

2023

Drought effects on biofuel feedstock production by *Populus trichocarpa*

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Drought effects on biofuel feedstock production by *Populus trichocarpa*

Marvin Wright

**Thesis submitted to the
Eberly College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Master of Science
in
Biology**

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Dr. Jennifer Hawkins, Ph.D.**

Department of Biology

**Morgantown, West Virginia
2023**

Keywords: Drought Stress, Osmotic Adjustment, Climatype, Genetic Variability, Stomatal Control

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Abstract

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Marvin Wright

As the world population continues to increase, so does the need for sustainable sources of fuel. Biofuels are of particular interest and could be an economically feasible fuel source given the right conditions. *Populus trichocarpa*, is a rapidly growing plantation species that, in addition to having a fully sequenced genome available for study, displays a wide range of phenotypic traits among genotypes. By analyzing these differences in both plantation and more controlled greenhouse settings, we aimed to discover which genotypes performed the best under drought conditions, and which physiological mechanisms granted them that high performance. In the field, differences in heights and stress tolerance among genotypes were observed, and 60 genotypes of differing water-limitation resistance were selected for further measures. No differences between resistance groups were seen in the physiological measures taken, yet the more resistant genotypes had higher stress tolerance indices and grew taller than susceptible genotypes from similar latitudes. The greenhouse study confirmed the water-limitation resistance rankings for 80% of the genotypes and found that resistant genotypes expressed greater midday stomatal control, enabling them to conserve water. Despite this temporary shutdown to photosynthesis, resistant genotypes assimilate carbon at a higher rate than the susceptible genotypes and can maintain their growth advantage. The quick response rate to water-limited conditions correlates with latitude and water availability of the collection site for the clones, suggesting that clones that do not regularly experience water-limitation are more sensitive to it and are able to make short-term adaptations to avoid such conditions. Further evaluation will be needed to examine if these short-term adaptations can maintain growth over extended periods of drought or on marginal lands in order for these genotypes to be a viable candidate for a rotational crop used for biofuel production.

Acknowledgements

I would first like to thank West Virginia University for providing me opportunities to learn and develop my skills as a biologist, to find my passion for teaching science, and of course for funding me along the way. I would also like to thank the Center for Bioenergy Innovation for allowing me to join the team and collaborate with inspired scientists from around the globe and for funding my small part of the project. I will forever be grateful for my advisor, Dr. Jonathan Cumming, and all the guidance, wisdom, and entertainment he has provided along this journey. I am further grateful for the other faculty members on my committee, Dr. Stephen DiFazio and Dr. Jennifer Hawkins, and for their guidance in my learning and research, as well as to the faculty members who taught me how to teach: Dr. Katrina Stewart, Dr. Kevin Barry, and Dr. Stephanie Young. There are too many to name, but I could not have completed my research without the aid of the other biology graduate students in the department or the many undergraduate students who have helped in our lab. Finally, I would like to thank my family for their love and support along the way: my awesome parents and siblings, lovely wife, and even the dog.

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1. Background and hypothesis

1.1 Background

1.1.1 Drought as a global issue

Mean global temperatures have risen 0.8°C since 1880 (Hansen et al., 2010). With environmental effects from the rising average such as changes in phenology and shifting ranges (Walther et al., 2002) and shifting in genotype frequencies (Root et al., 2003) of both plants and animals already being observed, it is essential to evaluate future effects of predicted continual temperature increases on ecosystems. A consequence of increasing temperatures is changes in precipitation patterns, and how this may contribute to effects on ecosystems. Models indicate increases in both severe weather events as well as droughts, with long-term droughts becoming as high as three times more likely to occur (Sheffield & Wood, 2008).

Drought stress effects plants at both the macro and micro level, hindering their likelihood of survival if the plant cannot cope with these effects. At the whole plant level, reductions in turgor pressure in plant cells due to limited water availability limit leaf expansion and root elongation and reduce overall growth (Farooq et al., 2009). Water limitation will also negatively impact nutrient acquisition and transport, and while a general decline of absorption with increased drought is expected, the magnitude of this response will vary between species and genotypes within species (Garg, 2003). As increased incidence and severity of droughts begin to occur, species or genotypes with greater drought tolerance will become more competitive than those without such tolerances, leading to changes in ecosystem and community composition and function.

1.1.2 Plant adaptations to drought stress

Plants with an ability to cope with drought can be placed into two categories: avoiders or tolerators. Avoiders employ mechanisms to avoid the stresses associated with low water availability. One mechanism to avoid drought is having a short life cycle or growing season that avoids peak drought times (Araus et al., 2002). Another mechanism of avoidance involves adjusting water loss through stomatal control. As the vapor pressure differential (VPD) changes with increased temperatures outside the plant, water loss due to transpiration increases if the plant does not close its stomata, either partially or completely. A plant which has greater control over its stomata is likely to lose less water through transpiration, and therefore have a higher water use efficiency (WUE). WUE is the relationship between amount of water used and the amount of biomass produced, with a plant using less water to produce the same amount of biomass being the more efficient (Kramer, 1983). A deeper or more extensive root system may also allow a plant to avoid drought (Turner et al., 2001). A plant with a root system that can reach down to the water table will have access to water that plants with shallower roots cannot reach, thereby increasing its competitiveness, while a more expansive root system will allow increased water uptake by accessing a greater volume of soil.

Morphological differences or changes in a plant may also increase the ability to avoid drought. These include changes in leaf anatomy of the plant, such as leaf area (Jones & Corlett, 1992) or leaf orientation (Zlatev & Lidon, 2005). Smaller leaves reduce solar heat loading and increase thermal dissipation, and cooler leaves lead to less transpiration and therefore a higher WUE of the plant. Similarly, the orientation of a leaf avoids direct sunlight, and some species may even curl their leaves during peak light hours. Plants with a waxy outer barrier on leaves and stems reduce water lost through trans-cuticular transpiration.

Drought tolerant species have developed physiological means to cope with the effects of water limitation. These include osmotic adjustment, cell stability and elasticity, and through other molecular controls. Osmotic adjustment is a process by which a plant may maintain water relations, driving water uptake to aid in overcoming the effects of drought. The accumulation of compatible solutes such as sugars, organic acids, or salts within the plant cells reduces the osmotic potential to maintain gradients to the plant, thereby maintaining water uptake and the turgor pressure necessary within the cells (Osmolovskaya et al., 2018). The stability of a plant's plasma membranes is another important factor in drought tolerance and can be used to score an individual plant's tolerance (Bajji et al., 2001). A membrane that is more tolerant of changing will better cope with the decrease in turgor pressure associated with drought. This helps the plant under drought conditions and speeds up recovery after the drought period has ended.

Molecular adaptations also play an important role in a plant's tolerance. These occur via the process of gene regulation, by which the expression of certain genes may be up or down regulated. Many genes have been observed to be differentially regulated under drought conditions, and this regulation aids in the plant's ability to cope with drought conditions (Kavar et al., 2008). The plants ability to up or down regulate genes encoding aquaporins, which increase the conductivity of water within a plant by providing a shorter, easier pathway through the cells (Maurel & Chrispeels, 2001), or stress proteins, such as heat shock proteins, that aid in maintaining the stability of other proteins when under temperature stress (Gorantla et al., 2007), play a major role in the plant's ability to cope with limited water conditions. Indeed, all the traits of drought stress tolerant plants are under transcriptional control, thus finding plants with greater and more sensitive controls is an important step in identifying which species or even genotypes within species will better tolerate the rising global temperatures.

1.1.3 *Populus* as a feedstock for biofuel production

Identifying more drought tolerant species/genotypes is critical in the face of growing global demand for two resources: food and fuel (Barnes et al., 2011). The combination of a growing population and decreased crop yields due to climate factors could have severe ramifications. In some cases, maize (*Zea mays*) for example, the crop is used for both food and biofuel production, and changes in production due to climate change will place strain on its demand (Edgerton, 2009). Fortunately, there are other sources of biofuels that would not take land away from food production. Understanding the growth process and drought coping mechanisms of these species would be doubly beneficial in aiding in the production of biofuels on less land and allowing the remainder to be used exclusively for food production. One such genus is that of poplar (*Populus*).

Species in the genus *Populus* exhibit extensive genetic variation and capacity to respond to their environment (Wilkins, et al., 2009). Poplar is an ecologically and economically important species, as it is used not only for biofuel production, but also for wood, paper, and pulp (Tuskan, 1998). The genome of black cottonwood (*P. trichocarpa*) has been fully sequenced, allowing it to be used as a model species and to examine the molecular controls that it may have to cope with drought.

Our study utilizes this knowledge and the vast number of genotypes within *P. trichocarpa* to identify genes and pathways responsible for various drought tolerance or avoidance strategies. Our study utilizes two studies: one of a plantation of *P. trichocarpa* in Boardman, OR, and the other in the greenhouse at West Virginia University in Morgantown, WV. The plantation in OR consists of over 1000 genotypes of *P. trichocarpa*, where 60 had been deemed as either drought “resistant” or “susceptible” (30 of each) based on growth differences

between well-watered and droughted individuals. All measurements were taken on these selected individuals. The WV study further refined this subset to the 10 high and low performers that exhibited the greatest overall height, to gain further knowledge on the largest producing genotypes.

1.2 Hypotheses

Study 1 – A common garden experiment in Boardman, OR with well-watered and water-limited treatments of 358 genotypes of Populus trichocarpa were examined for physiological and morphological differences that could explain differences in resistance to drought stress.

1. *P. trichocarpa* lines will express genotypic differences in height in response to drought stress, leading to some genotypes growing better than others.
2. *P. trichocarpa* lines will vary in physiological and morphological measurements taken.
3. The more drought resistant genotypes will express drought avoidance traits, namely the accumulation of compatible osmolites, decreased stem water potentials, and elevated stomatal control.

Study 2 – A greenhouse experiment using the tallest genotypes from the Boardman study from both the drought resistant and drought susceptible groups further examined the physiological traits that could separate the groups and compared growth and physiological measures with climatic variables from the collection sites of the clones.

4. Rankings of drought resistant or susceptible from the field study will be consistent with growth performance in the greenhouse.

5. Differences in growth will be due to the resistant genotype's abilities to control water loss through osmotic adjustment and stomatal closure.
6. Climatic variation in the sites of origin of the clones could further explain adaptations seen in some genotypes but not others.

2. Genotypic variation in *Populus trichocarpa* in response to water limitation in a field plantation in eastern Oregon, USA

2.1 Introduction

Global climate change is expected to alter patterns of precipitation worldwide (Hansen et al., 2010). Of particular concern is a predicted increase in the severity and duration of droughts (DABANLI, 2019; Sheffield & Wood, 2008). Drought stress affects many plant processes, including photosynthesis and transpiration (Rao & Chaitanya, 2016), turgor pressure for growth, the partitioning of fixed carbon (C) among metabolic demands, and the generation of reactive oxygen species that broadly damage cell structure and function (Shumilina et al., 2018). As a result, plants experiencing water limitation often have reduced growth and yield. While irrigation may offset some of the impacts of drought, numerous production systems, including woody biomass plantations, cannot be sustainably irrigated and will exhibit diminished productivity under water limitation.

Populus trichocarpa (black cottonwood) Torr. & A. Gray ex. Hook. is a common western North American hardwood species that is commonly grown in plantations for use as wood, pulp, and as a biofuel feedstock (Tuskan, 1998)). It is also a model tree species due to its ease of propagation, genetic variation, and fully sequenced genome (A. M. Brunner et al., 2004). While ideal as a wood-producing crop species due to its rapid growth, *P. trichocarpa*, along with many of the other cottonwoods, is a riparian species that is particularly susceptible to drought stress (Rood et al., 2003). Many studies, both physiological and genetic, have focused on *Populus* (spp.) responses to drought (Wilkins et al., 2009; Yan et al., 2012; C. Yin et al., 2005), yet our understanding

of the physiological and genetic linkages underlying responses to water limitation of *P. trichocarpa* and how that might affect future yields is still limited.

Plant adaptations to drought conditions help maintain growth or limit drought-induced injury despite limited access to water. A genotype that exhibits greater stomatal responsiveness, for instance, will limit transpirational water loss (Li et al., 2017), whereas one with a deeper root system may access water in deeper soil horizons (Pushpam et al., 2018). Leaf morphology can also aid in the ability to cope with drought, with smaller leaves remaining cooler and reducing leaf area experiencing water loss (Geeske et al., 1994). Plants may also reduce cell water potential through the accumulation of compatible solutes, such as proline or sugars, thereby increasing their ability to take up water from the soil (Chaves et al., 2003). These mechanisms of resistance/avoidance maintain greater water acquisition and higher water use efficiency (WUE), allowing for greater biomass accumulation per unit of water acquired.

Previous studies on *Populus* have highlighted extensive interspecific and intraspecific variation in response to water limitation. Many of these acclimation mechanisms are the result of local adaptation to the environment in which the tree existed. For example, a study on *P. deltoides* and *P. trichocarpa* and their hybrid progeny found that osmotic adjustment occurred in the hybrid offspring as well as the *P. deltoides* parent, but not in *P. trichocarpa*, suggesting that osmotic adjustment may not be a mechanism used by *P. trichocarpa* (Tschaplinski & Tuskan, 1994). Two clones of *P. nigra* originating from contrasting environments exhibited differing rates of photosynthesis and superoxide dismutase expression in response to water-limited conditions (Regier et al., 2009). Characterization of drought responses of *P. kangdingensis* included a decrease in biomass due to a reduction in photosynthesis, with the reduction caused by a decrease in stomatal conductance, an adaptation to control water loss and increase WUE.

Additionally, *P. kangdingensis* exhibited the ability to osmotically adjust to increase water uptake from the soil (C. Yin et al., 2005).

Populus trichocarpa is an upland and riparian species found growing broadly across western North America. Its range encompasses a variety of environments, from California through British Columbia into Alaska. While this region exhibits extensive variation in daylength, temperature, and water availability, its growth in river valleys and its obligate outcrossing nature may limit the evolution of climatypes and the expression of traits conferring adaptation. Gornall and Guy (2007) found that photosynthesis (A) and stomatal density increased with latitude among *P. trichocarpa* provenances and speculated that these differences were the result of the shorter growing season experienced in northern climes. However, there were no patterns in water use efficiency (WUE) and height growth was negatively correlated with both A and stomatal conductance (g_s) in these genotypes. McKown et al. (2014) noted that many phenological traits that would influence biomass accretion covaried with latitude, daylength, and/or temperature, with daylength and summer heat:moisture index at location of origin being the main variables separating 461 genotypes of *P. trichocarpa*. Associations of ecophysiological variables and geoclimate were not strong.

The present study aimed to evaluate genetic variation among *P. trichocarpa* lines in response to sustained water limitation in the field with the long-term goal of identifying genotypes for production in water-limiting environments. We assessed a suite of variables linked to drought resistance in an association population grown under controlled water availability in eastern Oregon, USA to identify traits for future selection of drought-resistant lines for biomass production.

2.2 Methods

2.2.1 Site establishment

Cuttings of poplar clones were collected from a common garden in Corvallis, OR in January 2015, in addition to collection sites across Oregon, Washington, and California. Cuttings were propagated in a greenhouse at Northern Arizona University and then stuck in Cornell potting mix (1:1:1 sphagnum peat moss, coarse vermiculite, horticultural perlite) in D-40 containers (Stuewe and Sons, Tangent, OR, USA) and watered as needed as clones developed. Plants were maintained in a greenhouse with maximum temperatures of 21°C days and 13°C nights. Fertilization began as soon as buds broke with Peter's Professional 20-20-20 water soluble formula at a rate of 30 ppm twice weekly. Following 4 months of growth in the greenhouse, plants were moved outside to harden over the summer and winter.

Clones were outplanted in the Boardman site in May 2016. Descriptions of the soil (Gebre et al., 1998) and climate (Tschaplinski et al., 2006) at this site have been previously given. Clones were randomly planted into four blocks (one replicate in each block) with two each to receive full and reduced irrigation. Differential water treatments commenced immediately, delivered through a drip irrigation system with emitters placed at the base of each clone

Differential irrigation treatments were established based on weekly potential evapotranspirational demand for Boardman, OR using coefficients scaled for poplar (Gochis and Cuenca 1998). Calculated irrigation treatments delivered 100 and 59% of PET in 2017 and this treatment regime was maintained through 2019. Differential water delivery began in late June and ceased in mid-October each year and was delivered starting at 1800h daily. Daytime gravimetric soil water content was measured at a depth of 6" in August 2018 was 4.49 ± 0.76 and $2.29 \pm 0.41\%$ for full- and reduced-water treatments, respectively ($n = 13$, $P = 0.018$ for the

difference, Table 2.1). Pre-dawn water potentials of trees in 2018 were -0.238 ± 0.011 and -0.336 ± 0.012 MPa for full- and reduced-water treatments, respectively ($n = 124$, $P < 0.001$ for the difference, Table 2.1).

2.2.2 Determination of extreme genotypes (Drought Resistant/Susceptible)

The relative change in total tree height, measured in winter 2018, between the drought and control treatments was used as a proxy to evaluate the response to water limitation of the clones in the trial. A correction was made for fine-scale spatial heterogeneity by regressing the raw ramet heights to the spatial coordinates using a thin plate spline (TPS) function with the R package “Fields” (Douglas et al. 2017). This correction was performed independently for the two treatments. A linear regression was then conducted with the corrected average height in the control treatment as the independent variable and the same estimate for the water limitation treatment as the response (Figure 2.1). The residuals of this model for each genotype were used as an estimator of the response in growth to water limitation, with positive residuals representing water-limitation resistant genotypes and clones with negative residuals water-limitation susceptible genotypes. After discarding the genotypes with less than two measured ramets in each treatment, the remainder were ranked and the top and bottom 30 were chosen as the “high” (resistant) and “low” (susceptible) performing genotypes, respectively. In addition to this approach, we used the TPS corrected heights to calculate two additional values for each genotype. The tolerance index (TI) was calculated from the ratio of growth under water-limited conditions to fully watered conditions. The stress tolerance index (STI, Negrao et al. 2017) was calculated as:

$$\text{Stress Tolerance Index} = \frac{H_{\text{WW}}}{H_{\text{WW average}}} \times \frac{H_{\text{WL}}}{H_{\text{WW average}}}$$

Where H_{WW} and H_{WL} are the heights of the genotype on the wall-watered and water-limited sides of the plantation, respectively, and $H_{WW \text{ average}}$ is the height of all trees on the well-watered side. A genotype with a higher STI demonstrates a greater ability to tolerate stress due to water limitation, as the STI compares the genotypic response to the population response to water stress (Negrao et al. 2017). The selected genotypes with their TI and STI values and the geographic origin of the parent tree from which they were cloned are given in Table 2.2.

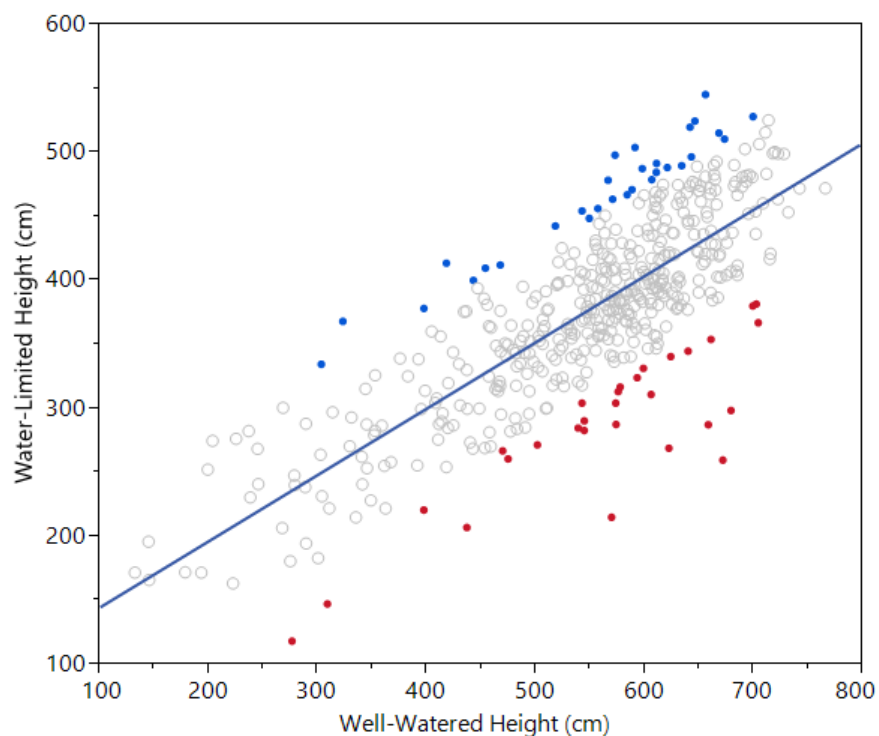


Figure 2.1. TPS-corrected height responses to drought after two growing seasons. Drought resistant genotypes appear in blue while drought susceptible genotypes appear in red.

2.2.3 Physiological and morphological measurements

In July of 2019, a suite of physiological and morphological measures was taken on leaves on the west side of trees over a three-day period. Stomatal conductance (METER SC -1 porometer, METER Group, Pullman, WA, USA) and leaf chlorophyll (SPAD 502Plus meter, Konica

Minolta, Inc., Osaka, Japan) were taken from the same leaf. Leaf temperatures were collected using an infrared thermometer (Extech Instruments Dual Laser InfraRed Thermometer, Extech Instruments, Nashua, NH, USA). Two additional lower leaves were taken to later determine the leaf area and dry weight, which were then used to calculate specific leaf area (SLA) of the leaves. Another leaf was collected and frozen on dry ice until they could be stored at -80°C . These leaves were used to test for the concentration of osmolites in the leaves using a Wescor Vapro osmometer (ELITechGroup, Logan, UT, USA). Briefly, a sample of this leaf was repeatedly freeze-thawed and placed into an Eppendorf tube with a hole in the bottom nested in a second tube and centrifuged to extract cell sap. Sap samples were analyzed for osmotic potential.

Daytime stem water potentials were taken between 1000 and 1600 h daily (time was recorded as a covariate). A leaf was sealed in a plastic bag and covered in aluminum foil for 30 minutes to bring the leaf water potential into equilibrium with the stem water potential, following the procedure of Meron et al. (Meron et al., 1987). This leaf was then excised and, while the leaf remained in the bag, the water potential was taken using a Scholander pressure bomb (SoilMoisture Equipment Corp., Goleta, CA, USA). In order to test the equilibrium leaf water potential with the soil, pre-dawn water potentials were taken between 0200 and 0600 h using an additional lower leaf from the same tree and using the same approach as the stem water potential leaves.

In December of 2019, core samples were collected from the trees. Cores were dried and the half of the core representing the growth during the final half of the last season was ground to homogenize with a Wiley mill until fine enough to pass through a 20-mesh screen. 1 mg of the ground cores were packed into tin capsules for carbon isotope analysis. Samples were then analyzed for stable C isotopes ($\Delta^{13}\text{C}$) with a ThermoFisher Delta V+ isotope ratio mass

spectrometer by the Central Appalachians Stable Isotope Laboratory, University of Maryland Center for Environmental Science. Intrinsic water-use efficiency (iWUE) was then calculated using the following formulas from Farquhar et al (1989):

$$\Delta^{13}\text{C} = a + (b - a) * \left(\frac{c_i}{c_a}\right)$$

Where a is the fractionation due to CO₂ diffusing through the stomata (4.4‰) and b is the fractionation of CO₂ by Rubisco (27‰). c_i/c_a can then be used to estimate iWUE with the following formula, where c_a is ambient CO₂ concentration (409 ppm):

$$iWUE = c_a \left(1 - \frac{c_i}{c_a}\right) * 0.625$$

2.2.4 Statistical analysis

Effects of water limitation on physiology were compared using nested analyses of variance (ANOVA) with genotype nested within resistance groups, with Tukey's HSD post hoc tests used to determine differences between genotypes and water treatments. For variables collected over a prolonged daily period (stomatal conductance, water potentials), time-of-day was used as a covariate in the analyses. Variables were log transformed when necessary to meet ANOVA criteria. Principle component analysis (PCA) was used to identify relationships among physiological measures and to determine to what degree each measure affected the overall tolerance of the genotype. Statistical analyses were performed using SAS JMP 16 (SAS Institute, Raleigh, NC, USA).

2.3 Results

The well-watered side of the plantation had an increased SWC 47% higher than that of the water-limited side ($4.49 \pm 0.76\%$ vs. $2.29 \pm 0.41\%$, $p=0.018$, Table 1), giving an effective difference in treatment levels. While no height differences were seen among well-watered genotypes due to resistance rankings, water-limited resistant genotypes were taller than their susceptible counterparts ($463.91 \pm 11.24\text{cm}$ vs. $277.98 \pm 11.44\text{cm}$, $p<0.0001$, Table 2) and had higher TIs (0.843 ± 0.015 vs. 0.503 ± 0.016 , $p<0.0001$, Table 2) and STIs (0.853 ± 0.039 vs. 0.513 ± 0.039 , $p<0.0001$, Table 2). Regressing the STI of each genotype vs. the average height of well-watered trees of that same genotype showed a clear separation of the resistant genotypes from the susceptible genotypes (Figure 2).

Table 2.1. Soil Water Content (SWC) and pre-dawn water potentials for well-watered and water-limited treatments.

Treatment	SWC %	Predawn Ψ MPa
Well-Watered	4.49 ± 0.76	-0.238 ± 0.011
Water-Limited	2.29 ± 0.41	-0.336 ± 0.012

Table 2.2. Genotype ids with their associated heights, resistance rankings, and geographical location of origin.

Genotype	Resistance	WW Height (cm)	WL Height (cm)	TI	STI	Latitude	Longitude
56	Resistant	658	544	0.827	1.153	46.099	-122.878
275	Resistant	593	503	0.848	0.96	47.862	-121.781
409	Resistant	568	477	0.84	0.873	48.506	-122.022
422	Resistant	636	488	0.768	1	48.523	-122.014
425	Resistant	520	441	0.849	0.739	48.525	-122.016
819	Resistant	675	509	0.754	1.108	47.087	-122.175
856	Resistant	608	477	0.785	0.936	47.861	-121.791
1016	Resistant	648	523	0.808	1.092	44.034	-123.862
1025	Resistant	572	462	0.807	0.852	42.553	-123.546
1036	Resistant	643	518	0.806	1.075	42.413	-123.125
1038	Resistant	455	408	0.896	0.599	42.43	-123.056
1042	Resistant	444	399	0.897	0.571	42.238	-123.071
1069	Resistant	325	367	1.129	0.384	41.233	-122.645
1079	Resistant	305	333	1.093	0.327	40.872	-121.552
1103	Resistant	399	377	0.944	0.485	40.774	-123.327
1156	Resistant	574	497	0.864	0.919	42.85	-123.182
1181	Resistant	559	455	0.814	0.819	43.917	-123.008
1201	Resistant	544	453	0.832	0.794	46.448	-116.862
CA-04-03	Resistant	551	447	0.812	0.794	39.331	-120.395
CA-05-01	Resistant	469	411	0.875	0.621	39.317	-120.502
CMBF-28-4	Resistant	586	466	0.795	0.879	49.95	-125.25
GW-9577	Resistant	645	495	0.768	1.029	47.167	-122.383
GW9578	Resistant	590	470		0.893	47.167	-122.383
GW-9587	Resistant	599	486	0.811	0.939	47.867	-122.633
GW-9589	Resistant	670	514	0.767	1.109	47.45	-123.033
GW-9591	Resistant	622	487	0.782	0.977	47.45	-123.033
GW-9860	Resistant	612	483	0.789	0.953	47.683	-121.917
GW-9861	Resistant	701	527	0.751	1.19	47.683	-121.917
SLMB-28-4	Resistant	613	490	0.8	0.968	50.217	-125.817
YALE-27-3	Resistant	420	412	0.982	0.558	49.567	-121.4
207	Susceptible	673	258	0.383	0.561	47.096	-122.22
368	Susceptible	681	297	0.436	0.652	48.492	-122.16
1024	Susceptible	595	323	0.542	0.618	42.541	-123.501
1031	Susceptible	575	286	0.497	0.531	42.623	-123.601
1032	Susceptible	471	265	0.563	0.403	42.43	-123.261
1061	Susceptible	546	281	0.515	0.495	41.284	-122.848
1082	Susceptible	278	117	0.42	0.105	40.811	-121.507
1093	Susceptible	399	219	0.549	0.282	40.675	-122.827

1098	Susceptible	579	315	0.544	0.589	40.693	-122.931
1121	Susceptible	439	206	0.469	0.291	39.945	-120.952
1134	Susceptible	311	146	0.47	0.146	39.571	-120.732
1138	Susceptible	310	90	0.292	0.09	39.567	-120.602
1145	Susceptible	575	303	0.526	0.561	41.847	-122.905
1150	Susceptible	541	283	0.524	0.494	42.683	-123.35
1160	Susceptible	571	214	0.374	0.393	42.94	-123.264
1167	Susceptible	546	289	0.529	0.509	43.217	-123.369
1171	Susceptible	663	353	0.532	0.753	43.415	-123.325
1207	Susceptible	503	270	0.537	0.438	46.126	-115.787
1212	Susceptible	476	259	0.544	0.398	46.708	-120.468
BLCG-28-1	Susceptible	601	330	0.549	0.639	49.833	-125.183
DENB-17-2	Susceptible	577	312	0.541	0.58	52.833	-126.7
GS-018-12	Susceptible	704	380	0.54	0.863	NA	NA
GW-9583	Susceptible	660	286	0.433	0.608	47.167	-122.383
GW-9899	Susceptible	701	379	0.54	0.855	47.117	-122.117
GW-9950	Susceptible	626	339	0.542	0.684	46.05	-121.933
GW-9953	Susceptible	642	343	0.535	0.71	46.15	-123.333
GW-9964	Susceptible	706	366	0.518	0.832	45.95	-121.95
KLNA-20-3	Susceptible	544	303	0.556	0.531	51.117	-125.583
LILC-26-4	Susceptible	510	363	0.712	0.596	50.5	-123
SLMD-28-3	Susceptible	624	268	0.429	0.538	50.283	-125.867

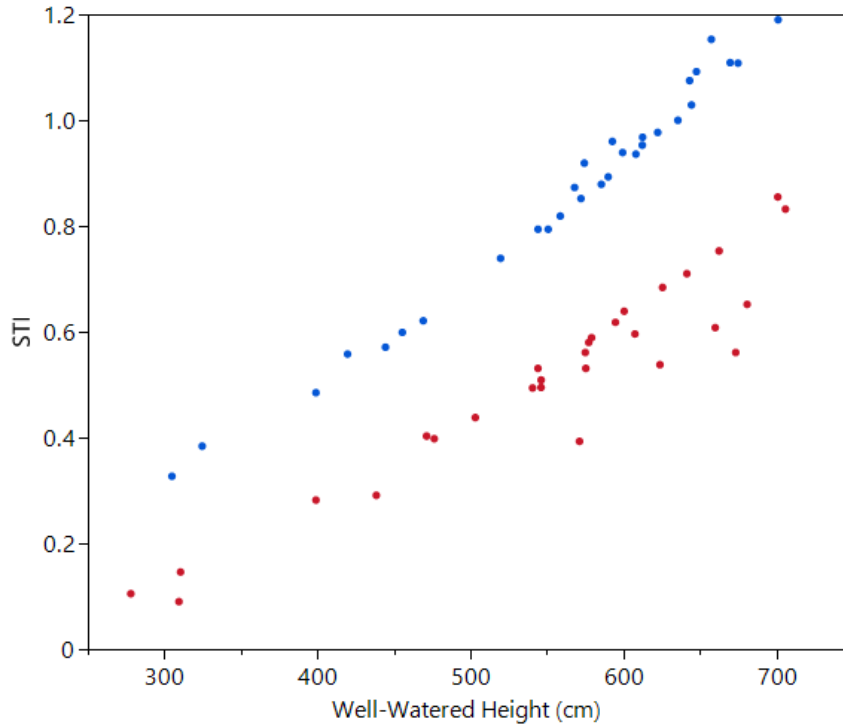


Figure 2.2. Stress Tolerance Index (STI) by height of genotypes in the well-watered treatment. Susceptible genotypes appear in red and resistant genotypes in blue.

For physiological measures, there were differences due to water-stress treatment observed for several variables (Table 3). Water-limited trees had higher osmolalities ($p=0.0002$), leaf temperatures ($p=0.0391$), specific leaf areas ($p=0.0455$), and lower stem water potentials ($p<0.0001$) and $iWUE$ ($p<0.0001$). None of the measures, however, varied between resistance groups, although osmolality ($p<0.0001$), SPAD ($p<0.0001$), SLA ($p=0.0331$), $iWUE$ ($p<0.0001$), stem water potential ($p=0.0016$) and stomatal conductance ($p=0.0496$) all showed variation among genotypes. There was no observed interaction between resistance group and water treatment for any of the measurements.

Table 2.3. Effect of water limitation on growth and physiological parameters of water-limitation resistant and susceptible genotypes of *P. trichocarpa*.

Water Treatment ¹	Resistance Group ²	MO ³ mOsm kg ⁻¹	Leaf Temp °C	SPAD	SLA cm ² g ⁻¹	iWUE μmol mol ⁻¹	Ψ MPa	g _s mmol m ⁻² s ⁻¹
WW	Resistant	764.7 ^{ab}	29.78 ^a	50.94 ^a	10.12 ^a	596.9 ^a	-5.33 ^a	447.9 ^a
	Susceptible	737.2 ^b	30.16 ^a	50.15 ^a	10.10 ^a	603.0 ^a	-5.74 ^a	411.4 ^a
WL	Resistant	796.3 ^a	31.12 ^a	51.19 ^a	10.62 ^a	581.5 ^b	-6.49 ^b	445.1 ^a
	Susceptible	802.5 ^a	31.01 ^a	50.44 ^a	10.55 ^a	585.8 ^b	-6.59 ^b	443.4 ^a
	<i>P</i> _{Treatment} ⁴	0.0002	0.0391	0.7106	0.0455	<0.001	<0.001	0.5954
	<i>P</i> _[High/Low] ⁵	0.5690	0.8127	0.5214	0.8561	0.1061	0.2377	0.4951
	<i>P</i> _{Genotype} ⁶	<0.001	0.0796	<0.001	0.0331	<0.001	0.0016	0.0496
	<i>P</i> _{Interaction} ⁷	0.1801	0.6501	0.9722	0.9221	0.6064	0.1307	0.4274

¹WW = well-watered, WL = water-limited

²Resistant and susceptible according to genotype Stress Tolerance Index (Table 1).

³MO = osmolality.

⁴Significance of the Water Treatment effect.

⁵Significance of the High/Low group effect.

⁶Significance of Genotype within resistance [High/Low] group effect according to the Wald test.

⁷Significance of the [High/Low] group × water treatment interaction.

Principle component analysis of measures that do not express short-term variability showed two clear clusters (Figure 3). These groupings do not, however, seem to show any patterns of following either water treatment or resistance groupings. Separation along the first principal component is driven positively by iWUE and TPS predicted height and driven negatively by latitude of collection (Table 4). The second principal component was positively driven by latitude, TPS predicted height, and stem water potentials, while being negatively driven by SPAD. These components explain a combined 54.1% of the variation among these variables.

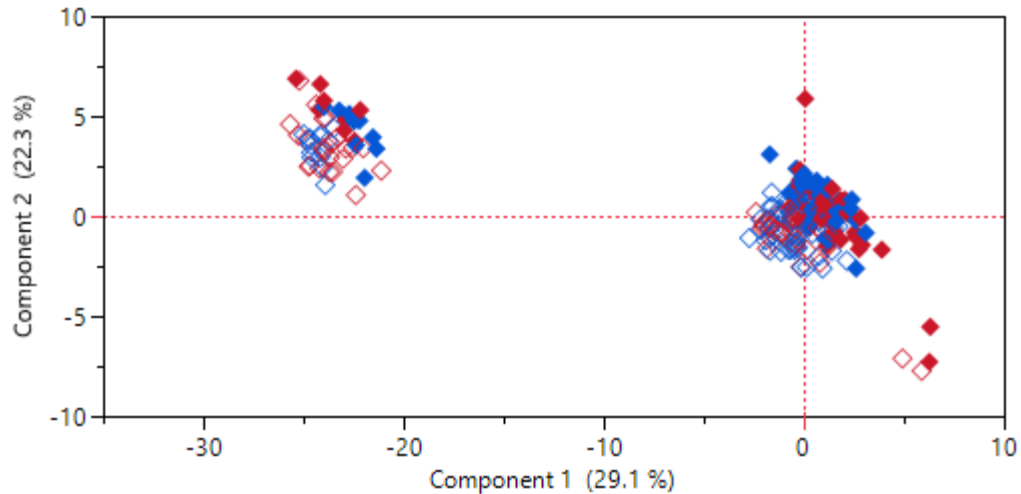


Figure 2.3. PCA of physiological measurements. Red indicates susceptible genotypes and blue indicates Resistant, while filled diamonds represent individuals on the well-watered side of the plantation and empty diamonds show individuals on the water-limited side.

Table 2.4. Principle component loadings for responses of *P. trichocarpa* genotypes to water limitation.

Component	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Variation %	29.1	22.3	17.0	13.9	11.9	5.89
TPS Predicted Height	0.4885	0.4945	-0.0540	0.1455	0.4133	-0.5672
Latitude	-0.4069	0.5137	-0.1524	0.4573	0.3362	0.4743
SLA	-0.1894	0.1537	0.8531	-0.3305	0.3193	0.0374
iWUE	0.6346	-0.1133	-0.0859	-0.2433	0.3343	0.6372
Ψ	0.2978	0.5599	0.2265	0.0139	-0.7084	0.2105
SPAD	0.2613	-0.3760	0.4327	0.7752	-0.0221	0.0388

Further examination of the influence of the latitude of collection site showed (1) a positive correlation of water-limited height with latitude ($p < 0.0001$, $R^2 = 0.1396$, Figure 4), (2) a separation of genotypes by resistance level, with resistant genotypes being taller than susceptible genotypes from similar latitudes ($p < 0.0001$, figure 4), and (3) negative relationships of latitude with SPAD ($p = 0.0011$, $R^2 = 0.0477$, figure 5) and iWUE ($p < 0.0001$, $R^2 = 0.1601$, Figure 5).

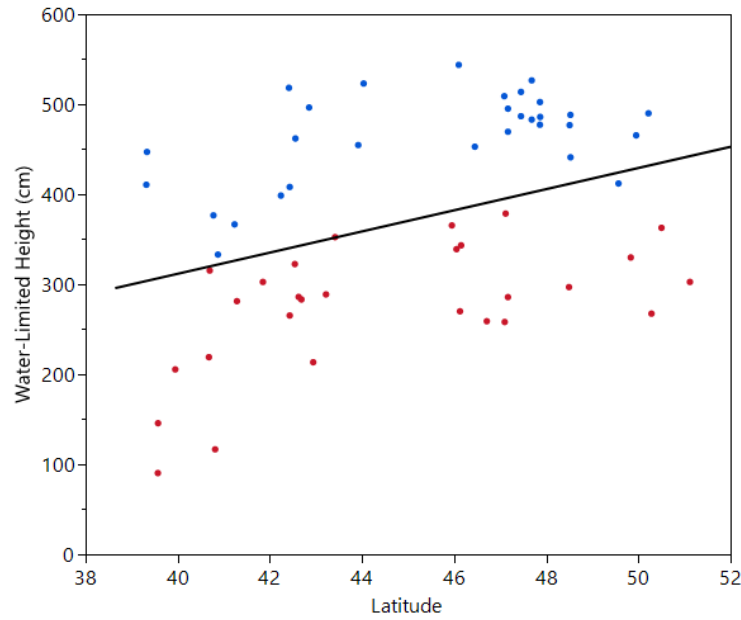


Figure 2.4. Relationship between latitude of collection site and height of genotypes in the water-limited treatment. Colors separate resistant (blue) and susceptible (red) genotypes.

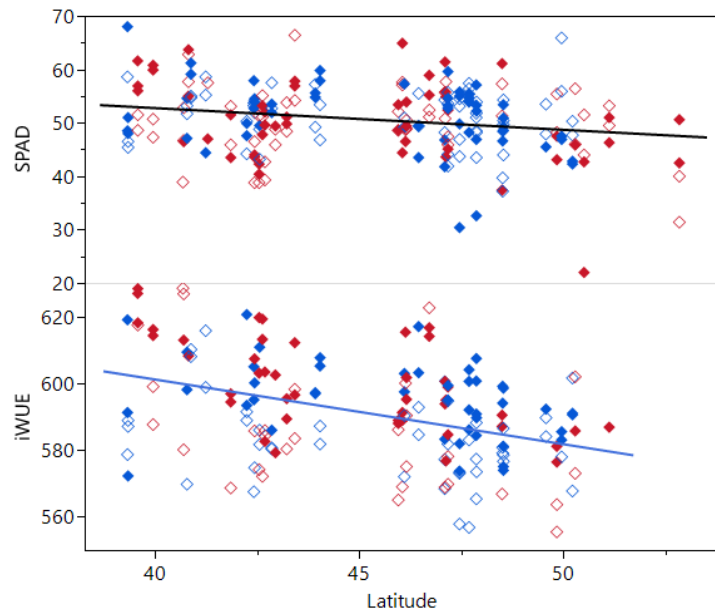


Figure 2.5. Relationships of latitude of collection site with SPAD and intrinsic water use efficiency. Colors separate resistant (blue) and susceptible (red) genotypes, while filled diamonds show well-watered genotypes and open diamonds show water-limited genotypes.

2.4 Discussion

This study aimed to find if different lines of *P. trichocarpa* would express genotypic variation in response to drought stress, and if so, if we could identify physiological variables that could be selected for to find viable candidate genotypes for use in plantations for biofuels production. We saw a large range of heights of the genotypes, with a similar range of reductions in heights between well-watered and water-limited individuals (table 2). This difference in height changes allowed us to select for drought resistant and drought susceptible genotypes based on the ratio of their water-limited and well-watered heights. These ranking were originally made based on 2018 heights and TIs, but heights, TIs, and STIs from this growth season after coppice confirm these ranking and suggest they will hold true through harvests and regrowth. The results further indicate that for any given growth rate, resistant genotypes will have a higher STI (figure 2).

While genotypic differences were seen in several of the physiological traits measured (osmolality, SPAD, SLA, iWUE, Ψ , and g_s), no difference was seen in these variables, or any other variable based on resistance rankings. Plant adaptations were made in response to drought, where water-limited trees accumulated osmolytes that in turn decreased their stem water potentials, a response that has been previously noted in *Populus* species (Silim et al., 2009; Tschaplinski et al., 2006, 2019). Specific leaf area, the ratio of leaf area to mass, another phenotypic trait that *Populus* species have been shown to use as an adaptation to drought stress (Marron et al., 2003), was increased for water-limited genotypes. While a reduced specific leaf area can result in increased water retention in the leaf, the increased surface area of a leaf with an increased specific leaf area can lead to increased rates of photosynthesis. This larger leaf area, in addition to the reduction of leaf coverage from the smaller trees in the water-limited treatment, is likely the cause for the increase in leaf temperature among these trees.

The lack of separation between resistant and susceptible groups goes against our hypothesis that we would see physiological adaptations that led to the genotype's differential growth rates and STIs. There are possible explanations as to why we did not see such differences in this study: (1) the site established in Boardman had low replication (n=2), (2) the site had variability in watering efficiency, particularly on the droughted side, and (3) field studies are inherently more variable due to the lack of control over all variables. The most influential of explanation is likely the low replication, where a dead replicate or minor effect of variability in watering schema or other unpredictable variation can cause low confidence in statistical results. A study with more replication and greater control over watering and outside variables would likely yield significant results in regard to physiological effects on resistance rankings.

The PCA analysis showed two distinct clusters, with a few outliers (figure 3). Clusters included both resistant and susceptible genotypes from both treatment levels and were therefore not driven by either of these variables. Inclusion of group and treatment as Z-variables in the analysis confirmed this result. The main separation, that along PC1, was largely driven by TPS predicted height, intrinsic water use efficiency, and latitude. An examination of the effect of latitude on water-limited heights showed a positive relationship, where genotypes from more northern climates grew taller on average than the lower latitude genotypes (figure 4). Furthermore, resistance ranking had a significant effect here, showing that resistant genotypes would grow taller under water-limited conditions than their susceptible counterparts from similar latitudes. We can conclude that while latitude plays an important role in the growth capabilities and drought resistance of *P. trichocarpa*, there are still other factors at play – be they physiological or otherwise – that influence the growth and resistance ability of the genotypes.

Intrinsic water-use efficiency has been previously examined in both water limitation and *Populus*-focused studies. Relationship of iWUE with latitude in *Populus* species is not clear, where some studies (i.e. Soolanayakanahally et al. 2009) report an positive relationship between iWUE and latitude, and others (i.e. (Gornall & Guy, 2007) observed no relationship between the

variables. Our site showed a negative correlation between the two variables, as well as a decline in iWUE for genotypes under drought stress. iWUE is a function of carbon assimilation to stomatal conductance, and a decrease in iWUE can be caused either by decreased assimilation or increased conductance (Urrutia-Jalabert et al., 2015). Here, it is likely that decreased iWUE is due to the lower rates of assimilation in the droughted treatment, indicated by the shorter heights of these trees. It is also worth noting that we only took conductance readings over a two-day period mid-summer, while iWUE is an integrated measurement over the entire season. It is possible that we would see discrepancies between these variables that do not follow the general trend of the literature. While it has been shown that iWUE has been increasing over the last century (Leonardi et al., 2012; Linares & Camarero, 2012; Marchand et al., 2020), the increase in iWUE is not always accompanied by increased growth for trees not experiencing drought stress (Gentilesca et al., 2021). The common trend, however, is that trees experiencing drought stress will have increased iWUE and lowered growth rates (Fichot et al., 2009; Linares & Camarero, 2012; Sun et al., 2018). We saw the expected lowering of growth rates, but not the increased iWUE. The negative correlation of iWUE with latitude, however, does support the increased growth rates of genotypes from higher latitudes (figures 4 and 5).

While we saw the expected genotypic variation in physiological traits and confirmed our resistance groups with plant height and STI, we were not able to determine which factors drove the increased growth of the resistant genotypes. Site complications such as low replication and inherent variability of field sites could have had significant effects here, and a follow up study in a more controlled greenhouse setting could be utilized to better focus on the selected variables. Regardless of understanding the exact mechanisms involved, we can conclude that *P*.

trichocarpa does vary in its responses to water limitation, and that consideration must be made when selecting genotypes for use as a plantation crop.

3. Variation in water limitation resistance in *Populus trichocarpa* is associated with a syndrome of physiological plasticity

3.1 Introduction

Global climate change is altering agricultural and forest landscapes through elevated temperatures and vapor pressure deficits and changes in the intensity and duration of drought (Masson-Delmotte et al., 2019). Understanding species- and genotype-specific effects of these changes is critical in maintaining reliable and sustainable agricultural and agroforestry yields as the environment continues to change. *Populus* genotypes are commonly grown as rotational crop species in Europe, North America, and Asia (Dickmann et al., 2001; Kumar, 2006) due to their multiple uses as feedstocks for wood pulp and fiber, biofuels, and timber (Zalesny et al., 2004). Plantations of poplar are generally started from cuttings, and clonal genotypes/hybrid crosses may be selected to maximize site-specific productivity. Extensive variation exhibited throughout *Populus* genera/hybrids (Mckown et al., 2014; Smith et al., 2011; Tschaplinski et al., 2006) may provide opportunities to establish plantation resilience in the face of future climate uncertainty (Bernier & Schoene, 2009) and allow for identification of genotypes that can more successfully be utilized on marginal lands in order to alleviate pressure on lands currently used for agriculture.

Climate-induced reductions in water availability are already impacting terrestrial ecosystems (Clark et al., 2016; Füssel, 2017; Lobell & Gourdji, 2012). Exposure to drought induces a suite of physiological impacts and metabolic changes in plants that reflect both stress and acclimation responses (Farooq et al., 2009; Krasensky & Jonak, 2012; Osmolovskaya et al., 2018). Water limitation and associated osmotic stress reduces cell turgor and cell expansion, with concomitant reductions in stomatal conductance that reduce photosynthetic carbon (C) fixation.

Perturbations to metabolism and electron transport lead to the production of reactive oxygen species (ROS) that subsequently damage organelles and cells (Krasensky & Jonak, 2012; Zhou et al., 2007). Water limitation impairs cell homeostasis, reduces C fixation, shifts C partitioning away from primary to secondary metabolism, and induces premature senescence, all of which constrain plant productivity (Blum, 2017; Clark et al., 2016; Farooq et al., 2009b).

There are a variety of responses plants have evolved to cope with water limitation (Araújo et al., 2011; Blum, 2017; I. Brunner et al., 2015; Chen & Jiang, 2010; Comstock, 2002). The suite of physiological and growth adjustments of species to water limitation have been used to categorize species as isohydric stress avoiders, those exhibiting behaviors that maintain steady-state plant water potentials, or anisohydric stress tolerators, those which allow water potentials to decline under water scarcity with limited impacts on plant physiology (Aroca, 2013; Polle & Rennenberg, 2019; Roman et al., 2015). Drought stress responses of various *Populus* species and crosses have been previously examined and indicate that *Populus* species vary in their responses to drought, with some able to maintain their water balance through the accumulation of sugars and secondary compounds (Tschaplinski et al., 2006, 2019), others exhibiting phenotypic changes in specific leaf area (Marron et al., 2003), while others show altered stomatal control to limit transpirational water loss (Monclus et al., 2006; Silim et al., 2009).

This variation in *Populus* response to water limitation may reflect the extensive variation in traits among species as well as within species across their broad geographical and climatic ranges. Given its role as a model woody feedstock species, evaluating the genetic variation in water-limitation tolerance in *P. trichocarpa* and its association with adaptation across the species' range may identify genotypes and the underlying genetic attributes that would contribute to sustainable feedstock production on marginal lands prone to drought and under changing climates.

Although limited to western North America, the range of *P. trichocarpa* extends from Mexico to Alaska, USA, across 30 degrees of latitude, and from sea level in Alaska to 2100 m elevation in British Columbia, Canada, with extensive variation in environmental variables across this range (Burns and Honkala 1990), providing potential to evaluate genetic variation in this species. In drier environments, however, *P. trichocarpa* is restricted to protected river valleys and other sites where water is more plentiful, suggesting that adaptation to water scarcity may be limited.

In this study, we compared the response of 20 *P. trichocarpa* genotypes, 10 “high performers” and 10 “low performers” under water-limiting conditions in a previous field trial, to a drydown period in a greenhouse setting. These genotypes were subjected to an acute drought period during which physiological measures were assessed that may identify traits contributing to water-limitation resistance in this species. We hypothesized that the high performing genotypes would exhibit a combination of stomatal limitation of water loss and osmotic adjustment, which would reduce transpirational water loss and increase water uptake during the drydown period and contribute to resistance in these lines.

3.2 Methods

3.2.1 Cutting propagation and establishment

The genotypes used in the current experiment were from the Center for Bioenergy Innovation (CBI) clone collection and were selected using height growth tolerance (TI = height under water-limited conditions/height under well-watered conditions) indices from a field water-limitation experiment in Boardman, OR, USA, where clones were experimentally subjected to irrigation regimes representing 100 or 60% of evapotranspirational demand (Table 1). Stem cuttings of these

genotypes were taken from a clone bank in Clatskanie, OR in December 2019. Cuttings were treated with a 0.5% Zeroto[®] (BioSafe Systems, East Hartford, CT, USA) solution and refrigerated until the trial began.

Propagation began in August of 2020, when the cuttings were once again treated with Zeroto[®] solution and placed in a flowing nutrient (0.5 mM Ca(NO₃)₂, pH 5.6) hydroponic system in a greenhouse for two weeks to allow the cuttings to root. After two weeks, rooted cuttings were transplanted into 10 cm × 7 cm × 24 cm pots containing SunGro[®] Professional Growing Mix (Sun Gro Horticulture, Agawam, MA, USA) and were watered three times a week for four weeks to ensure adequate water was provided for establishment. There were nine plants for each genotype.

The *P. trichocarpa* clones in the CBI collection have been collected from known locations over the past 15 years and each has a referenced geospatial location. From these geospatial locations, we extracted a suite of 15 climatic variables (Supplementary Table S1) at monthly resolution from the TerraClimate data repository (Abatzoglou et al., 2018). Each climatic variable was aggregated by season (winter, spring, summer, fall) over the past 30 years (from January 1990 to December 2019). For example, “spring precipitation” was derived by computing the average over all precipitation values observed in March, April, and May from 1990 through 2019. This process was repeated similarly for each climatic variable and season, creating a total of 60 climatic features.

3.2.2 Dry down

For the drydown, each genotype was split into three subsets of three plants each. Subset 1 was measured at the start of the drydown (t = 0) and at the end of the drydown (t=3). This subset was watered throughout the course of the experiment and served as the well-watered (WW) control.

Subset 2 was measured for the next two measurement periods ($t = 1, 2$), and subset 3 was measured at the end of the drydown ($t = 3$). Subsets 2 and 3 served as water-limited (WL) treatments. Measurement times were one week apart, for a total of three weeks of drydown.

Soil water content was measured with a Campbell Scientific Hydrosense II soil moisture probe (Campbell Scientific, Logan, UT, USA) for each pot, as well as soil water potential using TDR probes (Teros 21, METEK, Pullman, WA, USA) on a subset of plants. Soil water content was measured throughout the application of the drought.

3.2.3 Plant measurements

Plant height was measured from the base of the plant to the tip, and leaf count was taken by counting all fully developed leaves. SPAD values were recorded on the third fully developed leaf from the top of the plant using a SPAD-502 meter (Konica Minolta, Ramsey, NJ, USA). A CIRAS-3 Photosynthesis System (PP Systems, Amesbury, MA, USA) was used to record photosynthetic CO_2 assimilation (A), stomatal conductance (g_s), transpiration (E), and leaf internal CO_2 concentration (C_i) on the same leaf. Gas exchange data from the $t = 1$ were inadvertently lost. Stem water potentials (Ψ_{stem}) were measured on the fourth fully developed leaves from the tops of plants by sealing them in a plastic bag and covering them in aluminum foil (Meron et al., 1987). These leaves were left for ~30 min to equilibrate with the stem, and then removed for water potential measurement using a Scholander pressure bomb (Soil Moisture Systems 3005, Goleta, CA, USA). After the water potential was taken, the leaf was removed from the plastic bag, the petiole was recut under water to re-establish the transpiration stream, and the petiole was submerged in water for 24 hours to rehydrate in the dark. Following rehydration, leaves were frozen in liquid N_2 and stored at -80°C . To measure leaf osmolyte concentrations, frozen leaves were thawed at room temperature and then crushed using the

method of Clifford et al. (1998) to extract osmolytes. Osmolyte samples were assessed using a 5520 Vapro vapor pressure osmometer (Wescor, Inc., South Logan, UT, USA).

3.2.4 Statistical analysis

Effects of water-limitation treatment on growth and physiological parameters were compared using nested analyses of variance (ANOVA) with genotypes nested within resistance groups. Variables were log or square root transformed as needed to meet the assumptions of ANOVA. Post hoc Tukey's HSD tests were performed to determine significant differences between treatment means.

Stress Tolerance Index (STI) – The stress tolerance index (STI, Negrao et al. 2017) was calculated for growth responses (aboveground biomass, belowground biomass, height). The STI was calculated as:

$$\text{Stress Tolerance Index} = \frac{Y_{WW}}{Y_{WW \text{ average}}} \times \frac{Y_{WL}}{Y_{WW \text{ average}}}$$

Where Y_{WW} and Y_{WL} are measured responses for well-watered and water-limited treatments for each genotype, and $Y_{WW \text{ average}}$ is the growth response under control conditions for the entire population evaluated. A greater STI for a genotype indicates a greater degree of water-limitation tolerance. The STI accounts for genotypic variation to water stress across the population response to identify superior genotypes (Negrao et al., 2017).

Cluster and principal component analyses (PCA) were undertaken to determine relationships between responses and which parameters had the greatest influence on final grouping

of individuals. These statistical analyses were undertaken using SAS JMP 15 (SAS Institute, Raleigh, NC, USA).

Associations between clone response and climate variables were undertaken using the explainable artificial intelligence method, iterative random forest (iRF) (Basu et al., 2018). iRF is chosen as a predictive model since (i) it can discover high-order nonlinear interactions between inputs, (ii) rank each feature by the amount of variance explained in the outputs, and (iii) scale to supercomputing systems that enable the training of large statistically robust models (Cliff et al., 2019). In this case, seasonal averages of climatic values are used as inputs to predict clonal response outputs. For each response variable, we considered two sets of samples to train iRF models, control and water limited. Each iRF model consisted of 10,000 decision trees for prediction stability, and 100 iterations to ensure convergence of feature importance values. Further, samples were split into 80% training and 20% test sets using 5-fold cross validation to prevent overfitting, and cross validation fold sets were resampled 100 times for additional robustness. Each iRF model was trained using one compute node (taking ~120 seconds per model) on the Oak Ridge Leadership Computing Facility's Summit supercomputer at Oak Ridge National Laboratory, USA. Normalized feature importance scores were then derived by computing the average importance score of each input variable divided by the sum of all feature importance scores at the last iteration of iRF. The normalized feature importances were then used to rank each input by how much variance it explains in the output, thereby drawing associations between clone response and climatic variables.

3.3 Results

3.3.1 Soil drydown

A significant decline in soil water content (SWC) occurred over the three-week drydown period, with the greatest decline occurring during week 1 (Figure 1). Over the course of the experiment, SWC of pots containing water-limitation resistant genotypes declined by 98.1% from 38.3 to 0.7% H₂O, while pots containing susceptible genotypes decreased by 93% from 40.4 to 2.6% H₂O. The difference in SWC between water-limitation resistance groups was evident as early as 7 d after water withholding, with resistant lines extracting more water from pots than the susceptible lines (Figure 1). This was explored further by including plant height as a covariate in the analysis, which indicated that this difference in SWC may be driven by plant height ($P = 0.015$), which was greater in the water-limitation resistant lines, and not necessarily due to differences in water-limitation sensitivity of the two groups ($P = 0.789$). However, differences in SWC of resistant and susceptible groups in the well-watered controls were significant even with plant height as a covariate, indicating that the resistant clones were more effective than susceptible clones at extracting water from the rhizosphere (Table 2).

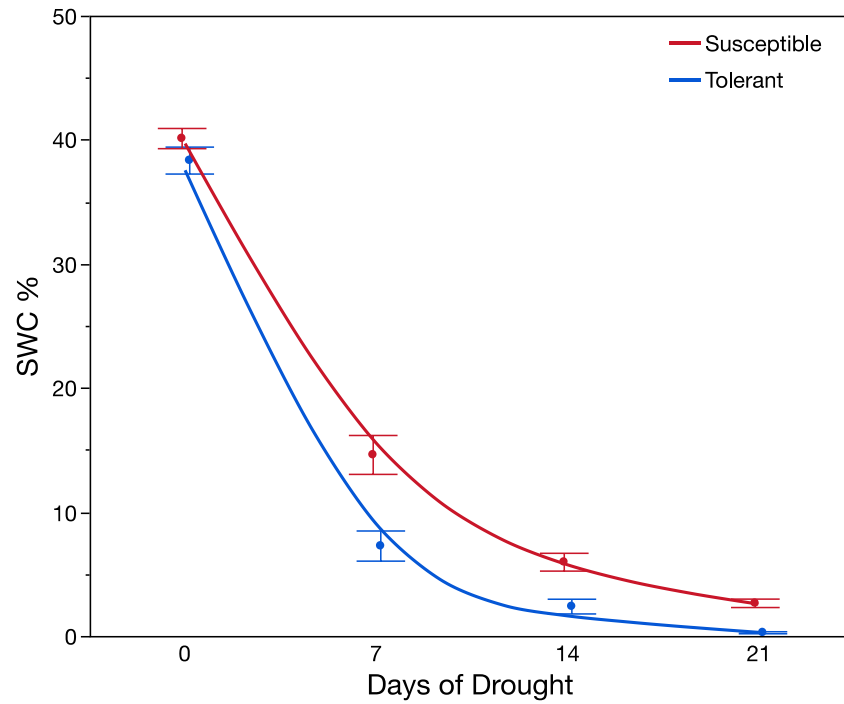


Figure 3.1. Change in soil water content of pots containing water-limitation resistant and susceptible genotypes during the 21-day drydown period. Error bars represent standard errors of the means for $n = 6$ plants.

Table 3.1. *Populus trichocarpa* genotypes and their growth responses to water limitation in the field and greenhouse.

Genotype	Field		Greenhouse					
	Height TI ¹	Height STI ²	Height TI	Height STI	AGB ³ TI	AGB STI	BGB ⁴ TI	BGB STI
BESC-56	0.827	0.876	0.672 ^{bc}	1.472 ^{ab}	0.544 ^{abc}	3.292 ^{abc}	0.242 ^{bc}	0.405 ^{bc}
BESC-275	0.848	0.730	0.650 ^{bc}	1.087 ^{bcd}	0.273 ^c	1.231 ^{de}	0.301 ^{abc}	0.196 ^c
BESC-422	0.768	0.760	0.716 ^{bc}	1.544 ^{ab}	0.546 ^{abc}	4.301 ^{ab}	0.196 ^c	0.328 ^{bc}
BESC-819	0.754	0.842	0.722 ^{bc}	1.435 ^{ab}	0.568 ^{abc}	2.601 ^{cd}	0.460 ^{abc}	0.297 ^{bc}
BESC-1156	0.864	0.699	0.737 ^{bc}	1.130 ^{bc}	0.465 ^{bc}	2.854 ^{bc}	0.326 ^{abc}	1.685 ^a
GW-9577*	0.768	0.782	0.897 ^{abc}	0.533 ^{ef}	0.540 ^{abc}	0.211 ^e	0.401 ^{abc}	0.321 ^{bc}
GW-9589	0.767	0.843	0.944 ^{abc}	1.877 ^a	0.533 ^{abc}	4.596 ^a	0.571 ^{abc}	1.171 ^{ab}
GW-9591	0.782	0.742	0.909 ^{abc}	1.122 ^{bc}	0.635 ^{abc}	2.856 ^{bc}	0.229 ^{bc}	0.433 ^{bc}
GW-9861*	0.751	0.904	0.756 ^{bc}	0.455 ^{ef}	0.586 ^{abc}	0.779 ^e	0.371 ^{abc}	0.465 ^{bc}
SLMB-28-4	0.800	0.735	0.675 ^{bc}	1.894 ^a	0.478 ^{abc}	3.726 ^{abc}	0.398 ^{abc}	0.850 ^{abc}
BESC-1024	0.542	0.470	1.153 ^{abc}	0.272 ^{ef}	1.045 ^{ab}	0.426 ^{de}	0.780 ^{abc}	0.201 ^{bc}
BESC-1098	0.544	0.447	0.812 ^{abc}	0.249 ^{ef}	1.036 ^{ab}	0.211 ^e	1.746 ^{ab}	0.177 ^{bc}
BESC-1145	0.526	0.426	0.806 ^{abc}	0.253 ^{ef}	0.440 ^{bc}	0.624 ^e	0.447 ^{abc}	0.367 ^{bc}
BESC-1171	0.532	0.572	1.045 ^{abc}	0.235 ^f	0.584 ^{abc}	0.217 ^e	0.671 ^{abc}	0.144 ^c
BLCG-28-1	0.549	0.485	0.999 ^{abc}	0.562 ^{ef}	1.023 ^{ab}	0.597 ^e	0.659 ^{abc}	0.315 ^{bc}
GS-18-12*	0.540	0.656	0.671 ^{bc}	1.083 ^{bcd}	0.537 ^{abc}	4.240 ^{ab}	0.297 ^{abc}	0.341 ^{bc}
GW-9899	0.540	0.650	1.093 ^{ab}	0.769 ^{cd}	0.998 ^{ab}	0.966 ^e	0.994 ^{abc}	0.549 ^{bc}
GW-9950*	0.542	0.520	0.822 ^{abc}	1.078 ^{bcd}	0.698 ^{abc}	3.650 ^{abc}	0.288 ^{abc}	1.091 ^{ab}
GW-9953	0.535	0.539	1.249 ^a	0.437 ^{ef}	1.257 ^a	0.518 ^e	1.520 ^a	0.483 ^{bc}
GW-9964	0.518	0.632	0.620 ^c	0.601 ^{def}	0.559 ^{abc}	0.361 ^e	0.564 ^{abc}	0.575 ^{bc}
$P_{\text{High/Low}}^5$			0.191	0.002	0.039	0.049	0.039	0.376
P_{Genotype}^6			0.018	0.004	0.037	0.004	0.056	0.012

¹Tolerance Index (TI) is the ratio of growth under water-limited conditions to fully watered conditions for each genotype. Means followed by different letters differ at $P < 0.05$ by Tukey's HSD.

²Stress Tolerance Index (STI) is product of the ratios of growth under water-limited conditions for each genotype to the population mean response under fully-water conditions and the growth under control conditions for each genotype to the population mean response under fully-water conditions. Means followed by different letters differ at $P < 0.05$ by Tukey's HSD.

³ABG = above ground biomass.

⁴BGB = below ground biomass.

⁵Significance of the High/Low group effect.

⁶Significance of Genotype within resistance [High/Low] group effect according to the Wald test.

*Genotypes that exhibited divergent field and greenhouse STIs.

3.3.2 Plant growth

The genotypes used in the current experiment were selected based on height growth TIs in a field water-limitation experiment in Boardman, OR (Table 1). Height STIs of these genotypes in the field were consistent with the height growth TIs. Height growth TIs were also used to evaluate genotype response to drydown in the greenhouse. Water-limitation susceptibility genotype TIs did not separate as categorized in the field ($P = 0.191$), and there were notable differences between genotypes within groups ($P = 0.018$), suggesting that group assignments may not be consistent, or that simple TIs are not reliable derivations (Table 1). STI analysis, which accounts for genotypic response to water limitation compared to a population response to reveal genotypes that are performing superior to others, did segregate as expected with the exception of four genotypes, two identified as water-limitation resistant (GW-9577 and GW-9861) and two susceptible (GS-18-12 and GW-9950) in the field (Table 1). These four genotypes behaved in other measures consistent with height STIs in the greenhouse. Given these large, but consistent, discrepancies (Table 1), these four genotypes were recategorized in their resistance designations for the current analysis of *P. trichocarpa* responses to water limitation.

Table 3.2. Effect of water limitation physiological parameters of water-limitation resistant and susceptible genotypes of *P. trichocarpa* following 21 d of dry down in the greenhouse. Means followed by different letters are significantly different at $P < 0.05$ by Tukey's LSD.

Water Treatment	Genotype Group ¹	SWC %	SPAD	Ψ MPa	MO ²	A $\mu\text{mol m}^{-2} \text{s}^{-2}$	Ci $\mu\text{mol mol}^{-1}$	E $\text{mmol m}^{-2} \text{s}^{-1}$	g_s $\text{mmol m}^{-2} \text{s}^{-1}$
Well-Watered	High	2.06 ^b	31.0 ^a	-0.372 ^a	219 ^c	2.85 ^a	279 ^{bc}	2.14 ^a	64.1 ^a
	Low	6.17 ^a	24.3 ^b	-0.388 ^a	250 ^{bc}	2.21 ^a	286 ^{ab}	1.61 ^a	46.7 ^{ab}
Water-Limited	High	0.36 ^c	32.3 ^a	-0.810 ^b	368 ^{bc}	0.21 ^b	322 ^a	0.36 ^b	7.0 ^c
	Low	2.18 ^b	23.1 ^b	-0.658 ^a	390 ^c	2.66 ^a	245 ^c	1.68 ^a	38.7 ^b
	SEM	1.5	1.7	0.069	43	0.29	11	0.18	5.6
	P_{Duration} ³	<0.001	0.987	<0.001	<0.001	0.001	0.908	<0.001	<0.001
	$P_{\text{[High/Low]}}$ ⁴	<0.001	0.003	0.066	0.962	0.011	0.019	<0.001	<0.001
	P_{Genotype} ⁶	0.036	0.007	0.540	0.129	0.192	0.096	0.465	0.751
	$P_{\text{Interaction}}$ ⁹	0.399	0.159	0.051	0.427	<0.001	<0.001	<0.001	<0.001

¹Resistant (High) and susceptible (Low) according to genotype Stress Tolerance Index (Table 1).

²MO = osmolality.

³Significance of the dry down Duration effect.

⁴Significance of the High/Low group effect.

⁵Significance of Genotype within resistance [High/Low] group effect according to the Wald test.

⁶Significance of the [High/Low] group \times dry down Duration interaction

Significant reductions in the heights of the plants due to the imposed drydown were evident, with reductions in growth of the water-limitation resistant lines (24.6%) being greater than those of sensitive lines (7.5%) (Fig. 2). Water availability influenced both above-ground and below-ground biomass in the same manner as height, with water-limitation induced reductions in growth that were significant in the resistant genotypes but not in the susceptible lines (Table 2).

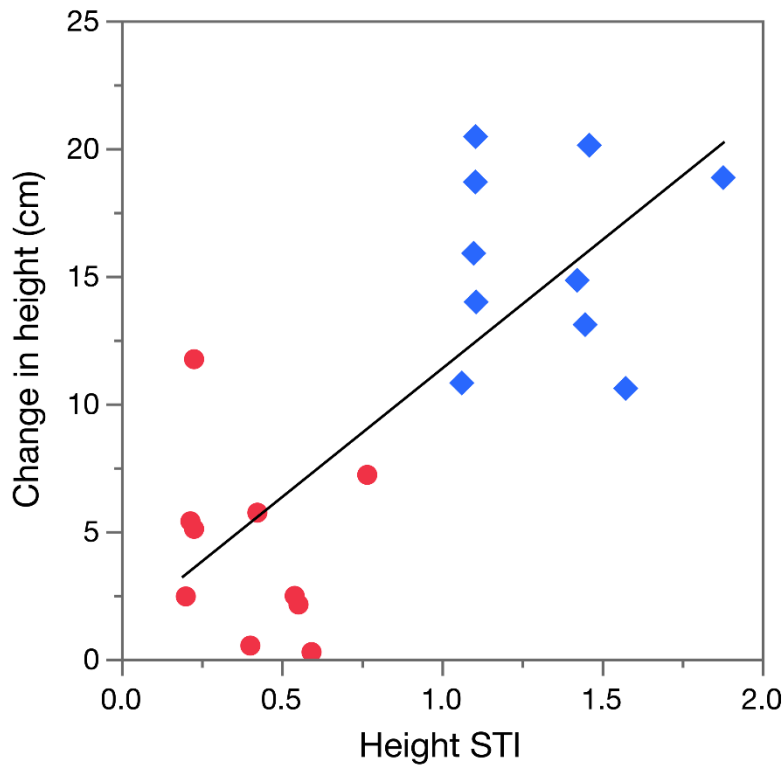


Figure 3.2. Reductions in height growth of poplar genotypes in relation to genotype water-limitation stress-tolerance index (STI). Red points represent susceptible genotypes, while blue represent resistant.

3.3.3 Physiological responses

Stem water potentials became more negative over the course of the drydown (Fig. 3), with resistant genotypes declining from -0.37 mPa to -0.81 mPa and susceptible lines from -0.39 mPa to -0.66 mPa by the third week of drydown (Table 2). The well-watered controls maintained high

Ψ_{stem} that did not differ between resistance groups (Table 2). SPAD levels of resistant clones were significantly higher than those of susceptible plants, although no difference was observed due to watering treatment and there was not an interaction between the two variables. Well-watered plants had lower concentrations of osmolytes than the water-limited plants, while no differences were noted between resistance groups (Table 2). Across all genotypes, A , E , and g_s were significantly reduced by water limitation (Table 2). However, the water-limitation susceptibility groups differed in their gas exchange responses to water limitation. Resistant lines consistently and substantially reduced g_s during dry-down compared with susceptible lines, with concomitant reductions in A and E (Table 2). Interestingly, C_i was higher in the resistant lines under water limitation (Table 2), suggesting that A was limited by both stomatal and non-stomatal factors in these genotypes.

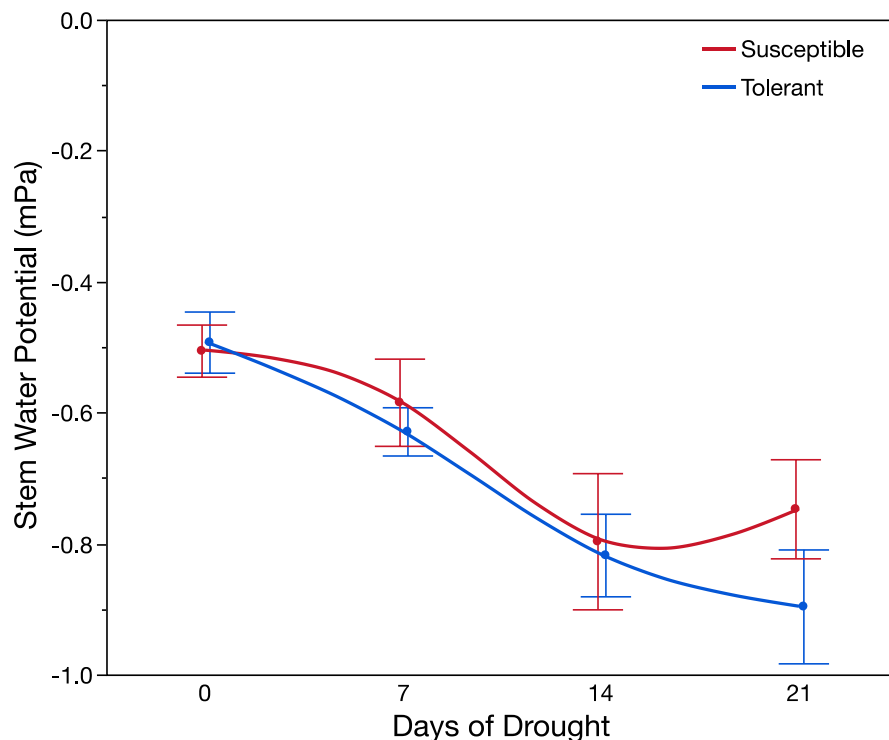


Figure 3.3. Change in stem water potentials of water-limitation resistant and susceptible genotypes during the 21-day drydown period. Error bars represent standard errors of the means for $n = 6$ plants.

Recognizing that reductions in g_s in the resistant clones may have been driven by lower SWC in these lines, we evaluated stomatal behavior across a broader Ψ_{stem} range by combining the control plants and harvests 2 and 3 responses. Assessment of stomatal response curves (Fig. 4) indicated that, over similar ranges of Ψ_{stem} , stomatal closure of resistant lines was more responsive to reductions in Ψ_{stem} than that of susceptible lines ($P = 0.035$ for the interaction).

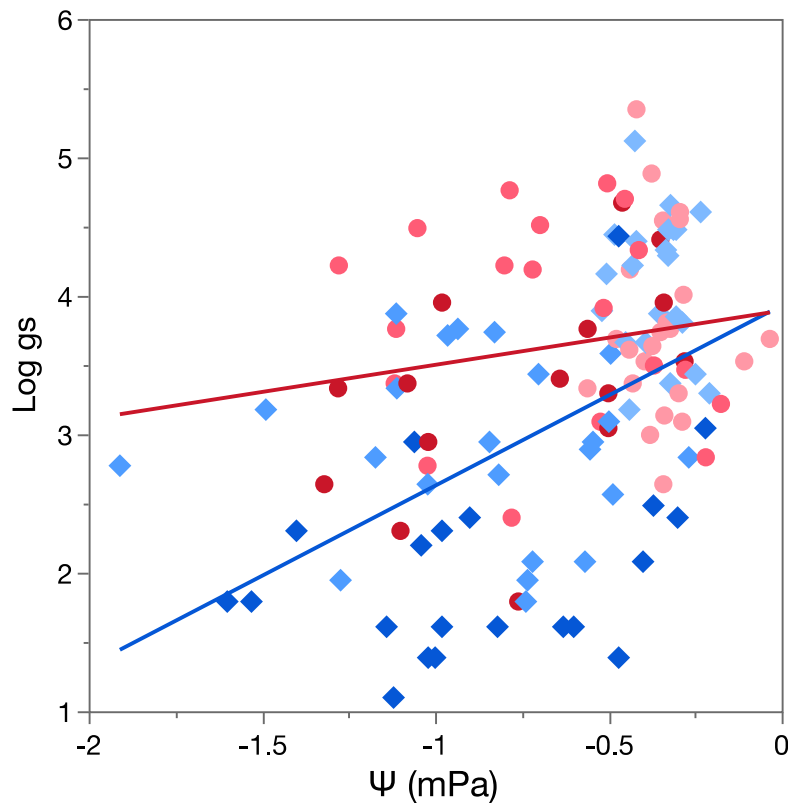


Figure 3.4. Stomatal response curves of water-limitation resistant (diamonds, blue) and sensitive (circles, pink) *P. trichocarpa* genotypes. Color intensity reflects intensity of water limitation: lightest are well-watered, medium obtained at 14 d of drydown, darkest colors at 21 d of drydown.

3.3.4 Contributions of physiological responses to water-limitation resistance

Cluster and PCA analyses confirmed resistance group responses to water limitation.

There were three clusters among the data when growth and physiological responses were

combined (Fig. 5). In comparison to susceptible genotypes, which clustered together no matter the water treatment, the control and water-limited treatments within the resistant genotype group separated based on differential responses to water availability (Fig. 5). Separation along the first PC axis was positively weighted by growth and gas exchange and negatively by osmolality and C_i , reflecting reductions in stomatal conductance and osmotic adjustment in the stress-resistant lines accompanying reductions in growth (Table 3). Separation on the second PC axis was positively weighted by final plant growth and negatively influenced by gas exchange responses, separating the resistant and sensitive genotypes (Fig. 5).

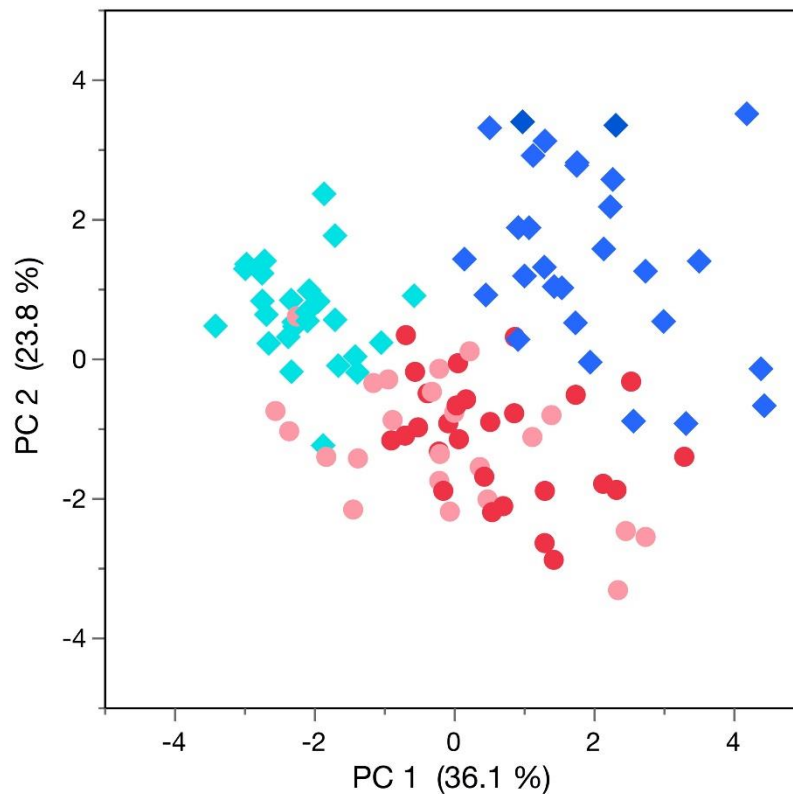


Figure 3.5. Principal components analysis of growth and physiological responses of water limitation resistant (blue diamonds) and sensitive (red circles) poplar genotypes under well-watered (darker hue) and water-limited (lighter hue) conditions. Data formed three clusters, with the water limitation sensitive genotypes clustering together under both treatments while the water limitation resistant genotypes separated into two clusters depending on water availability.

Table 3.3. Principle component loadings for responses of *P. trichocarpa* genotypes to water limitation.

Component	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6
Variation %	34.6	25.8	12.6	9.1	7.1	6.0
Height	0.295	0.864	0.041	0.014	-0.034	-0.130
Aboveground biomass	0.347	0.890	0.120	-0.061	-0.002	-0.018
Belowground Biomass	0.448	0.607	0.448	-0.091	-0.061	-0.145
SPAD	0.019	0.513	-0.353	0.652	0.137	0.387
Ψ	0.593	-0.092	0.274	-0.419	0.098	0.613
Osmolality	-0.353	-0.126	0.653	0.221	0.609	-0.080
<i>A</i>	0.866	-0.339	0.040	0.253	0.023	-0.030
<i>C_i</i>	-0.339	0.375	-0.572	-0.421	0.465	-0.045
<i>E</i>	0.902	-0.264	-0.201	0.022	0.175	-0.156
<i>g_s</i>	0.903	-0.195	-0.250	-0.010	0.190	-0.152

3.3.5 Climatype-clone response associations

The responses of *P. trichocarpa* genotypes were evaluated as functions of both latitude and the climate variables from the sites from which each clone originated. Clone STI was positively correlated with latitude, with resistant and susceptible clones clustering separately but exhibiting parallel behavior ($P = 0.007$ for the latitude effect, $P = 0.356$ for the group-by-latitude interaction) (Figure 6). STI was not correlated with longitude.

With respect to the climatological variables examined, clone STI was negatively associated with spring water deficit (DEF) and the summer Palmer Drought Severity Index (PDSI) and positively associated with summer precipitation (PPT) at the clones' home sites (Figure 6, top). Shoot biomass growth during the experiment under both full watering and water limitation was

positively associated with spring PPT. Growth was negatively associated with winter potential and actual evapotranspiration (PET and AET, respectively) under well-watered conditions but positively associated with winter PET and AET and negatively associated with spring PET in the water-limited condition (Figure 7, middle). Physiologically, stomatal conductance was strongly and positively associated with winter PDSI in the water-limited treatment only (Figure 7, bottom). Conductance was also negatively associated with the fall minimum temperature (TMIN) and positively associated with summer AET in the well-watered condition (Figure 7, bottom).

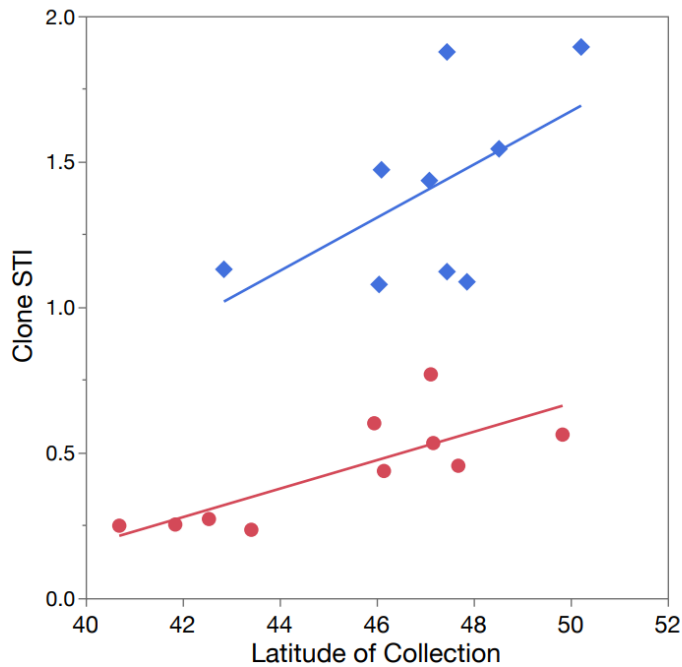


Figure 3.6. Increases in stress-tolerance index (STI) with increased latitude of collection site for each genotype. Blue diamonds represent resistant genotypes and red circles represent susceptible genotypes.

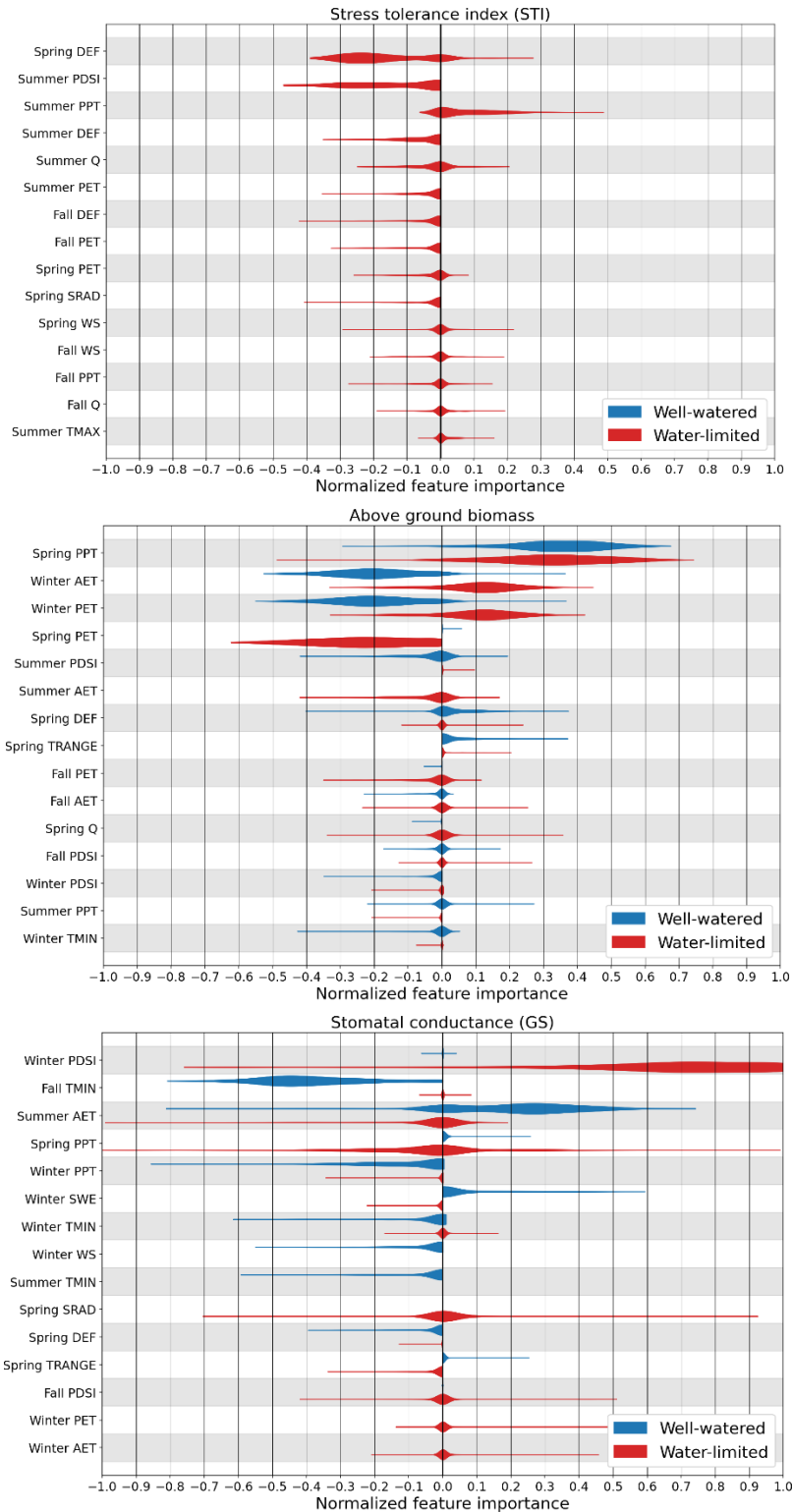


Figure 3.7. Stress response and growth associations (STI, above-ground biomass, and stomatal conductance) with climatic variables from the collection sites of the clones

3.4 Discussion

Populus trichocarpa is an important tree species, playing significant biological roles in western North American ecosystems and important economic roles as a timber and biofeedstock species. While genotypic variation to water limitation has been reported in this species, the physiological underpinnings of such variation have not been well described. Here, we evaluated the water-limitation responses of 20 *P. trichocarpa* genotypes differing in water-limitation resistance to elucidate the basis of drought resistance in this species and probed potential associations between adaptive responses and the geographic origins and climatypes of the clones.

Given the original water-limitation response designations were generated from a field trial with low replication ($n = 2$), that the plantation exhibited significant spatial variation in soil water content (Tschaplinski unpublished data), and that plant response to chronic water limitation may differ from that to episodic drydown, it was surprising that the 20 selected genotypes had an 80% concordance under field and greenhouse experimental conditions. Variation in the field and/or other discrepancies leading to categorization, for example variation in cutting vigor and performance of genotypes under early growth in the greenhouse, most likely underlie the divergent responses of four out of 20 genotypes evaluated (Table 1). We chose to analyze the current experiment based on the growth responses in greenhouse and not from the field, although the agreement between the field and greenhouse STIs was surprisingly high.

Notable differences were seen in the heights and biomass accumulation between resistant and susceptible genotypes (Table 2). Under well-watered conditions, resistant genotypes had greater than 100% increases in each of these growth variables compared to the susceptible genotypes, suggesting that they are more productive in general when resources are plentiful. This may be due in part to the resistant genotypes having higher SPAD values, indicating higher chlorophyll

concentrations in their leaves, although that did not translate into elevated photosynthesis under well-watered conditions (Table 2). We note that one-time measurements of photosynthesis do not capture diurnal or long-term differences in assimilation that may exist between genotypes (Kets et al., 2010; C. Y. Yin et al., 2006) that may underly differences in growth efficiencies between the high and low resistance genotypes of *P. trichocarpa*. Resistant genotypes did show a significant decrease in growth when water was limited, but still grew taller and produced more above-ground biomass than the water-limitation susceptible genotypes, which did not have significant reductions in growth (Table 2). The reduction in growth of the resistant genotypes in response to water limitation was driven by stomatal closure to conserve water (Martin-StPaul et al., 2017), a trait that other species of *Populus* have been shown to exhibit (Attia et al., 2015; C. Y. Yin et al., 2006). The lack of stomatal response in the susceptible genotypes would, in the long term, lead to desiccation, damage to the photosynthetic machinery, hydraulic failure, and greater loss of productivity (Henry et al., 2019; Martin-StPaul et al., 2017).

The separation of resistant/susceptible genotypes by PCA supported the divergent growth and stomatal control of gas exchange between the resistance groups (Figure 5). While both the well-watered and water-limited susceptible genotypes clustered together, the well-watered resistant genotypes clustered separately based on higher photosynthetic rates, stomatal conductance, and SPAD values (Table 3). Water-limited resistant genotypes separated from their well-watered counterparts and clustered based on low stomatal conductance, reduced photosynthetic rates, and more negative stem water potentials, demonstrating the resistant group's behavior to conserve water at the leaf level while increasing water extraction from soils (Figure 1). While this leads to a decrease in photosynthesis and, therefore, growth in the short term (Figure 2), these genotypes evidently maintain a growth advantage over the susceptible genotypes in the longer term as

evidenced in the field. This may reflect altered daily or seasonal patterns of photosynthesis of genotypes between resistance groups. The divergent stomatal behavior apparently reflects the safety-efficiency trade-off (Attia et al., 2015; Henry et al., 2019) operating in *P. trichocarpa*, where genotypes with greater stomatal conductance under high water availability exhibit greater sensitivity to leaf dehydration and close stomates at higher leaf Ψ . The significantly different gas exchange responses of the resistant and sensitive lines to water limitation in the current study reflect one component of a stress avoidance syndrome of the resistant lines to water limitation. Stomatal closure occurred sooner and at higher Ψ_{stem} than susceptible lines, thereby reducing A and E (Fig. 4) and reducing C fixation and growth (Fig. 3), which would reduce water demand. These clones also developed lower stem water potentials (Table 2), which led to greater soil water extraction (Table 2). Thus, water limitation resistance in faster growing *P. trichocarpa* clones appears to balance short-term (stomatal closure), mid-term (reduced Ψ_{stem}), and longer-term (growth cessation) responses to balance water supply and demand. Slower growing clones do not follow this syndrome and may not successfully balance water supply and demand, leading to the poorer performance in the field.

This pattern of drought avoidance in *P. trichocarpa* may also reflect earlier attainment of critical soil water content, resulting from greater E , leading to stomatal closure in resistant genotypes. The earlier and more substantial closure of stomata in the resistant lines may reflect greater sensitivity of guard cells to root-derived signals based on low SWC. It is well known that abscisic acid (ABA) derived from roots in water-deficit soils modulates stomatal closure and improves water use efficiency (Aroca, 2013; Osakabe et al., 2014). While the pathways leading to variation in ABA response are genetically complex, variation within species, as evidenced here

for *P. trichocarpa*, suggest that such variation may underlie differences in resistance to water limitation (Sanguineti et al. 1999, Monclus et al. 2006, Wu et al. 2021).

Poplars may follow either isohydric or anisohydric behavior (Attia et al., 2015), with *P. deltoides* showing lowered leaf osmotic potential, allowing for greater rates of water uptake (Tschaplinski et al., 2019). However, these responses may depend on the nature of water limitation. In *P. deltoides*, patterns of osmotic adjustment differ between cyclic or acute drought, both of which induced osmotic adjustment, although through different metabolic pathways (Tschaplinski et al., 2019). Silim et al. (2009) noted reductions in leaf water potential (Ψ_{leaf}) and g_s among nine poplar clones, which led to reductions in net photosynthesis. However, tolerant clones had lower Ψ_{leaf} and g_s , and maintained higher A at lower Ψ_{leaf} . Thus, more drought tolerant poplar species or genotypes may symptomatically maintain a higher level of A , even at lower levels of g_s , and osmotically adjust and reduce plant Ψ . While in our experiment we did not observe greater A in tolerant genotypes, the greater rate of biomass accumulation suggests a higher A on average among these genotypes coupled with greater stomatal control at low Ψ_{stem} compared with sensitive genotypes. In addition, the lower Ψ of the resistant genotypes points to osmotic adjustment as also playing a role in acclimation in these lines, which has not been previously noted in *P. trichocarpa* (Tschaplinski et al., 1994). These combined responses show, rather than isohydric and anisohydric behavior, a syndrome of drought tolerance in *P. trichocarpa*. The greater sensitivity of stomates to plant and soil water status complemented by osmotic adjustment provide candidate traits for use in future feedstock line development and production under environments with increasing and variable water limitation.

In the current study, water-limitation resistant *P. trichocarpa* genotypes were derived from environments with greater water availability during the growing season and have higher growth

rates. These clones, however, are more sensitive to short-term water limitation: they close their stomates, temporarily halting photosynthesis and ceasing growth under non-ideal conditions. In contrast, clones with lesser STI values, which maintain photosynthetic gas exchange and growth under water limitation, were derived from drier environments, as indicated by the positive association between stomatal conductance and winter PDSI. This seems to demonstrate that *P. trichocarpa* genotypes vary and express both drought avoiding (stomatal closure, growth cessation) and tolerating (continued photosynthetic gas exchange and growth, albeit at lesser rates) characteristics depending on the environment to which they are adapted. That *P. trichocarpa* exhibits only limited changes in leaf water potentials limits our using the terms “isohydric” and “anisohydric”, as have been used for many tree species, and highlights one potential limitation for the selection of *P. trichocarpa* genotypes for production in water-limited environments.

4. Evaluation of Hypothesis and Conclusions

4.1 Evaluation of hypotheses

Study 1 – A common garden experiment in Boardman, OR with well-watered and water-limited treatments of 358 genotypes of *Populus trichocarpa* were examined for physiological and morphological differences that could explain differences in resistance to drought stress.

1. *P. trichocarpa* lines will express genotypic differences in height in response to drought stress, leading to some genotypes growing better than others.

Supported. There was extensive variation in height among the trees at the Boardman site. From these differences we were able to select for the genotypes that experienced the lowest reduction in height from the well-watered treatment to the water-limited treatment as well as the genotypes that experienced the greatest reduction in height between treatments

2. *P. trichocarpa* lines will vary in physiological and morphological measurements taken.

Supported. There were significant differences due to genotype in all measures taken aside from leaf temperature.

3. The more drought resistant genotypes will express drought avoidance traits, namely the accumulation of compatible osmolites, decreased stem water potentials, and elevated stomatal control.

Unsupported. Despite the differences in growth capabilities observed, no measures taken aligned with the ranking groups. PCA analysis did show clustering driven by latitude and iWUE, but both ranking groups were present in each cluster.

Study 2 – A greenhouse experiment using the tallest genotypes from the Boardman study from both the drought resistant and drought susceptible groups further examined the physiological traits that could separate the groups and compared growth and physiological measures with climatic variables from the collect sites of the clones.

4. Rankings of drought resistant or susceptible from the field study will be consistent with growth performance in the greenhouse.

Partially supported. Two genotypes from each ranking group functioned more like the other group when grown in the greenhouse, but the remaining genotypes were consistent in their growth patterns in both the field study and greenhouse study.

5. Differences in growth will be due to the resistant genotype's abilities to control water loss through osmotic adjustment and stomatal closure.

Partially supported. Genotypes in the resistant group mainly used stomatal closure during midday to conserve water during times when photosynthesis is inefficient, but no differences were seen in accumulated osmolites between resistance groups.

6. Climatic variation in the sites of origin of the clones could further explain adaptations seen in some genotypes but not others.

Supported. Resistant genotypes are fast growers that are from wetter growing environments. Due to acclimation to these environments, they are more sensitive to water-limitation than the susceptible genotypes from drier climates, leading to more short-term changes to their physiology in an effort to avoid the drought.

4.2 Conclusions

The field study in Boardman, OR allowed us to analyze growth and drought susceptibility of several hundred genotypes and to down-select for 60 genotypes of differing water-limitation resistance. The trade-off for examining this many genotypes was a sacrifice in replication within the study, which we believe ultimately led to there being no observed differences in physiological measures between the resistance groups. When examining the growth and physiological responses of 20 of the selected clones of *P. trichocarpa*, they exhibited differing syndromes of response to a 21-day drydown in the greenhouse study, with resistant clones exhibiting a high degree of stomatal limitation of water loss and concomitant reductions in photosynthesis and growth and slight stem water potential and osmotic adjustment not exhibited by susceptible clones. Although these resistant clones had greater growth reductions under water limitation, they were more productive overall regardless of water treatment. While in both studies we saw the tolerance index of clones increased with latitude of collection, the two sensitivity groups clustered differently, suggesting other environmental variables may underly water-limitation resistance. The associations between clone stress response index, growth, and physiological variables under well-watered and water-limited conditions and climate variables from their collection sites indicated that resistant lines were derived from more mesic environments, suggesting that clones from environments with sufficient water resources are, overall, faster growing and more responsive to water deficit, allowing rapid growth under optimal conditions and rapid response to water limitation that protects the long-term health and productivity of these clones. Evaluation of these responses under long-term water limitation may identify additional acclimation systems supporting sustainable productivity on marginal soils and under future variable environmental conditions.

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