

**The Combined Effects of Fertilization and Relative Water Limitation on  
Tissue Water Relations, Hydraulic Parameters and Shallow Root Distribution  
in Loblolly Pine (*Pinus taeda* L.)**

**Edward Morgan Russell**

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**John R. Seiler, Chair**

**Chris A. Maier**

**Erik T. Nilsen**

**R. Quinn Thomas**

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**Academic Abstract**

One goal of this research was to characterize shoot tissue-level responses in loblolly pine to soil moisture limitation in combination with fertilization as well as to more severe soil moisture limitation. We found that neither fertilization alone, nor fertilization in combination with soil moisture limitation resulted in changes to shoot tissue water relations parameters classically characterized in drought response studies. More severe water limitation was necessary to elicit responses, and those responses had not been fully described previously. The more severe water limitation resulted in increased capacitance beyond turgor loss, increased relative water content at turgor loss, a more negative turgor loss point, an increased bulk modulus of elasticity, more negative osmotic potential at 100% relative water content, and an increased apoplastic water fraction. As there were indications of reduced water use and moisture stress in the absence of shoot level responses under less severe drought, such parameters are insufficient alone to characterize moisture stress in fertilized and in less severely water limited loblolly trees. Additionally, we sought a morphological or physiological explanation for the reduced transpiration and increased water use efficiency reported for fertilized trees in the Virginia Piedmont. Our characterizations of the responses of root distribution and hydraulics to limited soil moisture here complement existing research, which demonstrated changes to root distribution and hydraulics in response to fertilization. The responses we discovered in fertilized trees that accompanied reduced transpiration and increased water use efficiency that differed from responses to reduced

soil moisture alone were primarily large decreases to shallow root presence. We found this to be readily quantified using measures of root length density. Decreases to whole-tree hydraulic conductivity were also shown to occur with fertilization and were shown not to occur in shoot tissue, suggesting limitation via rhizosphere or root xylem conductance. Our results support the supposition that fertilization narrows hydraulic safety margins and potentially predisposes loblolly trees to moisture stress, particularly prolonged, severe water limitation following fertilization. Finally, we tested the validity of throughfall exclusion for simulating reduced rainfall using a greenhouse ‘split-pot’ study, which applied spatially fixed heterogeneous soil moisture to young, well-watered loblolly pines. The ‘split-pot’ experiments demonstrated that spatially fixed soil moisture heterogeneity does not confound drought effects; needle area specific transpiration was not decreased, nor was water use efficiency increased. This supports the validity of inferences taken from drought simulation experiments with loblolly pine where throughfall exclusion troughs reduce soil moisture content in a consistent, spatially heterogeneous manner.

**The Combined Effects of Fertilization and Relative Water Limitation on Tissue Water Relations, Hydraulic Parameters and Shallow Root Distribution in Loblolly Pine (*Pinus taeda* L.)**

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**General Audience Abstract**

We investigated various effects of soil moisture limitation alone, and in combination with common fertilization practices in loblolly pine production. Responses at the shoot and needle level to different levels of soil moisture limitation produced new findings concerning how tissues respond to more severe water limitation. A 30% decrease in throughfall precipitation alone, or in combination with fertilization did not elicit drought related shoot tissue responses despite the presence of other indications of moisture stress and reduced water use. We also sought to explain why fertilized trees experiencing water limitation had environmental sensitivities that were different from unfertilized tree receiving ambient rainfall amounts or from trees only experiencing water limitation without fertilization. We found that changes to shallow root presence, especially root length density, accompanied the different patterns of environmental sensitivity and water use. Also, the water conducting ability of roots changed unevenly in soil with uneven moisture levels. The ability of roots to resist loss of conductivity to water did not change unevenly in the same way. We did another set of experiments to determine if using impervious troughs to catch rain is a valid approach to reducing soil moisture for the purpose of testing how loblolly responds to water limitation. These throughfall exclusion troughs create uneven soil moisture reduction, which can have effects on plant water use that are separate from water limitation alone. We found that in well-watered young trees, uneven soil moisture alone did not produce responses that

could be confused with the effects of water limitation. This finding indirectly validates the use of throughfall exclusion troughs to simulate reduced rainfall.

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# Chapter 1: Introduction

## 1.1 Overview

The widespread use of fertilization in intensive silviculture, coupled with changes in precipitation patterns in the southeastern U.S. beg a greater understanding of their interactive effects on attributes related to water use in loblolly pine (*Pinus taeda L.*), the most widely planted tree in the southeastern U.S. (Baker and Langdon, 1990). Recent sap flow research carried out in the southeastern U.S. to test effects of fertilization and drought using partial throughfall-precipitation exclusion to simulate reduced rainfall has suggested fertilization may lead to increased vulnerability to drought (Bartkowiak et al., 2015; Ward et al., 2015). Since the use of fertilizer in loblolly plantations generally leads to increased annual basal area increment and subsequent yield, it is important for the forestry industry and the scientific community to understand if these gains accompany other less desirable effects. The research detailed here addresses these concerns and characterizes changes to loblolly pine tissue water relations, root distributions, as well as root and shoot hydraulics, largely focusing on trees grown in the VA piedmont.

The aforementioned research on loblolly in the southeastern U.S. based on sap flow data has suggested that fertilization may increase drought induced mortality due to decreases in transpiration per unit ground area ( $E_c$ ), which is further decreased in trees in throughfall exclusion plots that have been fertilized (Ward et al., 2015). Although the potential for increased mortality where fertilized trees experience drought is suggested, no further work had been done to characterize any particular physiological changes occurring in addition to the changes seen in  $E_c$ . In the absence of drought, fertilization has been shown to increase  $E_c$ , while fertilization followed by drought has been shown to decrease  $E_c$  (Samuelson et al., 2008; Ward et al., 2015). The simple statement that decreased  $E_c$  suggests increased drought susceptibility was not satisfactorily elucidated previously.

One of the major goals of this research was to address gaps in the body of existing knowledge of the response of loblolly pine to water limitation both alone, and in combination with common fertilization practices. There has been work to characterize physiological responses of loblolly pine to moderate water stress, but no known work had been done to characterize the shoot and needle tissue responses to prolonged, more extreme drought (Albaugh et al., 2004; Barnes, 2002; Bongarten and Teskey, 1986; Domec et al., 2010; Ewers et al., 2000; Hacke et al., 2000; Meier et al., 1992; Seiler and Johnson, 1985; Wakamiya-Noborio et al., 1999). A portion of the results reported here complement recent research that detailed morphological responses to multi-year 100% throughfall-precipitation exclusion, but omitted some shoot and needle tissue level responses (Maggard et al., 2016).

Insufficient evidence is available on what specific morphological or physiological changes are responsible for observed differences in stomatal conductance and environmental sensitivity during the combination of fertilization and drought (Bartkowiak et al., 2015; Ward et al., 2015). Additionally, throughfall exclusion as a method for drought simulation has been used in loblolly pine stands, but has not been validated (Maggard et al., 2016; Samuelson et al., 2018, 2014; Ward et al., 2015; Wightman et al., 2016; Will et al., 2015). Specifically, the effects of the heterogeneity of soil moisture caused by the use of throughfall exclusion troughs on root distribution, hydraulics, and stomatal behavior is unknown. More generally, investigations into the effects of heterogeneous soil moisture at the tree to stand scale on water use in loblolly pine are lacking (Coleman and Aubrey, 2018; Parker and Van Lear, 1996).

Partial throughfall-precipitation exclusion reduces the volume of rain that reaches the soil using impervious troughs, which shunt away the rain that falls on them, creating drier conditions below. The use of throughfall exclusion troughs leads to local heterogeneity of soil moisture and could unrealistically represent conditions of reduced rainfall. A greenhouse 'split pot' study, along with data collected from a 2 by 2 drought\*fertilizer field study was used to investigate if, and how, the use of throughfall

exclusion modifies water use characteristics of loblolly pine. The results of this research have implications for the interpretation of data collected from throughfall exclusion experiments. Additionally, results presented here also augment basic knowledge of the physiology of loblolly pine as well as inform models of productivity in the Southeastern United States and elsewhere.

Broadly, the research detailed here characterizes shoot level physiological responses to fertilizer and different degrees of drought, root distribution and shoot and root hydraulics in response to fertilization and soil moisture at the tree to stand scale, and examines effects of controlled partial root zone drying (PRZD) in the absence of volumetric water limitation. PRZD is an irrigation technique where only a portion of the root zone is irrigated, and may be static or dynamic. Together, these findings address questions about throughfall exclusion itself, as well as inferences based on results obtained from throughfall exclusion experiments on the interactive effects of nutrient and soil moisture limitations.

## **1.2 Background**

### *1.2.1 Needle-level Responses to Extreme Drought*

When trees are subjected to moisture stress there are a number of ways that they may acclimatize physiologically and morphologically. These changes can vary over time, and may or may not carry over from one season to the next. These responses may include changes to: osmotic potential at 100% relative water content, the bulk modulus of elasticity, the symplastic water fraction, the turgor loss point, leaf area to root area ratio, and capacitance (Bartlett et al., 2012; Johnson et al., 2011; Kozlowski and Pallardy, 2002; Kramer and Boyer, 1995; Meinzer et al., 2009; Pallardy, 2008; Pfautsch et al., 2015). In loblolly pine, water relations are known to vary with provenance, changes in stand density, fertilization, and history of soil moisture availability (Albaugh et al., 2004; Bongarten and Teskey, 1986; Ewers et al., 2000, 1999; Ginn et al., 1991; King et al., 2008; Seiler and Johnson, 1985).

Loblolly pine trees that have been fertilized according to regionally optimal recommendations generally display increased stem growth and an increased leaf area index (LAI) concomitantly with a decreased root area to shoot area ratio (Stovall et al., 2012). Also, one author found decreases in mid-day leaf water potential ( $\Psi_{md}$ ) in response to fertilization, but no changes due to irrigation (Samuelson et al., 2008). Additionally, fertilized trees exposed to drought stress in a throughfall reduction experiment in Georgia did not show significantly different pre-dawn or mid-day leaf water potentials (Samuelson et al., 2014). So, it seems that there are conflicting results on the effect of fertilization on  $\Psi_{md}$ , which almost certainly interacts with water availability. Mid-day water potential is only one aspect of the water status of a tree, many other factors interact to affect it (Cruiziat et al., 2002; Sperry et al., 1998; Williams et al., 1996; Wullschleger et al., 1998). For example, loblolly pine has been shown to exhibit osmotic adjustment in response to water stress, allowing for maintenance of turgor to more negative  $\Psi_{md}$ . However, the majority of this evidence is for seedlings (Meier et al., 1992; Seiler and Johnson, 1985). There is also evidence of changes to needle capacitance under elevated  $CO_2$ , but not in response to drought (Domec et al., 2009).

### *1.2.2 Root and Shoot Hydraulics and Root Distributions*

Recent research in the Virginia Piedmont has demonstrated reduced stomatal conductance and increased WUE in response to fertilization. Decreases in stomatal sensitivity to vapor pressure deficit and an increase in sensitivity to bulk volumetric soil moisture were also reported (Bartkowiak et al., 2015; Ward et al., 2015). It has been shown that in addition to root redistribution and changes in WUE, various forms of deficit irrigation can induce long-term changes in the hydraulic properties of the roots, with roots in consistently wetter soil showing higher hydraulic conductivity (Ewers et al., 2001; McLean et al., 2011; Sperry et al., 2002). Changes have also been characterized in the terminal shoots of trees exposed to experimentally varied water availability (Bucci et al., 2006). Thus, it is expected to observe differences in root presence and hydraulic conductivity when there is significant, consistent variation in the spatial distribution of



available soil moisture. Since the acropetal movement of abscisic acid from dry roots to needles leads to stomatal constriction and resultant increased WUE, one might expect to observe changes in WUE alongside changes in root distribution and conductivity in response to the heterogeneity of soil moisture created by throughfall exclusion troughs (Kang et al., 2003; Kang and Zhang, 2004).

Models of water use in trees generally include root parameter(s), and the importance of the area of roots to leaves or needles is widely recognized. Whether a tree is limited by rhizosphere conductance or limited by xylem hydraulics is a proposed consequence of the aforementioned ratio (Jackson et al., 2000; Smallman et al., 2013; Sperry et al., 2002, 1998; Williams et al., 1996). One question that remains is what root parameter can be used to characterize root distribution changes accompanying changes to transpiration associated with fertilization in loblolly pine. Whole tree conductance is also known to change in response to resource availability, but the location within the tree of those changes most significantly affecting whole tree conductance is unclear in loblolly pine. Some believe that root conductance is most important while others believe that shoot conductance is paramount (Cruiziat et al., 2002; Ewers et al., 2000; Scoffoni et al., 2017; Wullschleger et al., 1998).

### *1.2.3 Soil Moisture Heterogeneity and Water Use*

When the root zone of a woody plant is subjected to distinct variation in the spatial distribution of available soil moisture, e.g. is subject to partial root-zone drying (PRZD), abscisic acid is synthesized in the drying roots and is then transported acropetally to leaf or needle tissues, resulting in reduction of the stomatal aperture. The pH of the xylem sap is also reduced, which is believed to contribute to the magnitude of the effect of the abscisic acid signal (Davies et al., 2002). The constriction of the stomatal aperture generally leads to increases in water use efficiency (WUE), which is broadly defined as a decrease in water consumption for a given amount of carbon fixation (Davies et al., 2002; Kang and Zhang, 2004; Stoll et al., 2000). According to theory, a set quantity of net

biomass may then be produced using less water, or more net biomass may be produced with the same amount of water. Generally, effects of PRZD are greater when irrigation is applied to different sides of the root-zone in an alternating fashion, but are also documented for fixed or static PRZD (Abrisqueta et al., 2008; Perez-Perez and Dodd, 2015).

In addition to changes in WUE, PRZD and other types of controlled deficit irrigation generally result in concurrent redistribution of roots. Sustained spatial heterogeneity of soil moisture usually leads to higher root densities in the moister soil as a result of loss of root mass in the dry soil and more new growth in the wetter soil zones (Abrisqueta et al., 2008; Ansley et al., 2014). This is intuitive, as it is to be expected that woody plants will not retain or produce large volumes of new roots in very dry soil. Under more spatially uniform soil moisture conditions loblolly pine has been shown to have higher root densities when growing in relatively drier soils (Parker and Van Lear, 1996).

### **1.3 Research Objectives and Hypotheses**

One goal of this research was to characterize how, at a needle level, mature loblolly pine trees in the field adapt to fertilization along with prolonged soil moisture limitation, as well as to prolonged more severe soil moisture limitation. Using analyses of pressure-volume relationships in shoot tissues, this work demonstrates that compensatory physiological changes to tissue water relations occur and are dependent upon the magnitude of the soil moisture limitation. Significant responses were observed only under more severe soil moisture limitation. Osmotic adjustment, changes to the turgor loss point, increases in the bulk modulus of elasticity, and increases to capacitance beyond turgor loss were observed and quantified. It was hypothesized that (1) the bulk modulus of elasticity would contribute to the adaptive fitness of loblolly pine via maintenance of relative water content at turgor loss, (2) moderate to extreme drought would induce increases in tissue capacitance, and (3) industry recommended levels of fertilization

would not significantly affect shoot level water relations attributes characterized by pressure-volume analysis. (Chapter 2).

In forestry research, throughfall reduction is a technique that can be used to reduce the volume of rainwater reaching the soil. As the presence of troughs, especially low and wide ones, creates banding of soil moisture one can reasonably expect to see the aforementioned differences in root density and conductivity, which could co-occur with some degree of change in WUE. The described field research quantified the changes in root length density and root and shoot hydraulic conductivity in a throughfall reduction experiment. It was hypothesized that (1) changes to stomatal conductance seen in fertilized trees was a result of changes to whole-tree conductivity, not the root to shoot water potential gradient. Furthermore, changes to whole-tree conductance in fertilizer + throughfall exclusion trees were hypothesized to (2) occur primarily in roots or the rhizosphere. It was also hypothesized that (3) changes to shallow root presence would co-occur with the reduced transpiration seen in the fertilizer + throughfall exclusion trees, (4) native hydraulic conductivity would be decreased with respect to position relative to through position in the throughfall exclusion only plots. Also, it was thought that root tissue density, as a proxy for increased cavitation resistance, would increase differentially under and between exclusion troughs in response to the soil moisture content of the immediate soil environment (Chapter 3).

Since throughfall exclusion creates heterogeneous soil moisture conditions similar to those created with fixed partial root zone drying, a greenhouse ‘split-pot’ study was also undertaken. Its purpose was to characterize any differences in the transpiration of fertilized trees subjected to PRZD under well-watered conditions. Deficit irrigation volume was not combined with the PRZD treatment during the first phase of the experiment so that effects of the fixed PRZD alone could be elucidated. Also of interest were differences in the response to imposed soil drought of the trees that had received the PRZD treatment. We hypothesized that (1) the PRZD treated trees would exhibit decreased transpiration and increased WUE (either intrinsic water use efficiency or water

use efficiency of production or both). Also, more broadly, it was thought that (2) the PRZD treated trees would exhibit responses that could possibly confound effects documented in response to throughfall exclusion in recent studies, i.e. responses generally attributed to water limitation but that were actually due to soil moisture heterogeneity. These hypotheses were generally disproven. However, there were effects of the treatment (Chapter 4).

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## **Chapter 2: Multiyear soil moisture limitation, unlike fertilization, induces changes to cell elasticity, the turgor loss point, and shoot capacitance in *Pinus taeda* L. while relative water content is maintained**

### **Abstract**

Loblolly pine (*Pinus taeda* L.) is both environmentally and economically important in the southeastern U.S. As summer dryness is expected to increase throughout much of the Southeast in the coming century, a greater understanding of the response of Loblolly pine to prolonged water deficits is desirable. Both theoretical and applied perspectives can benefit from such an improvement to current knowledge. This study uses pressure-volume analysis to quantify changes in shoot level physiological attributes in response to drought and fertilizer treatments. Soil moisture limitation was imposed for several years using a 30% and a 100% reduction in throughfall precipitation at two different experimental sites within the native range of loblolly pine. At the 30% throughfall reduction site the main and interactive effects of optimal fertilization were also examined. We found that at the shoot level, the response to fertilizer or to mild drought is insignificant alone, or in combination. More extreme soil moisture limitation was necessary to induce significant changes. In response to 100% throughfall exclusion we noted significant active and passive osmotic adjustment, resulting in maintenance of turgor to more negative water potentials. This was attributable to decreases in osmotic potential at 100% relative water content and increases to the apoplastic water fraction. We found that increases in the bulk modulus of elasticity maintained relative water content at turgor loss relative to the control trees. This finding supports the cell water conservation hypothesis. Additionally, significant increases to relative capacitance beyond turgor loss were documented and may serve to prolong the survival of foliage after stomatal closure and turgor loss.

## 2.1 Introduction

Loblolly pine (*Pinus taeda L.*) is the most widely planted tree for the production of lumber and wood products across the southeastern U.S. It dominates approximately 29 million acres and accounts for over half of all standing pine biomass in the Southeast (Baker and Langdon, 1990). Periodic droughts are relatively common in the southeastern U.S. and variability of precipitation has been increasing for 60 years (Wang et al., 2010). Additionally, recent climate models predict future increases in variability of summer rainfall in the southeastern U.S. (Li et al., 2013), and that begs a greater understanding of how loblolly pine responds to drought conditions for both environmental and economic reasons. In particular, summer dryness is expected to increase monotonically over the coming century for many parts of the Southeast (“PINEMAP Decision Support System v. 1.5.,” 2015).

When trees are subjected to moisture stress there are a number of ways that they may acclimatize physiologically and morphologically. These changes can vary across temporal scales, and may or may not carry over from one season to the next. These responses may include changes to: osmotic potential at 100% relative water content, bulk modulus of elasticity, apoplastic water fraction, turgor loss point, relative capacitance, and leaf area to root area ratio (Kozlowski and Pallardy, 2002; Kramer and Boyer, 1995; Pallardy, 2008). In loblolly pine, water relations may vary with provenance, changes in stand density, fertilization, and history of soil moisture availability (Albaugh et al., 2004; Bongarten and Teskey, 1986; Ewers et al., 2000, 1999; Ginn et al., 1991; King et al., 2008; Seiler and Johnson, 1985).

The idea that woody plants may acclimatize to drought conditions to avoid excessive loss of cellular water is known as the cell water conservation hypothesis (Cheung et al., 1975). A widely documented cellular response to drought is osmotic adjustment and the resultant lowering of the leaf or needle water potential at turgor loss. Taken alone, these changes would lead to a decrease in relative water content at turgor loss. In support of the cell water conservation hypothesis, researchers have demonstrated that many woody

plants increase their bulk modulus of elasticity in order to maintain relative water content at turgor loss (Bartlett et al., 2012). Also, the conservation of cell water content may avoid harm to the biochemical machinery of photosynthesis in chloroplasts resulting from toxic concentrations of magnesium (Rao et al., 1987).

Classically, osmotic adjustment may occur as an active process whereby a plant produces and accumulates additional metabolically compatible solutes to lower the osmotic component of its symplastic water potential. Osmotic adjustment can also occur passively, which occurs when a plant's cells' sap is concentrated by water loss. There may also be a longer term transfer of water from cells into the surrounding apoplast. Both types of passive osmotic adjustment concentrate cell sap. The difference is that in longer term passive osmotic adjustment the water that has left the symplast is not gone, but has been moved to the apoplast. Frequently, changes are also noted in the elasticity of cells as characterized by the bulk modulus of elasticity which may either maintain relative water content at the point of turgor loss, or alternately decrease how much water is lost for a given decrease in water potential (Kozlowski and Pallardy, 2002; Pallardy, 2008). In addition to changes in osmotic attributes, water partitioning, and cell elasticity there are potential changes to relative capacitance of plant tissues. Capacitance represents a means by which the transpirational loss of water from xylem may be buffered, and in leaf or needle tissue capacitance can prolong tissue survival after stomatal closure and turgor loss (Kozlowski and Pallardy, 2002; Kramer and Boyer, 1995; Pallardy, 2008; Pfautsch et al., 2015; Sack et al., 2003).

When others have investigated the leaf level physiological responses of loblolly pine to prolonged or periodic soil moisture limitation there have been differing and sometimes conflicting responses documented, potentially attributable to the magnitude and duration of water limiting conditions, or the dearth of field experiments employing controlled drought and pressure-volume analysis. In a controlled lab experiment where treatment trees were watered every other week resulting in exposure to soil water potentials of -1.0 to -1.2 MPa, it was reported that no large-scale adjustment of osmotic potential at 100%

relative water content was observed. However, the relative water content at turgor loss was lowered in the treatment trees (Tschaplinski et al., 1993). In a different set of controlled lab experiments where water was withheld until soil water potentials reached -1.5 or -2.0 MPa before irrigation, the researchers found the occurrence of osmotic adjustment in shoots and roots attributable to the accumulation of proline and monosaccharides, increases in the bulk modulus of elasticity, and no statistically significant changes in the partitioning of water between the symplastic and apoplastic compartments. There were also no differences noted between the two families of loblolly pine investigated (Meier et al., 1992). Bongarten and Teskey (1986) carried out a lab experiment, which subjected treatment seedlings from six seed sources within the natural range of loblolly pine to five months of cyclical drought conditions by only watering to field capacity when predawn wilting was observed. They observed that seed source had no effect on water relation characteristics in the treatment trees, only in the trees grown as controls under a moist soil regime. Also, they found that the recurring severe drought led to significantly decreased osmotic potential at 100% relative water content, lower relative water content at turgor loss, lower whole plant hydraulic conductance, and decreased sensitivity to absolute humidity deficit, which is akin to vapor pressure deficit (Bongarten and Teskey, 1986).

The current study was undertaken to clarify the means by which shoots and foliage of plantation grown loblolly pine adapt to soil moisture limitation at the multiyear scale, and to investigate if fertilization affected the responses to soil moisture limitation. Earlier studies only quantified some of the possible shoot level physiological attributes related to loblolly pine drought response. Results varied with respect to the severity and duration of drought imposed (Bongarten and Teskey, 1986; Meier et al., 1992; Seiler and Johnson, 1985; Tschaplinski et al., 1993). Additionally, many of the aforementioned studies used greenhouse or growth chamber grown juvenile trees. Recently, investigators considering loblolly pine have largely focused on hydraulic conductance and gas exchange, and tree to stand level water use using sap flux data, needle gas exchange and modeling

techniques (Aspinwall et al., 2011; Bartkowiak et al., 2015; Campoe et al., 2013; Domec et al., 2009; Ewers et al., 1999; Gonzalez-Benecke and Martin, 2010; Samuelson et al., 2008, 2014; Sperry et al., 1998; Tang et al., 2004; Wakamiya-Noborio et al., 1999; Ward et al., 2015, 2008; Wightman et al., 2016). Subsequently, no clear picture of all the drought-stress tolerance mechanisms employed by loblolly pine at the shoot level has yet emerged.

We decided that a comparison between differing levels of long-term soil moisture limitation could provide a more comprehensive understanding, and that considering the effect of fertilization, a very common management practice, under soil moisture limitation could also improve the current state of knowledge. We used tissue samples from two experimental sites with unique methods of throughfall exclusion to qualitatively compare responses to differing levels of soil moisture limitation. One site was in the VA Piedmont, which attempted 30% throughfall precipitation exclusion, and the second site was in the Coastal Plain of Oklahoma and attempted 100% throughfall exclusion.

Based on previous work and water relations theory we tested using pressure volume analysis three hypotheses with this study. We hypothesized that 1) increases to the bulk modulus of elasticity in response to soil moisture limitation would conserve cellular water and maintain relative water content at turgor loss, 2) more severe soil moisture limitation would induce increases in tissue capacitance, and 3) industry recommended levels of fertilization would not significantly affect shoot level water relations attributes that changed in response to soil limitation.

## **2.2 Materials and Methods**

### **2.2.1 Study Sites and Experimental Designs**

This study was carried out using two existing experiments, one in Virginia, and one in Oklahoma. The Virginia site was a 2 by 2 factorial field experiment established in an upland, 13-year-old, mid-rotation, loblolly pine plantation located in the Appomattox-Buckingham State Forest. Specifically, the site is located at latitude 37.443 N, and

longitude -78.664 W, in the Piedmont physiographic region. The soil at the site is fine-textured, shallow, and acidic, consisting mostly of 1:1 low CEC clay, overlain with an approximately 10 cm O-A layer. The dominant soil series present are Spears Mountain and Littlejoe, which are loams that range from sandy loam to clay loam. From observation of a soil pit onsite, it is evident that there is a hardpan layer roughly 1 meter from the surface in some, if not all areas. Historically, summer rainfall is lowest in August, and mean annual rainfall is ~1400mm (“NCDC Climate Data Online,” 2017). The site was initially planted in 2003 with a seed orchard mix at 1200 stems ha<sup>-1</sup>. Following eight years of growth, natural mortality and removal of volunteer trees had reduced the planted density to ~790 stems ha<sup>-1</sup>. This research site is part of a larger regional network of experimental sites, namely the *Pine Integrated Network: Education, Mitigation, and Adaptation project*, known as PINEMAP (“PINEMAP,” 2011; Will et al., 2016).

The layout of the experiment is a completely randomized block design with four blocks. The first treatment factor was fertilization; none vs. one-time application of 224 kg N ha<sup>-1</sup>, 27 kg P ha<sup>-1</sup>, 52kg K ha<sup>-1</sup>, and 1.12 kg ha<sup>-1</sup> micronutrient mix (6% S, 5% Bn, 2% Cu, 6% Mn and 5% Zn), all applied in April 2012. The second treatment factor was throughfall reduction; none vs. ~30% removed, concurrently initiated in April 2012 with the fertilization treatments after understory vegetation was removed mechanically, and chemically with glyphosate, imazapyr, and metsulfuron applied at recommended rates. The throughfall reduction was accomplished in eight of the experimental plots by building troughs roughly 1 to 1.5 meters high made of wood and polyethylene. Exclusion troughs covered ~30% of the ground area and carried water away and downhill from the plots. Each square plot is approximately 0.356 ha, including a perimeter buffer strip, which leaves a square internal measurement plot of 0.141 ha. The total number of plots is 16, as there are four treatment combinations, with one replicate per block.

The Oklahoma site was a randomized complete block design field experiment established on private land in an upland, 8-year-old loblolly pine plantation near Broken Bow, Oklahoma (OK). The experiment was located at latitude 34.030 N, and longitude -



94.823 W in southeastern OK. The soil onsite is a deep, fine sandy loam characterized as Ruston series. The site was initially planted in 2008 at  $\sim 1650$  stems  $\text{ha}^{-1}$  using a mix of half-sib bare root seedlings. Prior to planting, in the summer of 2007, the site was subsoiled to a depth of  $\sim 60$  cm, and glyphosate and imazapyr were applied at recommended rates. A controlled burn to remove existing vegetation was also carried out that fall. Immediately prior to planting in the winter of 2008, sulfometuron methyl, metsulfuron methyl, and imazapyr were applied at recommended rates to control unwanted vegetation. As with the VA site, summer rainfall is historically lowest in August, and mean annual rainfall is  $\sim 1300$ mm (“NCDC Climate Data Online,” 2017).

The experiment was established in the late winter of 2013 in five experimental blocks of three trees each, where each of the two treatments were applied to a single tree with one buffer tree in between. The five experimental blocks were situated along a broad ridge to minimize any subsurface flow. The treatments were control (C), and 100% throughfall exclusion (TE). The 100% TE was accomplished by building impermeable, rectangular, 3.7 x 2.7m excluder platforms around each of the trees subjected to that treatment. The excluders were  $\sim 1.1$ m tall, and were topped with heavy duty polyethylene. They were angled slightly to allow for throughfall to run off and away from the soil underneath. Within the periphery of each excluder a trench was mechanically excavated to  $\sim 60$ cm depth, which was then filled with heavy duty plastic sheeting and back-filled. This was done in order to sever any existing subsurface roots extending beyond the excluders, and also to inhibit growth of new roots outside of the footprint of the individual excluders. The excluders extended  $\sim 0.3$ m beyond the filled, lined trenches. Polyethylene collars were fitted around each trunk to intercept rain moving downward as stemflow and to convey that water to the main body of the excluders. The trees receiving the control treatment also had excluders with collars constructed around them, but the excluder covers were liberally perforated to allow all throughfall to reach the ground beneath. All trees in the experiment were trenched in the same manner. For further details on the design of the OK site see Maggard et al., 2016.

Predawn needle water potentials for 100% TE trees in OK were in the -1.2 to -1.8 MPa range during the growing season prior to sample collection for pressure-volume analysis, while control trees were between -0.9 and -1.1 for the same period (Maggard et al., 2016). In VA, the growing season predawn needle water potentials in the 30% TE trees ranged between -0.3 and -0.85 MPa over the course of the summer, while the controls were between -0.3 and -0.65. As vapor deficit increases exponentially with temperature, and the OK site was hotter on average than the VA site, the OK trees experienced higher vapor pressure deficits (Maggard et al., 2016, Ward et al., 2015). The trees in VA and in OK also came from different genetic sources, however, researchers have demonstrated that seed source does not affect needle level water use in response to drought (Wakamiya-Noborio et al., 1999). It has also been shown that seed source didn't affect the canopy stomatal conductance response to changes in soil water content (Gonzalez-Benecke and Martin, 2010).

### *2.2.2 Sample Collection, Data collection and Analysis*

At the VA site, one terminal shoot was taken from each of two trees per experimental plot during mid-August of 2015. This resulted in the collection of a total of 32 shoots from the VA site. The shoots were placed in labeled plastic zipper bags with deionized water-soaked paper towels surrounding them. They were subsequently transported to the lab where the proximal ~10cm of each shoot was removed underwater to eliminate any embolized xylem that may have resulted from their initial removal. Shoots were then placed in a high humidity chamber with their stems submerged in deionized water overnight to allow them to reach maximum potential water content. Pressure-volume analysis was carried out the following day for each sample according to the methods first outlined by Tyree and Hammel (1972). Shoots were allowed to desiccate naturally on the lab bench and were periodically reweighed to characterize water loss at each of the time points where water potential was measured. Thirteen or fourteen data points were collected per shoot. Parameter values derived from the pressure-volume curves were collected using a digital analytic tool (Sack and Pasquet-Kok, 2011). Seven physiological

parameters were quantified. Abbreviations for and brief descriptions of these attributes are available in Table 1. The derived parameter values for the VA site were analyzed using mixed models employing Satterthwaite's approximation of degrees of freedom with the lme4 package in R to account for the random effect of experimental block and subsampling (Bates et al., 2015). The physiological attributes which were analyzed as response variables in the mixed models described above were first transformed by inspection to normalize their distributions using quantile-quantile plots and optimizing the results of Shapiro-Wilk tests of normality.

At the OK site, one terminal shoot was collected from each of the ten experimental units during mid-August 2015. The shoots were shipped overnight to the lab in plastic zipper bags with water soaked paper towels surrounding them, as with the VA samples. Upon arrival, they were treated in the same manner as the shoots from the VA site in preparation for data collection. A small validation experiment had been previously carried out to verify that the overnight shipping would not affect values of tissue water relations parameters for loblolly pine shoots. This was accomplished by keeping shoots in plastic bags overnight before bringing them to maximum potential water content and performing pressure-volume analysis and comparing the results obtained to shoots that spent approximately four hours in bags prior to being brought up to maximum potential water content and having pressure-volume analysis performed on them. There was no significant difference (unpublished data).

The pressure-volume analyses carried out on the samples from the OK site were done in the same manner as the analyses done on the samples from the VA site. All inferential statistical analyses on the OK data were performed using the base package in R, as there was no subsampling to account for (R Core Team, 2016). One pressure-volume curve was generated per experimental unit, and the parameters derived from each were tested using one-way ANOVA. As with the data from the pressure-volume curves generated from the VA site, all physiological attributes analyzed were first individually transformed to normalize their respective distributions. As before, this was done visually by

inspecting quantile-quantile plots, and quantitatively using optimization of the results of Shapiro-Wilk tests of normality.

## 2.3 Results

At the Virginia site none of the measured attributes were significantly different from the control treatments when the effects of fertilizer application and drought were considered as interactions or as main effects (Table 2). At the Oklahoma site relative capacitance beyond turgor loss, water potential at the point of turgor loss, the bulk modulus of elasticity, osmotic potential at 100% relative water content, and apoplastic water fraction were all significantly different from the control treatment. The attributes relative capacitance at full turgor and relative water content at turgor loss were not significantly different.

At the Oklahoma site the trees that were subjected to 100% TE showed mean capacitance beyond turgor loss that was almost four standard errors above that of the control trees. Mean values were 0.22 and 0.12 MPa<sup>-1</sup>, respectively. The 100% TE trees also had a mean turgor loss point that were approximately 8.5 standard errors below the control trees, with mean values of -1.86 and -1.52 MPa, respectively. The mean bulk modulus of elasticity of the 100% TE trees was almost 3.2 standard errors above the mean of the control trees at 5.17 MPa, while the 100% TE trees' mean bulk modulus of elasticity was 6.95 MPa. Additionally, mean osmotic potential at 100% relative water content for the 100% TE trees was approximately 5.7 standard errors below that of the control trees with mean values of -0.94 and -1.34 MPa, respectively. These differences can be seen in figure 2 in panels A, D, B, and C respectively. Also, the available water fraction of the 100% TE trees was just over 3.5 standard errors above the mean of the control trees with mean values of 66% and 44%, respectively. The mean relative water content at turgor loss for the 100% TE trees was very slightly lower than for the control trees, but was not significantly different at 81.6% and 83.4%, respectively.

## 2.4 Discussion

At the Virginia site, throughfall reduction removed approximately 30% of soil volumetric water content in the upper 12 cm of soil, and had less pronounced effects in deeper soil (Will et al., 2015). Since treatments were imposed in the spring of 2012 and samples were collected in the summer of 2015, the shoots taken for analysis were in their 4<sup>th</sup> summer of reduced shallow soil moisture. At the Oklahoma site, throughfall exclusion removed approximately 50% of soil moisture to 12 cm and approximately 35% of soil moisture from 12 to 45 cm (Maggard et al., 2016). There, treatment was imposed in the late winter of 2013 and sampling was done in the summer of 2015; the trees were in their 3<sup>rd</sup> summer of more pronounced shallow soil moisture reduction. It is possible that deep roots at both sites were accessing soil horizons where soil moisture was unaffected by the treatment, decreasing the magnitude of observed responses. As loblolly is capable of hydraulic redistribution (Domec et al., 2010), the effects of the excluders on shallow soil moisture may have also been lessened in that manner. Regardless, the levels of shallow soil moisture limitation were different at the different sites, with the OK site posing more severe limitation.

Several researchers have previously documented osmotic adjustment in loblolly pine in response to drought; our documentation of decreased osmotic potential at 100% relative water content and subsequent decreased turgor loss point at the OK site further supports those findings (Bongarten and Teskey, 1986; Meier et al., 1992; Seiler and Johnson, 1985). The increases in apoplastic water fraction we recorded suggest that in addition to the active osmotic adjustment previously described by Meier and others, there is also a passive component to the osmotic adjustment of loblolly pine in response to prolonged water limitation that occurs via concentration of fluid in the symplastic compartment. It is also possible that increases in the apoplastic water fraction may buffer water lost from the xylem on a diel scale (Pfausch et al., 2015). Also we found an increased bulk modulus of elasticity in the shoots from the 100% TE treatment, which is believed to maintain relative water content at turgor loss, despite more negative needle

water potential. This benefit of this means of acclimatization has been referred to as the cell water conservation hypothesis, which is largely predicated upon avoidance of toxic concentrations of ions in the symplast (Bartlett et al., 2012; Cheung et al., 1975; Rao et al., 1987). Although increased bulk modulus of elasticity has been previously documented by loblolly pine in response to drought (Meier et al., 1992), our study is the first to characterize it alongside maintenance of relative water content at turgor loss, strengthening the cell water conservation hypothesis. We suggest that the demonstrated increases to capacitance beyond turgor loss in the 100% TE treatment confer extended survival after stomatal closure and turgor loss (Sack et al., 2003). Also, the lack of response in the VA trees to fertilization shows that fertilization alone or in combination with reduced soil moisture doesn't induce change to tissue water relations attributes. The lack of response to the 30% TE treatment indicates that more severe prolonged drought is necessary to induce the changes we characterized in the OK trees.

## 2.5 Conclusion

The results presented here are the most detailed and complete characterization of the shoot level physiological responses of plantation-grown loblolly pine to drought. We were able to demonstrate that not only is there active but also passive osmotic adjustment. Additionally, increases in bulk modulus of elasticity do occur and present along with the maintenance of relative water content at turgor loss, protecting needle mesophyll from desiccation induced loss of metabolic function due to toxic ion concentrations (Rao et al., 1987). This last finding soundly supports the cell water conservation hypothesis. We also quantified the increase of capacitance beyond turgor loss and suspect that this has adaptive merit, engendering prolonging tissue survival following drought induced stomatal closure and turgor loss. This finding supports the benefit of increased capacitance beyond turgor loss that was suggested by Sack and others (Bartlett et al., 2012; Sack et al., 2003). Our initial hypotheses that 1) increases to the bulk modulus of elasticity would conserve cellular water and maintain relative water content at turgor loss, and that 2) more severe soil moisture limitation would induce

increases in capacitance were both substantiated by our results. The hypothesis that 3) fertilizer application would have little effect on shoot level physiology was also supported by our findings. The difference in the results from the 30% TE trees and the 100% TE trees indicate that loblolly pine requires relatively strong shallow soil water limitation for extended periods to induce the physiological changes we saw in this study. This makes intuitive sense, as loblolly pine naturally exhibits relatively strong isohydricity. It then follows that it would require more severe soil moisture stress to induce the quantifiable shoot level changes we found in the 100% TE trees because stomatal control alone would limit the need for additional responses under less extreme soil moisture conditions.

Employing a broader conceptual perspective, we note that despite the more severe soil moisture limitation experienced by the 100% TE trees in the upper soil profile, there was effectively no drought induced mortality. There were notable changes in morphology documented (Maggard et al., 2016) in addition to the shoot level physiological attributes we've described here. Also, despite lower predawn and midday needle water potentials, the 100% TE trees still maintained midday water potentials above -1.8 MPa and avoided stomatal closure (Maggard et al., 2016). This suggests that the morphological changes, such as reduced foliage biomass and decreased stem growth, worked in concert with the shoot level physiological changes to avoid drought induced mortality. Despite experiencing soil moisture reductions of approximately 40% in the upper 45 cm of soil for almost three years (Maggard et al., 2016) the 100% TE trees in OK combined shoot-level physiological changes with morphological changes to acclimatize.

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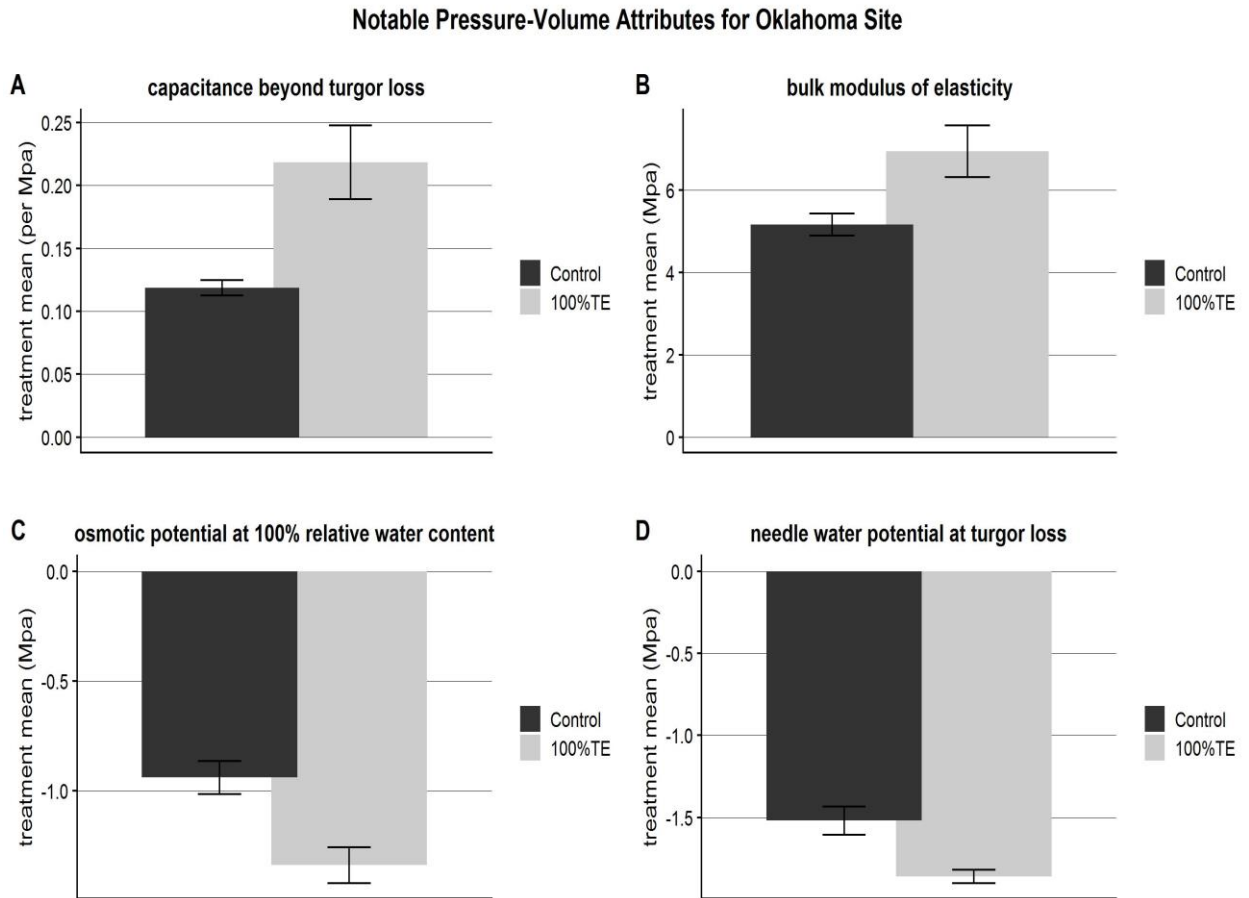


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## 2.7 Figures and Tables



**Figure 2.1.** Select water relations parameters of seven-year-old loblolly pine shoots from a site near Broken Bow, Oklahoma. The trees received 100% throughfall exclusion (TE) or control. Error bars show  $\pm 1$  standard error.

**Table 2.1.** Mature loblolly pine shoot water relations parameters for Oklahoma trees receiving 100% throughfall exclusion (TE) and Virginia trees receiving 30% throughfall exclusion. CTL – capacitance beyond turgor loss (MPa<sup>-1</sup>), TLP – turgor loss point (MPa), BME – bulk modulus of elasticity (MPa), OP – osmotic potential at 100% relative water content (MPa), AWF – apoplastic water fraction (percent), CFT – capacitance above turgor loss point (MPa<sup>-1</sup>), RWCTL – relative water content at turgor loss (percent)

| Physiological Attribute | Oklahoma Experiment |                      | Virginia Experiment |                       |
|-------------------------|---------------------|----------------------|---------------------|-----------------------|
|                         | Control             | 100% TE (p-value)    | Control             | 30% TE (p-value)      |
| <b>CTL</b>              | 0.12 ± 0.006        | 0.22 ± 0.026 (0.002) | 0.14 ± 0.01         | 0.11 ± 0.01 (0.075)   |
| <b>TLP</b>              | -1.52 ± 0.08        | -1.86 ± 0.04 (0.007) | -1.49 ± 0.05        | -1.49 ± 0.07 (0.945)  |
| <b>BME</b>              | 5.17 ± 0.24         | 6.95 ± 0.56 (0.048)  | 4.90 ± 0.21         | 4.62 ± 0.27 (0.510)   |
| <b>OP</b>               | -0.94 ± 0.07        | -1.34 ± 0.07 (0.008) | -0.94 ± 0.05        | -0.89 ± 0.06 (0.634)  |
| <b>AWF</b>              | 44.0 ± 5.89         | 66.6 ± 6.38 (0.033)  | 46.75 ± 2.42        | 39.81 ± 1.83 (0.116)  |
| <b>CFT</b>              | 0.11 ± 0.006        | 0.10 ± 0.004 (0.138) | 0.124 ± 0.004       | 0.122 ± 0.006 (0.719) |
| <b>RWCTL</b>            | 83.4 ± 1.75         | 81.6 ± 0.98 (0.369)  | 83.06 ± 0.54        | 83.31 ± 0.71 (0.765)  |

Values shown are the mean ± one standard error, with the ANOVA p-value in parentheses.

### **Chapter 3: Soil moisture limitation and fertilization differentially affect root and shoot hydraulics as well as root distribution in loblolly pine (*Pinus taeda* L.) subjected to throughfall exclusion**

#### **Abstract**

This study investigated the effects of drought and fertilization on loblolly pine (*Pinus taeda* L.) hydraulic architecture. The study was carried out employing an existing experiment on a plantation in the Piedmont physiographic region of Virginia planted in 2003, with treatments applied in 2012. The treatments had been implemented in a full factorial design with soil drought and complete fertilization as presence or absence factors. Soil drought had been simulated in half of the 16 plots using throughfall exclusion troughs standing over approximately 30% of the ground area. Root and shoot hydraulic and physical properties, root distribution, and pre-dawn and mid-day water potentials were characterized. It was shown that decreases in water use were not associated with differences in trees' internal water potential gradients. Instead, decreases to whole tree conductance were responsible. Decreases to shallow root length density were greatest in fertilized and soil moisture limited trees. This mirrored the observed decreases in transpiration. Root length density may be a relatively easy to measure characteristic of root presence associated with decreased transpiration when fertilized trees are limited by soil moisture. Native root hydraulic conductivities were lowered by the soil moisture limitation imposed by the throughfall exclusion treatment. The degree of loss of conductivity under and between exclusion troughs was not buffered differentially by increased cavitation resistance. This suggests a more systemic response of cavitation resistance to heterogeneous soil moisture limitation. In shoot xylem, fertilization largely mitigated drought induced decreases to needle area normalized native conducting efficiency.

### 3.1 Introduction

Researchers have strived to understand how trees use water and respond to constraints made by changing environmental conditions and resource availability for centuries, if not millennia. The body of research in woody plant water relations is extensive. Previous research has built upon the cohesion-tension theory and the Ohm's law analogy of plant water relations and has moved on to focusing on the hydraulic architecture of trees (Cruiziat et al., 2002; Scholander et al., 1973; van den Honert, 1948; Wullschleger et al., 1998). This paradigm is a subset of the soil-plant-atmosphere continuum framework and allows for a more holistic understanding of the tree and its environment but is markedly more complex than previous reductionist hydraulic conceptualizations (Sperry et al., 1998; Williams and Rastetter, 1996). There are many questions that remain to be answered, including how management practices and environmental constraints affect species of economic and environmental importance such as loblolly pine (*Pinus taeda* L.) (Baker and Langdon, 1990; Li et al., 2013; Stovall et al., 2012). Understanding dominant causative factors and characterizing measurable attributes that dictate the hydraulic architecture of trees expands understanding and actionability with respect to regulation of plant water loss, mortality, acclimation to resource availability, and productivity in general (Bartlett et al., 2012; Ewers et al., 2001; Hacke et al., 2000; Jackson et al., 2000; Mencuccini, 2003; Santiago et al., 2014; Skelton et al., 2015; Whitehead, 1998).

As both the application of complete fertilization and changes to the availability of soil moisture during the growing season are likely to increasingly co-occur in the near future (Fox et al., 2007; Li et al., 2013; "PINEMAP Decision Support System v. 1.5.," 2015), a need for better understanding of their effects has emerged. Researchers have investigated the effects of fertilization on the physiology of loblolly pine over several decades (Albaugh et al., 2004; Bartkowiak, 2015; Campoe et al., 2013; Ewers et al., 1999; Gough et al., 2004; King et al., 1999, 2008; Murthy et al., 1996; Samuelson et al., 2014; Tang et al., 2004, 1999) but relatively few have focused primarily on the interpretation from a hydraulic architecture perspective ( Domec et al., 2009a, 2009b, Ewers et al., 1999, 2000;

Meinzer et al., 2009; Sperry et al., 1988; Tyree et al., 1995). The ratio of area of roots to area of needles ( $A_r:A_l$ ) is known to decrease with fertilization (Ewers et al., 2000) but there is little evidence demonstrating a relatively easy to measure index of root presence that correlates to the changes seen with fertilization. There is also relatively little documentation of the combined hydraulic effects of fertilization and drought. Conflicting results and a lack of effect of soil moisture manipulation have also been reported (Ewers et al., 2000; Samuelson et al., 2008a, 2008b; Ward et al., 2015; Wightman et al., 2016). Additionally, various methods of rainfall manipulation used to simulate drought, such as throughfall exclusion, which intercepts a portion of throughfall precipitation, have been questioned (Beier et al., 2012).

In order to characterize whole tree water use and hydraulic architecture, researchers have utilized many different approaches (Abrisqueta et al., 2008; Alder et al., 1996; Ansley et al., 2014; Cruiziat et al., 2002; Domec et al., 2009a; Ewers et al., 2000, 1999; Meinzer et al., 2009; Samuelson et al., 2008b; Sperry et al., 2002; Ward et al., 2015; Williams et al., 1996; Wullschleger et al., 1998). A foundational characteristic for modeling water use at the whole tree level is the whole tree conductance, which is related to transpiration and the water potential gradient between the bulk soil and the needles (Sperry et al., 2002; Wullschleger et al., 1998). Consider an equation describing transpiration based on Darcy's Law (Ewers et al., 2000; Whitehead, 1998):

$$E_l = k_{tl} (\Psi_s - \Psi_l - h\rho_w g), \quad (1)$$

Here  $E_l$  is needle area normalized transpiration,  $k_{tl}$  is needle area normalized whole tree conductance,  $\Psi_s$  and  $\Psi_l$  are soil and needle water potentials, and  $h\rho_w g$  is a constant term accounting for gravitational pull on xylem sap with  $h$  as height,  $\rho_w$  as the density of water, and  $g$  as the acceleration downward due to gravity. By inspection one can see that  $E_l$  equates to the product of  $k_{tl}$  and  $\Delta\Psi$  minus a constant term, assuming tree height is constant. This means that  $E_l$  is proportional to both  $k_{tl}$  and  $\Delta\Psi - h\rho_w g$  when the values of  $k_{tl}$  and  $\Delta\Psi$  can be assumed to be fixed, e.g. over a relatively short period of time.



$$E_l \propto k_{tl}; \quad E_l \propto \Delta\Psi - \text{constant} \quad (2)$$

Other researchers working at the same VA experimental site used for the experiments described here found large differences in transpiration during the growing season using measurements of sap flux (Ward et al., 2015). The experimental site had four treatment combinations. They were control, 30% throughfall exclusion, complete macro and micro nutrient fertilization, and 30% throughfall exclusion + fertilization. They reported that ground area normalized canopy transpiration ( $E_c$ ) was approximately 29% lower than control for the fertilization + 30% throughfall exclusion treatment combination and was approximately 36% lower for needle area normalized canopy conductance ( $G_l$ ). The similarity in magnitude of these two values can be explained by the fact that conifers exhibit a close coupling between stomatal conductance and transpiration such that when stomatal conductance changes, transpiration changes almost proportionally. This concept is described by Jarvis'  $\Omega_l$  decoupling factor, which ranges from 0 to 1 and indicates the degree of decoupling between stomatal conductance and transpiration in the field. For conifers,  $\Omega_l$  takes on a value of approximately 0.05, i.e. transpiration and stomatal conductance show very little decoupling (Jarvis and Mcnaughton, 1986). Additionally, there was no significant difference in leaf area index (LAI) between treatments at the experimental site which supports the apparent similarity between measurements normalized to leaf area and those normalized to ground area (Ward et al., 2015).

Since LAI wasn't different between treatments at the experimental site, then ground area normed transpiration ( $E_c$ ) can be thought of in terms of equations (1) and (2). We investigated if differences in transpiration could be explained by a difference in the water potential gradient between root and shoot, as embodied by the difference between pre-dawn and mid-day water potentials ( $\Delta\Psi$ ). If differences in  $\Delta\Psi$  could not explain the differences in transpiration, then whole tree conductance ( $k_{tl}$ ) must be responsible. If that is the case, then where in loblolly pine trees do contributing changes occur? Whole tree hydraulic conductance decreases as a result of decreases in conductance at the soil root interface, and as xylem cavitates. These changes occur as water potentials within the tree

decrease as water use exceeds water uptake (Jackson et al., 2000; Sperry et al., 2002, 1998).

In one review of whole tree water use studies, it was determined that almost half of all the resistance to flow occurs below ground (Wullschleger et al., 1998). Similarly, it has been suggested in the past that the contribution of smaller shoot resistance in response to water stress is relatively minor (Granier et al., 1989). Others, more recently, have suggested that a loss of conductivity in small shoots and needles can act as a hydraulic bottleneck and therefore has a significant role in reducing whole tree conductance (Cruiziat et al., 2002; Scoffoni et al., 2017). We investigated this possibility. Alternately, others have argued convincingly that either the rhizosphere conductance or the xylem conductance may be the primary limiting factor, largely dependent upon the ratio of root area to needle area, with lower root to needle area leading to limitation primarily due to decreased rhizosphere conductance (Sperry et al., 2002).

The ratio of the area of roots to the area of needles ( $A_r:A_l$ ) is known to decrease with fertilization in loblolly pine along with reduced root conducting efficiency (Ewers et al., 2000). Identifying a simple metric that captures changes to root presence as a result of fertilization in the face of reduced soil moisture would be beneficial. For this reason, we characterized root distributions in throughfall exclusion + fertilization plots, throughfall exclusion only plots, and in control plots.

The study described herein was carried out with two general questions in mind. The first was what was different in the throughfall exclusion + fertilization plots as compared to the throughfall exclusion only plots that could potentially explain differences in  $E_c$ . We hypothesized that (1) in the absence of extreme drought, changes in transpiration in plantation grown, mature loblolly pine are driven not by large increases in  $\Delta\Psi$ , but instead by changes to whole tree conductance ( $k_{tl}$ ). This was tested by measuring  $\Delta\Psi$  at different time points during the growing season in trees exposed to the full factorial combination of 30% throughfall exclusion and fertilization. Furthermore, (2) when  $k_{tl}$  decreases in loblolly pine due to soil moisture limitation or fertilization, those decreases

are not primarily ascribed to changes in conductivity of terminal shoots. This was tested by characterizing hydraulic efficiency of all treatments. We also hypothesized that (3) decreases in shallow root presence caused by decreased soil moisture in plots with exclusion troughs would be further compounded by fertilization. It was hoped that a suitable metric could be found to quantify the expected changes in root presence associated with the changes to transpiration seen in fertilized loblolly pine experiencing soil moisture limitation (Ward et al., 2015). The second general question was how do shallow root distribution and hydraulic efficiency in non-sand soils respond to the heterogeneity of soil moisture created by throughfall exclusion troughs. We hypothesized that (4) shallow root presence in the throughfall exclusion only plots would increase between troughs to levels above those in the control plots. We also expected that (5) native hydraulic conducting efficiency would be differentially decreased by location relative to troughs in the throughfall exclusion only plots despite any changes to cavitation resistance. Also, root tissue density, as a proxy for cavitation resistance (Jackson et al., 2000; Kozlowski and Pallardy, 2002; Kramer and Boyer, 1995; Pallardy, 2008), would increase differentially. This would demonstrate a response to surrounding soil moisture content, not a root system-wide, systemic response.

## **3.2 Material & Methods**

### *3.2.1 Study Site and Experimental Design*

This study was undertaken using an existing field experiment in the Piedmont physiographic region of Virginia. The experiment was of a 2 x 2 factorial design and was established in an upland, mid-rotation, 13-year-old loblolly pine plantation located in the Appomattox-Buckingham State Forest. Explicitly, the site is located at latitude 37.443° North, and longitude 78.664° West. At the site the soil is fine textured, shallow and acidic. It consists largely of 1:1 low CEC clay and possesses an O-A layer approximately 10cm thick. The dominant soil series present are loams, namely Little Joe and Spear's Mountain, which range from sandy to clay loams. Also, there is a hardpan layer approximately 1 meter below the land surface, which was directly observed from an

onsite soil pit, although the precise lateral extent of the layer remains uncharacterized. Collectively, these are relatively common soil characteristics of the Piedmont physiographic region of central Virginia. Summer rainfall is historically the lowest in the month of August and the mean annual rainfall is ~1400mm (“NCDC Climate Data Online,” 2017). The site was planted in 2003 with an orchard mix and following eight years of growth, had a density of ~790 stems ha<sup>-1</sup>. The site where this research was undertaken is part of a larger regional study with multiple experimental sites (Will et al., 2015). The network of sites was referred to as the *Pine Integrated Network: Education, Mitigation, and Adaptation project*, colloquially known as PINEMAP (“PINEMAP,” 2011).

The experiment was implemented as a randomized complete block design comprising four blocks delineated topographically. The two-level treatment factors were crossed in each block thereby requiring four experimental plots per block. This led to the creation of 16 plots in four blocks, where each block was a replicate. The two treatment factors were complete fertilization and reduction of throughfall precipitation. The fertilization factor was imposed as none vs. one-time application of 224 kg N ha<sup>-1</sup>, 27 kg P ha<sup>-1</sup>, 52kg K ha<sup>-1</sup>, and 1.12 kg ha<sup>-1</sup> micronutrient mix (6% S, 5% Bn, 2% Cu, 6% Mn and 5% Zn), all applied in April 2012. The throughfall reduction factor was imposed as none vs. ~30% removed, instituted in April 2012 concurrently with the fertilization treatments after understory vegetation was removed. The throughfall reduction treatment was realized in eight experimental plots using 1 to 1.5 meters wide troughs on both sides of each tree row. The troughs were made from polyethylene sheeting suspended approximately 1 to 1.5 meters high on wooden frames. The exclusion troughs covered ~30% of the ground area and directed intercepted throughfall precipitation away and downhill from the experimental plots. Each square plot measured approximately 0.36 ha, including a perimeter buffer, which left an internal measurement plot of 0.14 ha.

### 3.2.2 *Sample Collection, Data collection and Analysis*

Several physiological and morphological attributes were characterized over two growing seasons. Data was collected on early and late season pre-dawn and mid-day

needle water potentials, as well as on shoot hydraulic conductivity. Root hydraulic conductivity and distribution was also investigated, along with needle level stomatal conductance, transpiration, and net photosynthesis.

Root distribution data was collected over two seasons. In May of 2015 four soil cores were collected per plot for the eight plots that had throughfall exclusion troughs. Two soil cores were taken randomly from under troughs and another two cores were taken randomly from between troughs in each of the 8 plots sampled. Each soil core was collected using a large, thin walled steel pipe hammered into the ground to a depth of 16 cm. Each soil sample's volume was approximately 1600 cm<sup>3</sup>. In July of 2016 the same sized soil cores were collected from each of four control plots and from between excluders from each of the four plots only subjected to throughfall exclusion. Three soil cores were collected randomly from each plot sampled for a total of 24 samples. All soil cores were placed in labeled sealed plastic bags for transportation. When 2015 and 2016 root data was combined, subsampling was averaged out. In September of 2016, root sections of approximately 10 cm length and 2 mm diameter were collected from the same plots sampled in July of that year. Two samples were collected randomly from each control plot, from under the troughs of the throughfall only plots, and from between the troughs of the throughfall only plots. This resulted in 24 total root sections, which were placed into sealed plastic bags with deionized water for transport.

The soil cores were kept refrigerated overnight and the roots they contained were analyzed for morphological characteristics using WinRHIZO software (Regent Instruments, Quebec, Canada) the following day. The root sections for characterization of hydraulic properties were transported to the lab immediately following collection and both ends of each section were re-cut under deionized water resulting in sections of approximately 5 cm length. The re-cutting was done to avoid any embolisms created when they were initially cut and taken from the soil. The re-cutting occurred for each sample immediately prior to their installation into the apparatus fashioned to test their hydraulic conductivity.

Measurements of hydraulic conductivity were made using instrumentation similar to that used by Tyree and others (Tyree et al., 1983). This approach incorporates an elevated reservoir containing water and the measurement of water flow through the root segments over time. Instead of a pipette on the downstream portion of each root section an analytic balance and micropipettes filled with tissue paper were used for quantification of flow. A manifold was fashioned which allowed six samples to be measured simultaneously, which allowed for more rapid characterization of all samples. As Sperry found that there was little difference in the reduction of hydraulic conductivity over time when comparing various salt solutions to deionized water, deionized water was used (Sperry et al., 1988). Although dilute formaldehyde solutions and solutions with low pH inhibit the reduction of hydraulic conductivity beyond perfusion times of 4 to 10 hours there is no appreciable effect prior to that threshold. Samples were attached to the perfusion manifold and flow rates were allowed to reach steady state, which took approximately 8 minutes. Following stabilization, water passing through the segments was collected for 10 minutes and conductivity was determined by dividing the gravimetric flow rate by the pressure differential, which was 11 kPa, and multiplying by the lengths of the sections to arrive at hydraulic conductivity values. Sapwood area was taken to be the cross sectional area inside the root bark. After drying at 70°C for 48 hours, the mass of each root sample was recorded and used to calculate root tissue density.

The data collected on shoots included pre-dawn and mid-day needle water potentials, and shoot hydraulic conductivities. The needle water potentials were collected in mid-June and early August of 2015. For each sampling date, two shoots were collected from the NE quarter of different randomly selected trees at mid-canopy level in each of the 16 experimental units. The same trees were sampled at pre-dawn and again at mid-day. This resulted in a total of 64 samples per sampling date. Different trees were randomly chosen by sampling date. Shoots were placed into sealed plastic bags lined with deionized water moistened paper towels. After collection, the samples were tested onsite for needle water potential using a pressure chamber (PMS Instrument Company, Albany, OR). The

method used for determination of needle water potential was that of Tyree (Tyree and Hammel, 1972). The original values of pre-dawn and mid-day water potential for each shoot were recorded, along with their calculated differences ( $\Delta\Psi$ ).

Shoot hydraulic conductivity was measured on two random subsamples from each experimental unit, for a total of 32 samples. The subsamples, which included needle tissue, were collected in mid-April of 2015 when the local monthly precipitation was 100 mm (“NCDC Climate Data Online,” 2017). In April of 2013, the monthly precipitation was 106 mm and the fertilizer + throughfall exclusion trees transpired ~75% of control, and the fertilizer alone and the throughfall exclusion alone both transpired ~90% of control (“NCDC Climate Data Online,” 2017; Ward et al., 2015). The shoots were cut under deionized water in the field and were transported to the lab with the cut ends submerged in deionized water inside of zipper bags. Upon arrival, and immediately prior to installation into the manifold constructed for the purpose, the shoots’ proximal ends were re-cut under water and the distal portions with needles were also removed leaving sections of 5-6 cm. The needle tissues from each subsample were measured, dried, and weighed for determination of surface area. This was accomplished using specific leaf area (SLA) calculated from 3-fascicle subsamples of each shoot. Needle area per fascicle was calculated as number of needles per fascicle\*diameter\*length +  $\pi$ \*diameter\*length. If any needles were lost in transport it was assumed to be consistent between samples and was ignored. The method and instrumentation employed for determination of hydraulic conductivity was the same as described for measuring root conductivities. As before, the samples were allowed to reach steady state flow before measurements were taken, and measurement of native hydraulic conductivity proceeded for 10 minutes. Conductivity was calculated and standardized on both a cross sectional and needle area basis. Cross sectional area was measured as all area inside the bark. The area of sapwood was calculated as  $\pi$ \*radius<sup>2</sup> and was divided by total needle area per shoot to arrive at area of sapwood per area of needles ( $A_s:A_l$ ).

The statistical analysis of all data was conducted using linear mixed models in R to address blocking and any subsampling by partitioning the random error utilizing the lme4 and LmerTest packages. For example, with the data collected from under and between exclusion troughs from fertilizer + throughfall exclusion and throughfall exclusion only plots, the trough factor was treated as a split plot factor. The appropriate splitting of the error variance was accomplished by adding a random error term to the mixed model specification that acts as denominator in the F test for the effect of trough presence, e.g.  $y \sim \text{fert} + \text{trough} + \text{fert}:\text{trough} + (1|\text{block}) + (1|\text{block}:\text{fert})$ . See Bates et al., 2015 and Crawley, 2013 for further details. If a fixed interaction term was insignificant, it was subsequently removed and the model was re-run (Crawley, 2013). Any p-values obtained were the result of ANOVA with type three sums of squares, and either Satterthwaite's or the Kenward-Roger approximation was implemented for the determination of degrees of freedom for F tests. Statistical models, once the final specification was determined, were fit using either maximum likelihood (ML) or restricted maximum likelihood (REML). ML estimation was attempted first since it produces unbiased estimates of the fixed model effects, but if estimation of any of the variance components was not possible with ML then REML was used for parameter estimation (Bates et al., 2015; Crawley, 2013; Kuznetsova et al., 2017). In addition, custom contrasts that appropriately weighted group means according to ground area were used to make inferences when comparing control with the overall throughfall exclusion treatment when measurements from both below and between exclusion troughs were considered (Table 3.4). These contrasts were implemented with general linear hypothesis testing in the multcomp package (Hothorn et al., 2008). Weighted means and weighted standard errors presented in Table 3 were calculated using the Hmisc package (Harrell and DuPont, 2017). Due to the expected extreme variability of root characteristics, an  $\alpha$  of 0.075 was used for root data inferences, while an  $\alpha$  of 0.05 was used for inferences on the data collected from shoots.



### 3.3 Results

#### 3.3.1 *Root Distribution and Hydraulics*

In the 30% throughfall exclusion only and the 30% throughfall exclusion + fertilizer plots, root length density, root surface area per volume soil, and root volumetric density in the upper 16 cm of soil were all different depending on whether samples were taken under or between troughs (Table 3.2). In addition to the main effect of root location relative to trough seen for all attributes, there was also an additional main effect of fertilizer limited to the root length density attribute (Figure 3.1). The marginal effect of trough presence on root length density, i.e. ignoring fertilization, was a decrease from  $2642 \text{ m m}^{-3} \pm 259$  between troughs to  $1330 \pm 197$  under troughs ( $p = 0.0007$ ). The marginal effect of fertilization on root length density was a decrease from  $2405 \text{ m m}^{-3} \pm 322$  in unfertilized throughfall exclusion plots to  $1507 \pm 173$  in fertilized throughfall exclusion plots ( $p = 0.01$ ). The marginal effect of trough presence on root surface area, which was only significantly affected by trough presence, was to decrease root surface area from  $7.73 \text{ m}^2 \text{ m}^{-3} \pm 0.95$  between troughs to  $3.51 \pm 0.41$  under troughs ( $p = 0.0003$ ). The marginal effect of trough presence on root volume density, which was only significantly affected by trough presence, was to lower root volume density from  $0.193 \text{ percent m}^3 \text{ m}^{-3} \pm 0.03$  between troughs to  $0.078 \pm 0.01$  under them ( $p = 0.02$ ).

There were no statistically significant differences in root length density, root surface area, or root volumetric density when control plots were compared with the same attributes measured between troughs of the TE treatment (Table 3.2). Data from under and between troughs in the throughfall exclusion only plots were weighted by ground area to allow the comparisons to control displayed in Table 3.4. Root length density was not different between control and overall throughfall exclusion only plots. Root surface area was greater in control plots, and root volumetric density was not different.

Values of conductivity normalized for root sapwood cross sectional area ( $K_{Rs}$ ) and values of root tissue density as a proxy for cavitation resistance are shown in Table 3.3. The data come from control plots, and from both under and between troughs in the throughfall exclusion only plots.  $K_{Rs}$  was greater for roots from control plots than for roots taken from under exclusion troughs. It was also greater for roots collected between troughs compared to roots taken from under troughs. Root tissue density was smaller in control plots than in positions either under or between troughs. There was no difference in root tissue density for roots under troughs compared to roots between troughs in the throughfall exclusion only plots. Whole-plot hydraulic data for the 30% throughfall exclusion only plots compared to control is shown in Table 3.4. The whole-plot data is composed of data from under and between troughs weighted by relative ground area within the plots. Overall, both  $K_{Rs}$  and root tissue density were different.  $K_{Rs}$  was reduced in the 30% throughfall exclusion only plots, and root tissue density was increased.

### 3.3.2 *Shoot and Needle Level Attributes*

Water potential gradients ( $\Delta\Psi$ ) measured in June and in August of 2015 were not significantly different between any of the treatment combinations of fertilization and 30% throughfall exclusion. Data collected from shoots used for measurements of native hydraulic properties showed an interaction between throughfall exclusion and fertilization for sapwood specific conductivity ( $p = 0.04$ ), and also for needle area specific conductivity ( $p = 0.01$ ). The relationship for sapwood specific conductivity ( $K_s$ ) is illustrated in Figure 3.2. Fertilizer application reduced  $K_s$  less in the trees also subjected to throughfall exclusion. Needle area specific conductivity ( $K_l$ ) was decreased by fertilization only in plots without throughfall exclusion. Means and standard errors for both measures of conductivity for all treatment combinations are shown in Table 3.5. The ratio of sapwood area to needle area of small shoots was not different for any treatment combination, and specific needle area was increased by fertilization (Table 3.5).

### 3.4 Discussion

The first general question that prompted this research was what was different in the throughfall exclusion + fertilization plots compared to the throughfall exclusion only plots that could potentially explain the large differences in  $E_c$  seen by other researchers at the same site. Our first hypothesis was that differences in transpiration were driven not by differences in the water potential gradient ( $\Delta\Psi$ ) but instead by differences in whole-tree conductivity. When we measured  $\Delta\Psi$  during June and August of the 2015 growing season there were no significant differences between any of the treatment combinations (Table 3.5). This validated our first hypothesis, since by equations (1) and (2), if  $\Delta\Psi$  was not responsible for differences in transpiration, then whole-tree conductivity ( $k_{\text{tl}}$ ) must be responsible.

Our second hypothesis was that any reductions in  $k_{\text{tl}}$  would not be explained by differences in small shoot conductivity. We found that, for both sapwood specific conductivity ( $K_s$ ) and needle specific conductivity ( $K_l$ ), shoots from the control plots had the greatest conductivities (Table 3.5). Shoots from the fertilizer only plots had the lowest conductivities. The significant interactions between fertilization and throughfall exclusion meant that the lowering of  $K_s$  and  $K_l$  produced by fertilization were significantly less when combined with throughfall exclusion (e.g. Figure 3.2). This means that conductivities for shoots from fertilization + throughfall exclusion plots were significantly higher than those from throughfall exclusion only. Hence, we can confidently state that conductivities for small shoots were highest for the control treatment, and that fertilization only was the lowest, significantly lower than throughfall exclusion alone. The small shoots were sampled in mid-April of 2015, when the monthly rainfall was 100 mm (“NCDC Climate Data Online,” 2017). In April of 2013, the monthly rainfall was 106 mm (“NCDC Climate Data Online,” 2017) and the lowest transpiration per ground area ( $E_c$ ) was seen in the throughfall exclusion + fertilization trees. They transpired 75% of control. At the same time, both the fertilizer only and the throughfall exclusion only trees transpired 90% of control (Ward et al., 2015). Since we

found that the conductivities of the fertilization only shoots were significantly less than the conductivities of the fertilization + throughfall exclusion shoots, shoot conductivity was not the main factor limiting  $k_{tl}$ . This validated our second hypothesis by elimination.

Our third hypothesis was that shallow root presence in the fertilization + throughfall exclusion plots would be significantly lower than shallow root presence in the throughfall exclusion only plots. This was predicated upon others' findings that fertilization lowered the ratio of the area of roots to the area of shoots ( $A_r:A_l$ ) (Ewers et al., 2000; King et al., 1999). Ewers et al. (2000) also found that fertilization lowered the sensitivity of transpiration to vapor pressure deficit, a finding echoed at the site used for our experiments (Ward et al., 2015). Since the extent of roots relative to the extent of needles has been demonstrated to be a major determinant of hydraulic limitation in woody plants (Sperry et al., 1998, 2002), we suspected that the fertilizer + throughfall exclusion trees might have decreased root presence compared to the throughfall only trees, which contributed to their lower transpiration rates. We found that fertilization did lower root length density when combined with throughfall exclusion, as compared to throughfall exclusion alone (Table 3.1). This effect occurred both between and under throughfall exclusion troughs (Figure 3.1). Shallow root surface area and volumetric density were both lower under troughs than between them in the throughfall exclusion only and in the throughfall exclusion + fertilization plots. The main effect of fertilization was not significant for shallow root surface area or shallow root volumetric density.

The second general question that motivated this research was how do shallow root distribution and hydraulic efficiency in non-sand soils respond to the heterogeneity of soil moisture created by throughfall exclusion troughs. We limited that question to non-sand soils because others have observed that loblolly favors deep roots when grown on sandy soils without perched water tables (Wightman et al., 2016). As there is a hard pan layer at the experimental site where deeper water may perch, it follows that overall, the throughfall exclusion only and fertilizer + throughfall exclusion trees may be relying more on roots near the hardpan layer but are also prioritizing shallow roots between

troughs (Ansley et al., 2014). Additionally, the presence of any living roots remaining under the troughs may serve as an indirect validation of the presence of hydraulic redistribution in loblolly pine (Domec et al., 2010a). From observations made on-site, it was clear that the shallow soil under troughs was notably drier than the shallow soil between troughs in the throughfall exclusion only and the fertilizer + throughfall exclusion plots. If there was hydraulic redistribution occurring, the effects on shallow soil moisture of the exclusion troughs was still pronounced.

Our fourth hypothesis was that shallow root presence would increase between troughs in the throughfall exclusion only plots as compared to control. Roots are known to redistribute under soil moisture limitation to areas with relatively wetter soil (Abrisqueta et al., 2008; Ansley et al., 2014). If this were true, it would demonstrate that the throughfall exclusion only trees were depending more on soil between troughs for water. Furthermore, any decrease to root presence between troughs caused by the addition of fertilization would further support the assertion that decreased root presence in the fertilizer + throughfall exclusion plots limited the water uptake capabilities of the trees and could cause lead to decreased hydraulic efficiency and transpiration (Sperry et al., 2002). We did not find that shallow root presence was greater between troughs of the throughfall exclusion only plots compared to control (Table 3.2). However, when we compared root presence in throughfall exclusion only and fertilization + throughfall exclusion plots, both the main effect of sample location, i.e. under or between troughs, and the main effect of fertilization were significant. This means fertilization significantly lowered root presence regardless of sampling location relative to trough location in the fertilizer + throughfall exclusion only plots (Figure 3.1).

Our fifth hypothesis was that native hydraulic conductivity would decrease relative to trough position in the throughfall exclusion only plots, despite any changes to cavitation resistance that might occur. It has been shown that roots of loblolly pine lose conductance in response to homogeneous reductions to soil moisture content (Domec et al., 2010b), but the response to heterogeneous soil moisture is less clear. Additionally, we thought

that root tissue density, as a proxy for cavitation resistance, would increase differentially under and between troughs in the throughfall exclusion only plots. This would demonstrate a response specific to the immediate root environment, and not a root system-wide response. We found that native sapwood area specific root hydraulic conductivity was significantly lower under troughs than between them in the throughfall exclusion only plots; it was lowered by ~69%. Despite being lowered by ~24%, on average, the native sapwood specific root conductivity between troughs was not significantly lower than in control plots (Table 3.3). Root tissue density was lower both under and between troughs in the throughfall exclusion only plots compared to control. It was not significantly different when roots from under the troughs were compared to roots from between them. Considering the observed changes to native conductivity and root tissue density together, it seems that although cavitation resistance increased overall in the throughfall exclusion only plots, it did not do so differentially with respect to trough position. That means that losses to shallow root native conductivity as a result of decreased shallow soil moisture were not differentially buffered, leaving root sapwood specific native conductivity much lower under troughs. So, the cavitation resistance response was not linked to immediate soil environment, and native root sapwood specific conductivity was. These findings did not support our fifth hypothesis, and possibly point to a more systemic cavitation resistance response in trees subjected to soil moisture limitation alone.

Since the roots and shoots sampled for native conductivity were collected in late summer and spring, respectively, we do not directly compare their numeric importance as drivers of reductions to whole-tree conductance. Instead, we propose qualitatively that by elimination, if shoot conductivities could not explain relative reductions in transpiration then root or rhizosphere limitation of conductance must be responsible. This is based on the belief of many plant physiologists that the primary limits to whole-tree conductance occur either in small shoot xylem or in root xylem or in the rhizosphere (Cruiziat et al., 2002; Ewers et al., 2000; Granier et al., 1989; Scoffoni et al., 2017; Sperry et al., 1998,

2002; Wullschleger et al., 1998). Since loblolly pine is known to locally deplete soil moisture in the rhizosphere more so than in the bulk soil (MacFall et al., 1990), it is difficult to separate embolism-induced loss of root xylem conductivity due to a dry rhizosphere from limitation due to decreased soil moisture conductance in a dry rhizosphere. Sperry et al. showed that the ratio of the area of roots to the area of shoots is a major factor that determines the nature of the limitation to whole-tree conductance (1998, 2002). When the ratio is high, xylem conductivity is more likely to limit hydraulic function. When it is low, primarily rhizosphere conductance is limiting.

We found evidence eliminating water potential gradient and small shoot conductivities from the pool of possible explanations of the greatly reduced transpiration of the fertilizer + throughfall exclusion trees at the VA experimental site. We also found that root length density was significantly reduced in the fertilizer + throughfall exclusion plots compared to the throughfall exclusion only plots. In the absence of changes to leaf area index, root presence is known to be a large factor determining limitations to whole-tree conductance (Sperry et al., 1998, 2002), we propose that decreases in shallow root length density in non-sandy soils may be a significant contributing factor to the reduction in transpiration in fertilized trees also experiencing soil moisture limitation. Since throughfall exclusion only did not significantly lower shallow root length density relative to control, the combination of fertilization and soil moisture limitation was uniquely associated with the changes to shallow root length density among the physiological and morphological attributes we measured in this study. Fertilization alone caused changes to the patterns of sensitivity of transpiration to vapor pressure deficit and bulk soil moisture relative to control trees and to throughfall exclusion only trees (Ward et al., 2015). This was more extreme in the fertilizer + throughfall exclusion trees. The fertilization alone treatment decreased shallow root length density, and when soil moisture limitation was imposed in addition to fertilization, the loss of root length density was significantly more pronounced. This pattern mirrors that seen in the environmental sensitivity of

transpiration, which reduced long-term transpiration in the fertilizer + throughfall exclusion trees.

### 3.5 Conclusion

Our first general question was what could help explain the differences in transpiration observed between loblolly trees subjected to soil moisture limitation only and loblolly trees subjected to fertilization in addition to soil moisture limitation at our experimental site. We found that at the site in the Virginia Piedmont, transpiration was not limited by the root to shoot gradient of water potential. By elimination, it was instead limited by decreases to whole-tree conductance. Differences in whole-tree conductance inferred from differences in canopy transpiration per ground area were not solely explained by decreases to the hydraulic conductivity of small shoots. This finding was based on comparisons of the relative magnitude of transpiration and shoot hydraulic conductivity in the same month, but in different years where rainfall differed by only 7 mm. We also found that shallow root length density was decreased by both the soil moisture limitation caused by the throughfall exclusion treatment, and by fertilization in an additive fashion when treatments were combined. This indicates that shallow root length density may be a useful metric to help explain why the transpiration of fertilized trees that are chronically limited by reduced soil moisture display reduced vapor pressure deficit sensitivity and increased sensitivity to bulk soil moisture. This could be complicated, and likely exacerbated, by concomitant increases to leaf area index that are usually expected when loblolly trees are fertilized. There were no increases to leaf area index at the experimental site described here.

Our second general question was how would heterogeneous soil moisture caused by the throughfall exclusion troughs affect shallow roots. To address this, we examined shallow root presence, native hydraulic conductivity, and root tissue density in control and throughfall exclusion only plots. We found that there was not an increase in root presence between troughs relative to control. Also, native shallow root hydraulic conductivity did decrease relative to trough position in the throughfall exclusion only



plots. When data was weighted by ground area under and between troughs there was still an overall effect of soil moisture limitation, despite the heterogeneous nature of its reduction. Changes to root tissue density, as a proxy for cavitation resistance, did not occur differentially in shallow roots relative to trough position in the throughfall exclusion only plots. This meant that cavitation resistance did not buffer the effect of lower shallow soil moisture under the troughs, allowing for the much lower native hydraulic conductivities that developed there. This could suggest that increases to cavitation resistance are not in response to the immediate soil environment, and may be more systemic in nature, when soil moisture is limiting in a heterogeneous manner.

Future work could investigate the effects of fertilization in combination with soil moisture limitation on cavitation resistance in shallow roots, as well as the effects on shallow root conductivity. Also, investigating rooting depth and the distribution of deeper roots could also help to identify more general changes to the root systems of loblolly pine subject to the combination of soil moisture limitation and fertilization. Future work based on this study could also benefit from more fully elucidating the precise physical and biochemical effects that fertilization has on the hydraulic efficiency of root tissue in plantation grown loblolly pine. Additionally, studies fully characterizing the effects on water use efficiency of partial root zone drying by directly comparing trees growing with uniformly reduced bulk VWC to those with long-term, spatially distinct bulk VWC would be highly informative.

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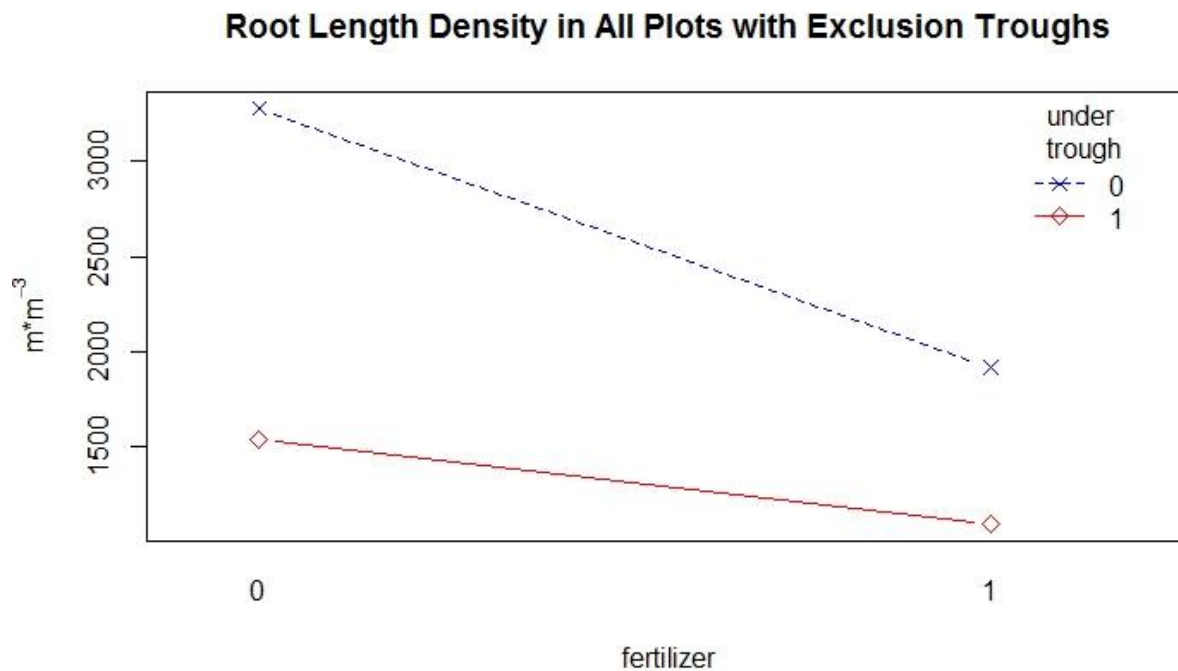
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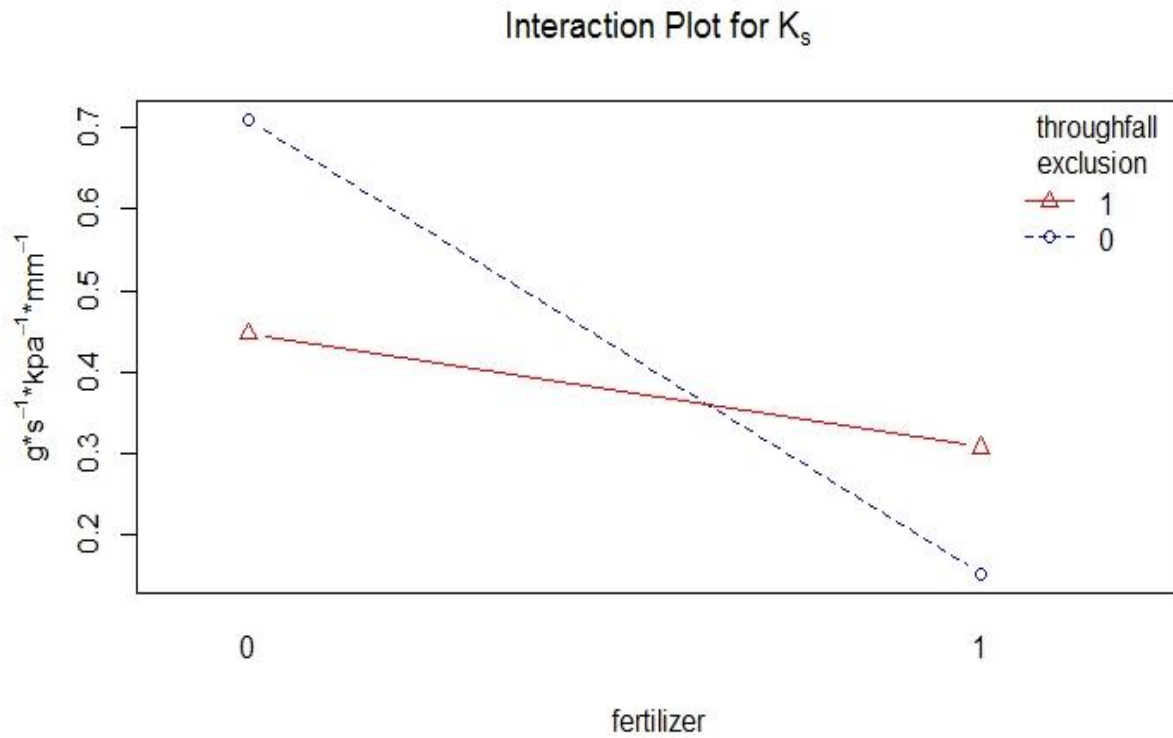
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### 3.7 Figures and tables



**Figure 3.1.** Mean values of root length density in the upper 16cm of soil as influenced by fertilization and by position under or between troughs in plots with exclusion troughs at the 12-year-old loblolly pine plantation located in the Piedmont of Virginia. This figure presents data from throughfall exclusion only and throughfall exclusion + fertilization plots, which indicated significant main effects for fertilizer and sampling position relative to troughs. Absence or presence of a factor is indicated by a 0 or a 1, respectively.





**Figure 3.2.** Small shoot sapwood normalized hydraulic conductivity ( $K_s$ ) from all factorial combinations of fertilizer and 30% throughfall exclusion in a 12-year-old loblolly pine plantation located in the Piedmont of Virginia. Fertilizer application lowered  $K_s$  significantly less in the trees not subjected to throughfall exclusion ( $p = 0.04$  for interaction). Labels of 0 or 1 indicate absence or presence of the treatment factors, respectively.

**Table 3.1.** Root distribution attributes for all roots collected from the upper 16 cm of soil in a 12-year-old loblolly pine plantation in the Piedmont of Virginia. Data comes from 30% throughfall exclusion (TE) only plots and 30%TE + fertilizer plots. In addition to the different treatments, sampling position relative to troughs within each treatment is also indicated. Root length density has units of  $\text{m m}^{-3}$ , root surface area has units of  $\text{m}^2 \text{m}^{-3}$ , and root volume density has units of  $\text{m}^3 \text{m}^{-3}$ , as a percentage.

| Attribute           | 30%TE Only             |                        | Fertilized + 30%TE     |                        |
|---------------------|------------------------|------------------------|------------------------|------------------------|
|                     | Under Troughs          | Between Troughs        | Under Troughs          | Between Troughs        |
| Root Length Density | 1535 ± 323 <b>B(2)</b> | 3274 ± 309 <b>A(2)</b> | 1096 ± 196 <b>B(1)</b> | 1919 ± 211 <b>A(1)</b> |
| Root Surface Area   | 3.39 ± 0.60 <b>B</b>   | 8.74 ± 1.17 <b>A</b>   | 3.35 ± 0.59 <b>B</b>   | 5.88 ± 1.23 <b>A</b>   |
| Root Volume Density | 0.070 ± 0.018 <b>B</b> | 0.218 ± 0.066 <b>A</b> | 0.085 ± 0.016 <b>B</b> | 0.161 ± 0.054 <b>A</b> |

Values shown are means ± 1 standard error. Different letters indicate a significant main effect of sampling location (either under or between troughs), and the parenthetical numbers indicate an additional significant main effect of fertilization.

**Table 3.2.** Root distributional attributes for all roots collected from the upper 16 cm of soil in a 13-year-old loblolly pine plantation on the Piedmont of Virginia in control plots and between exclusion troughs in 30% throughfall exclusion only plots. Root length density has units of  $\text{m m}^{-3}$ , root surface area has units of  $\text{m}^2 \text{m}^{-3}$ , root volume density has units of  $\text{m}^3 \text{m}^{-3}$  as a percentage.

| Attribute           | Control                | Between TE Troughs     |
|---------------------|------------------------|------------------------|
| Root Length Density | 3001 ± 177 <b>A</b>    | 3193 ± 349 <b>A</b>    |
| Root Surface Area   | 9.65 ± 0.67 <b>A</b>   | 10.98 ± 1.25 <b>A</b>  |
| Root Volume Density | 0.257 ± 0.033 <b>A</b> | 0.312 ± 0.046 <b>A</b> |

Values shown are means ± 1 standard error. Different letters indicate significant difference within a row.

**Table 3.3.** Root hydraulic attributes collected from small roots in the upper 16 cm of soil in a 13-year-old loblolly pine plantation in the Piedmont of Virginia. Data is organized by sampling position relative to troughs for the 30% throughfall exclusion (TE) only plots. Root sapwood specific hydraulic conductivity ( $K_{Rs}$ ) has units of  $\text{Kg m s}^{-1} \text{MPa}^{-1} \text{m}^{-2}$ . Root tissue density has units of  $\text{Kg m}^{-3}$ .

| Attribute           | Control               | Under TE Troughs      | Between TE Troughs    |
|---------------------|-----------------------|-----------------------|-----------------------|
| $K_{Rs}$            | 2.46 ± 0.49 <b>A</b>  | 0.58 ± 0.21 <b>B</b>  | 1.88 ± 0.35 <b>A</b>  |
| Root Tissue Density | 427.4 ± 18.1 <b>B</b> | 529.0 ± 47.3 <b>A</b> | 513.5 ± 32.0 <b>A</b> |

Values shown are means ± 1 standard error. Different letters indicate significant difference within a row.

**Table 3.4.** Root distribution (all roots) and hydraulic attributes (small roots) collected over two seasons (12<sup>th</sup> and 13<sup>th</sup>) from the upper 16 cm of soil in a loblolly pine plantation on the Piedmont of Virginia in control plots and under and between troughs in 30% throughfall exclusion only plots. Root sapwood specific hydraulic conductivity ( $K_{Rs}$ ) has units of  $\text{Kg m s}^{-1} \text{MPa}^{-1} \text{m}^{-2}$ . Root tissue density has units of  $\text{Kg m}^{-3}$ . Root length density has units of  $\text{m m}^{-3}$ , root surface area has units of  $\text{m}^2 \text{m}^{-3}$ , root volume density has units of  $\text{m}^3 \text{m}^{-3}$ , as a percentage. (the overall 30% throughfall exclusion data means and standard errors presented here come from data weighted by ground from areas under and between troughs).

| <b>Attribute</b>           | <b>Control</b>         | <b>30% Exclusion</b>   |
|----------------------------|------------------------|------------------------|
| <b>Root Length Density</b> | 3001 ± 177 <b>A</b>    | 2752 ± 141 <b>A</b>    |
| <b>Root Surface Area</b>   | 9.65 ± 0.67 <b>A</b>   | 7.13 ± 0.45 <b>B</b>   |
| <b>Root Volume Density</b> | 0.257 ± 0.033 <b>A</b> | 0.173 ± 0.020 <b>A</b> |
| <b><math>K_{Rs}</math></b> | 2.46 ± 0.49 <b>A</b>   | 1.48 ± 0.21 <b>B</b>   |
| <b>Root Tissue Density</b> | 427.4 ± 18.1 <b>B</b>  | 518.2 ± 20.1 <b>A</b>  |

Values shown are means ± 1 standard error. Different letters indicate significant difference within a row.

**Table 3.5.** Shoot hydraulic and morphological attributes, as well as whole-tree water potential gradients ( $\Delta\Psi$ ) collected from mid-crown height in a 12-year-old loblolly pine plantation on the Piedmont of Virginia as affected by throughfall exclusion in fertilized and unfertilized plots. Shoot sapwood specific hydraulic conductivity ( $K_s$ ) has units of  $\text{Kg m s}^{-1} \text{MPa}^{-1} \text{m}^{-2}$ , shoot needle area specific hydraulic conductivity ( $K_l$ ) has units of  $\text{Kg m s}^{-1} \text{MPa}^{-1} \text{m}^{-2}$ . Shoot sapwood to needle area ratio ( $A_s:A_l$ ) is dimensionless and is presented as sapwood area per needle area, and specific needle area (SLA) has units of  $\text{cm}^2 \text{g}^{-1}$ . Water potential gradients ( $\Delta\Psi$ ) have units of  $-\text{MPa}$ .

| <b>Attribute</b>                        | <b>Control</b>                                    | <b>30% Exclusion</b>                               | <b>Fertilized</b>                                  | <b>30% Exclusion + Fertilized</b>                  |
|---|---|--|--|--|
| <b><math>K_s</math> (April) *</b>       | 0.71 ± 0.21                                       | 0.45 ± 0.10  | 0.15 ± 0.08  | 0.31 ± 0.02  |
| <b><math>K_l</math> (April) *</b>       | 5.1E <sup>-5</sup> ± 1.8E <sup>-5</sup>           | 3.0E <sup>-5</sup> ± 0.5E <sup>-5</sup>            | 1.1E <sup>-5</sup> ± 0.6E <sup>-5</sup>            | 4.0E <sup>-5</sup> ± 1.1E <sup>-5</sup>            |
| <b><math>A_s:A_l</math> (April)</b>     | 0.8E <sup>-4</sup> ± 0.1 E <sup>-4</sup> <b>A</b> | 0.7 E <sup>-4</sup> ± 0.1 E <sup>-4</sup> <b>A</b> | 1.0 E <sup>-4</sup> ± 0.3 E <sup>-4</sup> <b>A</b> | 1.3 E <sup>-4</sup> ± 0.4 E <sup>-4</sup> <b>A</b> |
| <b>SLA (April)</b>                      | 126.5 ± 7.9 <b>B</b>                              | 124.7 ± 2.3 <b>B</b>                               | 143.2 ± 3.0 <b>A</b>                               | 140.1 ± 2.3 <b>A</b>                               |
| <b><math>\Delta\Psi</math> (June)</b>   | 0.75 ± 0.05 <b>A</b>                              | 0.90 ± 0.08 <b>A</b>                               | 0.89 ± 0.04 <b>A</b>                               | 0.83 ± 0.06 <b>A</b>                               |
| <b><math>\Delta\Psi</math> (August)</b> | 1.00 ± 0.01 <b>A</b>                              | 0.96 ± 0.04 <b>A</b>                               | 1.03 ± 0.04 <b>A</b>                               | 0.93 ± 0.03 <b>A</b>                               |

Values shown are means ± 1 standard error. Different letters indicate significant difference within a row. An asterisk following an attribute label indicates a significant interaction between fertilization and throughfall exclusion.

## **Chapter 4: The effects of soil moisture heterogeneity on young, well-watered loblolly pine trees: A series of static partial root zone drying experiments**

### **Abstract**

Loblolly pine (*Pinus taeda* L.) is extremely important to the economy and environment of the Southeastern United States. A changing climate demands a better understanding of the response of loblolly to drought conditions. Some studies undertaken for this purpose utilize impermeable throughfall exclusion troughs to reduce throughfall precipitation. This generally leads to heterogeneity of soil moisture, along with reducing available soil moisture overall. There is a similar set of soil moisture conditions created by the use of partial root zone drying in agricultural production. Partial root zone drying generally decreases transpiration, and increases the water use efficiency of biomass production. These effects have also been documented in throughfall exclusion studies. When plants are subjected to partial root zone drying, decreased transpiration and increased water use efficiency occurs due to stomatal constriction in response to increased abscisic acid concentrations in leaves. The abscisic acid is transported from roots in drying soil to leaves. This study was designed to investigate the effects of fixed soil moisture heterogeneity without concurrent water limitation. This was accomplished by ensuring that the trees subjected to partial root zone drying had ample water to satisfy transpirational demand. Loblolly pine seedlings were grown in large pots with two distinct soil zones in a greenhouse for 29 weeks. Half of the trees were then subjected to static partial root zone drying for 60 days to simulate the heterogeneous soil moisture conditions created by throughfall exclusion troughs. Subsequently, all experimental groups were exposed to their initial drought experience via two cycles of zero irrigation. Sap flow, physiological and morphological data were recorded and analyzed. It was found that static partial root zone drying did not reduce transpiration relative to the

control when the trees were well watered, nor did it increase water use efficiency. An increase in the ratio of sapwood area to needle area was observed, as was an increase in whole-tree hydraulic conductance in the treatment trees. There were also small differences in the response of transpiration to low volumetric soil moisture content. It appears that the heterogeneity of soil moisture created by throughfall exclusion troughs does not confound the effects of the soil moisture limitation they create. These findings support the use of throughfall exclusion troughs to simulate drought.

#### **4.1 Introduction**

Loblolly pine (*Pinus taeda* L.) is the dominant tree species across some 29 million acres in the American Southeast. Of all the standing pine biomass in the region, loblolly pine accounts for more than half (Baker and Langdon, 1990). Climate modelers have recently predicted an increase in the variability of summer rainfall patterns in the coming decades in the Southeast (Li et al., 2013). A measure of summer dryness is expected to consistently increase for at least the remainder of the 21<sup>st</sup> century (“PINEMAP Decision Support System v. 1.5.,” 2015). Numerous researchers have set out to better understand how the growth, productivity, and physiology of loblolly pine will be affected, given current management practices and the expected changes to the growing environment in the Southeastern United States (Domec et al., 2012; Ewers et al., 2000; Maggard et al., 2016; Meier et al., 1992; Samuelson et al., 2014; Stovall et al., 2011; Wightman et al., 2016; Will et al., 2015; Xiao et al., 2011).

Throughfall exclusion has been used as a means of simulating reduced rainfall in a number of the aforementioned studies. Depending on the width of the excluders and their height above the ground, throughfall exclusion can create heterogeneous soil moisture under and between the impervious troughs used to exclude a portion of throughfall precipitation. This is in addition to their intended purpose, which is to limit soil moisture. This phenomenon is akin to the edaphic moisture heterogeneity produced by an agricultural deficit irrigation technique known as partial root zone drying (PRZD), wherein irrigation is only applied to a portion of the root zone of orchard or row crops.

The deficit irrigation may be applied in alternation to different sides of the root zone or consistently on one side only, the latter approach being known as static or fixed PRZD. Static PRZD tends to have significantly less pronounced physiological effects (Abrisqueta et al., 2008; Perez-Perez and Dodd, 2015). PRZD has been shown to increase water use efficiency via the action of increased acropetal abscisic acid delivery to leaf or needle tissue accompanied by increased xylem sap pH. Increased concentrations of abscisic acid in guard cells and increased pH of xylem sap have been demonstrated to reduce stomatal conductance, contributing to observed improvement in water use efficiency (Davies et al., 2002). Overall, water consumption is reduced while higher relative productivity is realized, given the deficit in irrigation volume (Kang et al., 2003; Kang and Zhang, 2004). Increases to the hydraulic conductance of the portion of trees' root systems in moist soil has also been described in response to PRZD (McLean et al., 2011).

Loblolly pine trees have been shown to increase their water use efficiency in response to uniform soil moisture limitation (Bongarten and Teskey, 1986). In experiments using throughfall exclusion troughs to reduce rainfall, this increased water use efficiency resulting from decreased soil moisture could potentially be confounded by increased water use efficiency resulting from the heterogeneity of soil moisture created by the troughs. Where throughfall exclusion has been used to simulate reduced rainfall, researchers have noted increased water use efficiency in loblolly pine stands. This effect accompanied reduced stomatal conductance and transpiration (Maggard et al., 2017; Samuelson et al., 2018; Ward et al., 2015). It is unclear if those effects are due solely to soil moisture limitation, or if soil moisture heterogeneity plays a role.

When throughfall exclusion is employed, heterogeneous soil moisture and soil moisture limitation are created together. We applied conditions of soil moisture heterogeneity, but ensured that the volume of water and the dryness of the soil was not limiting in the irrigated portion of the root zone. We accomplished this by using 56.8 liter pots, which when divided into two root zones, had a volume of at least 19 liters on each

side. During the static PRZD treatment, irrigation to field capacity at 25-30% volumetric water content in the single irrigated root zone occurred every 6 hours. Before the treatment was imposed, 24-hour mean transpiration was less than 1 liter. All trees were maintained with volumetric soil moisture above 15% at all times by increasing irrigation duration as trees grew.

We hypothesized that (1) static PRZD would result in some degree of reduced transpiration relative to control trees. We further hypothesized that (2) the experimental trees subjected to static PRZD would exhibit increased water use efficiency, either intrinsic or water use efficiency of productivity or both. Lastly, and more broadly, we hypothesized that (3) the trees exposed to static PRZD would exhibit responses that could potentially confound the observed effects of throughfall exclusion documented in recent simulated drought studies. For this work, loblolly seedlings were cultivated in a way that created two distinct root zones per tree and allowed for the application of static PRZD. Sap flow was measured in well-watered PRZD trees and in control trees, and both treatment groups were ultimately exposed to their first simulated soil droughts. Physiological and morphological responses were characterized for each phase of experimentation.

## **4.2 Material & methods**

The containerized loblolly seedlings used in this experiment were from a single family of control mass pollinated trees (IFCO, Moultrie, GA). Sixteen loblolly pine seedlings were grown in a greenhouse on a single bench in 56.8-liter pots (~ 0.4 m by 0.4 m each). Two portions of each pot were separated vertically by a section of fine gravel ~ 8 cm wide. Blank newsprint paper initially separated the central gravel divider from the soil zones. The bottom 5 cm of each pot was first filled with pea (1 cm particle size) gravel to avoid moisture wicking from one side to the other, and to avoid ponding in general, in an attempt to restrict root systems to the soil zones (Figure 1). Additional drainage holes were made at the outer lower edge of the pots at 6-8 cm intervals to

eliminate standing water. The soil used to fill the root zones of each pot was a 3 to 2 mixture by volume of Promix HP (Premier Tech Horticulture, Quakertown, PA) and field soil taken from a loblolly pine plantation located in the Appomattox-Buckingham State Forest of Virginia. The field soil was taken from the upper 15 cm of the mineral soil profile and was crushed and sieved to 5 mm before use. The field soil texture was characterized as a clay loam. When the soil was prepared in February of 2016, Osmocote Plus (The Scotts Miracle-Gro Company, Marysville, OH) was added to supply 0.68 Kg nitrogen (N) per pot. Four months later, in June, additional Osmocote Plus was added to the surface of the pots at 0.14 Kg N per pot. At an initial height of ~ 2 meters above the bench, five high pressure sodium lamps totaling 2000 watts were evenly spaced and used to create a 16-hour photoperiod. As trees grew, the lamps were raised to maintain their distance from the tops of the trees.

When the seedlings were first potted they received 0.65 l every six hours via a single dripper placed over the root plug. After 3 weeks, they were each switched to two drippers, which were placed just to the outside of the root plug on opposite sides. Each dripper applied 0.4 l every six hours. Over the course of 4 weeks, the two drippers were moved weekly in small increments to the center of the separate soil zones within each pot. Soil moisture was checked regularly using a TRASE System 1 time domain reflectometry (TDR) unit with 20 cm wave guides (Soil Moisture Corporation, Santa Barbara, CA). The wave guides remained in place in the pots to avoid root disturbance. Irrigation was periodically adjusted to consistently reach 25-30% volumetric water content 15 minutes after each irrigation event. Pots were maintained above a minimum daily soil moisture of 15% in both root zones of each pot until the partial zoot-zone drying (PRZD) treatment was imposed. The treatment began in mid-August of the same year (Figure 2). Of the 16 trees, 8 randomly chosen trees were subjected to static PRZD. When the PRZD treatment was initiated, trees chosen for the PRZD treatment had one dripper moved so that both drippers were then positioned on one side only of the PRZD pots. Irrigation of all trees continued to be monitored and adjusted such that wet side soil



moisture was brought up to field capacity at 25-30% after each irrigation, and a daily minimum water content of 15% was maintained.

Before the PRZD treatment was imposed, all of the trees were fitted with thermal dissipation type (TDP) sap flux probes to a depth of 10 mm. This was done simultaneously for all trees after the tree with the smallest diameter was greater than 16 mm in outer diameter. The probes were constructed in the USFS Research and Development lab in Research Triangle Park, NC. They were constructed according to the design of Granier, using type T thermocouples (Granier, 1985). The sensor data was converted to what Granier refers to as sap flux density along a radius, quantified in  $\text{m s}^{-1}$ , according to methods from later work (Granier, 1987). Alternate parameter values (Sun et al., 2012) for the conversion from voltage differential to sap flux density along a radius were tested, but are not reported on here, as none of the statistical inference gleaned from their use was different from inference derived from Granier's original parameter values. Also, some research has indicated that the original parameter values are accurate for yellow pine species (Renninger and Schäfer, 2012). The alternate parameter values resulted in reduced estimates of transpiration on the order of  $\sim 30\%$ . Flow inside the main stems of the trees was assumed to be consistent in the radial direction, as the probes were short and the trees were young and without heartwood (Clearwater et al., 1999; Wullschleger et al., 2011). Before probes were installed in the experimental trees, all components of the data logging system (Campbell Scientific, Logan, UT) and the voltage regulation (DROKing, Hong Kong, China) system were tested on other potted trees not used in the study. The geometric mean of starting and final stem diameter corrected for 1.5 mm bark thickness was used to calculate sapwood cross-sectional area for determining sap flow rates. Sapwood area was assumed to occupy the entire stem volume inside the bark. Flow rates in units of  $\text{cm}^3 \text{s}^{-1}$  were calculated for every 15-minute time step and then the rates were numerically integrated using Simpson's Quadrature to arrive at total volumes transpired. All calculations, numerical integration, statistical modeling and analyses were carried out using the R language for statistical computing (R Core

Team, 2018). When different types of models were compared for the same data, selection was made using Akaike Information Criteria and content knowledge.

Data was collected during a pre-treatment period, and during two phases of experimentation. After the trees were fitted with the TDP probes, and satisfactory data quality and equipment performance was demonstrated, sap-flux-based transpiration was monitored for four consecutive, mostly cloudless days to establish a pretreatment baseline. Immediately following the pre-treatment period, the PRZD treatment was imposed upon the 8 randomly selected trees. During phase one of experimentation, sap flow data was collected for 60 days from all trees after the initiation of the treatment. Additionally, needle-level physiological and water potential measurements were also made during the third and fifth weeks of the first phase of the experiment using a LiCor 6400XT (LiCor Bioscience, Lincoln, NE) and a Scholander-type pressure chamber (PMS Instrument Comp., Albany, OR). Measurements of photosynthesis taken using the LiCor 6400XT utilized 400 ppm CO<sub>2</sub>, 800 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux density, chamber temperature of 25°C, and relative humidity in the 40-50% range. Needle area inside the cuvette for each single fascicle subsample measured was calculated as: length inside the cuvette\*fascicle diameter\*fascicle needle count + π\*fascicle diameter\*length inside the cuvette.

The second phase of experimentation involved ceasing all irrigation. Following the cessation of irrigation, soil moisture and needle-level physiology and water potential were all monitored daily over the course of a week during which time late morning transpiration approached zero for all trees. This imposed drought was repeated again, following two days of resumed irrigation. After completion of the second phase of the experiment, the trees were destructively sampled for needle area and root mass determination. Using specific needle area from subsamples and the total needle dry weight per tree obtained after final sampling, transpiration per needle area was calculated for all trees for statistical analysis. Specific needle area was measured on three fascicles per tree by first calculating area as length\*diameter\*needle count + π\*diameter\*length,

and then oven drying samples to obtain dry weights. An average specific needle area per tree was calculated as area per mass. Then the dried total mass of needles from each tree was multiplied by its respective specific needle area to derive total needle area per tree. Needle area normed transpiration was calculated for each tree individually as transpiration for that tree divided by its respective total needle. Sapwood normed transpiration was calculated from intact stem diameter measurements made 5-8 cm above the soil line, just below the sap flux probes; transpiration values per tree were divided by respective calculated sapwood areas.

### 4.3 Results

Total root mass was very similar between control and PRZD trees, while the PRZD trees had significantly less root mass in their dry root zones. The PRZD trees had approximately two times the mass of roots in their wet soil zones as compared to their dry zones. Control trees had equal root mass in their two root zones. When roots were collected at the end of all experimentation for analysis, the trees were not root-bound, nor were there noteworthy volumes of roots in the gravel at the bottom of pots. Although sapwood cross sectional area and total needle surface area were not significantly different between treatments, their ratio of sapwood area to needle area ( $A_S:A_L$ ) was significantly different ( $p = 0.04$ ). The PRZD trees had  $A_S:A_L$  mean values of  $0.67 \text{ cm}^2 \text{ m}^{-2} \pm 0.04$ , while the mean for control trees was  $0.56 \pm 0.03$ . The increases in sapwood area normalized by starting area during the combined phases of the experiment were not significantly different, nor were the absolute increases in sapwood area (Table 4.1).

The pre-treatment data collection period suggested that the trees randomly selected for PRZD were slightly larger and transpiring slightly more water daily, albeit insignificantly. The PRZD trees had transpired 961 ml daily, on average, while the control trees had transpired 753 ml. Following the completion of phase one of the experiment, the PRZD treatment phase, analysis of variance was performed on day 21 and day 60 sapwood area specific total transpiration per tree, total needle area specific transpiration per tree, and on mean daily transpiration normed with pre-treatment values.

We analyzed day 21 values to determine if there were early differences that were obscured in the longer term. Only the needle area specific transpiration was significantly different between treatments, by a factor of  $\sim 1.4$ . This was true for both the day 21 ( $p = 0.008$ ) and the day 60 analyses ( $p = 0.02$ ). Water was transpired on a per needle area basis by the PRZD trees at  $0.44 \text{ ml cm}^{-2} \pm 0.04$  versus  $0.30 \pm 0.03$  for the control trees after 21 days of treatment. This difference was also significant after 60 days. However, all of the aforementioned analyzed attributes exhibited the same general pattern of higher transpiration for the PRZD trees. The total volume transpired by all PRZD trees calculated from sap flow data combined over 60 days was  $\sim 562$  liters, while the control trees transpired a combined total of  $\sim 414$  liters. Saliently, the mean daily transpiration values normed by the corresponding pre-treatment values demonstrate that there was no decrease in transpiration in the PRZD trees relative to their pre-treatment levels after either 21 or 60 days of treatment (Table 4.2).

Instantaneous sap flow data for the first three weeks of the PRZD treatment is shown in Figure 3. The control and PRZD time series have been smoothed using an 8<sup>th</sup> order moving average model and are shown with bootstrapped 95% confidence intervals added by 15-minute time step. Despite the PRZD treatment, the PRZD trees continued to consistently transpire more than the control trees. The same pattern continued for the remainder of the 60-day treatment period of phase one. The gas exchange and needle water potential measurements made at 3 and 5 weeks after the start of phase one showed no significant differences in transpiration, stomatal conductance, net photosynthesis or mid-day needle water potential between groups. There was also no difference in intrinsic water use efficiency, nor in water use efficiency of productivity characterized as 60-day stem growth per total transpiration.

In the second phase of the experiment where all irrigation was ceased, and late morning transpiration was allowed to approach zero, there were differences noted between groups. Figure 4A shows 8<sup>th</sup> order moving average time series for the PRZD and control groups over two drought and recovery periods. Figure 4B shows the calculated

difference between the groups. It can be seen that transpiration in the PRZD group fell below that of the control group during both of the two drought periods during phase two as transpiration declined in both groups (Figure 4.4). Mean daily transpiration data from phase two was normalized by dividing average daily values by the corresponding pre-treatment mean daily values. This gave a normed metric that provided a quantification of transpiration relative to what each tree was transpiring before any treatments were imposed. When ANOVA was performed on pre-treatment normed mean daily transpiration from phase two, the PRZD trees were transpiring significantly less ( $p = 0.007$ ). This is in contrast to the aforementioned consistently higher transpiration in the PRZD group during phase one. Intrinsic WUE was unaffected by treatment during phase two. There was a main effect of treatment ( $p = 0.002$ ) on the relationship between mid-day needle water potential ( $\Psi_{md}$ ) and soil volumetric water content measured on the wet sides of the trees' pots during phase two.

The PRZD trees had significantly more negative  $\Psi_{md}$  values at all volumetric soil water contents (Figure 4.5). The converse was true for the relationship between  $\Psi_{md}$  and volumetric water content when volumetric water content was calculated as an average of both sides of each pot (data not presented). The relationship between transpiration and mid-day needle water potential remained unchanged by the PRZD treatment. Overall, the PRZD trees transpired less when irrigation was withheld, and PRZD  $\Psi_{md}$  values were greater or less than the control values for a given soil moisture dependent on whether 'wet-side' or mean volumetric water content was considered.

When the relationship between volumetric water content and transpiration during phase two was modeled with a 3-parameter exponential asymptote, the best fit of numerous nonlinear models tested, the model fit was significantly better with a treatment term. This was true for the relationship between mean volumetric water content and transpiration ( $p = 0.03$ ), and also for 'wet side' volumetric water content and transpiration ( $p = 0.002$ ) (Figures 4.6 & 4.7). The parameter estimates for the asymptote term in the 'wet-side' volumetric water content versus transpiration model were significantly

different, with the PRZD trees having higher transpiration values (Figure 4.7). This inference was based on non-overlapping 95% confidence intervals for the asymptote parameter estimates for data from the two treatments. From inspection of plots of volumetric water content versus transpiration during phase two, it was suspected that the relationship between volumetric water content and transpiration changed at low soil moisture contents. When volumetric water content values below 10% were modeled using OLS regression on monotonically transformed transpiration data, different patterns presented (Figures 4.8 & 4.9). The ‘wet-side’ data set was characterized with main effects of volumetric water content and treatment ( $p = 0.0004$ ), and an interaction ( $p = 0.001$ ), all of which were significant (Figure 4.8). Effective intercepts were therefore significantly different between treatments and the slopes of the regressions were also significantly different, producing a disordinal interaction. The mean volumetric water content data set was characterized with the same model. The main effect of treatment was not significant, while the interaction term ( $p = 0.04$ ) and the volumetric water content main effect were. This suggests a shared intercept and different slopes by treatment. This model and data set demonstrate an ordinal interaction, an interaction where the crossover point occurs at or beyond the lower boundary of the domain of the volumetric water content variable (Figure 4.9). In short, models confirmed higher transpiration at elevated volumetric water content for the PRZD trees during the dry-down cycles, and differences in the nature of the relationship between transpiration and low volumetric water content that depended on the consideration of mean or ‘wet-side’ water content.

#### **4.4 Discussion**

In studies characterizing the response to PRZD in other species there has been a documented increase in abscisic acid delivery to the shoots and resultant increases in water use efficiency (Dodd et al., 2008; Kang et al., 2003; Kang and Zhang, 2004; Perez-Perez and Dodd, 2015; Stoll et al., 2000). Abscisic acid is recognized as the primary hormone affecting stomatal closure by decreasing the concentration of  $K^+$  in the guard cells, hence elevated abscisic levels in leaves or needles generally leads to decreased

stomatal aperture. The water status of shoots also interacts with abscisic acid and its effect on stomata (Kramer and Boyer, 1995). As soil dries, xylem sap nitrate concentration is reduced and pH is increased. This leads to reduced uptake of abscisic by leaf mesophyll cells and is thought to increase the concentrations of abscisic acid penetrating the guard cells, potentiating the signal received by the stomata as soil dries (Davies et al., 2002). As a portion of the soil dries and root xylem abscisic acid increases, the magnitude of sap flow from the affected roots greatly affects the abscisic acid concentrations in the transpirational stream of sunflowers (*Helianthus annuus* L.), which are isohydric like loblolly pine (Dodd et al., 2008). This means that when the PRZD treatment is fixed, i.e. only one side of the root zone is irrigated, there is very little water uptake from the dry portion of the root zone and therefore little increase in the abscisic acid reaching the guard cells. It has been shown that when combined with deficit irrigation, static PRZD has less of an effect than alternating PRZD combined with deficit irrigation (Kang and Zhang, 2004; Perez-Perez and Dodd, 2015). Since we did not measure abscisic acid concentrations in xylem sap and needle tissue in our study it is unclear if the observed lack of an increase in WUE in the PRZD trees was associated with unchanged needle abscisic acid concentrations due to the fixed nature of the treatment. Since the PRZD treatment was fixed spatially, it is likely that very little water was transpired from the dry portion of the root zone and therefore very little abscisic acid from those roots would have entered the transpirational stream and reached the needles.

Morphologically, the experimental trees were similar, with two basic exceptions. The root mass of the PRZD trees was significantly biased towards the wet side of their pots, but total root masses were the same as those of the control trees. This redistribution of root mass is a known phenomenon and agrees with the observations of other researchers studying soil moisture heterogeneity (Abrisqueta et al., 2008; Ansley et al., 2014; Kozlowski and Pallardy, 2002; Kramer and Boyer, 1995); it also serves to indirectly validate our methods used to apply the PRZD treatment. Lower root mass in conjunction

with the lower soil moisture measured on the dry side of the PRZD pots also confirms the success of the methods we used to create two distinct soil zones within all of the pots.

In addition to the differences in root distribution, the ratio of sapwood area to needle area ( $A_S:A_L$ ) of the PRZD trees were significantly greater (Table 1). Although the ratio of sapwood area to needle area was originally promulgated as a simple metric of hydraulic supply, this interpretation has been criticized due to the stem diameter independent effects of tracheid diameter on sap flow (Cruiziat et al., 2002). In Scots pine, an isohydric species like loblolly pine, it was found that changes to the ratio of sapwood area to needle area observed with differing soil moisture and evaporative demand at different sites co-occurred with changes to whole-tree conductance (Mencuccini and Grace, 1994). Those changes to sapwood area to needle area ratio resulted in the maintenance of the same root-to-shoot water potential gradient across sites. In addition to the different values of sapwood area to needle area ratio observed between treatments in the current study, the needle area normed mean daily transpiration was also significantly greater in the PRZD trees during phase one by a factor of  $\sim 1.4$  (Table 2). Transpiration can be characterized as a flux equal to the product of water potential gradient and whole-tree conductance (Whitehead, 1998; Wullschleger et al., 1998). As such, the observed lack of any difference in  $\Psi_{md}$  at multiple time points during phase one, coupled with a very modest assumption of equal or more negative mean root water potential in the PRZD trees, indicates that the whole-tree conductance was greater for the PRZD trees, which transpired more than control trees under well-watered conditions.

It has been demonstrated that root ground tissue and xylem conductances in different parts of trees play major roles in the hydraulic limits to whole-tree water supply (Sperry et al., 2002). In loblolly pine, some researchers suggest that root conductance is the major driver of changes to whole-tree conductance under conditions of low soil moisture (Domec et al., 2009). Elevated ratios of sapwood area to needle area have been observed to accompany elevated whole-tree hydraulic conductivity in fertilized and irrigated loblolly pine compared to trees that only received fertilization (Ewers et al., 2000). It has



also been shown that in as little as 24 hours, some woody species can increase aquaporin expression and increase the hydraulic conductivity of roots remaining in well-watered soil when other portions of their root system are subjected to PRZD (McLean et al., 2011). Longer term, structural changes are also likely to occur. Since the trees in the current study only experienced very transient drought relative to the much longer period of well-watered PRZD, we propose that the significant differences we observed between treatments were caused by the PRZD treatment.

During the simulated droughts of phase two, the PRZD trees transpired significantly less than the control trees. Once irrigation was stopped, the PRZD trees had only half the volume of water available to them as compared to the control trees. As each of the two periods of withheld irrigation progressed during phase two, the PRZD trees more quickly developed more negative  $\Psi_{\text{md}}$  values, lower stomatal conductance, and decreased their transpiration. Also, the PRZD trees more rapidly depleted the soil moisture available to them as phase two progressed. This is how studies that use throughfall exclusion troughs to simulate reduced rainfall hope to elicit drought responses (Bartkowiak et al., 2015; Maggard et al., 2016; Ward et al., 2015; Wightman et al., 2016; Will et al., 2015). In other words, throughfall exclusion troughs are known to reduce the volume of water available for uptake within a given volume of soil. This has been proven for the shallower portions of the soil profile (Will et al., 2015). When PRZD is implemented, and the total irrigation volume is less than potential evapotranspiration for a region or crop evapotranspiration ( $ET_c$ ) for a given crop species and location, there is a combined effect of drought and the alternating drying itself. This has been documented to result in increased water use efficiency largely via reduced water consumption and lower stomatal conductance, increased acropetal abscisic acid signaling, higher sap pH, and altered root growth dynamics (Abrisqueta et al., 2008; Davies et al., 2002). During phase two, we saw no evidence of any of the classic abscisic acid mediated responses in the PRZD trees, such as increases in intrinsic water use efficiency.

There were differences noted between the control and PRZD trees during phase two, independent of time. Regardless of whether soil volumetric water content was represented as a mean value across soil zones per pot or as the wet-side volumetric water content, the PRZD trees decreased their transpiration more sharply than the control trees at low volumetric water contents (Figures 6, 7, 8 and 9). Researchers have demonstrated that xylem more susceptible to cavitation loses conductivity at less negative water potentials than more resistant xylem (Domec and Gartner, 2001; Kozlowski and Pallardy, 2002; Kramer and Boyer, 1995; Pallardy, 2008). This potentially explains why, when considering wet-side volumetric soil water content, the PRZD trees' transpiration dropped to near zero at a higher volumetric water content than the control trees. Although not always the case, frequently xylem with higher maximum conductance is more susceptible to cavitation (Cruiziat et al., 2002; Sperry and Saliendra, 1994). This would agree with the higher whole-tree conductance observed in the PRZD trees during phase one. If the PRZD trees' wet-side root xylem was more prone to cavitation, they could have a narrower water use envelope at low soil  $\Psi$  and would stop transpiring at higher wet-side soil volumetric water content than the control trees, which is what was observed (Sperry et al., 2002). It is also possible that bulk soil moisture measured in the PRZD pots was less representative of rhizosphere water content than in the control pots. However, this is unlikely because the TDR rods used to measure soil volumetric water content passed directly through the center of the root zones where root density was highest.

The PRZD treatment also affected the relationship between  $\Psi_{md}$  and soil volumetric water content during phase two, with more negative  $\Psi_{md}$  observed in the PRZD trees relative to wet-side volumetric water content. This is not surprising, as the PRZD trees had less root mass in moist soil at a given wet-side volumetric water content compared to the control trees; without more conservative stomatal control the PRZD trees developed more negative  $\Psi_{md}$ . When soil volumetric water content was represented as the mean of the soil zones of each pot the PRZD trees had less negative  $\Psi_{md}$ . This also follows because at a given mean soil volumetric water content value, the majority of the roots of

the PRZD trees were in soil with a higher moisture content than that represented by the mean value, while the control trees' mean soil volumetric water content values were representative of the soil environment of all of their roots (Sperry et al., 1998; Williams et al., 1996). It would seem that the apparent effect of the PRZD treatment on the  $\Psi_{md}$  versus soil volumetric water content relationship was largely, if not entirely, due to the difficulty of representing heterogeneous soil moisture in simple models and was likely not due to a physiological effect of the treatment itself.

Loblolly trees that have been exposed to prior drought conditions have well known responses such as decreased stomatal conductance, decreased whole-tree conductance, and decreased stomatal sensitivity to vapor pressure deficit, which generally act to increase drought tolerance (Bongarten and Teskey, 1986; Seiler and Johnson, 1985). In the current study, the PRZD trees did not display the aforementioned responses. They had received adequate irrigation prior to the imposition of the dry periods in phase two. The volume of plant available water in the wet portion of their root zones was more than sufficient to satisfy daily transpirational demand without inducing moisture stress. This supports the assertion that the responses we documented are those of consistently sufficient, but heterogeneous soil moisture and not of drought conditioning or deficit irrigation. During phase two there was no effect on intrinsic water use efficiency, nor on the relationship between  $\Psi_{md}$  and transpiration over the full range of soil volumetric water content. Also, as noted previously, transpiration was not decreased by PRZD during phase one, WUE did not increase, and whole-tree conductance increased, not decreased. Taken together, this further supports the assertion that the effects documented are those of heterogeneous soil moisture alone, and not prior drought conditioning or soil moisture limitation. This means we were able to test the effects of static imposed soil moisture heterogeneity on established trees with existing root masses without confounding those effects with the additional overall soil moisture reduction throughfall exclusion troughs cause. If soil moisture heterogeneity alone doesn't cause increases in WUE or decreases in transpiration then the effects seen in studies using throughfall exclusion troughs can be

attributed to the reduction of soil moisture they create, and not to the heterogeneity of soil moisture that goes along with it. This validates the use of throughfall exclusion troughs to simulate reduced rainfall.

## 4.5 Conclusion

The hypotheses this research set out to test were (1) PRZD would result in some degree of decreased transpiration in the treatment trees, (2) water use efficiency would be increased by the PRZD treatment, and more broadly, (3) there would be static PRZD effects that could potentially confound the effects of decreased soil moisture in throughfall exclusion experiments in the field, i.e. there would be drought-like effects from well-watered PRZD alone. Interestingly, all three of these hypotheses were generally disproven by the results. The PRZD trees did not decrease their transpiration during the first phase of experimentation. In fact, intrinsic water use efficiency and the relationship between  $\Psi_{md}$  and transpiration remained unchanged for PRZD trees compared with the control. This is generally in line with development under adequate soil moisture and a lack of drought conditioning or soil moisture limitation. Despite the lack of abscisic acid mediated effects on water use seen in other species subjected to PRZD, there were still clear effects of the treatment. The PRZD trees changed their root distribution and increased their sapwood area to needle area ratio relative to the control treatment. Also, the PRZD trees increased their whole-tree hydraulic conductance during phase one and had different transpirational responses to low soil volumetric water content during soil drought. These findings indicate that the effects of static PRZD alone are tangible and distinct from drought effects in young loblolly pine, and do not include reduced transpiration or increased WUE. Furthermore, this serves to validate the use of throughfall exclusion troughs in field experiments to simulate drought since the effects we reported are unlikely to influence or obscure the moisture limitation and drought responses of trees subjected to throughfall exclusion. Thus, experiments employing throughfall exclusion appear to document effects associated with a soil drought response,

unlike the effects we described here (Bartkowiak et al., 2015; Samuelson et al., 2018; Ward et al., 2015; Wightman et al., 2016; Will et al., 2015).

Further research to characterize any effects of PRZD in loblolly pine would benefit from combining static PRZD with long-term deficit irrigation and comparing those effects to uniform, long-term deficit irrigation alone. Additionally, research into the interactive effects of tree age and degree of root establishment on the responses to PRZD could be informative.

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## 4.7 Figures and tables

**Table 4.1.** Physical attributes of greenhouse-grown, young loblolly pine trees either subjected to 60 days of static partial root zone drying treatment (PRZD) or to control treatment

| <b>Morphological Attribute</b>   | <b>PRZD</b>          | <b>Control</b>       |
|--|----------------------|----------------------|
| <b>total root mass (below root crown, grams)</b>                         | 52.3 ± 4.4 <b>A</b>  | 48.9 ± 4.4 <b>A</b>  |
| <b>root mass ('dry' side, grams)</b>                                     | 17.0 ± 1.9 <b>B</b>  | 24.6 ± 2.7 <b>A</b>  |
| <b>`` total needle area (m<sup>2</sup>)</b>                              | 4.97 ± 0.47 <b>A</b> | 5.34 ± 0.42 <b>A</b> |
| <b>SLA (cm<sup>2</sup> g<sup>-1</sup>)</b>                               | 207.8 ± 4.6 <b>A</b> | 215.1 ± 5.4 <b>A</b> |
| <b>sapwood area to needle area ratio (cm<sup>2</sup> m<sup>-2</sup>)</b> | 0.67 ± 0.04 <b>A</b> | 0.56 ± 0.03 <b>B</b> |
| <b>geometric mean sapwood area (cm<sup>2</sup>)</b>                      | 3.26 ± 0.33 <b>A</b> | 2.95 ± 0.28 <b>A</b> |
| <b>sapwood increment proportion (cm<sup>2</sup> cm<sup>-2</sup>)</b>     | <b>0.93 ± 0.10 A</b> | <b>0.86 ± 0.10 A</b> |

Values reported are sample means ± one standard error (SE). Different letters in a row indicate statistically significant differences. `` indicates inference on a Box-Cox transformed variable; the reported means and SEs for total needle area on the original scale are therefore nonparametric bootstrapped values (R=1,000)

**Table 4.2.** Transpirational attributes of greenhouse-grown, young loblolly pine trees either subjected to 60 days of static partial root zone drying (PRZD) or to control treatment. Results are reported for 21 days of treatment, and for the full duration of treatment.

| <b>Attribute (per tree)</b>                              | <b>Day 21 of Treatment</b> |                      | <b>Day 60 of Treatment</b> |                       |
|--|----------------------------|----------------------|----------------------------|-----------------------|
|  | <b>PRZD</b>                | <b>Control</b>       | <b>PRZD</b>                | <b>Control</b>        |
| <b>total E (liters)</b>                                  | 22.14 ± 3.31               | 15.74 ± 1.40         | 70.19 ± 11.07              | 51.67 ± 4.41          |
| <b>˘ sapwood normed total E (l cm<sup>-2</sup>)</b>      | 6.69 ± 0.54 <b>A</b>       | 5.39 ± 0.31 <b>A</b> | 21.28 ± 1.76 <b>A</b>      | 17.86 ± 1.24 <b>A</b> |
| <b>˘ needle area normed total E (ml cm<sup>-2</sup>)</b> | 0.44 ± 0.04 <b>A</b>       | 0.30 ± 0.03 <b>B</b> | 1.43 ± 0.14 <b>A</b>       | 1.00 ± 0.10 <b>B</b>  |
| <b>˘ pre-treatment normed daily E</b>                    | 1.09 ± 0.04 <b>A</b>       | 1.01 ± 0.04 <b>A</b> | 1.23 ± 0.08 <b>A</b>       | 1.17 ± 0.06 <b>A</b>  |

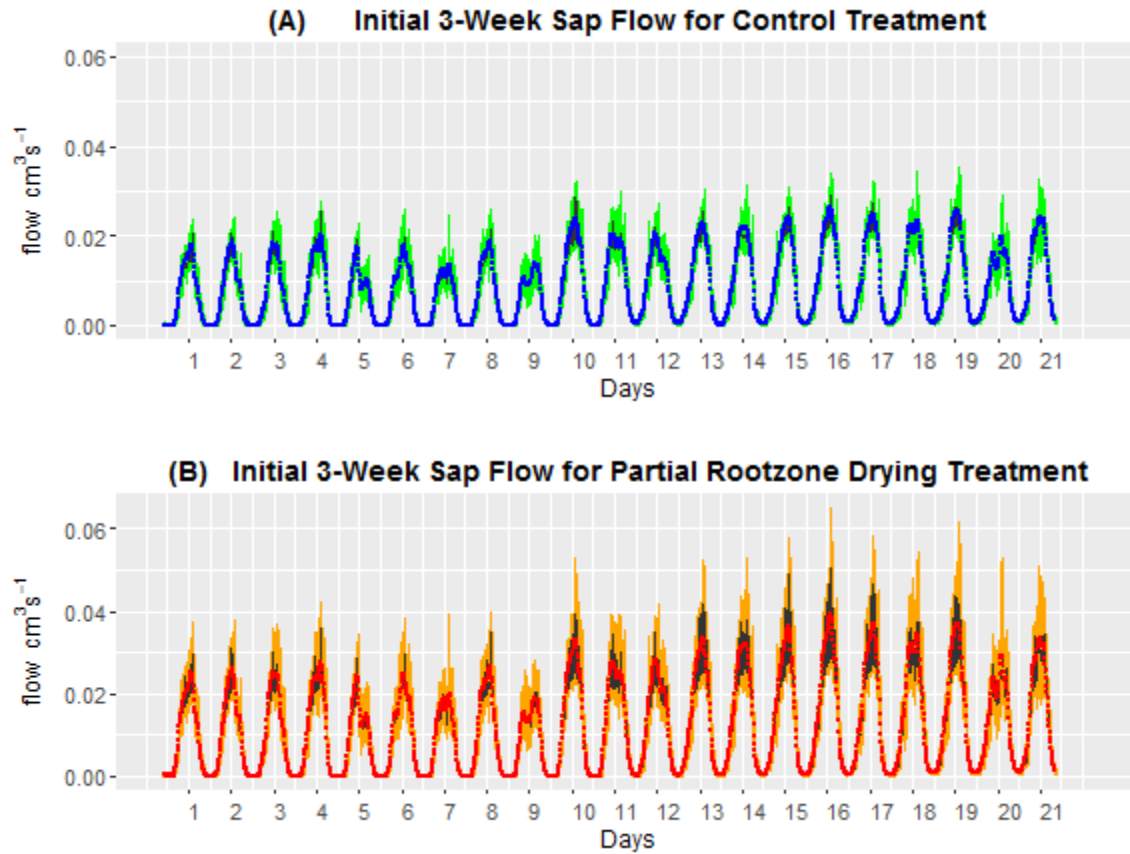
Values reported are sample means ± one standard error (SE). Different letters in a row indicate statistically significant differences for the given treatment duration. ˘ indicates inference on log transformed variable; reported means and SEs on the original scale are bootstrapped maximum likelihood estimates for lognormal data (R=1,000)



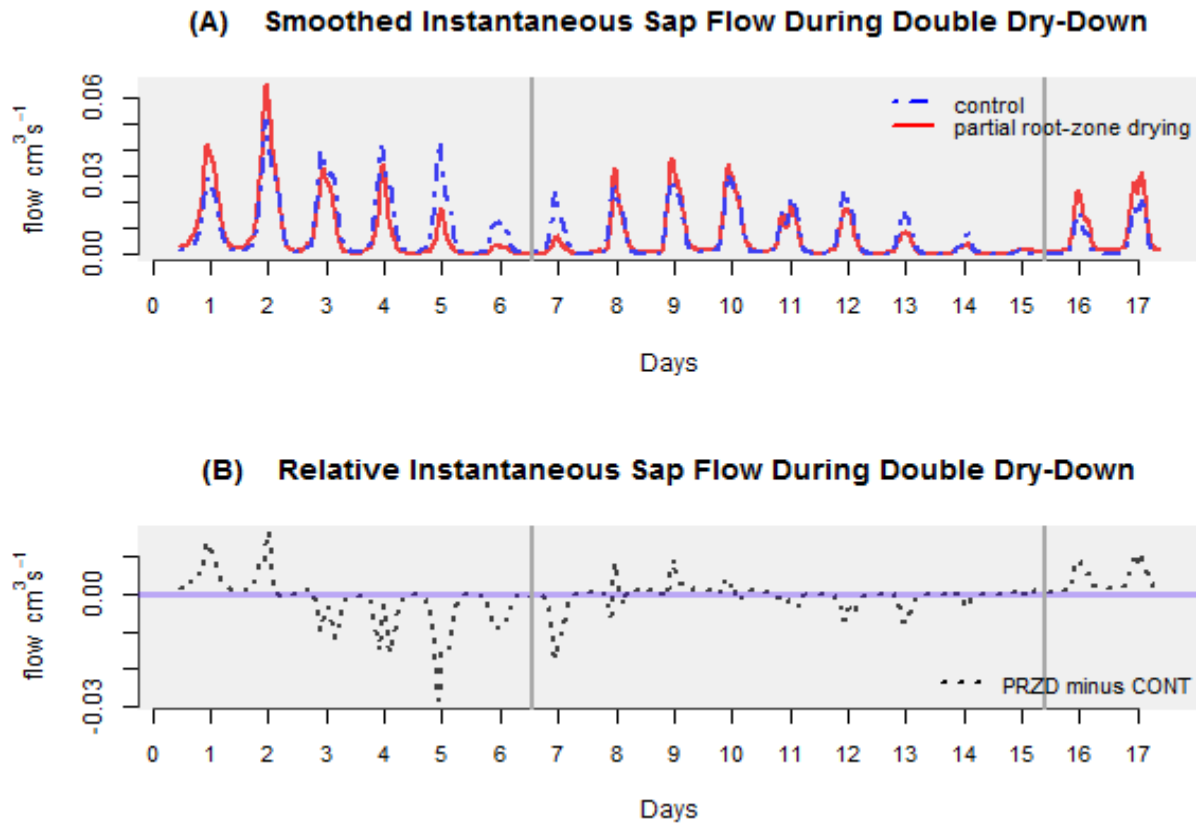
**Figure 4.1.** Each pot was filled with a pea-gravel base, a central dividing region of same gravel, and two distinct soil zones, see Methods for further details (Picture taken on 02/02/2016, three weeks prior to the switch to two drippers per pot)



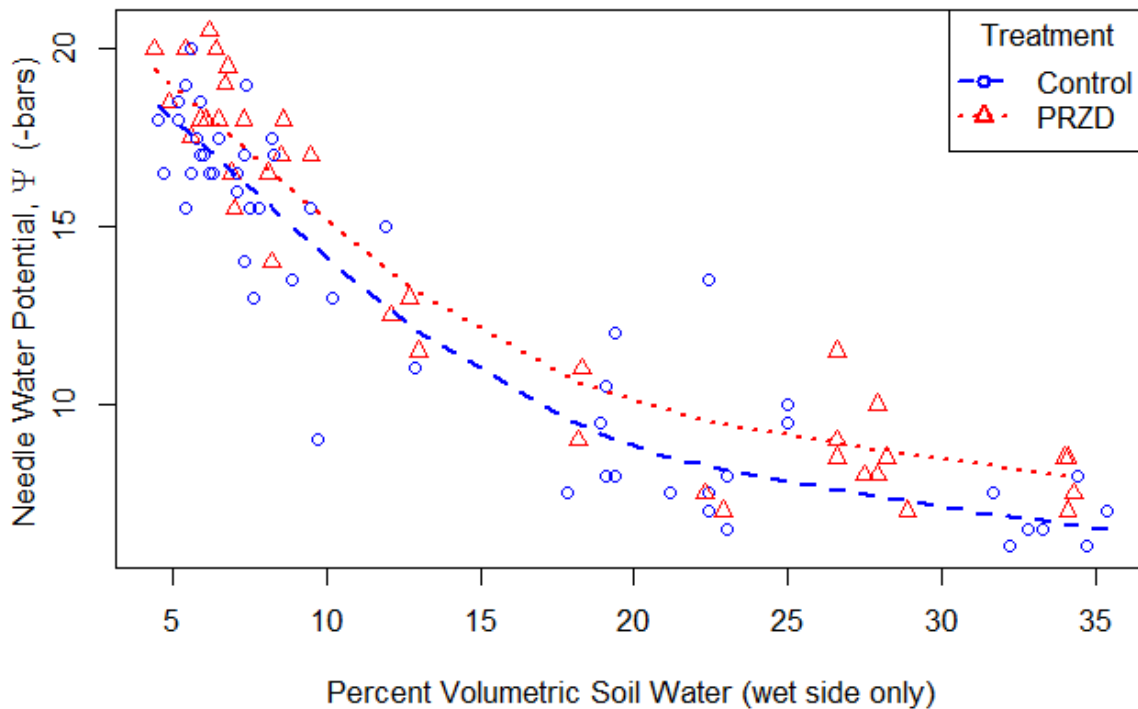
**Figure 4.2.** The experimental trees after six months of growth, and two weeks prior to the initiation of the static partial root zone drying treatment (picture taken on 07/30/2016)



**Figure 4.3.** Instantaneous sap flow presented as 8<sup>th</sup> order moving averages (2-hr window) separated by treatment. Sub-plot (A) shows the control treatment and sub-plot (B) shows the static partial root zone drying treatment. Only the first 21 days of treatment are shown. The dotted lines represent the moving averages while the colored bands indicate bootstrapped 95% confidence intervals by 15-minute time point. Flow rates are per tree.

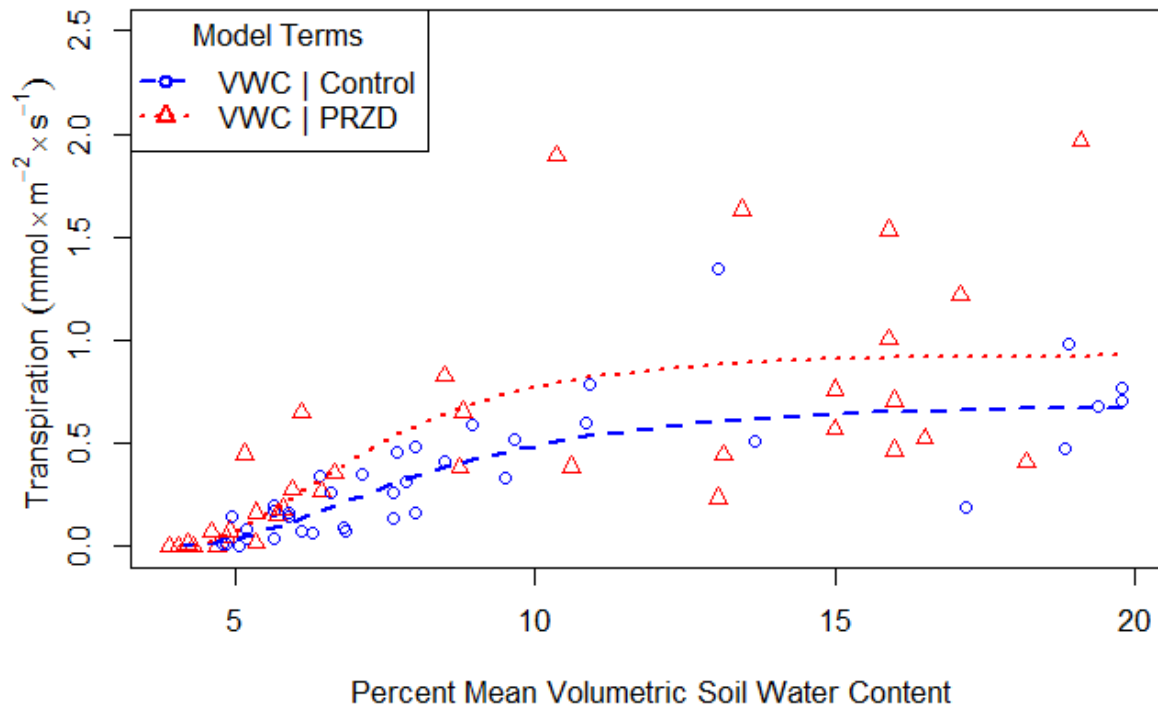


**Figure 4.4.** Instantaneous sap flow as 8<sup>th</sup> order moving averages (2-hr window) during the two cycles of withheld irrigation are shown in sub-plot (A). Sub-plot (B) shows the difference between treatments, with control transpiration subtracted from the static partial root zone drying treatment's transpiration. Flow rates are per tree. Rewatering occurred at the vertical lines.

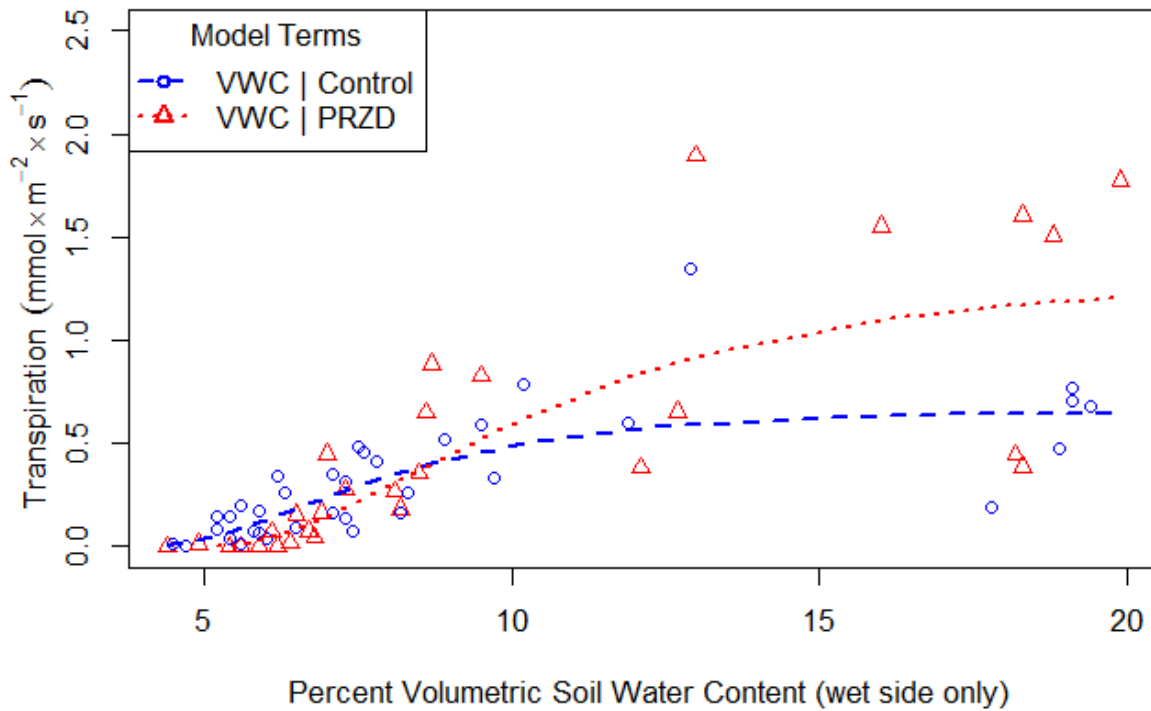


**Figure 4.5.** Mid-day needle water potential shown in response to soil moisture on the wet side only of the static partial root zone drying trees and a randomly chosen side of the control trees. Measurements were taken during phase two where irrigation was withheld twice in sequence for both the control trees, and the trees that had been previously subjected to fixed partial root zone drying. A generalized additive model fit to the power transformed water potential data, with soil moisture as the smoothed term, indicated a significant difference between treatments. The smoothing parameter estimation method used was generalized cross validation (GCV) with basis function dimension  $k = 9$ . The converse relationship between treatments was seen for mean soil moisture data. The backtransformed fit is shown here on the original scale for context.

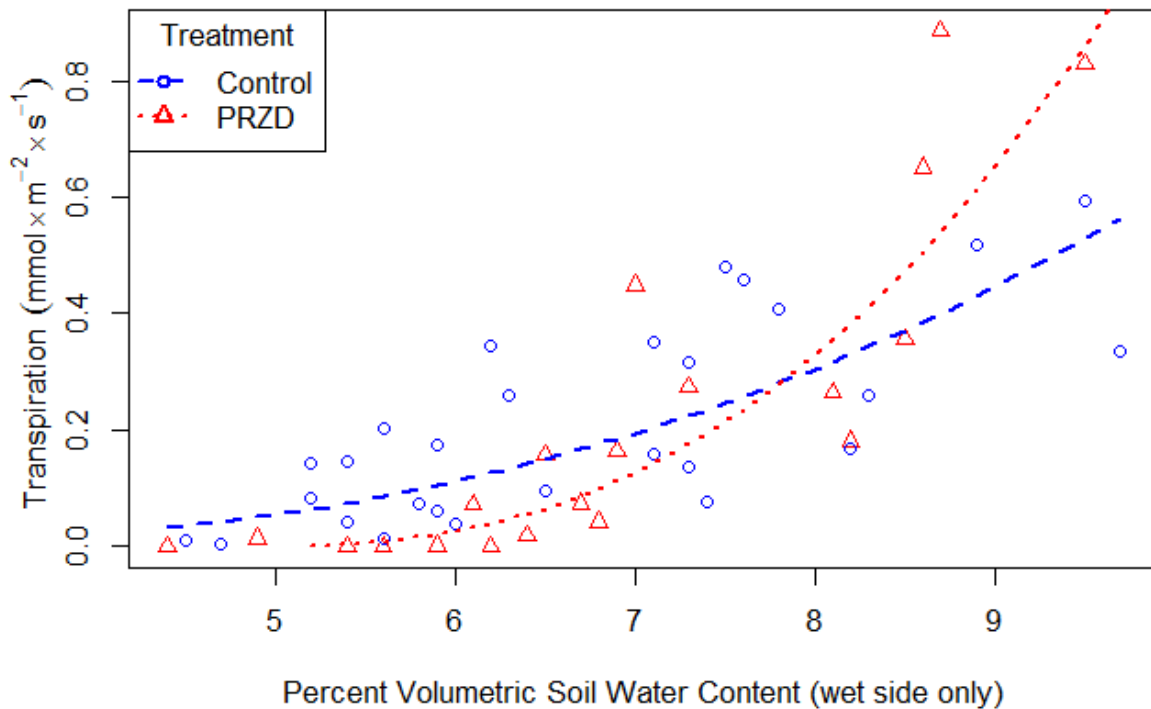




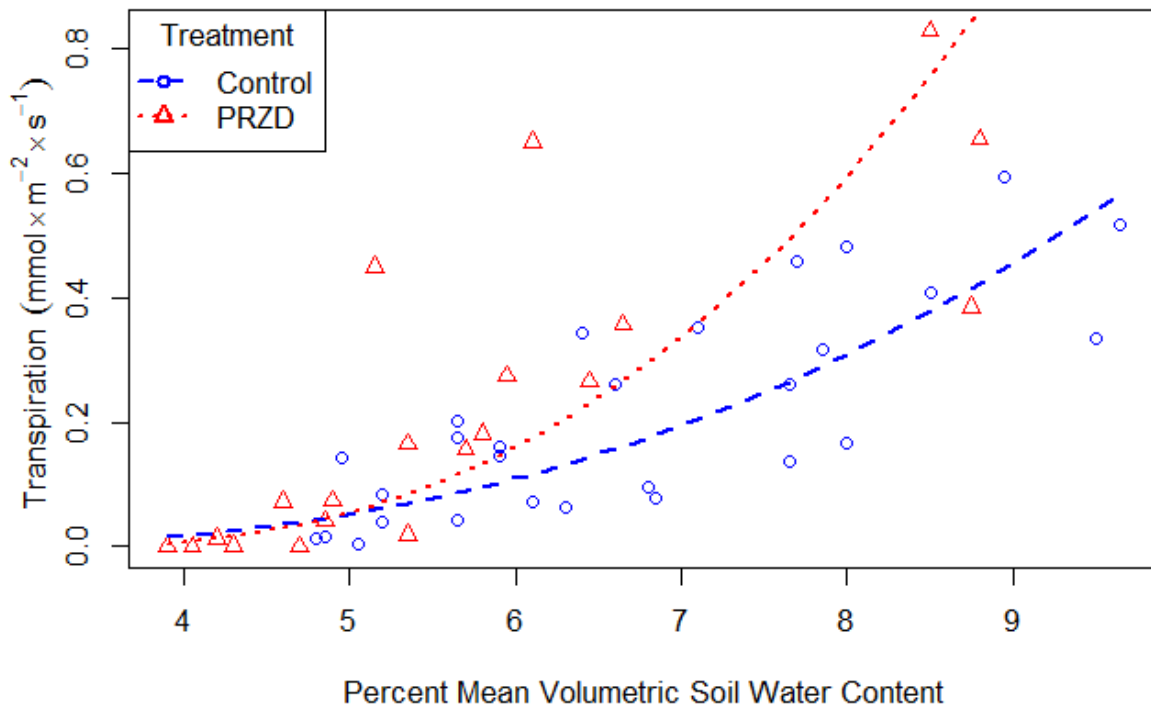
**Figure 4.6.** Transpiration is shown in response to mean volumetric soil moisture averaged across both halves of the root zone of each tree. Measurements were taken during phase two, where irrigation was withheld twice in sequence for both the control trees and the trees that had been previously subjected to fixed partial root zone drying. A 3-parameter asymptotic exponential model fit to the power transformed transpiration data in response to mean (across root zones) soil moisture was a significantly better fit with a treatment term. The backtransformed fit is shown here on the original scale for context.



**Figure 4.7.** Transpiration is shown in response to volumetric soil moisture on the wet side only of the static partial root zone drying trees and a single, randomly chosen side of the control trees. These measurements were taken during phase two, where irrigation was withheld twice in sequence for both the control trees and the trees that had been previously subjected to fixed partial root zone drying. A 3-parameter asymptotic exponential model fit to the power transformed transpiration data in response to wet side soil moisture was a significantly better fit with a treatment term. The asymptote term was also significantly different by treatment. The backtransformed fit is shown here on the original scale for context.



**Figure 4.8.** Transpiration is shown in response to the lower range of volumetric soil moisture on the wet side only of the static partial root zone drying trees and a randomly chosen, single side of the control trees. These measurements were taken during phase two, where irrigation was withheld twice in sequence for both the control trees and the trees that had been previously subjected to fixed partial root zone drying. A linear model fit to the power transformed transpiration data in response to ‘wet’ side soil moisture had a significant interaction term and significant effective intercepts, producing a classic disordinal interaction. The backtransformed fit is shown here on the original scale for context.



**Figure 4.9.** Transpiration is shown in response to the lower range of mean volumetric soil moisture averaged across both halves of the root zone of each tree. Measurements were taken during phase two, where irrigation was withheld twice in sequence for both the control trees and the trees that had been previously subjected to fixed partial root zone drying. A linear model fit to the power transformed transpiration data in response to mean soil moisture had a significant interaction term. The intercepts were not significantly different from zero. This produced an ordinal interaction. The backtransformed fit is shown here on the original scale for context.

## Chapter 5: Synthesis

This research was carried out with the intention of answering a number of questions related to the drought sensitivity of plantation grown loblolly pine. Recent research in the Virginia Piedmont has suggested that fertilization leads to increased drought susceptibility. Summer drought is a common occurrence where loblolly pine is grown commercially. Summer dryness is expected to increase in Virginia in coming decades; therefore, it seemed critical to further investigate this assertion (PINEMAP Decision Support System v. 1.5. 2015, Bartkowiak et al. 2015, Ward et al. 2015). Specifically, does drought have to be relatively severe to elicit the type of adaptive responses classically characterized at the shoot level in tree water relations research? Are there other indications of moisture stress in loblolly trees exposed to combinations of soil moisture limitation and fertilization? What measureable physiological or morphological attributes are associated with the previously described differences in stomatal response to vapor pressure deficit and bulk soil moisture reported in fertilized trees? How do fertilization and drought affect the hydraulics of loblolly, as hydraulic characteristics are known to be tightly linked to stomatal conductance and hence, to transpiration? Do root hydraulic properties differ under and between throughfall exclusion troughs? Were the methods used to simulate drought in the studies, which characterized fertilization as promoting drought induced mortality valid? Specifically, does the heterogeneity of soil moisture seen where throughfall exclusion troughs are deployed have an effect on the magnitude of transpiration and on water use efficiency? Does that banding affect total shallow root presence relative to control trees? Can the effects of consistently heterogeneous soil moisture be considered separate from those of soil moisture limitation or deficit in loblolly plantations? These questions are presented conceptually in Figure 5.1, which shows where in this work they are addressed and how they are related.

We found that 30% throughfall exclusion or fertilization + 30% throughfall exclusion did not lead to significant changes in water relations of shoots of mature,

plantation-grown loblolly trees. More severe soil moisture limitation was necessary to elicit notable responses. In trees where 100% throughfall removal was attempted (Maggard et al. 2016), active and passive osmotic adjustment occurred leading to the maintenance of turgor at more negative needle water potential values. The trees subjected to the more severe soil moisture limitation also increased their bulk modulus of elasticity, increasing their relative water content at turgor loss. Also, capacitance beyond the turgor loss point was significantly increased, likely serving to prolong the survival of foliage after turgor loss. These findings include the most detailed characterization of the response of mature loblolly trees to relatively extreme drought known. They demonstrate that less severe soil moisture limitation in mature trees, despite leading to other moisture stress responses such as decreased water potentials and decreased growth and transpiration, does not elicit classic water relations adaptations at the shoot level (Chapter 1, Chapter 2, Ward et al. 2015). Furthermore, such adaptations can occur, but require relatively extreme water limitation to be expressed. It seems that there are several layers to the way in which loblolly pine responds to water limitation. For example, before tissue water relations are significantly affected there are changes to transpiration, growth rates, mid-day needle water potential, and root and shoot hydraulics.

Mid-day shoot water potential values in June and August demonstrated that there was a main effect of 30% throughfall exclusion, and no effect of fertilization. There was no significant effect on the difference between pre-dawn and mid-day water potentials; this shows that the water potential gradient driving transpiration was unchanged by fertilization, 30% throughfall exclusion, or a combination of both factors (Chapter 2). There were also no differences in leaf area index (LAI) at the field site (Ward et al. 2015). Since transpiration can be estimated as the product of whole tree conductivity and the water potential gradient from root to foliage, there must have been changes to whole tree conductivity, which reduced transpiration in the exclusion, fertilization, and fertilization plus exclusion treatments. It was determined that those changes did not occur in the shoots. There was also a significant loss of shallow root length density as a result

of fertilization, likely leading to a lower root area to needle area ratio ( $A_r:A_l$ ) and contributing to hydraulic constraints on water use. Root length density was deemed a functional indicator of root presence, and decreased additively with fertilization and soil moisture limitation. When root presence data for throughfall exclusion only plots was weighted by respective ground area under and between troughs, there were no differences in root length density compared to control plots; only root surface area was different. The decreases to root presence caused by soil moisture limitation occurred differentially under and between exclusion troughs. Root presence was not higher between exclusion troughs in the throughfall exclusion only plots compared to control. Decreases to native conducting efficiency of roots was documented in the exclusion only plots and accompanied increased root tissue density. The changes to native conductivity were markedly more pronounced under the exclusion troughs. This was not true for root tissue density, which was similar under and between troughs. This means that cavitation resistance did not buffer the loss of conductivity that occurred as a result of the soil moisture heterogeneity caused by the exclusion troughs in the throughfall exclusion only plots. The decreased sensitivity to vapor pressure deficit and decreased transpiration at higher bulk soil volumetric water content levels seen in the fertilized trees in Ward et al., 2015 at the same site co-occur with decreased root presence, which can be quantified using root length density. The decreases to sapwood specific root conductivity and increased root tissue density seen in the exclusion only plots were also documented by other researchers in response to fertilization (Ewers et al. 2000). Thus, the decreased root length density seen in the current study was the most salient attribute associated with the changes to environmental sensitivity and decreased transpiration seen in the fertilized trees. This supports the idea that rhizosphere limitation is likely primarily responsible for the changes in hydraulics shown by the fertilized trees with their reduced shallow root presence (Sperry et al. 1998, 2002).

The 'split-pot' greenhouse study demonstrated that there were no changes to water use efficiency or reductions to transpiration exhibited by the young trees subjected to

well-watered, spatially fixed partial root zone drying (PRZD). This means fixed soil moisture heterogeneity alone doesn't cause the effects seen in throughfall exclusion experiments where soil moisture heterogeneity and soil moisture limitation are inextricably created together by the throughfall exclusion troughs. The root distributions in the PRZD trees were shifted such that there was approximately twice the root mass on the wet sides of their pots compared to their dry sides. The total root mass was not different between the PRZD trees and the control trees. Therefore, the total root mass in moist soil was higher for the control trees. The ratio of sapwood area to needle area was significantly increased in the PRZD trees. The PRZD trees had larger trunks and lower needle area at the end of the experiments, which led to their increased sapwood area to needle area ratios. As the PRZD trees transpired more per needle area without larger water potential gradients, it was inferred that the PRZD trees exhibited increased whole-tree conductivity relative to control. At low levels of soil moisture, the PRZD trees transpired less. This fits with the possibility that the PRZD trees possessed narrower hydraulic safety margins at low volumetric soil moisture. Narrower hydraulic safety margins can occur if increased hydraulic conductivity is accompanied by increased sensitivity to cavitation (Ewers et al., 2000; Sperry et al., 1998, 2002). Overall, there were no responses to well-watered PRZD that would be likely to confound the responses to water limitation seen in the throughfall exclusion trees in field experiments. This supports the use of throughfall exclusion troughs to simulate reduced rainfall and indirectly validates the results of the other researchers working at the PINEMAP sites where throughfall exclusion was employed. It seems that the responses seen in this 'split-pot' study utilizing young, fast growing trees are distinct from the drought responses seen as a result of throughfall exclusion.

In summary, these findings indicate that loblolly pine trees require relatively severe soil moisture limitation to exhibit shoot tissue level responses, which have been classically characterized in investigations of tree water relations. Even in cases where stomatal conductance and transpiration decrease along with more negative mid-day

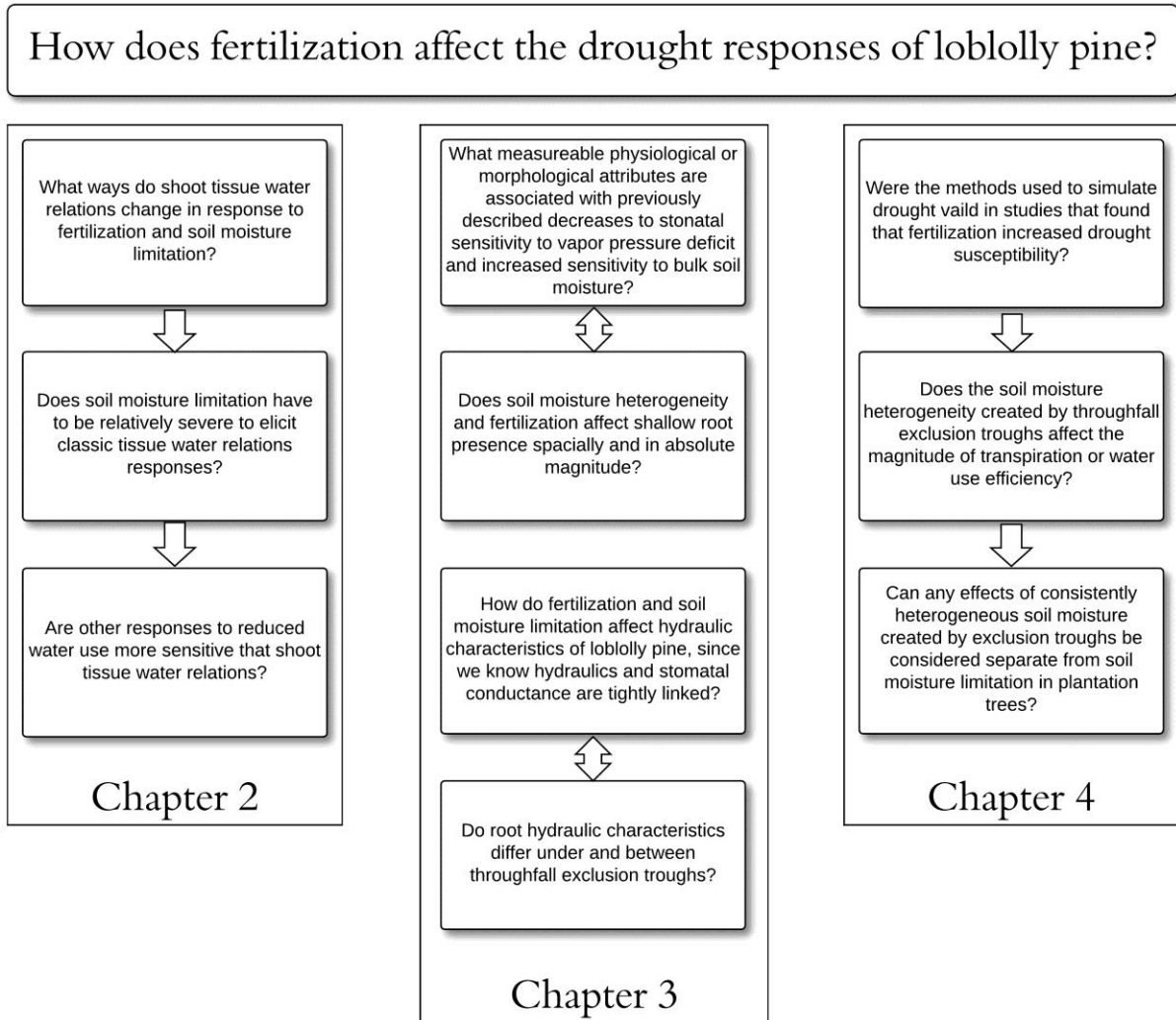


needle water potentials, classic shoot tissue-level water relations parameters do not change. Additionally, fertilization decreases transpiration and increases water use efficiency while greatly lowering the measured root length density. This likely narrows hydraulic safety margins and the area of roots to the area of needles, which leads to hydraulic limitation via reduced rhizosphere conductance. As a result, it could indeed lead to increased tree mortality under prolonged, extreme drought via carbon starvation, or less likely, hydraulic failure (Sperry et al. 2002, Klein 2015). When considering the lack of mortality at the 100% throughfall exclusion experiment in Oklahoma, it seems that if fertilization will lead to increased mortality during soil drought, it will only do so under relatively severe conditions that continue for numerous years. In particular, prolonged, extreme droughts which begin the season following fertilization are likely the most dangerous (Linder et al. 1987). Also, as the PRZD trees did not exhibit decreased transpiration or increased WUE, the drought responses seen in the throughfall exclusion treatments in the field are likely attributable to the decreased precipitation reaching the soil, and not the consistent soil moisture heterogeneity. It is possible that the results of static PRZD are different in established stands with closed canopies as opposed to young, quickly growing trees. Further research could serve to address this possibility. Barring the aforementioned caveat, this means that the results of experiments utilizing throughfall exclusion troughs to simulate reduced rainfall do appear to be valid.

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## Figures



**Figure 5.1.** A conceptual diagram which shows major questions that led to the investigations described in this work and their chapter groupings and relationships.