INVESTIGATION OF DROUGHT RESISTANCE IN THE GRANITE OUTCROP SUNFLOWER, *HELIANTHUS PORTERI*, COMPARED TO THREE NON-OUTCROP CONGENERS

by

ELISE MICHELLE BARTELME

(Under the Direction of Lisa A. Donovan)

ABSTRACT

Helianthus porteri is a sunflower endemic to drought-prone granite outcrops, suggesting that this species possesses some combination of morphological and physiological traits which confer resistance to drought. We compared *H. porteri* to three other sunflowers from habitats with varying local water availability (*H. agrestis*, *H. annuus* and *H. carnosus*) in a series of experiments with well-watered, sustained mild drought, and soil dry-down treatments. Under well-watered conditions, *H. porteri* exhibited a root system that allows for greater water uptake per unit mass as compared to the other species. In response to mild drought *H. porteri* maintained photosynthetic rates while decreasing water loss. Finally, in response to a dry-down *H. porteri* wilted at a less-negative water potential. Compared to the other *Helianthus* species, *H. porteri* possess a unique combination of traits that provide increased water absorption and water conservation, supporting an avoidance strategy for drought resistance.

INDEX WORDS: Drought stress, sunflower, ecophysiology, granite outcrop, *Helianthus porteri*

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Limitation to water availability has been a major research focus because it is recognized as an influential factor shaping plant diversity and ecological performance (Levitt 1980, Ludlow 1989). Understanding how a plant is able to resist drought will inform predictions of species responses to climate change, especially if drought frequency and duration increases. Here, drought resistance refers to the ability of a plant to prevent severe water stress and ultimately plant death. Drought resistance can be expressed as a continuum of plant strategies or trait combinations that can be conceptually divided into three categories: escape, avoidance, and tolerance. On one extreme of the spectrum, plants escape drought by completing reproduction (as is the case of many annuals) or going dormant (as is the case of many perennial species) prior to the onset of drought. With this strategy, the plants limit their exposure to drought stress. Plants in the middle of this spectrum avoid drought stress by maintaining a plant-water status which decreases exposure to water stress. Avoidance trait combinations include those which increase water uptake, such as deeper rooting and increased root mass ratio (RMR), and minimize water loss by decreasing stomatal conductance (g_s) and increasing water-use efficiency (WUE).Together these traits help a plant avoid drought by maintaining a more favorable balance between water uptake and water loss. Finally, at the other extreme, plants tolerate drought by physiologically lowering the turgor loss point through osmotic adjustment (Levitt 1980, Ludlow 1989, Verslues, Agarwal et al. 2006). This drought resistance spectrum can be used to as a conceptual framework for investigating species responses to drought and understanding of how species are adapted to their native habitats.

Rocky outcrops are found all over the world and are home to unique assemblages of plants endemic to these extreme habitats (Poot, Hopper et al. 2012). These plants live in shallow soils, endure high amounts of sunlight, and the soils can either be water-logged due to low drainage (Poot, Bakker et al. 2008) or drought prone due to high amounts of run-off (Lugo and McCormick 1981, Poorter, Niklas et al. 2012). The granite outcrops of the Southeastern U.S. are located in the Piedmont region spanning from Alabama to Virginia and experience periods of drought during the summer months (Baskin and Baskin 1998). Species endemic to these outcrops are found in shallow depressions on the rock surface. These depressions are formed as lichens cover the bare rock helping to erode the surface and capture soil particles. Over time, other organisms are able to establish: mosses, then annuals and finally perennials (Burbanck and Platt 1964, McCormick and Platt 1964). There are four successional stages on these particular granite outcrops which are defined by both soil depth and the species which are found there. The first is the *Diamorpha* community with soils 2-6cm deep. Second is the lichen-annual herb community with soils 7-15cm deep. The third stage is the annual-perennial herb community with soils 16-39cm deep. The final stage is the perennial-shrub community with soils 40-50cm deep (Burbanck and Phillips 1983). Of the species which grow on these Southeastern granite outcrops, eighteen are endemic to this rocky outcrop. Studies have shown that they all require high light for growth and survival (Mellinger 1972, Baskin and Baskin 1988), and they must also possess some combination of traits which allow them to resist short periods of drought.

One species unique to these granite outcrops is *Helianthus porteri* (A. gray) Pruski (Pruski 1998). This is the only species of sunflower living in the Piedmont outcrop habitat, and is primarily found within the lichen-annual and annual-perennial communities (7-39cm deep). *Helianthus porteri* is unique compared to many other outcrop species because it neither

completes its life cycle nor goes into dormancy prior to the drought-prone summer months. Instead, it germinates in late March, begins flowering in August and continues to flower until first frost. During the summer months, it has been observed to wilt earlier than the other outcrop species in response to drought, persist in a wilted state for two weeks and then recover quickly after rain (Shelton 1963, Mellinger 1972). In addition there is some speculation as to whether or not this species has the ability to root deeply into cracks at the rock surface in search of water (Shelton 1963). This species survives in a drought-prone habitat and has been observed to persist through periods of low water availability, suggesting that it is likely more drought resistant than sunflower species from less drought prone habitats. A study by Gevaert (2011) found evidence to suggest that this species exhibits a drought avoidance strategy through increased photosynthetic water-use efficiency when exposed to drought. However, there have been no explicit tests to determine which traits allow *H. porteri* to resist drought. Therefore, the objective of this research was to investigate traits that may confer *H. porteri* drought resistance on drought-prone granite outcrops. *Helianthus porteri* was contrasted with three other sunflower species from habitats differing in soil moisture availability.

Two of the study species, *H. agrestis* and *H. carnosus*, are closely related to *H. porteri* and together these three species form a single, monophyletic clade that is basal to the genus (Timme, Simpson et al. 2007). *Helianthus agrestis* is an annual found in mucky, wet soils in wetland areas in Florida and *H. carnosus* is an endangered perennial found in wet, sandy soils in Northeastern Florida (Heiser 1969). The final species, *H. annuus*, is the wild progenitor of the cultivated sunflower. *Helianthus annuus* is part of a separate clade which is comprised of mostly annual sunflower species (Timme, Simpson et al. 2007). This species is widespread and typically occupies mesic clay-based soils (Rosenthal, Schwarzbach et al. 2002), in addition to desert areas

(Heiser 1969, Donovan, Rosenthal et al. 2010). Together, these four sunflowers can be thought of as a species continuum which experience different edaphic conditions. On one extreme, *H. porteri* occupies the most drought-prone habitat and on the other extreme, *H. agrestis* can be found in the least drought-prone habitat. Using this suite of species, we compared drought resistance traits of *H. porteri* with those of three closely related congeners. The following questions were posited in order to determine if *H. porteri* has traits associated with a drought avoidant strategy on the drought resistance spectrum:

1. Prior to drought, does *H. porteri* have a fast rooting depth rate and higher root growth rate?
2. Does *H. porteri* possess traits which allow it to avoid declines in plant water status during a mild drought?
3. During a soil dry down, at what plant water potential does *H. porteri* wilt during a decline in soil moisture and does it have a greater ability to withstand a wilted state?

In order to address these questions, four greenhouse studies were conducted using a comparative approach with three species of sunflower from different habitats compared to *H. porteri*. All four wild sunflower species were assessed for gas exchange characteristics, biomass accumulation, root growth rates, and recovery from drought. The first question was addressed using well-watered conditions (i.e. conditions where water is not limited) in order to assess inherent root growth traits. The second question was addressed in a study that used a datalogger and a customized irrigation system to control well-watered and drought treatments to assess species response to mild drought. The final question was addressed in an experiment where water was withheld to initiate an uncontrolled dry-down in soil moisture. As a whole, these

studies were designed in order to determine if *H. porteri* has a greater ability to resist drought in comparison to its non-outcrop congeners.

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CHAPTER 2

ROOT GROWTH CHARACTERISTICS OF A GRANITE OUTCROP SUNFLOWER, *HELIANTHUS PORTERI,* COMPARED TO THREE WILD CONGENERS[1]

[1] Bartelme, E.M. and L.A. Donovan. To be submitted to *Southeastern Naturalist*

Abstract

Fast root development and growth is important at the juvenile stage for species survival in habitats that are prone to drought. *Helianthus porteri* is a sunflower endemic to drought-prone shallow soils of granite outcrops in the Southeastern United States. *Helianthus porteri* was compared to three other wild species of *Helianthus* under well-watered conditions in order to assess root system and biomass characteristics hypothesized to contribute to drought resistance.

Methods: Using two greenhouse studies, root characteristics were investigated for four sunflower species grown under well-watered conditions. In a shallow pot study (30 cm), each plant was harvested when its roots reached the bottom of the 30 cm deep pot, i.e. standardized for rooting depth. In a deep pot study (120 cm), plants were all harvested when the fastest growing species reached the bottom of the 120 cm pots, i.e. standardized by time. The relative ranking of species root system growth rates remained the same across both greenhouse studies, with *H. porteri* ranking intermediate between faster-rooting *H. annuus* and slower-rooting *H. carnosus*. In the shallow pot experiment, *H. porteri* produced the highest total root length, highest frequency of lateral roots and higher specific root length (SRL) than *H. annuus.* In the deep pot experiment *H. porteri* had the highest RMR. Compared to two other species at the same age, *H. porteri* is able to allocate more total biomass towards root production, which is essential for water uptake in the shallow soils of the granite outcrops and suggests that this species may utilize a drought avoidant strategy to resist drought. In addition, results of these experiments suggest that each species exhibits root morphology consistent with the water availability in their native habitats.

Index Words: *Helianthus*, root mass ratio, inherent root growth, specific root length, root morphology, drought avoidance, drought resistance

Introduction

Drought is one of the most influential abiotic stresses that can limit plant productivity throughout the world. This stress has the potential to severely affect the growth and survival of rare and endangered species found in specialized habitats, particularly those which are already prone to drought. Because of this, rare and endangered species in these habitats may be at risk in the future if drought increases. Therefore, an investigation of the traits associated with drought resistance may provide insight into methods necessary to conserve these species in the face of increasing drought due to climate change.

Simply stated, drought can be defined as a decline in the amount of soil moisture available to a plant, typically caused by an absence of precipitation (Boyer 1995). To survive during drought, a plant must resist drought, which is the ability of a plant to prevent severe water stress (Kramer and Boyer 1995). Drought resistance to water stress can be conceptualized into three categories, defined by trait combinations along a continuum, which allow a plant to either escape, avoid or tolerate drought (Levitt 1980, Ludlow 1989, Verslues, Agarwal et al. 2006). Inherent traits under well-watered conditions can also be placed along this continuum. Fast growing annual species that often exhibit an escape strategy are associated with high stomatal conductance (g_s), lower water use efficiency (WUE), and a low root mass ratio (RMR). Avoidance strategy is often associated with intermediate values of g_s, WUE and RMR. On the other end of the spectrum, plants exhibiting drought tolerance will have and inherently low g_s and higher WUE and RMR under well-watered conditions. While inherent traits cannot fully explain how a species will resist drought, these traits can be assessed in order to predict how a species might respond when exposed to drought (Levitt 1980, Ludlow 1989).

Plant root characteristics are thought to play a key role in adaptation to drought-prone habitats. The ability to allocate high amounts of carbon towards root production in the early stages of plant growth, and prior to the onset of drought, could be beneficial for maintaining plant access to soil moisture in deeper horizons (Padilla, Miranda et al. 2007, Seiler 2008). A study investigating the root architecture and anatomy of perennial seedlings from both high and low rainfall habitats under well-watered conditions found that seedlings from high rainfall sites had an overall faster elongation rate for the main root axis (Nicotra, Babicka et al. 2002). However, species from low rainfall habitats showed a greater proportional allocation to the main root as well as a greater elongation rate of the main root, which may result in faster access to the water table despite their overall slower growth of the whole plant. Another study comparing seedling survival of Mediterranean shrub species found that earlier root establishment led to higher survival later in the summer when drought became more prevalent (Lloret, Casanovas et al. 1999). However, species from these studies were not restricted by soil depth in their native habitats. Plants endemic to rock outcrops across the world are adapted to growth and survival in shallow soils that limit rooting depth (Poot and Lambers 2008). Therefore, we might expect that species from these types of habitats to exhibit inherently shallow rooting, even when given the ability to root deeply (i.e. in a deep pot). However, these shallow soils lead to a habitat that is extremely drought-prone due to water run-off and high evapotranspiration, especially during summer months when temperatures are higher (Cumming 1969, Sharitz and McCormick 1973, McCormick, Lugo et al. 1974). It is possible that deep rooting does occur if plants are able to extend roots deep into cracks in the underlying rock where water may be available (Poot, Hopper et al. 2012).

Granite outcrops of the Southeastern United States are one such habitat where endemic species grow in these restricted shallow soils. Plant communities are established in succession as soil begins to accumulate in shallow depressions after lichen establishment. Successional stages on these particular rock outcrops are traditionally grouped into four classifications, and they are defined by soil depth and the vegetation characteristic to each of those communities: (1) a *Diamorpha* community with a soil depth of 2-6cm; (2) a lichen-annual herb community with a soil depth of 7-15cm; (3) an annual-perennial herb community with a soil depth of 16-39cm; and (4) a perennial-shrub community with a soil depth of 40-50 cm (Burbanck and Platt 1964). *Helianthus porteri* is an herbaceous, annual sunflower endemic to these Southeastern granite outcrops which typically grows in soils from 7-39 cm deep. This species germinates in the spring, lives throughout the drought-prone summer months, and then flowers and sets seed in the fall. Therefore, this species may possess some combination of drought resistant traits allowing it to survive to the reproductive stage. One hypothesis for this species' survival is its ability to extend roots deeply into cracks within the rock surface leading to drought avoidance through increased water uptake (Shelton 1963). However, this hypothesis has not been explicitly tested in the field because identifying where these fissures are located is difficult. In addition, many of the outcrop habitats on which this species is found are protected due to habitat decline. Therefore, we used two greenhouse studies to investigate root growth rate and biomass allocation of *H. porteri* compared to three congeners that do not grow on granite outcrops.

The three other *Helianthus* species used in this study were chosen because their habitats differ greatly with respect to soil moisture availability (Heiser 1969). *Helianthus agrestis* is an erect, branching, herbaceous annual found in mucky wet soils in central Florida, and it has been observed growing in standing water (Personal observation, Heiser 1969). The second species, *H.*

carnosus, is an endangered herbaceous perennial species which grows in the moist sandy soils of Northeastern Florida (Heiser 1969). This species grows in a basal rosette form. *Helianthus agrestis* and *H. carnosus* are closely related to *H. porteri*, forming a monophyletic clade containing just these three species (Timme, Simpson et al. 2007). The final species chosen for this study, *H. annuus*, is an erect, branched annual, found throughout the U.S., Canada and Mexico. The widespread distribution of this species, suggests that it has the ability to occupy a wide range of habitats with respect to soil moisture ability, including desert habitats.

Together these four sunflower species provide a novel study system for studying inherent root growth strategies since we can place them on a spectrum from more drought-prone habitat to least drought-prone habitat. *Helianthus porteri* is on the more drought-prone end of the spectrum followed by *H. annuus* then *H. carnosus* and finally *H. agrestis*, which is found in the least drought prone-habitat. In addition, the two species on the drought-prone habitat end of the spectrum grow in very different habitats: *H. porteri* is restricted by how deep roots can grow, while *H. annuus* has no such restriction. Therefore, the goal of this research was to investigate the root growth characteristics of *H. porteri* in comparison to three other wild species of *Helianthus* both at the same stage and the same age. We expected to see faster root growth rates for *H. porteri* since it is from the most drought-prone environment. In addition, we expected to see a higher biomass allocation towards root production (RMR) for *H. porteri* since this is considered to be an essential inherent drought avoidance trait.

Materials and Methods

The objectives of this study were addressed in two greenhouse experiments. The first study compared the time it took for the root systems of the four species, each represented by 2-3 different seed sources (wild populations), to reach the bottom of a 30 cm deep pot. All of these

individuals were grown under well-watered conditions, and once an individual plant's roots reached the bottom of the pot, it was harvested. The second study compared three species for daily changes in root depth for two months under well-watered conditions in 120 cm deep pots. Based on the original experimental design (see below), one species had roots which were compared both under a soil moisture decline and well-watered conditions, while the others were only compared under the well-watered treatment. After the two months, all of the plants in the deep pots were harvested at the same time.

Shallow pot experiment

Achenes (hereafter seeds) were either collected from the wild, or obtained from the U.S.D.A. Germplasm Resources Information Network (GRIN) from sites that span the range of each species. Seeds of *H. agrestis* were wild collected from two populations in Florida (FB: 28°21'N, -80°51'W and SC: 28°47'N, -81°51'W) and a third population was obtained from GRIN (GL: AMES 30848). Seeds of *H. annuus* were collected from one wild population in Utah (LS: 39°41'N, -112°22'W) and two more populations were obtained from GRIN (TX: PI494567 and NE: PI586870). Seeds of *H. carnosus* were wild collected from one population in Florida (FC: 29°30'N, -81°15'W) and the two other populations were obtained from GRIN (DE: AMES28375 and FE: PI64956). Seeds for *H. porteri* were wild collected from three populations in Georgia (CR: 33°14'N, -85°8'W; HR: 33°32'N, -82°16'; and PM: 33°38'N, -84°10'W).

Seeds were germinated by removing the blunt end and placing the seed on a wet filter paper in a Petri dish on April 2, 2012. Seeds were kept in the dark for 48 hours and then moved to a 12h day/12h night light schedule for three days. Six replicates from each population (72 plants total) were transplanted into 30 cm deep Treepots (Stuewe and Sons, Inc., Tangent, OR) containing 1:1 sand and turface (Turface Athletics, MVP ®, Buffalo Grove, IL) mixture in the

greenhouse at the University of Georgia. A screen mesh was placed at the bottom of the pot before adding the soil in order to allow for observation of root growth. In addition, the bottom 2.5 cm of each of the four edges of each pot was sliced open along the corners so that the sides could be peeled open to facilitated observation root growth along the corners and side of the pot. Pots were arranged in a randomized complete block design with two spatial blocks. All seedlings were misted twice a day for two weeks to ensure seedling establishment in the pot, followed by thorough watering to capacity once a day throughout the duration of the experiment. Fifteen grams of slow-release fertilizer (Osmocote Plus 15-9-12, Scotts, Marysville, OH) was applied to the top of the soil one week after transplant in the pots. There was a low germination rate for the CR population for *H. porteri*, so extra replicates for HR and PM were planted into the available pots (N=6 for each population of all species examined except N=9 for HR and PM populations of *H. porteri*).

Each day, pots were checked for the presence of roots in all four corners and the bottom of the pot. When roots were observed, seedlings were measured for shoot height and then harvested for aboveground and belowground biomass. Aboveground biomass was separated into stems, leaves and cotyledons (if still present). Leaves were scanned and assessed for total leaf area using the freeware Image J (NIH; http://imagej.nih.gov/ij/). Roots were carefully rinsed, spread out in a thin layer of water in a transparent plastic tray, and scanned using a desktop scanner at 300dpi. Root system images were then analyzed using WinRHIZO (v. 2002c, Regent Instruments, Quebec) with a threshold value of 115 for each image, and assessed for total root length and total surface area. In addition, a 5 cm section starting at the top of the root was assessed for the number of lateral roots off the main taproot. All biomass was oven-dried at 60°C for 3 days before weighing. Root mass ratio (RMR) was calculated as total root biomass/total

plant biomass and specific root length (SRL) was then calculated as total root length/total root biomass.

Data were analyzed using a nested ANOVA with populations nested within species using SAS® v.9.3 (SAS Institute Inc. 2001). The SLICE command was used in order to partition species differences and comparisons among populations of each species for time to root to the bottom of the pot, biomass, and root characteristics. Significant differences among populations for each species were assessed by comparing the LS means, where a significant difference was determined if $P < 0.05$.Data was transformed using a natural log transformation when necessary in order to fit the normality assumptions of an ANOVA, and normality was determined by a Shapiro-Wilk test.

Deep pot experiment

The deep pot study compared the four study species for rooting characteristics in 120 cm deep pots using a single population per species (*H. agrestis* – SC, *H. annuus* – LS, *H. carnosus* – FE, and *H. porteri* – PM). A single population was chosen because there were no significant differences among populations for the traits collected from the shallow pot experiment (see results section).The study was initially designed to compare the species for rooting characteristics under both well-watered and droughted conditions with a randomized complete block design (4 species x 2 treatments x 6 replicates x 2 blocks). However, the study encountered several challenges that necessitated altering the design. One species, *H. agrestis*, had low seedling survival and was removed from the study. Additionally, the drought treatment was only applied to *H. annuus* (See Appendix 1 for original design and results for drought treated *H. annuus*). Therefore, results are presented for root growth characteristics for three species of

Helianthus under well-watered conditions in a 120 cm deep pot (N=24 for *H. porteri* and *H. carnosus* and N=12 for *H. annuus*).

Seeds were germinated on October 3rd, 2013 and seedlings were transplanted into clear tubes (Uline, Pleasant Prairie, WI – hereafter pots) containing 1:1 sand and turface (Turface MVP ®, Buffalo Grove, IL) on October 10th. Pots were placed at a 45° angle to the floor so that plant roots would grow along the bottom side of the pot to facilitate viewing (Latta, MacKenzie et al. 2004). Black plastic sheeting was placed over the bench to ensure that the roots were not exposed to daylight, and small openings were cut so that the top 10 cm of each pot was exposed above ensuring that the roots were not exposed. An automatic irrigation system was used to supply water through six drip emitters per pot, located every 15cm starting at the top, which were placed along the top side of each pot. The system supplied water for 5 minutes every six hours, (12 am, 6 am, 12 pm, 6 pm; 0.63L each watering cycle).

Each day, every plant's roots were observed after sundown using a green/blue light so that roots could be seen without inducing leaf stomatal opening. The deepest root observed along the bottom side of the pot was marked with a permanent marker (Sharpie ®, Chicago, IL) and dated for a total of 62 days. Plants were harvested between December 11th and 15th after two months of growth when five *H. annuus* individuals reached the bottom of the deep pots (120 cm). The marks along the pots were measured from the top of the soil line to assess daily changes in root growth. Aboveground biomass for each plant was dried to a constant mass at 60 °C and weighed. For belowground biomass roots were extracted by rinsing away the rooting substrate, dried to a constant mass at 60 °C and weighed. Root mass ratio (RMR) was calculated as total root biomass divided by total plant biomass.

Root depth data were analyzed using a repeated measures ANOVA for six evenly spaced days during the time in which roots were measured (every 11 days: Oct 22, Nov 1, Nov 11, Nov 21, Dec 1, Dec 11) using SAS® v.9.3 (SAS Institute Inc. 2001). This method was chosen since a repeated measures ANOVA cannot handle 62 days of data; therefore, choosing evenly spaced dates was more appropriate in order to capture root growth rate differences. A one-way ANOVA was performed using the PROC GLM statement to investigate species differences in overall taproot growth rate, which was measured as total growth/62 days (cm/day). Biomass was transformed using the natural log in order to meet the assumptions of normality as assessed by a Shapiro-Wilk test.

Results

Shallow pot study: Plants harvested at the same stage, 30 cm rooting depth

Species differed significantly for the number of days it took to reach the bottom of the 30 cm pot before harvesting ($F=34.47$, $P<0.0001$; Figure 2.1A). For each species, however, there were no significant differences among the populations for time to reach the bottom of the pot. *Helianthus annuus* had a significantly faster root growth rate than any of the other species and reached the bottom of the 30 cm pot in 15 (± 1) days. There was no difference in the time it took for *H. porteri* and *H. agrestis* to reach the bottom of the pot, as both reached the bottom in 22 days (± 2 and ± 1, respectively). Finally, *H. carnosus* had the slowest growing roots and took 41(± 7) days to reach the bottom of the pot (Figure 2.1A). There were also significant differences in species height at harvest ($F=75.18$, $P<0.0001$, Figure 2.1B). *Helianthus porteri* was significantly taller than *H. annuus,* which was in turn taller than both *H. carnosus* and *H. agrestis*. Species were significantly different for aboveground biomass, belowground biomass and total biomass ($F=15.63$, $P<0.0001$; $F=21.12$, $P<0.0001$; $F=17.48$, $P<0.0001$, Figure 2.1C, D

and E respectively). *Helianthus carnosus* had the highest aboveground, belowground, and total biomass while *H. annuus* had the lowest when harvested at 30 cm rooting depth. The RMR at harvest significantly differed among the four species ($F=12.81$, $P<0.0001$, Figure 2.1F). *Helianthus agrestis* had a significantly higher RMR than all other species. *Helianthus porteri* had a significantly higher RMR than *H. annuus*, but it was not significantly different from *H. carnosus*.

Entire root systems (Figure 2.2) were analyzed for total root length, surface area and mean diameter. There were significant species differences in total root length ($F=12.38$, $P<0.0001$, Figure 2.3A), with *H. carnosus* and *H. porteri* exhibiting a longer root length and *H. annuus* exhibiting a shorter total root length. In addition, there was a significant species difference in root surface area ($F=6.66$, $P=0.0006$, Figure 2.3C). *Helianthus carnosus* had the highest root surface area while *H. porteri* and *H. annuus* had the lowest. Significant species differences were also found for mean root diameter ($F=7.37$, $P=0.0003$, Figure 2.3B). *Helianthus carnosus* had the largest root diameter, and the other three species did not significantly differ from one another. Finally, there were also significant species differences in specific root length (SRL, $F=38.90$, $P<0.0001$, Figure 2.3D). *Helianthus porteri* and *H. annuus* exhibited a higher SRL than the other two species when harvested at 30 cm rooting depth.

Deep pot study: plants harvested at same age.

Under well-watered conditions, *H. annuus* grew roots deeper than the other two species after two months of growth (Figure 2.4). In addition, there were significant species differences for total biomass, aboveground biomass, and belowground biomass at harvest ($F=20.99$, $P<0.0001$, $F=20.26$, $P<0.001$; $F=21.67$, $P<0.0001$; Figure 2.5A, B and C, respectively). *Helianthus annuus* had the highest biomass and *H. carnosus* had the lowest biomass. There were

also significant species differences for RMR (F=6.55, P=0.0029, Figure 2.5D). *Helianthus porteri* had an almost two-fold higher RMR in comparison to *H. annuus*, which did not significantly differ from *H. carnosus*.

Discussion

The ability to survive in the face of a drought is important for species which are found in drought-prone habitats. Early root growth characteristics are important for an individual's establishment before the onset of drought and are essential for water uptake. Therefore, the aim of this study was to compare root growth and root biomass characteristics of a sunflower endemic to the drought-prone shallow soils on granite outcrops with three other wild species of sunflower from differing habitats. We evaluated these root growth characteristics at both the same stage (when roots reached the bottom of a 30 cm pot) and at the same age (2 months of growth).

In order to maximize capture of available soil moisture, Seiler (2008) predicted that in comparison to cultivated *H. annuus*, wild sunflower species from drier habitats should both show rapid root development as well as a high proliferation into the surrounding soil under well-watered conditions. Therefore, we expected that *H. porteri* would have a faster root growth rate because it is from the most drought-prone habitat. Instead, *H. annuus* reached the bottom of the 30 cm pot first. Similar results were found during a study which compared *H. annuus* with *H. niveus* ssp. *tephrodes*, a species also expected to have a faster root growth rate due to its drought-prone desert habitat (Milton 2013). In that study, *H. annuus* reached the bottom of a 30 cm pot at 23 days while *H. niveus* ssp. *tephrodes* reached the bottom of the pot at 38 days. *Helianthus annuus* is a common sunflower found throughout Northern America and can be found in many different habitats, including the deserts of the Southwest US and our population collected in

Little Sahara in Utah (Heiser 1969, Ludwig, Rosenthal et al. 2004). Therefore, fast root growth could be indicative of *H. annuus* ability to root quickly prior to the onset of drought, which is consistent with an avoidance strategy (Levitt 1980, Verslues, Agarwal et al. 2006).

Both *H. porteri* and *H. annuus* had a similar specific root length (SRL). This parameter is thought to characterize the economic aspects of root systems whereby, long thin roots (high SRL) are indicative of a resource acquisitive species (Ostonen, Puttsepp et al. 2007). *Helianthus porteri* has a higher root proliferation than *H. annuus*, as evidenced by a higher number of lateral roots and higher total root length. This high amount of lateral roots may be important for both the anchorage of this species due to the fact that it grows in shallow soils of the granite outcrops (Burbanck and Phillips 1983) and for the uptake of available soil moisture in those shallow soil layers.

Helianthus agrestis and *H. carnosus* are native to habitats that are much wetter than those of *H. porteri* and *H. annuus* for the majority of the year (Heiser 1969). However, these species have different life histories, as *H. agrestis* is an annual and *H. carnosus* is a perennial. Even though *H. carnosus* produced a much higher total root biomass in the shallow pot study, this was due to the fact that it was growing for a longer period of time before reaching the bottom of the pot than *H. agrestis*. Both species produced the same amount of root biomass when grown for the same amount of time, two months, in the deep pots. However, when the RMR from the shallow pot experiment for these two species is compared, *H. agrestis* is the highest. This is surprising because *H. agrestis* has been placed on the least drought-prone habitat end of our spectrum and we did not expect to find inherently high RMR for this species.

When the three species in the deep pot experiment were harvested at the same age (two months of growth), we found that the relative ranking of root growth rates for these individuals

remained the same as in the shallow pot experiment. *Helianthus annuus* had the fastest taproot growth rate followed by *H. porteri* and finally *H. carnosus*. In addition to likely being a more resource acquisitive species as evidenced by its higher root system SRL, *H. annuus* also rooted the deepest. Deep root growth for *H. annuus* may be a survival strategy as water sources can be found deep within the soils, and access to this water may aid in avoiding drought in a range of habitats, whereas deep water sources may not be accessible for *H. porteri* on the shallow granite outcrops. A similar study comparing twenty xeric and twenty mesic genotypes of *Avena barbata,* an annual species in California, demonstrated that the seedlings with the xeric genotype expressed a greater RMR than the mesic genotypes (Latta, MacKenzie et al. 2004). In addition, seedlings with the xeric genotype allocated a higher proportion of roots deeper in the soil. The results of this study are similar to those we found for *H. annuus*, which had the highest aboveground biomass and deepest rooting. Despite *H. annuus* having a greater biomass production, *H. porteri* had a higher RMR which suggests that this outcrop species allocates a greater amount of carbon towards root production. This is a classic strategy that drought avoiders utilize in order to delay the effects of drought (Levitt 1980). Therefore, the results of the deep pot experiment suggest that both species may be equally likely to avoid drought either by deep rooting or by increased allocation to root biomass, frequency of lateral roots, and total root length.

Helianthus porteri is endemic to granite outcrops where it grows in shallow soils of 7-39 cm deep (Burbanck and Platt 1964), and is therefore restricted in how deep the roots are able to penetrate through the soil. Many studies have suggested that coarse roots are necessary to access fissures in rocks where water may be located (Graham, Schoeneberger et al. 1997, Poot, Hopper et al. 2012). Shelton (1963) posited that *H. porteri* may also be extending roots into fissures in

the underlying granite. Although we did not explicitly test the outcrop sunflower's ability to access water stores in rock fissures, the results of our study suggest that *H. porteri* may not be accessing these fissures. A combination of shallow rooting, high RMR, high total root length, and high SRL indicate that this granite outcrop species allocates more carbon towards root production. This high SRL means that *H. porteri* produces the highest root length for water absorption per unit mass in comparison to the other three sunflowers. Similar conclusions were made by Nicotra et al. (2002), whereby increased SRL is typical for species in response to drought stress and is used as a mechanism to increase water uptake.

High SRL may be a key trait which allows *H. porteri* to acquire high amounts of water during summer rains which allows it to produce more roots which increases the surface area that is essential to extract water as soil moisture declines. Similar results were found for ironstone endemics in Australia (Poot and Lambers 2008). Ironstone *Hakea* endemics allocated more biomass to root production compared to aboveground biomass, exhibited a greater total root length, and had a higher SRL during early development under well-watered conditions. The differences between outcrop *Hakea* species, and those found in deeper soils may be attributed to an evolutionary tradeoff, whereby the endemic species must increase the chance of getting access to water before the onset of drought using specialized root system morphology which may not allow them to compete successfully off of the ironstone outcrop (Poot and Lambers 2008). A previous study has shown that *H. porteri* is dependent upon a low level of competition and high light requirement lead to *H. porteri* endemism on the outcrops (Shelton 1963). Although this study did not look at root competition, it is possible that the root system morphology of *H. porteri* may have a similar evolutionary tradeoff as the *Hakea* species from the ironstone

outcrops. Future studies *in situ* would be necessary to determine if this tradeoff further explains *H. porteri* endemism to the granite outcrops.

This study has provided the unique opportunity to study root growth characteristics of a sunflower species which grows on drought-prone granite outcrops. Drought is one of the most influential abiotic stresses that can limit plant productivity. It is expected that changes in temperatures and precipitation patterns due to global warming will result in an increase in the frequency and severity of drought (Sheffield and Wood 2008). This increasing stress has the potential to severely affect the growth and survival of species restricted to drought-prone habitats, such as *Helianthus porteri.* Results of our studies suggest that *H. porteri* exhibits rooting characteristics observed under well-watered conditions that are consistent with a drought avoidance strategy. In addition, we have shown that each of the four species exhibits different root morphologies which likely attribute to their adaptation to the native habitats. In the future, these species could face increasingly drier habitats if climate change results in less precipitation. Therefore, studies which focus on how these species are affected by drought in their native habitats, potentially using rain-out shelters, will be useful in understanding their rooting response under water stress.

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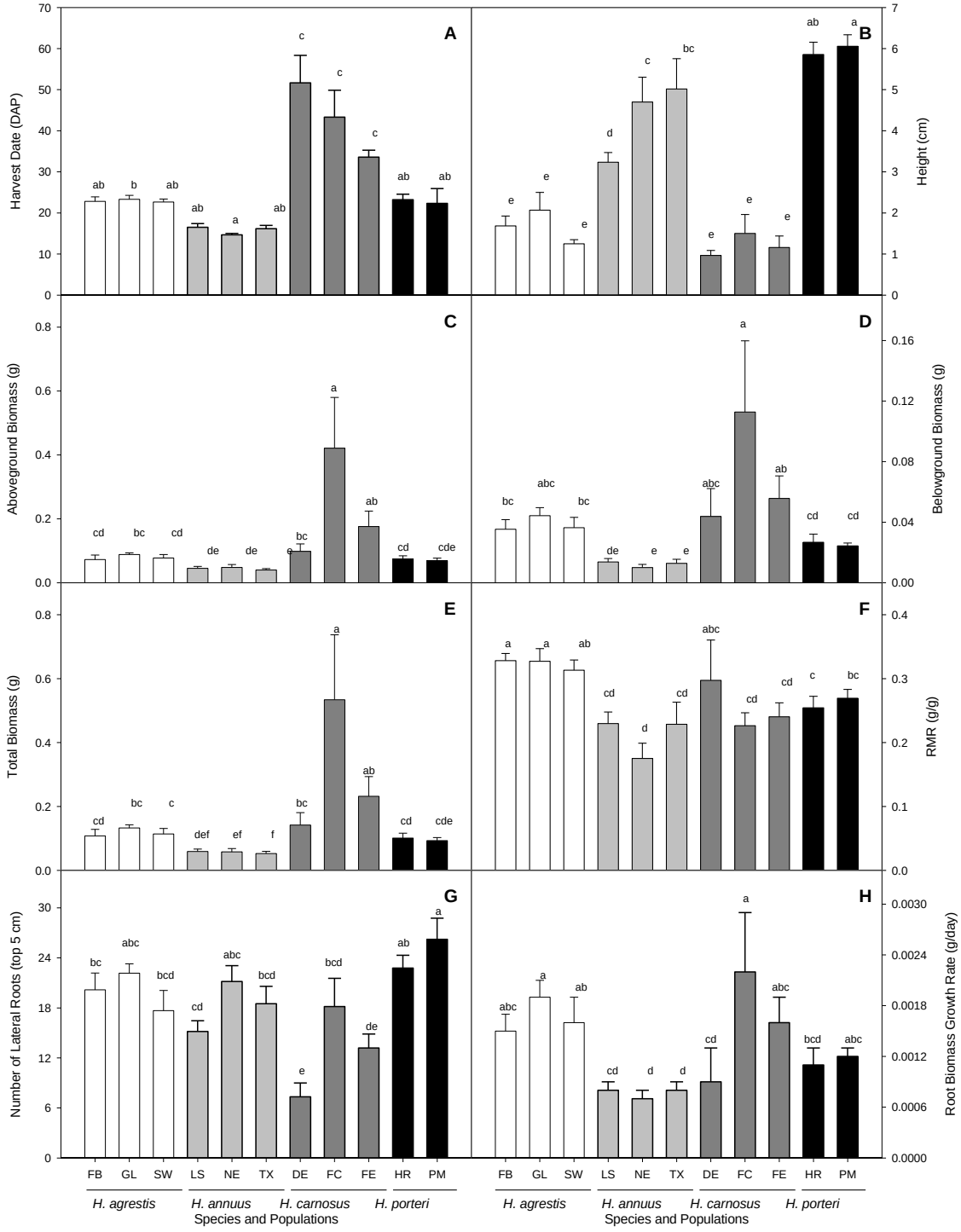

FIGURE 2.1: Comparison of *H. porteri* to three other *Helianthus* species for traits (mean ± SE) for when harvested at 30 cm rooting depth. Harvest date is represented as days after planting

(DAP, A). Measured traits at harvest include height (B), aboveground biomass (C), belowground biomass (D), total biomass (E), Root mass ratio (RMR, F), the number of lateral roots in the top 5cm of soil (G), and root biomass accumulated per day (g/day; H).

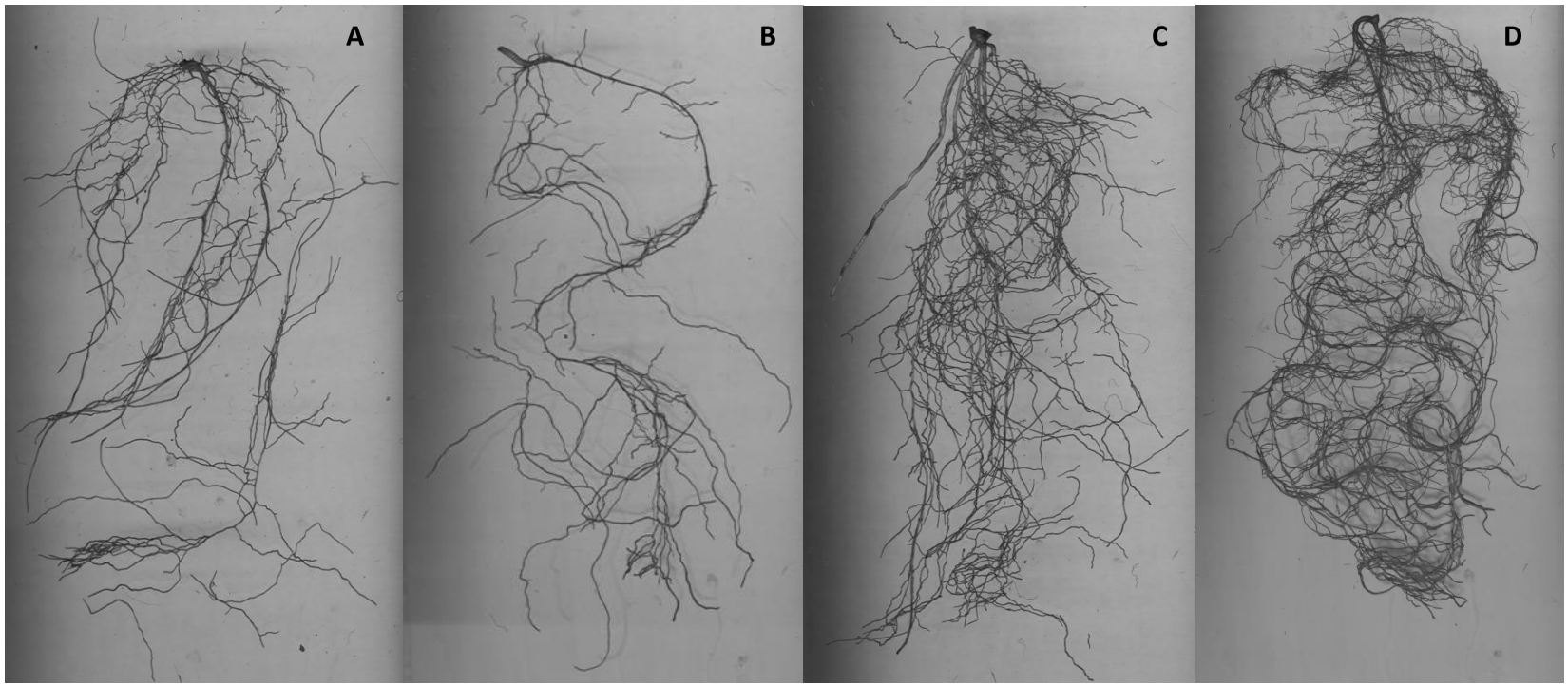

FIGURE 2.2: Entire root system scans for one representative of each species: *H. agrestis* (A, 22 ±1 days), *H. annuus* (B, 15 ± 1 days), *H. carnosus* (C, 41 ± 7 days), and *H. porteri* (D, 22 ± 2 days).

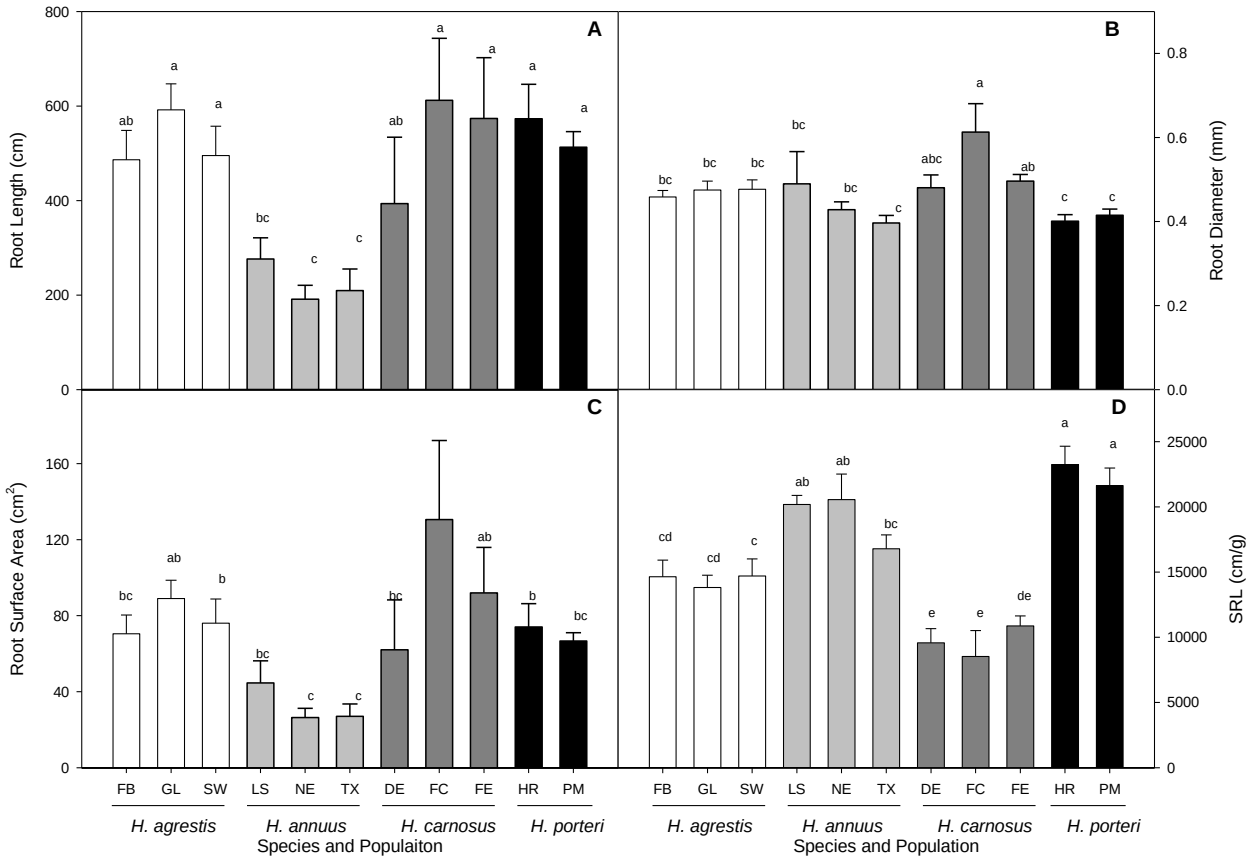

FIGURE 2.3: Comparison of *H. porteri* to three other species of *Helianthus* for root traits (mean ± SE) when were harvested at 30 cm rooting depth. Root analysis traits include total root length (A), root diameter (B), root surface area (C), and specific root length (SRL, D).

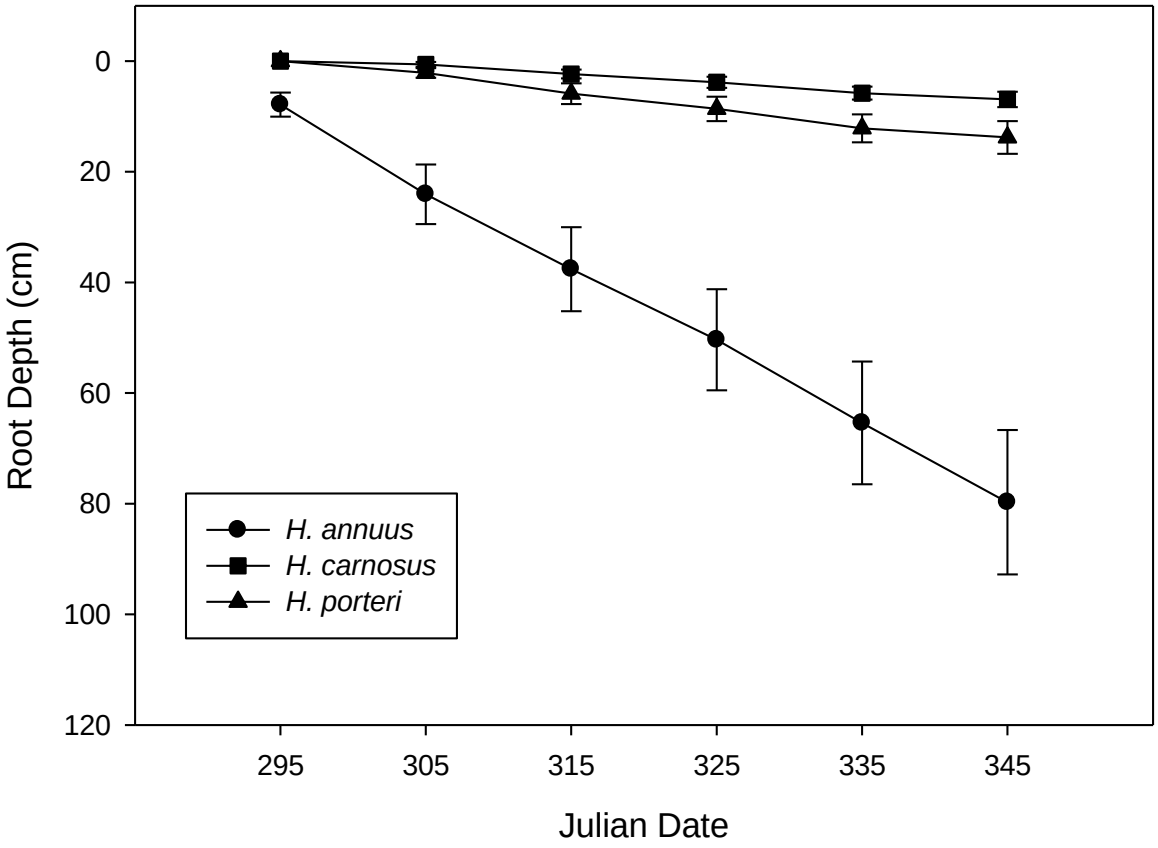

FIGURE 2.4: Root depth (mean ± SE) on six different dates over a two-month growing period for *H. porteri* compared to *H. annuus* and *H. carnosus* grown under well-watered treatment (n=12 for *H. annuus*, n=23 for *H. carnosus*, and n=20 for *H. porteri*).

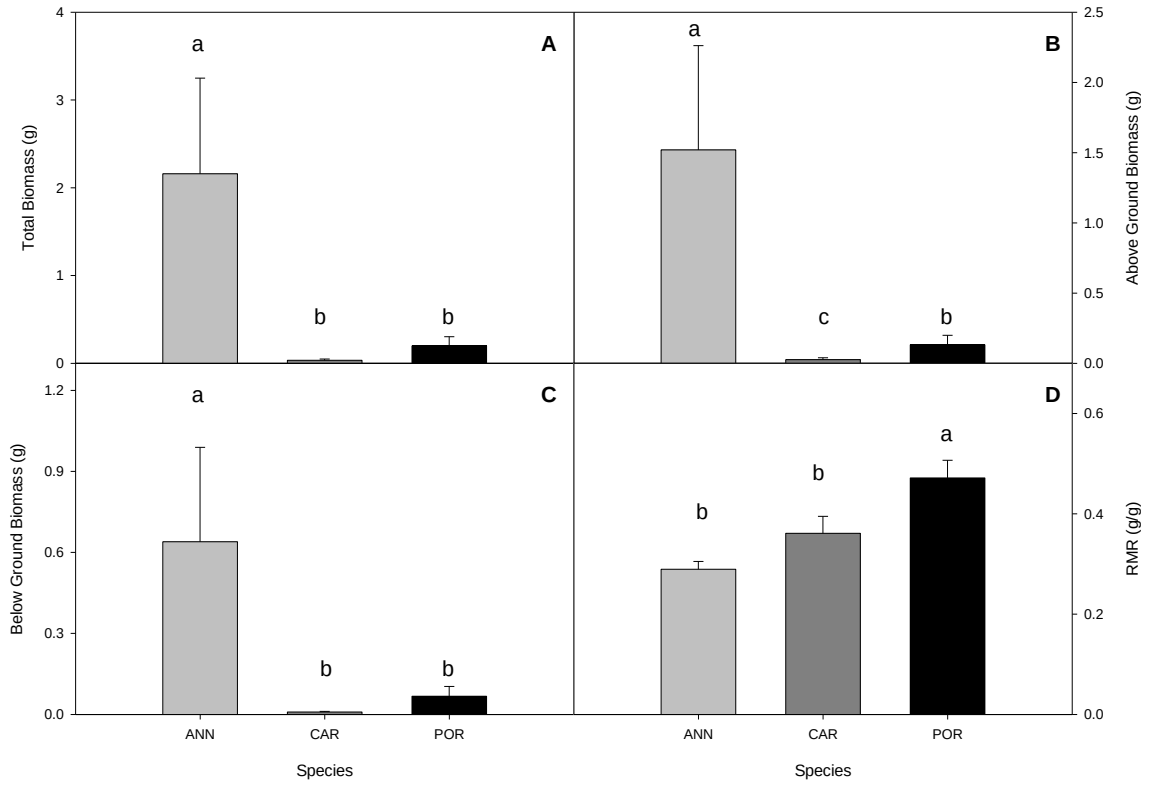

FIGURE 2.5: Root traits (mean ± SE) of *H. porteri* compared to two other species of *Helianthus* after two months of growth in a 120 cm deep pot under well-watered conditions. Measured traits include total biomass (A), aboveground biomass (B), belowground biomass (C), and root mass ratio (RMR, D).

CHAPTER 3

COMPARISON OF MILD DROUGHT RESISTANCE OF FOUR *HELIANTHUS* SPECIES FROM VARYING HABITATS[2]

[2] Bartelme, E.M., A.J Pilote and L.A. Donovan. To be submitted to *Southeastern Naturalist*

Abstract

Protecting unique habitats and their rare, endemic species in the face of a predicted increase in drought is of ecological concern. In this effort, studying the wild granite outcrop sunflower, *Helianthus porteri*, which experiences varying levels of drought during the summer, can provide insight into its water stress resistance strategy. The purpose of this study was to determine if *H. porteri* responds to a mild drought through greater increases in water use efficiency (WUE) and root mass ratio (RMR) in comparison to three other wild species of *Helianthus*. In a greenhouse study, juvenile plants were subjected to two watering treatments, well watered and sustained mild drought, using an automated irrigation system. After two weeks under treatment, species were assessed for the response of gas exchange, biomass and osmotic adjustment to mild drought. *Helianthus porteri* responded to the mild drought treatment with lower total biomass, no change in root mass ratio (RMR), lower stomatal conductance (g_s) and higher photosynthetic WUE. *Helianthus agrestis* responded to the mild drought treatment with no change in total biomass, but higher RMR. Both *H. agrestis* and *H. annuus* responded with decreased g_s, thereby increasing WUE. *Helianthus carnosus* is the only species that did not alter gas exchange rates, but it did respond by increasing RMR. None of the species showed evidence for osmotic adjustment. Overall, *H. porteri* exhibits trait responses to mild drought consistent with a drought avoidance strategy of decreasing water loss through the stomata. Further testing of this species response to severe drought could determine if *H. porteri* utilizes traits such as osmotic adjustment and cell wall stiffening; these traits may also be important in *H. porteri*'s persistence during prolonged summer drought on the rock outcrops.

Index Words: *Helianthus*, wild congener species, drought, stomatal conductance, root mass ratio, osmotic adjustment, automatic irrigation

Introduction

Scientists studying global climate change predict that precipitation patterns will change world-wide causing drought in areas that do not currently experience drought (Povilitis and Suckling 2010). These precipitation changes have the potential to impact species distribution and diversity around the world, especially in arid and semi-arid regions. Because of this, it is important to understand how species respond to drought so that conservation efforts can be made to protect naturally occurring populations before water shortages become more prevalent.

Drought resistance refers to the ability of a plant to prevent severe water stress and ultimately plant death. These trait responses can be conceptually divided into three non-mutually exclusive categories which can be placed along a spectrum of strategies: escape, avoidance and tolerance (Levitt 1980, Verslues, Agarwal et al. 2006). On one end of the spectrum, plants utilizing the escape strategy either complete their reproductive cycle or undergo dormancy before the onset of drought. Drought avoidance is a plant's ability to delay declines in plant-water potential by maximizing water uptake while minimizing water loss. Plants which utilize this strategy may allocate more resources to root biomass production (increasing ratio of root mass to total mass RMR), enhancing water uptake. Plants may also conserve water by decreasing water lost through stomata (lower stomatal conductance, g_s), which leads to a higher water use efficiency (WUE). On the far end of the spectrum, plants which use a tolerance strategy for drought resistance have traits which allow them to continue metabolic function at lower (more stressful) plant water potential. Plants can decrease the turgor loss point (the osmotic potential where turgor is zero) through osmotic adjustment, which is the accumulation of compatible solutes in tissues that do not disrupt normal cellular function (Levitt 1980, Ludlow 1989). Another trait associated with a tolerance strategy is cell wall stiffening, assessed as the elastic

modulus (ε, MPa) of the cell wall. (Verslues, Agarwal et al. 2006). When the cells have a high ε, the cell walls are stiffer. As water is lost from the cells, the cell walls remain stiff and the cytoplasm can no longer push up against the cell wall. When this occurs, the cells lose turgor, but maintain a high relative water content (RWC, %).

In order to understand the drought resistance, many studies have assessed plant response to a soil dry down imposed by withholding water (Zavalloni, Gielen et al. 2008, Curran, Clarke et al. 2013, Perez-Ramos, Volaire et al. 2013). For example, a dry-down study investigating the drought response of Mediterranean tree seedlings imposed the drought by using an automatic irrigation system to administer water once per week. This study demonstrated that water stressed seedlings exhibited lower biomass, lower specific leaf area and lower photosynthetic rates compared to the unstressed plants (Valladares and Sanchez-Gomez 2006). Another study investigating drought resistance strategies of six willows (*Salix*) by withholding water found that the species which occurred within drier habitats exhibited high WUE and faster growth rates as compared to species from wetter habitats (Savage and Cavender-Bares 2011). However, very few studies have investigated species' response to a sustained, mild drought treatment in order to determine which traits are most important for drought resistance; therefore, we use a sustained, mild drought administered using an automatic irrigation system and datalogger in order to investigate trait response to drought.

Rocky outcrop habitats in the Piedmont region of Southeastern U.S. are extremely drought prone due to shallow soil and high surface temperatures, yet they are home to many rare, endemic species (Baskin and Baskin 1988, Damschen, Harrison et al. 2012, Curtis, Stirton et al. 2013). These habitats are of great conservation concern due to habitat destruction and the potential for increased frequency and duration of drought due to climate change (Sheffield and

Wood 2008). *Helianthus porteri*, a wild relative of the agriculturally important sunflower, *H. annuus*, is the only sunflower species which is native to the shallow soils of granite outcrops in the Southeastern United States. *Helianthus porteri* germinates in the spring and survives on the granite outcrops throughout the hot summer, and then flowers and sets seed in the fall (Shelton 1963, Mellinger 1972). During the summer when temperatures and evapotranspiration are extremely high on these outcrops (Cumming 1969, McCormick, Lugo et al. 1974, Burbanck and Phillips 1983), this species has been observed to respond to a drought by wilting sooner than co-occurring outcrop species, persisting in that wilted state for up to two weeks, and recovering after it rains (Shelton 1963, Mellinger 1972). This suggests that, to some extent, *H. porteri* is able to resist drought, provided the localized drought is not too severe, in which case, the entire population may not survive to reproduction. Therefore, investigating *H. porteri* response to a sustained, mild drought could help explain the responses observed on the granite outcrops.

In order to assess *H. porteri*'s ability to resist drought, three congeners were chosen based on their differing native habitats. Two species, *H. agrestis* and *H. carnosus*, are closely related to *H. porteri*, forming a monophyletic clade (Timme, Simpson et al. 2007). In addition, both of these species are found on habitats which are more mesic than *H. porteri*. *Helianthus agrestis* is an erect, branched annual found throughout central Florida. This species grows in mucky, wet soils and can be found in standing water in some areas (Personal observation, Heiser 1969). *Helianthus carnosus* is a rare, perennial species with basal rosettes found in Northeastern Florida on wet, sandy soils. For this perennial species, bolting and flowering begins in late June and occurs until first frost. The final species chosen for this study was *Helianthus annuus*, a member of a different clade comprised of annual species (Timme, Simpson et al. 2007). *Helianthus annuus* is an erect annual found throughout the central portion of North America from Canada to

Mexico. This species was chosen because it occupies a wide range of habitats, including desert regions of the Southwestern U.S., as it is the wild progenitor of cultivated *H. annuus*. Together, we can place these four species on a continuum from the most drought-prone habitat to least drought-prone habitat: *H. porteri, H. annuus, H. carnosus,* and finally *H. agrestis.*

For this study, we ask whether *H. porteri*, a sunflower that grows on granite outcrops, exhibit traits which confer greater drought resistance in response to a mild drought when compared to three other *Helianthus* species. Three physiological characteristics were assessed: gas exchange, biomass production and allocation, and osmotic adjustment. We predict that *H. porteri* species will have the greatest ability to decrease its stomatal conductance and increase its water use efficiency under mild drought stress in order to decrease water loss, a strategy consistent with drought avoidance. In addition, we expect to see a greater increase in root mass ratio for *H. porteri* in comparison to the other three species of sunflower. Finally, under mild drought stress, we predict that *H. porteri* will exhibit less of an ability to osmotically adjust as compared to the other species due to the fact that this species has been observed to wilt early during a decline in soil moisture compared to other outcrop species.

Materials and Methods

Seed germination and automatic irrigation

Seed was either wild collected (*H. annuus* - Little Sahara, UT and *H. porteri* – Panola Mtn., GA) or obtained from the U.S.D.A. National Genetic Resources Program (GRIN; *H. agrestis*: AMES30851 and *H. carnosus*: PI649956) and was scarified for germination on May 15th 2013. Briefly, the blunt end of each seed was cut off and seeds were placed on filter paper inside of a petri dish. After 48 hours in the dark, seeds were placed on the lab bench under a 12h/12h light/dark cycle until seedlings were transplanted into permanent pots containing a 1:1

sand to turface mixture (Turface, MVP ®, Profile Buffalo Grove, IL). These pots were arranged in a randomized block design with three blocks each containing four replicates of each species and treatment combination (4 species x 3 blocks x 2 treatments x 4 replicates = 96 pots total).

Plants were grown for approximately 40 days under well-watered conditions (watered to capacity twice per day) and fertilized with a slow release fertilizer with micronutrients (Osmocote Plus 15-9-12, Scotts, Marysville, OH) in order to reach a size that would facilitate measurements before the reproductive stage. On July 1st, two treatments were implemented using an automatic irrigation system to maintain soil moisture (SM) at 20% for the well-watered treatment and 14% for the drought treatment. This mild drought set-point was chosen because it allowed plants to persist under drought without wilting and death. Percent water content was monitored using soil moisture probes (ECH_2O-5 probes, Decagon Devices, Pullman, WA). The soil moisture for each treatment of each species was averaged (12 pots total), and this average was compared to the set-point every 30 seconds. When the average soil moisture for a group of pots dropped below the set point, a signal was sent to a solenoid valve that would open and allow watering to occur for 30 seconds (Nemali and van Iersel 2006) (See Appendix 3 for datalogger program).

A problem occurred with the irrigation system which interfered with the water delivery for all twelve well-watered *H. annuus* causing them to experience a soil dry down to about 12% early in the experiment, before recovering to the well-watered level of 20% (Figure 1). Since biomass is integrated over the plants life, we did not include *H. annuus* in our analyses. However, this species has been shown to quickly recover from a soil dry-down (Cechin, Rossi et al. 2006), and the water treatments were maintained at target levels for 11 days before

physiological measurements, so we were able to include *H. annuus* gas exchange and osmotic adjustment measurements in our analyses.

Construction of the pressure-volume curve and gas exchange

Pressure chamber measurements were collected between July 15th and 19th for the construction of pressure-volume curves in order to get osmotic potential at full hydration (π_o, MPa), which is used to calculate osmotic adjustment. These data were collected using rehydrated stems for *H. agrestis*, *H. annuus* and *H. porteri*. Stems were chosen since *H. porteri* and *H. agrestis* have little to no petiole. In addition, since *H. carnosus* grows in a basal rosette with leaves having a prominent mid-rib, it was not possible to collect more than three or four measurements before the mid-rib was crushed making it impossible to continue data collection. Data for the pv-curves was collected using a Scholander pressure chamber following the bench dry method (Boyer 1995). Briefly, stem water potential was measured with the pressure chamber, the sample was weighed and then dried on the bench before measuring again. At least 10 measurements were collected for each stem in order to construct each pv-curve. Pressure volume curves were constructed using the Pressure Volume Curve analysis spreadsheet provided by Dr. Lawren Sack (http://prometheuswiki.publish.csiro.au). The negative inverse of water potential data was plotted against 100-Relative Water Content (RWC). These curves were used to determine osmotic potential at full hydration (π_o), water potential at turgor loss (π_{tlp}), and RWC at turgor loss point (RWC_{tlp}).

After stems were sampled for pv-curves, gas exchange was measured for two replicate plants of each species in each treatment and each block (4 species x 3 blocks x 2 treatments x 2 replicates = 48 plants). Due to cloudy conditions at the time of measurement, plants were moved to a growth chamber set at 25°C and a 55% relative humidity. The light condition in the chamber

was a photosynthetically active radiation (PAR) of 580 watts*m^{-2}, and the vapor pressure deficit (VPD) was 2.07 kPa. After the plants had been given at least an hour to acclimate to the conditions in the growth chamber, gas exchange measurements were collected using the Li-Cor 6400 (Lincoln, NE) on the most recently fully expanded leaf with a PPFD set to 2000 μmol m^{-2} s^{-1}. This leaf was chosen for this measurement due to the fact that it was developed under treatment conditions. When the chamber was not clamped on to the leaf, flow was set to 500 μmol s^{-1} and CO_2R was set to 400 μmol CO_2 mol^{-1}air, and when a leaf was in the chamber the parameters were switched so that CO_2S was set to 400 μmol CO_2 mol^{-1}air and the H_2OS was set to 25 mmol H_2O mol^{-1}. Gas exchange data collected include photosynthetic rate (A_{max}), stomatal conductance (g_s) and water-use efficiency (WUE, A_{max}/g_s)After gas exchange data was collected, all plants were harvested by bulking the aboveground biomass for each individual (pv-curve sample + remaining biomass) and washing the soil from the roots to collect belowground biomass. All biomass was dried to a constant mass at 60°C and weighed.

Statistical analyses

Pressure-volume curve parameters, harvest data, and gas exchange data were all evaluated using a 2-way ANOVA in SAS (9.3). The LSMEANS statement was used to calculate the LS-mean of each fixed effect (species and treatment) where significant differences between species for a given treatment was determined if $P<0.05$. Data were transformed for WUE (natural log), total biomass (natural log) and RMR (arcsine) to meet assumptions of normality.

Results

Gas Exchange

Gas exchange rates were collected 22 days after the treatment began. There was a significant species and treatment effect for A_{max} ($F=7.44$, $P=0.0006$ and $F=4.19$, $P=0.0488$,

respectively), but no significant interaction (F=1.15, P=0.3446; Figure 3.2a). In the well-watered treatment, *H. annuus* had a significantly higher photosynthetic rate than the other species under well-watered conditions, which did not significantly differ from each other. In addition, *H. annuus* had a significantly higher A_{max} under the drought treatment in comparison to the other three species.

Significant species, treatment, and species by treatment interactions were found for stomatal conductance (g_s) (F=13.17, P<0.0001; F=37.06, P<0.0001; F=7.17, P=0.0008, respectively; Figure 3.2b). Under well-watered conditions, *H. annuus* had a significantly higher g_s than any of the other three species. Additionally, *H. agrestis* and *H. porteri* had a significantly higher g_s than *H. carnosus*, but were not significantly different from each other under the well-watered treatment. When comparing each species response to a mild drought, *H. annuus*, *H. agrestis*, and *H. porteri* significantly decreased their g_s, but *H. carnosus* did not show a treatment response. There were no species differences for g_s under the mild drought treatment.

The water-use efficiency showed significant species and treatment effects, and there was a significant interaction (F=4.16, P=0.0132; F=23.26, P<0.0001; and F=2.87, P=0.0249, respectively; Figure 3.2c). *Helianthus carnosus* had a significantly higher WUE in the well-watered treatment compared to the other three species. *Helianthus porteri*, *H. agrestis*, and *H. annuus* responded to the drought treatment with an increase in WUE, but there was no significant change for *H. carnosus*. There were also no species differences in WUE under the mild drought treatment.

Biomass

Helianthus annuus biomass data were excluded from analyses due to a problem with irrigation early in the experiment (see methods). Comparing *H. porteri*, *H. agrestis* and *H.*

carnosus there is a significant species effect and a marginal treatment effect (F=56.96, P<0.0001 and F=3.62, P=0.0627 respectively; Figure 3.3a), but no significant species by treatment interaction (F=0.49, P=0.6139). Specifically, *H. porteri* had a significantly higher total biomass than the other two species under well-watered conditions. In addition, *H. porteri* is the only species which shows significant decreases in total biomass in response to a mild drought.

There were significant treatment and species by treatment interaction effects for RMR (F=8.40, P=0.0055 and F=3.48, P=0.0380, respectively; Figure 3.3b) but no significant species effect (F=0.46, P=0.6329). None of the species showed significant differences in RMR under well-watered conditions. However, both *H. agrestis* and *H. carnosus* responded to the drought treatment with significant increases in RMR when compared to the well-watered treatment. In addition, there is a significant treatment effect for aboveground biomass (F=5.22, P=0.0261) but not for belowground biomass (F=0.34, P=0.5640).

Osmotic adjustment

Pressure volume curves were used to obtain the following parameters: osmotic potential at full hydration (π_o), water potential at turgor loss point (π_{tlp}), and the relative water content at the turgor loss point (RWC_{tlp}). Under well-watered conditions, there were no significant differences among the three species for any of the pv-curve parameters (π_o, π_{tlp}, and RWC_{tlp}, Table 3.1). In addition, there were no treatment differences between the well-watered and drought treatments (See Table 3.1 for statistics).

Discussion

We compared the drought response of *Helianthus porteri*, a species found on drought-prone granite outcrops, with three wild congeners. Specifically, we investigated inherent traits under well-watered conditions and those same traits under a sustained, mild drought. Using this

approach, we were able to determine that each of these four species exhibits different inherent trait combinations under the well-watered conditions, as well as a differential response to mild drought.

Traits of well-watered plants

Comparison of species traits under well-watered conditions can help determine trait differences that may provide an advantage in native habitats such as differences in maximum biomass production and photosynthesis (Arntz and Delph 2001). *Helianthus porteri*, the granite outcrop species, had the highest total biomass production compared to its congeners. This result is consistent with the observations of high growth rates for *H. porteri* when water availability was high on granite outcrops (Mellinger 1972, Lugo and McCormick 1981). Both *H. agrestis* and *H. carnosus* had lower biomass production under well-watered conditions. *Helianthus carnosus* was the only perennial species in our study, and it was shown to have the lowest photosynthetic rate and stomatal conductance rate under well-watered conditions, which resulted in higher WUE. The lower total photosynthesis rates are consistent with lower biomass accumulated by *H. carnosus*.

Despite the initial well-watered irrigation problems for *H. annuus* (Figure 1), this species exhibited the highest A_{max} and $g_{s,}$ after proper irrigation was resumed in comparison to the other four species. Our gas exchange measurements were collected ten days after irrigation was resumed, which allowed plenty of time for *H. annuus* to recover its gas exchange rates (Cechin, Rossi et al 2006). In addition, our values are consistent with values previously measured for this species under well-watered conditions (Schwarzbach, Donovan et al. 2001). This recovery ability and high gas exchange rate is consistent with the live fast, die young strategy of many desert annuals (Donovan, Dudley et al. 2007).

Drought-prone species response to drought

Overall, the four wild sunflower species chosen for this study showed different responses to mild drought stress. Under a sustained level of mild drought controlled with an automated irrigation system, *H. porteri* significantly decreases its total biomass without a significant change in RMR, suggesting that under mild drought, allocations to above and belowground decrease proportionally. In addition, this species decreased water loss by lowering its g_s, and increasing WUE, although there was no significant decrease A_{max}. Although there is a trend that suggests that photosynthetic rate was affected. The construction of an A/C_i curve (Carbon assimilation (A)/ intercellular CO_2 (C_i)) under both well-watered and drought treatments would be necessary to determine the relative contributions of stomatal limitation and decreases in photosynthetic capacity.

Under mild drought, *H. annuus* responded with a stomatal closure to a value similar to other species, demonstrating its ability to conserve water in response to mild drought. In addition, *H. annuus* still had a significantly higher photosynthetic rate under mild drought in comparison to the other three species. Similar results were observed by Schwarzbach and Donovan et al. (2001), whereby when water was withheld, *H. annuus* exhibited a significantly higher photosynthetic rate and stomatal conductance as compared to two other species of sunflower at the point of wilting. Together, this suggests that this sunflower is a drought avoidant species.

Osmotic adjustment is a trait response indicative of a species which utilizes a drought tolerant strategy during drought. We expected to find evidence that *H. porteri* osmotically adjusts under mild drought, but we found no evidence of osmotic adjustment under a sustained mild drought. It is possible that the stress level imposed was not strong enough to cause a

significant response, but a more severe drought stress is necessary to determine if this is a trait *H. porteri* uses in response to drought.

Wetland species response to drought

Under mild drought, *H. agrestis* significantly increased RMR which is a trait typically associated with an avoidance strategy. In addition, this species conserved water by decreasing stomatal water loss, but A_{max} did not significantly decrease. Once again there are trends for a decrease in photosynthetic response, and A/C_i curves should be constructed in order to explain the trends we see. In addition, it is possible that *H. agrestis* response to mild drought is consistent with frequent, short-term periods of drought that may occur during the summer months in Florida (Martinez, Maleski et al. 2012). High WUE and RMR can help this sunflower maintain a plant-water status at an unstressed level until it rains again. However, without specifically testing if the observations we have observed are consistent with traits collected in native habitats, it is not possible to confirm whether or not this species utilizes a drought avoidance strategy.

Significant increases in RMR under the mild drought treatment without significant changes in total biomass, suggests that *H. carnosus* was able to shift carbon allocations toward root biomass production. However, the mild drought stress did not significantly affect the gas exchange rates. This impressive response to mild drought suggests that this particular species may be able to increase water uptake through increased root production while maintaining metabolic function in the face of drought. A drought study comparing the growth rates, biomass allocation and root distribution for three perennial legumes (*Medicago sativa*, *Dorycnium hirsutum*, and *D. rectum*) found that the slower growth rates of *Dorycnium* seedlings make them more prone to establishment problems due to competition from faster growing species (Bell

2005). However, despite its slower growth rate, *D. hirsutum* may be more adapted to drought-prone habitats, in comparison to the other two species, due to its deep rooting which is important for accessing water in deeper soil horizons. This supports the possibility that despite slower growth rates, *H. carnosus* may respond to drought using the avoidance strategy of higher root production for increased water uptake.

Another study investigating the effects of drought on tree growth found similar results. Tree growth decreased under drought regardless of the species group; however, the most shade-tolerant (low-potential growth rate) of these tree species exhibited a resource conservative strategy leading to decreased sensitivity to drought (Ouedraogo, Mortier et al. 2013). Similarly, the slower growing eucalyptus tree, *Eucalyptus sideroxylon*, showed greater resistance to drought compared to the faster growing, *E. saligna*, which wilted at less negative water potentials due to faster dry down of soil due to water uptake (Lewis, Smith et al. 2013). Again, we show further support for slower growing species that may have an advantage in water-stressed habitats due to high biomass allocation to root production.

Conclusions

Overall, our findings suggest that *H. porteri* utilizes a drought avoidant strategy under mild drought stress by decreasing g_s and transpirational water loss. We were surprised that this outcrop species did not respond by increasing RMR in order to increase water uptake. These traits may be important on the granite outcrops as they would allow *H. porteri* to avoid exposure to water stress by maintaining plant-water status. Studies focusing on how *H. porteri* responds to a more severe water stress could test for the ability to osmotically adjust, which was not detected in the present study. If *H. porteri* were to exhibit osmotic adjustment, this would lower the turgor loss point of a plant which allows the plant to remain turgid despite a decline in soil water

potential. However, since *H. porteri* has previously been observed to wilt earlier than co-occurring species on the granite outcrops during a summer drought (Shelton 1963, Mellinger 1972), it is possible that this sunflower uses an alternative strategy of either inherently stiff cell walls or induced cell wall stiffening to maintain a high relative water content and metabolism while wilted (Verslues, Agarwal et al. 2006). Additional studies are needed to provide insight into this species' ability to resist severe drought using tolerance strategies.

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TABLE 3.1 A comparison of Pressure-volume curve parameters collected on stems for three species of *Helianthus* grown under well-watered and mild drought treatments. Traits include osmotic potential at full hydration (π_o), osmotic potential at turgor loss point (π_{tlp}), and relative water content at turgor loss point (RWC_{tlp}). The value in *italics* following each mean is its associated standard error (N=6 for every species/treatment except for N=5 for POR WW). F and P values resulting from the 2-way ANOVA are also presented where * P < 0.05, ** P <0.01, *** P <0.0001.

		Control Plants (WW)	Droughted Plants (DR)	Species		Treatment		Species x Treatment	
	Species			*F*	*P*	*F*	*P*	*F*	*P*
π_o (MPa)	AGR	-0.663 *0.051*	-0.680 *2.053*	0.39	0.6825	2.75	0.1083	0.52	0.6023
	ANN	-0.538 *0.077*	-0.689 *0.065*						
	POR	-0.585 *0.043*	-0.688 *0.087*						
π_{tlp} (MPa)	AGR	-0.829 *0.060*	-0.884 *0.048*	0.51	0.4844	1.50	0.2383	0.16	0.6963
	ANN	-0.768 *0.097*	-0.884 *0.053*						
	POR	-0.807 *0.029*	-0.867 *0.093*						
RWC_{tlp} (%)	AGR	91.316 *0.048*	92.967 *1.369*	3.78	0.0696	1.36	0.2611	0.55	0.4705
	ANN	89.412 *1.932*	90.839 *0.631*						
	POR	89.848 *0.459*	90.294 *0.629*						

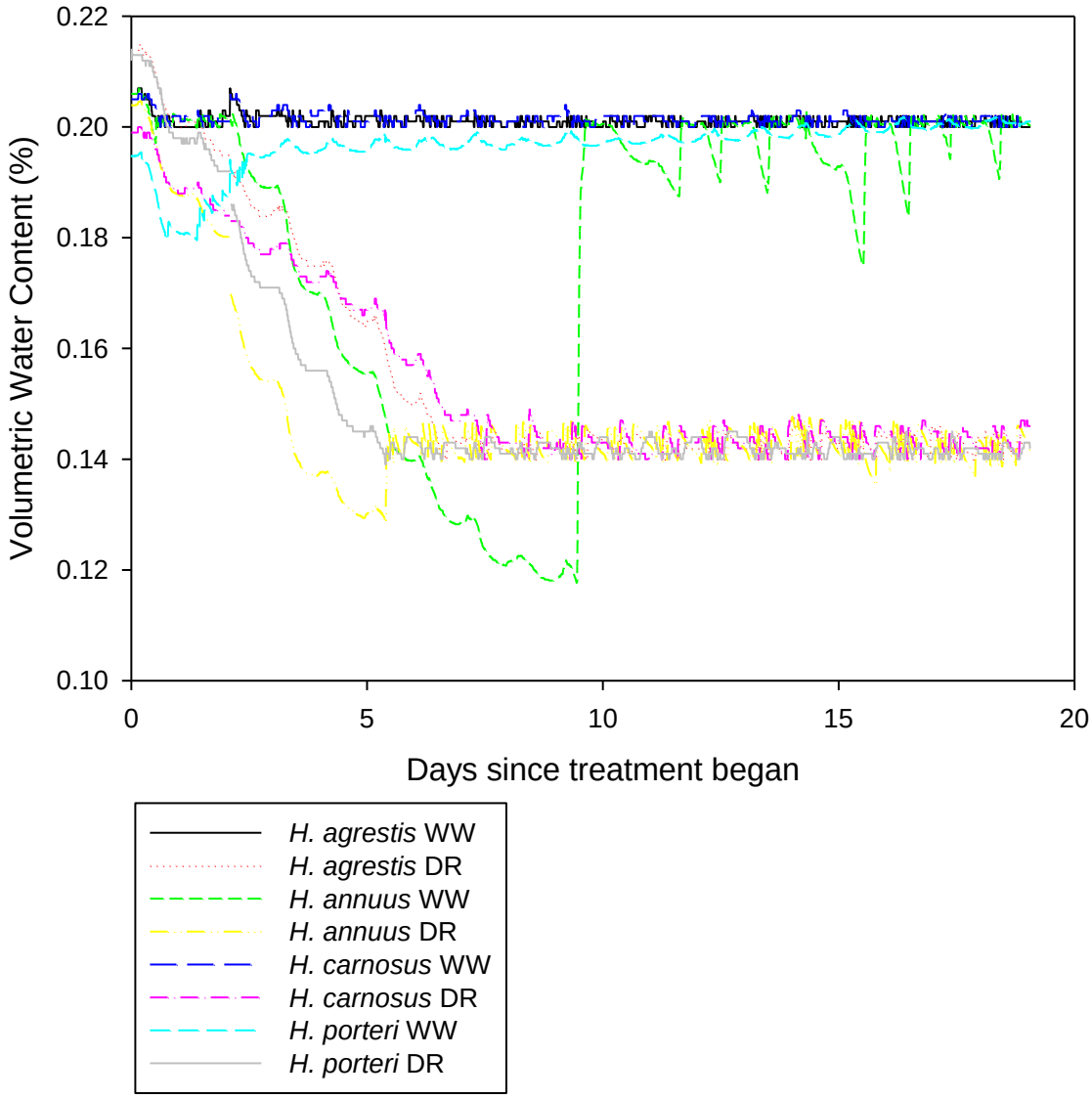

FIGURE 3.1: Soil moisture data collected using a datalogger during a two week period in which an automated irrigation system maintained a well-watered soil moisture of 20% and a mild drought treatment of 14% for four species of *Helianthus.*

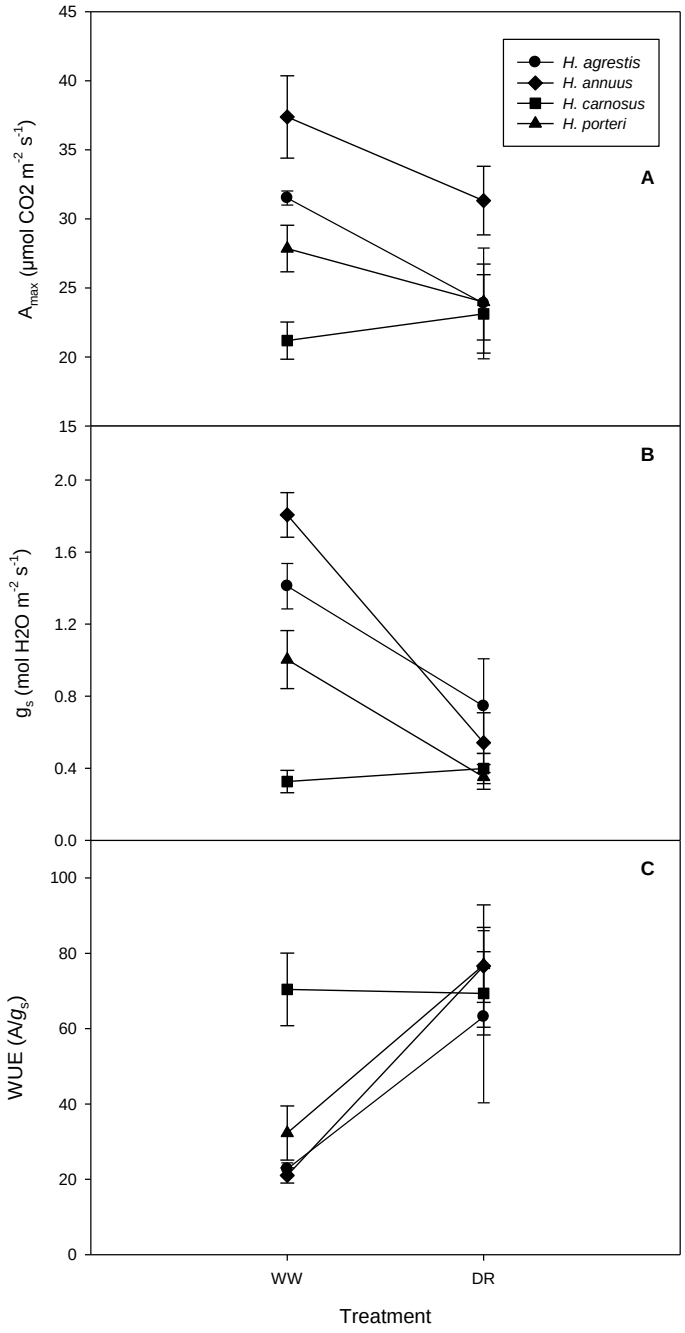

FIGURE 3.2: A comparison of *H. porteri* gas exchange rate response (mean ± SE) to mild drought compared to the response of three other species of *Helianthus* after two weeks under treatment (N=6). Traits measured include photosynthetic rate (A), stomatal conductance (B) and instantaneous water-use efficiency (C).

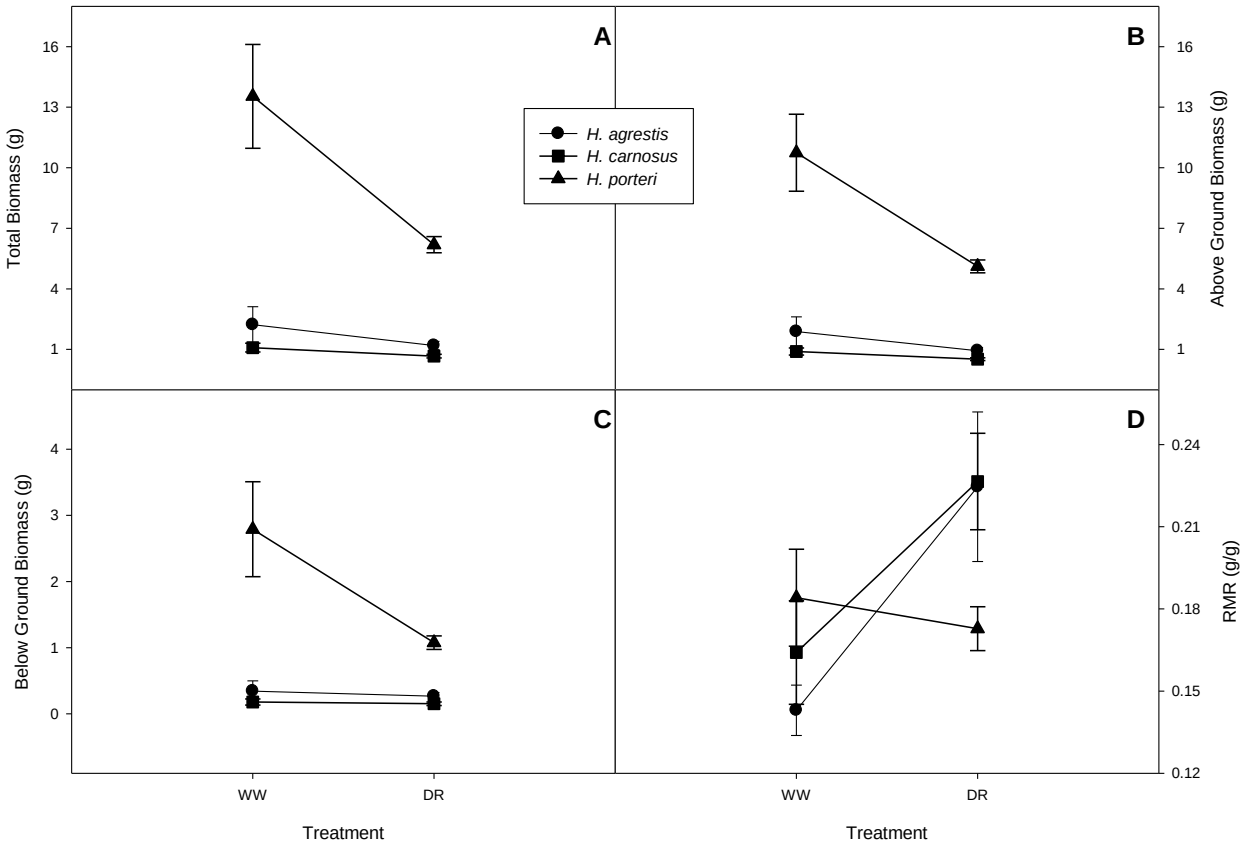

FIGURE 3.3: A comparison of biomass (mean ± SE) for *H. porteri* response to mild drought compared to the response of two other species of *Helianthus* after two weeks under treatment (N=6). Traits measured include total biomass (A), aboveground biomass (B), belowground biomass (C) and RMR (D) at harvest (N=12 for *H. annuus* and *H. carnosus* for both treatments; N=11 for *H. agrestis* DR and *H. porteri* WW; N=10 for *H. porteri* DR; and N=9 for *H. agrestis* WW).

CHAPTER 4

DRY-DOWN AND SUBSEQUENT RECOVERY COMPARISON OF A GRANITE OUTCROP SUNFLOWER WITH THREE NON-OUTCROP CONGENERS[3]

[3] Bartelme, E.M. and L.A. Donovant. To be submitted to *Southeastern Naturalist*

Abstract

Rock outcrops across the world are home to a unique assemblage of species endemic to these drought-prone habitats, but relatively little research has focused on drought resistance traits associated with survival in these conditions. *Helianthus porteri* is a sunflower species which can be found on granite outcrops in the Southeastern US. We compared *H. porteri* to three non-outcrop congeners for the response to soil moisture decline and re-watering in order to assess traits hypothesized to be associated with drought resistance and recovery. In a glasshouse study, four *Helianthus* species were subjected to a drought (withholding water) and re-watering treatments. Biomass allocation was determined with harvests prior to initiation of drought. At wilting, pre-dawn water potential, soil moisture, and functional cuticular conductance were measured. After a period of 3, 5, 7, 9, 11 or 13 days in a wilted state, plants were re-watered to field capacity and assessed for one week. Prior to the drought treatment, there were differences in total biomass with *H. annuus* having the highest and *H. carnosus* having the lowest, but there were no differences in root mass ratio (RMR). *Helianthus porteri* wilted at a much less negative water potential than *H. annuus* and wilted at the same water potential as *H. agrestis*. The functional cuticular conductance for *H. porteri* was not different from *H. agrestis* and *H. carnosus*, and all three species were lower than *H. annuus*. All species were able to recover from a persistent wilted state, but *H. agrestis* suffered the most with dead apical meristems after 13 days in a wilted state. *Helianthus porteri* and *H. agrestis* did not differ for pre-dawn water potential at wilting and functional cuticular conductance, which is surprising since *H. porteri* is from a relatively drought-prone habitat and *H. agrestis* is from a mesic habitat. In addition we found evidence suggesting that high functional cuticular conductance may be contributing to pre-dawn disequilibrium for *H. annuus* which may contribute to this species more negative predawn

water potential at wilting. Overall, it appears that *H. porteri* is exhibiting a drought avoidance strategy in response to the soil dry-down; however, future studies investigating cell wall stiffening could explain the predawn water potentials we observed.

Index Words: *Helianthus*, water potential, wilting, drought, stomatal conductance

Introduction

Water availability is a key factor influencing species distribution throughout the world. Global temperatures are expected to increase due to climate change; however, it is much harder to predict how climate change will alter precipitation patterns (IPCC 2013, Povilitis and Suckling 2010). Therefore, understanding how species are able to resist drought has become more important. Drought resistance can be defined as the ability of a plant to respond to a decrease in soil water availability (either lower percentage or more negative water potential) by both morphological and physiological changes.

Drought resistance spectrum can be divided into three strategies based on the combination of traits exhibited during drought: escape, avoidance and tolerance (Levitt 1980, Ludlow 1989, Verslues, Agarwal et al. 2006). Drought escape occurs when a plant completes its growth and reproductive cycle prior to the onset of drought. In contrast, drought avoidant species are able to survive through a drought by maintaining a relatively high water potential (Ψ) despite a decrease in soil moisture (Levitt 1980). Plants avoid drought by allocating carbon to increase root mass ratio (RMR) in order to acquire water deep within the soil. Plants also decrease their stomatal conductance (g_s) in order to decrease water loss at the leaf surface which increases water use efficiency (WUE) (Verslues, Agarwal et al. 2006). How quickly a plant closes its stomata and the extent to which a plant is able to do this determines the amount of water that is conserved in plant tissues. Even if the stomata are functionally fully closed, water can still be lost through the leaf cuticle (functional cuticular conductance, g_c) and through inability to completely seal the stomatal pore. Drought tolerant plants utilize mechanisms such as osmotic adjustment and cell wall stiffening to continue metabolic function despite low internal water potentials (Levitt 1980, Verslues, Agarwal et al. 2006). These strategies of drought escape, avoidance and tolerance are

not mutually exclusive, and plants tend to use a combination of these traits in order to resist drought. Studying species which are endemic to drought-prone habitats can help describe plant adaptations to drought.

Across the globe, there are many species which occupy relatively open habitats consisting of shallow, rocky soils which tend to be drought prone when precipitation is limiting (Poot and Lambers 2008). Uncontrolled dry-down studies, such as withholding water and intermittent watering with periods of soil moisture decline, are commonly used to determine which traits species express when faced with declining soil moisture. A study which investigated the competition between the outcrop species *Talinum calcaricum* and a non-outcrop grass, *Poa pratensis* grown in the same pot, found that when water was intermittently administered every two, four, or six days, both species incrementally decreased their total biomass production, but this decrease was most severe in the grass (Ware 1991). There is also some evidence suggesting that some species that grow on these granite outcrops have the ability to avoid drought by extending their roots deep within cracks at the rock surface, allowing them to acquire deep water stores when soil moisture declines (Rabaioli da Silva and Dillenburg 2007, Poot and Lambers 2008, Poot, Hopper et al. 2012).

In the Southeastern United States, granite outcrops occur in the piedmont region of Alabama, Georgia and the Carolinas (Baskin and Baskin 1988). Here, granite and gneiss have become exfoliated due to years of weathering, causing the overlying rock to erode away. Initially, only bare rock is exposed, but over time, plant communities are established. Lichens are the pivotal on these outcrops by covering bare rock, eroding it, and accumulating soil particles. Slowly, these soil particles and organic matter begin to fill in depressions on the rock surface and provide the basis for succession to begin (McCormick and Platt 1964, Burbanck and Phillips 1983).

Succession on the granite outcrops consists of a series of four major plant communities which are defined by the characteristic vegetation and soil depth. The first stage is the *Diamorpha* community (2-6cm), the second is the lichen-annual herb community (7-15cm), the third stage is the annual-perennial herb community (16-39cm), and the fourth stage is the perennial-shrub community (40-50cm) (Burbanck and Platt 1964). All of the species found in this unique habitat share a common trait: they survive from year to year in this drought-prone habitat.

Helianthus porteri is a particularly interesting species because it is the only member of the genus that occupies rocky outcrops. This species exhibits high growth rates during times of adequate rainfall and high soil water potentials (Mellinger 1972, Lugo, McCormick. 1981), but it has been suggested to withstand extended periods of drought by remaining in a wilted condition (Shelton 1963, Mellinger 1972). In fact, Shelton observed that *H. porteri* responded to drought by wilting before other co-occurring species on the granite outcrops leading to the hypothesis that this species wilts at a less negative water potential. In addition, this species has been reported to persist in this wilted state for 14 days without dying and recovers when rains return (Shelton 1963). This is not to say that this particular species is able to survive the most severe drought because 100% mortality has been observed in years where precipitation is particularly limiting (Mellinger 1972, Houle and Phillips 1989, Gevaert 2011). However, to date, there have been no studies which examine specific drought resistant traits for this unique species, specifically its water potential at wilting.

Using a soil dry down imposed by withholding water, we compared *H. porteri* to three congeners from contrasting habitats in order to test the hypothesis that this outcrop sunflower wilts at a less negative plant water potential. Here we assessed the following: soil moisture at wilting, plant pre-dawn water potential at wilting, functional cuticular conductance at wilting,

and a recovery after re-watering. We predict that. *H. porteri* will wilt at a less negative water potential compared to three other sunflowers. In addition, we predict that this species will survive the longest while in a wilted state before significant plant death is observed.

Materials and Methods

Study System and seed collection

The genus *Helianthus* is a diverse group of 51 species, of which 14 are of annual habit and 37 are perennial, occupying a broad range of habitats throughout North America (Heiser 1969). *Helianthus porteri* is an erect, branched annual endemic to Southeastern U.S. granite outcrops of Alabama, Georgia and the Carolinas. This species grows in a relatively drought-prone environment whereby it endures hot, dry summer conditions before flowering and setting seed in the fall (Mellinger 1972, Shelton 1963). Two species that form a clade with *H. porteri*, *H. agrestis* and *H. carnosus*, were chosen for comparison. (Timme, Simpson et al. 2007). *Helianthus agrestis* is an erect, branched annual found throughout central Florida. This species is a wetland sunflower and it can even be found in standing water in some areas (Personal observation, Heiser 1969). *Helianthus carnosus* is a rare, perennial species with basal rosettes found in Northeastern Florida on moist sandy soils. Bolting and flowering begins in late June and occurs until first frost. A fourth species, *Helianthus annuus*, has been chosen as a comparison to these three closely related species. *Helianthus annuus* is an erect annual found throughout the central portion of North America from Canada to Mexico. This species was chosen because it occupies a wide range of habitats with respect to soil moisture availability (Heiser 1969). Together, these four species provide an excellent system with which to study drought resistance because they occupy a wide range of habitats, with respect to soil moisture availability.

Achenes (hereafter seeds) were either collected from the wild, or obtained from the U.S.D.A. Germplasm Resources Information Network (GRIN) from sites that span the range of each species. Seeds for *H. porteri* were wild collected from three populations in Georgia (CR: 33°14'N, -85°8'W; HR: 33°32'N, -82°16'; and PM: 33°38'N, -84°10'W). For *H. agrestis*, seeds were both wild collected (FB: 28°21'N, -80°51'W and SC: 28°47'N, -81°51'W) and the third population was obtained from GRIN (GL: AMES 30848). Seeds of *H. annuus* were collected from one wild population (LS: 39°41'N, -112°22'W) and two more were obtained from GRIN (TX: PI494567 and NE: PI586870). For the fourth species, *H. carnosus*, seed was collected from one wild population (FC: 29°30'N, -81°15'W) and the other two were obtained from GRIN (DE: AMES28375 and FE: PI64956).

Experimental set up and pre-treatment harvest

This experiment, conducted during the fall of 2012, was carried out in three separate stages: growth under well-watered conditions prior to dry down, a dry down period and a recovery period. In order to make soil moisture dry down rates more similar for all four species, germination was timed so that the plants would be approximately the same size at the start of the dry down. Therefore, *H. carnosus* was germinated on July 30th two weeks prior to the other three species (germinated August 13th) due to its slower growth rate. All species were germinated by removing the blunt end each seed and then placed on a Petri dish for four days, transferred to seedling trays for two weeks, and finally transplanted into permanent pots (25.4 cm diameter, 6.1 L; August 27th), containing a 1:1 sand and turface mixture (Turface MVP ®, Profile, Buffalo Grove, IL), in the greenhouse using a randomized complete block design (9 replicates, 4 species, 3 populations each for a total of 108 plants). Plants were allowed to grow for two and a half weeks in the greenhouse with daily watering to full capacity (soil saturation). Three replicates

per species and population (36 plants total) were randomly chosen for a harvest on September 12, 2012 in order assess plant size at the start of the dry down. Harvested plants were measured for height, stem diameter, and noted for the presence of buds. The whole plant was divided into aboveground biomass (leaves, stems, buds) and belowground biomass. All biomass was dried in the oven at 60°C for three days and then weighed. Root mass ratio (RMR) was calculated as the total root biomass divided by the total plant biomass.

Dry down and wilting point

Once the pre-dry down harvest was complete, water was withheld from remaining plants to allow for a gradual dry down within the pot. A layer of pine bark was placed on top of the sand and turface to decrease the amount of water loss through evaporation at the soil surface. Each morning three pre-dawn soil moisture readings (volumetric water content, VWC %) were taken from each pot using a Theta Probe (Dynamax, Houston, TX) and averaged per pot, and each plant was assessed visually for wilting, defined as having curled leaves and a petiole that was no longer stiff (i.e. the leaves were floppy). A pre-dawn pressure chamber measurement (PMS Instrument Company; Albany, OR; (Scholander, Hammel et al. 1965)) was collected to determine Ψ_{leaf} of the wilted leaf as our estimate of turgor loss point.

A soil core (15.24 cm deep and 0.375 cm wide) was also collected halfway between the stem and the side of the pot and was used to determine percent soil moisture using the following equation: [(wet mass – dry mass)/wet mass]*100. This gravimetric water content was then converted to volumetric water content (VWC) in order to compare the two methods for collecting soil moisture. Three replicate plants per population (36 plants total) were randomly assigned to be assessed for changes in gas exchange rates during the dry down period using a Li-Cor 6400 (Lincoln, NE). We use a final gas exchange measurement taken on the day of initial

wilting to estimate functional cuticular conductance, which include water loss through the cuticle and fully closed stomata (Caird, Richards et al. 2007, Howard and Donovan 2007). The remaining three replicate plants per species and population that were not measured for gas exchange were assessed for pre-dawn water potential at wilting and recovery.

Assessment of duration of wilting and recovery ability

Each of the six replicates subjected to the dry down was randomly assigned to one of six wilting recovery treatments, consisting of re-watering after 3, 5, 7, 9, 11 or 13 days. This range of days before re-watering was chosen based on preliminary study results demonstrating that *H. agrestis* showed almost total whole plant death after 4 days and reports that *H. porteri* can persist in a wilted state in the field for two weeks (Shelton 1963). In this experiment, the day that the plant wilted is represented as day 1, after which each plant was re-watered to field capacity according to the assigned treatment (i.e. after 3, 5, 7, 9, 11 or 13 days of being wilted). After initial re-watering, plants were watered daily and assessed for recovery (afternoon of initial re-watering, and at 24 hours, 48 hours and one week). At each time point, plants were assessed for complete wilt, partial wilt, turgidity (i.e. not visually wilted), tissue death and new growth.

Statistical analyses

Pre-dry down biomass was analyzed using an ANOVA with the PROC GLM command in SAS® v.9.3 (SAS Institute Inc. 2001). A slice command was used in order to compare multiple species as well as multiple populations within species. This same procedure was conducted for soil moisture at wilting, stomatal conductance at wilting, and pre-dawn water potential at wilting. A *t*-test was used to compare the two methods of estimating soil moisture (Theta probe and soil core). The initial stomatal conductance and functional cuticular

conductance was plotted against their respective soil moistures for a visual representation of how the stomata were affected by a decline in soil moisture.

Results

Prior to the initiation of the drought treatment, a sub-set of species were harvested in order to compare plant size. There were significant differences in species height ($F=21.18$, $P<0.0001$, Figure 4.1A) with *H. porteri* and *H. annuus* being taller than *H. agrestis* and *H. carnosus*, but there were no differences among the populations for each species ($F= 1.95$, $P=0.1021$). There were no significant differences in stem diameter both among species and among populations ($F=2.36$, $P=0.8099$ and $F=0.55$, $P=0.8099$, respectively; Figure 4.1B). For the biomass, there were significant differences between aboveground biomass, belowground biomass, and total biomass among species ($F=13.21$, $P<0.0001$, Figure 4.1C; $F=14.18$, $P<0.0001$, Figure 4.1D; and $F=13.68$, $P<0.0001$, Figure 4.1E, respectively), but not among populations ($F=0.57$, $P=0.7938$; $F=0.91$, $P=0.5273$ and $F=0.62$, $P=0.7524$, respectively). Specifically, both *H. annuus* and *H. porteri* had greater biomass on average compared to both *H. agrestis* and *H. carnosus,* which had the lowest. RMR did not differ among species or among populations ($F=0.86$, $P=0.4745$ and $F=1.73$, $P=0.1476$, respectively; Figure 4.1F).

On average, *H. annuus* wilted at seven days, *H. porteri* wilted at nine days, *H. agrestis* wilted at eleven days, and finally *H. carnosus* wilted at twenty-five days after water was withheld. There were no significant differences in percent soil moisture at wilting both within and among species for both methods of soil moisture collection (Theta probe: among species $F=1.20$, $P=0.3183$ and within species $F=0.62$, $P=0.7599$; Soil core: among species $F=0.69$, $P=0.5610$ and within species $F=1.49$, $P=0.1825$). When both soil moisture collection methods were compared using a *t*-test, it was found that there were significant differences between the

two (P=0.0095), whereby the core method had a 2% (± 0.5%) higher VWC compared to the probe method.

There were significant differences among species for plant pre-dawn water potential at wilting (F=92.37, P<0.001; Figure 4.2) but not among populations (F=1.26, P=0.2815). At wilting, *H. annuus* had a significantly more negative predawn water potential than *H. carnosus*. *Helianthus carnosus* wilted at a significantly more negative water potential than either *H. agrestis* or *H. porteri*. There were significant differences among species for functional cuticular conductance but not among populations (g_c, F=27.17, P<0.0001 and F=2.74, P=0.0266, respectively; Figure 4.3), whereby *H. annuus* had a significantly higher g_c than the other three species. However, for all species, the functional cuticular g_c was much lower than stomatal conductance measured prior to the dry-down (Figure 4.4)

Every plant survived the wilted state, even after 13 days. However, most individuals did show some leaf death which steadily increased with the length of time in the wilted state. *Helianthus porteri* and *H. agrestis* both showed leaf death prior to wilting and more leaves died with a longer period in the wilted state. *Helianthus agrestis* was more affected by the thirteen day wilting period than *H. porteri* since the apical meristems died. Neither *H. annuus* nor *H. carnosus* showed leaf death prior to wilting, but when wilted for a prolonged period of time, some leaf death occurred. All individuals recovered to leaf turgidity after one week of re-watering. Plants which were only in the wilted state for a three and five days showed turgidity 12-24 hours after re-watering. In addition, after one week of re-watering, all species showed evidence of either new leaf or branch growth. Even the *H. agrestis* individuals that had dead apical meristems had new growth on lateral branches. Flower buds began forming for *H. annuus* and *H. porteri* prior to wilting, and by the time they reached the recovery stage, many were

flowering and even more buds were forming. *Helianthus carnosus* individuals began bolting and budding during the period of wilting but it did not flower during the wilting or recovery period.

Discussion

In this study, we conducted a soil dry down to determine how three species of *Helianthus* compared to the outcrop sunflower, *H. porteri,* for drought resistance traits and recovery ability. Each of these four sunflower species exhibited a different rate of soil moisture decline with *H. annuus* being the fastest, *H. porteri* and *H. agrestis* being intermediate, and *H. carnosus* being the slowest. This can be attributed to differences in plant size at the initiation of the drought. The differences in dry down rates were something that we could not control leading to the different dry-down rates. Thus, it was not possible to eliminate differences in dry-down rates and they may have affected the ability to acclimate. However, we don't believe that the differences in dry-down rates explain the observed species differences because a slower soil dry-down would be expected to enhance a plants ability to acclimate via osmotic adjustment and achieve a lower predawn water potential, which is not what we observed.

Leaf predawn water potential

Of the four species, *Helianthus porteri* wilted at the least negative water potential, along with *H. agrestis*, which is consistent with observations of *Helianthus porteri* wilting before other species on the outcrops during a soil moisture decline (Shelton 1963, Mellinger 1972). Even though we did not test plant predawn water potential at wilting on the granite outcrops, our results provide some evidence that *H. porteri* does wilt at a less negative plant water potential in response to soil drying compared to *H. carnosus* and *H. annuus*. This species may have inherently stiffer cell walls, or be adjusting the elasticity of its cell wall in response to drought. The stiffness of cell walls for a plant, or the modulus of elasticity, is a physiological response of

a species which utilizes a drought tolerance strategy for resistance to water stress (Kramer and Boyer 1995). When cell walls stiffen (high modulus of elasticity), the cell wall cannot compress around the cytoplasm of the cell, which is necessary for a cell to remain turgid, and therefore rapid decreases in turgor cause wilting (Verslues, Agarwal et al. 2006). Therefore, a high modulus of elasticity may be the mechanism explaining the loss of turgor and wilting at a less negative water potential for *H. porteri*.

Helianthus carnosus wilted at a leaf predawn water potential that was more negative than *H. porteri* and *H. agrestis* but less negative than *H. annuus*. This species also grew the slowest as evidenced by low biomass prior to the drought treatment. Slower growth is often associated with a lower photosynthetic rate and a lower stomatal conductance, and these traits combined lead to a slower draw down of available soil moisture within the *H. carnosus* pots. *Helianthus annuus* wilted at the most negative leaf predawn water potential in comparison to the other three species. Our results are similar to a previous study which looked at leaf predawn water potential at stomatal closure in *H. annuus* (Turner, Begg et al. 1978). In that study, stomatal closure occurred at leaf pre-dawn water potentials ranging from -1.7MPa to -2.7MPa. These results suggest that *H. annuus* may be accumulating compatible solutes, which would indicate a greater ability to osmotically adjust and lower the turgor loss point in comparison to the other *Helianthus* species in our study.

Predawn plant water status and stomatal closure

In our study, we found that *H. annuus* wilted at the most negative predawn water potential and it had the highest functional cuticular conductance as compared to the other species, even though percent soil moisture did not differ. This suggests that *H. annuus* may have been experiencing predawn disequilibrium. Predawn disequilibrium occurs when the plant

predawn water potential is more negative than that of the soil water potential at predawn (Donovan, Richards et al. 2003). Night-time transpiration is thought to lead to predawn disequilibrium whereby the stomata are open at night, causing a more negative plant predawn water potential in comparison to the surrounding soil. A previous study found evidence of predawn disequilibrium for wild *H. annuus* under well-watered conditions (Donovan, Linton et al. 2001).

Drought resistance of H. porteri

Very few studies have investigated the differences in drought resistance between an outcrop endemic and its congeners, and those that have, have focused on the unique root morphology necessary for survival on the outcrops (Poot and Lambers 2003, Poot, Bakker et al. 2008). Our study is unique in that we have tested an outcrop species response to a soil dry down with its non-outcrop congeners. Many studies have used a dry-down by withholding water in order to compare drought resistance between congeners from contrasting habitats (Savage and Cavender-Bares 2011, Curran, Clarke et al. 2013, Fang, Turner et al. 2014). For example, a comparison of three congeneric pairs of rainforest seedlings and their mortality rate during a soil dry-down found that species from the relatively drier sites had lower mortality than their mesic congeners (Curran, Clarke et al. 2013). Those results parallel this study in that the wetland sunflower species exhibited severe tissue death in comparison to our outcrop species under a prolonged drought. However, a very severe drought has the ability to inflict 100% mortality on even the most drought resistant species.

Overall, this experiment investigating *H. porteri*, a species from a drought-prone habitat, response to a soil dry-down, has shown that this species wilts at a less negative water potential, has low functional cuticular conductance at wilting and can survive in a wilted state for a

thirteen-day period. These same traits were seen for *H. agrestis*, a species from a less drought-prone habitat, with the only difference being that *H. agrestis* exhibited greater leaf and meristem death than *H. porteri*. These two species were much more similar than we expected on the basis of source habitat.

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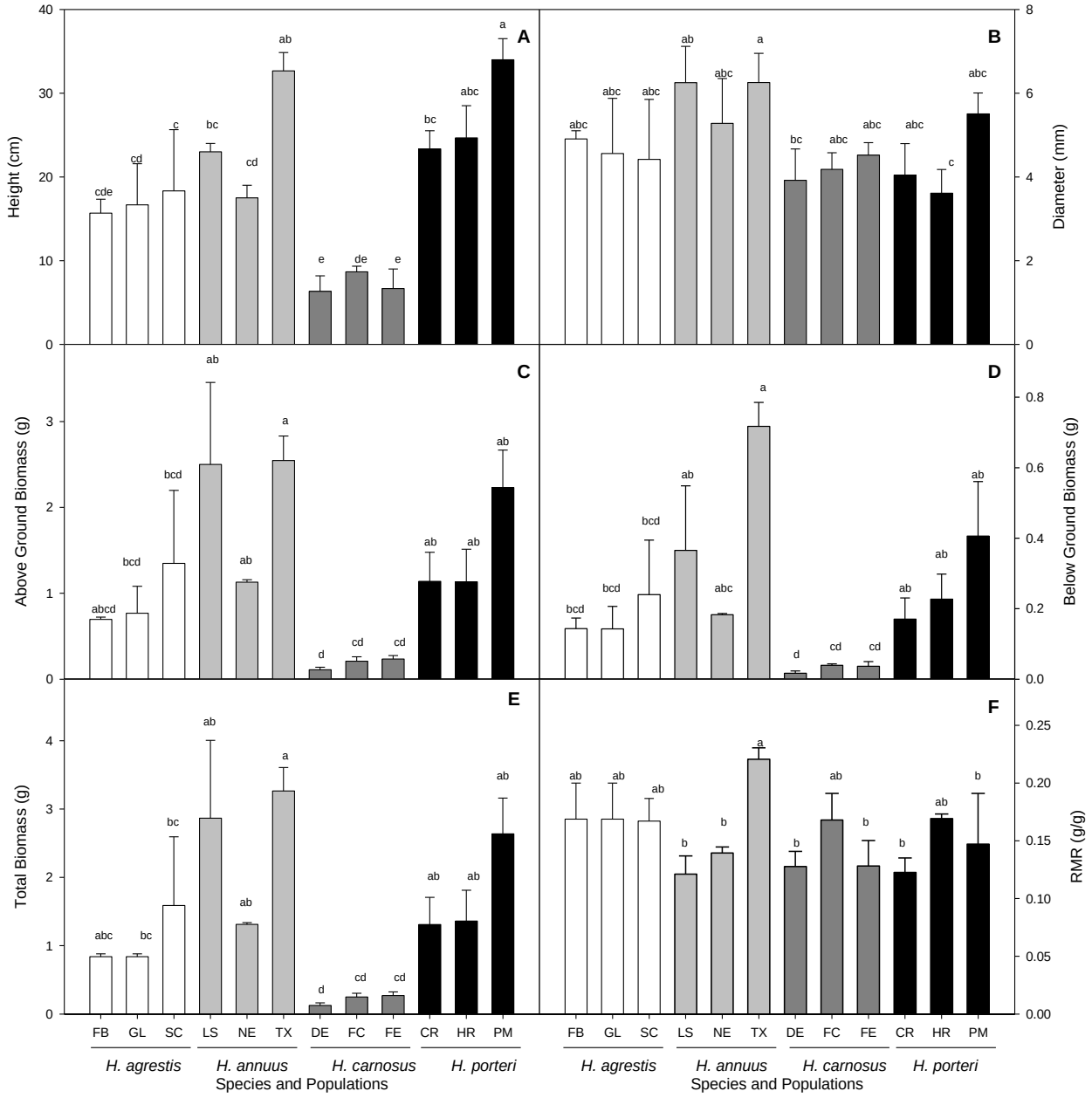

FIGURE 4.1: A comparison of *H. porteri* to three other *Helianthus* species for harvest traits (± SE) collected for a sub-set of plants prior to a dry-down treatment (N=3). Traits measured

include for height (A), stem diameter (B); aboveground biomass (C); belowground biomass (D); total biomass (E); and RMR (F).

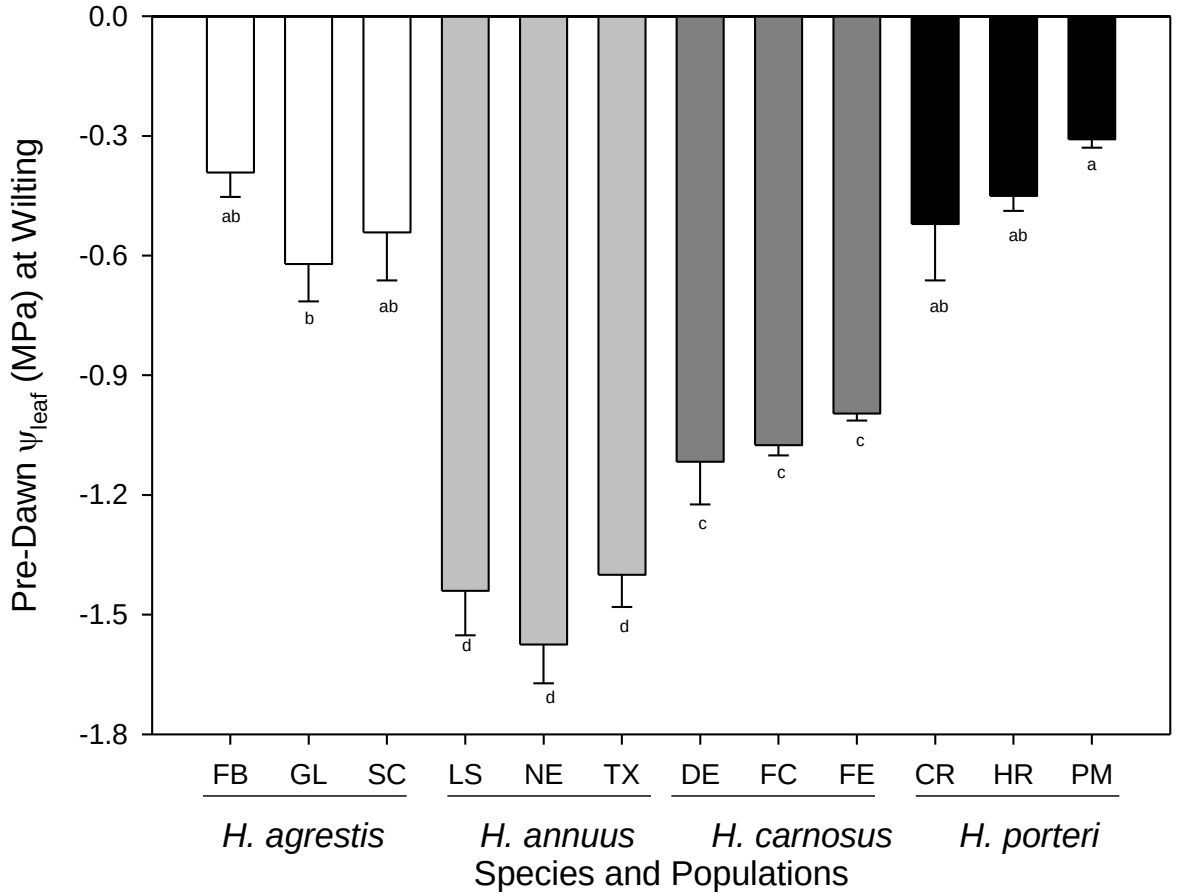

FIGURE 4.2: Predawn water potential (ψ_{leaf}, mean ± SE) at wilting of *H. porteri* compared to that of three other species of *Helianthus* (N=6 for each population, except N=5 for LS; and N=4 for NE).

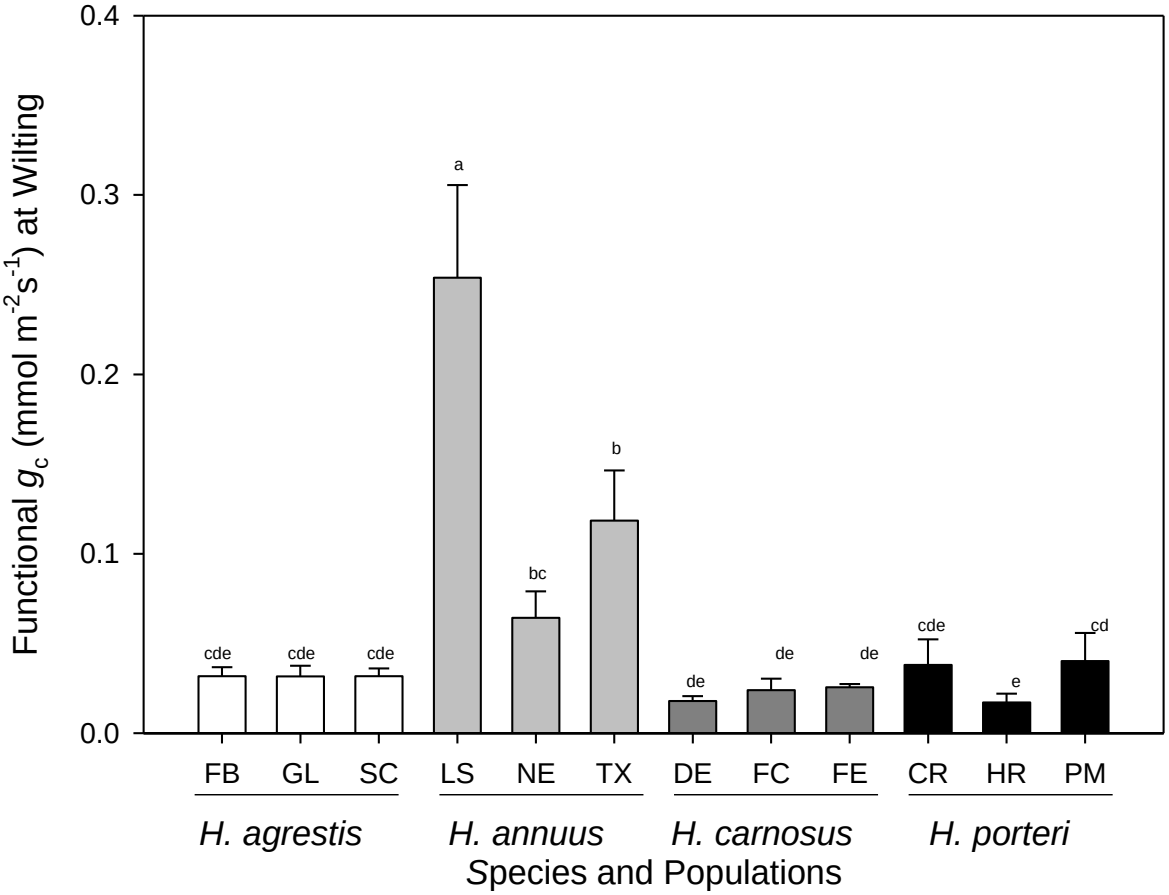

FIGURE 4.3: Functional cuticular conductance (mean ± SE) of *H. porteri* compared to rates of three other species of *Helianthus* when wilting is first observed (N=3 for each population).

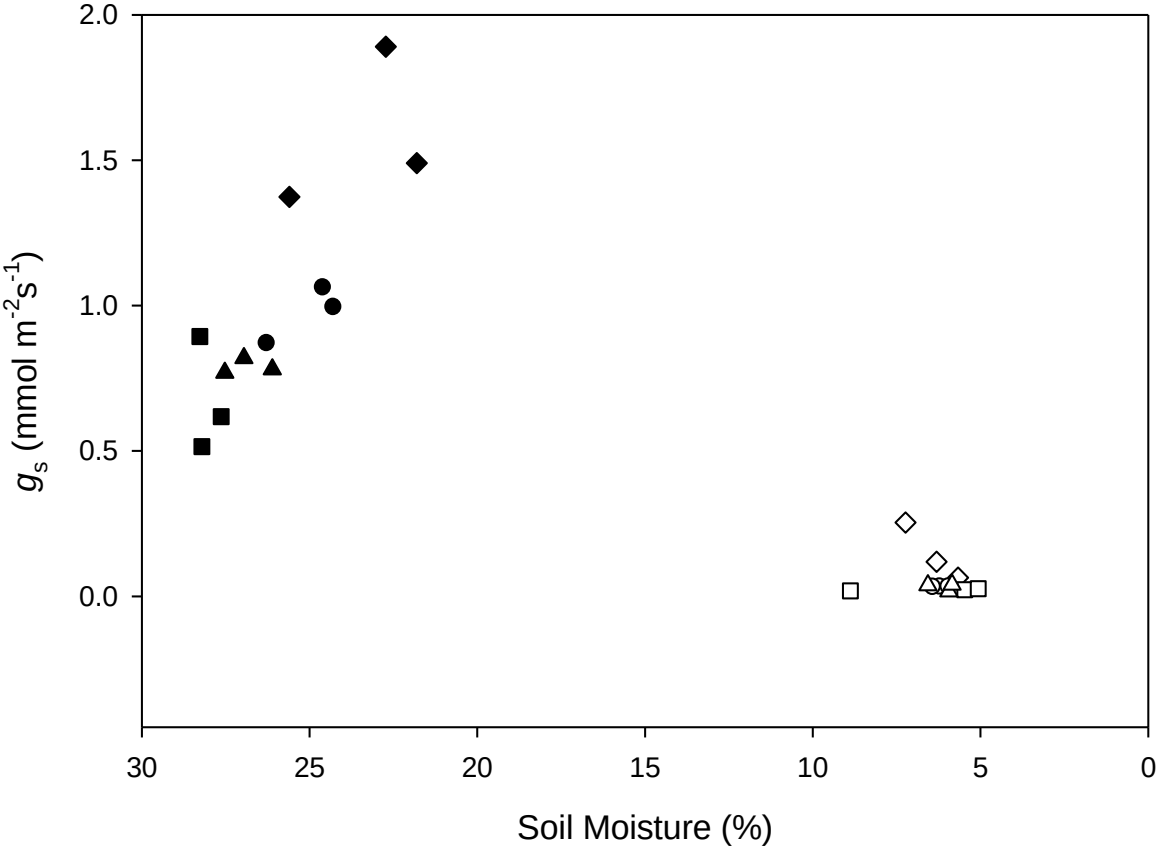

FIGURE 4.4: Initial stomatal conductance (black symbols) prior to withholding water and functional cuticular conductance (white symbols) at wilting, both plotted against soil moisture for four sunflower species: *H. agrestis* (circle), *H. annuus* (diamond), *H. carnosus* (square) and *H. porteri* (triangle).

CHAPTER 5

CONCLUSIONS

Granite outcrops are unique habitats that are home to a number of endemic species which survive on shallow drought-prone soils (Shure 1999, Poot, Hopper et al. 2012). This research investigated the possible drought resistance of *Helianthus porteri*, a sunflower endemic to the granite outcrops of the Southeastern U.S by comparing this species to three non-outcrop congeners. By choosing congeneric species which are found across a broad spectrum of habitats, we used a comparative approach to study drought response. This has been previously useful in determining species response to drought because it gives the advantage of explaining the distribution of closely related species which are known to grow in different native habitats (Savage, Cavender-Bares et al. 2009, Rosenthal, Stiller et al. 2010, Daniels, Mabusela et al. 2013). We compared *H. porteri* to *H. annuus*, another species that grows in some drought-prone habitats but is not restricted by how deep roots can grow in the soil. We also compared *H. porteri* to two species that grow in relatively wetter habitats: *H. agrestis* and *H. carnosus* (Heiser 1969). Together, these four species create a spectrum from most drought-prone to least drought-prone with which we could investigate the morphological and physiological traits associated with greater drought resistance. On one end of this spectrum, we have the most drought-prone habitat (*H. porteri*) and on the other extreme the least drought-prone (*H. agrestis*). We hypothesized that *H. porteri* would display a number of traits which suggest that it has the greatest ability to resist drought compared to the other species. Using the specific questions posed in the first chapter, we have summarized our findings.

Prior to drought, does H. porteri *have a fast rooting depth rate and a higher root growth rate?*

No, *Helianthus porteri* did not have the fastest taproot growth rate, nor did it root the deepest. Instead, *H. annuus* exhibited the fastest taproot growth rate in addition to rooting the deepest. *Helianthus porteri* was consistently intermediate in taproot growth rate and how deeply it rooted with the perennial *H. carnosus* being the slowest and shallowest. Even though we did not specifically test for deep rooting through cracks on the granite outcrops, shallower rooting seems consistent with the soil depth limitations *H. porteri* experiences in its native granite outcrop habitat. Full root system analysis also indicated that *H. porteri* had the highest root mass ratio, very high total root length and high specific root length compared to both *H. annuus* and *H. carnosus*. A high specific root length suggests that *H. porteri* produces the highest root length for water absorption per unit mass in comparison to the other three sunflowers. Similar conclusions were made by Nicotra et al. (2002), whereby higher inherent specific root length was observed for species from low rainfall habitats when grown under well-watered conditions. They concluded that high SRL is typical for species from drought-prone habitats and is used as a mechanism to increase water uptake. Therefore, high specific root length may be a key trait which allows *H. porteri* to acquire high amounts of water during intermittent rain events in the summer. In addition, this high SRL may allow *H. porteri* to explore a larger volume of soil in order find untapped stores of water during intermittent drought events.

Does H. porteri *possess traits which allow it to avoid declines in plant water status during a mild drought?*

Yes, *H. porteri* does possess traits that allow it to avoid drought, which is important given that this species lives on drought-prone rock outcrops. *Helianthus porteri* responds to a mild drought by increasing its water-use efficiency (WUE) which is a trait typically associated with a

drought avoidance strategy (Levitt 1980, Ludlow 1989, Verslues, Agarwal et al. 2006). *Helianthus porteri* had an inherently higher RMR in comparison to both *H. agrestis* and *H. annuus* under well-watered conditions; however, *H. porteri* did not increase its RMR in response to the mild drought treatment as expected, possibly because it already had a high RMR. It may be advantageous for *H. porteri* to maintain a high RMR given the frequency and unpredictability of drought in granite outcrop habitats. Overall, it appears that *H. porteri* exhibits a drought avoidant strategy under mild drought through stomatal regulation which increases WUE.

During a soil dry down, does H. porteri *respond early to a decline in soil moisture and does it have a greater ability to withstand a wilted state?*

Wilting is a visual sign that a plant is water stressed (Boyer 1995). During the granite outcrop studies in the early 1970's, it was observed that *H. porteri* was the first of the co-occurring species to wilt with the onset of drought (Shelton 1963, Mellinger 1972), suggesting that it is wilting at a less negative soil water potential than surrounding species. We used a soil dry down by withholding water in order to determine the plant predawn water potential and functional cuticular conductance at wilting for these four species. We hypothesized that *H. porteri* species would respond to a decline in soil moisture by wilting at a less negative predawn water potential.

Helianthus porteri did wilt a less negative predawn plant water potential compared to both *H. annuus* and *H. carnosus*, but it did not differ from *H. agrestis*. *Helianthus porteri* and *H. agrestis* responded to a decline in soil moisture by wilting at a less negative water potential compared to the other species. Once again, this is surprising because we would not expect that a wetland species exhibit the same water potential at wilting as *H. porteri*. In addition, *H. porteri, H. agrestis* and *H. carnosus* all have a lower functional cuticular conductance at wilting

suggesting that at wilting, these three species lose less water through the cuticle and fully closed stomata. *Helianthus annuus*, on the other hand, wilted at the most negative plant water potential and exhibited the highest functional cuticular conductance, which suggests that this species was exhibiting predawn disequilibrium due to transpirational water loss from incompletely closed stomata.

Final conclusions

Overall, we found evidence that *H. porteri* exhibits a drought avoidant strategy in response to drought by investigating inherent root growth and responses to mild drought. *Helianthus porteri* has a root system that may allow for greater water uptake per unit mass which is important when soil moisture is limiting on the granite outcrops. When exposed to a mild drought, this sunflower is able to decrease water loss via stomatal closure. However, during a soil dry-down, *H. porteri* wilted at a less-negative water potential in comparison to *H. carnosus* and *H. annuus*.

In order to further our understanding of how *H. porteri* responds to and survives during the drought-prone summer months, future studies should focus on experiments directly on the granite outcrops. A comparison between *H. porteri* and species that co-occur with this sunflower on the granite outcrops for pre-dawn water potentials at wilting can confirm our observation and those of Shelton (1963). In addition, studies should also focus on determining if *H. porteri* exhibits any combination of drought tolerance strategies, such as osmotic adjustment or cell wall stiffening. It is likely that cell wall stiffening contributes to our observations of *H. porteri* wilting at a less-negative predawn water potential, which subsequently maintains high relative water content; however, specifically testing this hypothesis is necessary in order to determine the extent cell wall stiffening aids *H. porteri* drought resistance. Other traits that could help clarify

the drought resistant strategy of *H. porteri* include compatible solute quantification, abscisic acid signaling to induce stomatal closure, A/C_i curves under varying levels of drought to help understand photosynthetic limitation, resistance to cavitation, hydraulic failure and embolism repair. Finally, it would be interesting to use rain out shelters on the granite outcrops to simulate more frequent and prolonged drought, potentially providing insight into how *H. porteri* and co-occurring species may respond to climate change. Results from these studies have the potential to inform future management plans in order to protect this unique sunflower.

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APPENDIX 1

ROOT GROWTH RATE OF WILD *HELIANTHUS ANNUUS* UNDER DROUGHT

Introduction

With drought becoming increasingly important to overcome, both in crop management and in the protection of wild plants, understanding how a species responds to drought is crucial. One way plants can avoid drought is by allocating biomass towards the production of roots. In doing so, plants can increase surface area for water absorption and extend roots deeper into the soil to reach available water. Here we ask the question, does *Helianthus annuus* respond to a soil dry down by increasing its root biomass (increased RMR) and rooting deeper to reach available water?

Methods

Twenty-four seedlings of *Helianthus annuus* were transplanted into 120cm deep pots (2 treatments x 2 blocks x 6 replicates). The same automatic irrigation system as the deep pot study in Chapter 2 was used to supply water to the plants, and two EC5 soil moisture probes (Decagon Devices, Inc., Pullman, WA) placed in half of the pots were used to monitor changes in soil moisture (See Appendix 2 for program). For the *H. annuus* plants which were given a drought treatment, the treatment was applied by removing the first (top) trip emitter when the roots reached the depth of the second. Similarly, when roots reached the depth of the third drip emitter, the second was removed. The sixth drip emitter was never removed and represented the plant reaching the "water table." Each day, every plant's roots were observed after sundown. The

deepest roots observed along the bottom side of the pot were marked and dated with a Sharpie for a total of 62 days (2 months). Plants were harvested when five *H. annuus* reached the bottom of the 120cm pots. Aboveground biomass for each plant was dried to a constant mass at 60 ℃ and weighed. For belowground biomass, the pots were then cut into 30cm sections. Roots were extracted from each section by washing away the rooting substrate, dried to a constant mass at 60°C and weighed. A 1-way ANOVA was used to compare the differences in rooting depth and biomass allocation for the two treatments.

Results

There were no differences between well-watered and droughted *H. annuus* for root growth rate (Figure A1). In addition, there were no statistically significant differences between the two treatments for above and belowground biomass, total biomass, RMR and portion of roots within the soil, both total and percentage of roots (Table A1, Figure A2 A-F). However, there were trends for the well-watered *H. annuus* to have higher biomass accumulation. In contrast there was a trend for the droughted *H. annuus* to have a higher RMR as well as a higher percentage of biomass allocated to deeper portions of the soil (Figure A2D and A2F, respectively). At sixty-two days, three droughted *H. annuus* reached the bottom of the 120cm pot while only two well-watered individuals reached the same depth.

Discussion

Overall, it was surprising that there were no differences between the well-watered and droughted *Helianthus annuus*. We expected that the droughted plants would reach the bottom of the pot quicker since one way a plant can avoid drought is to extend roots deeper into the soil in search of deep water stores. In addition, we expected that there would be differences in biomass allocation between the two treatments. Specifically, we expected to see an increase in root

biomass in the droughted plants since increased carbon is allocated to root production under drought conditions. There was a trend for increased root mass ratio (RMR) under the drought conditions, but this was not significant. This is surprising since previous research has characterized *H. annuus* as a drought avoider. Instead, these results suggest that the droughted *H. annuus* may be able to grow just as fast as the well-watered plants. Alternatively, it is possible that the method in which the drought was implemented was not sufficient to observe significant differences between these two treatments.

TABLE A1: Results of a One-way ANOVA comparing root biomass traits between well-watered and droughted *H. annuus* (n=12 for each treatment).

Traits	DoF	F-Value	P-Value
Total Biomass (g)	1, 11	1.23	0.2796
Aboveground Biomass (g)	1, 11	1.56	0.2253
Belowground Biomass (g)	1, 11	0.92	0.3492
RMR (g/g)	1, 11	2.24	0.1564
Portion of Total Biomass at Four Different Depths (g)			
0-30 cm	1, 11	1.49	0.2351
30-60 cm	1, 11	0.04	0.8385
60-90 cm	1, 11	0.63	0.4455
90-120 cm	1, 11	0.62	0.4546
Percentage of Total Biomass at Four Different Depths (%)			
0-30 cm	1, 11	0.54	0.4694
30-60 cm	1, 11	0.03	0.8751
60-90 cm	1, 11	0.04	0.8465
90-120 cm	1, 11	0.01	0.9389

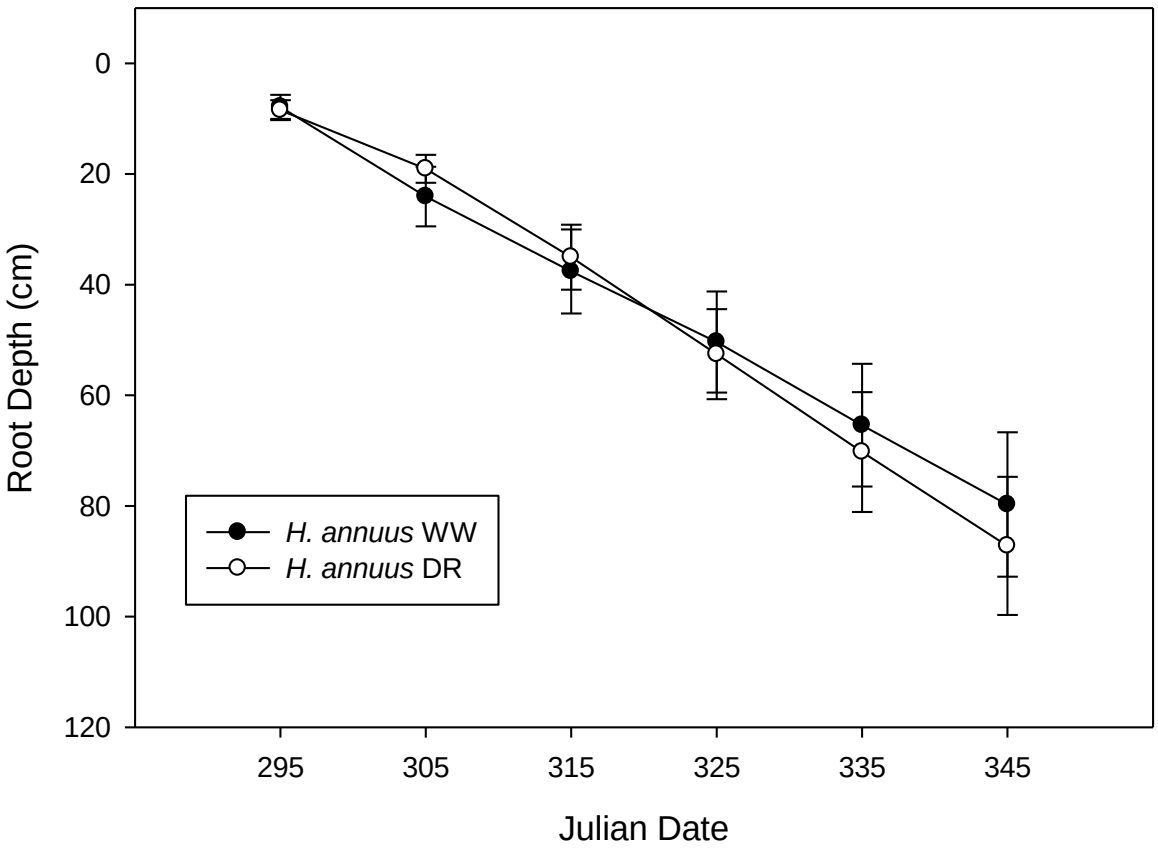

FIGURE A1: Root depth (mean ± SE) at six different times over a two-month period for both well-watered and droughted *H. annuus* (n=12).

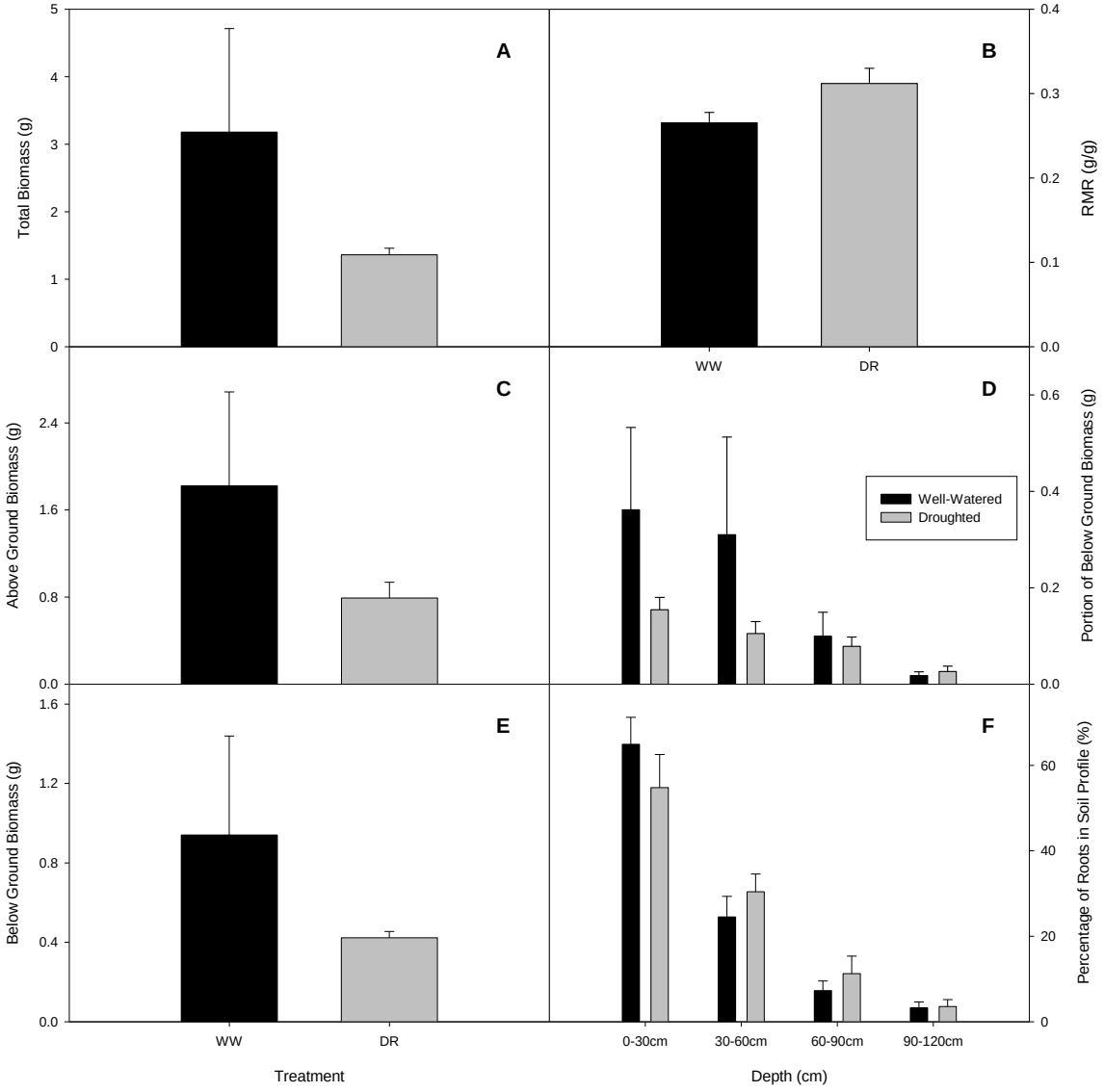

FIGURE A2: A comparison of well-watered and droughted *H. annuus* growing in a 120cm deep pot for the following traits (mean ± SE): total biomass (A), RMR (B), aboveground biomass (C), total root biomass in 30cm sections of soil (D), belowground biomass (E), and the percentage of total root volume within each 30 cm section of soil (F).

APPENDIX 2

DATALOGGER PROGRAM CODE FOR THE DEEP POT EXPERIMENT

```
EBartelme Avoidance Study Fall 2013 v1.csi, Table 1

;CR23X
;
;Elise Bartelme
;
;This program will monitor changes in soil moisture (volumetric water
content) as the soil moisture within 48 tall clear tubes
;The program will measure 96 sensors, with two sensors in each of the 48 pots
;In addition, the program will include a scheduled irrigaiton for eight lines
of irrigation
;The first four lines are AGRESTIS 1, ANNUUS 1, CARNOSUS 1 AND PORTERI 1 -
they correspond to the first four solenoids
;The second four lines are AGRESTIS 2, ANNUUS 2, CARNOSUS 2 AND PORTERI 2 -
they correspond to the last four solenoids,
;*************************************************************************
;A CR23X Datalogger (Campbell Scientific) will be used
;Two AM416 Multiplexers (Campbell Scientific) will be used
;96 EC-5 Soil Moisture Probes (Decagon Devices) will be used to measure
changes in %soil moisture
;One Relay driver will be used to control 4 solenoid valves
;*************************************************************************
**********************
;The first AM416 is connected to the CR23X using this configuration:
;WIRING: MP 12V wired to 12V on DL
;WIRING: MP GND wired to G on DL
;WIRING: MP CLK wired to CP 4 on DL - switches to the next channel
;WIRING: MP RES wired to CP 5 on DL - turns the multiplexer on and off
;WIRING: MP COM port H1 to EXCITATION 1 on DL
;WIRING: MP COM port L1 to SE1 on DL
;WIRING: MP COM port H2 to SE2 on DL
;WIRING: MP COM port L2 to SE3 on DL
;WIRING: MP COM SHIELD to G on DL
;*************************************************************
;The second AM416 is connected to the CR23X using this configuration:
;WIRING: MP 12V wired to 12V on DL
;WIRING: MP GND wired to G on DL
;WIRING: MP CLK wired to CP 6 on DL red wire
;WIRING: MP RES wired to CP 7 on DL white wire
;WIRING: MP COM port H1 to EXCITATION 2 on DL white wire
;WIRING: MP COM port L1 to SE4 on DL black wire
;WIRING: MP COM port H2 to SE5 on DL red wire
;WIRING: MP COM port L2 to SE6 on DL green wire
;WIRING: MP COM SHIELD to G on DL
;*************************************************************************
*************
;ECH2O EC-5 Sensors are connectedto the AM416 using this wiring:
```

```
;WIRING (white - EXCITATION): 16 groups of 3 white wires attached to the H1
ports 1-16 (1-6) on the AM416
;WIRING (red - VOLTAGE SIGNAL): 16 groups of 3 wires each wired into L1, H2,
and H3 ports 1-16 (1-6) on the AM416
;WIRING (silver): 48 wires connected together and wired into SHILED port on
the AM416
;this is done for both multiplexers 48 sensors each for a total of 96 sensors
;*************************************************************************
*******************************
;The relay driver is connected to the CR23X using this configuration:
;WIRING: C1 on RD to C1 on DL
;WIRING: C2 on RD to C2 on DL
;WIRING: C3 on RD to C3 on DL
;WIRING: 12V on RD to 12V on DL
;WIRING: G on RD to G on DL
;*************************************************************************
;An LED is wired to Control Port 8 on the Datalogger
;*************************************************************************

;********************** START PROGRAM ************************************
*Table 1 Program
01: 600 Execution Interval (seconds)
;The execution interval for this program is every 10 minutes
;*************************************************************************
************************
;This command turns on the multiplexer
1: Do (P86)
1: 45 Set Port 5 High

;built-in delay as per CSI manual and Sue!
2: Delay w/Opt Excitation (P22)
 1: 1       Ex Channel
 2: 0       Delay W/Ex (0.01 sec units)
 3: 15      Delay After Ex (0.01 sec units)
 4: 0000    mV Excitation

;This command is for the 16 groups of three probes on the multiplexer
;It shows that there are 16 groups of three wires in each loop
;There are a total of 48 probes (#1-48)

3: Beginning of Loop (P87)
1: 0000 Delay
 2: 16       Loop Count

;This command switches to the next channel on the multiplexer by pulsing port
4
4: Do (P86)
 1: 74      Pulse Port 4

;This indicates that three probes are grouped together
5: Step Loop Index (P90)
 1: 3       Step

;Measuring 48 EC-5 sensors using 2500mV excitation
;The three reps represents each group of 3 wires
;Multiply the measured mV output by 0.001 to covert millivolts to volts
6: Delay w/Opt Excitation (P22)
```

```
 1: 1       Ex Channel
 2: 0       Delay W/Ex (0.01 sec units)
 3: 10      Delay After Ex (0.01 sec units)
 4: 0000 mV Excitation

7: Excite-Delay (SE) (P4)
 1: 3       Reps
 2: 15      5000 mV, Fast Range
 3: 1       SE Channel
 4: 1       Excite all reps w/Exchan 1
 5: 1       Delay (0.01 sec units)
 6: 2500    mV Excitation
 7: 1       -- Loc [ Volt_1 ]
 8: 0.001   Mult
 9: 0.0     Offset

;Ends the loop
8: End (P95)

;This command turns off the first multiplexer
9: Do (P86)
 1: 55      Set Port 5 Low

;*************************************************************************
;This command turns on the second multiplexer

10: Do (P86)
 1: 47      Set Port 7 High

;built in delay
11:   Delay w/Opt Excitation (P22)
 1: 1       Ex Channel
 2: 0       Delay W/Ex (0.01 sec units)
 3: 15      Delay After Ex (0.01 sec units)
 4: 0       mV Excitation

;This command is for the 16 groups of three probes on the multiplexer
;It shows that there are 16 groups of three wires in each loop
;There are a total of 48 probes (#1-48)

12: Beginning of Loop (P87)
 1: 0000 Delay
 2: 16      Loop Count

;This command switches to the next channel on the multiplexer by pulsing port
4
13: Do (P86)
 1: 76      Pulse Port 6

;This indicates that three probes are grouped together
14: Step Loop Index (P90)
 1: 3       Step

;Measuring 48 EC-5 sensors using 2500mV excitation
;The three reps represents each group of 3 wires
;Multiply the measured mV output by 0.001 to covert millivolts to volts
```

```
15: Delay w/Opt Excitation (P22)
 1: 1       Ex Channel
 2: 0       Delay W/Ex (0.01 sec units)
 3: 10      Delay After Ex (0.01 sec units)
 4: 0000 mV Excitation

16: Excite-Delay (SE) (P4)
 1: 3       Reps
 2: 15      5000 mV, Fast Range
 3: 4       SE Channel
 4: 2       Excite all reps w/Exchan 2
 5: 1       Delay (0.01 sec units)
 6: 2500    mV Excitation
 7: 4       -- Loc [ Volt_4 ]
 8: 0.001   Mult
 9: 0.0     Offset

;Ends the loop
17: End (P95)

;This command turns off the second multiplexer
18: Do (P86)
 1: 57      Set Port 7 Low
;*************************************************************************
;Converting the measured voltage output into substrate water content using
the following calibration:
;VWC = 0.6855 x Voltage - 0.1487
;This is the calibration used for the 50:50 sand:turface mixture used for
this study

19: Beginning of Loop (P87)
 1: 0000 Delay
 2: 96        Loop Count

   20: Z=X*F (P37)
    1: 1          -- X Loc [ Volt_1 ]
    2: 0.6855     F
    3: 97         -- Z Loc [ WC_1 ]

   21: Z=X+F (P34)
    1: 97         -- X Loc [ WC_1 ]
    2: -0.1487    F
    3: 97         -- Z Loc [ WC_1 ]

;Ends the loop
22: End (P95)

;*************************************************************************
*****************************
;The following commands will turn on an LED to indicate a failed sensor (i.e.
sensor is reading above 35% and below 0%)

23: Beginning of Loop (P87)
 1: 0000 Delay
 2: 96     Loop Count

   24: If (X<=>F) (P89)
```

```
    1: 97   X Loc [ WC_1 ]
    2: 3    >=
    3: 0    F
    4: 48   Set Port 8 High

   25: If (X<=>F) (P89)
    1: 97 X Loc [ WC_1 ]
    2: 4    <
    3: 1    F
    4: 58  Set Port 8 Low

   26: If (X<=>F) (P89)
   1: 97    X Loc [ WC_1 ]
   2: 4     <
   3: 35    F
   4: 48    Set Port 8 High

;Ends the loop
27: End (P95)

;******************** DATA STORAGE
*********************************************************************
*********
;The following commands will store the voltage and volumetric water content
data
28: Do (P86)
 1: 22 Set Flag 2 Low

29: If time is (P92)
 1: 0       Minutes (Seconds --) into a
 2: 10      Interval (same units as above)
 3: 10      Set Output Flag High (Flag 0)

30: Real Time (P77)
 1: 1110    Year,Day,Hour/Minute (midnight = 0000)

;This command stores the 10 minute average for raw voltage readings from each
sensor
31: Sample (P70)
1: 96       Reps
2: 1        Loc [ Volt_1 ]

;This command stores the 10 minute average water content for each sensor
32: Average (P71)
 1: 96      Reps
 2: 97      Loc [ WC_1 ]
;********************************** END TABLE1*****************************

;********************************** START TABLE 2 ************************
;The execution interval for this part of the program is every hour.
*Table 2 Program
02: 900 Execution Interval (seconds)
;*****************************************************************
;This command will cause the four irrigation lines to be watered four times
each day at 12am, 6am, 12pm and 6pm
;This command resets al of the CTL locations (defined later) to zero (no
irrigation)
```

```
1: Beginning of Loop (P87)
 1: 0000    Delay
 2: 16        Loop Count

   2: Z=F x 10^n (P30)
    1: 0.0   F
    2: 0 n,  Exponent of 10
    3: 193   -- Z Loc [ CTL_AGR1 ]

3: End (P95)

;This command irrigates at 12am for AGRESTIS 1
4: If time is (P92)
 1: 0       Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   5: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 193  Z Loc [ CTL_AGR1 ]

6: End (P95)

;This command irrigates at 12am for ANNUUS 1
7: If time is (P92)
 1: 0       Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   8: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 194  Z Loc [ CTL_ANN1 ]

9: End (P95)

;This command irrigates at 12am for CARNOSUS 1
10: If time is (P92)
 1: 0       Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   11: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 195  Z Loc [ CTL_CAR1 ]

12: End (P95)

;This command irrigates at 12am for PORTERI 1
13: If time is (P92)
 1: 0       Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   14: Z=F x 10^n (P30)
```

```
    1: 1    F
    2: 00   n, Exponent of 10
    3: 196  Z Loc [ CTL_POR1 ]

15: End (P95)

;This command irrigates at 12am for AGRESTIS 2
16: If time is (P92)
 1: 0       Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   17: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 197  Z Loc [ CTL_AGR2 ]

18: End (P95)

;This command irrigates at 12am for ANNUUS 2
19: If time is (P92)
 1: 0       Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do


   20: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 198  Z Loc [ CTL_ANN2 ]

21: End (P95)

;This command irrigates at 12am for CARNOSUS 2
22: If time is (P92)
 1: 0       Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   23: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 199  Z Loc [ CTL_CAR2 ]

24: End (P95)

;This command irrigates at 12am for PORTERI 2
25: If time is (P92)
 1: 0       Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   26: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 200  Z Loc [ CTL_POR2 ]
```

```
27: End (P95)

;*****************************************
;This command irrigates at 6am for AGRESTIS 1
28: If time is (P92)
 1: 360     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   29: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 193  Z Loc [ CTL_AGR1 ]

30: End (P95)

;This command irrigates at 6am for ANNUUS 1
31: If time is (P92)
 1: 360     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   32: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 194  Z Loc [ CTL_ANN1 ]

33: End (P95)

;This command irrigates at 6am for CARNOSUS 1
34: If time is (P92)
 1: 360     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   35: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 195  Z Loc [ CTL_CAR1 ]

36: End (P95)

;This command irrigates at 6am for PORTERI 1
37: If time is (P92)
 1: 360     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   38: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 196  Z Loc [ CTL_POR1 ]

39: End (P95)

;This command irrigates at 6am for AGRESTIS 2
40: If time is (P92)
```

```
 1: 360     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   41: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 197  Z Loc [ CTL_AGR2 ]

42: End (P95)

;This command irrigates at 6am for ANNUUS 2
43: If time is (P92)
 1: 360     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   44: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 198  Z Loc [ CTL_ANN2 ]

45: End (P95)

;This command irrigates at 6am for CARNOSUS 2
46: If time is (P92)
 1: 360     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   47: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 199  Z Loc [ CTL_CAR2 ]

48: End (P95)

;This command irrigates at 6am for PORTERI 2
49: If time is (P92)
 1: 360     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   50: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 200  Z Loc [ CTL_POR2 ]

51: End (P95)
;****************************************
;This command irrigates at 12pm for AGRESTIS 1
52: If time is (P92)
 1: 720     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   53: Z=F x 10^n (P30)
```

```
    1: 1    F
    2: 00   n, Exponent of 10
    3: 193  Z Loc [ CTL_AGR1 ]

54: End (P95)

;This command irrigates at 12pm for ANNUUS 1
55: If time is (P92)
 1: 720     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   56: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 194  Z Loc [ CTL_ANN1 ]

57: End (P95)

;This command irrigates at 12pm for CARNOSUS 1
58: If time is (P92)
 1: 720     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   59: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 195  Z Loc [ CTL_CAR1 ]

60: End (P95)

;This command irrigates at 12pm for PORTERI 1
61: If time is (P92)
 1: 720     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   62: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 196  Z Loc [ CTL_POR1 ]

63: End (P95)

;This command irrigates at 12pm for AGRESTIS 2
64: If time is (P92)
 1: 720     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   65: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 197  Z Loc [ CTL_AGR2 ]

66: End (P95)
```

```
;This command irrigates at 12pm for ANNUUS 2
67: If time is (P92)
 1: 720     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   68: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 198  Z Loc [ CTL_ANN2 ]

69: End (P95)

;This command irrigates at 12pm for CARNOSUS 2
70: If time is (P92)
 1: 720     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   71: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 199  Z Loc [ CTL_CAR2 ]

72: End (P95)

;This command irrigates at 12pm for PORTERI 2
73: If time is (P92)
 1: 720     Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   74: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 200  Z Loc [ CTL_POR2 ]

75: End (P95)
;*****************************************
;This command irrigates at 6pm for AGRESTIS 1
76: If time is (P92)
 1: 1080    Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   77: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 193  Z Loc [ CTL_AGR1 ]

78: End (P95)

;This command irrigates at 6pm for ANNUUS 1
79: If time is (P92)
 1: 1080    Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
```

```
 3: 30      Then Do

   80: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 194  Z Loc [ CTL_ANN1 ]

81: End (P95)

;This command irrigates at 6pm for CARNOSUS 1
82: If time is (P92)
 1: 1080    Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   83: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 195  Z Loc [ CTL_CAR1 ]

84: End (P95)

;This command irrigates at 6pm for PORTERI 1
85: If time is (P92)
 1: 1080    Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   86: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 196  Z Loc [ CTL_POR1 ]

87: End (P95)

;This command irrigates at 6pm for AGRESTIS 2
88: If time is (P92)
 1: 1080    Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   89: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 197  Z Loc [ CTL_AGR2 ]

90: End (P95)

;This command irrigates at 6pm for ANNUUS 2
91: If time is (P92)
 1: 1080    Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   92: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
```

```
    3: 198  Z Loc [ CTL_ANN2 ]

93: End (P95)

;This command irrigates at 6pm for CARNOSUS 2
94: If time is (P92)
 1: 1080    Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   95: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 199  Z Loc [ CTL_CAR2 ]

96: End (P95)

;This command irrigates at 6pm for PORTERI 2
97: If time is (P92)
 1: 1080    Minutes (Seconds --) into a
 2: 1440    Interval (same units as above)
 3: 30      Then Do

   98: Z=F x 10^n (P30)
    1: 1    F
    2: 00   n, Exponent of 10
    3: 200  Z Loc [ CTL_POR2 ]

99: End (P95)
;*********************************************
;This command turns off the solenoid control ports
100: SDM-CD16 / SDM-CD16AC (P104)
 1: 16   Reps
 2: 00   SDM Address
 3: 193  Loc [ CTL_AGR1 ]

;This command collects data for a rainguage which will be used to monitor the
irrigation
101:     Pulse (P3)
 1: 1    Reps
 2: 1    Pulse Channel 1
 3: 02   Switch Closure, All Counts
 4: 209  Loc [ RainGuage ]
 5: 1.0  Multiplier
 6: 0.0  Offset

;These next two instructions collect the data from the rain guage
102: Do (P86)
 1: 10   Set Output Flag High (Flag 0) ;

103: Totalize (P72)
 1: 1    Reps
 2: 209  Loc [ RainGuage ]

*Table 3 Subroutines

End Program
```

APPENDIX 3

DATALOGGER PROGRAM CODE FOR THE MILD DROUGHT EXPERIMENT

```
;CR23X
;
;Elise Bartelme

;This program will monitor changes in soil moisture (volumetric water
content) of 96 pots in the greenhouse
;there will be two treatments: well watered (20%sm) and droughted (14%sm)
;eight groups of 12 pots will be monitored for each species and treatment
combination
;for example, all 12 pots containing H. porteri under the drought treatment
will be irrigated with its own solenoid

;*************************************************************************
*********************
;A CR23X Datalogger (Campbell Scientific) will be used
;Two AM416 Multiplexers (Campbell Scientific) will be used
;96 EC-5 Soil Moisture Probes (Decagon Devices) will be used to measure
change in %soil moisture
;One relay driver will be used to control 8 solenoids
;*************************************************************************
*********************
;*****************************************************************
;The First AM416 is connected to the CR23X using this configuration:

;WIRING: MP 12V wired to 12V on DL
;WIRING: MP GND wired to G on DL
;WIRING: MP CLK wired to CP 4 on DL - switches to the next channel
;WIRING: MP RES wired to CP 5 on DL - turns the multiplexer on and off
;WIRING: MP COM port H1 to EXCITATION 1 on DL
;WIRING: MP COM port L1 to SE1 on DL
;WIRING: MP COM port H2 to SE2 on DL
;WIRING: MP COM port L2 to SE3 on DL
;WIRING: MP COM SHIELD to G on DL
;*****************************************************************
;The second Am416 is connected to the CR23X using this configuration:

;WIRING: MP 12V wired to 12V on DL
;WIRING: MP GND wired to G on DL
;WIRING: MP CLK wired to CP 6 on DL red wire
;WIRING: MP RES wired to CP 7 on DL white wire
;WIRING: MP COM port H1 to EXCITATION 2 on DL white wire
;WIRING: MP COM port L1 to SE4 on DL black wire
;WIRING: MP COM port H2 to SE5 on DL red wire
;WIRING: MP COM port L2 to SE6 on DL green wire
;WIRING: MP COM SHIELD to G on DL
;*************************************************************************
*************
```

```
;Sensors ECH2O EC-5 connection on to AM416

;WIRING (white - EXCITATION): 16 groups of 3 white wires attached to the H1
ports 1-16 (1-6) on the AM416
;WIRING (red - VOLTAGE SIGNAL): 16 groups of 3 wires each wired into L1, H2,
and H3 ports 1-16 (1-6) on the AM416
;WIRING (silver): 48 wires connected together and wired into SHILED port on
the AM416
;this is done for both multiplexers 48 sensors each
;*************************************************************************
**************
;The relay driver is connected to the CR23X using this wiring:
;WIRING: C1 on RD to C1 on DL
;WIRING: C2 on RD to C2 on DL
;WIRING: C3 on RD to C3 on DL
;WIRING: 12V on RD to 12V on DL
;WIRING: G on RD to G on DL
;*************************************************************************

*Table 1 Program
  01: 30        Execution Interval (seconds)

1:  Z=Z+1 (P32)
 1: 1         Z Loc [ COUNTER   ]


2:  If time is (P92)
 1: 0         Minutes (Seconds --) into a
 2: 10        Interval (same units as above)
 3: 30        Then Do

     3:  Z=F x 10^n (P30)
      1: 0.0      F
      2: 0        n, Exponent of 10
      3: 1        Z Loc [ COUNTER   ]


4:  End (P95)


;This command turns on the multiplexer
5:  Do (P86)
 1: 45        Set Port 5 High

;built-in delay as per CSI manual and Sue!
6:  Delay w/Opt Excitation (P22)
 1: 1         Ex Channel
 2: 0         Delay W/Ex (0.01 sec units)
 3: 15        Delay After Ex (0.01 sec units)
 4: 0000      mV Excitation

;This command is for the 16 groups of  three probes on the multiplexer
;It shows that there are 16 groups of three wires in each loop
;There are a total of 48 probes (#1-48)

7:  Beginning of Loop (P87)
 1: 0000      Delay
```

```
 2: 16       Loop Count


;This command switches to the next channel on the multiplexer by pulsing port
4
     8:  Do (P86)
      1: 74       Pulse Port 4

;This indicates that three probes are grouped together
     9:  Step Loop Index (P90)
      1: 3        Step

;Measuring 48 EC-5 sensors using 2500mV excitation
;The three reps represents each group of 3 wires
;Multiply the measured mV output by 0.001 to covert millivolts to volts

     10:  Delay w/Opt Excitation (P22)
      1: 1        Ex Channel
      2: 0        Delay W/Ex (0.01 sec units)
      3: 10       Delay After Ex (0.01 sec units)
      4: 0000     mV Excitation

     11:  Excite-Delay (SE) (P4)
      1: 3        Reps
      2: 15       5000 mV, Fast Range
      3: 1        SE Channel
      4: 1        Excite all reps w/Exchan 1
      5: 1        Delay (0.01 sec units)
      6: 2500     mV Excitation
      7: 2     -- Loc [ Volt_1    ]
      8: 0.001    Mult
      9: 0.0      Offset

12:  End (P95)

;This command turns off the first multiplexer

13:  Do (P86)
 1: 55       Set Port 5 Low


;*****************************************
;This command turns on the second multiplexer

14:  Do (P86)
 1: 47       Set Port 7 High

;built in delay

15:  Delay w/Opt Excitation (P22)
 1: 1        Ex Channel
 2: 0        Delay W/Ex (0.01 sec units)
 3: 15       Delay After Ex (0.01 sec units)
 4: 0        mV Excitation

;This command is for the 16 groups of  three probes on the multiplexer
;It shows that there are 16 groups of three wires in each loop
```

```
;There are a total of 48 probes (#1-48)

16:  Beginning of Loop (P87)
 1: 0000     Delay
 2: 16       Loop Count


;This command switches to the next channel on the multiplexer by pulsing port
4
     17:  Do (P86)
      1: 76       Pulse Port 6

;This indicates that three probes are grouped together
     18:  Step Loop Index (P90)
      1: 3        Step

;Measuring 48 EC-5 sensors using 2500mV excitation
;The three reps represents each group of 3 wires
;Multiply the measured mV output by 0.001 to covert millivolts to volts

     19:  Delay w/Opt Excitation (P22)
      1: 1        Ex Channel
      2: 0        Delay W/Ex (0.01 sec units)
      3: 10       Delay After Ex (0.01 sec units)
      4: 0000     mV Excitation

     20:  Excite-Delay (SE) (P4)
      1: 3        Reps
      2: 15       5000 mV, Fast Range
      3: 4        SE Channel
      4: 2        Excite all reps w/Exchan 2
      5: 1        Delay (0.01 sec units)
      6: 2500     mV Excitation
      7: 50    -- Loc [ Volt_49   ]
      8: 0.001    Mult
      9: 0.0      Offset

21:  End (P95)

;This command turns off the second multiplexer


22:  Do (P86)
 1: 57       Set Port 7 Low


;*************************************************************************
************************
;Converting the measured voltage output into substrate water content using
the following calibration:
;VWC = 0.6855 x Voltage - 0.1487
;This is the calibration used for the 50:50 sand:turface mixture used for
this study


23:  Beginning of Loop (P87)
 1: 0000     Delay
```

```
 2: 96       Loop Count


     24:  Z=X*F (P37)
      1: 2     -- X Loc [ Volt_1    ]
      2: 0.6855   F
      3: 98    -- Z Loc [ WC_1      ]

     25:  Z=X+F (P34)
      1: 98    -- X Loc [ WC_1      ]
      2: -0.1487  F
      3: 98    -- Z Loc [ WC_1      ]


26:  End (P95)


;*************************************************************************
;These commands will turn any extreme value into the appropriate set point
(i.e. 20% or 14%) to avoid
;biasing when irrigations will occur


;Well-watered AGRESTIS
27:  Beginning of Loop (P87)
 1: 0000     Delay
 2: 12       Loop Count

     28:  If (X<=>F) (P89)
      1: 98    -- X Loc [ WC_1      ]
      2: 4        <
      3: 0.0      F
      4: 30       Then Do

          29:  Z=F x 10^n (P30)
           1: .20      F
           2: 00       n, Exponent of 10
           3: 98    -- Z Loc [ WC_1      ]


     30:  End (P95)

31:  End (P95)



;Droughted AGRESTIS
32:  Beginning of Loop (P87)
 1: 0000     Delay
 2: 12       Loop Count

     33:  If (X<=>F) (P89)
      1: 110      X Loc [ WC_13     ]
      2: 4        <
      3: 0.0      F
      4: 30       Then Do
```

```
          34:  Z=F x 10^n (P30)
           1: 0.14     F
           2: 0        n, Exponent of 10
           3: 110      Z Loc [ WC_13     ]

     35:  End (P95)

36:  End (P95)

;Well-watered ANNUUS
37:  Beginning of Loop (P87)
 1: 0000     Delay
 2: 12       Loop Count

     38:  If (X<=>F) (P89)
      1: 122      X Loc [ WC_25     ]
      2: 4        <
      3: 0.0      F
      4: 30       Then Do

          39:  Z=F x 10^n (P30)
           1: 0.20     F
           2: 00       n, Exponent of 10
           3: 122      Z Loc [ WC_25     ]

     40:  End (P95)

41:  End (P95)


;Droughted ANNUUS
42:  Beginning of Loop (P87)
 1: 0000     Delay
 2: 12       Loop Count

     43:  If (X<=>F) (P89)
      1: 134      X Loc [ WC_37     ]
      2: 4        <
      3: 0.0      F
      4: 30       Then Do

          44:  Z=F x 10^n (P30)
           1: 0.14     F
           2: 0        n, Exponent of 10
           3: 134      Z Loc [ WC_37     ]

     45:  End (P95)

46:  End (P95)


;Well-watered CARNOSUS
47:  Beginning of Loop (P87)
 1: 0000     Delay
 2: 12       Loop Count
```

```
     48:  If (X<=>F) (P89)
      1: 146      X Loc [ WC_49     ]
      2: 4        <
      3: 0.0      F
      4: 30       Then Do

          49:  Z=F x 10^n (P30)
           1: 0.20     F
           2: 0        n, Exponent of 10
           3: 146      Z Loc [ WC_49     ]


     50:  End (P95)

51:  End (P95)

;Droughted CARNOSUS
52:  Beginning of Loop (P87)
 1: 0000     Delay
 2: 12       Loop Count

     53:  If (X<=>F) (P89)
      1: 158      X Loc [ WC_61     ]
      2: 4        <
      3: 0.0      F
      4: 30       Then Do

          54:  Z=F x 10^n (P30)
           1: 0.14     F
           2: 0        n, Exponent of 10
           3: 158      Z Loc [ WC_61     ]

     55:  End (P95)

56:  End (P95)


;Well-watered PORTERI
57:  Beginning of Loop (P87)
 1: 0000     Delay
 2: 12       Loop Count

     58:  If (X<=>F) (P89)
      1: 170      X Loc [ WC_73     ]
      2: 4        <
      3: 0.0      F
      4: 30       Then Do

          59:  Z=F x 10^n (P30)
           1: 0.20     F
           2: 00       n, Exponent of 10
           3: 170      Z Loc [ WC_73     ]

     60:  End (P95)

61:  End (P95)
```

```
;Droughted PORTERI
62:  Beginning of Loop (P87)
 1: 0000     Delay
 2: 12       Loop Count

     63:  If (X<=>F) (P89)
      1: 182      X Loc [ WC_85     ]
      2: 4        <
      3: 0.0      F
      4: 30       Then Do

          64:  Z=F x 10^n (P30)
           1: 0.14     F
           2: 00       n, Exponent of 10
           3: 182      Z Loc [ WC_85     ]

     65:  End (P95)

66:  End (P95)

;*************************************************************************
;These commands avereage the water content values for 8 groups of 12 probes
for each treat/spp combo


67:  Spatial Average (P51)
 1: 12       Swath
 2: 98       First Loc [ WC_1      ]
 3: 194      Avg Loc [ AVG_AGR_W ]

68:  Spatial Average (P51)
 1: 12       Swath
 2: 110      First Loc [ WC_13     ]
 3: 195      Avg Loc [ AVG_AGR_D ]

69:  Spatial Average (P51)
 1: 12       Swath
 2: 122      First Loc [ WC_25     ]
 3: 196      Avg Loc [ AVG_ANN_W ]

70:  Spatial Average (P51)
 1: 12       Swath
 2: 134      First Loc [ WC_37     ]
 3: 197      Avg Loc [ AVG_ANN_D ]

71:  Spatial Average (P51)
 1: 12       Swath
 2: 146      First Loc [ WC_49     ]
 3: 198      Avg Loc [ AVG_CAR_W ]

72:  Spatial Average (P51)
 1: 12       Swath
 2: 158      First Loc [ WC_61     ]
 3: 199      Avg Loc [ AVG_CAR_D ]

73:  Spatial Average (P51)
```

```
 1: 12       Swath
 2: 175      First Loc [ WC_78     ]
 3: 200      Avg Loc [ AVG_POR_W ]

74:  Spatial Average (P51)
 1: 12       Swath
 2: 182      First Loc [ WC_85     ]
 3: 201      Avg Loc [ AVG_POR_D ]


;This resets all of the CTRL locations (defined later) to zero (no
irrigation)

75:  Beginning of Loop (P87)
 1: 0000     Delay
 2: 16       Loop Count

     76:  Z=F x 10^n (P30)
      1: 0.0      F
      2: 00       n, Exponent of 10
      3: 202   -- Z Loc [ CTL_AGR_W ]

77:  End (P95)

;This makes sure that our COUNTER is at 1 before irrigation, and makes sure
that I only irrigate once
;per 10 minute interval

;This commmand sets the CTRL locations to 1 that are below the set points.
These commands also set our
;minimum set points of 0.20 or 0.14 VWC depending on the treatment for each
group of sensors.

;Well-watered AGRESTIS
78:  If (X<=>F) (P89)
 1: 1        X Loc [ COUNTER   ]
 2: 1        =
 3: 1        F
 4: 30       Then Do

     79:  If (X<=>F) (P89)
      1: 194      X Loc [ AVG_AGR_W ]
      2: 4        <
      3: 0.20     F
      4: 30       Then Do


          80:  Z=F x 10^n (P30)
           1: 1        F
           2: 00       n, Exponent of 10
           3: 202      Z Loc [ CTL_AGR_W ]

     81:  End (P95)

82:  End (P95)

;Droughted AGRESTIS
```

```
83:  If (X<=>F) (P89)
 1: 1        X Loc [ COUNTER   ]
 2: 1        =
 3: 1        F
 4: 30       Then Do

     84:  If (X<=>F) (P89)
      1: 195      X Loc [ AVG_AGR_D ]
      2: 4        <
      3: 0.14     F
      4: 30       Then Do

          85:  Z=F x 10^n (P30)
           1: 1        F
           2: 00       n, Exponent of 10
           3: 203      Z Loc [ CTL_AGR_D ]

     86:  End (P95)

87:  End (P95)

;Well-watered ANNUUS
88:  If (X<=>F) (P89)
 1: 1        X Loc [ COUNTER   ]
 2: 1        =
 3: 1        F
 4: 30       Then Do

     89:  If (X<=>F) (P89)
      1: 196      X Loc [ AVG_ANN_W ]
      2: 4        <
      3: 0.20     F
      4: 30       Then Do

          90:  Z=F x 10^n (P30)
           1: 1        F
           2: 00       n, Exponent of 10
           3: 204      Z Loc [ CTL_ANN_W ]

     91:  End (P95)

92:  End (P95)

;Droughted ANNUUS
93:  If (X<=>F) (P89)
 1: 1        X Loc [ COUNTER   ]
 2: 1        =
 3: 1        F
 4: 30       Then Do

     94:  If (X<=>F) (P89)
      1: 197      X Loc [ AVG_ANN_D ]
      2: 4        <
      3: 0.14     F
      4: 30       Then Do

          95:  Z=F x 10^n (P30)
```

```
           1: 1        F
           2: 00       n, Exponent of 10
           3: 205      Z Loc [ CTL_ANN_D ]

     96:  End (P95)

97:  End (P95)

;Well-watered CARNOSUS
98:  If (X<=>F) (P89)
 1: 1        X Loc [ COUNTER   ]
 2: 1        =
 3: 1        F
 4: 30       Then Do

     99:  If (X<=>F) (P89)
      1: 198      X Loc [ AVG_CAR_W ]
      2: 4        <
      3: 0.20     F
      4: 30       Then Do

          100:  Z=F x 10^n (P30)
           1: 1        F
           2: 00       n, Exponent of 10
           3: 206      Z Loc [ CTL_CAR_W ]

     101:  End (P95)

102:  End (P95)

;Droughted CARNOSUS
103:  If (X<=>F) (P89)
 1: 1        X Loc [ COUNTER   ]
 2: 1        =
 3: 1        F
 4: 30       Then Do

     104:  If (X<=>F) (P89)
      1: 199      X Loc [ AVG_CAR_D ]
      2: 4        <
      3: 0.14     F
      4: 30       Then Do

          105:  Z=F x 10^n (P30)
           1: 1        F
           2: 0        n, Exponent of 10
           3: 207      Z Loc [ CTL_CAR_D ]

     106:  End (P95)

107:  End (P95)

;Well-watered PORTERI
108:  If (X<=>F) (P89)
 1: 1        X Loc [ COUNTER   ]
 2: 1        =
 3: 1        F
```

```
 4: 30       Then Do

     109:  If (X<=>F) (P89)
      1: 200      X Loc [ AVG_POR_W ]
      2: 4        <
      3: 0.20     F
      4: 30       Then Do

          110:  Z=F x 10^n (P30)
           1: 1        F
           2: 00       n, Exponent of 10
           3: 208      Z Loc [ CTL_POR_W ]

     111:  End (P95)

112:  End (P95)

;Droughted PORTERI
113:  If (X<=>F) (P89)
 1: 1        X Loc [ COUNTER   ]
 2: 1        =
 3: 1        F
 4: 30       Then Do

     114:  If (X<=>F) (P89)
      1: 201      X Loc [ AVG_POR_D ]
      2: 4        <
      3: 0.14     F
      4: 30       Then Do

          115:  Z=F x 10^n (P30)
           1: 1        F
           2: 00       n, Exponent of 10
           3: 209      Z Loc [ CTL_POR_D ]

     116:  End (P95)

117:  End (P95)

;This command turns off the solenoid control ports


118:  SDM-CD16 / SDM-CD16AC (P104)
 1: 16       Reps
 2: 00       SDM Address
 3: 202      Loc [ CTL_AGR_W ]


;*************************************************************************
************************
;DATA STORAGE

;These commands store the data every 15 minutes

;119:  Do (P86)
; 1: 22       Set Flag 2 Low
```

```
119:  If time is (P92)
 1: 0        Minutes (Seconds --) into a
 2: 10       Interval (same units as above)
 3: 10       Set Output Flag High


120:  Real Time (P77)^21488
 1: 1110     Year,Day,Hour/Minute (midnight = 0000)

121:  Sample (P70)^13579
 1: 96       Reps
 2: 2        Loc [ Volt_1    ]

;This command stores the 10 minute average water content for each pot


122:  Average (P71)^31986
 1: 96       Reps
 2: 98       Loc [ WC_1      ]


;This command records the averages for each of the species treatment
combinations
;Starting with well-watered agrestis

123:  Average (P71)^10517
 1: 8        Reps
 2: 194      Loc [ AVG_AGR_W ]

;This command totalizes the number of times each group of pots are irrigated
;Starting wiht the well-watered agrestis

124:  Totalize (P72)^17727
 1: 8        Reps
 2: 202      Loc [ CTL_AGR_W ]

*Table 2 Program
  02: 0.0000    Execution Interval (seconds)

*Table 3 Subroutines

End Program
```