substrates, the highest number of viable bacteria was detected in the soil, followed by clay and then sand (Figure 2.5). Within soil, the SC56 plant treatment had a slightly lower microbial presence than the no-plant control. Within the clay and sand substrates, both plant treatments had substantially greater viable microbial counts than respective no-plant controls. Among substrates, both genotypes kept a relatively consistent microbial presence. However, the microbial presence for the SC56 plant treatment displayed lower trends than that of BTx623 within each substrate.

DISCUSSION

This study utilized non-targeted metabolomics to understand variation in rhizosphere-associated exudation due to substrate and genotype in sorghum. Unlike traditional approaches that detect root exudation in artificial media, our protocol can compare how plant-environment interactions differ among substrates and genotypes. This platform is especially powerful moving forward, as we can now effectively study how manipulating below ground environment (*e.g.*, nutrient deficiencies, toxicities, microbial inoculations, exogenous biochemical applications) mediates plant-environment interactions via metabolite exudation across a variety of genotypes.

Here we find that exudate variation was largely driven by substrate, but plant genotypes also played a role. Our findings in exudate variation reflect trends in the microbial literature, as microbial

communities in the rhizosphere of *Arabidopsis thaliana* were also largely influenced by substrate and to a lesser degree by genotype (Lundberg et al. 2012). More recently, a study conducted using a hydroponic system found genetic variation in root exudate composition of *A. thaliana* utilizing non-targeted metabolomics profiling with UPLC-MS (Mönchgesang et al. 2016 a). In our study utilizing substrates that represent various soil conditions, we found mostly quantitative differences among metabolites existed between substrates and genotypes. Other studies have found variation in root exudates in response to growth substrate; root exudates from a single variety of lettuce (*Lactuca sativa*) grown in three different substrates that differed in previous plant cultivation exhibited mostly quantitative differences in metabolites exuded between the substrates using GC-MS analysis (Neumann et al. 2014). Several of the annotated metabolites in our study represented known root exudates; these metabolites suggest that our method is a valid approach to characterizing rhizosphere-associated exudation. Past studies have identified functional roles of root exudates, but offer limited inference about how plants interact with their surroundings due to their artificial environments (e.g. hydroponic systems, sterile media). Here, we evaluated a method to extract rhizosphere-associated metabolites of two sorghum genotypes grown in complex substrates that represent growing conditions. Furthermore, we illustrate this method's utility by discussing a subset of annotated exudates in each substrate and how these metabolites may serve in their respective environments.

Rhizosphere-associated exudation responds to stressful abiotic conditions

Of all the substrates, sand represented the poorest conditions for plant growth; it had the highest mechanical impedance due to its high bulk density and lowest soil moisture content (Table 2.1). Additionally, it had the highest Ph and the lowest nutrient availabilities. Furthermore, sand had the largest number of detected rhizosphere-enhanced metabolites among the substrates, especially on the UPLC-MS platform. As root exudates fluctuate in response to environmental conditions (Bais et al.

2006), many of these exudates were likely involved in buffering the harsh abiotic conditions of the sand environment.

Mechanical impedance limits root growth, and results in enlarged root diameters to facilitate growth within a dense substrate (Boeuf-Tremblay et al. 1995). After growth in sand, root lengths were the smallest while average root diameters were the largest (Figure 2.1). In addition, plant roots increase root exudation of viscous compounds such as mucilage to reduce friction (Bengough et al. 2006; Boeuf-Tremblay et al. 1995). Although we were unable to annotate many of the exudates present in the sand environment, some may be involved in overcoming mechanical impedance. Furthermore, increased exudation of mechanically impeded roots is predicted to increase the microbial presence within the rhizosphere, aiding in nutrient acquisition (Watt, McCully & Kirkegaard 2003). We detected more rhizosphere-enhanced metabolites and rhizosphere-abated metabolites in the clay and sand substrates compared to the soil substrate (Figure 2.3); in these substrates, we also found a significant increase in viable microbial presence in plant treatments grown relative to their bulk substrate controls (Figure 2.5). This suggests that an increase in the number of exudates enriches the microbial presence.

We detected large $\log_2$ FC in metabolites that may be involved in plant stress tolerance. For instance, trehalose is a disaccharide especially associated with abiotic stress such as drought, high salinity or extreme temperatures in both plants and microorganisms (Fernandez et al. 2010; Glick 2012). We found trehalose to be enriched in the plant treatments in the sand substrate (Table 2.3). Although the detected presence of trehalose in the sand no-plant controls and plant treatments were not as high as detected in the clay or soil substrates, trehalose did not significantly differ nor display a trend between either plant treatment grown in the clay or soil substrates in comparison to respective no-plant controls (Table 2.3; Figure 2.4E). Therefore, trehalose in the plant treatments of the sand substrate likely serves to cope with the stressful abiotic environment.

Additionally, other metabolites largely varied in sand in comparison to the clay or soil substrates. Organic acids are associated with buffering environmental conditions such as nutrient toxicities or deficiencies, particularly in environments with a high Ph as was the case with the sand substrate (Table 2.1) (Bais et al. 2006; Lopez-Bucio et al. 2000). Furthermore, organic acids can be released by the plant to attract specific microorganisms, as well as be released by microorganisms themselves in unfavorable environmental conditions to act as chelators to increase nutrient availability (Jones et al. 1998). A major organic acid in our system was quinic acid, which was detected with the highest $\log_2$ FC for each plant treatment in the sand substrate (Table 2.3). We cannot conclude whether its origin is from the plant or microbe, however quinic acid has been found to increase bacterial richness in the soil as a root exudate in 50radiata pine (*Pinus radiate*) (Shi et al. 2011). Additionally, quinic acid serves as a precursor to many secondary metabolites utilized by both plants and microorganisms (Guo et al. 2014; Minamikawa 1976). Chaparro et al. (2013) found that secondary metabolites such as phenolics found in root exudates of *A. thaliana* were associated with an increased number of microbial functional genes that correspond to secondary metabolism. These secondary metabolites serve several functions including growth promotion and defense against abiotic and biotic stressors (Weston & Mathesius 2014).

Another organic acid that increases in the unfavorable sand environment, malic acid, was also detected with the highest $\log_2$ FC for each plant treatment in the sand substrate, although not found to be significant in this substrate (Table 2.3; Figure 2.4D). The lack of significance for this metabolite in sand is likely due to the large variation between plant replicates particularly in comparison to other substrates. However, the large $\log_2$ FC for each plant treatment in sand suggests that malic acid is a rhizosphere-associated exudate. Malic acid has been implicated in attracting beneficial bacteria and improving nutrient availability (Hunter et al. 2014; Rudrappa et al. 2008). As other organic acids have been associated with improving nutrient availabilities (Jones et al. 1998), it is likely that a portion of the

un-annotated metabolites in the sand substrate include organic acids among other metabolites that directly or indirectly improve nutrient availabilities.

Root exudates serve to enlist plant growth-promoting bacteria

Sugars function to provide microorganisms with readily available sources of energy (Behera & Wagner 1974). We detected high $\log_2$ FC for sucrose, glucose and fructose among other sugars for both plant treatments, especially in the clay and sand substrates (Table 2.3). This may serve to recruit a general population of microorganisms as both clay and sand no-plant controls had significantly lower viable microbial presences than the soil bulk substrate control (Figure 2.5). In particular, it has been indicated that the exudation of sugars early in the development of *A. thaliana* may be effective in enlisting a general population of microorganisms (Chaparro et al. 2013). However, it is the exudation of amino and organic acids that may attract more specific microorganisms that help to promote plant growth (Yang et al. 2015).

One of many ways that PGPM serve the plant is by producing the growth-stimulating phytohormone auxin (Spaepen & Vanderleyden 2011). More than 80% of rhizosphere bacteria are estimated to produce IAA (indole-3-acetic acid), a dominant form of auxin that promotes plant growth (Barea et al. 1976). The primary biosynthetic pathway to IAA is through tryptophan metabolism, which can be conducted by plants or soil microorganisms (Frankenberger and Arshad 1995). We found tryptophan to be present with the highest $\log_2$ FC in the sand substrate, followed by the clay and soil substrates (Table 2.3; Figure 2.4B). Additionally, plants grown in sand were the smallest in regards to both leaf area and total root length (Figure 2.1). This finding suggests that plants grown in sand increased production of tryptophan in an attempt to promote plant growth in the sand substrate through auxin synthesis.

Metabolites can be abated by the rhizosphere environment

Of particular interest is the ability of this method to determine rhizosphere-abated metabolites (RAMs). Although log_2 FC among these metabolites were not especially large in comparison to some of the detected rhizosphere-enhanced metabolites in the rhizosphere, there were still several significant metabolites in the clay and sand substrates that were detected at lower amounts in the plant treatments than respective controls (Table 2.2). Only one metabolite, glycerol, was able to be annotated that was rhizosphere-abated in the clay and sand substrates (Table 2.3; Figure 2.4F). Glycerol is a sugar alcohol that can be produced by plants or microorganisms to act as an 52smolytes to protect against osmotic stress (Bolen 2001; Shen et al. 1999). Glycerol can also be a carbon and energy source for microorganisms (Nikel et al. 2015). However, the presence of glycerol in the rhizosphere has been found to have negative effects on plant root growth in *A. thaliana* where it affects auxin distribution (Hu et al. 2014). Although other studies have detected glycerol as a root exudate (Chaparro et al. 2013; Neumann et al. 2014), our study provides the novel perspective of glycerol in the belowground plant-environment interaction. Glycerol may be produced in the bulk substrates of clay and sand by microorganisms. Furthermore, glycerol dissimilation may be occurring by both microorganisms and/or plants, serving as a carbon source or counteracting its effects as root growth inhibitor.

We were able to annotate one of the rhizosphere-abated metabolites in the soil substrate as a sugar alcohol (Table 2.3). Sugar alcohols such as sorbitol or mannitol are utilized as substrates by microorganisms and enrich the soil microbial functional diversity when added as a soil amendment (Yu et al. 2016). This sugar alcohol may be consumed by a diverse group of microorganisms in the rhizosphere of the soil substrate as the soil no-plant control already has a high viable microbial presence (Figure 2.5).

Rhizosphere-associated metabolite detection and analysis considerations

In metabolomics, it is well known that the extraction and analytical methods implemented largely influences the detected metabolites (Johnson et al. 2016). Therefore, considerations when utilizing this method to determine rhizosphere-associated metabolites within a substrate include evaluating 1) the large plant replicate variation that may impact detecting metabolites of interest, 2) edaphic factors that likely affect the metabolite extraction/presence and 3) the ability of the platform chosen to detect metabolites.

First, there was a relatively low number of significant metabolites detected within the soil substrate using our criteria in comparison to the clay or sand substrates, but several annotated metabolites were likely produced by the plant as evidenced in $\log_2$ FC (Table 2.3). For example, sucrose was among the metabolites having one of the largest $\log_2$ FC detected within the soil substrate, but was not considered significant for the SC56 plant treatment (Table 2.3). Furthermore, $\log_2$ FCs for sucrose were high for all plant treatments in every substrate, even though not all were considered significant. As sucrose is a well-established component of the root exudate profile, it is reasonable to conclude that it was detected at a higher presence in both plant treatments within the soil substrate. It is likely that the large plant-to-plant variability (biological variability) contributes to the lack of significance (as we similarly found for malic acid in the sand substrate). Indeed, plant-to-plant variability represents a large portion of total variation in root metabolite profiles, with the amount of variation differing between different classes of metabolites (e.g. sugars, organic acids, amino acids, phenylpropanoids, flavonoids) (Mönchgesang et al. 2016 b). Due to this variation, a large number of replicates are needed to maintain statistical power particularly when analyzing a broad range of classes of metabolites as in non-targeted metabolite profiling (Mönchgesang et al. 2016 b). Additionally, choosing the appropriate extraction buffer and altering extraction buffer concentrations should be considered. A recent study has shown that plant replicate variation increases when using a higher concentration of methanol buffer, which

may be attributable to minor root damage (Petriacq et al. 2017). We also propose that future metabolite analyses should incorporate total root lengths to standardize total root exudation across plants and account for variation in plant size.

Second, there are several intrinsic factors of the soil substrate that likely obscured the number of significant metabolites detected in this substrate. For instance, soil had a high percentage of organic matter, a high cation exchange capacity (CEC), and an initial high viable microbial presence which may contribute to binding and turnover of compounds (Table 2.1; Figure 2.5). Furthermore, some rhizosphere-associated metabolites (i.e. phenylalanine) were detected at higher levels and with more variation in the bulk substrate controls of soil compared to the clay and sand controls (data not shown). Therefore, it is likely that several other metabolites were not considered significant within this substrate due to their pre-existence, but are of interest. Although our analyses indicate that sand and clay substrates have more detected metabolites in common (Figures 2.2A and 2.3), this may be due to the intrinsic properties of soil that mask the number of detectable metabolites that were both significant and had a $\log_2$ FC greater than one. We propose that future studies should consider metabolites of interest with more liberal p-values and $\log_2$ FC. However, implementing a combination of visual tools such as volcano plots with multivariate and univariate statistical analyses and z-score test statistics to determine metabolites of interest will also help to determine rhizosphere-associated metabolites. Advantages and disadvantages of several aspects of univariate analyses in non-targeted metabolomics profiling can be reviewed in Vinaixa et al. (2012).

Lastly, our study utilized the UPLC-MS platform in addition to the GC-MS platform, which provided insight into a wide range of metabolites of interest. For instance, on the UPLC-MS platform, we detected the aromatic amino acids (phenylalanine, tryptophan and tyrosine) (Table 2.3), which serve as precursors to many secondary metabolites and hormones that aid in plant abiotic or biotic stress tolerance (Davies 2010; Moe et al. 2013; Tzin & Galili 2010). Although GC-MS is an effective tool in

detecting sugars and various amino and organic acids that are prevalent in the root exudate profile such as these aromatic amino acids, the inability to annotate these on the GC-MS platform in our study reflects the value of using multiple platforms. Additionally, the identification of a dhurrin, a species-specific cyanogenic glycoside associated with sorghum (Busk & Møller 2002) was identified using the UPLC-MS platform. Therefore, using both platforms allowed for a more comprehensive understanding of the root exudate profile.

Several metabolites could not be annotated that were of interest between both platforms. However, the continual addition of metabolites to databases will contribute to the progression of metabolite identifications. Furthermore, the root exudate profile likely contains secondary metabolites that are more specialized or species-specific, such as the allelopathic compounds juglone exuded by black walnut or sorgoleone exuded by sorghum (Bertin et al. 2003). As these metabolites are not as commonly quantified as sugars, amino and organic acids that are prevalent throughout metabolomics studies, the development of standards is required to annotate these secondary metabolites and their derivatives.

This study demonstrated an effective method to determine rhizosphere-associated metabolites involved in belowground plant-environment interactions using non-targeted metabolomics profiling. Among the substrates examined, we suggest fritted clay as an appropriate substrate for future greenhouse studies. When trying to make inferences about the natural environment, fritted clay simulates more representative conditions than the sand or soil substrates. Plant growth was not as limited in the clay substrate compared to the sand substrate. Additionally, although plant above ground traits may vary between clay and soil, below ground traits are generally more similar. Furthermore, a higher number of metabolites are detected in clay and sand while only a few were differentially detected in soil. Future studies should explore the utility of this method in examining rhizosphere-associated metabolites in response to varying environmental conditions (abiotic and biotic stress) and

within field soils. Exploring root exudation in the context of the soil ecosystem allows for a more accurate representation of the belowground plant-environment interaction and therefore may serve as a useful tool in designing more sustainable cropping systems.

TABLES AND FIGURES

Table 2.1. Characteristics of bulk substrates used in this study. Characteristics were analyzed using no-plant controls at 21 days after sowing and included the addition of Hoagland solution as a fertilizer.

Substrate	**Sand**	**Clay**	**Soil**
Bulk Density (g/cm^3)	1.55	0.66	0.33
Organic Matter (LOI[a] %)	0.2	<0.1	50.1
Cation Exchange Capacity (me/100g)	2.6	8.0	16.8
Gravimetric Soil Moisture (%)	25.9	92.7	135.7
Organic Carbon (%)	0.019	0.067	24.537
pH	8.4	5.2	5.8
Nitrogen (ppm)	19	98	6178
Phosphorous (ppm)	12	50	107
Potassium (ppm)	44	604	315
Sulfate (ppm)	7	55	136
Zinc (ppm)	0.14	0.35	2.72
Iron (ppm)	3.9	32.4	25.2
Manganese (ppm)	0.5	9.3	4.2
Copper (ppm)	0.18	0.05	0.35
Calcium (ppm)	404	491	998
Magnesium (ppm)	62	175	346
Sodium (ppm)	12	27	35

[a] loss on ignition

Table 2.2. Number of metabolites of interest detected within sand, clay and soil used in this study. Metabolites of interest were determined after adjusting p-values for false discovery rate and using $p < 0.05$ and a $\log_2$ fold change of >1 (REMs) or <-1 (RAMs).

Substrate	Number metabolites of interest	GC-MS	UPLC-MS	REMs	RAMs
Sand	162	119	43	105	57
Clay	135	113	22	73	62
Soil	13	9	4	11	2

Table 2.3. List of annotated metabolites grouped by carbohydrates, amino acids, organic acids, vitamins and other along with the platform detected, GC- or UPLC-MS. Metabolites that were annotated at a chemical class level are numbered if there are multiples (*i.e.* disaccharide 01, disaccharide 02). Associated log_2 fold changes and FDR adjusted p-values for each genotype within each substrate are displayed. Bolded p-values are less than 0.1000.

		Sand				**Clay**				**Soil**			
Metabolite	**Platform Detected**	**BTx623**		**SC56**		**BTx623**		**SC56**		**BTx623**		**SC56**	
Carbohydrates		***log_2 FC***	***p-value***	***log_2 FC***	***p-value***	***log_2 FC***	***p-value***	***log_2 FC***	***p-value***	***log_2 FC***	***p-value***	***log_2 FC***	***p-value***
disaccharide 01	UPLC-MS	6.01	**<0.0001**	5.54	**0.0101**	3.04	**0.0343**	2.69	**0.0289**	0.49	0.5546	0.40	0.5050
disaccharide 02	UPLC-MS	6.90	**0.0005**	5.09	**0.0080**	1.23	**0.0293**	1.59	**0.0560**	-0.49	0.6437	-0.16	0.8950
fructose	GC-MS	7.72	**0.0008**	7.14	**0.0006**	7.29	**0.0034**	6.80	**0.0241**	2.06	0.1461	2.18	0.2888
hexose sugar acid	GC-MS	1.10	**0.0330**	0.70	0.1017	4.13	**0.0093**	3.66	**0.0627**	1.15	0.1156	1.24	0.4533
hexose + glutamine	UPLC-MS	5.93	**<0.0001**	4.97	**0.0010**	3.48	**0.0137**	3.17	**0.0259**	0.94	0.1785	0.33	0.5323
glucose	GC-MS	8.09	**0.0042**	7.88	**0.0067**	6.65	**0.0025**	6.11	**0.0247**	0.24	0.2436	0.29	0.7694
glycerol	GC-MS	-2.84	**0.0010**	-2.10	**0.0015**	-1.48	**0.0041**	-0.90	**0.0261**	-0.49	0.7143	-0.38	0.7425
hexose 01	GC-MS	7.54	**0.0013**	7.56	**0.0026**	6.24	**0.0190**	5.60	**0.0498**	0.05	0.2892	0.12	0.7679
hexose 02	GC-MS	4.39	**0.0012**	4.04	**0.0051**	3.93	**0.0254**	3.70	**0.0368**	-0.08	0.7143	0.04	0.9964
inositol-like	GC-MS	3.31	**0.0508**	2.84	**0.0121**	1.91	**0.0237**	1.41	**0.0188**	-0.07	0.6744	0.10	0.8236
myo-inositol	GC-MS	4.60	**0.0006**	4.96	**0.0004**	4.39	**0.0120**	4.03	**0.0113**	0.31	0.2587	0.30	0.4533
pentose	GC-MS	3.67	**0.0004**	3.54	**0.0158**	3.74	**0.0025**	3.60	**0.0244**	1.05	0.1785	0.99	0.3661
sucrose	GC-MS	6.53	0.1135	6.20	**0.0666**	8.56	**<0.0001**	8.32	**0.0200**	5.21	**0.0635**	4.93	0.3333
sugar alcohol 01	GC-MS	5.16	0.1614	5.68	**0.0486**	3.25	0.5230	3.94	0.2763	-0.13	0.8860	0.65	0.8556
sugar alcohol 02	GC-MS	5.94	0.2057	7.22	**0.0382**	0.19	0.9370	1.64	0.3052	-1.31	0.1206	-1.75	0.0286
sugar alcohol 03	GC-MS	0.58	0.2693	0.37	0.2932	0.89	0.2100	0.64	**0.0955**	0.69	0.1557	2.01	0.2259
trehalose	GC-MS	3.95	0.2333	5.51	**0.0489**	-1.46	0.2067	0.24	0.9132	-0.33	0.9717	0.02	0.7694
trisaccharide	GC-MS	-0.21	0.7571	0.07	0.9323	-0.67	0.3383	-0.58	0.3475	0.49	0.6368	2.01	0.4472
Amino Acids													
alanine	GC-MS	4.28	**0.0002**	4.24	**0.0820**	3.21	**0.0137**	2.71	**0.0150**	1.29	**0.0895**	1.05	0.3161
B-alanine	GC-MS	3.67	**0.0012**	3.97	**0.0379**	1.37	0.4369	1.96	**0.0851**	0.78	0.1206	0.69	0.6791
aminobutyric acid	GC-MS	2.50	**0.0026**	2.51	**0.0724**	0.87	0.3710	1.25	0.1438	0.80	0.2942	0.85	0.6579
$C_5H_{11}NO_2$ (betaine or valine)	UPLC-MS	5.14	**0.0008**	2.45	**0.0379**	0.63	0.3200	0.53	0.2153	-0.88	0.5546	-1.59	0.4187
choline + glutamic acid	UPLC-MS	5.20	**0.0065**	5.04	**0.0018**	2.40	**0.0145**	2.19	**0.0259**	1.35	**0.0645**	1.06	0.1726
glycine	GC-MS	2.04	**0.0100**	2.02	**0.0617**	0.54	0.7521	1.08	**0.0631**	0.49	0.3633	0.52	0.7180
leucine	UPLC-MS	5.25	**0.0001**	5.59	**0.0215**	4.26	**0.0258**	3.90	**0.0251**	0.87	0.3550	0.51	0.7694
phenylalanine	UPLC-MS	7.48	**0.0256**	7.34	**0.0205**	4.56	**0.0134**	4.73	**0.0042**	1.43	0.2979	1.46	0.2670
pyroglutamate	GC-MS	3.45	0.2487	2.57	0.4107	3.36	0.4140	1.43	0.2219	1.01	0.5871	2.09	0.4284

Compound	Platform												
serine	GC-MS	4.02	**0.0002**	3.85	**0.0219**	3.59	**0.0020**	2.90	**0.0261**	1.99	0.1103	1.76	0.2145
threonine	GC-MS	4.42	**0.0010**	4.90	**0.0995**	2.45	0.3479	3.69	**0.0851**	0.71	0.2389	0.55	0.7657
tryptamine	UPLC-MS	4.97	0.1013	5.22	**0.0213**	5.68	**0.0121**	5.70	**0.0113**	1.25	**0.0797**	2.36	0.1350
tryptophan	UPLC-MS	6.88	**0.0033**	7.76	**0.0122**	4.73	**0.0469**	6.27	**0.0113**	2.96	0.2371	3.70	0.1547
tyrosine	UPLC-MS	6.12	**0.0030**	5.47	**0.0280**	4.54	**0.0469**	4.15	**0.0127**	2.76	**0.0645**	1.92	0.3013
valine	GC-MS	4.33	**0.0007**	4.40	**0.0811**	2.76	0.3001	3.71	**0.0241**	1.33	**0.0645**	0.90	0.7087
Organic Acids													
aconitic acid	GC-MS	4.51	0.5198	1.70	**0.0802**	0.44	0.8463	0.54	0.7111	2.18	0.6733	1.74	0.4837
glyceric acid	GC-MS	1.59	**0.0198**	0.99	0.1468	2.29	**0.0818**	1.50	**0.0749**	0.55	0.3271	0.77	0.4444
malic acid	GC-MS	6.02	0.2159	6.25	0.1816	3.17	**0.0559**	2.43	**0.0599**	2.39	0.4720	2.07	0.2145
quinic acid	GC-MS	5.11	**0.0085**	4.36	**0.0136**	4.40	**0.0015**	3.85	**0.0276**	3.53	**0.0927**	3.25	**0.0320**
threonic acid	GC-MS	5.42	**0.0053**	5.68	**0.0234**	5.88	**0.0015**	5.82	**0.0379**	3.01	0.1206	2.68	0.1350
Vitamins													
pantothenic acid	UPLC-MS	5.44	**0.0269**	4.72	**0.0176**	5.31	**0.0249**	4.49	**0.0181**	4.64	0.1206	3.78	0.1753
Other													
dhurrin	UPLC-MS	8.27	**0.0235**	7.59	**0.0214**	7.33	**0.0015**	6.50	**<0.000**	5.55	**0.0348**	5.11	**0.0286**
prolyl-histidine-like	UPLC-MS	2.41	0.2219	2.07	**0.0529**	8.23	**0.0134**	8.65	**0.0045**	0.84	**0.0927**	0.97	0.1753
tyrosyl-histidine-like	UPLC-MS	7.98	**0.0079**	6.81	**0.0200**	6.77	**0.0172**	5.01	**0.0149**	5.49	**0.0645**	3.63	0.2259

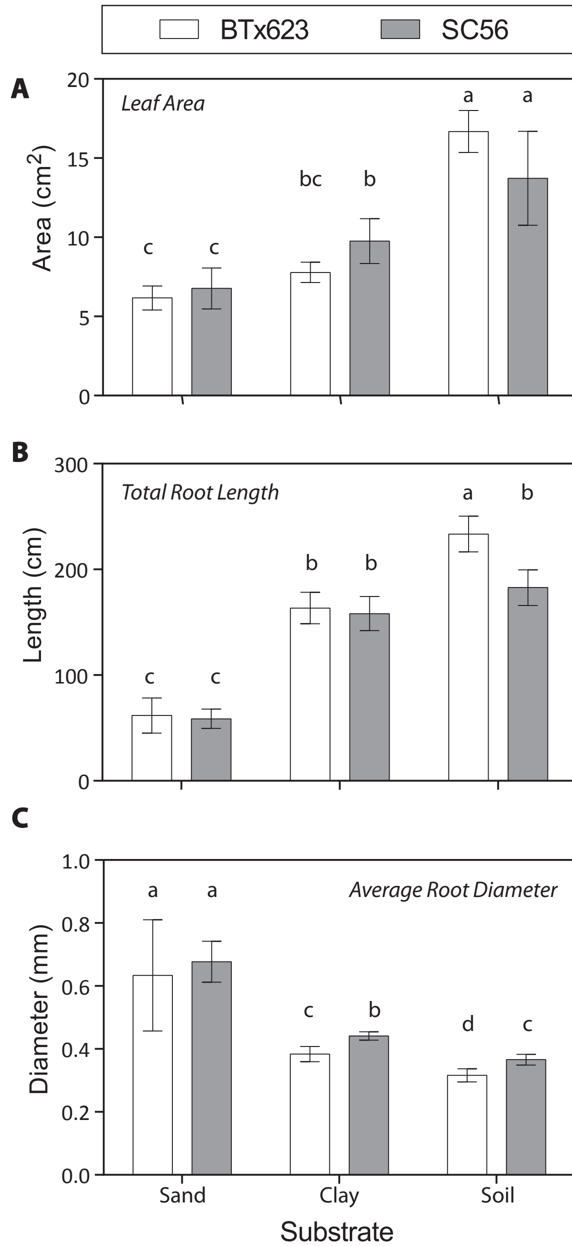


Figure 2.1. Morphological trait variation. Least square means for **A)** leaf area, **B)** total root length and **C)** average root diameter. Vertical bars represent standard error of means. Lowercase letters indicate statistical significance (Student's t) between means assessed within each trait measured.

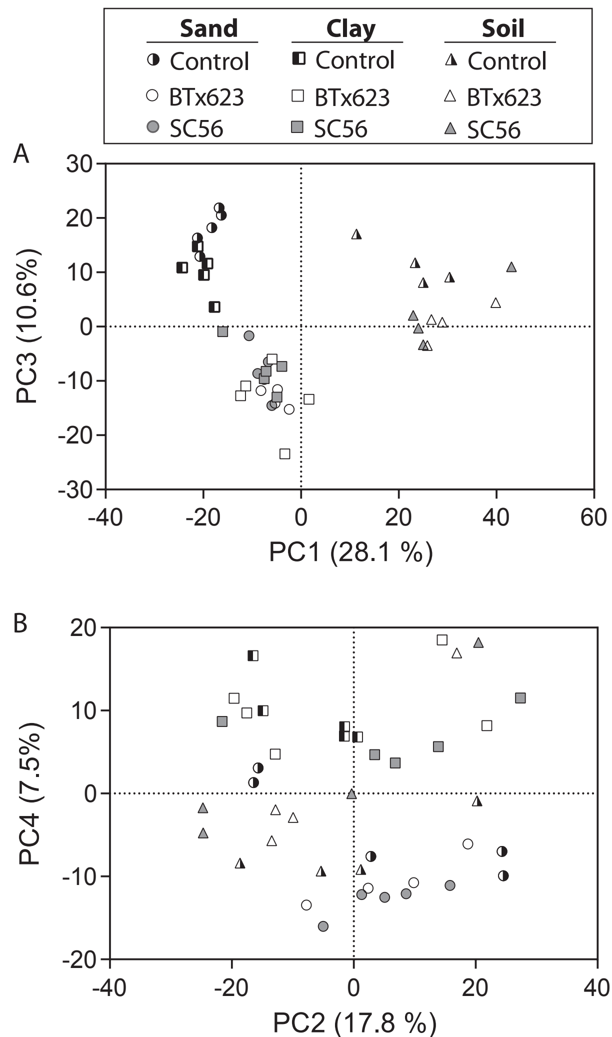
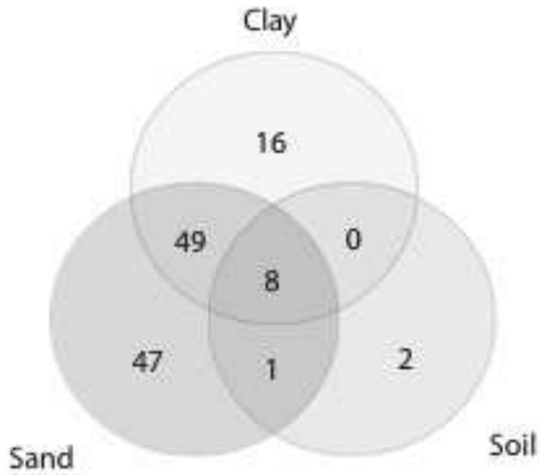


Figure 2.2. PCA plots for metabolite profiles. Scores plot for **A)** PCs 1 and 3 and **B)** PCs 2 and 4. Data from GC- and LC-MS analyses were combined and the analysis is based on 1,304 metabolites. No-plant controls are represented by half-shaded symbols, genotype BTx623 by open symbols, and genotype SC56 by closed symbols. Circles represent the sand substrate, squares the clay substrate and triangles the soil substrate.



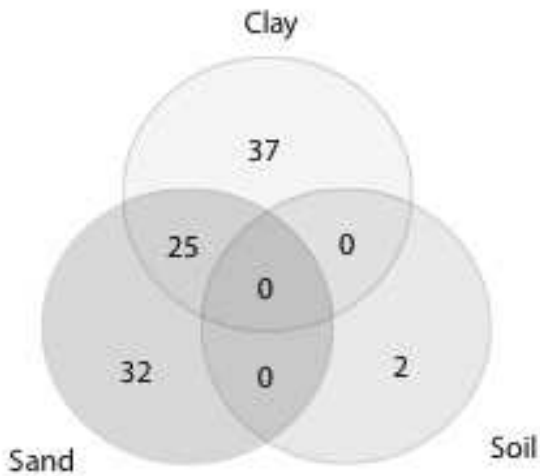


Figure 2.3. Venn diagram for the number of significant metabolites that were either **A)** rhizosphere-enhanced metabolites (log_2 FC >1) or **B)** rhizosphere-abated metabolites (log_2 FC <-1). Shading indicates different substrates and the numbers in the overlapping regions represent the number of significant metabolites that are in common.

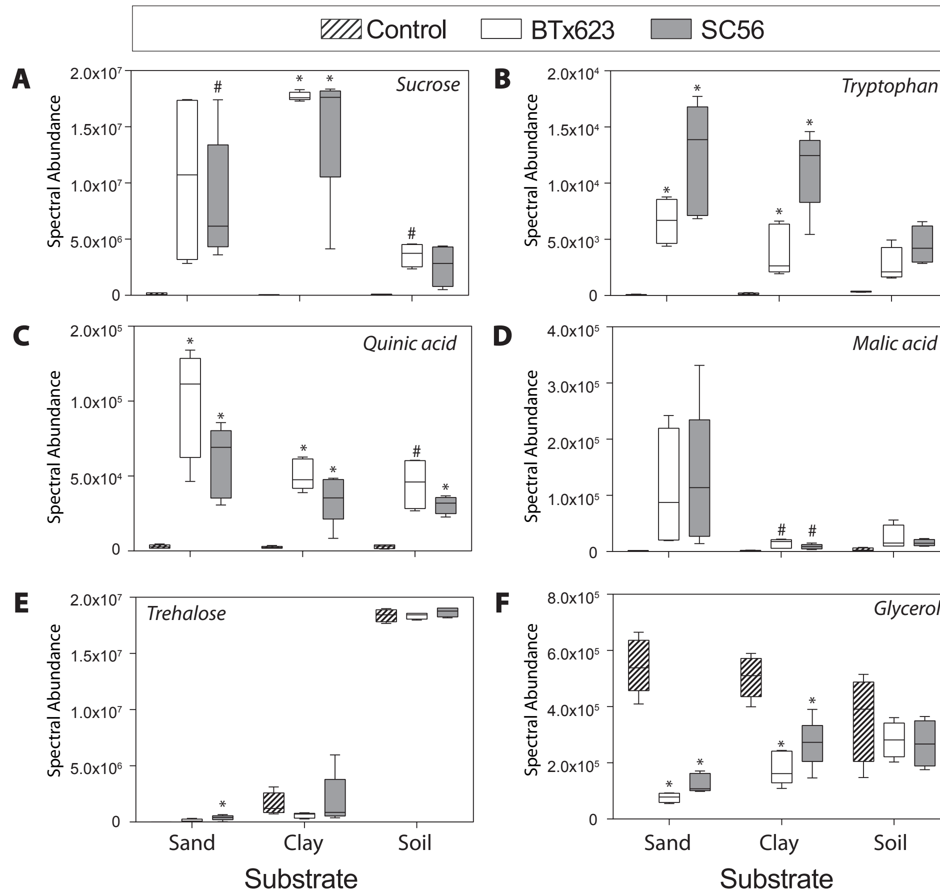


Figure 2.4. Box plots of selected metabolites **A)** sucrose, **B)** tryptophan, **C)** quinic acid, **D)** malic acid, **E)** trehalose, and **F)** glycerol within sand, clay and soil. Asterisk (*) indicates a significant difference between the genotype and no-plant control ($p < 0.05$); pound sign (#) indicates $p < 0.10$.

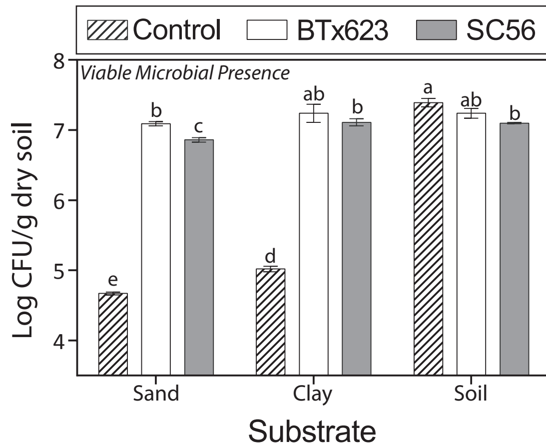


Figure 2.5. Viable microbial presence. Least square means and standard error of means (vertical bars) for the detected, culturable microorganisms for each treatment within each substrate. Lowercase letters indicate statistical significance (Student's t) between means assessed among all substrates and genotypes.

Supplementary Figure 2.S1

See Miller Dissertation Supplementary Tables & Figures.xlsx

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CHAPTER 3: RHIZOSPHERE-ASSOCIATED EXUDATION IN SORGHUM UNDER DROUGHT STRESS AND SUBSEQUENT RECOVERY DIFFERS BY LINE AND THROUGH TIME

SUMMARY

Drought stress is considered one of the most important environmental stressors limiting crop yields worldwide. Under water stress, it essential for plants to maintain internal water status by limiting water loss and continuing water uptake. However, drought stress is complex and elicits many other stressors such as nutrient deficiencies. Root exudation serves to buffer environmental changes and is key component to acquiring nutrients. However, little attention has been given to genotypic variation in root exudation under water stress. We characterized root exudation of sorghum (*Sorghum bicolor*) lines under water stress varying in pre-flowering drought tolerance and water management strategy (water-savers and water-spenders). We utilized a non-targeted metabolomics approach using gas-chromatography-mass spectrometry. Plants were grown in fritted clay and a dry down commenced at 21 days after sowing. Exudates were extracted at two time points: four days after withholding water and 24 h after reinstituting watering. Exudate adjustments were determined by comparing lines under water stress to those under well-watered conditions. Variation in exudation was driven by treatment (water-stressed or well-watered conditions), however variation in exudation between lines, drought tolerance groups and water management groups was observed at both time points. Although the composition of exudate adjustments differed between the tolerant lines evaluated, both drought tolerant lines more quickly responded to water stress and rewatering as evidenced through the number of exudate adjustments. These findings indicate that root exudate adjustments play a key role in pre-flowering drought tolerance in sorghum that can potentially be implemented into breeding programs.

INTRODUCTION

Drought stress is a major global concern, and is considered one of the most important environmental stressors limiting crop growth and yield (Boyer 1982; Cativelli et al. 2008; Mittler and

Blumwald 2010). In response to limited water availability, plants regulate stomatal closure to reduce water loss, which directly reduces carbon assimilation and plant growth (Chaves et al. 2003; Flexas and Medrano 2004). However, water deficit also exposes plants to other stressors, including excessive heat and light, soil resistance to root penetration, and nutrient deficiencies or toxicities (Langridge et al. 2006; Alam 1999). In response to these added pressures induced by drought, many changes simultaneously occur within the plant which are interconnected at the morphological, physiological, biochemical and molecular levels (Mitra 2001). Plant responses to drought stress are, simply put, complex.

Despite the complexity of the drought stress response, plants generally must maintain plant water status by moderating water uptake by the roots and water loss through transpiration within the plant under water stress (Aroca et al. 2012; Barea 2015). This is partially accomplished through internal metabolic adjustments, such as the production of osmolytes including the amino acid, proline, and the soluble sugars, sucrose and glucose, that aid cells in water retention and maintaining structural integrity (Hare et al. 1998). However, there is variation in the mechanisms utilized among drought tolerant genotypes to defend against water deficit. For example, plants can be categorized by their water management strategy under water stress as either water-savers (isohydric plants) or water-spenders (anisohydric plants) (Sade et al. 2012). Water-savers that reduce stomatal conductance under water stress are considered conservative with their growth whereas water-spenders that maintain stomatal conductance and carbon assimilation under water stress are considered productive with their growth (Sade et al. 2012). Furthermore, it has been suggested that water-savers are better suited for environments that experience severe terminal drought while water-spenders are more beneficial under more moderate drought stress (Blum 2015; Sade et al. 2012). Yet, both water-savers and water-spenders within rapeseed (*Brassica napus* L.) can be considered drought tolerant in the Mediterranean climate (Urban et al. 2017). Therefore, it is essential to investigate mechanisms employed by both

water-savers and water-spenders under specific water stress conditions to determine unique and common strategies that support water management under drought stress.

Many traits directly mitigate the effect of water deficit such as reducing leaf area while maintaining the root system (Farooq et al. 2009). However, few studies have considered evaluating traits that aid in overcoming various other abiotic stressors that occur under water stress. For example, nutrients that are required for plant functioning are limited under water stress due to the lack of diffusion to the plant, but also because nutrient uptake by the plant is reduced (Alam 1999). As there is genetic variation for plant nutrient uptake (Hu and Schmidhalter 2005), exploring traits that contribute towards nutrient uptake under water stress may be valuable in understanding plant drought tolerance.

The root system is directly involved with water and nutrient uptake and has been studied for its size, distribution, allometry and structural components that contribute towards maintaining plant water status (Comas et al. 2013). However, few drought studies have incorporated evaluating root exudates, small molecules exuded by roots into the adjacent soil, that fluctuate in response to environmental changes (Badri and Vivanco 2009). For instance, plant roots secrete mucilage, a viscous polysaccharide, to overcome soil resistance that is induced by drought stress (Bengough et al. 2006). Furthermore, root exudation is a key component to acquire nutrients that are required to sustain carbon metabolism (Hu and Schmidaler 2005; Nunes-Nesi et al. 2010). Plant roots exude compounds, often organic compounds such as malic and citric acid, to directly bind recalcitrant nutrients in the soil under nutrient deficiency (Marshner 1995, Jones 1998). Additionally, plants can use exudates to attract plant growth promoting rhizobacteria (PGPR) that aid in the growth of plants via nutrient acquisition, microbial antagonism, modulation of phytohormones or release of microbial hormones (Glick 2012). Root exudation is therefore a key component for nutrient acquisition, particularly under nutrient deficiency.

Tolerance to several nutrient deficiencies has been found to rely on root exudation and often involve only a few dominant genes (Rengel 2002). As nutrients become limited under drought stress and

root exudation is under genetic control, it may be that drought tolerance could be achieved through modifying select root-exuded metabolites. Drought studies should thus compare the root exudate profile of tolerant and susceptible lines to target root-exuded metabolites that may impart drought tolerance (Rengel 2002). Furthermore, studies often simulate droughts using artificial systems (polyethylene glycol (PEG) in hydroponic systems) that neglect the dynamic drying soil environment. Therefore, evaluating exudate adjustments between water-stressed and well-watered conditions of plants that vary in drought tolerance and grown in substrates that represent soil conditions can potentially inform plant breeding to improve drought tolerance.

Sorghum (*Sorghum bicolor* L. Moench) is an ideal crop for exploring the mechanisms behind drought tolerance. It is a model crop species known for its uses in food and forage, as well as a source for both grain and cellulosic biofuel production. Unlike other crops, sorghum is naturally adapted to drought stress and known to be nutrient efficient (Kimber et al. 2012; Staggenborg et al. 2008). However, sorghum can still can be considered susceptible to drought stress as water deficit can reduce its grain and biomass yields (Assefa and Staggenborg 2010). Several lines have been identified as sources for either pre-flowering or post-flowering drought tolerance that maintain their yields across environments (Rosenow et al. 1983). Furthermore, mechanisms employed by lines to cope with drought stress in each category vary (Rosenow et al. 1983, Fracasso et al. 2016). Pre-flowering drought tolerance is particularly important as water stress during seedling establishment limits plant development, reducing productivity at the end of the season (Richards 2000). Therefore, investigating root exudates in sorghum that sustain water and nutrient acquisition during pre-flowering drought stress will provide insight into the maintenance of plant development that is critical to preserve our yields.

In this study, we use gas chromatography-mass spectrometry (GC-MS) and a non-targeted metabolite profiling approach to evaluate a broad spectrum of rhizosphere-associated metabolites in sorghum lines that vary in pre-flowering drought tolerance and differ in water management strategy

(water-savers and water-spenders). Our main goals were to identify responses in exudation that were universal to pre-flowering drought stress, specific to tolerant lines and unique to individual tolerant lines that differed in water management strategy. To this end, exudates were sampled at two time points: one representing pre-flowering water stress conditions (limited water) and one representing the readdition of water into the system (24-h recovery). Specifically, we sought to 1) determine the quantitative and qualitative similarities and differences in exudate adjustments among the lines at each time point and 2) evaluate the temporal differences in exudate adjustments between limited water and 24-h recovery. We hypothesize that exuded metabolites will vary quantitatively and qualitatively among the lines evaluated in response to water stress and recovery, some of which may be integral for pre-flowering drought tolerance in sorghum.

MATERIALS AND METHODS

Plant Materials & Growth Conditions

Four grain sorghum lines were chosen due to their differences in morphophysiological responses to pre-flowering drought stress as shown in chapter one. BTx623 and Tx7000 have both been identified as pre-flowering drought tolerant (T) lines (Rosenow et al. 1983) while BTx642 and SC56 are both stay-green lines that are pre-flowering drought susceptible (S) (Kebede et al. 2001; Tuinstra et al. 1996). Seeds were first germinated in Petri dishes with fungicide (Maxim XL, Syngenta) for two days in the dark followed by one day in the light. Seedlings were then transplanted into 1.4 L pots lined with a porous cloth and filled with fritted clay (Profile® Field & Faraway™ fritted clay) for a greenhouse experiment (20°C – 30°C daily temperature; 16-h photoperiod; 55% relative humidity).

Plants were watered every other day to 100% field capacity (FC). The weight that represents 100% FC was obtained prior to the experiment by soaking each individual pot (lined with cloth and filled with fritted clay) in water overnight and allowing to drain for one h. Plants were fertilized weekly by watering to 100% FC with a nutrient solution (75% Hoagland's solution which consisted of: KH_2PO_4;

KNO_3; $Ca(NO_3)_2$; $MgSO_4$; H_3BO_3; $MgCl_2$-$4H_2O$; $ZnSO_4$-$7H_2O$; $CuSO_4$-$5H_2O$; MoO_3-H_2O; and Sequestrene 138 iron chelate) until the onset of the treatments.

Experimental Details

The experimental design included a randomized complete block design with five replicates for each line subjected to either a water-stressed treatment or a well-watered treatment, each beginning at 21 days after sowing (DAS). For the water-stressed treatment, plants were subjected to a dry down where water was withheld for six days. This dry down in fritted clay mimics water stress that slowly develops in agricultural fields and plants experience moderately stressful drought conditions (Des Marais et al. 2012; Lovell et al. 2015). After six days of water stress, water-stressed plants were rewatered back to 100% FC. For the well-watered treatment, plants were watered to 100% FC each day during the dry down. Two time points were sampled for morphology, physiology and root exudation: 1) limited water (four days of withholding water) and 2) 24-h recovery (24 h after rewatering).

Assessment of Plant Physiology & Morphology

Leaf gas exchange was assessed using the youngest fully expanded leaf of each replicate using the LI-6400XT (LI-COR, Inc, Lincoln, NE, USA) with leaf chamber attachment. The following conditions were used to take measurements: block temperature (ambient), photosynthetically active radiation (400 umol $m^{-2}s^{-1}$), CO_2 (400 umol mol^{-1}), and relative humidity (ambient).

Evaluated morphological traits included leaf area and root morphological traits. Leaf area was determined first using the LICOR LI-3100C leaf area meter (LI-COR, Inc, Lincoln, NE, USA). Root traits were assessed using the root-scanning equipment (Epson Expression 1100 XL, Epson America, Inc., Long Beach, CA, USA) and WinRHIZO software (Regent Instruments, Inc. Quebec, QC, CA).

Categorization of water use strategy for each line

Morphophysiological traits including leaf area, total root length, carbon assimilation and stomatal conductance were evaluated in response to water stress (Figure 3.1). Morphophysiological variation was used to classify water use strategy for each of the lines under water stress as either water-savers or water-spenders. Water-savers reduce stomatal conductance and limit growth under water stress whereas water spenders continue growing under drought stress (Urban et al. 2017). Thus, significant differences in stomatal conductance at limited water and leaf area at 24-h recovery between well-watered and water-stressed lines were used to categorize water use strategy (Figure 3.1G and 3.1B). Leaf area at 24-h recovery is a product of water stress as indicated in chapter one and was used as indicator of plant growth. The lines BTx623 (T) and SC56 (S) reduce stomatal conductance and reduce leaf area under water stress in comparison to well-watered controls and were therefore categorized as water-savers (Figure 3.1). In contrast, both BTx642 (S) and Tx7000 (T) maintain stomatal conductance and leaf area under water stress, representing water-spenders.

Metabolite Extraction

Metabolite extraction from the rhizosphere of each plant followed the protocol established in chapter two. Roots were removed from substrate with any fritted clay particles still clinging to the roots. Extracted roots were then placed into a 50 Ml conical tube which contained 10 Ml of 70% methanol and shaken for 10 sec by hand. Roots were then removed and a five-gram subsample of mixed bulk soil (obtained from the same pot and mixed on pulse in a sanitized food processor for 10 sec was placed into the same 50 Ml tube. Tubes were then shaken for 2 h on their sides on a shaker at 24°C. Afterwards, samples were centrifuged at 23°C, 4750 rpm for seven minutes. A two Ml sample of the liquid portion was then transferred into a microcentrifuge tube which was dried using Thermo Savant™ AES 2010 Speedvac® system (Thermo Fisher Scientific, Waltham, MA, USA).

Derivatization for and Metabolite Detection by Gas Chromatography – Mass Spectrometry

Total root exudation is likely to be dependent on the amount of root tissue. To account for this, the volume of exudate extract and the derivitization solution were adjusted by the total root length of each replicate. That is, when more root tissue was present, proportionally less extract was used for GC-MS analysis. The dried samples were resuspended in 25 Ml of pyridine containing 25 mg/Ml of methoxyamine hydrochloride, incubated at 60°C for 45 min, vigorously vortexed for 30 sec, sonicated for 10 min, and incubated for an additional 45 min at 60°C. Next, 25 Ml of N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS, Thermo Scientific) were added, and samples were vigorously vortexed for 30 seconds and incubated at 60 °C for 30 minutes. Metabolites were detected using a Trace 1310 GC coupled to a Thermo ISQ mass spectrometer. Derivatized samples (1 µL) were injected at a 10:1 split ratio to a 30 m TG-5MS column (Thermo Scientific, 0.25 mm i.d., 0.25 µm film thickness) with a 1.2 Ml/min helium gas flow rate. GC inlet was held at 285°C. The oven program started at 80°C for 30 s, followed by a ramp of 15°C/min to 330°C, and an 8 min hold. Masses between 50-650 m/z were scanned at 5 scans/sec under electron impact ionization. Transfer line and ion source were held at 300 and 260°C, respectively. Pooled QC samples were injected after every 6 experimental samples.

Metabolomics Data Analysis

For each sample, raw data files were converted to .cdf format, and matrix of molecular features as defined by retention time and mass (m/z) was generated using XCMS software in R (Smith et al. 2006) for feature detection and alignment. Raw peak areas were normalized to total ion signal in R, outlier injections were detected based on total signal and PC1 of principle component analysis, and the mean area of the chromatographic peak was calculated among replicate injections (n=2). Features were grouped based on a novel clustering tool, RAMClustR (Broeckling et al. 2014), which groups features into

spectra based on coelution and covariance across the full dataset, whereby spectra are used to determine the identity of observed compounds in the experiment. Metabolites were annotated by searching against in-house and external metabolite databases including NIST v12, Massbank, Golm, and Metlin. The peak areas for each feature in a spectrum were condensed via the weighted mean of all features in a spectrum into a single value for each compound.

Statistical Analysis

Morphological and physiological traits within a time point were statistically analyzed by using an Analysis of Variance (ANOVA) for line, treatment and their interaction term as fixed effects using JMP Pro 11 (SAS Institute, Cary, NC, USA). Data were box-cox transformed prior to analysis if the data did not pass the Shapiro-Wilk Normality Test and the analysis was sliced by line to examine treatment differences.

To determine variation in metabolite data, ANOVAs were performed within each time point by using the aov function in R (R Development Core Team, 2012). ANOVAs included water management strategy, pre-flowering drought tolerance, line, treatment and the interaction terms, water management strategy * treatment and pre-flowering drought tolerance * treatment, as fixed effects. A false discovery rate (FDR) adjustment was used on the p-values using p.adjust function (Benjamini and Hochberg 1995). A principle components analysis (PCA) was performed using SIMCA v14.0 (Umetrics, Umea, Sweden) and unit variance (UV) scaling. Metabolite z-scores were calculated for each line using the well-watered treatments as controls. Z-score values were used to determine exuded metabolites that adjusted in response to water stress, termed exudate adjustments. Z-score values that represent two standard deviations above the well-watered treatment mean (> 1.96) represented exuded metabolites detected at greater levels in the water-stressed treatments. Z-score values that represent

two standard deviations below the well-watered treatment mean (< -1.96) were metabolites detected at lower levels in the water-stressed treatments.

RESULTS

Variation in rhizosphere-associated metabolites was driven by lines and water condition

Variation in rhizosphere-associated metabolites was evaluated for four sorghum lines that differed in pre-flowering drought tolerance and water management strategy under well-watered and water-stressed conditions that began at 21 days after sowing. We evaluated root exudation at two time points: 1) limited water (four days of withholding water) and 2) 24-h recovery (24 h after rewatering). Non-targeted metabolite profiling using gas chromatography-mass spectrometry (GC-MS) was utilized to evaluate variation in metabolites. GC-MS analyses determined 5,376 molecular features that were clustered into 328 spectral clusters, which represented distinct compounds. Of the 328 compounds, 50 were annotated as metabolites.

Principal component analyses (PCA) were performed on the 328 compounds at limited water and at 24-h recovery. At limited water, nine principal components (PC) explained 72.3% of the variation (Table 3.1A). The second PC shows variation between water management strategy (water-savers and water-spenders) whereas PC 3 reflects variation in treatment (well-watered and water-stressed conditions) (Figure 3.2A). Two PCs varied by water management strategy (PCs 2 and 7), one PC varied by tolerance group (PC 8), two PCs varied by treatment (PCs 3 and 5), and four PCs varied by line (PCs 1, 2, 6 and 8) (Table 3.1A). At 24-h recovery, seven PCs explained 67.4% of the variation (Table 3.1B). PC 1 varied by water management strategy, tolerance group and line and PC 2 varied by treatment (Figure 3.2B; Table 3.1B). One PC varied by water management strategy (PC 1), two PCs varied by tolerance group (PCs 1 and 4), two varied by treatment (PCs 2 and 6) and two varied by line (PCs 1 and 4) (Table 3.1B). In general, this supports that individual lines have a large effect on root exudation along with water management strategy and drought tolerance, but that treatment also plays a role.

At limited water, there were no compounds that varied by line, drought tolerance, water management strategy (water-savers and water-spenders) or the interaction between water management strategy and treatment (ANOVA, FDR adjusted $p < 0.05$) (Table 3.S1A). One metabolite varied by drought tolerance and treatment interaction and there were 98 compounds out of the 328 compounds (29.9%) that varied by treatment. At 24-h recovery, 29 compounds (8.8%) varied by line, no compounds varied by drought tolerance, 20 compounds (6.1%) varied by water management strategy and 103 (31.4%) varied by treatment (Table 3.S1B). Three compounds varied for the water management strategy by treatment interaction and three compounds also varied for the drought tolerance by treatment interaction. Overall, more compounds varied at 24-h recovery than at limited water and the majority of metabolites varied by treatment.

Exudate adjustments vary qualitatively and quantitatively among lines

To determine metabolic variation among lines, metabolites were z-transformed within each line using well-watered treatments as controls. Z-scores were used to determine metabolites detected at higher levels in water-stressed lines compared to well-watered lines (> 1.96 in value) and those detected at lower levels in water-stressed lines (< -1.96). Additionally, greater magnitudes in exudate adjustments (greater increases in water-stressed lines compared to well-watered lines) are indicated by a z-score of greater than three. In general, fewer exudate adjustments occurred at limited water compared to 24-h recovery (Figure 3.3A).

At limited water, there were more metabolites that increased in water-stressed tolerant lines than those of susceptible lines (Figure 3.3A). Between water-savers, more metabolites increased in the water-stressed tolerant line (BTx623), but fewer metabolites decreased. Between water-spenders, more exudate adjustments increased and decreased in the water-stressed tolerant line (Tx7000). However, when comparing the composition of exudate adjustments between lines of tolerance groups (tolerant

and susceptible) or water management groups (water-saver and water-spender), few exudate adjustments were similar (Figure 3.3B and 3.3C).

At 24-h recovery, fewer metabolites increased in water-stressed tolerant lines than those of susceptible lines regardless of water management strategy (Figure 3.3A). Furthermore, tolerant lines made fewer major exudate adjustments when comparing the number of metabolites with a z-score greater than three. At 24-h recovery, several metabolites that increased in water-stressed lines were common among all the lines, but few exudate adjustments were common between lines in either tolerance or water management groups (Figure 3.3D and 3.3E).

Z-scores of annotated metabolites are visualized as heatmaps displaying line variation and organized with hierarchical clustering (Figure 3.4). At limited water, several metabolites were universally higher among all the water-stressed lines including several organic acids (citric, malic and quinic) and the amino acid, proline (Figure 3.4A). The magnitude of these metabolic changes differed between lines including greater changes in quinic acid for both tolerant lines and a large change in citric acid for the tolerant water-spender Tx7000. Although few metabolites decreased in the water-stressed lines, 5-hydroxy-tryptamine (serotonin) was detected at a lower presence in the water-stressed Tx7000 (T) compared to its well-watered control. This also was the trend for BTx623 (T).

At 24-h recovery, metabolites that were universally higher among all the water-stressed lines compared to respective well-watered controls included sucrose, myo-inositol, proline, valine and asparagine (Figure 3.4B). Many exudate adjustments occurred in all of the lines except the water-spender, Tx7000 (T), including the amino acids leucine, isoleucine, threonine and the nitrogen-rich compound urea. Between water management groups, glucose decreased in the water-stressed lines of water-spenders and not in water-savers. Two metabolites, cis-aconitic acid and quinic acid, trended lower in all water-stressed lines.

DISCUSSION

Under drought stress, it is essential that plants allocate their limited resources appropriately. In this study, we found that the composition of exudate adjustments largely varied between the tolerant lines under water stress that likely support their water management strategy (water-saver or water-spender), representing different mechanisms that can be used to tolerate drought stress. However, regardless of water management strategy, we found that both drought tolerant water-stressed lines increased more metabolites under water stress and fewer metabolites after rewatering. This suggests that a response in exudation to altered environmental conditions does play a role in drought tolerance. Additionally, as increasing overall root exudation allocates carbon assimilates to roots instead of reproduction, thus limiting yields (Rengel 2002), smaller modifications in root exudation may contribute towards pre-flowering drought tolerance in sorghum. Therefore, appropriate modifications in both the quantitative and qualitative composition of exudate adjustments under water stress and in response to rewatering that sustain a plant's water use strategy may be essential to pre-flowering drought tolerance in sorghum.

Lines respond differently to both water stress and rewatering

To our knowledge, this study is one of the first to characterize the rhizosphere-associated metabolite profile under water stress and after the readdition of water within a substrate using non-targeted metabolite profiling. Here, we found genotypic variation at both limited water and 24-h recovery (Figures 3.3 and 3.4). In general, we found few adjustments were detected among the lines in response to water stress, but many more adjustments were detected in response to rewatering (Figure 3.3). We cannot rule out that the detected exudate adjustments at both time points may be cumulative as exudates can persist in the soil (Curl and Truelove 1986).

However, we did find that the composition of exudate adjustments among the lines differed through time, suggesting that the detected exudate adjustments at each time point reflect separate responses to water stress and rewatering (Figure 3.4). For instance, there were several metabolites that increased under water-stressed conditions at limited water, but decreased or displayed no change under water-stressed conditions at 24-h recovery such as citric acid and malic acid (Figure 3.4). Therefore, characterizing the adjustments made by each line representing different pre-flowering drought tolerance and water management strategies at both limited water and 24-h recovery will identify mechanisms employed by lines that may serve in drought tolerance.

Common exudate adjustments among all lines under water stress match known endogenous metabolic alterations

Some metabolites displayed universal responses to water stress among the lines observed, irrespective of drought tolerance or water management strategy (Figure 3.4). These included metabolites that are noted to respond intrinsically within the plant to water stress such as sugars, polyols, amino acids and organic acids (Witt et al. 2012). Proline, sucrose and myo-inositol were among metabolites that increased at 24-h recovery that likely act as osmolytes to drive water transport to the roots serving in water acquisition and may also function to maintain structural integrity of the root (Hare et al. 1998; Aroca et al. 2012). In addition to maintaining water balance, sugars and polyols are common substrates for cellular respiration for microbes and may also elicit a general population of microbes that aid in plant growth (Chaparro et al. 2013).

We also saw a general increase of organic acids such as malic and citric acids at limited water (Figure 3.3A); these metabolites increase under water stress and may aid in nutrient acquisition (Henry et al. 2007; Song et al. 2011). However, the adjustments in these metabolites differed in magnitude

among the lines evaluated, suggesting that quantitative differences in exudation may serve a role in drought tolerance and water management strategy (Figure 3.3A).

Tolerant lines differ in qualitative composition of exudate adjustments, but share in the number of moderate, quantitative responses to water stress and rewatering

Despite differences in the composition of exudate adjustments, we found that a larger number of metabolites increased in water-stressed tolerant lines than those of water-stressed susceptible lines at limited water (Figure 3.3A). Guo et al. (2017) evaluating gene expression found that a drought tolerant variety of *Brassica rapa* grown in a hydroponic system under PEG-induced osmotic stress activated more pathways that were proactive in defending against water stress such as ABA signaling than that of a susceptible variety. Additionally, gene expression of stress-response genes evaluated at 4, 8 and 12 hs after initially inducing osmotic stress indicated a quicker response to osmotic stress in the tolerant variety. Therefore, it may be that the greater number of exudate adjustments detected at limited water in our study serve in defending against water stress. These changes in exudation may also indicate a faster response to water stress and future studies should evaluate metabolite production on a finer time scale to determine if drought tolerant lines of sorghum respond more quickly to water stress.

Fewer differences in the number of exudate adjustments were observed between well-watered and water-stressed conditions at 24-h recovery in tolerant lines, indicating a return to normal development (Figure 3.3A). In chapter one, we found that upon the readdition of water, tolerant sorghum lines previously under water stress were better able to recover leaf area and development to that of well-watered controls than susceptible lines, thus supporting that tolerant sorghum lines quickly return to normal developmental conditions upon rewatering. This rapid return to normal development after rewatering was also demonstrated in the nodules of peanuts (*Arachis hypogaea*) where metabolites of tolerant cultivars promptly returned back to well-watered control values after the

readdition of water (Furlan et al. 2017). Therefore, regardless of the composition of exudate adjustments made under drought stress, drought tolerant sorghum lines make more modifications under water stress that may buffer altered environmental conditions and fewer upon rewatering to return back to normal developmental conditions.

Changes in metabolism can be regarded as energetically costly (DeWitt et al. 1998). Furthermore, diverting resources to increased root exudation reduces the amount of assimilates that can be utilized for growth and reproduction, reducing yields (Rengel 2002). At 24-h recovery, we found that tolerant lines modify the magnitude of exudate adjustments more moderately than susceptible lines (Figure 3.3A). Similarly, Fracasso et al. (2016) found that a drought tolerant sorghum genotype under pre-flowering water stress increased the expression of drought-related genes in leaf tissue less than that of a drought susceptible genotype. Thus, the ability to make minor adjustments to defend against water stress may be indicative of pre-flowering drought tolerance in sorghum. As the tolerant lines in our study shared in the number of quantitative responses to water stress and rewatering, but shared few common exudate adjustments, it is likely that the composition of exudate adjustments are involved with water management strategy.

Differences in exudation between tolerant lines suggest multiple pathways are used to cope with water stress

It was particularly evident that tolerant lines in this study exhibited different patterns in exudate adjustments at both time points (Figures 3.3 and 3.4), which supports different strategies to defend against water stress. Fracasso et al. (2016) compared gene expression of two pre-flowering drought tolerant genotypes of sorghum and similarly found that pre-flowering drought tolerance strategies in sorghum can vary under water stress, but likely support water management strategy. Lines that reduced stomatal conductance in response to water stress increased expression of dehydrin, a stress tolerance

protein that aids in maintaining cell membrane stability. Additionally, there was an increase in a protein involved in limiting stomatal conductance in one of the tolerant lines evaluated. Therefore, it may be that mechanisms employed by drought tolerant sorghum lines follow their water management strategy. For instance, abscisic acid (ABA) enhancement and subsequent osmotic adjustments within the plant to defend against abiotic stress are more important for water-savers that limit transpiration than water-spenders (Blum 2015). Indeed, Bowne et al. (2012) found that a drought tolerant wheat (*Triticum aesivum*) genotype representing a water-saver (termed conservative) displayed larger adjustments in leaf metabolite responses to water stress than a drought tolerant genotype representing a water-spender (termed productive).

In rapeseed, Urban et al. (2017) found genotypes that were drought tolerant and susceptible among both water-savers and water-spenders. Although genotypes in each water management category shared in conserved protein responses to drought stress, many leaf proteome adjustments were specific to the tolerant genotype, potentially relaying its drought tolerance. In this study, we found that lines with the same water management strategy and not pre-flowering drought tolerance follow more similar approaches in exudate adjustments to water stress (Figure 3.2A; Table 3.1A), but the composition of these exudate adjustments varied between the lines within each water management category (Figure 3.4). Therefore, it may be that this variation in the composition of exudate adjustments contributes towards drought tolerance according to each water management strategy. Overall, our findings in exudate adjustments support that drought tolerant lines under water stress can respond using a variety of mechanisms, but likely need to the water management strategy employed by the line under water stress.

Specific exudate adjustments are required according to water management strategy

As water-spenders continue to grow under water stress, it essential that they acquire the appropriate nutrient resources required for carbon metabolism. Urban et al. (2017) found that rapeseed water-spenders increase proteins related to carbohydrate/energy and photosynthesis to maintain metabolism and growth under water stress. Here, we found that drought tolerant water-spender Tx7000 made many exudate adjustments under conditions of limited water, which may serve in acquiring water and resources for its continued growth (Figures 3.3A and 3.4A; Figure 3.1B). In contrast, the susceptible water-spender BTx642 (S), made few exudate adjustments at limited water, but also continued to grow (Figures 3.3A and 3.4A; Figure 3.1B). This lack of change in BTx642 (S) may lead to its susceptibility as it continued to grow under water stress, but did not alter its exudation that may support this growth. Therefore, altering root exudation under water stress to acquire water and nutrients may be essential for water-spenders.

Many of the exudate adjustments that were exclusive to the tolerant water-saver, BTx623, could not be annotated. Therefore, we could not make inferences about the metabolites that likely play a role in this line's drought tolerance. We did find a similar pattern in BTx623 (T) as in Tx7000 (T), where both increased several unique metabolites in their water-stressed lines at limited water (Figures 3.3A and 3.3B). However, the susceptible water-saver SC56 made many exclusive exudate adjustments that either increased or decreased at limited water. This reflects that there are adjustments in root exudation that are beneficial under drought stress for water-savers. Urban et al. (2017) found that rapeseed water-savers alter leaf proteins related to nitrogen assimilation and defense, particularly against reactive oxygen species (ROS). Furthermore, water-savers that experience carbon starvation and limit their growth under water stress are more susceptible to biotic stressors (McDowell et al. 2008; Sade et al. 2012). Thus, perhaps drought tolerant water-savers also increase secondary metabolites that serve in defense against biotic agents. Future research should explore root-exuded metabolites related to these

functions that may confer drought tolerance in water-savers as well as utilize other metabolomics platforms to detect a broader range of secondary metabolites.

The tolerant water-spender line displays many unique exudate adjustments

At limited water, citric acid was universally higher among all the lines under water-stressed conditions. However, the increase was the most dramatic for Tx7000 (T) (Figure 3.4A). In a study conducted by Song et al. (2012) observing organic acid root exudation of maize (*Zea mays*) grown in a hydroponic system under extreme drought stress simulated by polyethylene glycol (30% PEG), similar results were found between a tolerant and susceptible variety of maize. Citric acid has been implicated in the solubilization of phosphorous, iron and other micronutrients essential for carbon metabolism (Jones 1998). Phosphorous is especially important under drought stress as it is required for many processes such as photosynthesis, energy transfer and the regulation of enzymes and is involved in the phosphorelay system that senses and signals drought stress to the plant (Hu and Schmidhalter 2005; Osakabe et al. 2013). Furthermore, its addition to soil has been found to increase plant growth under drought stress (Ackerson 1985; Garg et al. 2004; Hu and Schmidhalter 2005). As Tx7000 (T) is a water-spender that continues carbon assimilation and growth (Figure 3.1B), it requires many nutrient resources involved in growth and maintenance. Therefore, it may be that this increase in citric acid in the water-stressed Tx7000 (T) is a critical component to its drought tolerance.

Tx7000 (T) made several other unique exudate adjustments at limited water. Of interest is the decrease in serotonin (5-hydroxytryptamine) under water-stressed conditions in Tx7000 (T), a pattern also observed for BTx623 (T) (Figure 3.4A). Serotonin has been implicated in regulating root development in *Arabidopsis thaliana* as it likely inhibits auxin, a root growth promoting hormone (Pelagio-Flores et al. 2011). Both serotonin and indole-3-acetic acid (IAA), a common form of auxin, are synthesized from tryptophan. Therefore, by decreasing the level of serotonin, auxin production may be

promoted (Pelagio-Flores et al. 2011). This may be the case in the water-stressed Tx7000 (T) as it was the only water-stressed line at 24-h recovery that did not see a decrease in total root length (Figure 3.1D). Thus, a decrease in serotonin likely serves to promote auxin production to support increased root growth.

At 24-h recovery, we saw an increase in several amino acids among all lines under water stress (Figure 3.4B). In particular, the branched-chain amino acids increased at 24-h recovery, which increase within the plant under water stress and may be a product of protein degradation (Bowne et al. 2012). However, the tolerant line (Tx7000) often displayed similar levels in amino acids between its water-stressed and well-watered counterparts as evidenced by leucine, isoleucine, threonine, alanine as well as the nitrogen-rich compound urea at 24-h recovery (Figure 3.4B). This effect may be due to less protein degradation within this line. Both isoleucine and leucine are among the amino acids that result from protein breakdown under abiotic stress as indicated by an increase in catabolic enzymes under abiotic stress conditions (Less and Galili 2008). Furthermore, mature leaf senescence occurs under water stress to support nitrogen remobilization of amino acids to other organs including the roots (Masclaux-Daubresse et al. 2010). Therefore, as leaf area did not decrease for the line Tx7000 that displayed few increases in amino acids (Figures 3.1B and 3.4B), protein degradation in leaf tissue may not be occurring in this line. Overall, future research should explore endogenous protein, metabolite and hormone levels in drought tolerant water-spenders to better understand the mechanisms employed to sustain their growth.

In this study, we found that pre-flowering drought tolerant lines of sorghum make more exudate adjustments in response to water stress and fewer adjustments upon rewatering indicating a return to normal exudation. This may suggest that pre-flowering drought tolerant lines more quickly respond to environmental changes. Therefore, future studies should evaluate proteins, metabolites and hormones over time that may be involved with sensing, signaling and responding to both water stress and

rewatering. Furthermore, many exudate adjustments were unique to drought tolerant lines within each water use category that are potential targets for improving drought tolerance and should therefore be further explored. However, several of these metabolites that could not be identified that may play essential roles. Targeting metabolites and hormones that may not be readily annotated through non-targeted metabolomics and that may serve in drought tolerance should thus be evaluated to better understand the role of root exudation in drought tolerance. Further research should also explore the functional roles of root-exuded metabolites of both water-savers and water-spenders by incorporating leaf and root transcriptomics and considering the functional capacity of the soil microbiome.

Table 3.1. ANOVA on metabolite principal components by water management strategy (water-savers and water-spenders), drought tolerance, treatment (well-watered and water-stressed) and by line for **A)** Limited Water and **B)** 24-H Recovery.

A) Limited Water

Principal Component	% Variation explained	p-value by line	p-value by drought tolerance	p-value by water management	p-value by treatment
1	22.8	0.0133	0.1168	0.1488	0.0886
2	11.2	0.0045	0.5801	0.0003	0.323
3	9.7	0.4246	0.9325	0.1021	0.0026
4	6.5	0.1569	0.1388	0.1045	0.9721
5	5.6	0.1528	0.1781	0.7887	0.0066
6	5	0.0012	0.4049	0.2365	0.9245
7	4.6	0.0992	0.4637	0.0262	0.5166
8	3.8	0.0074	0.0005	0.6276	0.3762
9	3.2	0.4842	0.1451	0.5915	0.7048

B) 24-H Recovery

Principal Component	% Variation explained	p-value by line	p-value by drought tolerance	p-value by water management	p-value by treatment
1	17.4	0.0003	0.0012	0.0113	0.1008
2	15.9	0.4935	0.3223	0.2936	<.0001
3	11.4	0.1483	0.7656	0.1434	0.3621
4	6.4	0.0123	0.0028	0.2577	0.5249
5	6.3	0.2143	0.6398	0.1585	0.7078
6	5.3	0.0838	0.5195	0.1088	0.0443
7	4.7	0.4032	0.1488	0.3762	0.4283

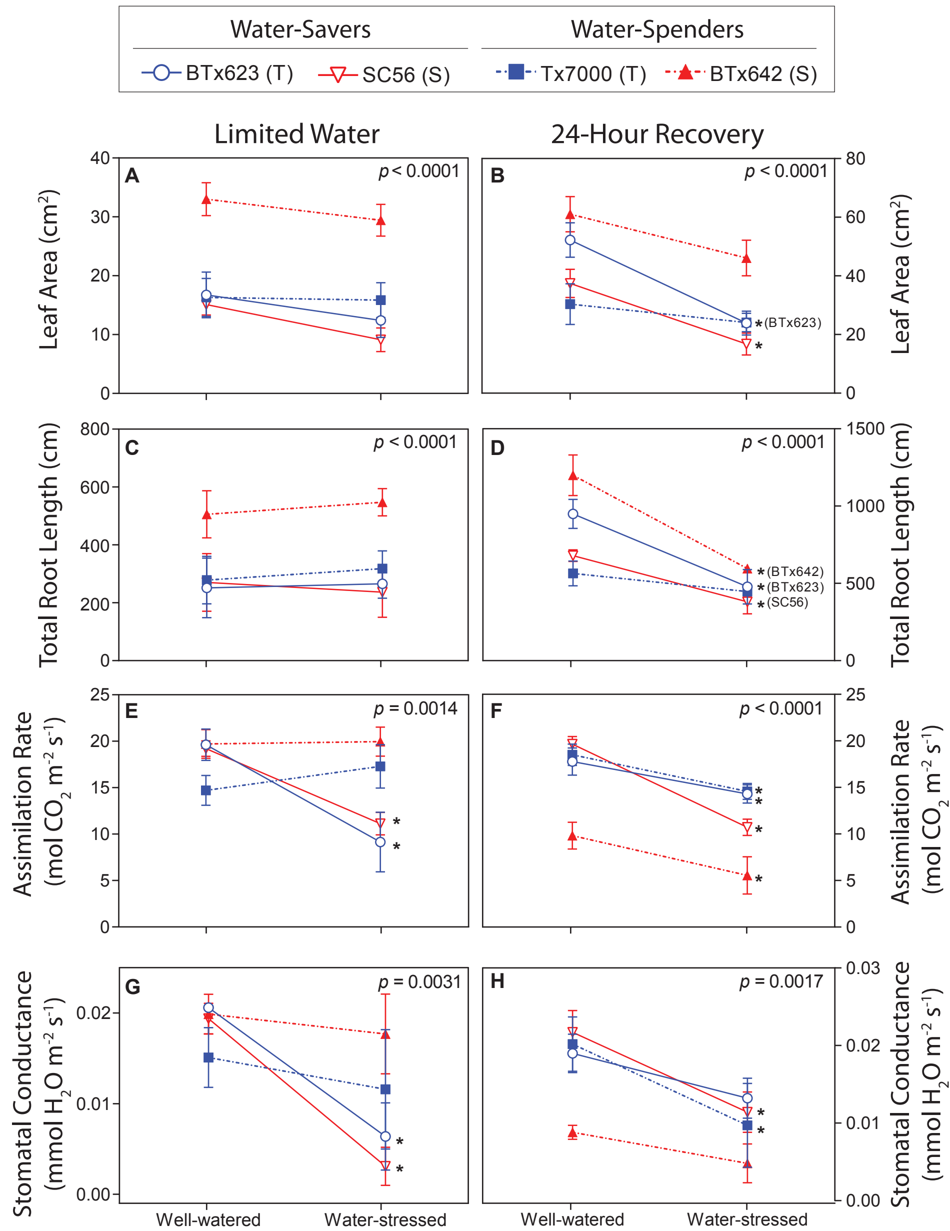

Figure 3.1. Reaction norms illustrating phenotypic plasticity of each of the sorghum lines between well-watered and water-stressed conditions. Least square means and standard error of means (vertical bars) are displayed for each line and water condition. Asterisk (*) indicates statistical significance from well-watered control. Colors delineate tolerant lines (blue) from susceptible lines (red).

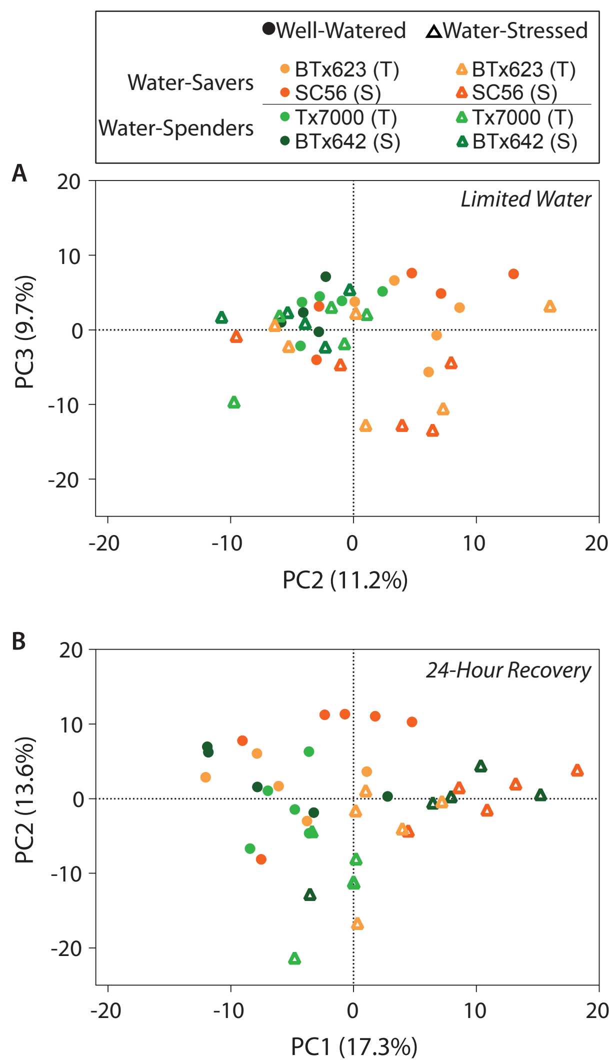

Figure 3.2. Scores plots from PCA of the GC-MS metabolomics analysis of four sorghum lines under water-watered (closed symbols) and water-stressed (open symbols) conditions at **A)** Limited Water and **B)** 24-H Recovery. Colors indicated water-savers (orange) and water-spenders (green).

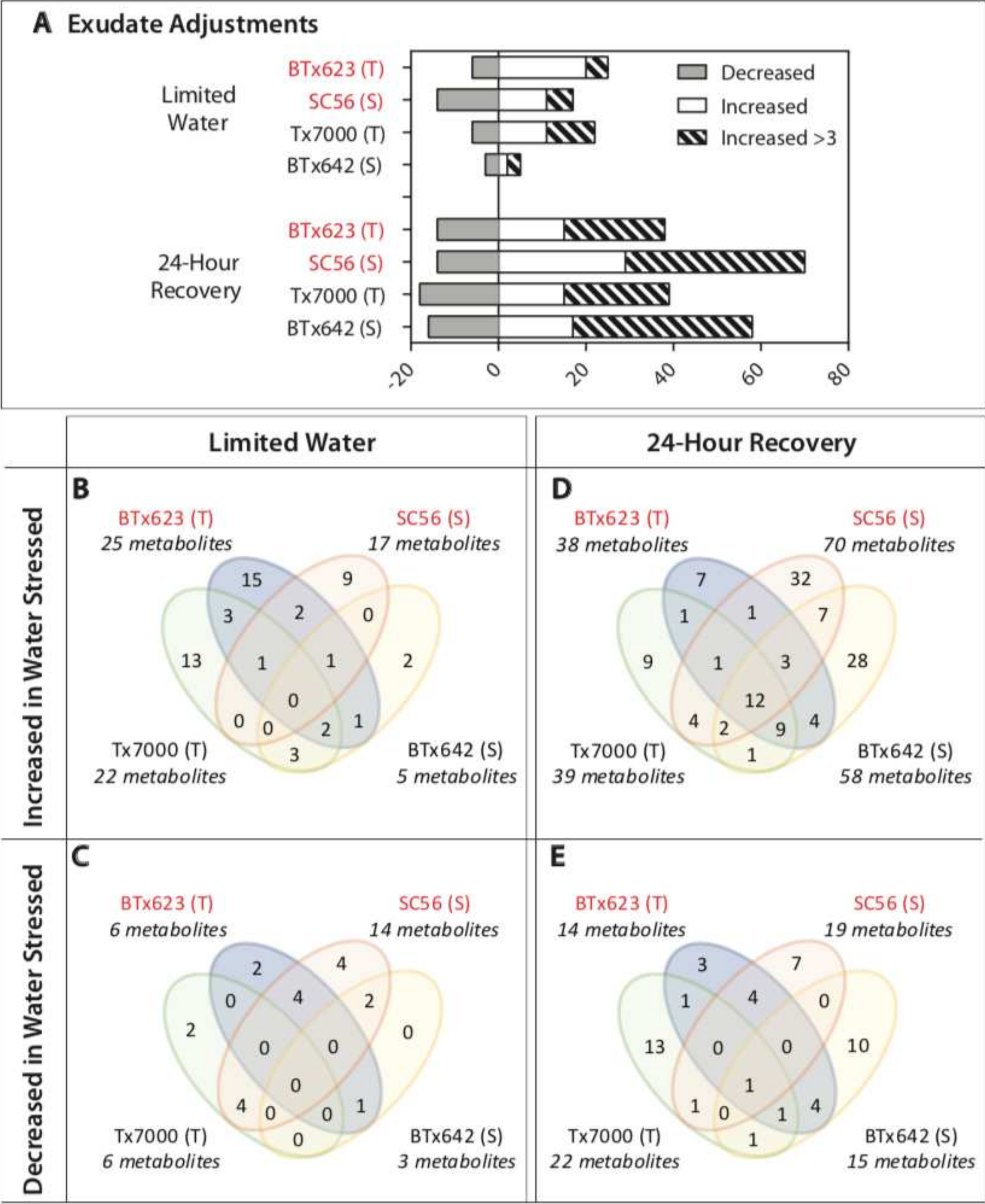

Figure 3.3. Comparisons of total exudate adjustments among the lines. Sorghum lines in red represent water-savers and sorghum lines in black indicate water-spenders. **A**) Stacked bar chart displaying the total number of exudate adjustments in each line. Exudate adjustments that decreased are shaded gray, adjustments that increased are white, and adjustments that increased with a z-score greater than three are indicated by hatched bars. Venn diagrams reflect the number of exudate adjustments that are shared or different among the lines at each time point. At limited water, exudate adjustsments are shown that **(B)** increased and **(C)** decreased under water-stressed conditions. At 24-h recovery, exudate adjustments are displayed that **(D)** increased and **(E)** decreased under water-stressed conditions. Colored circles indicate different lines and numbers in overlapping regions reflect common exudate adjustments.

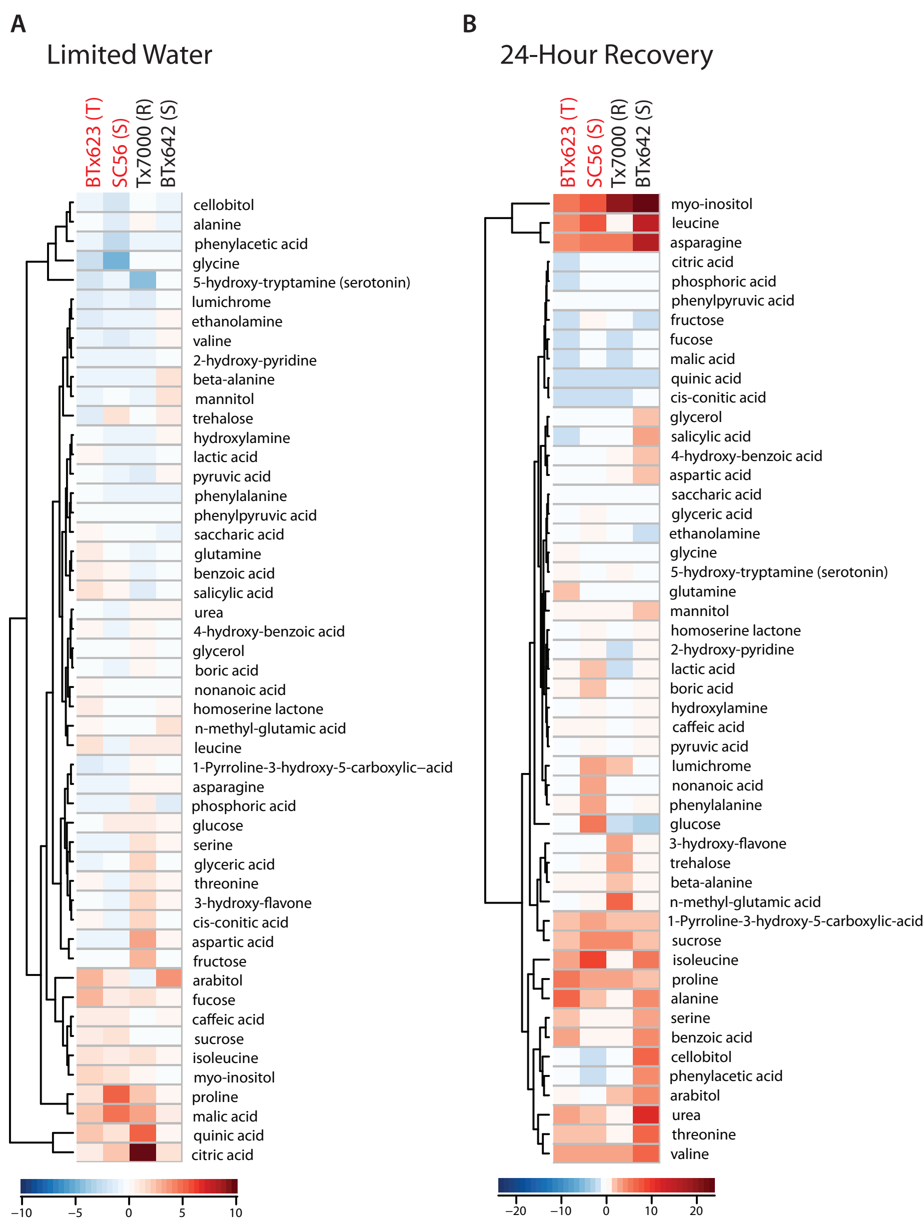

Figure 3.4. Z-scores of annotated metabolites that increased (red) and decreased (blue) under water-stressed conditions of each line. The relative abundance of each metabolite is normalized to the well-watered control of each line. Sorghum lines in red represent water-savers and sorghum lines in black indicate water-spenders.

Supplementary Table 3.S1

See Miller Dissertation Supplementary Tables & Figures.xlsx

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CHAPTER 4: FUTURE DIRECTIONS

The overall goal of this dissertation was to describe phenotypes that indicate sorghum pre-flowering drought tolerance that can be utilized in plant breeding and sustainably increase agricultural productivity. For example, leaf area recovery indices can be utilized early in the season to indicate pre-flowering drought tolerance and predict end-of-season traits, thus serving in variety selection. Furthermore, the model crop species sorghum and its rhizosphere-associated exudation was used to provide a novel framework to better understand belowground plant-environment interactions. We found genotypic variation for sorghum rhizosphere-associated exudation in response to water stress and rewatering suggesting that the plant is intimately involved with its environment under water deficit and can potentially be utilized in plant breeding. However, a more comprehensive understanding of endogenous plant mechanisms and the plant-environment interaction under water stress is required to improve our knowledge of plant drought tolerance. Future research should be conducted to determine the functional roles of metabolites and their extension to other crop species and soil types. As the field of metabolomics is rapidly advancing to encompass a broader range of metabolites and hormones (Hong et al. 2016, van Dam and Bouwmeester 2016), the identification and quantification of metabolites can be utilized in determining how endogenous metabolites and root exudation aids in drought tolerance.

Further studies are required to uncover specific plant mechanisms that confer drought tolerance as there are many successful plant responses to water deficit as evidenced in this dissertation. Combining metabolomics, proteomics and transcriptomics of lines that differ in water management strategy (water-savers and water-spenders) under water stress is thus critical for determining mechanisms that are universally employed as well as vary between water management groups that may serve as potential targets in plant breeding. We found that drought tolerant sorghum genotypes largely vary in the composition of exudate adjustments that may follow water management strategy.

Therefore, it is essential to assess the functional roles of these metabolites. For example, whether and how these metabolites mitigate the multi-dimensional effects of water stress either directly such as through chelation of nutrients or indirectly by enlisting plant growth-promoting bacteria (PGPB). Furthermore, we found that regardless of water management strategy, drought tolerant lines of sorghum return to normal development sooner than susceptible lines, suggesting that sensing and signaling of altered environmental conditions may play a key role in drought stress. Thus, evaluating gene expression, metabolites, hormones and proteins involved in sensing, signaling and responding over a fine time scale may provide a better understanding of the mechanisms employed. Our evaluation of genotypic variation in root exudation in response to water deficit was not exhaustive; future studies should evaluate additional genotypes among other species and determine other exudate constituents that might influence drought tolerance.

Targeted exudates that are not readily annotated in non-targeted metabolomics and that may serve in drought tolerance should also be evaluated under realistic soil conditions to better understand their role in combating water deficit. For example, ethylene is a hormone that regulates plant growth that is produced by plants and rhizosphere microorganisms. However, ethylene also increases in response to stress and triggers leaf senescence (Abeles et al. 1992). Thus, its reduced presence may be important for continued plant growth under water stress. Methionine and 1-aminocyclopropane-1-carboxylate (ACC) are precursors to ethylene produced by both the microbial population of the rhizosphere and the plant (Arshad and Frankenberger 1992). Both of these metabolites were unable to be annotated in the root exudate profiles represented in our datasets using the available standards on databases. By targeting and quantifying the presence of these exudate compounds in the rhizosphere of genotypes that vary in drought tolerance, we may be able to determine if the reduced presence of specific metabolites is of importance under water deficit. Furthermore, technologies such as the CRISPR/Cas9 system have developed for targeted genome editing (Feng et al. 2014). This system could

potentially alter the plant pathways that produce metabolites implicated in drought tolerance. These technologies, however, require an established transformation system. Currently, there are few laboratories that can routinely create sorghum transformants.

Analyzing how specific metabolites interact with distinct microbial communities and among soil types will further provide an improved understanding of the plant-environment interaction that can potentially be utilized in sustainable agricultural practices. For example, PGPB that produce the enzyme ACC deaminase to reduce ACC and subsequent ethylene levels in the rhizosphere can help plants tolerate water stress (Glick et al. 2007). However, it is still unclear how root exudation elicits these particular ACC deaminase-containing PGPB along with the many other PGPB that aid in overall plant health. Is it the synthesis of specific compounds or perhaps is it the quantity and diversity of chemical classes produced? As plant genotypes have a selective effect on the rhizosphere microbiome (Bakker et al. 2012), it is essential to investigate these questions for their potential uses in plant breeding and sustainable agriculture. Thus, genotypic variation in the composition of exudates should be evaluated along with root gene expression of genes involved with exudate transport and biosynthesis under water stress. Furthermore, studies should also evaluate the functional capacity of the soil microbiome to better understand which traits are enriched in the rhizosphere microbiome among drought tolerant genotypes under water stress. It may be of benefit to assess the microbial composition of the rhizosphere to better understand if increasing exudate quantity and/or diversity is more beneficial to improving drought tolerance. The investigation of both plant and microbial traits that confer drought tolerance among varying soil types to determine overlapping traits will also inform ubiquitous sustainable agricultural practices. If specific exudates do improve drought tolerance, their implementation as biochemical inoculants or their increased expression in plants can help to reduce the amount of chemical inputs and water required to alleviate water stress. As drought stress has been shown to alter microbial communities (Santos-Medellin et al. 2017), the identification of specific

microorganisms that aid in drought tolerance can potentially be used as microbial inoculants to enhance plant growth under water deficit.

The overall goal of this study was to identify plant traits that may confer drought tolerance. As the improved affordability and timing of sequencing has resulted in a vast amount of genomic data, phenotypic data to accompany this genomic data is quickly becoming a limiting factor in plant breeding (Kuijken 2015). Furthermore, drought tolerance is a quantitative trait involving many genes, serving as a particular challenge for plant breeders (Lopes et al. 2011). By combining information across multiple levels of morphological, physiological, biochemical and molecular organization of plant drought tolerance responses, we can better understand the mechanisms that underlie drought tolerance. Using high-throughput technologies to identify phenotypic traits that elucidate genes involved in drought tolerance can thus narrow the number of candidate genes targeted in molecular breeding (Fernie and Schauer 2009). Overall, it is becoming increasingly important that we take a systems approach using realistic environmental conditions to evaluate plant responses to drought stress as endogenous plant mechanisms along with the plant's interaction with its environment play key roles in complex traits such as drought tolerance.

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