# IMPACTS OF POLYPLOIDY AND SUBSEQUENT NATURAL SELECTION ON THE ECOPHYSIOLOGY OF *SOLIDAGO ALTISSIMA*

A Thesis
by
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**Abstract**

The evolutionary importance of polyploidy in plants is still a subject of much research. Polyploidy could be an evolutionary dead end, or it could lead to reproductive isolation and creation of new species. Goldenrod (*Solidago altissima*) is a North American herbaceous perennial with diploid, tetraploid, and hexaploid populations: diploids (MWD) and tetraploids are restricted to the Midwest while hexaploids occur in both the Midwest (MWH) and East (EH). Plants were grown in a common outdoor garden at Appalachian State University, and aspects of their morphology measured. EH had larger and more vertically oriented leaves than MWD and MWH, but MWH leaves had a higher specific leaf mass. Stomatal guard cells were larger in both hexaploids, but abaxial densities were not different between MWD and MWH, and were lowest in EH. Hydraulic flow rates were potentially higher in the hexaploids. A split-plot greenhouse drought experiment was performed. Photosynthetic rates ($A$) were initially highest in MWH, followed by MWD, and lowest in EH. $A$ and stomatal conductance ($g_s$) declined with time in both treatments, but more so in droughted plants after cessation of watering. MWD stomata displayed a threshold response to drought before closing whereas MWH did not EH was intermediate. MWH had the lowest water use efficiency, while during peak drought it was highest for EH. By the end of the experiment, cytotype differences for $A$, $g_s$, and water potential were absent in both treatments. Results show substantial morphological and physiological differences among the hexaploids, indicating significant natural selection following polyploidization.

**Acknowledgements**

I would like to thank my primary thesis advisor Howard S. Neufeld for patiently introducing me to plant ecophysiology, to various scientific methodologies, and to scientific writing. I thank my committee members Ray Williams and Matt Estep who helped me obtain the plants and resources used for this project, as well as knowledge on the subject matter. I also thank my lab mates Erica Pauer, Sarah McCoy, and Anthony Percival for their help in potting, experiment set-up, and taking measurements, as well as the rest of the Neufeld lab for their moral support. Funding and support for research, and conference travel came from the Office of Student Research, and the Association of Southeastern Biologists. Thank you to ASU greenhouse manager Jerry Meyer for logistical support and space. Finally, thank you to the other students in the Neufeld lab who also provided encouragement and practical support throughout this project.

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## Foreword

This thesis was completed with help from my lab mates Erica Pauer and Sarah McCoy, to ascertain the impacts polyploidy may have on *Solidago altissima* and why the cytotypes within this species complex are distributed inconsistently throughout the United States distribution. The format and references follow the guidelines of the scientific journal American Journal of Botany, in anticipation of submitting this project for publication.

# INTRODUCTION

For over a century, scientists have worked to better understand the role of genomics and its implications for plant evolution and ecology. From this research, polyploidy, or whole-genome duplication, has become a hot topic for debate due to its high prevalence among angiosperms (Adams and Wendel, 2005; Weiss-Schneeweiss et al., 2013) and its potential role in speciation (Stebbins, 1971; Lewis, 1979). Polyploids are classified into two categories based on their genetic origin: autopolyploids, which arise from multiplication of genetically identical chromosome sets within a single species, and allopolyploids, which are formed by the multiplication of chromosome sets between two genetically different species (Ramsey and Schemske, 1998; Weiss-Schneeweiss et al., 2013). These processes not only affect the genotype of a species, but also the phenotype, resulting in observable changes in plant anatomy, morphology, phenology, and physiology (Maherali et al., 2009). As natural selection acts on the phenotype, any traits that are altered have the potential to change the course of evolution once polyploidization occurs (Halverson et al., 2008; Weiss-Schneeweiss et al., 2013; Cheng et al., 2020).

Despite this understanding, the ultimate long-term fate of polyploids still presents many questions for the scientific community (Maherali et al., 2009; Otto and Whitton, 2000; Weiss-Schneeweiss et al*.,* 2013; Soltis et al., 2014; Ramsey and Ramsey, 2014). Some researchers have argued that polyploidy is a "*hindrance to the evolutionary success of higher plants*" (Stebbins, 1971), while others propose that it leads to reproductive isolation and speciation events in the

long-term (Soltis et al., 2014), although it may not be frequent (Levin, 2019). For speciation to happen, new cytotypes would have to overcome obstacles such as difficulties in establishment, small population sizes, competition with their diploid progenitors, and even diploidization or genome size reduction (Lewis, 1979; Soltis et al., 2015; Baniaga et al., 2020; Escudero and Wendel, 2020; Bowers and Patterson, 2021). Yet, even with these hurdles to overcome, genomic data show that globally, most vascular plants, regardless of current genome size and chromosome number, have undergone polyploidization; some of them repeatedly (Weiss-Schneeweiss, 2013; Del Pozo and Ramirez-Parra, 2015; Soltis et al., 2015; Baniaga et al., 2020). Some research suggests that there may also be an ecological cost to having too many or too few copies of the genome, so a lowering of ploidy number may be a stabilizing event (Bowers and Patterson, 2021). Once polyploids have established, the longer-term process of diploidization may help further their evolutionary speciation process (Dominguez-Delgado et al., 2021; Liqin et al., 2019). Diploidization commonly occurs shortly after polyploidization by downsizing the genome through the loss of duplicate genes and small genomic fragments (Cheng et al., 2018) or by rendering one copy of essential genes nonfunctional (Bowers and Patterson, 2021). Other evolutionary processes, such as gene subfunctionalization and neofunctionalization, gene silencing, and/or the activation of transposable elements or genome rearrangements, can shape the genomic and phenotypic structure of the mesopolyploids to allow their functional diploidization (Del Pozo and Ramirez-Parra, 2015), and ultimately aid in their adaptive evolution (Dominguez-Delgado et al., 2021).

Although most polyploid lineages eventually go extinct (Lewis, 1979; Levin, 2019; Bowers and Patterson, 2021), a few survive by overcoming their ecological obstacles (Bowers and Patterson 2021; Dominguez-Delgado et al., 2021). The adaptive shifts needed for

neopolyploids to persist may include the ability to inhabit new ecological niches due to incremental changes in gene expression (e.g., phenotype) that allow them to "*escape evolutionary stasis*" (Wright, 1932; Liqin et al., 2019; Bowers and Patterson, 2021), at least in the short-term (Soltis et al., 2015; Dominguez-Delgado et al., 2021). Speciation through polyploidization can then be accomplished through pre- or post-zygotic reproductive isolation. Prezygotic mechanisms would include geographic or ecological isolation, flower morphology and phenological differences, and pollinator consistency that eliminates cross-pollination due to morphological and/or physiological changes in the flowers. Postzygotic mechanisms include such factors as triploid hybrid inviability and inbreeding depression (Bretagnolle and Lumaret, 1995; Ramsey and Schemske, 1998; Seagraves and Thompson, 1999; Maherali et al., 2009; Soltis et al., 2014; Barker et al., 2016; Certner, 2020).

For polyploids, reproductive isolation means less mixing with diploid progenitors or self-fertilization, and therefore an increase in viable offspring, and successful establishment of the population (Bowers and Patterson, 2021). The genomic changes that occur immediately after polyploidization may lead to the creation of this isolation through long-term genetic, physiological, and morphological differentiation resulting in novel phenotypes, the "*raw material for evolution*" (Weiss-Schneeweiss et al., 2013; Dominguez-Delgado et al., 2021), but they may also have ecological consequences. For example, polyploidization has an immediate effect on plant anatomy via enlarged cell sizes (known as the "G*igas effect*"; Certner, 2020) due to a higher DNA content and a larger genome size (Muntzing, 1936; Stebbins, 1971; Warner and Edwards, 1993; Del Pozo and Ramirez-Parra, 2015). This can alter the cell architecture, leading to a lower cell surface area to volume ratio that can affect the distance and efficiency of exchange of materials across cell surfaces, although not much is known about this at present

(Doyle and Coate, 2019). Since molecules must be moved across cell membranes, they will take longer to traverse the cell if the cell has increased in size (Doyle and Coate, 2019). Therefore, cell-cell signaling within or among tissues, and responses to pathogens, as well as volatilization of chemical constituents into the atmosphere or rhizosphere, which are involved in attracting symbionts or functioning as plant-to-plant warnings against herbivores, could all be affected by cell size and dosage effects arising from polyploidization (Doyle and Coate, 2019). Other implications include a reduced potential for exchange of materials relative to cell volume, which could affect the time course and degree to which cells can adjust to environmental changes. For example, it is not known if the density of aquaporins, which allow for the exchange of water and $CO_2$ across cell membranes, is altered by polyploidization.

This basic change in cell size has significant implications at higher scales of organization that can lead to morphological, developmental, and physiological changes at the organ, tissue, whole plant, and even ecosystem level (Del Pozo and Ramirez-Parra, 2015). However, phenotypic differences may be reduced due to subsequent changes in the complexity of traits at higher levels of organization that offset such changes at lower levels (Ježilová et al., 2015). Thus, prediction of the ultimate effects of polyploidy are difficult unless analyzed at multiple scales of organization.

At the tissue level, larger cells affect the degree of cell packing, density, and function (Doyle and Coate, 2019). Intercellular distances may be reduced in some polyploids due to the tighter packing of larger cells (Warner and Edwards, 1989) and this could affect the tortuosity of gaseous diffusion within the leaf, which in turn, would affect gas exchange at the leaf level (Earles et al., 2018; Borsuk and Broderson, 2019; Roddy et al., 2020; Harwood et al., 2021). Cell packing and density may also affect the rate at which water is supplied to guard cells, with

consequences for stomatal opening and closing time constants (Dudits et al., 2016). Such stomatal responsiveness may play a role in water use efficiencies under conditions of low humidity or restricted water supply (Li et al., 2017). This could also have implications for how plants deal with heat stress and sunflecks. If hexaploids have larger and/or fewer guard cells they could be at a disadvantage if their stomata respond slower than those of diploids, leading to energy balance problems and greater heat stress (Li et al., 2009; Kardiman and Ræbild, 2017). Curiously, Ježilová et al. (2015) reported that hexaploids had higher induction rates upon sudden increases in light compared to plants with lower ploidy levels, but in this case, there were no differences in stomatal sizes. In contrast, Del Pozo and Ramirez-Parra (2014) reported larger cells and lower stomatal density in a synthetic autopolyploid *Arabidopsis* relative compared to its diploid progenitors. They also found that the stomatal index (ratio of stomata to epidermal cells) decreased with ploidy, suggesting stomatal development was altered in tetraploids (Del Pozo and Ramirez-Parra, 2014). In this case, the tetraploids, despite having larger and less dense stomata, still showed a greater tolerance to drought stress due to enhanced stomatal control through abscisic acid (ABA) signaling and reactive oxygen species homeostasis (Del Pozo and Ramirez-Parra, 2014).

Drought stress tolerance could be enhanced by larger mesophyll cell sizes because of increased cell capacitance, which would buffer these plants against times with limited soil water (Pozo et al., 2014; Baerdemaeker et al., 2018). Stomata may determine rates of water loss and gas exchange, but vein size and density determine water supply throughout plant leaves. If the cells and tissues that make up these veins (i.e., xylem) are affected by polyploidization, then the balance of water uptake, use, and loss will be impacted, and could influence functional (Maherali et al., 2009) and adaptive plasticity (Brodribb et al., 2013). Increased xylem cell size could affect

the sensitivity of polyploids to water stress differently from diploids (Maseda and Fernandez, 2006). Larger xylem conduits would result in an increase in whole-plant hydraulic conductivity, thereby raising the capacity for roots and stems to supply leaves with water (Zimmermann, 1983). This could ultimately result in increased stomatal opening (Mencuccini, 2003) and higher transpiration rates (Maherali et al., 2009; Guo et al., 2016) in the absence of drought stress, and higher photosynthetic rates (Hubbard et al., 2001), although there can be exceptions (Gao et al., 2017). At the same time, higher hydraulic conductivity may come at the expense of hydraulic safety and protection against embolisms arising from drought stress, and under drought stress, diploids may exhibit higher WUE and transpiration (Guo et al., 2016).

Plants of different ploidy levels can also differ in photosynthetic rates ($A$), leading to variation in growth, development, and competitive status (Warner and Edwards, 1993; Liao et al., 2016; Etterson et al., 2016; Gao et al., 2017; Williams and Oliviera, 2020). However, the relationship between ploidy and photosynthetic rate varies widely among species. For example, Vyas et al. (2007) looked at diploids (2x) and synthetic tetraploids (4xsyn) of *Phlox drummondii* in the initial generation (4x0) and those 11 generations later (G11). They found RUBISCO and other biochemical parameters were highest in tetraploids, with stomatal conductance ($g_s$) higher in 4x-G11 (Vyas et al., 2007). $A_{max}$ was also higher in leaves of 4x-G11 than 4x0 even though RUBISCO amounts were the same, suggesting that selection for different $A_{max}$ occurred many generations after polyploidization as the genome stabilized (Vyas et al., 2007). This higher $A_{max}$ in 4x plants could be due to higher RuBP regeneration capacity and higher chloroplast surface area adjacent to airspaces as well as higher $g_s$ (Vyas et al., 2007). In another study, Zhang et al. (2020) investigated photosynthetic rates of the autotetraploid pak choi (*Brassica rapa* ssp. *Chinensis*) and found that tetraploids had thicker leaves with larger cells, larger intercellular

spaces, and more granal height, which may have contributed to higher electron transport rates and overall higher $A_{max}$ (Zhang et al., 2020).

Greater cell sizes can result in polyploids having larger leaves (Li et al., 2009; Dudits et al., 2016; Zhang et al., 2019), which will affect their leaf energy balance due to increases in boundary layer thickness and a subsequent reduction in convective heat exchange (Gates, 1965; Levin, 1983). Leaf size is important because whole-plant carbon assimilation rates are closely related to individual and whole-plant leaf area (Sefton et al., 2002). Some modern wheat cultivars, for example, have higher rates of whole-plant carbon assimilation because they have larger leaves than older wheat cultivars, even though on a per unit area basis rates are lower (Richards, 2000). In another example, Chen et al. (2021) looked at diploid and tetraploid *Liridendron sino-americanum* and found 4x plants to have larger, thicker, and deeper green leaves, and thicker stems than 2x plants. Tetraploids also had larger stomata, but lower stomatal density (Chen et al., 2021). These morphological changes resulted in higher $A$ and $g_s$ in tetraploids than diploids (Chen et al., 2021). However, not all species show this pattern and, in fact, some polyploids can have smaller leaves than diploids, regardless of cell size, like that of the autotetraploid dwarf apple (*Malus domestica*) and Rangpur lime (*Citrus limonia* Osbeck) (Allario et al., 2011; Ma et al., 2016).

If polyploids develop more total leaf area per plant, they may also have higher whole-plant assimilation rates than diploids (Warner and Edwards, 1993), either by developing larger and/or more leaves than a diploid progenitor. If they develop leaf area more rapidly and earlier, they may avoid the deleterious effects of a late-season drought or heat wave (Warner and Edwards, 1993). Higher whole-plant assimilation could lead to larger plants too (Otto and Whitton, 2002; Lavania et al., 2012; Liquin et al., 2019; Doyle and Coate, 2019). Differences in

growth rates can have implications for phenology and seasonal climate interactions as well (Liao et al., 2016). Slower growth has been observed in polyploids and this may cause reproductive isolation by shifting flowering times. For example, *Ocimum kilimandscharicum* tetraploids flower 30 - 45 days later than diploids (Bose and Choudhury, 1962).

Additionally, for some species, polyploids have shown to be more competitive in stressful environments and in times of rapid environmental change through wider and/or differing geographical distributions, novel ecological niches, and wider niche breadths than their diploid progenitors (Stebbins, 1971; Buggs and Pannell, 2007; Ramsey and Ramsey, 2014; Levin, 2019; Baniaga et al., 2020). Polyploids that spread into new environments may then undergo natural selective pressures and become locally adapted to their new habitat. This may allow for allopatric isolation of polyploids from their diploid progenitors and create a reproductive barrier to gene flow at range boundaries (Buggs and Pannell, 2007) and may also be a means for sympatric speciation (Stahlberg, 2007; Ramsey and Ramsey, 2014; Baack and Stanton, 2005; Soltis et al., 2007; Maherali et al., 2009). Cytotypes of *Betula papyrifera* Marsh. are associated with certain geographical locations resulting from their morphological and adaptive differentiation (Li et al., 1996). Diploids are found in moist, wet sites usually at the tops of mountains and have even been classified as a different species, heart-leafed paper birch (*Betula cordifolia* Regel), while tetraploids and pentaploids are found at lower elevations and latitude, in warmer and drier environments (Li et al., 1996).

Tall goldenrod (*Solidago altissima* L.) is a North American old-field herbaceous perennial found across most of the United States and Canada (Semple et al., 1984; Zlonis and Etterson, 2019). It can reproduce through seeding and by extensive rhizome production. This species has populations with three different ploidy levels (i.e., cytotypes; diploid, tetraploid, and

hexaploid), with each having different geographical distributions throughout the Midwest and Eastern regions of the United States (Halverson et al., 2008). Diploids and tetraploids are found almost exclusively in the Midwest, whereas hexaploids range from the Midwest to the East (Richardson and Hanks, 2011). Although populations may contain mixtures of cytotypes (Halverson et al., 2008; Richardson and Hanks, 2011; Etterson et al. 2016), diploids are restricted to open old-field environments (Etterson et al., 2016), and may be more drought tolerant (Zlonis and Etterson, 2019), while hexaploids are abundant throughout, but particularly at the edges of old fields near shadier, forest sites (Richardson and Hanks, 2011, Etterson et al., 2016).

Since diploids are found only in the Midwest while hexaploids are found throughout the species range, this suggests they may have different environmental tolerances (Etterson et al., 2016). At a continental scale, climate in the Midwest differs from that in the East with the former having greater temperature extremes (both high and low) and lower annual precipitation amounts (PRISM climate data 1981-2010). Furthermore, the presence of hexaploids in both regions suggests they may have broader environmental tolerances than diploids due to phenotypic plasticity, even though in the Midwest the hexaploids are more abundant near shadier, forested micro-sites, which implies reduced environmental tolerance, at least with respect to light and temperature. Given the large climatic differences between the Midwest and East, there is the possibility that hexaploids have undergone considerable selection and could differ substantially.

Because of the wider geographical distribution of hexaploids, I hypothesized that they might tolerate more variable environments than diploids and therefore undertook an investigation comparing the ecophysiology of diploids and hexaploids. I conducted morphological, phenological, and physiological measurements on each cytotype to determine if potential

changes resulting from polyploidization and/or natural selection might reflect adaptation to their current local environments. I also conducted a short-term drought experiment to ascertain their ability to tolerate physiological stress and to evaluate differences in their ecophysiology that might provide insights into their different habitat preferences.

## METHODS

***Study Site —***

*Solidago altissima* rhizomes were obtained from old-field locations across the Midwestern US and locally in Watauga County, North Carolina. The midwestern diploid (MWD) came from the Conrad Environmental Research Area (Iowa: N 41.68125, W 92.86002, 259 m), the midwestern hexaploid (MWH) from Johnson Sauk State Park (Illinois: N 41.32944, W 89.88278, 212 m), and the eastern hexaploid (EH) from Watauga County just west of Boone (N 36.23747, W 081.74692, 887 m). In September 2016 rhizomes from the Midwest collections were planted in 7.5 L pots containing Metro Mix general purpose soil at the Appalachian State University (ASU) greenhouse and overwintered. Plants from Watauga County were collected in spring of 2016 and propagated in the same manner. In May 2017, Midwestern and Watauga County plants were transferred to a common garden located at the ASU Robert Gilley field station, a natural area of 101 ha in Ashe County, NC. In August of 2018, 10 - 12 plants from each of the three cytotypes were moved from the Gilley property to the courtyard at the ASU greenhouse and planted into PVC pots (20 cm in diameter x 40.8 cm in height; V = 4.72 L) with Fafard #3B Mix (Sungro Horticulture, Agawam, Massachusetts, USA) potting soil. Plants were overwintered and used for experiments in the 2019 growth season.

In May 2019, sprouts were transplanted into two sets of pots with Fafard #3B Mix potting soil and Osmocote fertilizer (14, 14, 14; amounts of fertilizer varied with pot and are described below). One set was placed into large tubs (51 cm in diameter x 43 cm in height; V = ~75 L)

located inside the greenhouse for a drought experiment (see below), while the rest were placed into PVC pots (4.72 L) in the courtyard for morphological, phenological and hydraulic conductivity measurements. Courtyard pots had ~25 – 50 mL of Osmocote fertilizer, with half having 25 mL and half having 50 mL. The lower amount was measured and utilized based on the amount of soil present in each pot.

***Phenological Methodologies —***

I made phenological measurements on the plants in the courtyard during the 2019 & 2020 growing seasons. In 2019, these consisted of the dates for flower bud set, and onset of flowering. In 2020, I noted the sprouting date of plants for each cytotype. Measurements were not taken again until July 2020 due to restricted greenhouse access because of COVID-19. When plants began to flower in July 2020, observations were made on a weekly basis once more.

***Leaf Morphology of Courtyard Cytotypes —***

*Xylem Anatomy —*

Stem xylem anatomy measurements were made by Erica Pauer, an undergraduate student researching hydraulics of *S. altissima* for her senior capstone experience. In September of 2019, stem cuttings were taken from five plants within each cytotype grown outdoors in the courtyard. Cuttings were sampled 1 m from the base of each plant (which were on average ~ 1.6 m tall) and preserved in a solution of 2.5% glutaraldehyde and 0.1 M saline phosphate buffer. Samples were rinsed with buffer before placing on a Leica vibratome (Leica Microsystems Inc., Buffalo Grove, Illinois, USA). Sections were then cut and placed on slides for analysis. Fifty vessels from each cross section were measured in order of appearance, starting at the pith and moving outward toward the epidermis, using an Ix81 Light Microscope with an Olympus DP80 camera (Olympus Cor., Tokyo, Japan) that was paired with CellSens software (Olympus Cor., Tokyo, Japan).

Due to the mostly elliptical shape of each xylem vessel, major and minor axes were measured and the cross-sectional area ($\mu m^2$) of each vessel calculated using the equation for an ellipse:

$$A = \frac{\pi ab}{4}$$

where a and b are the major and minor axes. The frequency distribution of vessel sizes was also determined. Finally, percent vessel area within a randomly chosen 200 $\mu m^2$ within the area being analyzed was also calculated.

A theoretical hydraulic flow ($J_h$; $m^3\ s^{-1}$) that takes into consideration cells with elliptical lumens was calculated using the equation from Lewis and Boose (1995):

$$Jv = -\left(\frac{\pi}{64\mu}\right) * \frac{a^3 * b^3}{a^2 + b^2} * \frac{\Delta p}{\Delta x}$$

where $J_v$ is the volume flow rate; $\mu$ is the viscosity; $a$ and $b$ are the short and long axes of the lumen respectively; and $\Delta p/\Delta x$ is the pressure gradient. From a theoretical standpoint, the pressure gradient is understood as constant since the pressure decreases downstream naturally. Here, we used flow rate as a surrogate for actual conductivity. $J_v$ was calculated for each of the 50 vessels whose dimensions were measured and then summed to give the total $J_h$ for that cross section. These values allowed for a comparison of $J_h$ across the three cytotypes.

*Stomatal Density and Size —*

To compare stomatal densities and sizes among the cytotypes, I took leaf samples from the courtyard plants in late September 2019 and placed nontoxic dental sealant (low viscosity polysiloxane) on both the abaxial and adaxial sides of single leaves from five individuals in each cytotype. After drying, the sealant was peeled off, and clear fingernail polish was placed over the sealant molds to make positive impressions for microscopic analysis. The fingernail polish

impressions were mounted on slides, and I recorded the density and size of stomata on each leaf surface (adaxial and abaxial) using an Ix81 Light Microscope with an Olympus DP80 camera. Using CellSens software, I counted stomatal density within the field of view area (FOV). The area was then measured by tracing a square around the FOV using that same software. A low magnification (100x) was used to assess stomatal density for the adaxial sides since densities on this surface were so low. Higher magnification (200x) was used for the abaxial sides due to the higher densities on this surface. I measured the size of guard cells for three stomata on each surface using measuring lines in the software mentioned above. A line was drawn from the long axis and across the widest middle axis on each guard cell for the selected stoma.

*Leaf Dimensions & Angle of Display —*

In June 2020, I measured leaf area on 10 fully mature leaves from 10 plants of each cytotype growing in the courtyard. Leaves were clipped at ~21 cm from the top of each plant and placed into small envelopes for transport back to the lab. Each leaf was then scanned using a Canon 9600F (Canon Inc., Tokyo, Japan) to create a digital image which was converted to a black and white image using Blackspot (Varma and Osuri, 2013), a shareware imaging program. Pixels were then accumulated to determine leaf area.

The angle of leaf display was measured by Erica Pauer in July 2019. Five leaves from six plants within each cytotype were randomly chosen at 1 m height on each plant. The angle of display was recorded as degrees from vertical using an inclinometer app on a cell phone. A horizontal leaf was recorded as having an angle of 90$^{o}$ from vertical. A leaf whose abaxial surface was less than 90$^{o}$ from vertical was inclined downward and drooping toward the ground while a leaf with an angle greater than 90$^{o}$ was inclined above the horizontal.

*Light Response Curves —*

Light response curves were made in July 2019 by Erica Pauer and Sarah McCoy on four, courtyard-grown plants from each cytotype using an automated program on the LI-6800 gas exchange system (Li-Cor Inc., Lincoln, NE). Light curves were made from 9 am - 12 pm to minimize diurnal effects.

The light levels and the order in which they were used were: 1000, 2000, 1500, 1250, 1000, 750, 500, 300, 150, 50, and 0 μmol $m^{-2}$ $s^{-1}$. Leaves were first acclimated in the chamber to 1000 μmol $m^{-2}$ $s^{-1}$ before starting the automated program in the Li-6800 that produces step-changes in light once rates of photosynthesis stabilize. Temperature was set to reflect the ambient temperature of that day and ranged from 25 - 29 ° C. $CO_2$ within the chamber was set at 400 ppm and relative humidity was kept at 50%.

A three-parameter exponential rise to maximum equation was fit to each light response curve using SigmaPlot Ver. 14.0 (Systat Software Inc., San Jose, California, USA):

$$[y = y_0 + (1 - e^{-bx})]$$

where $y$ is the rate of net photosynthesis ($A_{net}$), $y_0$ is the respiration rate at 0 PAR, $a$ and $b$ describe the curvature and dependence of photosynthesis on PAR, and $x$ is the level of PAR. From this, I extracted the dark respiration rate (at 0 PAR), light compensation point (where $A_{net}$ = 0), apparent quantum efficiency (slope derived from the linear regression of the first three points), $A_{max}$ (average of four highest rates of $A_{net}$), and saturation light intensity (where $A_{net}$ = 97% of $A_{max}$).

***Drought Experiment —***

For the drought experiment, three sprouts, one from each cytotype, were placed into one of 20 tubs (75 L capacity) for a total of 20 plants per cytotype. I drilled five holes in the bottom of each tub and placed mesh screening over them to allow for adequate drainage. A ~5 cm layer of coarse gravel was then placed on top of each screen before filling the tubs with the potting soil. Each tub had ~ 50 mL of Osmocote fertilizer and ~ 90 g of Marathon insecticide mixed into the top ~16 cm of soil to ensure sufficient nutrients for growth and to protect against insect damage from aphids. The plant physiological effects from using this insecticide are unknown, but not expected to be large. Translucent plastic sheeting was hung near the greenhouse ceiling above the pots to protect them from water leaks in the roof and this shaded the plants an additional ~27% on average. Greenhouse temperatures were kept between 20 - 35˚ C and relative humidity was not controlled.

The large tubs chosen for this experiment allowed drought to proceed progressively over time but not too rapidly. This also helped to alleviate problems associated with roots contacting the container walls, which can affect responses of plants to drought when grown in small containers (Poorter et. al, 2012). Plants were placed in the tubs ~5 weeks before the start of the experiment, which commenced in mid-June, to allow time for the plants to acclimate to the greenhouse conditions.

Tubs were placed on two raised, perforated tables to allow for unrestricted water drainage. Five watered and five droughted tubs were placed alternately on each of two tables, for a total of 20 tubs. Control tubs were watered regularly at 3 - 4 day intervals, while water was withheld from those subjected to drought, beginning on June 24, 2019, the day the experiment started.

Watering times were determined by the decrease in volumetric water content (VWC) as measured at 20 cm depth using a Hydrosense II Soil Moisture Probe (CS658, Campbell Scientific, Inc., Logan, Utah, USA). The probe was placed into the soil in the center of each pot and the volumetric water content noted. Well-watered tubs ranged from 33% to 25% water content over the course of the experiment, while the droughted tubs dropped below the detection limits of the Hydrosense meter by the end of the experiment. The experiment lasted 24 days total, with plants experiencing drought for the first 14 days, after which they were watered and allowed to recover for 10 days.

*Gas Exchange —*

Gas exchange measurements were made using an Li-6800 portable gas exchange system (Li-Cor Inc., Lincoln, NE) and the 6 $cm^2$ cuvette equipped with the LED light source. I recorded net photosynthesis ($A$), transpiration ($E$), and stomatal conductance ($g_s$) every 3-4 days between the hours of 9 am and 1 pm. Most days were predominantly sunny, and environmental conditions on measurement days were relatively consistent throughout the experiment. All well-watered pots were watered the day before taking measurements. Leaves used for gas exchange were marked with a twist-tie to ensure that no leaf was used twice during the experiment, as prolonged exposure in the cuvette can damage leaves or result in leaf fatigue (Marler and Mickelbart, 1992).

I used fully mature leaves located in the upper 50 cm of the stem for all gas exchange measurements. New leaves for gas exchange were always chosen above the previously used leaf positions. This means that as the experiment progressed, relative leaf position moved up the plant as it grew but remained in the upper 50 cm of the stem. Therefore, each new leaf chosen was younger than the one previously used but had aged over the course of the experiment so that the

actual variation in leaf ages was minimized. However, leaves were older by the end of the experiment than at the beginning because of low rates of new leaf production.

I set conditions in the LI-6800 cuvette to near ambient conditions: (photosynthetically active radiation (PAR) at 700 μmol $m^{-2}$ $s^{-1}$ which was near or above the light saturation point for most of the cytotypes (MWH tended to saturate at higher PAR, but was within 10% of saturation at this PAR), $CO_2$ at 410 ppm, relative humidity at 65%, and temperature within 5˚C of ambient in the greenhouse (25 – 34 ˚C). Leaves were acclimated for 30-90 seconds to achieve stable readings before recording gas exchange measurements. For each plant's measurements, three data points were made every five seconds and the average was recorded.

*Water Potential —*

I measured water potentials ($\psi_w$) on individual leaves with a Scholander Pressure Chamber (PMS Instruments, Albany, Oregon, USA) equipped with the grass flange. Both pre-dawn (~6 am) and mid-day (~2 pm) $\psi_w$ measurements were made. I sampled five random individuals of each cytotype in each treatment at four specific times over the course of the experiment. This limited sampling was necessary to conserve leaf material during the experiment. I took readings at (a) the start of the experiment (day 1), (b) when $g_s$ rates decreased on average by $\geq$ 50% (day 10), (c) on the last day of drought (day 14), and finally, (d) three days after droughted plants were re-watered (day 18).

*Leaf Temperature —*

As the drought experiment progressed (June 24, 2019 – July 18, 2019), I measured leaf temperature on unshaded, individual leaves located at the mid-height of each plant in both the well-watered and droughted treatments using an OS532E IR Thermometer (OMEGASCOPE; Omega Engineering, Norwalk, Connecticut, USA).

*Biomass Assessments —*

I measured the above-ground biomass of plants in the greenhouse drought experiment and those growing outside in the courtyard in late August – early September 2019. Plants were cut once they began to set seed to prevent hybridization among the cytotypes. I separated the plant material into stems, leaves, flowers, and late season sprouts. Leaves were removed from the main stem until leaf sizes began to taper off at the top of each plant. At this point, the top was cut off and added to the flowers bag. I did this because *S. altissima* produces flowers near the top of each plant and on shoots that grow off the main stem. If these offshoots had flowers on them, they were considered reproductive material and the entire structure (flowers, leaves, small stems) included in the flower category. Plant material was dried to constant weight in a drying cabinet at 72 – 76˚C for a minimum of ~ 48 hrs. I calculated the ratio of each category to total biomass. Roots were not harvested because the plants were needed for the next field season.

*Stem Growth —*

Stem height and diameter were measured on greenhouse grown plants using a digital caliper at internodes just below leaves selected for gas exchange. Stem height was measured from the pot rim to the highest point of the main stem using meter sticks. These measurements were taken in June 2019 before the start of the experiment and on July 18, 2019, at the conclusion of the experiment.

*Specific Leaf Mass and Pigments —*

Chlorophyll content was measured after completion of the drought experiment for all cytotypes using leaf samples from well-watered plants only. In July 2019, three leaf punches per plant (0.84 $cm^2$ total leaf area) were removed and placed in 3 mL of DMF (*N, N*-Dimethylformamide), and placed in the dark in a refrigerator at 5˚C for a minimum of 24 hours.

Three more leaf punches from the same leaves were also collected to calculate weight to area ratios. Absorbances for leaf extracts were measured using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan) and chlorophyll and carotenoid concentrations calculated using equations in Porra (2002).

***Statistical Analyses —***

Statistical analyses and figures were completed using Sigmaplot Ver. 14.0, SAS Ver. 9.2 (SAS Inc., Cary, North Carolina, USA), Minitab 19 (Minitab LLC, State College, Pennsylvania, USA) and Microsoft Excel 2016 (Microsoft Corporation, Redmond, Washington, USA). One-way ANOVAs were used to analyze all morphological measurements, coupled with Tukey post-hoc tests. If data did not meet the assumptions for parametric analyses, I used a Kruskal-Wallis test instead. For the drought experiment, I used a repeated measure analysis in a split-plot design with cytotype being the split-plot. For all analyses, significance was assumed if $p \leq 0.05$.

## RESULTS

***Phenology of Courtyard Plants —***

In 2019, all cytotypes of *S. altissima* showed initial growth in the beginning of April with midwestern diploids (MWD) sprouting first, followed by midwestern hexaploids (MWH), and then eastern hexaploids (EH). Budding and flowering phenology followed the same pattern, with MWD plants budding first on Julian day 212. At this time, both MWH and EH plants only had mature leaves. MWD exhibited their first open flowers on day 219 and continued to flower until 36 days later when the last flowers began to brown (day 254; Fig. 1). On day 246, MWH had open flowers, slightly overlapping in flowering time with MWD, and continued flowering until day 268. At this point, MWH were harvested for biomass measurements and to avoid hybridization of the cytotypes. EH began flowering 8 days later than MWH on day 254 and these two cytotypes had considerable overlap in their flowering times. However, EH still had a higher percentage of open flowers towards the end of the season after MWD were almost fully done flowering (Fig. 2). EH flowers were browning by day 273, 19 days after the first onset of flowering and the population was also harvested on this date for biomass measurements. In 2020, data collection was not possible due to COVID.

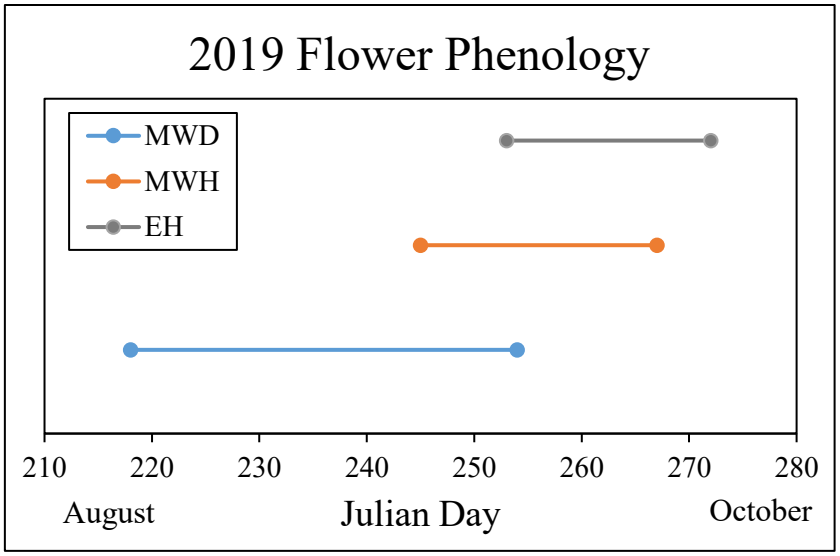


*Figure 1*: Comparison of 2019 flowering phenology for each cytotype.

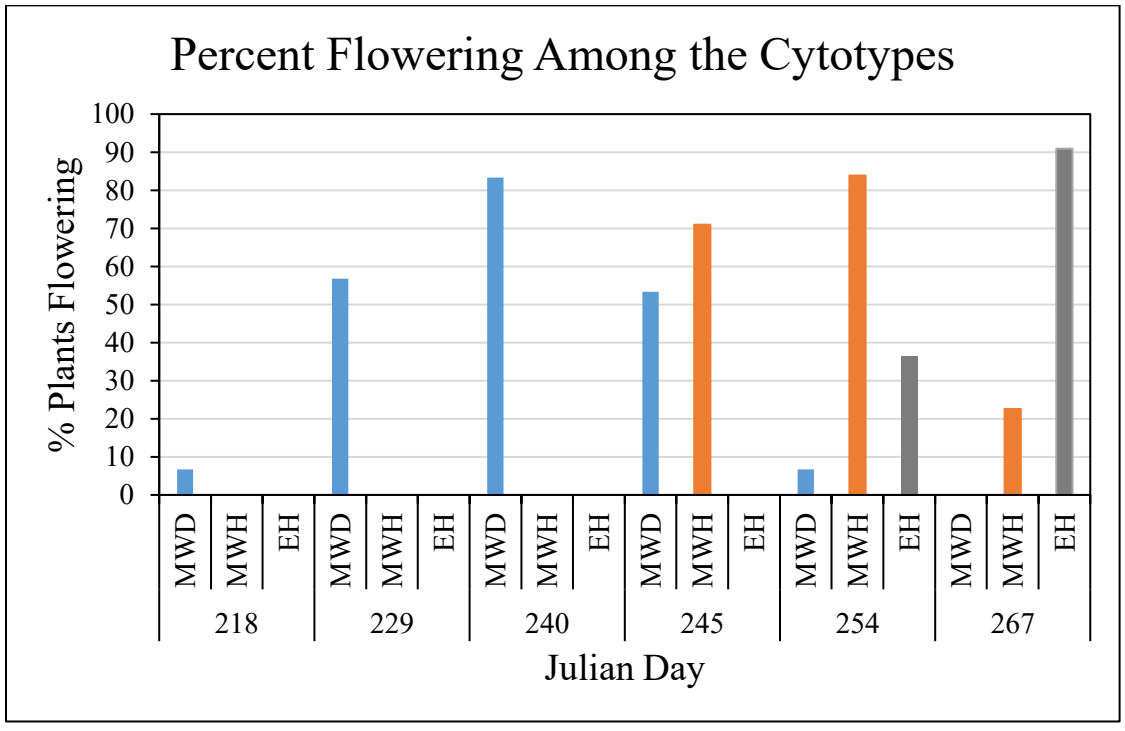


*Figure 2*: Percent of plants flowering among the cytotypes for each phenological measurement day. Julian day 218 is August 6th and day 267 is September 24th.

***Leaf Morphology, Anatomy, & Biomass —***

*Leaf Dimensions & Angle of Display —*

Leaf size differed significantly among all three cytotypes ($p < 0.001$; Fig. 3). EH had the largest leaves, which were 23% larger than those for MWH (15.0 $\pm$ 1.03 $cm^2$ vs 12.2 $\pm$ 0.57 $cm^2$. MWD had the smallest leaves, averaging 8.8 $\pm$ 0.58 $cm^2$. MWH had the highest leaf specific mass, indicating either thicker and/or denser leaves, while EH and MWD had lower values and did not differ ($p < 0.001$).

After log-normalizing the data and running a parametric analysis, leaf angles among the cytotypes were determined to be statistically different from one another ($p < 0.001$; Fig. 3). Leaf orientation, the angle between the abaxial leaf surface and the stem, was lowest for EH plants and characteristic of droopy leaves. MWD and MWH, on the other hand, had leaves oriented either horizontally or slightly above horizontal.

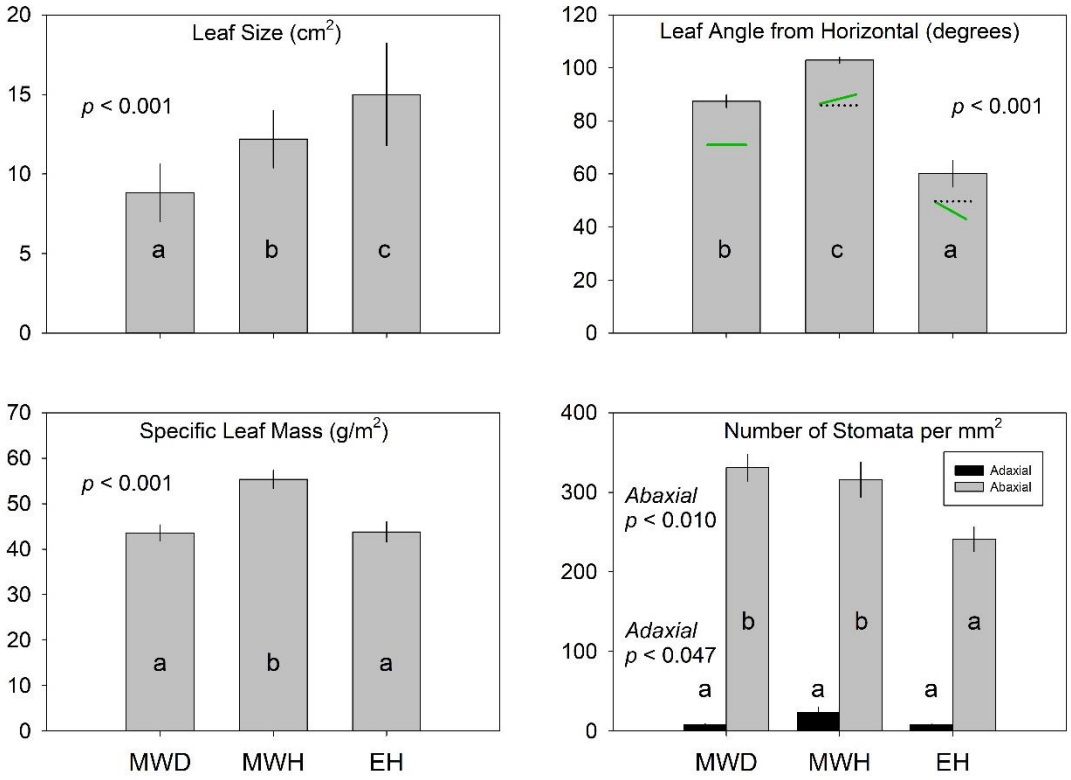


*Figure 3*: Leaf dimensions and angle of display measurements for each cytotype. Values shown are mean $\pm$ se; n=6 (leaf angles), n=5 (# of stomata), n=10 (leaf size and mass). Green lines in the leaf angle graph diagrammatically show leaf orientation.

*Stomatal Density and Size —*

*Solidago altissima* plants are mostly hypostomatous, with more than 92% of stomata on the abaxial leaf surface. Both MWH and MWD plants featured higher densities of stomata on their abaxial leaf surfaces than EH ($p = 0.001$), whereas there were no differences among the cytotypes for adaxial stomatal density (Fig. 4).

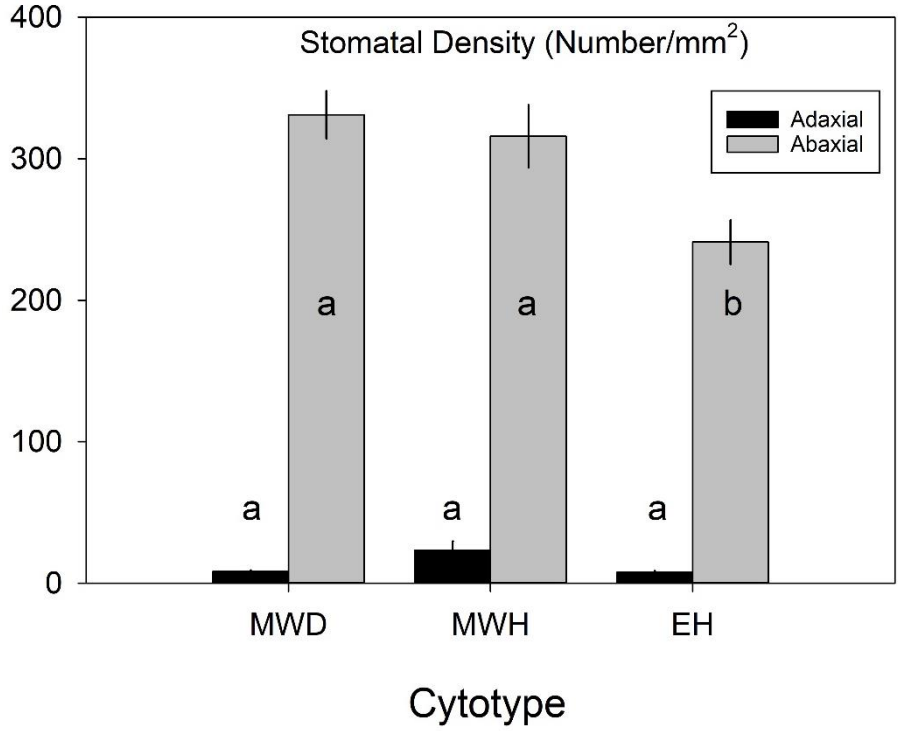


*Figure 4*: Stomatal density for adaxial and abaxial surfaces for each cytotype. Values are means + se; n = 5. Means within a surface not followed by the same letter differ statistically at $p \leq 0.05$.

EH had the largest stomatal lengths, followed by MWH, and then MWD with the smallest ($p < 0.001$; Fig. 5). Except for MWH, abaxial stomata were longer than adaxial stomata ($p < 0.001$).

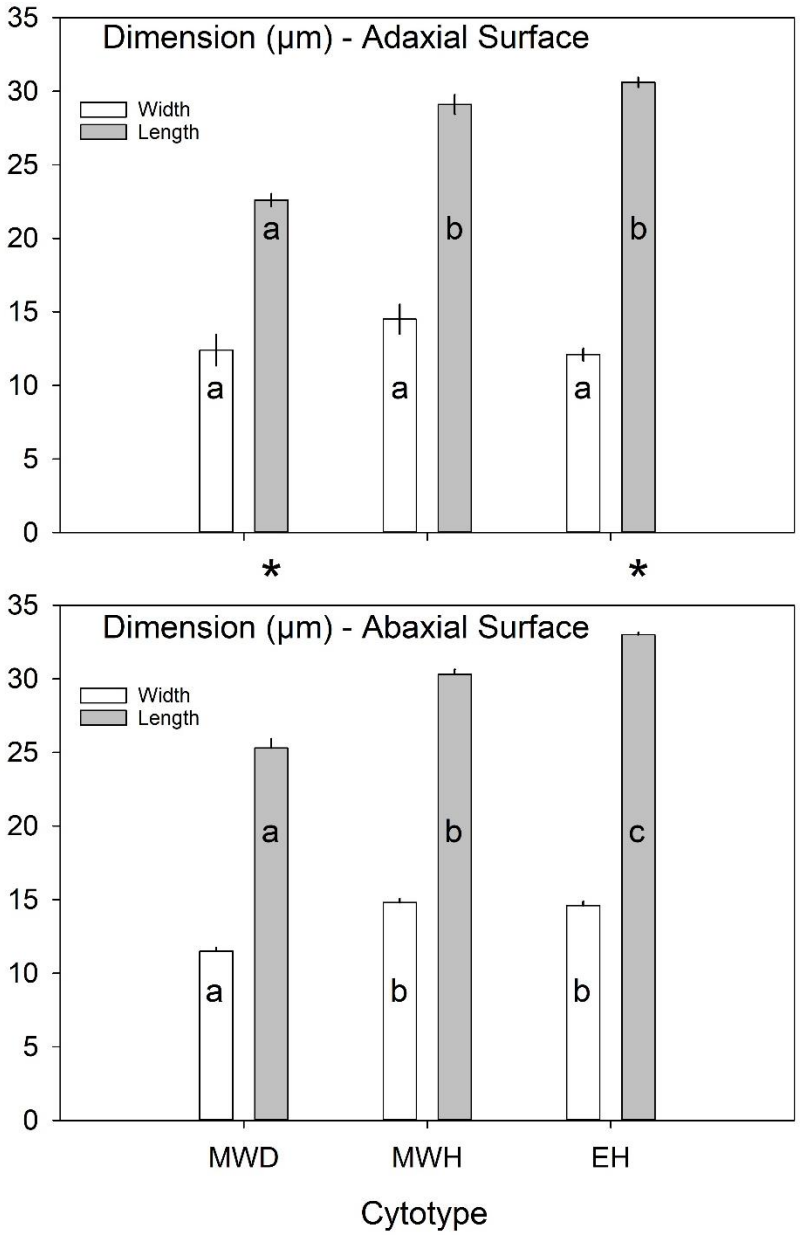


*Figure 5*: Stomatal lengths and widths on the adaxial and abaxial surfaces for each cytotype. Values are means + se; n = 5. Means within a surface not followed by the same letter differ statistically at $p \leq 0.05$. Asterisks indicate differences between adaxial and abaxial surfaces.

Stomatal widths showed a significant cytotype x surface effect ($p = 0.043$). There were no differences on the adaxial surface, but the two hexaploids, MWH and EH had wider stomata than did MWD on the abaxial surface (Fig. 5).

*Xylem Anatomy —*

Several aspects of xylem anatomy (Fig. 7) were measured and compared among the cytotypes (Fig. 6). These included: (a) cross-sectional vessel area ($\mu m^2$), (b) largest and smallest vessel areas ($\mu m^2$), (c) number of circular and elliptical vessels, (d) theoretical hydraulic flow ($J_h$; $m^3\ s^{-1}$), and (e) average percent coverage by vessel lumens in a specified cross-sectional area of stem ($\mu m^2$ of vessel area*100/$\mu m^2$ of stem vascular bundle area, which ranged from 40108 $\mu m^2$ to 40840 $\mu m^2$). No significant differences ($p > 0.05$) among the cytotypes were found for any of the measured parameters except for largest vessel size, which were largest for EH ($p = 0.007$) compared to either MWH or MWD, which did not differ. One EH plant had a maximum vessel area almost twice as large as those of any EH plants, but a Grubbs test showed that this value was not an outlier. Analysis with or without this vessel did not change the statistical relationships found. Most vessels were elliptical, indicating that the major axis was at least 10% longer than the minor axis.

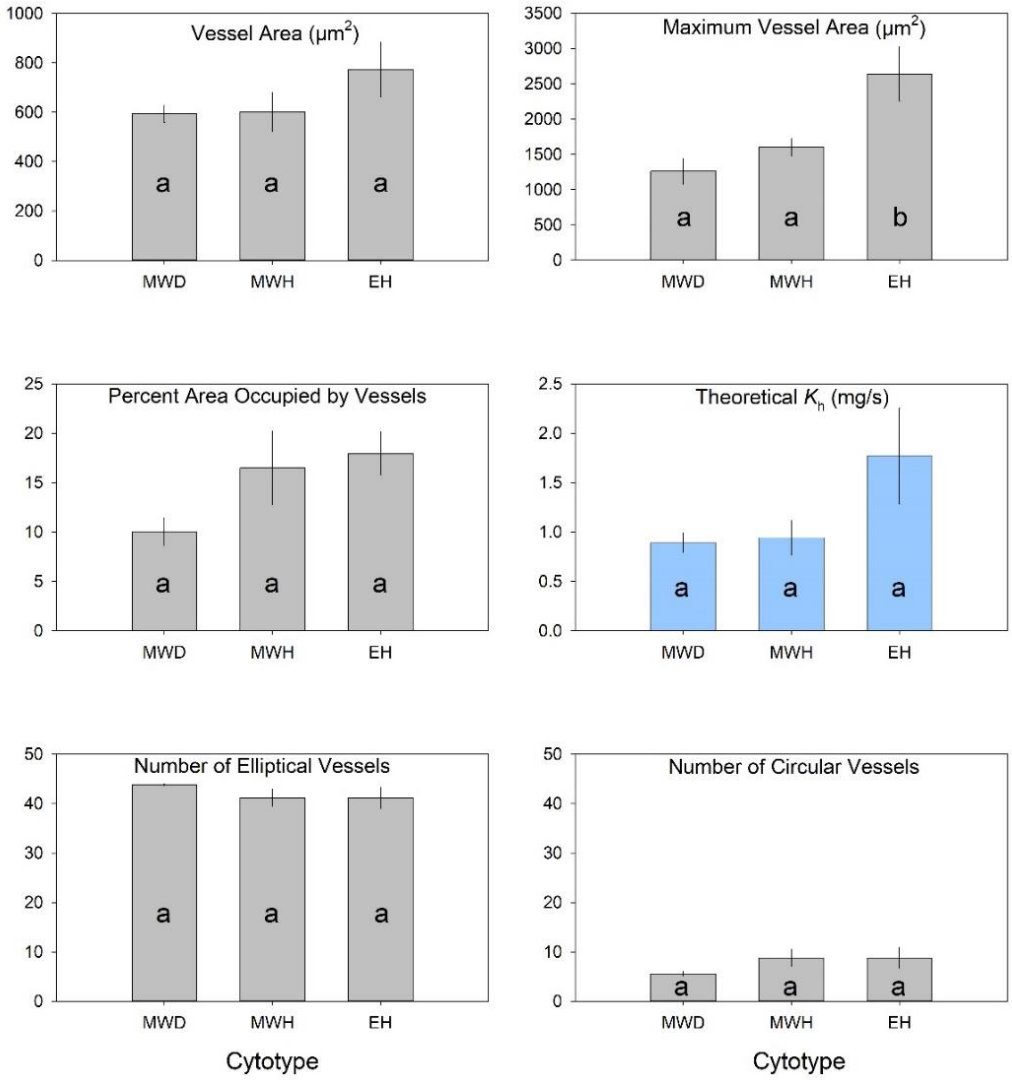


*Figure 6*: Leaf xylem anatomy measurements compared for each cytotype. Values are means ± se; n=5. Vessel area is the cross-sectional area of the lumen; Maximum vessel area is the cross-sectional area of the lumen of the single largest vessel found for each plant; Percent area occupied is the percent of a defined stem area (ranging from 40,108 µm$^2$ to 40,840 um$^2$) occupied by xylem vessel lumens; theoretical $K_h$ is the flow rate of the xylem vessel population in each defined area using equations in Lewis and Boose (1995); Elliptical vessels are those with the major diameter ≥ 10% longer than the minor diameter and circular vessels are those with similar major and minor diameters.

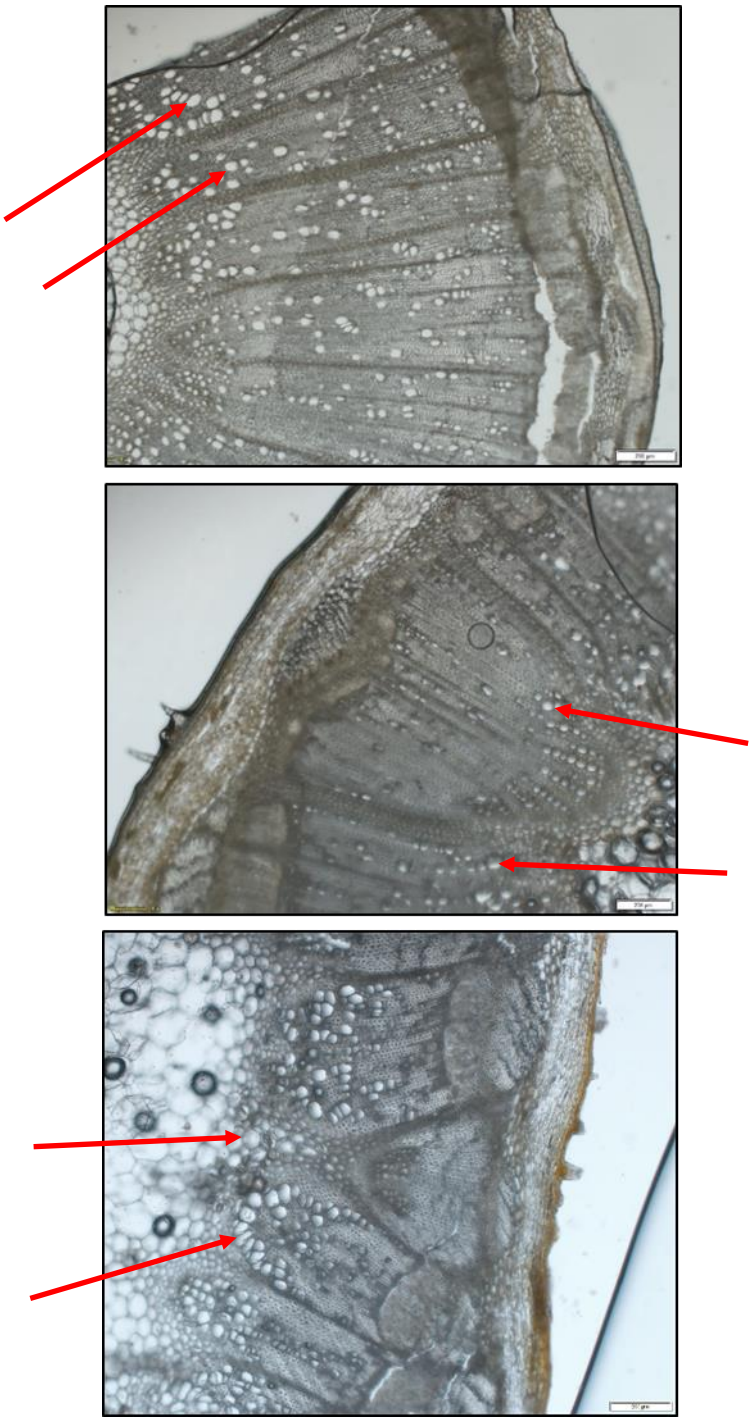

*Figure 7*: Stem cross-section at 400x magnification. (a) MWD, (b) MWH, (c) EH. Red arrows point towards xylem cells. Scale bar in bottom right of image shows 200 µm.

*Aboveground Biomass (Courtyard Plants) —*

Aboveground biomass of courtyard plants showed significant differences among the cytotypes ($p < 0.001$; Fig. 8). EH had the largest biomass which differed from both MWD and MWH, but these latter two cytotypes were not statistically different.

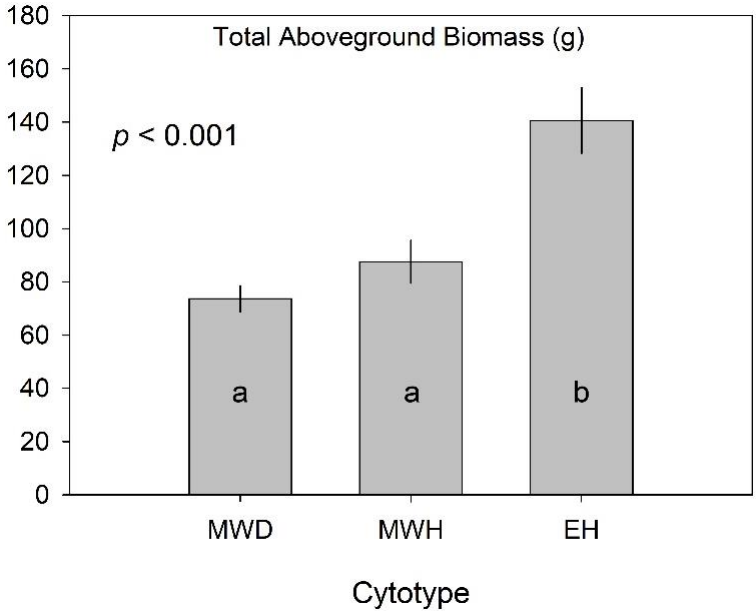


*Figure 8*: Total aboveground biomass of courtyard-grown plants for each cytotype. Values are means ± se; n=13 for EH, n=15 for MWD and MWH. Means with different letters are significantly different ($p < 0.001$: Tukey test).

*Light Response Curves —*

Light response curves (Fig. 9) were constructed for all the cytotypes. MWH had the highest rates of photosynthesis ($A_{max}$) at saturating PAR ($p < 0.001$), followed by MWD and EH which did not differ (Fig. 10). This represents a 4-fold difference in $A_{max}$ between the two hexaploids and it is nearly twice as high as the difference between MWH and MWD. In addition to the highest $A_{max}$, MWH also had the highest Light Saturation Point ($p < 0.001$). Dark Respiration Rate, Apparent Quantum Efficiency, and Light Compensation Point did not differ

among the cytotypes (Fig. 10). Stomatal conductance at $A_{max}$ was also highest in MWH ($p$ = 0.003), followed by MWD and EH, which did not differ.

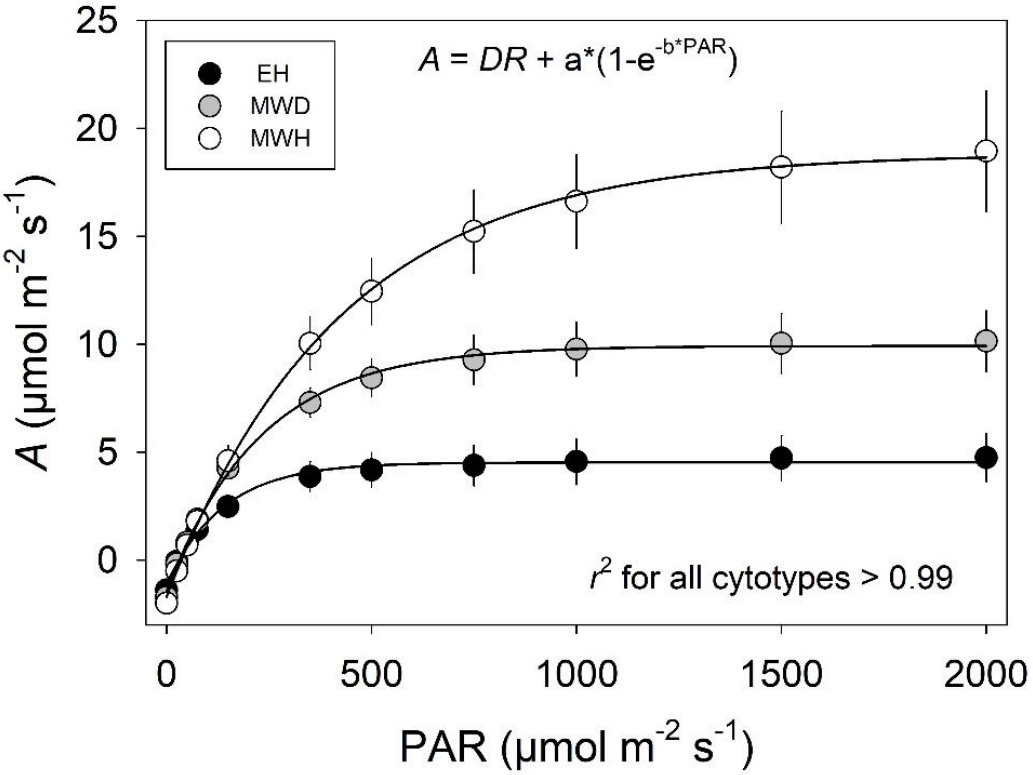


*Figure 9*: Fitted light response curves for each cytotype. Measurements made in July 2019 on well-watered plants. Symbols are means ± se; n=5 (MWD and EH), n=4 (MWH). A 3-parameter, exponential rise to maximum equation was used to fit the curves and is shown in the graph panel.

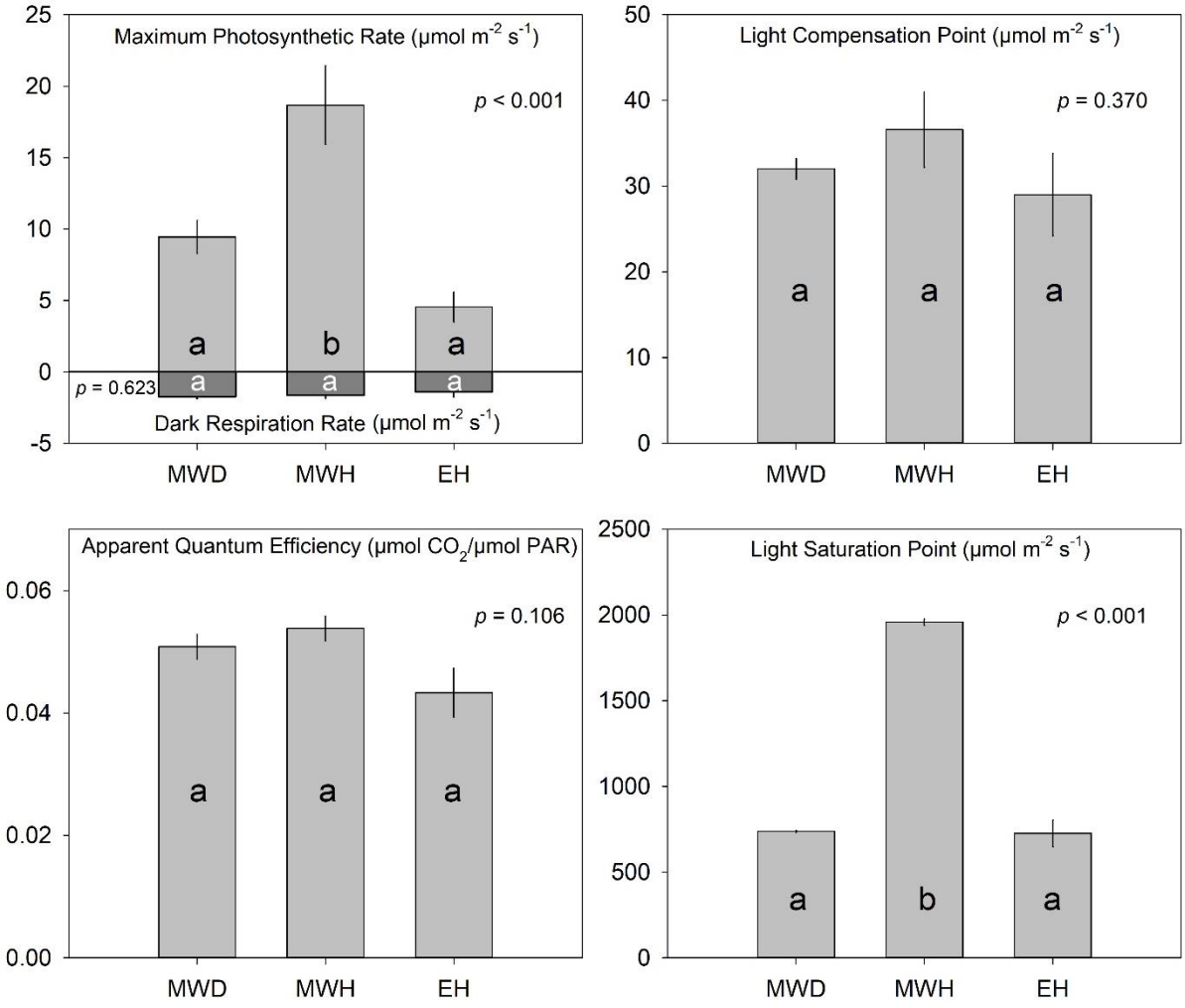


*Figure 10*: Photosynthesis rates at saturating PAR ($A_{max}$), dark respiration, apparent quantum efficiency, light saturation point, and light compensation point for each cytotype. Values are means ± se; n=5. Means with different letters are significantly different at $p \leq 0.05$. See text for details of cuvette environment.

***Drought Experiment —***

*Volumetric Soil Moisture Content —*

At the beginning of the experiment, all pots had volumetric soil water contents (VWC) that ranged between 25% – 33% (Fig. 11). On day 1 of the experiment (Julian day 175), watering was stopped in the drought treatment, and from that point on the pots dried rapidly until day 10 (Julian day 185), by which time the soil VWC had dropped below 9%. After this date, the rate of drying was much lower, due to both reduced water uptake by the plants and because of the very low water content of the soil.

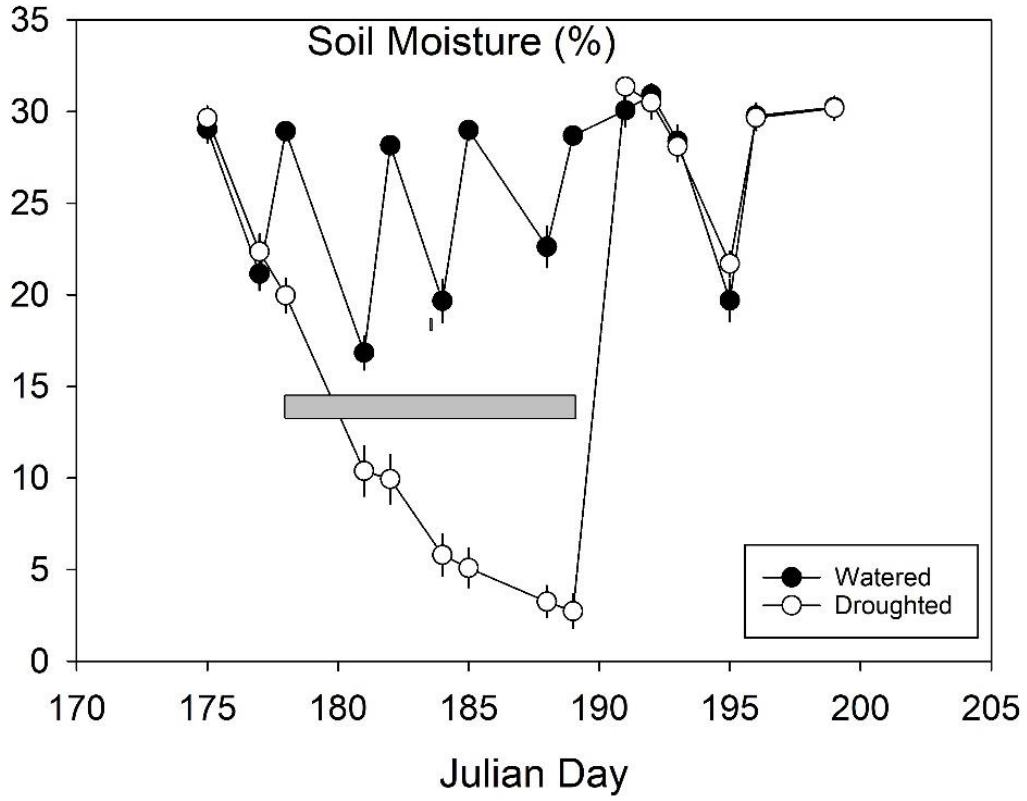


*Figure 11*: Volumetric soil moisture content for both watered and droughted treatments. Water was withheld starting on Julian Day 175 and droughted plants were rewatered late afternoon on Julian Day 189. Values are means $\pm$ se; n=10. Gray bar indicates days when soil moisture differed statistically between treatments at $p \leq 0.05$.

*Effects of Drought on Photosynthesis —*

At the start of the experiment on day 175, and before water was withheld from the plants assigned to the drought treatment, there were no differences in photosynthesis ($A$) or stomatal conductance ($g_s$) between plants assigned to the two watering treatments (Fig. 12; $p = 0.192$). However, there were cytotype differences ($p < 0.001$) as illustrated in the red boxes in Figure 12. Across treatments, MWH and MWD exhibited higher rates of $A$ than EH, which had the lowest rates. This pattern of differences held up to day 182, at which time increasing drought stress eliminated any cytotype differences ($p > 0.05$). Only after rewatering, on day 199, did cytotype differences re-appear. On this date, MWH still had the highest rates, although they were reduced by 57% compared to the starting rates, with EH intermediate and not different from either Midwestern cytotype. MWD moved down in the rankings with the lowest rates.

During the experiment, $A$ decreased with time within both the watered and drought treatments, suggesting a leaf aging effect. Since different leaves were measured on each date, with each leaf located adjacent to previously measured ones, this pattern of decreasing $A$, particularly in the non-stressed well-watered plants, does not represent an accumulation of mechanical stress effects, or cuvette "fatigue" (Marler and Mickelbart, 1992), but rather, probably represents leaf ageing with time.

Significant treatment differences in Midwestern cytotypes were not found until day 185, at which time rates were lower in the droughted plants ($p = 0.006$ and 0.008 for MWD and MWH, respectively). Four days later, the pattern was similar, with all three cytotypes showing lower photosynthetic rates in the drought treatment compared to the well-watered treatment ($p =$ 0.023, 0.005, and 0.0004, for EH, MWD, and MWH, respectively). With additional water stress and the possible leaf ageing effect, treatment differences disappeared by day 191 for the EH and

MWD plants but remained for MWH ($p = 0.009$). On day 196, rates for MWD plants in the watered treatment had fallen significantly lower than those for EH and MWH, which did not differ from each other ($p = 0.023$, 0.014, and 0.846, respectively), while there were no cytotype differences among the droughted plants ($p > 0.464$ for all comparisons). For the last measurement, on day 199 after rewatering, there were no cytotype differences within the droughted treatment, but in the watered treatment, MWH had significantly higher rates than MWD, but not EH, the latter two of which did not differ ($p = 0.004$, 0.189, and 0.094, respectively).

*Effects of Drought on Stomatal Conductance —*

As with photosynthesis, there were no treatment differences for $g_s$ at the start of the experiment ($p = 0.336$), with values averaging 0.441 mol $m^{-2}$ $s^{-1}$, although there were significant cytotype differences ($p < 0.001$; Fig. 12). MWH had much higher $g_s$ than either MWD or EH, which did not differ from each other. However, as the experiment progressed $g_s$ progressively declined in both watered and droughted plants, but the pattern of decline was cytotype specific. Well-watered MWH, which started off with the highest $g_s$ declined by 63% by the end of the experiment, whereas droughted MWH reached nearly this same level of decline (56%) much sooner, by day 182. Then, just three days later, $g_s$ in MWH dropped to 21% of its starting value. At the end of the experiment, even after rewatering, MWH $g_s$ remained low.

These temporal patterns were similar for the other two cytotypes, MWD and EH, especially among the well-watered plants, where values rarely differed statistically until the end, when $g_s$ for MWD dropped to the lowest of all three cytotypes. For droughted MWD plants, the decline was less severe up to day 185, and $g_s$ was similar between well-watered and droughted plants ($p > 0.05$). However, after this date, MWD stomata closed and $g_s$ remained below 0.100

mol $m^{-2}$ $s^{-1}$ for the remainder of the experiment, even after rewatering. In contrast, stomata on droughted EH plants began to close three days before those of MWD (Fig. 12) and by day 189 were of similar magnitude as MWD. On the last day of the drought, day 196, $g_s$ was consistently higher for well-watered vs droughted plants for cytotypes EH ($p = 0.027$) and MWH ($p < 0.001$), but not for MWD plants ($p = 0.755$). Three days after rewatering, the differences between well-watered and droughted EH and MWD had disappeared ($p = 0.062$ and 0.978, respectively), whereas $g_s$ was significantly higher in well-watered vs droughted MWH ($p = 0.001$).

*Effects of Drought on Water Use Efficiency —*

Water use efficiency (WUE), expressed as the ratio of $A/g_s$, showed distinct treatment and cytotype differences throughout the duration of the experiment (Fig. 12). The decline over time of both $A$ and $g_s$ in well-watered plants resulted in a steady increase in WUE for all three cytotypes as the experiment progressed. Whereas MWH had the highest $g_s$ initially, its WUE was the lowest ($p = 0.005$) while the other two cytotypes did not differ. By the end of the experiment, maximum WUE values for well-watered plants were 47.9 $\pm$ 7.43, 86.3 $\pm$ 6.06, and 75.0 $\pm$ 10.61 μmol/mol for MWH, MWD, and EH, respectively and all three cytotypes were statistically different from each other.

For the droughted plants, WUE initially rose as it did for the well-watered plants, but the sharp drop in $g_s$ resulted in a plateauing of WUE by day 185 for MWH and MWD (Fig. 12), whereas EH peaked on day 189 before exhibiting a steady decline to the end of the experiment. On this date, WUE was statistically higher for this cytotype ($p = 0.008$).

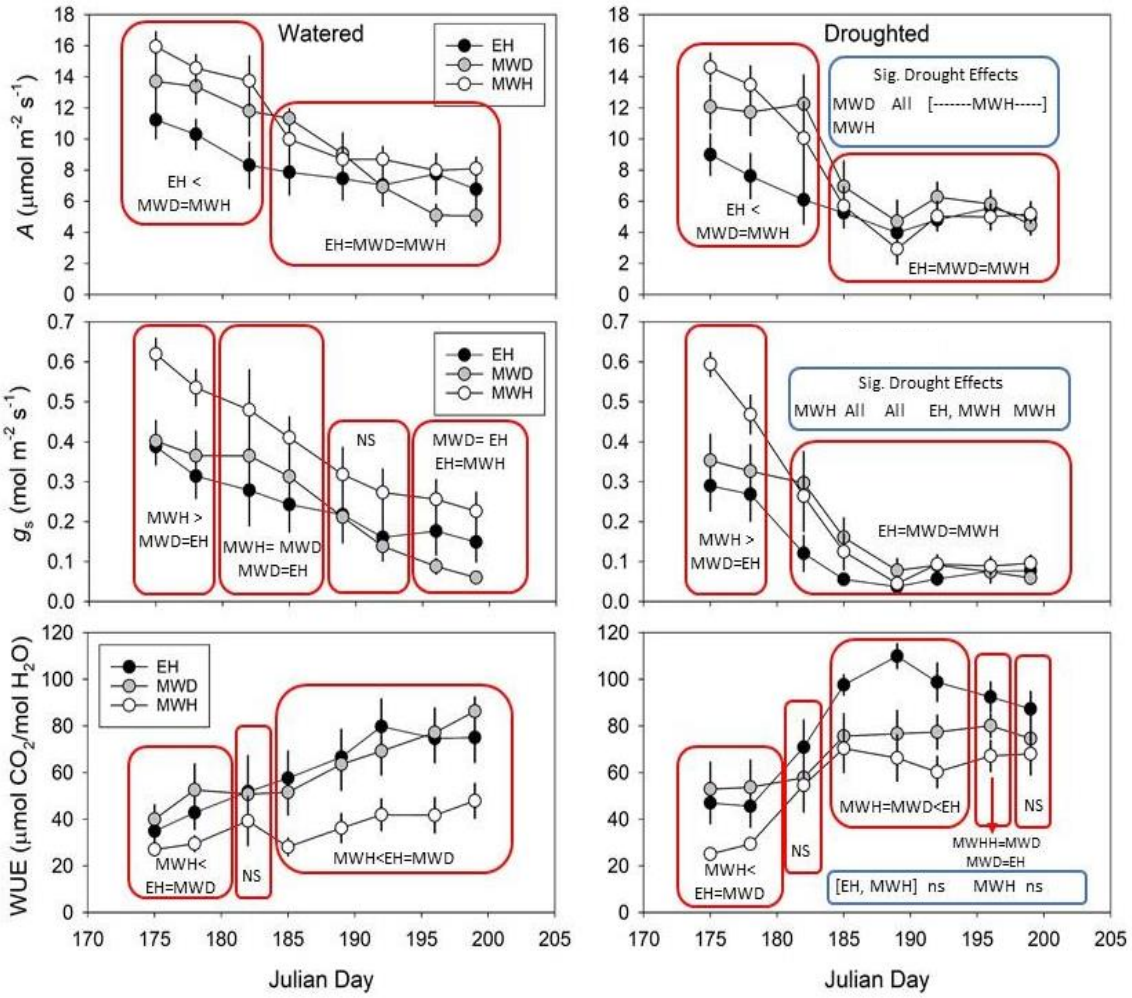


*Figure 12*: Gas exchange responses (photosynthesis (*A*), stomatal conductance ($g_s$), and water use efficiency (WUE) of each cytotype for well-watered (left column) and droughted plants (right column): MWD = Midwestern Diploid; MWH = Midwestern Hexaploid; EH = Eastern Hexaploid. **Comparisons in red boxes indicate within treatment cytotype differences. Comparisons in blue boxes indicate within cytotype treatment differences.** A split-plot design was used: no interaction effects were significant. Points are means ± se with significance assumed when $p \leq 0.05$; n =10.

At the end of the drought period on day 196, WUE did not differ between well-watered and droughted plants within any of the cytotypes, nor did it differ among any of the droughted cytotypes ($p > 0.05$; Fig. 12). Three days after rewatering, on day 199, there were no differences between well-watered and droughted plants for any of the cytotypes, but there were differences

among cytotypes, primarily in the well-watered treatment. EH and MWD, which did not differ, had the highest WUE, while MWH had the lowest ($p < 0.002$ for both comparisons).

*Stomatal Conductance as a Function of Soil Moisture —*

Stomatal conductance of plants subject to drought declined as soil moisture decreased, but the patterns for the hexaploids differed from that of the diploid (Fig. 13). A 3-parameter exponential rise to maximum equation was fit to the data for droughted plants during the period when water was withheld from the plants (days 175 to 189): $g_s = a + b(1 - e^{-c*S})$ where $S$ is soil moisture in percent (Table 1). All three equations had $r^2$ values at or above 0.95, indicating good fits. Conductance in both hexaploids decreased almost as soon as soil moisture began declining, whereas there was a threshold of 10% or above before $g_s$ declined in the diploids. After rewatering, $g_s$ remained low among all three cytotypes, with no evidence of recovery, even after 10 days.

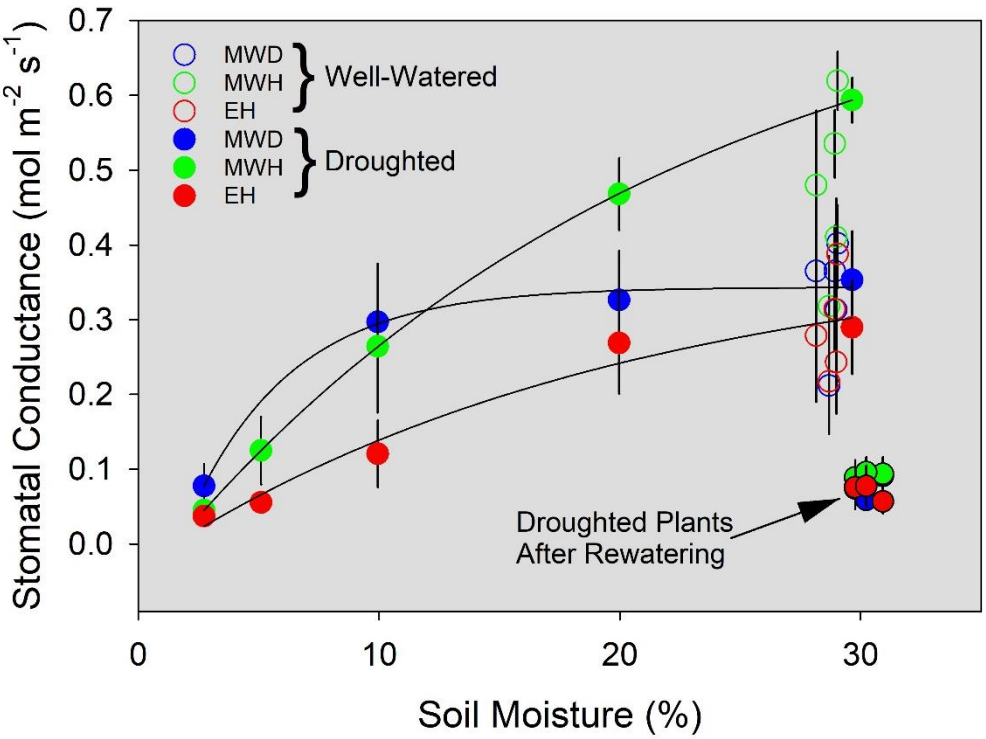


*Figure 13*: Soil moisture vs stomatal conductance ($g_s$) for both watered and droughted treatments during drought period and after rewatering. Values are means ± se; n=10. Plants subject to drought (filled circles) fitted with 3-parameter exponential rise to maximum curves (see Table 1 below for equation. $r^2$ for MWD, MWH and EH are 1.00, 0.98, and 0.95, respectively.

*Table 1*. Parameter fits of $g_s$ vs soil moisture for droughted plants during the phase of withholding water (from day 175 to 189). A 3-parameter exponential rise to maximum equation was used to fit the data: $g_s = a + b(1 - e^{-c*S})$ where $S$ = soil moisture (%).

| Cytotype | a | b | c | $r^2$ |
|---|---|---|---|---|
| **MWD** | -0.0587 | 0.8724 | 0.0464 | 1.00 |
| **MWH** | -0.1608 | 0.5042 | 0.2348 | 0.98 |
| **EH** | -0.0306 | 0.4299 | 0.0505 | 0.95 |

*Water Potential —*

Water potential readings (Table 2) were limited to just four times during the experiment because of the need to preserve leaf area. Measurements were made prior to imposition of water stress on day 175, two times during the drought (days 185 and 189), and once after rewatering at

the end of the experiment (day 193). Neither predawn nor midday leaf water potentials (ψ) differed between the watered and droughted treatments over the course of the experiment except on the last day, when predawn values were slightly lower in droughted vs well-watered EH ($p$ = 0.045). There were only two instances where cytotype differences were significant: on day 1 ψ were lower in MWD than either MWH or EH ($p$ = 0.038) and on day 2, when predawn ψ for MWH were lower than for the other two cytotypes ($p < 0.001$). The same cytotype order appeared at midday, but these means were not statistically different from each other.

By day 189, soil VWC readings in the droughted treatment had decreased to ~10%, and predawn leaf ψ did not differ either between watering treatments or cytotypes ($p$ = 0.793 and $p$ = 0.214). Droughted plants exhibited larger variances on this day compared to well-watered plants, due to variability in rates of drying among the pots and this obscured the ability to detect treatment differences. Across all cytotypes, predawn leaf ψ decreased to a range between -0.45 and -0.54 MPa, and again, variances were much higher among droughted than watered plants. By midday, leaf ψ no longer differed between treatments ($p$ = 0.553), nor among cytotypes ($p$ = 0.602). Interestingly, at midday, the variance of the two hexaploids was much greater than that for the diploid, and midday leaf ψ were the lowest over the course of the experiment, ranging from -1.00 $\pm$ 0.257 to -1.06 $\pm$ 0.282 MPa for MWH and EH, respectively.

***Table 2***: Predawn and midday water potentials (ψ) for the three cytotypes over the duration of the drought experiment. Values are means ± se; n = 5-7. Bolded means not followed by the same letter within a [treatment x time of day] combination are significantly different at $p \leq 0.05$. There were no significant treatment effects on either predawn or midday water potentials except on day 193 (see bolded italicized values), where midday droughted values for EH were lower than those for well-watered plants ($p = 0.045$).

| Treatment | Time of Day | Cytotype | Ψ (MPa) | | | |
|---|---|---|---|---|---|---|
| | | | Day 175 | Day 185 | Day 189 | Day 193 |
| Watered | Predawn | MWD | -0.31 ± 0.044[a] | -0.19 ± 0.028[a] | -0.17 ± 0.027[a] | -0.18 ± 0.010[a] |
| | | MWH | -0.24 ± 0.047[a] | **-0.40 ± 0.045[b]** | -0.24 ± 0.036[a] | -0.18 ± 0.033[a] |
| | | EH | -0.14 ± 0.043[a] | -0.13 ± 0.009[a] | -0.10 ± 0.016[a] | ***-0.13 ± 0.032[a]*** |
| | Midday | MWD | **-0.68 ± 0.078[b]** | -0.44 ± 0.100[a] | -0.68 ± 0.147[a] | -0.72 ± 0.093[a] |
| | | MWH | -0.50 ± 0.029[a] | -0.50 ± 0.135[a] | -0.70 ± 0.116[a] | -0.60 ± 0.053[a] |
| | | EH | -0.54 ± 0.035[a] | -0.31 ± 0.040[a] | -0.71 ± 0.200[a] | -0.63 ± 0.064[a] |
| Droughted | Predawn | MWD | -0.30 ± 0.079[a] | -0.19 ± 0.019[a] | -0.66 ± 0.228[a] | -0.20 ± 0.028[a] |
| | | MWH | -0.18 ± 0.047[a] | -0.22 ± 0.038[a] | -0.75 ± 0.285[a] | -0.17 ± 0.015[a] |
| | | EH | -0.14 ± 0.051[a] | -0.19 ± 0.024[a] | -0.80 ± 0.254[a] | ***-0.21 ± 0.016[a]*** |
| | Midday | MWD | -0.66 ± 0.045[a] | -0.31 ± 0.096[a] | -0.83 ± 0.326[a] | -0.63 ± 0.082[a] |
| | | MWH | -0.62 ± 0.036[a] | -0.38 ± 0.076[a] | -1.00 ± 0.257[a] | -0.63 ± 0.053[a] |
| | | EH | -0.58 ± 0.062[a] | -0.32 ± 0.053[a] | -1.06 ± 0.282[a] | -0.58 ± 0.046[a] |

After taking ψ measurements on Day 189, plants were rewatered and by day 193 there was some evidence of recovery to unstressed leaf ψ values. Each cytotype in the well-watered treatment recovered to either pre-drought or to even less stressful levels (Table 2), but in the droughted plants, only EH failed to recover as much. By midday, cytotype or treatment differences were no longer apparent.

*Leaf Temperature —*

There were no treatment effects on midday leaf temperatures throughout the experiment, but there were significant cytotype effects ($p < 0.001$) on days 175 and 182, with MWH having significantly lower leaf temperatures (by about 2 ℃) than either MWD or EH (Fig. 14).

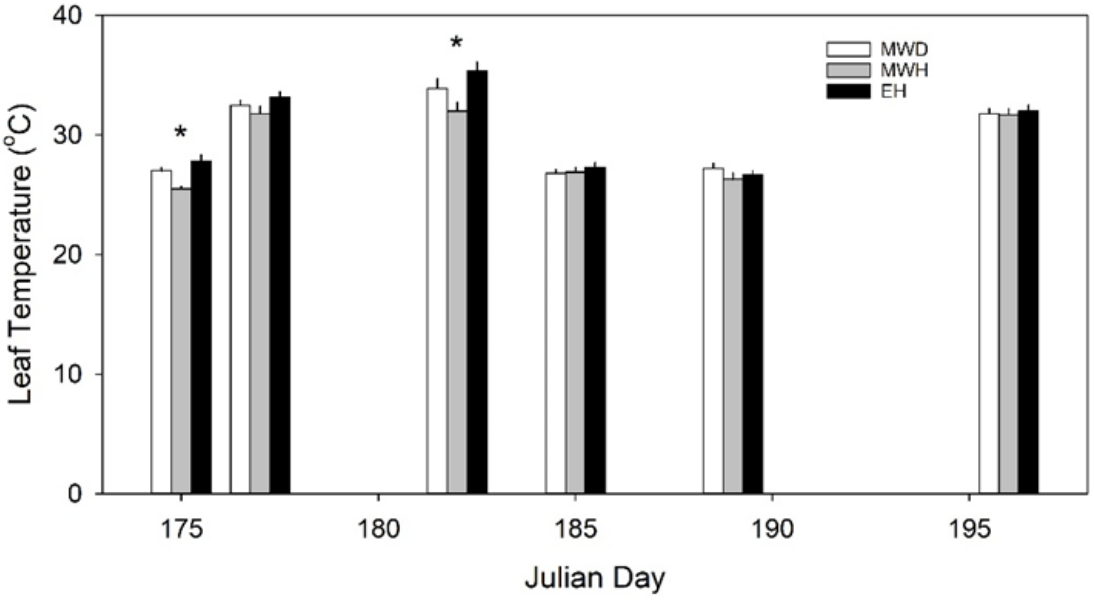


***Figure 14***: Midday leaf temperature across both treatments for each cytotype. Values are means ± se; n=20. Asterisks shown on days when MWH was significantly ($p \leq 0.05$) cooler than either MWD or EH, which did not differ from each other. No other cytotype comparisons were significant.

*Aboveground Biomass (Greenhouse) —*

Total aboveground biomass of plants in the greenhouse drought experiment was consistently higher in the watered plants than droughted plants (Fig. 15). MWH was the most vigorous of the cytotypes, having the highest overall biomass, regardless of experimental treatment. EH and MWD plants had lower, but equal biomass for each subset measured (flowers, leaves, stem). For leaf biomass, there were no significant treatment or cytotype effects ($p = 0.3225$). MWH had the highest flower biomass ($p < 0.0001$), followed by EH and MWD, which did not differ. The same pattern was seen for stem and total biomass ($p = 0.0001$ and $p < 0.0001$ respectively). However, the ratio of leaf biomass to total plant biomass was significantly higher in EH plants than MWH, with MWD intermediate ($p = 0.0047$). The flower biomass ratio to whole plant biomass did not show a treatment effect ($p = 0.5926$), but there were cytotype differences, with higher ratios in MWH than EH, while MWD had the lowest ratios.

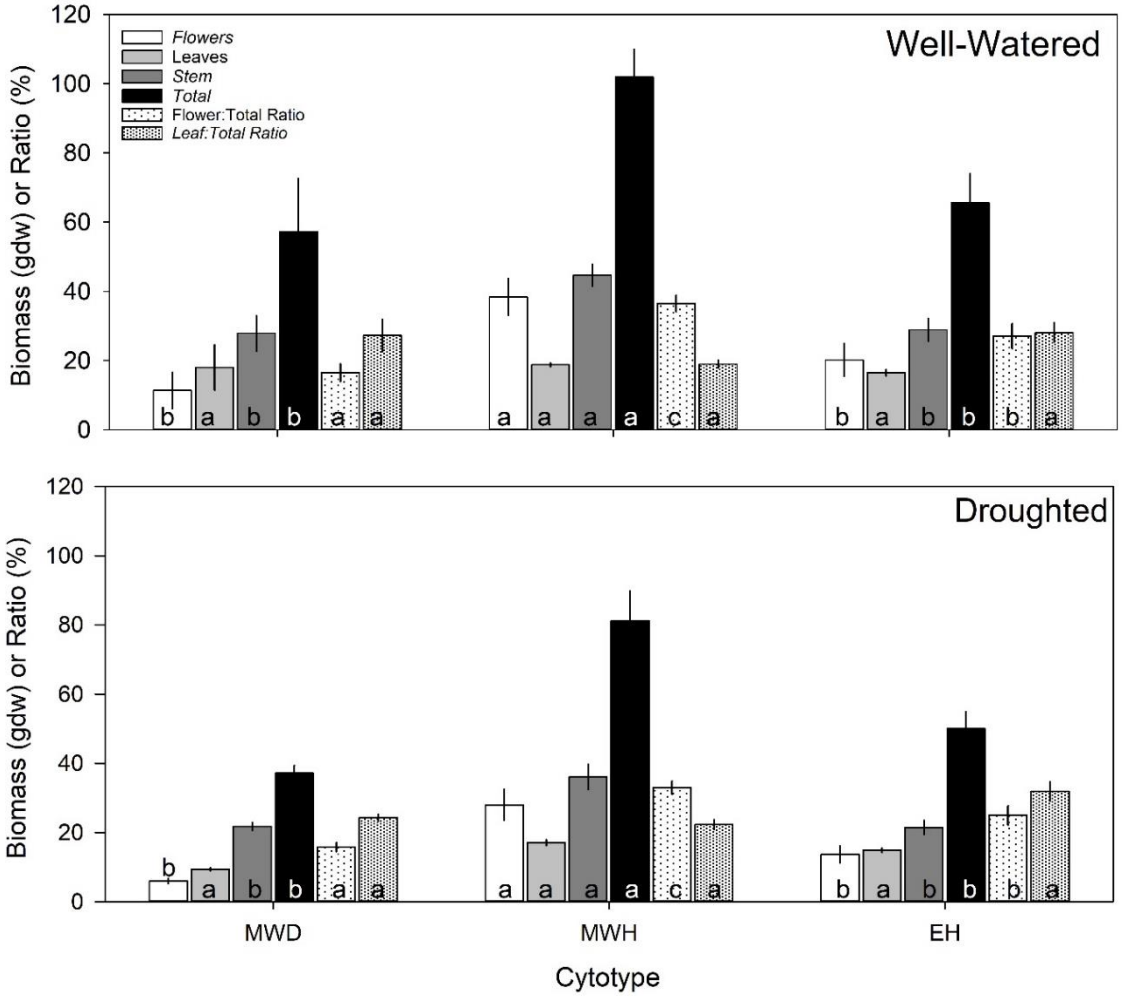


*Figure 15*: Aboveground dry weight biomass for each cytotype in the drought experiment. Values are means ± se; n=10. Cytotype means within a treatment not followed by the same letter are significantly different ($p \leq 0.05$). Italicized categories in the legend indicate a significant ($p \leq 0.05$) difference in that category between well-watered and droughted plants within a cytotype.

*Stem Height Growth —*

MWH had the most stem height growth throughout the course of the drought experiment, while EH and MWD were not significantly different ($p < 0.001$).

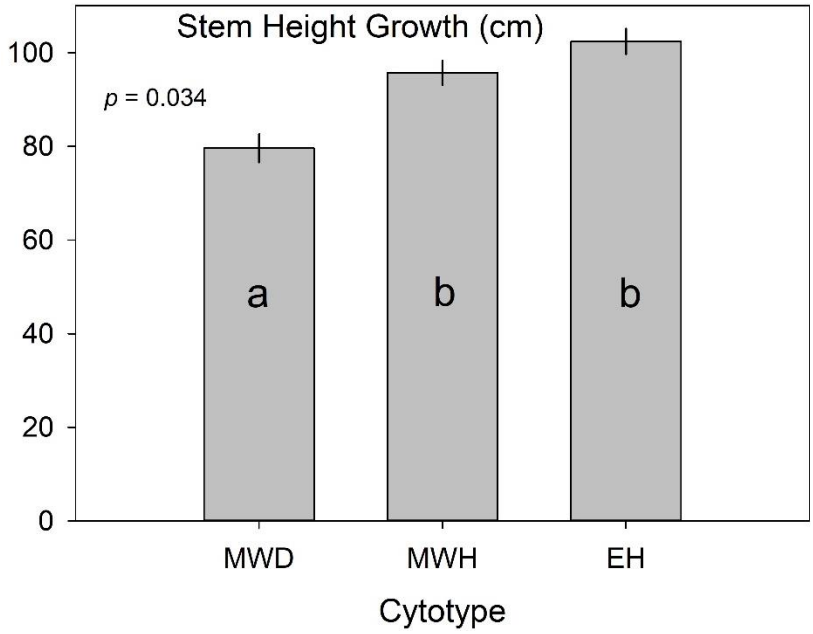


*Figure 16*: Stem height growth for each cytotype in the drought experiment. There were no statistically significant treatment differences, so samples were combined to better show the cytotype effect. Values are means ± se; n=20. Means with different letters are significantly different ($p \leq 0.05$).

*Leaf Pigments —*

Total chlorophyll and carotenoid contents were lowest ($p < 0.001$) in leaves from EH plants and significantly different from both midwestern cytotypes, which did not differ from each other (Fig. 17). If expressed on a mass/mass basis, MWD had more carotenoids ($p = 0.006$) than either MWH or EH, which did not differ. Patterns for chl a and b on an area basis were like those for total chlorophyll (data not shown). There were no differences among cytotypes for the chl a to b ratio ($p = 0.089$, data not shown).

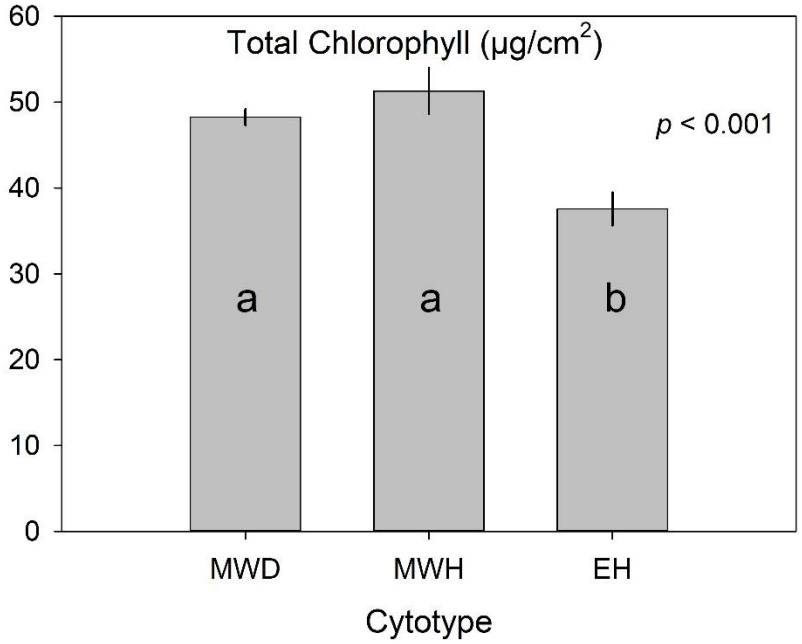


*Figure 17*: Total chlorophyll content for each cytotype. Samples taken only from well-watered plants. Values are means ± se; n=10. Means not followed by the same letter are statistically different at $p \leq 0.05$.

## DISCUSSION

In this study of Midwestern and Eastern populations of *Solidago altissima*, I found differences in morphology and physiology both within (diploid vs hexaploid) and between (Midwestern vs Eastern hexaploids) geographical locations. Some of the largest character differences occurred between the two hexaploids, e.g., for photosynthetic rate and stomatal conductance, while for other characters, such as stomatal density and stem xylem anatomy, the two Midwestern cytotypes were similar. In some cases, such as for light response parameters like light compensation and saturation points, the Eastern hexaploid was more like the Midwestern diploid than the Midwestern hexaploid. The disparity between the two hexaploids suggests substantial post-polyploidization selection and evolutionary divergence (Etterson et al., 2016).

My results are also consistent with studies that suggest Midwestern *S. altissima* diploids tolerate greater stress in open field environments, while hexaploids avoid such stresses and are more abundant adjacent to shadier, forested habitats (Richardson and Hanks, 2011; Etterson et al., 2016). My study is the first, though, to show that substantial differences exist between the Midwestern and Eastern hexaploids, which may shed some light on why diploid cytotypes are absent from the eastern portion of the United States. These differences also suggest that environmental tolerances differ among the cytotypes that could affect their ability to respond to competition and future climate change.

*Polyploidy and Cell Size Effects —*

Polyploidy initially results in increased in cell size (Cavalier-Smith, 1978; Gregory, 2001; Otto, 2007), known as the Gigas effect, which can cause physiological changes due to the reduction in the surface area to volume relationship governing the transfer of materials across the cell membrane. However, the physiological implications of increased cell size by themselves are still poorly understood (Doyle and Coate, 2019) and may be moderated by subsequent alterations in leaf morphology and development (Warner et al., 1987). Furthermore, not all cells in a leaf show similar increases in sizes with polyploidy. Katagiri et al. (2016) found that epidermal pavement cells in *Arabidopsis* leaves were larger in synthetic polyploids, but palisade mesophyll cells were not.

When increased cell sizes do occur, it is primarily because polyploidization enlarges genome size, which requires a larger nucleus, which is closely correlated with final cell size (Šímová and Herben, 2012). Although polyploidy and genome size are often positively correlated within closely related genera (Théroux-Rancourt et al., 2021), there is a tendency for angiosperms to undergo rediploidization, which results in a reduction in genome size over evolutionary time (Simonin and Roddy, 2018). Thus, in some cases, final cell sizes relate more to genome size than ploidy level (Théroux-Rancourt et al., 2021).

Aside from cell size, additional morphological and physiological changes can occur at higher levels of organization at the tissue, organ, and ultimately whole plant levels (John et al., 2017). Compensatory changes at each higher organizational level can moderate or offset some of the effects of increased cell size or packing such that the

effects of polyploidy may not be apparent (Husband and Schemske, 1998; Suda et al., 2007; Dauphin *et al*., 2014; Reis et al., 2014), a phenomenon referred to as "cryptic polyploidy" (Dauphin et al., 2014). For example, the porosity of a leaf, which is a measure of the internal airspaces relative to mesophyll cell volume, can change with both cell size and packing density. For a given cell size, denser cell packing in leaves would result in fewer intercellular airspaces and greater mass per area with (John et al., 2017) or without (Xiong et al., 2016) changes in leaf thickness.

The amount of mesophyll cell surface area exposed to airspaces inside the leaf governs the efficiency of internal diffusion of $CO_2$ (Nobel *et al*., 1975; Longstreth et al., 1981). High ratios of this parameter to mesophyll volume ($M_{esa}/V_{mes}$) facilitate liquid phase diffusion across the wet surfaces of these cells. Modeling shows that smaller cell sizes result in higher $M_{esa}/V_{mes}$ ratios, but that changes in porosity and tortuosity (Harwood et al., 2021), which depend on cell packing and size, do not (Théroux-Rancourt et al., 2021). Thus, the diffusion of $CO_2$ across cell walls in the liquid phase will increase when cell size decreases, and when cells are more densely packed, whereas such changes exert only minimal effects on gaseous phase diffusion, which is 10,000 times faster than in water. The $M_{esa}/V_{mes}$ ratio is strongly regulated by genome size (Théroux-Rancourt et al., 2021) so neo-polyploids, which have not yet undergone genome size reduction, should have reduced rates of gas exchange because of their larger cell sizes, unless other compensatory processes offset these reductions.

*Phenological Implications of Polyploidy —*

Increased cell sizes resulting from polyploidy can alter the phenology of plants (Ramsey, 2011) by altering developmental patterns and rates. Such phenological

asynchrony can reduce overlaps in flowering between plants that differ in ploidy (Bretagnolle and Lumaret, 1995; Bretagnolle and Thompson, 1996; Nuismer and Cunningham, 2005; Ramsey, 2011). This would decrease the possibility of hybridization (Husband and Schemske, 2000) that would otherwise result in "*gamete wastage*" (Levin, 1975; Herrera et al., 2004), since backcrossing between diploid progenitors and their polyploid congeners would be unsuccessful anyway. This would also reduce competition for pollinators between competing polyploids, resulting in improved seed set and reproductive success of each cytotype (Husband et al., 2002; Husband and Sabara, 2003).

In my study, in a common garden situation in the mountains of western North Carolina, Midwestern diploids flowered nearly three weeks earlier than their congener hexaploids, with only about a 10 day overlap in flowering (Figs. 1 and 2). Etterson et al. (2016) also found that diploids in MN flowered earlier, but only by about 8 days. Conversely, in Ramsey's study (2011) of wild yarrow, the opposite pattern was found, where hexaploids flowered before tetraploids, which suggests that the direction of phenological shifts in polyploids may be species and environment dependent.

There was substantial flowering overlap between the two hexaploids growing in the common garden in North Carolina though (Figs. 1 and 2). Given the difference in climate between the Midwest (93.7 cm annual precipitation; summer avg. highs 25 – 30 ˚C; summer avg. lows 12.2 – 18.3˚C; plantmaps.com, 52240 Zipcode Border, Zone 5a) and Eastern mountains (146.0 cm annual precipitation; summer avg. highs 21.7 – 25.6˚C; summer avg. lows 12.8 – 15.0˚C; plantmaps.com, 28607 Zipcode Border, Zone 6b), this suggests that the delay in flowering by the hexaploids is driven primarily by polyploidization, since both cytotypes had nearly the same phenology despite the

differences in their natural growing seasons. I had expected the Eastern cytotypes to flower sooner because they are adapted to a shorter growing season (PRISM Climate Group, accessed 6/22/2021), but this was not the case (Fig. 1). In fact, percent flowering was delayed more for EH than MWH (Fig. 2). The similarity between the Midwestern and Eastern hexaploids suggests that flowering might be responding in large part to daylength, which would be similar for both populations, since they are native to nearly the same latitude. Etterson et al. (2016) found that latitude exerted a strong effect on flowering phenology, with southern prairie plants flowering later than those from more northerly biomes, and diploids had a stronger latitudinal response than the polyploids, suggesting that they are genetically more differentiated along this gradient than the two polyploids (Etterson et al., 2016). Studies of *S. altissima* populations from the coastal plain and Piedmont of NC would help separate elevation from longitude in future studies, since they occur at a similar elevation as Midwestern populations and at nearly the same latitude.

*Morphological Implications of Polyploidy —*

There were substantial morphological differences among the cytotypes, as also found by Etterson et al. (2016). Both hexaploids had larger leaves than the diploid, with EH being larger than MWH (Fig. 3). However, MWH had a higher specific leaf mass than both EH and MWD, indicating thicker/denser leaves. Although I did not measure leaf thicknesses of the cytotypes, I think the differences in specific leaf mass are due primarily to cell size differences. John et al. (2017) found that specific leaf mass across many plant species was most highly correlated with increased cell sizes, greater main vein densities, more mesophyll cell layers and a higher cell mass density.

As expected, and found in other polyploids (Speckman et al., 1965; Melaragno et al., 1993), stomata were larger in the hexaploids, and largest in EH (Fig. 4). This usually means that stomatal densities should be lower in the hexaploids, which was true for EH but curiously, not for MWH, which had a abaxial density close to that of MWD. Etterson et al. (2016) found that the ratio of abaxial to adaxial stomatal densities differed among their cytotypes in Iowa, with tetraploids having the lowest ratio while diploids and hexaploids were similar. Ratios for my plants were much higher than those of Etterson et al. (2016) and ranged from 41.6 $\pm$ 4.49 for MWD to a minimum of 17.5 $\pm$ 4.23 for MWH, with EH intermediate at 33.9 $\pm$ 4.10 ($p$ = 0.005, One-Way ANOVA, Tukey post-hoc test). My ratios are 5-8 and ~3 times higher than the values found by Etterson et al. (2016) for the diploids and hexaploids, respectively, for reasons that are not apparent. Perhaps differences in growing conditions caused these patterns. Nonetheless, they show that in in NC, MWH had a lower ratio than either MWD or EH, in contrast to Etterson et al. (2016) findings where the diploid and hexaploid did not differ. Since densities did not differ among cytotypes for the adaxial surface (Fig. 3), these differences in the ratios primarily reflect the differences I found for the abaxial surface. When I compare the ratio of abaxial to adaxial stomatal lengths or widths, I also find ploidy differences, indicating a complementary effect, but this time based on size. These results point out that some traits directly follow from the increased cell size effect in polyploids (e.g., stomatal and leaf sizes) but that other traits may not because of compensatory responses at higher organizational levels. For example, there is no difference in specific leaf mass between EH and MWD, or in abaxial stomatal density between MWD and MWH, which differ in

ploidy, but there is between EH and MWH, which reinforces the concept that such traits can be subject to genic selection (Ramsey, 2011).

The direction of change in specific leaf mass is somewhat paradoxical, since at least two studies in the Midwest provide evidence that hexaploids prefer habitats adjacent to forest edge where light levels may be lower (Richardson and Hanks, 2011; Etterson et al., 2016). Plants grown in lower light often have lower SLM (Neufeld and Young, 2014), but Etterson (personal communication) has indicated that all cytotypes in the Midwest grow out in the open, but that the hexaploids are more frequent adjacent to forest edges. However, no one has measured whether light levels differ in these two habitats. Halverson et al. (2008) did not find such segregation; instead, diploids, tetraploids, and hexaploids all co-occurred within 5 to 10 m of each other in old-fields in Iowa. In both the Richardson and Hanks (2011) and Etterson et al. (2016) studies, diploids were reported as the least abundant in old-field habitats but absent from areas adjacent to forest edges. It would be interesting to redo my common garden study across a range of light levels to determine if the polyploids respond differently to shade.

Richardson and Hanks (2011) and Etterson et al. (2016) speculated that MWH are less tolerant than MWD of open-field conditions and that is why they are restricted to these locations. This distribution pattern also suggests that hexaploids may be less tolerant than MWD of high light, and of the hotter, drier conditions that occur away from forest edges. In the East though, EH primarily grow in full sun in high elevation old-fields and are not necessarily restricted to along forest edges. Mid-summer maximum temperatures in the Midwest are higher and rainfall amounts and humidity lower (PRISM Climate Group, accessed 6/22/2021) than in the mountains of western NC. Possibly, EH

can tolerate open fields in the East because they experience less physiological stress than plants growing in similar habitats in the Midwest.

Differences in leaf size and orientation may play roles in determining habitat preferences. For instance, I found differences in leaf display among the cytotypes, something that to my knowledge has not been reported previously in the polyploidy literature. EH plants tended to have "droopier" leaves than the Midwestern cytotypes. For Midwestern plants, horizontal leaves would have maximal light interception near or at midday, when air temperatures approach their daily maximum and vapor pressure deficits are high. These two conditions could result in higher leaf temperatures and exacerbate potential water loss. For the hexaploid, the larger leaf sizes would be linked to a larger leaf boundary layer and reduce the efficiency of heat dissipation (Gates, 1980). Leaves that droop, on the other hand, would avoid maximal radiation loads at midday and instead would experience high radiation loads early in the day when it is cooler and more humid, thus potentially limiting daily water losses. This pattern suggests that EH plants may be particularly sensitive to midday water stress compared to Midwestern cytotypes.

For MWH in the Midwest, the combination of larger and more horizontal leaves may lead to leaf overheating unless other means to dissipate excess energy are employed. MWH have the highest $g_s$ of the three cytotypes, and this would result in more evaporative cooling due to transpiration and may be a way to avoid harmful leaf temperatures, especially in midsummer. It should be noted that early in the drought study, when plants still had high gs, that MWH leaf temperatures were up to 2$^{o}$C cooler than the other cytotypes. This may not be as severe a problem for EH, even though its leaves are larger than those of MWH, because maximum temperatures are lower in the mountains

than in the Midwest (PRISM Climate Group, accessed 6/22/2021). I could test this by doing reciprocal transplant studies with EH plants in the Midwest, and measuring leaf angles, temperatures, and transpiration rates to see if leaf orientation in the hexaploids plays a role in their ability to cope with environmental conditions in old-field situations.

*Implications of Polyploidy for Hydraulic Functioning —*

Changes in leaf size and number are intimately related to the anatomy and functioning of the xylem network in plants (Sperry et al., 2002). The ability of xylem to supply leaves with water can determine the degree of stomatal opening and conductance (Sack and Holbrook, 2006; Brodribb et al., 2017), and may set limits on total plant leaf area that can be adequately supplied with water from the roots (Meinzer and Grantz, 1990). If polyploidy increases cell and leaf sizes, and whole plant leaf area, then there may be changes in xylem structure to supply leaves with more water. Such changes could include more xylem per unit stem area, increased xylem lumen diameters to lower resistance to flow (Gibson et al., 1985) or increased pit pore numbers and sizes (Gibson et al., 1985; Venturas et al., 2017). However, larger cell sizes might also lead to lower vein densities in leaves, reducing their capacity to supply the stomata with water (Brodribb et al., 2017), which would suggest that polyploids should have lower leaf, if not stem, xylem hydraulic conductance ($K_h$).

However, there are relatively few studies of polyploidy effects on xylem anatomy or $K_h$ in plants (Maherali et al., 2009; Hao et al., 2013; Zhang et al., 2017; Greer et al., 2018). Polyploidy effects on xylem anatomy and function are difficult to predict because of numerous interacting developmental processes during xylogenesis that could affect $K_h$.

Lumen size, for example, is critical for limiting maximum $K_h$. The Hagen-Poiseuille Equation predicts that water flow at a particular tension should be proportional to the fourth power of the radius (Tyree and Zimmermann, 2002). This means that small changes in lumen diameter will have large impacts on flow, since a doubling of the radius increases flow by $2^4$, or 16 times. Just a few larger vessel elements among more numerous smaller ones could substantially increase stem $K_h$. Increases in $K_h$ would, in turn, permit higher $g_s$ and higher rates of photosynthesis (Hubbard et al., 2001), leading possibly to greater net productivity by the plant. If so, such adaptations might provide one cytotype with a competitive advantage over another.

However, I found little to no variation in xylem anatomy or morphology among the three cytotypes (Fig. 5). The only significant difference was that EH occasionally had the capacity to produce much larger vessel elements than either MWD or MWH. However, these cells were quite rare and did not increase the theoretical $K_h$ enough to distinguish EH statistically from the other cytotypes, even though its mean $K_h$ was higher than the other two cytotypes. There was a trend, albeit non-significant, for the two hexaploids to have a higher percentage of stem area devoted to xylem, which would suggest that $K_h$ could be higher in these cytotypes. But the theoretical calculations did not support this when determined on a fixed cell count.

However, if xylem abundance and theoretical water flow are taken jointly into account, i.e., when you combine the percent of a fixed cross-sectional area occupied by xylem vessels with predicted water flow, then $K_h$ is predicted to be much higher in the two hexaploids than the diploid. For example, EH and MWH have a mean vessel occupancy 64% and 79% higher than MWD. When applied to the calculated $K_h$ (based on

50 cells), estimates of total water flow are 1.7x higher in MWH and 3.6x higher in EH compared to MWD. A higher flow rate would mean that hexaploids could transpire at the same rate as diploids, but at less negative water potentials (Maherali et al., 2009). The higher flow capacity in MWH is consistent with its higher $g_s$, but not for EH, which had the highest calculated flow but whose $g_s$ was of similar magnitude as MWD, which had the lowest flow. This suggests that factors additional to $K_h$ may act to determine maximum $g_s$ in EH.

Maherali et al. (2009) found that tetraploid *Chamerion angustifolium* plants had wider xylem lumens and higher $K_h$ than diploids, but without differences in rates of $A$ or $g_s$, even though they found larger but less dense stomata in the tetraploids. My situation with *S. altissima* was more complex. Only the Eastern hexaploid had a lower stomatal density than the diploid, but both hexaploids had larger stomata. MWH had the highest $g_s$ whereas EH and MWD did not differ, which makes comparisons of the influence of polyploidy on drought resistance difficult to interpret. Nonetheless, some patterns were apparent from the drought experiment. MWH showed immediate reductions in $g_s$ once water was withheld (Figs. 11 and 12), whereas EH appeared to tolerant slightly more stress before stomata closed and diploids appeared the most resistant to declines in soil moisture levels and were able to maintain a constant $g_s$ to lower soil moisture contents than the other two cytotypes. This suggests that moderate water stress causes relatively greater decreases in $A$ and $g_s$ for the hexaploids than the diploids, which may give the diploids an advantage in hot, dry environments, and may result in reduced competitive ability of the hexaploids when under moisture stress.

It would have been useful to obtain direct measurements of $K_h$ in *S. altissima* plants, but this proved difficult to do because the porous nature of the pith in their stems prevented accurate measurements of water flow through the xylem (personal observations). Instead, for future studies, I would calculate $K_h$ using an alternative method that makes use of the relationship between whole plant transpiration and water potential. For a plant undergoing steady-state transpiration, $K_h$ can be calculated as the ratio of $E$ to the driving force for water movement in the stem (ψ), the water potential gradient from the roots to the leaves:

$$K_h = \frac{E}{\psi_{root-leaf}} \quad (1)$$

where $K_h$ is hydraulic conductance (Kg $H_2O$ m $MPa^{-1}$ $s^{-1}$), $E$ is transpiration rate (Kg $H_2O$ $m^{-2}$ $s^{-1}$) and $\psi_{root\text{-}leaf}$ is the xylem tension gradient (MPa $m^{-1}$) from root to leaf (Tyree and Ewers, 1991). To do this, I would grow plants in pots, saturate the soil to obtain a soil water potential of 0 MPa, and then place them on a balance to calculate transpiration. Once I achieved a steady-state transpiration rate, I would measure leaf ψ and calculate $K_h$.

*Ecophysiological Differences in Gas Exchange among the Cytotypes —*

Polyploidization increases the number of chloroplasts per cell (Warner et al., 1987; Standring et al., 1990; Ewald et al., 2009) as well as several other cellular constituents related to photosynthesis, such as RUBISCO molecules and mitochondria (Warner and Edwards, 1993; Preuten et al., 2010). An increase in cell size may also change organelle sizes and/or number which could affect the functioning of individual cells (Doyle and Coate, 2019; Munzbergova and Haisel, 2019). There are scattered reports where chloroplast size increases in polyploids (see Sax and Sax 1937) and Warner

et al. (1987) speculate that in *Panicum virgatum* tetraploids, chloroplasts might be larger and/or contain higher amounts of chlorophyll than diploids.

As Warner and Edwards (1993) note in their review of the effects of polyploidy on photosynthesis, rates per cell correlate closely with the amount of DNA per cell. Nevertheless, rates per leaf depend in addition on changes in leaf structure, such as the number of cells per unit area and their packing, which as noted earlier in this discussion (Harwood et al., 2021; Théroux-Rancourt et al., 2021) affect the internal diffusion of $CO_2$ to the chloroplasts. Leaf level photosynthetic rates depend on the relative changes in number of cells per unit area. If leaf area increases exceed those resulting from larger cell size, rates per leaf may decline, and vice-versa if the opposite occurs (Warner and Edwards, 1993). As a result, rates can be either higher or lower for polyploids than diploids (Warner and Edwards, 1993; Harwood et al., 2021; Théroux-Rancourt et al., 2021). Numerous studies of gas exchange comparing diploids to autopolyploids show the full range of possible responses (Warner and Edwards, 1993), from lower rates per unit leaf area (Romero-Aranda et al., 1997), to no change (Frydrych, 1970), to higher rates (Greer et al., 2018).

In my study, both in the courtyard-grown and drought experiment plants, $A$ was lowest in EH and highest in MWH, with MWD being intermediate between the two (Figs. 8 and 11). Based on the light curves, $A_{max}$ was 4x higher for MWH than for EH, which was twice the difference between MWH and MWD. Plants in the drought experiment showed a similar ranking for $A$ as those used for the light curves. The large difference between the two hexaploids in $A_{max}$ shows that there has been considerable

selection and divergence, much more than between the Midwest hexaploid and its diploid progenitor.

The much higher *A* in MWH is due in large part to its higher $g_s$ and the converse is true for EH, but it is not clear why this should be so. Both hexaploids have nearly the same size stomata (Fig. 4) although MWH has a higher abaxial density than EH, which could lead to higher $g_s$ (Li et al*.,* 1996). One consequence of this is that MWH appears more capable of evaporative cooling than the other two cytotypes (Fig. 13). However, the difference in $g_s$ does not appear large enough to cause a four-fold difference in *A*. Furthermore, higher stomatal densities do not always result in higher $g_s$ (Ohsumi et al., 2007). Specific leaf mass was higher in MWH than EH and that could lead to higher *A* because of increased internal mesophyll cell surface area (Théroux-Rancourt et al., 2021), but again, the difference was only about 20% and doesn't seem high enough to account for the large difference in *A* between the hexaploids.

One of the most common results of polyploidy are larger guard cells at lower densities (Sax and Sax, 1937; Chaves et al., 2018; Hassanzadeh et al., 2020). Yet, Midwestern hexaploids and diploids had comparable densities (Fig. 3) even though stomatal sizes were larger in the hexaploids. This suggests that after polyploidization, there was selection for higher densities in the hexaploid plants. The combination of higher stomatal density and size could be responsible for the high $g_s$ in MWH, although high stomatal densities may lead to overlap of diffusion shells that lowers water loss rates (Cook and Viskanta, 1968; Lehmann and Or, 2015). If this happens for MWH, then higher stomatal density may moderate high water loss rates resulting from the larger stomatal pores while still allowing for $CO_2$ uptake, thus giving MWH a considerable

advantage over MWD. Why a comparable change in density did not happen for EH plants is unknown, but perhaps evaporative demand is lower in the Southern Appalachian Mountains and selection pressure is low for high stomatal density. However, these patterns do suggest that in this polyploidy complex, stomatal size may be more important than density for governing $g_s$ (Reich, 1984).

Another factor to consider regarding stomatal size is that within a genus, species with smaller stomatal guard cells tend to respond faster to environmental cues than those with larger cells (e.g., diploids vs. hexaploids; Dudits et al., 2016). This may confer advantages to diploids, because they could respond more rapidly to environmental variation than their congeneric polyploids. It may also allow diploids to better regulate water loss during drought through improved improving water use efficiency (Lawson and Blatt, 2014; Raven, 2014). Conversely, denser packing of mesophyll cells in MWH, which had a higher specific leaf mass, might allow for better cell signaling that could include cues to open or close stomata (Flütsch and Santelia, 2021), even if they react more slowly. More research on the anatomy and hydraulics of these plants, and on stomatal kinetics, would be a welcome addition to the polyploidy literature.

Differences in $A$ are partially correlated with chlorophyll amounts per unit leaf area (Fig. 16). For example, EH had the lowest $A$ and the lowest total chlorophyll amount of the three cytotypes. However, this does not explain the differences in $A$ between the two midwestern cytotypes, which had similar chlorophyll amounts.

An analysis of the light curve responses shows some correlations with the anatomical differences among the cytotypes, although apart from saturating PAR, and $A_{max}$, none of these parameters were statistically different. For example, MWH had the

highest specific leaf mass, indicative of thicker/denser leaves. Such leaves would intercept more light and require higher irradiances to saturate *A* (Akhkha et al., 2001; Johnson and Murchie, 2011), which is what I found in my study (Fig. 8). More cellular mass might also imply higher dark respiration rates, but no differences were apparent among the cytotypes. Thicker and denser leaves might also imply a higher apparent quantum efficiency and higher compensation point, as occurs for sun-grown plants (Boardman, 1977; Neufeld and Young, 2014), but again, the differences were not large enough to warrant statistical significance. The lack of differences among the cytotypes for the light response parameters confirms that these cytotypes should respond similarly to variations in light, and all are native to old-field habitats with high irradiances. That some cytotypes, such as MWH, segregate adjacent to forest edges, where it might be slightly shadier, may have more to do with their water relations than their ability to process light energy.

Although EH had the lowest *A* of the three cytotypes, its productivity in the courtyard was the highest, while in the greenhouse, MWH out-produced the other cytotypes (Fig. 14). The higher growth rates for greenhouse-grown MWH correlate with their higher gas exchange rates, but the same cannot be said for courtyard-grown EH, which had the lowest rates. For greenhouse-grown plants, there were no differences in the leaf:total biomass ratio, although there was a non-significant trend for slightly higher values for EH plants. Why EH achieved the highest biomass in the courtyard and MWH in the greenhouse experiment is perplexing. Plants in the courtyard were grown in smaller pots outdoors and were more likely root-bound than those in the greenhouse drought experiment, and it is possible that with differences in pot size, shape, and nutrient/water

contents, growth allocation aboveground changed (Thomas and Strain, 1991; McConnaughay et al., 1993). Further experiments on biomass accumulation and allocation patterns (Bush, 2020), including analyses of below ground production of roots and rhizomes, under standardized conditions, would help clear up these issues.

It is possible that the EH compensated for its low *A* by increasing leaf size, which was the largest of all the cytotypes (Fig. 3). Although I did not count leaves, whole plant productivity would be enhanced if EH produced more leaf area per plant. High productivity in modern hexaploid wheat varieties, for example, links more to larger leaf sizes, as *A* per unit leaf area is higher in the less productive ancestral varieties (Del Blanco et al., 2000 and references therein). However, as noted in my drought study, gas exchange rates decreased with time, even in well-watered plants, which suggests there are large differences in rates as leaves age and possibly as they change position relative to other leaves, as found to occur in other plant species (Constable and Rawson, 1980; Jung et al., 2021). Future studies of ontogenetic influences on leaf gas exchange, and whether these differ among the cytotypes, would further our understanding of the implications of polyploidy on gas exchange.

*Responses of the Cytotypes to Drought —*

A common paradigm is that some plants close their stomata under mild water stress to maintain higher leaf water potentials and to prevent cavitation events that result in embolisms. This occurs at the expense of taking up $CO_2$ for photosynthesis and plants that adopt this strategy are characterized as isohydric (Tardieu and Simonneau, 1998). Other plant species maintain open stomata and can tolerate lower water potentials without suffering embolisms, a strategy that allows them to continue to take up of $CO_2$ for

photosynthesis. These plants are characterized as anisohydric. Of course, plants do not just fall into one or the other category. Rather, they align themselves along the spectrum from isohydric to anisohydric. In my drought experiment, the cytotypes appear to adopt strategies that range along the spectrum from isohydric to anisohydric. Well-watered MWH responded to soil moisture depletion by rapidly closing its stomata, with no apparent threshold response (Fig. 12), and therefore would fall onto the isohydric portion of the spectrum, whereas MWD kept their stomata open until soil moisture levels reached ~10%, at which time they closed. That cytotype would fall onto the anisohydric side of the spectrum. EH had an intermediate response, with closure beginning once soil moisture decreased to ~20%.

Despite being on both sides of the hydric spectrum, I found no differences in midday water potentials among the cytotypes, indicating that despite disparate stomatal strategies for dealing with drought stress, all the cytotypes exhibited equivalent degrees of water stress. This might arise because the rapid closure of MWH and EH stomata, which started off at high rates, would reduce water losses at moderate soil moisture depletion while the delay in stomatal closure for MWD would have smaller effects on water loss because the initial rates of $g_s$ were so low. These contrasting strategies may play a role in determining microhabitat segregation among these cytotypes. Plants growing along forest edges, such as MWH in the Midwest, might tolerate moderate soil moisture stress but would quickly reduce $g_s$ to cope with severe water stress and be less competitive in open fields where irradiance levels are higher. MWD, which are primarily restricted to open fields, can maintain open stomata at lower soil moisture levels, and may outcompete the MWH in such situations.

In water-limited environments, such as Midwestern old-fields, and even occasionally in old-fields in the Southern Appalachians, maintenance of high water use efficiencies could be important. MWH had the lowest WUE despite having the highest *A*, due primarily to its much higher $g_s$, while WUE for the other two cytotypes were similar. All cytotypes showed increases in WUE with time, driven primarily by the sharper ontogenetic decreases in $g_s$ than *A*. This would be beneficial to this species because it would help these species cope with the hotter and drier conditions that occur later in the season, and where water would be more limiting.

After imposition of water stress, stomatal closure caused rapid increases in WUE for both Midwestern cytotypes and even more so for EH (Fig.11). Drastic decreases in $g_s$ were mostly responsible for the increased WUE, although *A* suffered large declines also. Nearly complete stomatal closure moderated further increases in WUE for the rest of the experiment. Ten days after rewatering, none of the droughted plants had recovered $g_s$ to pre-stress conditions, which resulted in the maintenance of high WUE right to the end of the experiment. This suggests that severe water stress imposes irreversible declines on gas exchange in these plants, but more work is necessary regarding the dynamics of drought stress to understand how this species may cope with future climate change.

A review of the literature shows that no generalizations concerning the impacts of polyploidy on water use patterns apply broadly to all species. Greer et al. (2018) reported that triploid clones of quaking aspen (*Populus tremuloides*) had higher WUE, but also higher $g_s$, making them potentially more susceptible to drought than diploids. For the temperate annual grass, *Brachypodium distachyon*, WUE was associated with the aridity

of the environment and tetraploids, which primarily occupied drier habitats, had higher WUE than diploids, especially under water stress conditions (Manzaneda et al., 2012).

Plants from dry environments also exhibit other adaptations, such as larger biomass allocation to root systems (Eziz et al., 2017). Buggs and Pannell (2007) studied tetraploid and diploid populations of *Mercurialis annua* in Spain and showed that diploids were superior competitors under drought stress and predominated in both mesic and dry areas. Furthermore, hexaploids were less drought tolerant than the diploids, despite being localized to more arid areas. In this case, the diploids appeared to be superior competitors and were projected to displace the hexaploids over time. This situation is comparable to what I found, where the diploids appear to be better tolerators of soil moisture stress, but the hexaploids do better in more mesic environments, contributing to habitat segregation among the cytotypes.

These results leave open the question of why diploids are absent in the East. Many studies suggest that polyploids should have wider environmental tolerances that predispose them to being able to colonize a wider array of habitats (Madlung, 2012). For instance, all invasive *S. altissima* in Asia and Europe are hexaploids (Etterson et al., 2008; Sakata et al., 2013; Verloove et al., 2017). In Japan, it was first introduced in 1897 as an ornamental and in the 1980s became naturalized throughout the country with most introductions arising from the Southern United States and just a few from the Midwest (Sakata et al., 2015). Surprisingly, relatively few studies have examined the innate physiological tolerances of such plants (but see Maherali et al., 2009 and Ramsey, 2011). If diploids, on the other hand, are more tolerant of stress than hexaploids, one might

speculate that they should outcompete them in old-field habitats, and yet even in the Midwest, they are the least abundant among the three cytotypes (Etterson et al., 2016).

One explanation for this apparently paradoxical finding is that there may be an evolutionary "cost" to greater stress tolerance that results in an inability to compete in environments where stress levels are lower. Plants produce Reactive Oxygen Species (ROS) in response to both temperature and drought stress (Suzuki and Mittler, 2006; Cruz de Carvalho, 2008) and these can damage cell membranes, resulting in cell death (Gechev et al., 2006; Van Breusegem and Dat, 2006). Plants have evolved a suite of enzymatic and non-enzymatic compounds to detoxify ROS before they can do damage to the leaf (Waszczak et al., 2018), but their upregulation and production come at a "metabolic cost" to the plant by diverting carbon away from growth processes. In stressful situations, such plants would perform better than less well-adapted genotypes and could therefore outcompete them. However, plants that have evolved in the absence of extreme stress may allocate fewer resources to detoxifying ROS because in such environments, competition with other species is more intense and favors allocation to growth instead (Grime, 2001). This would enhance the competitive status of hexaploids in these situations. For example, if high elevation mountain old-fields are less stressful than old-fields in the Midwest, then EH may have evolved to be more competitive against its diploid progenitor, thus preventing MWD from invading and establishing in the East. This may also explain the paucity of MWD from the potentially shadier habitats favored by MWH in the Midwest, where the two cytotypes co-exist (Etterson et al., 2016). An analogous situation was found for wild geraniums (*Geranium carolinianum*) exposed to $SO_2$ pollution in Georgia (Taylor et al., 1986). Plants adjacent to nearby power plants,

which at that time produced excessive amounts of atmospheric $SO_2$, were shown to have evolved resistance to $SO_2$ and had higher photosynthetic rates than geraniums native to unpolluted locations, but only when under $SO_2$ stress. In the absence of $SO_2$ stress, the reverse was true, and sensitive genotypes had higher photosynthetic rates, which Taylor *et al*. (1986) attributed to the innate costs of evolving $SO_2$ resistance.

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# VITA

Katie Rose Krogmeier was born in Wilmington, NC to Rita and Perry Krogmeier. She graduated from E.A. Laney high school in June 2014. The following autumn, she entered Appalachian State University (ASU) to study Biology, and in December of 2018 was awarded a Bachelor of Science degree with a minor in Geology. During her time as an undergraduate, she pursued many paths within the realm of Biology, but found her passion of plants through an apprenticeship with a local woman. This experience introduced her to the vast diversity of the Southern Appalachia flora and taught her the medicinal and practical qualities of local plant species. Through this, she began to volunteer many hours in the ASU Herbarium, eventually leading to her senior project digitizing the Tater Hill Plant Preserve Collection.

Before graduating, Katie wanted to learn more about the aspects of plants and decided to pursue a Master of Biology degree at ASU. Her graduate research led her to plant ecophysiology that expanded her understanding of plants and their importance in ecology and society. Her continued pursuit of knowledge opened several opportunities for her including teaching assistantships where she taught Botany and Introductory Biology labs within the Biology department, and a research assistantship funded by an NSF grant that funded the building and maintenance of a phenology garden for long-term flora data monitoring. During her second and final year working on her master's thesis, she also obtained an adjunct professor position at Lee's McRae College teaching Botany, as well as a conservation internship conducting biological surveys on Wilson Creek in North Carolina.

Katie received her Master's Degree in Biology in August of 2021. She is an active botanist, martial artist, and conservationist of natural ecosystems. She is always eager to continue in her pursuit of knowledge so that she may have a voice in changing the way humans treat the natural ecosystems that we are a part of.