

**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

Physiological responses of forest species to water stress

Marina Shinkai Gentil Otto

Thesis presented to obtain the degree of Doctor in Science.
Area: Plant Physiology and Biochemistry

**Piracicaba
2015**

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Physiological responses of forest species to water stress
versão revisada de acordo com a resolução CoPGr 6018 de 2011

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RESUMO

Respostas fisiológicas de espécies florestais ao estresse hídrico

Estresses abióticos e bióticos podem afetar o crescimento das árvores e desempenham um papel importante na determinação da distribuição geográfica das espécies. O objetivo deste estudo, foi elucidar as seguintes questões: (1) o aminoácido GABA e o controle estomático são bons indicadores da tolerância ao estresse hídrico em clones de *Eucalyptus*? E quais são as diferenças anatômicas entre clones de *Eucalyptus* tolerantes e sensíveis ao estresse hídrico? (2) existem diferenças de vulnerabilidade a cavitação do xilema entre famílias de *Pinus flexilis* suscetíveis e resistentes à ferrugem do pinho branco (WPBR) e com diferentes procedências (elevada e baixa altitudes)? Dois estudos foram desenvolvidos para elucidar as questões acima descritas. No capítulo 1, oito clones de *Eucalyptus* de diferentes procedências e condições climáticas, sendo três clones sensíveis ao estresse hídrico (CNB, FIB e JAR), três clones tolerantes ao estresse hídrico (GG, SUZ e VM) e dois clones plásticos (VER e COP), foram estudados sob duas condições distintas: sob adequado suprimento de água (tratamento controle) e sob condições de estresse hídrico (tratamento estresse). Do primeiro capítulo concluiu-se que o GABA é um aminoácido que possui alta sensibilidade ao estresse hídrico, no entanto, não houve relação entre a concentração de GABA e os níveis de tolerância ao estresse hídrico dos clones. Além disso, todos os clones reduziram a condutância estomática em relação ao aumento do déficit de pressão de vapor (DPV), sendo que, sob condições de estresse hídrico, os clones plásticos e tolerantes à seca (exceto o clone GG) apresentaram menor sensibilidade estomática ao DPV do que os clones sensíveis ao estresse hídrico. Além disso, todos os clones apresentaram diferenças anatômicas, sendo que, diferentemente dos demais, os clones COP (plástico) e SUZ (tolerante) apresentaram mesofilo homogêneo e folhas anfi-hipoestomáticas. Todos os clones aumentaram a quantidade de estômatos e reduziram a espessura foliar das folhas formadas após períodos de estresse hídrico. No segundo capítulo foram avaliadas 12 famílias de *Pinus flexilis* procedentes de regiões de baixa e alta altitudes, sendo seis famílias contendo um alelo dominante C4 (resistente à WPBR) e seis famílias sem o alelo C4 (suscetíveis à WPBR). Este estudo apresentou uma variação da pressão média da cavitação (MCP) para *Pinus flexilis* de -3,63 a -4,84 Mpa, e embora tenha havido uma diferença significativa da susceptibilidade a cavitação entre todas as famílias estudadas, esta variável não relacionou-se com a susceptibilidade a doença WPBR e com a região de procedência das famílias. Estes estudos comprovam que a avaliação das respostas fisiológicas das plantas sob condições de estresse hídrico são importantes ferramentas que podem ser utilizadas para complementar as estratégias da seleção de genótipos em programas de melhoramento florestal.

Palavras-chave: GABA, Anatomia; Condutância estomática; Cavitação do xilema; Tolerância ao estresse

ABSTRACT

Physiological responses of forest species to water stress

Abiotic and biotic stresses affect tree growth and play a major role in determining the geographic distribution of species. The objective of this study is to elucidate the following questions: (1) are GABA aminoacid and stomatal control good indicators of tolerance to water stress in *Eucalyptus* clones? In addition, what are the anatomical differences between drought-tolerant and drought-sensitive clones of *Eucalyptus*? (2) Are there differences of xylem vulnerability to cavitation in *Pinus flexilis* families susceptible and resistant to white pine blister rust (WPBR) and with different origins (high and low altitudes)? Two studies were carried out to elucidate the issues above. On chapters 1, eight *Eucalyptus* clones from different geographical and climatological conditions, three drought-sensitive (CNB, FIB and JAR), three drought-tolerant (GG, SUZ and VM), and two plastics (VER and COP), were studied in normal water supply (control treatment) and in water stress conditions (stress treatment). The first chapter concluded that GABA is an aminoacid very sensitive to water stress, but there was no relation between GABA concentration and tolerance to water stress of the clones. In addition, all clones decreased stomatal conductance with increasing vapor pressure deficit, and plastics and drought-tolerant clones (except GG) presented lower stomatal sensitivity to vapor pressure deficit under stress conditions than drought-sensitive clones. Besides, all clones showed differences on the anatomical parameters between, and only COP (plastic) and SUZ (drought-tolerant) showed homogeneous mesophyll and amphihypostomatic leaves. All clones increased the number of stomata and reduced leaf thickness of the leaves formed after water stress period. On the chapter 2, we studied 12 families of *Pinus flexilis* originating from high and lower altitudes, in which six families previously shown to contain the dominant C4 allele (resistant to WPBR) and six families without C4 allele (susceptible to WPBR). This study showed that the mean cavitation pressure (MCP) of *Pinus flexilis* varying between 3.63 a -4.84 Mpa, although there was a significant difference in vulnerability to cavitation comparing all families, this variable was not related to WPBR and origin region. These studies highlight that the physiological responses of plants under water stress conditions are important tools that can be used to complement the strategies of genotype selection in forest breeding programs.

Keywords: GABA; Anatomy; Stomatal conductance; Xylem cavitation; Stress tolerance

1 INTRODUCTION

Around the world, studies on drought-dependent responses of physiological variables have been conducted for different forest species. In ecology, understanding these interactions is essential in terms of species tolerance to different environments. In agricultural and forestry crop environments, these studies may assist in genotype selection and in the characterization of management strategies for cultivated areas.

By definition, the term stress is considered a significant deviation from the optimal conditions for vital maintenance processes and thereby induces changes in all functional levels of organisms, which at a first moment are reversible but may become permanent (LARCHER, 2006). Plants respond to biotic and abiotic stresses by physiological, biochemical, cellular, and molecular events that occur at the same time and very quickly (KEYS, 2009; SHAO et al., 2009).

The effect of each abiotic factor on plant growth depends on its quantity or intensity, and rarely does their natural plant environment present optimal intensity or amount of all the environmental factors simultaneously. Thus, most of the time, plants are exposed to a stressor agent (SCHULZE et al., 2005).

In this study, we addressed two topics related to the effect of biotic and abiotic stresses on two forest species important for economy and ecology in Brazil and United States.

***Eucalyptus* plantations in Brazil**

Brazilian forest sector benefits its society, environment, and national economy, by respectively creating jobs, reducing the pressure on natural forests, and participating in the national balance of trade. In 2012, for instance, it represented 28.1% of the total balance (ABRAF, 2014). Currently *Eucalyptus* plantation occupies over 5.5 million hectares, mainly in the states of Minas Gerais, São Paulo, and Bahia (ABRAF, 2014). Our preference for this genus is justified because of the high productivity and flexibility to different conditions of soil and climate.

In Brazil, in recent decades, the global area cultivated with *Eucalyptus* have increased considerably and hence spread to areas subject to water stress, thus increasing the necessity for strategies of selection of more tolerant genetic materials to grow in adverse conditions (ABRAF, 2013).

Clonal plantations are the standard for fast-growing *Eucalyptus* in Brazil; in traditional areas of production, these clones are well adapted, but expanding afforestation to new frontiers

poses higher risks to production due to environmental stresses different from those where the clones were selected (IPEF, 2012). Thus, it is essential to develop strategies for selecting genetic materials tolerant to water stress for planting in new regions.

In addition to being economically important for Brazil and for the world, *Eucalyptus* is the second forest genus, after *Populus*, with detailed functional genomic sequencing, highlighting the importance of studying the genotype-environment interactions of these species.

The distinction between drought-tolerant clones and drought-sensitive clones is particularly relevant to assist forest enhancement programs to select clones more productive under water stress. Thus, the objective of this study was to evaluate the performance of different *Eucalyptus* clones under water stress in order to find sensitive variables that could be used for a pre-selection of drought-tolerant genotypes.

The specific questions asked were:

Chapter 1: (1) Does the time required to recover photosynthesis differ in tolerant and sensitive clones? (2) Are there clonal differences in stomatal responses to vapor pressure deficit in water stress treatment? (3) Is γ -aminobutyric acid is a good indicator of water stress conditions? (4) If so, are there clonal differences of γ -aminobutyric acid accumulation that make a clone more favorable under drought conditions? (5) What are the anatomical differences between these clones in adequate conditions of water availability? (6) What anatomical adaptations occur after a period of water stress conditions?

***Pinus flexilis* forests in the United States**

Since 1910, the introduction of non-native fungal pathogen (*Cronartium ribicola* J.C Fisch) that causes a lethal disease, white pine blister rust (WPBR) has had a devastating impact on forests of North American white pine species. *Pinus flexilis* (Limber Pine) is one of the nine white pine species that are highly susceptible to WPBR.

In high altitudes, limber pine is a keystone species, given that it is the only tree that can live in these environmental extremes. This species plays several important ecological roles, such as: being one of the first species that colonize a site after a fire, facilitating the establishment of late successional species in high altitudes, mediating snow capture and snowmelt, controlling erosion, and providing diverse animals with food and habitat (SCHOETTLE, 2004; SCHOETTLE et al., 2014).

Once abiotic stress can act as agent of balancing selection, the development of genetic resistant trees is the main strategy that can provide this species with potential success of restoration. Since 1940s, breeding efforts have been developed to select families of white pine

species with heritable resistance to WPBR, aiming to restore devastated forest plantations (KING et al., 2010).

Schoettle et al. (2014) identified R gene in limber pine, named “Cr4”, which confers complete resistance to WPBR. Families from trees containing this R gene had greater cold hardiness and drought tolerance than families without the R gene, which implies that plants resistant to WPBR may have a different suit of stress tolerance (VOGAN and SCHOETTLE, 2015).

It is unclear if selection for rust resistance will result in the loss of some physiological traits in these species; we do not know if accelerating the establishment of WPBR-resistant genotypes across the landscape can affect the conservation of genetic diversity of this species. Therefore, analyses of vulnerability of xylem cavitation related with anatomical parameters of rust-resistant and rust-susceptible families can provide important insights that might assist in rapidly developing and implementing conservation programs.

The objective of this study is to investigate the variability in cavitation between rust-resistant (R) and rust-susceptible (S) and to relate with xylem anatomy of *Pinus flexilis*. We will test two hypotheses about these patterns: (1) there is no difference in 50% loss of conductivity pressure (P50) and mean cavitation pressure (MCP) between WPBR resistant and susceptible limber pine families; (2) families from higher altitudes will be more resistant to cavitation than families from lower altitudes.

To elucidate the issues above, this thesis was elaborated in the form of two independent chapters. Chapters will be presented according to the sequence below:

1. Responses to water stress of gas exchange, leaf anatomy and γ -aminobutyric acid concentration in *Eucalyptus* clones.
2. Xylem vulnerability to cavitation in *Pinus flexilis*: are there differences between white pine blister rust susceptible versus resistant families?

References

ASSOCIAÇÃO BRASILEIRA DOS PRODUTORES DE FLORESTAS PLANTADAS. **Anuário estatístico ABRAF 2014**: ano base 2013. Brasília, 2013. p. 74.

INSTITUTO DE PESQUISAS E ESTUDOS FLORESTAIS. Disponível em: <<http://www.ipef.br/techs/>>. Acesso em: 25 jun. 2015.

KING, J.N.; DAVID, A.; NOSHAD, D.; SMITH, J. A review of genetic approaches to the management of blister rust in white pines. **Forest Pathology**, Malden, v. 40, p. 292-313, 2010

LARCHER, W. **Ecofisiologia vegetal**. São Carlos: RiMa, 2006. 550 p.

SCHOETTLE, A.W. Ecological roles of five-needle pines in Colorado: potential consequences of their loss. In: SNIEZKO, R.A.; SAMMAN, S.; SCHLARBAUM, S.E.; KRIEBEL, H.B. (Ed.). **Breeding and genetic resources of five-needle pines: growth, adaptability and pest resistance**. Fort Collins: USDE, Forest Service, Rocky Mountain Research Station, 2004. p. 124-135.

SCHOETTLE, A.W.; SNIEZKO, R.A.; KEGLEY, A.; BURNS, K.S. White pine blister rust resistance in limber pine: evidence for a major gene. **Phytopathology**, St. Paul, v. 104, n. 2, p. 163-173, 2014.

SCHULZE, E.D.; BECK, E.; MULLER-HOHENSTEIN, K. **Plant ecology**. Berlin: Springer, 2005. 702 p.

SHAO, H.B.; CHU, L.Y.; JALEEL, C.A.; MANIVANNAN, P.; PANNEERSELVAM, R.; SHAO, M.A. Understanding water deficit stress-induced changes in the basic metabolism of higher plants: biotechnologically and sustainably improving agriculture and the eco environment in arid regions of the globe. **Critical Reviews in Biotechnology**, Abingdon, v. 29, p. 131-151, 2009.

VOGAN, P.J.; SCHOETTLE, A.W. Selection for resistance to white pine blister rust affects the abiotic stress tolerances of limber pine. **Forest Ecology and Management**, Amsterdam, v. 344, p. 110-119, 2015.

2 RESPONSES TO WATER STRESS OF GAS EXCHANGE, LEAF ANATOMY AND γ -AMINOBUTYRIC ACID CONCENTRATION IN *EUCALYPTUS* CLONES

Abstract

Drought is one of main abiotic factors that have a negative effect on survival, development, and productivity of plants. Identification of molecules involved in the perception of plants to water stress will be of interest to breeding programs. In this study, eight *Eucalyptus* clones from different geographical origins (three drought-sensitive, three drought-tolerant, and two plastics) were evaluated in two treatments: normal water supply (control) and under water stress conditions (stress). We test four hypotheses in this study: (1) drought-tolerant clones would be differently affected by water stress and require less time to recover after rewatering, namely at the photosynthetic level; (2) drought-tolerant clones would have lower stomatal sensitivity (g_s) to vapor pressure deficit (D) than drought-sensitive clones; (3) all clones under water stress treatment would accumulate GABA during stress days and would reduce GABA concentration after rewatering; (4) drought-tolerant clones would present lower GABA accumulation in stress treatment than drought-sensitive clones; (5) drought-tolerant clones will present anatomical differences compared to drought-sensitive clones; (6) anatomical adaptation will occur with all clones after a period of water stress. We measured gas exchange variables using the equipment LI-6400, stomatal quantity, water potential using a Scholander chamber, determined GABA concentration according to the method described by De diego et al. (2012) and we performed histological leaves analysis. All clones reduced ~60% of the photosynthesis after water stress days. The time required for recovering photosynthesis did not differentiate tolerant and sensitive clones. All clones increased the photosynthetic rate after rewatering until it exceeded or matched the photosynthetic rate in control treatment. All clones decreased g_s with increasing D in both treatments. All plastics and drought-tolerant clones (except GG) presented lower stomatal sensitivity to D under stress conditions than drought-sensitive clones. There was a significant difference in GABA concentration among all clones subjected to water stress, but there was no relation between these differences and tolerance to water stress. GABA concentration was very sensitive to water stress conditions, showing that it is a signal that form an important link between environment and plant. Clonal variation in anatomical parameters was evident; COP (plastic) and SUZ (drought-tolerant) presented different anatomical characteristics compared with the other clones, as homogeneous mesophyll and amphi-hipostomatic leaves. All clones increased the number of stomata and reduced leaf thickness after water stress, but there was no response to water stress for the other parameters. According to our results, response of g_s to D was the best physiological variable that can differentiate tolerant and sensitive clones, and GABA is an indicator of the beginning and the end of critical periods reached after the implementation of management strategies that can minimize drought situations.

Keywords: Stomatal conductance; Photosynthesis; γ -aminobutyric acid; Stress indicator; Rewatering

2.1 Introduction

Historically, studies on the physiological processes of plants due to environmental changes have been the focus of many researchers. To ecologists, understanding these

interactions is essential in terms of species tolerance to different environment. In agricultural and forestry crops, these studies may provide tools to select drought-tolerant genotypes as well as to help define management strategies that could alleviate stress conditions.

Different physiological responses occur once plants have a perception mechanism composed of a network of molecular signalizations with the capability of transferring and processing information about environmental changes (ROSHCHINA, 2001). Despite major progress in understanding how water stress affects plant functioning (SPERRY, 1998; BREDA et al., 2006; FLEXAS et al., 2009), the perception of plants to climatic variations remains poorly resolved, and this limits our ability to adequately predict drought tolerance varieties under stress conditions.

Several studies have identified substances in plants found in the nervous system of animals, such as histamine (BARGER; DALE, 1910), acetylcholine (EWIS, 1914), dopamine (BUELOW; GISVOLD, 1944), adrenaline (ASKAR et al., 1972), and serotonin (BOWDEN et al., 1954). The presence of γ -aminobutyric acid (GABA) in plants was discovered in 1949 in potato tuber. GABA is a non-protein amino acid that occurs at high levels in the brain of animals as a neurotransmitter (STEWART et al., 1949).

The interest in the study of GABA metabolism in plants has increased from experimental observations that this amino acid is rapidly produced in response to biotic and abiotic stresses (KINNERSLEY; TURANO, 2000). These situations have been reported in drought-stressed cotton (HANOWER; BRZOZOWSKA, 1975), bean (RAGGI, 1994), turnips (THOMPSON, 1996), *Eucalyptus* (WARREN et al., 2011), in heat-stressed cowpea cells (MAYER et al., 1990), in cold-stressed soybeans (WALLACE et al., 1984), mechanical damage in soybean leaves (WALLACE et al., 1984), and in strawberry subjected to a higher CO₂ concentration (DEEWATTHANAWONG et al., 2010). Furthermore, Bown et al. (2002) demonstrated that, when the insect larvae of tobacco simply walk on soybean plants, a stimulation of GABA synthesis occurs in minute intervals, indicating that it is a molecule of stress signaling.

GABA metabolism has been associated with many physiological responses, including the regulation of cytosolic pH (CARROLL et al., 1994; SNEDDEN et al., 1995; MAZZUCOTELLI et al., 2006), nitrogen metabolism (ROLIN et al., 2000; BUVE et al., 2004), biotic defense (Mc LEAN et al., 2003; MAC GREGOR et al., 2003), protection against oxidative stress (BOUCHÉ et al., 2003; FAIT et al., 2006), osmoregulation (SCHWACKE et al., 1999, SHELP et al., 1999), and signalization (BOUCHÉ; FROMM, 2004). Despite these reports, a direct function for GABA, such as diverse stresses, has not been demonstrated, and

more studies that link stress perception, GABA accumulation, and physiological responses is needed (KINNERSLEY; TURANO, 2000; WARREN et al., 2011).

The limitation of plant growth imposed by water stress is mainly due to reductions in photosynthesis. For this reason, photosynthesis responses to drought have been subject of studies and debate for decades (LAWLOR; CORNIC, 2002). There are many studies on gas exchange to water stress showing that stomatal control is an important mechanism of plant survival in stress conditions, and different species may exhibit different stomatal sensitivity to vapor pressure deficit (D) (OREN et al., 1999; MEDIAVILLA, 2004; ADDINGTON et al., 2004; HUAMAN, 2010; MOKOTEDI, 2010; EKSTEEN et al., 2013; OCHEL TREE et al., 2013). Species and individuals that present high g_s at a low D tend to present a more stomatal sensitivity to an increasing D (OREN et al., 1999; ADDINGTON et al., 2004; MAHERALLI et al., 2003).

Although there are many studies showing different concentrations of GABA in response to drought and differences in response of stomatal conductance in water stress situations, there are few studies that examine these physiological variables under water stress conditions simultaneously (WARREN et al., 2011).

Anatomical and physiological adaptations play a crucial role in plant survival. In general, plants are able to have short-term control of stomatal closure (hours or minutes) and long-term (weeks or months) stomatal development and morphology to adapt to environmental changes (CASSON; HETHERINGTON, 2010; COMPOSEO et al., 2011). Some of the most common anatomical traits are: density of the mesophyll and scarcity of intercellular spaces (CRAVEN et al., 2010), thick cuticles (ENGLAND et al., 2011), content of cuticular wax chemicals (YANG et al., 2011), trichome count (SHTEIN et al., 2011), stomata size and density (EKSTEEN et al., 2013).

Differences in morphological and anatomical parameters of leaves are important to understand the mechanisms related to plants living in water stress conditions. The stomata control and stomata density are important factors determining water relations. However, it is not completely elucidated if stomatal density increases or decreases, under hot and dry climate conditions (BEERLING; CHALONER, 2001; XU; ZHOU, 2008; FRASER et al., 2009; SHTEIN et al., 2011; COMPOSEO et al., 2011). Variations in leaf structure are related, in most of the cases, to climate conditions and represent an important plastic plants' response to water availability.

One way for plants to adjust to environmental conditions is the modification of the anatomy of leaves (CUTLER et al., 2011). For instance, the increased number of palisade layers with small cell volume, decreased number of spongy layers with small volume, and less intercellular spaces characterize plants that live in water shortage conditions (CHARTZOULAKIS et al., 2002). Thus, the quantification and sizing of the cell structures of the plant leaves can be an important tool to understand their behavior, presenting a potential use of the leaf anatomy for the zoning and plant breeding. However, insufficient sampling locations and few species examined may not accurately reflect an intrinsic relationship between anatomy and environmental factors.

Eucalyptus was studied in this project, because it is an important species that account for 8% of planted forests in the world (FAO, 2011). However, the global area planted with *Eucalyptus* in regions under water stress is increasing; therefore, it becomes necessary to improve our understanding about the molecules involved in stress perception and physiological processes triggered by plants under water stress conditions.

With the advancement of the global area planted with *Eucalyptus* to regions under water stress, it becomes necessary to increase our understanding about physiological and anatomical processes triggered by plants under water stress conditions. Development of varieties presenting increased drought tolerance to any species would result in a more stable yield under stress conditions (EKSTEEN et al., 2013), but breeding, specifically for drought tolerance, is still time-consuming and expensive (PIDGEON et al., 2006). Besides, the physiological and anatomical characteristics that lead to response of clones to drought can be investigated to allow us to examine how breeding can use physiological parameters to select more adapted clones to dry situations.

The distinction between drought-tolerant clones and drought-sensitive clones is particularly relevant to assist forest breeding programs in selecting clones more productive under water stress.

We will test six hypotheses about these patterns: (1) drought-tolerant clones would be less affected by water stress and require less time to recover after rewatering, namely at the photosynthetic level; (2) drought-tolerant clones would have higher stomatal sensitivity to water deficit pressure than drought-sensitive clones; (3) all clones under water stress treatment would accumulate GABA during stress days and would reduce GABA concentration after rewatering; (4) drought-tolerant clones would present lower GABA accumulation in stress treatment than drought-sensitive clones; (5) drought-tolerant clones will present anatomical

differences compared to drought-sensitive clones; (6) anatomical adaptation will occur with all clones after a period of water stress.

Considering these hypotheses, eight *Eucalyptus* clones from different regions (dry and humid) were used to study the effect of water stress on physiological traits, focusing particularly on photosynthesis, stomatal control to D, GABA concentration and leaf anatomy. The aim of this study was to evaluate the performance of different *Eucalyptus* clones to water stress and recovery in order to find sensitive variables that could be used for an early selection of drought-tolerant genotypes.

Therefore, six specific questions were asked: (1) Does the time required to recover photosynthesis differ in tolerant and sensitive clones? (2) Are there clonal differences in stomatal responses to D in water stress treatment? (3) Is GABA accumulation a good indicator of water stress conditions? (4) If so, are there clonal differences of GABA that make a clone more favorable under drought conditions? (5) What are the anatomical differences between these clones in adequate conditions of water availability? (6) What anatomical adaptations occur after a period of water stress conditions?

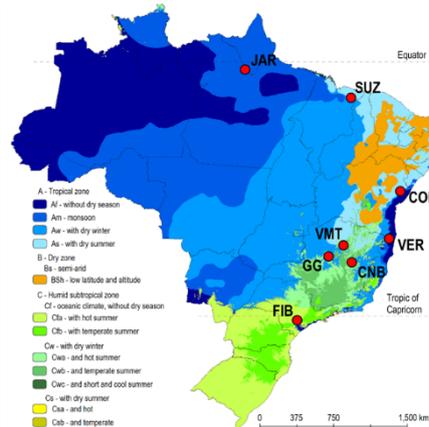
2.2 Material and Methods

2.2.1 Plant material and experimental design

Cuttings of eight *Eucalyptus* clones from different geographical and climatological breeds were evaluated in this study. Clones planted in humid regions were considered drought-sensitive to water stress, clones planted in dry regions were considered drought-tolerant, and clones planted in both (dry and humid regions) were considered plastics (Table 1). They were planted in pots (320l) containing soil (66%), sand (17%), and peat (17%), located in Piracicaba, in February 2013 (Figure 1a).

Table 1 - Origin of the eight *Eucalyptus* clones (Map modified from Alvares et al., 2013).

Clone	Híbrido/Especie	Water stress Classification
CNB	<i>E. urophylla</i> x <i>E. grandis</i>	Sensitive
FIB	<i>E. urophylla</i> x <i>E. grandis</i>	Sensitive
JAR	<i>E. urophylla</i> x <i>E. grandis</i>	Sensitive
GG	<i>E. urophylla</i>	Tolerant
SUZ	<i>E. urophylla</i> x <i>E. tereticornis</i>	Tolerant
VM	<i>E. urophylla</i>	Tolerant
VER	<i>E. urophylla</i>	Plastic
COP	<i>E. grandis</i> x <i>E. camaldulensis</i>	Plastic

Figure 1 - Experimental design with *Eucalyptus* clones in 320 liters pots (a), details of plastic cover to induce water stress (b) and irrigation system (c)

Piracicaba is a city located in São Paulo State, Brazil (22° 42' 30" S e 47° 38' 00" W), with average annual temperature (21.6°C), average precipitation (1230 mm yr⁻¹), and average potential evapotranspiration (1042 mm yr⁻¹). The sites climate is classified as Cfa (Humid temperate) under Koppen Classification (ALVARES et al., 2013), showing three winter months (June, July, and August), with dry season during the winter and a wet summer. Total rainfall for the study period (October 2013 to January 2014) was 684 mm and monthly average temperatures were 25°C.

The experimental design was completely randomized in a factorial 2 x 8: two treatments (water stress (Stress) and normal water supply (Control)) and eight clones, with six replicates, totaling 96 experimental units.

For the normal water supply treatment, plants were irrigated every day until complete water soil saturation (Figure 1c). Water stress treatment began on November 12th when cuttings were 9-month-old and about 10-15 meters tall. Water stress was imposed by non-irrigation of plants, and pots were covered using plastic sheeting to avoid rainfall and humidity (Figure 1b). Water was withheld during three cycles: two water stress days (cycle 1), four water stress days (cycle 2), and two water stress days (cycle 3); between cycle 1 and cycle 2, water-stressed plants were rewatered during 2 weeks to recover.

2.2.2 Leaf water potential

Leaf water potential (Ψ_{leaf}) were determined during water stress days (T1 and T2; T15 to T18; and T41 and T42) and after rewatering (R1d, R2d, and R3d; R22d, R28d, R30d). We measured four fully-expanded leaves per treatment, located in the middle position on the tree crown, at midday (11am to 12am) and predawn (4 to 5am) along the drought cycle using a Scholander chamber (SCHOLANDER et al., 1965).

2.2.3 GABA analysis

Extraction

Leaf samples were collected on the same days that we measured water potential. Leaves were collected between noon and 1pm on sunlit days, when water stress is most severe and to control possible diurnal variations in GABA concentration. We punched three leaves of four plants per treatment (12 repetitions) from the middle part of the crown, and they were immediately frozen in liquid N and subsequently stored at -80°C .

GABA concentration was extracted according to the method described by De diego et al. (2012). Plant material was pooled and homogenized in liquid N. Each pooled sample was weighted to the nearest 0.25 mg of fresh weight, was placed in a 50 mL teflon tube, and dropped in 12 mL of extraction mixture of methanol, chloroform, and water (12:5:3, v:v). Extracts were homogenized in vortex for 1 min, and then centrifuged at 7000 rpm for 10 min at 4°C . Pellets were re-extracted for 10 min with additional 12 mL of the same extract solution.

Extracts were combined and transferred to a round-bottom flask, and the solvent was evaporated to dryness in a rotary evaporator under vacuum at 45°C . The pellet was dissolved

in 4 mL solution of acetonitrile and water (1:1, v/v). Samples were filtered through 13-mm diameter teflon membrane Millex filters (0.22 μm , Millipore, Bedford, MA, USA).

Quantification

Analyses were carried out in the LC–ESI-MS/MS system: Liquid Agilent (Wilmington, USA) Chromatograph 1200. The chromatographic separations were carried out using a Thermo Scientific Hypersil GOLD C18 column (100 mm \times 2.1 mm, 3 μm particle size). Table 2 shows chromatographic parameters used for GABA detection.

The mobile phases were A – 0.1 % formic acid in Milli-Q®water (Millipore; Bedford, USA) and B – 0.1 % formic acid in acetonitrile. The elution was in isocratic mode at the proportion of A:B - 20:80, v/v. The flow remained constant at 0.40 mL min⁻¹, the column temperature was fixed at 30 °C, and the injection volume was 5 μL .

We used a mass spectrometer Quadruple Triple 6430 as a detector. The ESI parameters in the positive ionization mode was the following: gas flow of 10 L min⁻¹, gas nebulizer at 50 psi, gas temperature at 350 °C, and capillary voltage of 4000 V. Nitrogen 99.99 % was used as nebulizer and 99.9999% as collision gas. For data acquisition, we used the software Agilent Mass Hunter, and for detection in the MS/MS, we used the MRM mode.

Table 2 - Detection and chromatographic parameters

Transitions	Fragmentation energy (V)	Collision energy (V)
104.1 --> 87.2*	45	4
104.1 --> 69.2**	45	12

* Quantifier **Qualifier

2.2.4 Leaf gas exchange measurements

We evaluated gas exchange variables using the equipment LI-6400 system (Li-Cor, Lincoln, NE, USA) all days when we measured water potential and GABA concentration. Measurements were performed after 10-15 min of stabilization at a light saturation of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, ambient humidity above 50%, and CO₂ concentration of 400 ppm. We measured nine repetitions per treatment on fully expanded leaves located in the middle position on the tree crown. To observe variations between stomatal conductance and D, measurements were taken in the morning (8 to 12am) and afternoon (2 to 5pm).

2.2.5 Stomatal anatomy

Samples of fully expanded leaves, chosen at random from the middle third portion of shoots, were collected from each clone on two dates: (i) before water stress days (T1) and (ii) after all water stress cycles (T42). In order to capture the water stress effect, newly emergent leaves were tagged in the upper third of the crown after cycle 1 and collected after three water stress cycles, when they were fully expanded (Figure 2).

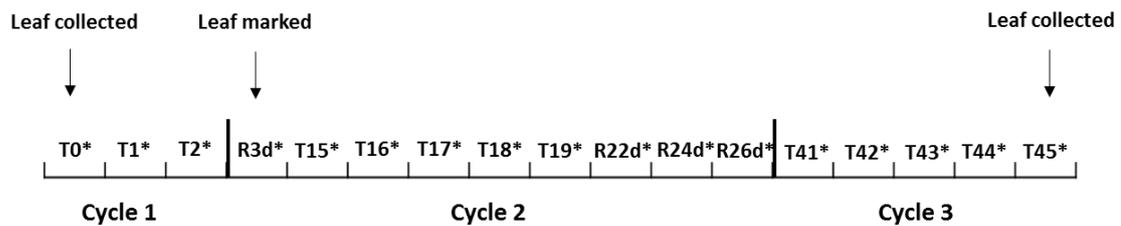


Figure 2 - Water stress cycles. T1 and T2, T15 to T19 and T41 and T42 are days with water stress, R3d and R22d to R26d are days with rewatering. Asterisk represents days with stomatal conductance and water potential measurements and arrows indicates days when leaves were collected and marked for anatomical evaluations

Leafsprings of the two sides were taken on samples of three leaves for each clone by means of the suplerglue technique adapted from Gulcan and Misirli (1990). This sampling was made at the middle portion of the leaf, because previous studies have shown that the highest stomatal frequency is found near the leaf tip, the lowest frequency near the leaf base, and intermediate frequency at the middle (SALISBURY, 1927; MIRANDA et al., 1981).

Stomatal frequency was studied on 3072 fields (8 clones x 3 leaves x 2 leaf sides (abaxial and adaxial) x 4 slides x 4 fields x 2 treatments x 2 periods) chosen at random in the middle of the blade. Stomatal quantity was measured under an optical microscope, and manual counts of the number of stomata were made on these digital images for each field. Around 27195 stomata were counted in total. From these measurements, mean stomatal frequency was determined for each leaf surface of each replicate. Stomatal frequency was calculated as the number of stomata per mm^{-2} ($n \text{ mm}^{-2}$).

2.2.6 Histological analysis of leaves

For the histological analysis, samples of three leaves per clone were collected. We studied 1536 fields (8 clones x 3 leaves x 4 slides x 4 fields x 2 treatments x 2 periods) chosen at random in the middle of the slide.

Small tissue samples of leaves were sectioned, fixed in Karnovsky solution, and stored refrigerated. Subsequently, leaf samples were dehydrated in alcohol-ethanol series at increasing concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% v / v) and immersed in hydroxyethyl methacrylate resin (Historesin was performed, Sigma®, Heidelberg, Germany).

The resin blocks containing the tissue samples were sectioned using knife steel C type coupled to the manual rotary microtome. Histological sections were colored with periodic acid-Schiff reagent and naphtol blue black.

The histological slides were photomicrographed from four microscope fields per leaf using the Image-Pro Plus software. We measured the thicknesses of adaxial (TAD) and abaxial epidermis (TAB), the palisade (PP), and spongy (SP) parenchyma in clones with heterogeneous mesophyll and the total parenchyma (TP) in clones with homogeneous mesophyll. With these variables we determined the leaf thickness (LT) by adding the epidermis and total parenchyma.

2.2.7 Statistical methodology

Relationships between stomatal conductance x water pressure deficit (D) and GABA x water potential

Generalized additive models (WOOD, 2006) were fitted for the relations between stomatal conductance x D and GABA x water potential. For the relation between stomatal conductance and D, sub-models included the effects of the cubic spline smoothing function over D (parallel linear predictors) and only the cubic spline over D (coincident linear predictors). Then, the same maximal model and sub-models were fitted to the conductance data split for each clone.

GABA concentration under stress and after rewatering

For comparison of GABA concentration under water stress and after rewatering, analyses of variance were conducted (ANOVA) and, when the test F presented significance ($P < 0.10$), Tukey test at 0.05 significance was applied.

Leaf anatomy measurements

The continuous variables associated with leaf anatomy were fitted in classical analysis of variance models with the effects of clone and treatment and the interaction between clone and treatment in the linear predictor. Multiple comparisons were made using Tukey's test ($P = 0.05$).

All analyses were carried out using the statistical software R (R CORE TEAM, 2014).

2.3 Results and discussion

2.3.1. Plant water status

In the control treatment, ψ_{pd} average was -0.2 MPa, with few variations during all period, and ranged from -0.06 to -1.2 MPa, suggesting that water availability the control treatment was adequate for all clones during the period of study. The minor ψ_{pd} in this treatment occurred on November 29th (T16) and 30th (T17), which were the hottest days during all the period, with 29°C and 28.7°C as average temperatures, respectively. Measured values of ψ_{pd} under good water supplies were in agreement with the values reported by Mielke et al. (1998) for *Eucalyptus grandis* plantation in Espirito Santo, Brazil and Dye (1996) in South Africa.

The changes in water potential are illustrated in Figure 3. The values of ψ_{pd} in stress treatment ranged from -0.6 to -2.7 MPa, and the minimum values occurred on T41 day, when the maximum temperature reached 34°C at noon, DPV was 3.2 kpa, and relative humidity was 41% .

On stress days, the values of ψ_{pd} were around -1.5 MPa and the lowest value of ψ_{pd} occurred during days with maximum water stress for each cycle, i.e., T2 (cycle 1), T18 (cycle 2), and T44 (cycle 3) for all clones. On those days the predawn leaf water potential ranged from -2.3 to -3.6 MPa, and one day after rehydration, all clones recovered and increased ψ_{pd} , ranging from -0.05 to -0.6 MPa. For the midday water potential (ψ_{md}), water stressed plants presented -1.8 MPa (it ranged -0.7 to -3 MPa) and -0.4 to -2 MPa after rewatering.

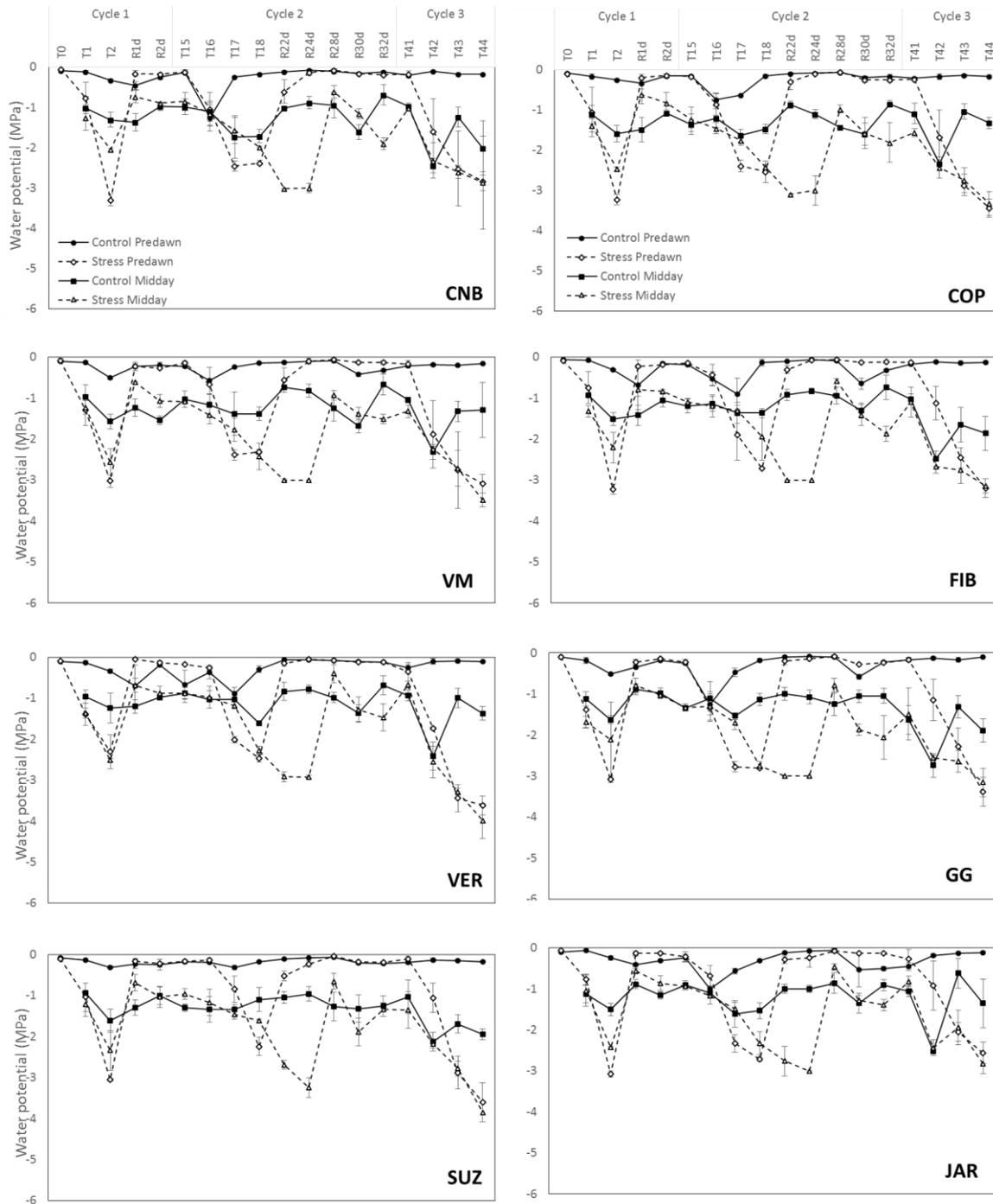


Figure 3 – Leaf water potential in eight *Eucalyptus* clones exposed to water stress along a drought period (T0 to T45) with subsequent recovery after rewatering (R1d to R32d) and control treatment

In the first cycle, all clones increased Ψ_{pd} and Ψ_{md} one day after rehydration, matching the values of Ψ_{pd} and Ψ_{md} of control treatment. The recovery of Ψ_{pd} in the second cycle also occurred one day after rewatering, but Ψ_{md} increased two days after rewatering, showing that water stress in the second cycle caused more damage to all the clones, which needed two days to recover Ψ_{md} completely.

This behavior of ψ_{pd} and ψ_{md} evidences that *Eucalyptus* is a species that exhibits a high resilience to water stress. Warren et al. (2012) observed complete recovery of ψ_{pd} two days after a severe water stress in two species of *Eucalyptus*. In another study comparing five species of *Eucalyptus*, the recovery of ψ_{pd} occurred 4 days after rewatering (WARREN et al., 2011).

2.3.2. Responses of gas exchange to water stress

The average rate of net photosynthesis in control plants varied among species and were 9.5, 12.8, 12.6, 10.1, 9.7, 10.9, 13.3, and 9.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to CNB, COP, FIB, GG, JAR, SUZ, VER, and VM, respectively. During the water stress period, photosynthetic rates were reduced in all clones, and the average was 3.4, 5.8, 5.3, 3.6, 6.7, 3.9, 2.9, and 1.8 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to CNB, COP, FIB, GG, JAR, SUZ, VER, and VM, respectively (Figure 4).

This photosynthesis reduction after water stress is consistent with the concept of dynamic stress when plants are under stress through a succession of phases, and the alarm phase occurs at the beginning of the disturbance (LARCHER, 2006). At this early stage, there is a loss of structure stability and functions that keep the vital activities of the plant. Photosynthesis is considered a vital function of the plant and is a non-specific indication of the stress state, once this reduction is not specific to the nature of water stress and may occur in different situations.

All clones reduced ~60% of the photosynthesis during water stress days. This reduction varied between 27 and 94%, as has been found in other studies (WARREN et al., 2004; GALMÉS et al., 2007; FLEXAS et al., 2009; GALLE et al., 2009). Warren et al. (2011) observed a photosynthesis reduction from 10 to 70% in five *Eucalyptus* species under water stress conditions.

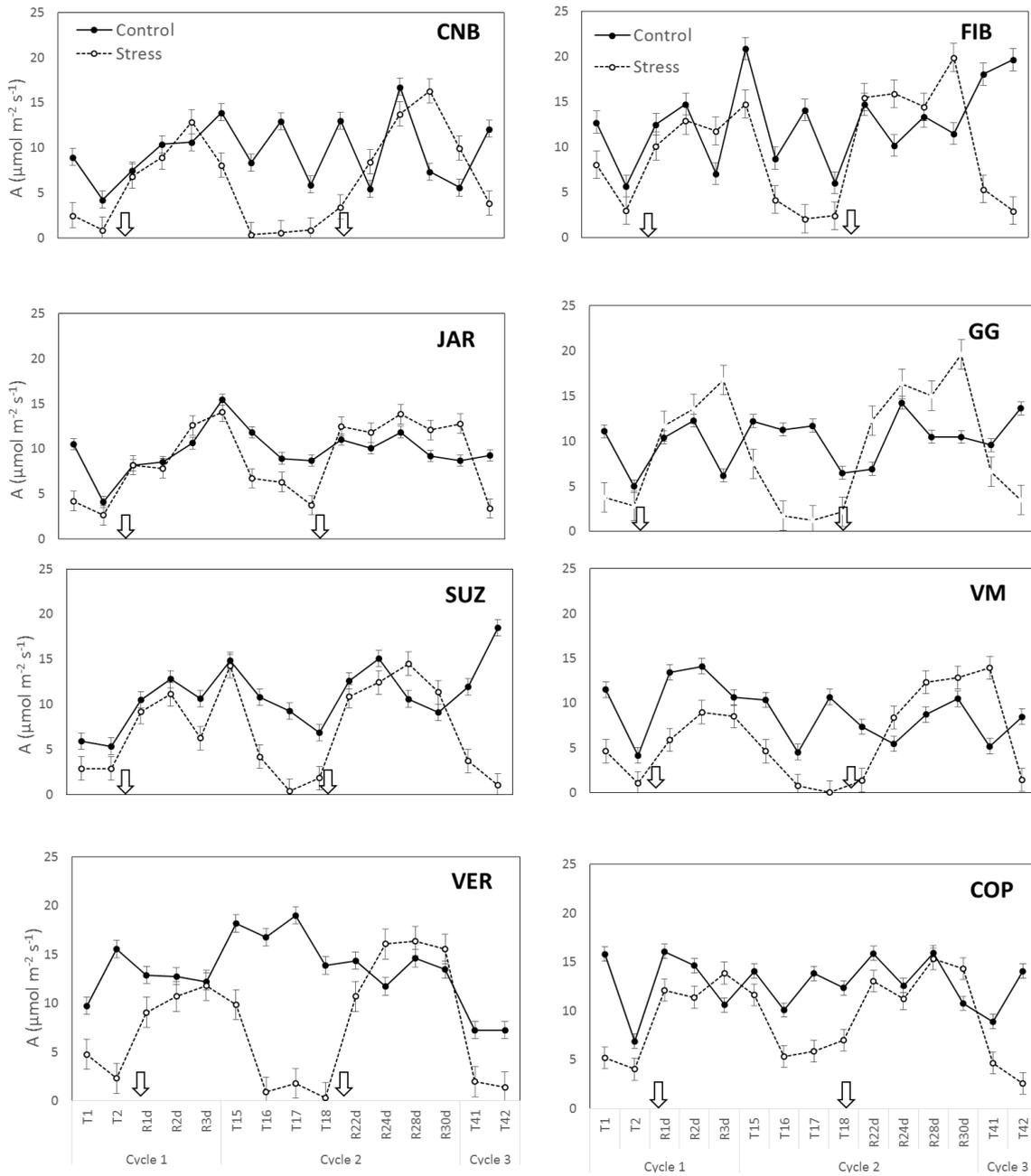


Figure 4 - Photosynthesis variation (A) of eight *Eucalyptus* clones under water stress and after rewatering. Arrows indicate days when there was irrigation. Bars indicate standard deviation for $n=4$.

In cycle 1, recovery of net photosynthesis of CNB, JAR, GG, and SUZ clones from water stress occurred immediately 1 day after rehydration, FIB recovered on the second day, and VM, VER, and COP clones recovered after 3 days of rewatering. In cycle 2, all clones recovered 1 day after rehydration, excluding SUZ, VER, and COP, which recovered after 2 days (Figure 2).

Other studies have shown that complete recovery of *Eucalyptus* species after drought stress takes several days and may differ among species (FAN; GROSSNICKLE,

1998; NGUGI et al., 2004). Warren et al. (2011) showed that five species of *Eucalyptus* needed 5-11 days to recover completely. However, these differences in rate of recovery from water stress depend on the severity of water stress before rewatering and are species-dependent (GALMÉS et al., 2007).

Although the time required to recover photosynthesis did not differentiate the clones, all of them increased the photosynthetic rate until it exceeded or matched the control treatment. This fast recovery after rehydration proves that all clones were tolerant to the tested conditions and presented efficient physiological responses. In general, there are species subjected to severe water stress that recover only 40-60% of maximum photosynthesis rate during the day after rewatering, and maximum photosynthesis rates are not always recovered (SOFO et al., 2004; FLEXAS et al., 2009).

Stomatal conductance decreased with increasing vapor pressure deficit in both treatments (Figure 5). One of the most significant environmental variable controlling g_s is the D, which is a stomatal response that prevents excessive dehydration and hydraulic failure (SCHULZE; HALL, 1982; MOTT; PARKHURST, 1991; OREN et al., 1999).

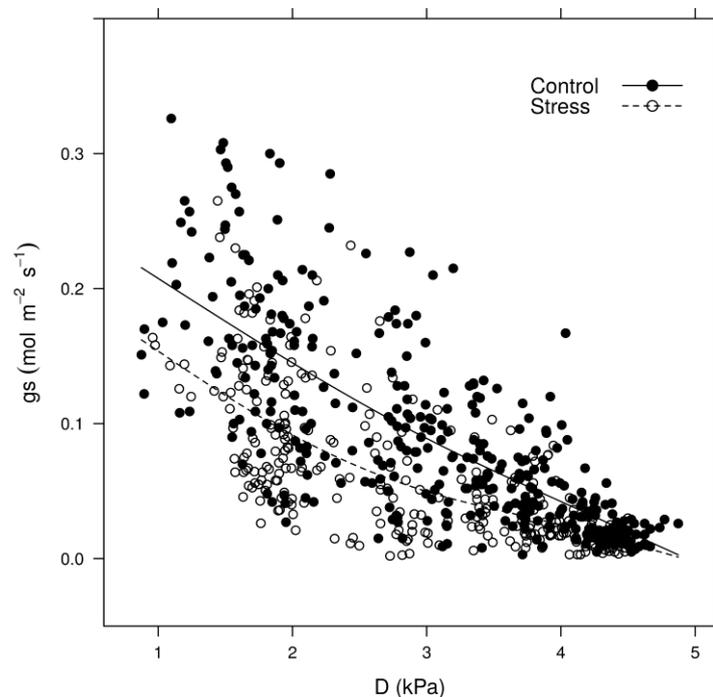


Figure 5 - Relationship between stomatal conductance (g_s) and D to stress (white circles) and control treatment (black circles)

Increased stomatal sensitivity to D is an indication of isohydric behavior, i.e., when stomata limit transpiration once D is increased, to prevent leaf water potential from

decreasing to levels that endanger the integrity of the hydraulic system (GHARUN et al., 2015).

Moreover, the likelihood-ratio test between the different linear prediction model versus the parallel linear predictor model was significant ($\chi^2_{1.6} = 21.27, p < 0.0001$); in this fashion, the stomatal conductance decreased with increasing D for both treatments (stress and control), but the equation for relationships was different comparing both treatments (Figure 5).

In the stress treatment, the relation between stomatal conductance and D was lower than in control treatment, showing that, in this case, in addition to D, water restriction in the soil also contributed to stomatal closure. Generally, plants in more drought-prone environments exhibit lower minimum stomatal conductance (CHRISTMAN et al., 2008).

Comparing all clones, we can see different allometric equations for relationships between g_s and D for each clone (Table 3). For instance, CNB, FIB, and JAR clones presented parallel curves between stress and control treatments, which means that the stomatal sensitivity to D was the same between treatments, but in stress treatment, stomata were more closed than in control treatment, a clear effect of water stress. Although these clones presented parallel curves, there was a different behavior when comparing them; for example, FIB showed a continuous curve in both treatments, but CNB and JAR clones changed the inclination curve (lower slope) after approximately 3.0 kPa of D in both treatments (Figure 6).

Table 3 - Equations for relationships between g_s and D for eight *Eucalyptus* clones

Clone	Test	
	Different vs. parallel	Parallel vs. coincident
CNB	$\chi^2_{2.7} = 1.49, p = 0.6275$	$\chi^2_{1.1} = 14.03, p = 0.0002$
COP	$\chi^2_{1.5} = 14.81, p = 0.0003$	-
FIB	$\chi^2_{1.3} = 1.30, p = 0.1586$	$\chi^2_{1.1} = 17.02, p < 0.0001$
GG100	$\chi^2_{3.0} = 5.47, p = 0.1427$	$\chi^2_{1.0} = 0.95, p = 0.3170$
JAR	$\chi^2_{1.2} = 2.69, p = 0.1310$	$\chi^2_{1.3} = 11.75, p = 0.0010$
SUZ	$\chi^2_{1.4} = 18.75, p < 0.0001$	-
VER	$\chi^2_{1.8} = 10.49, p = 0.0041$	-
VM	$\chi^2_{2.5} = 11.76, p = 0.0050$	-

Tolerant clones (except GG) and plastic clones showed concurrent curves, and hence stress and control treatments presented a different behavior. In the control treatment, SUZ and VM presented higher g_s values until ~2.0kPa and reduced the curve slope after that. In the stress treatment, the curve slope was lower than in control and was uniform for

variations of D. GG was the only clone that presented coinciding curves, so this clone showed no difference in g_s and D relation when comparing both treatments (Figure 4).

In contrast to SUZ and VM, plastic clones (COP and VER) in the control treatment had a lower slope at the beginning of the curve and higher after 2.5kPa of D. In the stress treatment, stomatal conductance was lower compared with the control treatment, and the curve slope was smaller and more uniform for all values of D, similar to SUZ and VM.

These results indicate that tolerant and plastic clones presented different stomatal sensitivity to D, an indication that these clones have greater ability to adapt in water stress situations. With these results, we can consider that relations between g_s and D provide a convenient tool for describing the sensitivity of *Eucalyptus* clones under water stress conditions (WHITEHEAD, 2004; GHARUN et al., 2015).

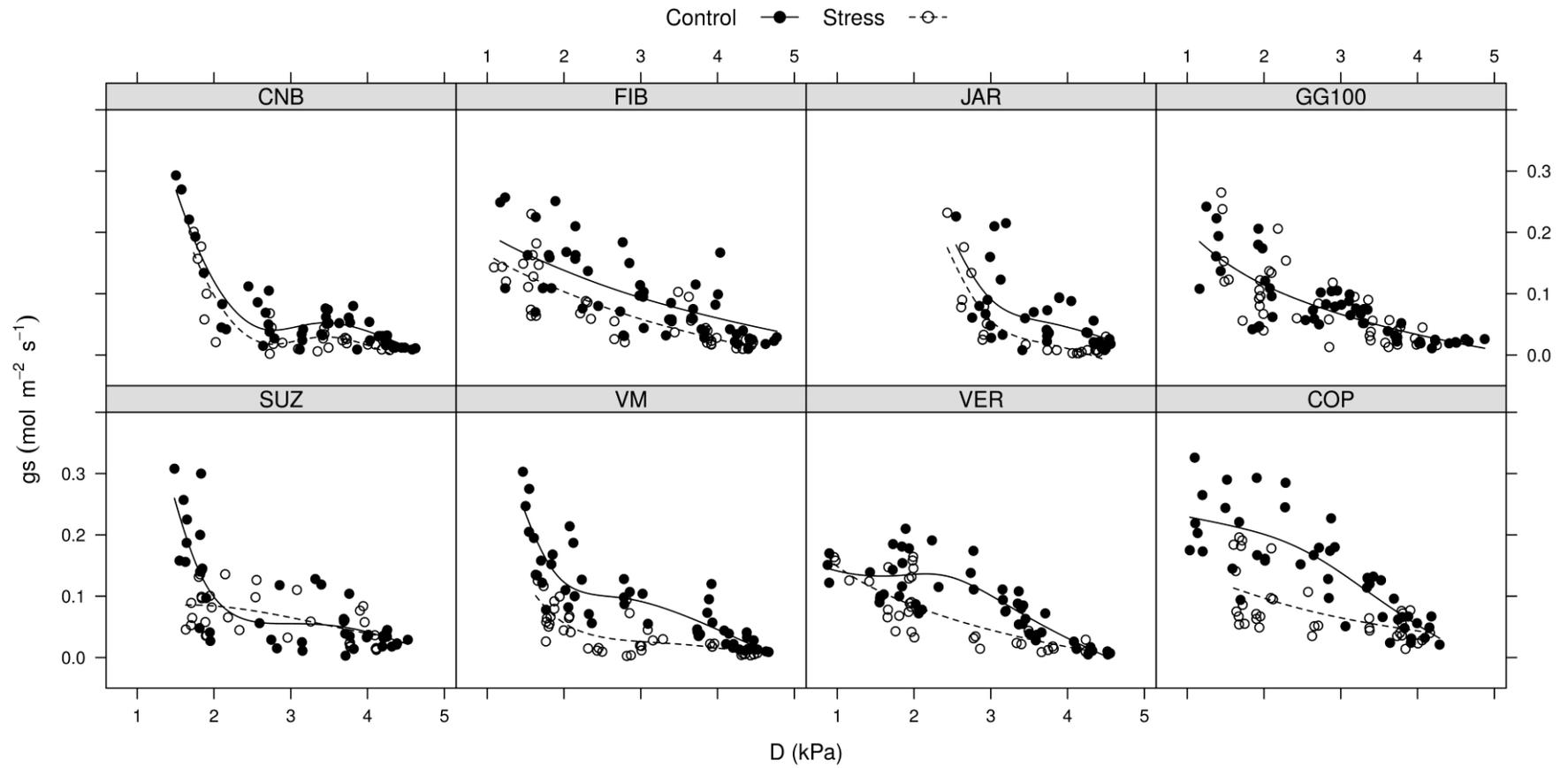


Figure 6 - Relationship between stomatal conductance (g_s) and vapor deficit pressure (D) of 8 *Eucalyptus* clones in control and water stress treatment.

2.3.3. GABA concentration

Water stressed plants increased GABA concentration during days with lowest midday leaf water potential (Figure 7).

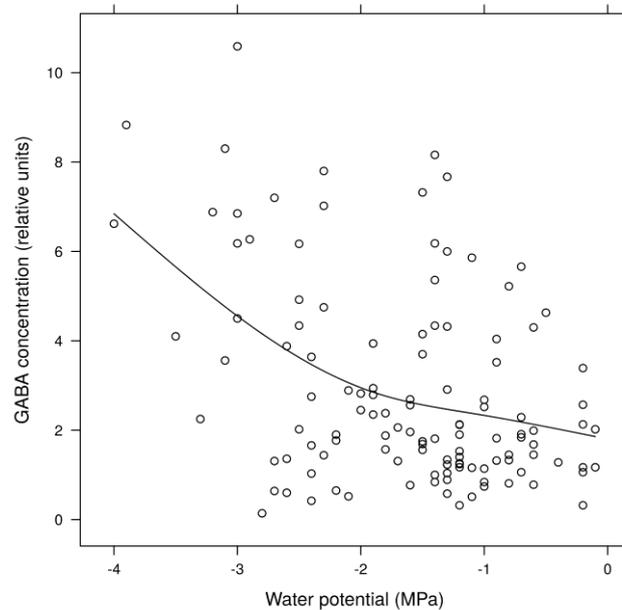


Figure 7 - Relationship between GABA concentration and midday water potential

Clones showed more GABA on the second stress day (T2) of cycle 1, except COP and GG, which showed a higher concentration on the first day (T1). In cycle 2, all clones presented a higher concentration on the fourth stress day, and in cycle 3, all clones concentrated GABA on the second stress day (T42), except JAR, which showed a lower GABA concentration (0.07) (Figure 8).

Water stress decreased photosynthesis and water potential; in addition, GABA levels were quantitatively significant and rose 0.1- to 10.6-fold after water stress among *Eucalyptus* clones (Figure 6). Similar to our findings, in response to drought stress, GABA levels in five species of *Eucalyptus* and two of *Acacia* leaves increased 5- to 16-fold (WARREN et al., 2011). High and rapid GABA accumulation was also reported in the leaves of bean (RAGGI, 1994), turnip (THOMPSON et al., 1996), sesame (BOR et al., 2009), and *Pinus* under drought stress (De DIEGO et al., 2013).

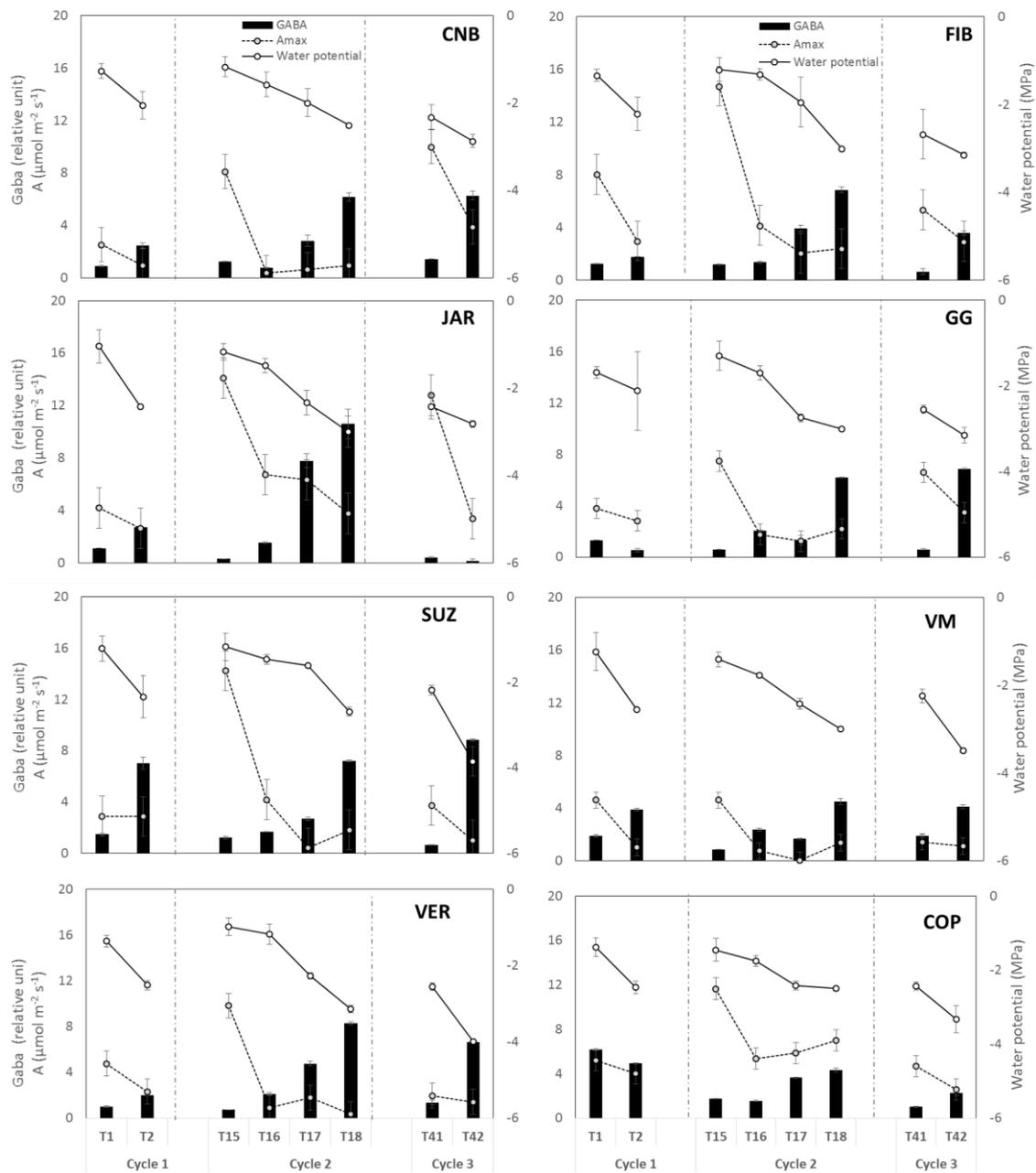


Figure 8 - Variations of water potential, photosynthesis (A) and GABA relation in 3 cycles of water stressed days for eight *Eucalyptus* clones. Errors bars indicate standard deviation for n=4

GABA is mainly metabolized via short pathway composed of three enzymes called GABA shunt. GABA shunt appears to be part of the metabolite pathways involved into in the C:N balance and the metabolism of nitrogen. The production of GABA is tightly linked to the glutamate content, and it is supposed that GABA has a dominant role of buffering the production of glutamate (MASCLAUX-DAUBRESSE et al., 2002). Glutamate-to-GABA conversion may be of considerable importance in the N economy and may function to rid plants of excess C (BOWN; SHELP, 1997).

In this sense, the high GABA ratio observed in all clones during stress days could be due to the GABA shunt being associated with carbon flux into the tricarboxylic acid cycle to provide carbon skeletons, which maintain normal cellular metabolism when carbon availability is reduced (photosynthesis decrease, Figure 6). Michaeli et al. (2011) showed that a mitochondrial GABA permease has been characterized and shown to bridge GABA metabolism and TCA cycle. The proper GABA transport into the mitochondria, where its degradation occurs, is required for plant growth upon carbon limitation, suggesting that GABA has a role in the respiration under low sugar conditions.

There was a significant difference in the GABA concentration among all clones on days of water stress, but there was no relation between these differences and tolerance to water stress (Table 4). This lack of correlation between GABA and tolerance to water stress does not support the hypothesis that GABA is associated with tolerance. In this study, we determined GABA content in plant leaves; however, it is known that the clones studied presented different quantity of leaves in the canopy and a specific dynamic leaf area index, thereby the total amount of GABA in the crown may vary for each clone, which was not studied in this work.

Table 4 - GABA concentration of eight *Eucalyptus* clones during water stress days and after rewatering

Clone	GABA (relative units)	
	Stress	Rewatering
		%
GG	2.05 aA	1.36 aA
CNB	2.53 aA	1.12 aB
VER	2.93 abA	1.66 aA
COP	3.72 abcA	1.18 aB
JAR	4.19 abcA	1.52 aB
SUZ	4.39 bcA	1.85 aB
VM	4.94 cA	2.58 aB
FIB	6.37 dA	0.95 aB
P<		0.3305
DMS		2.2514
CV (%)		0.5213

*Lowercase letters indicate differences in the column and capital letters differences in the line

Studies have reported GABA concentration as a compound related to stress that is able to directly protect or promote other benefits in leaves, increasing stress tolerance. Schaberg et al. (2011) associated the increase in GABA concentration with increased tolerance to cold stress in red spruce (*Picea rubens*) trees. Moreover, a study on *Arabidopsis thaliana* (BOUCHÉ; FROMM, 2004) suggests that GABA helps plants

survive stress. Hatmi et al. (2015) found more GABA concentration in drought-resistant than in sensitive grapevine genotype.

Besides, the effect of exogenous GABA can increase plant resistance to various pathogens (KINNERSLEY, 1998; YU et al., 2014), alleviate chilling injury under cold storage (SHANG et al., 2011; YANG et al., 2011; PALMA et al., 2014, WANG et al., 2014), and have a protective role in heat-stressed plants (NAYYAR et al., 2014).

However, there are studies that showed the opposite, once stress-tolerant plants presented lower GABA concentration than sensitive plants (BOLARIN et al., 1995; WARREN et al., 2012; De DIEGO, et al., 2013). Bor et al. (2009) suggested that GABA's role under stress conditions is more related to stress perception than protection, since growth was limited under stress conditions when GABA levels increased.

In all clones, except GG and VER, water stress led to reversible changes in GABA levels, that is, after rewatering GABA was rapidly reduced (Table 4); this occurrence was also observed by Warren et al. (2011). Shelp et al. (2011) suggested that GABA may, in turn, be used to rapidly generate succinate and energy via a tricarboxylic acid cycle upon removal of the stress.

According to our results, GABA concentration was very sensitive to water stress conditions, showing that it is a signal that form an important link between environment and plant. This amino acid could provide knowledge of the beginning and end of critical periods and the plant water stress levels reached after the implementation of management strategies that can minimize drought situations. In addition, we could conclude that GABA concentration is more related to stress perception than protection, since GABA was not associated with tolerance under water stress conditions.

2.3.4. Leaf anatomical characterization under adequate conditions of water availability

The GG clone (drought-tolerant) showed a thicker adaxial epidermis (AdE, 18.5 μ m), similarly to the other clones and significantly different from COP, SUZ, and JAR clones, which presented a thinner adaxial epidermis (14.7, 14.5, and 14.5 μ m, respectively). Likewise, GG clone showed a thicker abaxial epidermis (AbE, 14.5 μ m), similarly to other clones, except JAR clone, which presented a thinner adaxial epidermis (11.8 μ m) (Table 5).

All clones, except SUZ (drought-tolerant) and COP (plastic), presented heterogeneous mesophyll (Figure 9a), with similar palisade parenchyma thickness (PP), varying between 62.8

and 71.3 μm . However, the spongy parenchyma (SP) was thicker in VM clone (14.7 μm) and thinner in GG clone (12.1 μm). There was no differentiation between PP and SP for SUZ and COP clones (Figure 9b), and the homogeneous mesophyll (HM) thickness was similar between them (20.4 and 19.1 μm , respectively) (Table 5).

An important characteristic observed was the difference on properties of palisade and spongy layers. COP and SUZ clones have a homogeneous mesophyll, which might influence the dynamics of light and gases. Columnar palisade cells provide a deeper propagation of light into the mesophyll, and a small fraction of air space between cells decreases water loss through transpiration and changes the dynamics of CO₂ diffusion (TERASHIMA, 1992; VOGELMANN; MARTIN, 2001, MOORE et al., 1998).

Grisi et al. (2008) showed that a water stress tolerant species of coffee presented smaller intercellular spaces, thicker cuticle, and organized cells, characteristics that enable an adaptive advantage with higher photosynthetic rates and greater tolerance than the susceptible species.

Nevertheless, all clones, except COP and SUZ, have palisade and spongy layers. The cell wall of the spherical spongy mesophyll and the large fraction of air space in the leaf interior can increase light absorption by chloroplasts within the mesophyll (DE LUCIA et al., 2003).

All clones presented a similar leaf thickness (LT), varying between 21.9 and 23.9 μm (Table 5). Some studies have shown that plants from dry regions (xeric site) present leaves thicker than plants' from wet regions. This increase in leaf thickness is mainly due to a thicker palisade parenchyma and cuticle layer (BUSSOTTI et al., 2002).

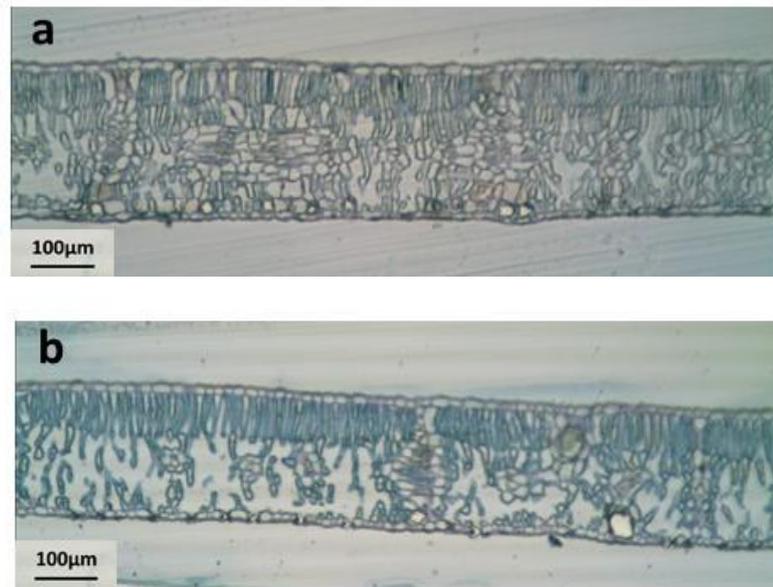


Figure 9 - Comparative leaf anatomy in blade cross-sections of COP with homogeneous mesophyll (a) and CNB with heterogeneous mesophyll (b)

Only COP and SUZ clones presented stomata on both leaf surfaces (Figures 3c and 3d), and stomata density was higher in the abaxial epidermis (DAb, average $42.9 \text{ n}^\circ \mu\text{m}^{-2}$) and lower in the adaxial epidermis (DAd), being 16.9 and $10.3 \text{ n}^\circ \mu\text{m}^{-1}$ to COP and SUZ, respectively (Table 5). The other clones showed stomata only in the abaxial epidermis (Figure 10a and 10b). James and Bell (1995) and Eksteen et al. (2013) studied *E. camaldulensis* and found amphistomatic leaves; similarly, we found that COP (which has *E. camaldulensis* in their genetic composition) has amphi-hipostomatic leaves.

Some savannah and xeric species, because of the high light intensity, adapted their leaves to this condition. In general, their leaves are amphistomatic and have a vertical position on the trees, reducing the damage caused by the direct sun (BROOKER, 2002).

Louro et al. (2003) performed an anatomical characterization of *Eucalyptus grandis* x *urophylla* and observed hypostomatic leaves, but occasionally found stomata on the adaxial side and restricted near the midrib of the leaf. Most of terrestrial plant species have more stomata on the lower leaf side than on the upper side, although a significant fraction (including most of grasses) have almost equal numbers of stomata on both leaf surfaces (MEIDNER; MANSFIELD, 1986). This restriction of stomata on the underside of the leaf may have been necessary to prevent photo-oxidative damage to the chlorophyll-containing guard cells in the epidermis (BAKER; BOWYER, 1994). Moreover, most species with stomata on the upper leaf surface that are exposed to direct sunlight have guard cells sunken in cavities and covered by epidermal projections (UPHOF; HUMMEL, 1962).

CNB clone, which is from a wet region, had the highest percentage of stomata ($678.7 \text{ n}^\circ \mu\text{m}^{-1}$) and COP clone, which is from a dry region, had the lowest amount of stomata ($413.7 \text{ n}^\circ \mu\text{m}^{-1}$) (Table 5). Some studies have shown a relationship between the amount of stomata and water stress tolerance. Camposeo et al. (2011) evaluated the adaptations of two species of almonds – tolerant (*A. webbii*) and susceptible to water stress (*A. communis*) – and observed that the susceptible species showed higher stomatal density compared with tolerant species.

Lower stomatal density may be a form of plant protection against water stress conditions, since fewer stomata reduce the leaf transpiration area, with a consequent reduction of water loss (SHTEIN et al., 2011; HAMAMISHI et al., 2012)

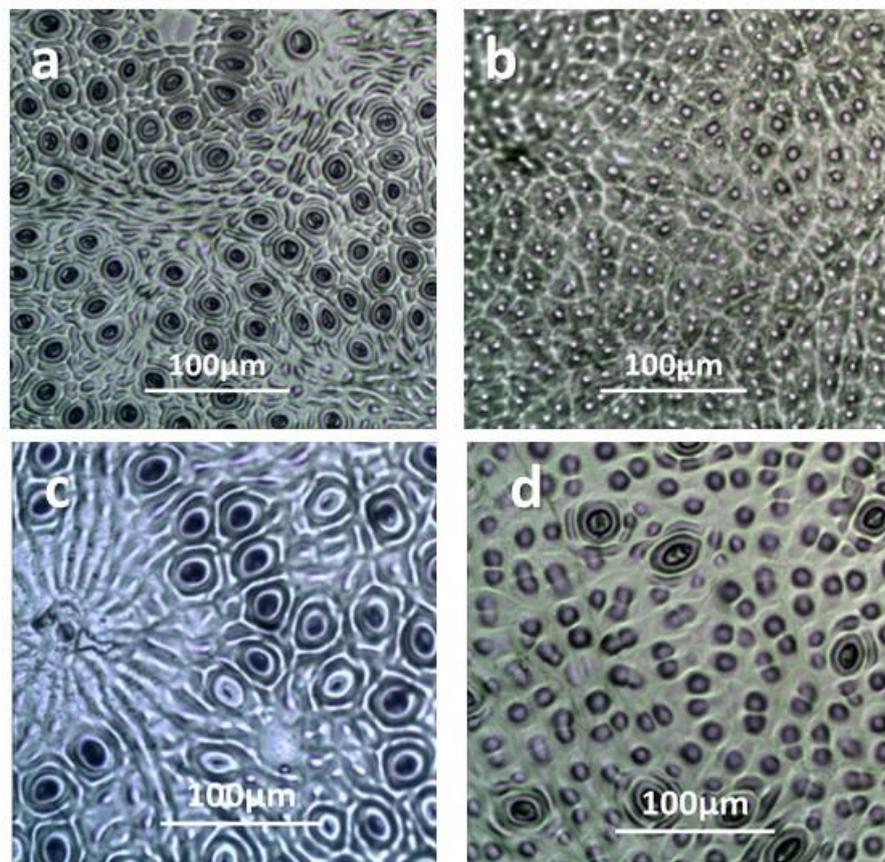


Figure 10 - Stomatal density on *Eucalyptus* leaves. Images of abaxial (a) and adaxial (b) epidermis of clone CNB and abaxial (c) and adaxial (d) of clone SUZ

Table 5 - Anatomical measurements in cross-sections of fully expanded leaves of eight *Eucalyptus* clones in control and water stress conditions

Clone	Abaxial epiderm Thickness	Adaxial epiderm Thickness	Palisade parenchyma	Spongy parenchyma	Homogeneous parenchyma	Leaf thickness	Abaxial stomatal density	Adaxial stomatal density
	— 10 ⁻² μm —	— 10 ⁻² μm —	— 10 ⁻² μm —	— 10 ⁻¹ μm —	— 10 ⁻¹ μm —	— x 10 ⁻¹ μm —	— x 10 ⁻¹ n mm ⁻¹ —	— x 10 ⁻¹ n mm ⁻¹ —
CNB	1.57 ab	1.38 a	6.55 a	1.32 ab	0.00	2.27 a	678.68 a	0.00
COP	1.47 b	1.30 a	0.00	0.00	2.04 a	2.32 a	413.77 d	169.17 a
FIB	1.63 ab	1.25 a	6.92 a	1.33 ab	0.00	2.31 a	560.43 abcd	0.00
GG	1.85 a	1.45 a	7.13 a	1.21 b	0.00	2.25 a	462.09 cd	0.00
JAR	1.43 b	1.18 a	6.58 a	1.30 ab	0.00	2.22 a	482.51 bcd	0.00
SUZ	1.45 b	1.28 a	0.00	0.00	1.91 a	2.19 a	443.72 d	103.15 b
VER	1.55 ab	1.30 a	6.82 a	1.40 ab	0.00	2.36 a	613.86 ab	0.00
VM	1.57 ab	1.30 a	6.28 a	1.47 a	0.00	2.39 a	605.18 abc	0.00
	p = 0.0105	p = 0.0096	p = 0.3934	p = 0.0568	p = 0.1210	p = 0.3357	p < 0.0001	p = 0.0276
Treatment								
Control	1.60 a	1.32 a	6.89 a	1.37 a	2.00 a	2.34 a	491.57 b	112.71 a
Stress	1.53 a	1.30 a	6.54 a	1.30 a	1.96 a	2.24 b	573.49 a	159.61 a
	p = 0.1898	p = 0.5125	p = 0.1525	p = 0.1269	p = 0.6169	p = 0.0313	p = 0.0011	p = 0.0927

2.3.5. Anatomical and physiological changes after water stress

There was a significant reduction of the leaf thickness (LT) and increase of abaxial stomatal density (AbD) in all clones after water stress (Table 5). Many abiotic stresses (such as drought and high temperatures) cause a reduction of water in the cells and consequent reduction of leaf thickness (BUSSOTTI et al., 2002). Chartzoulakis et al. (2002) observed a reduction of LT after water stress in two cultivars of avocado, which was attributed to the reduction of the size of the mesophyll cells. Cell size is related with cell wall elasticity; in general, bulk modulus of elasticity increases with cell size, and thus small cells can withstand negative pressure better than large cells (STEUDLE et al., 1977). This is evidenced by our results, according to which mesophyll thickness decreased in the stressed plants, indicating a reduction in cell size. This strategy can be considered as a drought adaptation mechanism (CLUTER et al., 1977; STEUDLE et al., 1977).

All clones increased their number of stomata after water stress (Table 5, Figure 11). The stress treatment had $\sim 573.5 \text{ n}^\circ \text{ mm}^{-2}$, and control treatment had $491.6 \text{ n}^\circ \text{ mm}^{-2}$. In addition, there was also an increase in the adaxial stomatal density of COP and SUZ clones with an average of 112.7 in the control treatment, compared with 159.6 $\text{n}^\circ \text{ mm}^{-2}$ in water stress (Table 5).

The higher number of stomata in leaves is very common in xerophytes plants; this strategy is called “get-it-while-you-can” and might increase their photosynthetic rates (MOORE et al., 1998). Having stomata on both sides, like COP and SUZ, may increase the supply of carbon dioxide to the mesophyll cell area (MOTT et al., 1982, PARKHURST, 1994; PARKHURST; MOTT, 1990).

Modification of stomatal density in response to drought varies between plant species and depends on the severity of water deficit. For example, a drought-induced reduction in stomata numbers was observed in *Eucalyptus camaldulensis x tereticornis* (NAUTIYAL et al., 1994), almonds (COMPOSEO et al., 2011), olives (BOSABADILIS; KOFIDIS, 2002), apples (SLACK, 1974; ELIAS, 1995), and umbu trees (SILVA et al., 2009). In contrast, increased stomatal density was observed in grass (XU; ZHOU, 2008), Acacia (CRAVEN et al, 2010), olives (ENNAJEH et al., 2010), and *Eucalyptus* (EKSTEEN et al., 2013). Species that have unchanged stomatal characteristics in response to drought are reported for groundnut (CLIFFORD et al., 1995), grape (BARBAGALLO et al., 1996), and olive (GUCCI et al., 2002).

Higher stomata density and smaller stomata size are forms of adaptation to drought, because these features enable plants to regulate water transport and transpiration more

effectively (FAHN; CUUTER, 1992; DICKISON, 2000; ENNAJEH et al., 2010). Besides stomatal density, stomatal behavior is very important in controlling different gas-exchange parameters. For instance, opening and closing stomata and stomatal orientation on leaf surfaces may prove vital (NEJAD et al., 2006).

There was no significant change in other anatomical variables after water stress for all clones. Although GG100 clone had presented a thicker abaxial and adaxial epidermis compared with the other clones – and this characteristic is considered as part of the plants' control against desiccation (JAMES; BELL, 1995) – there was no significant difference between treatment and stress control.

These changes in the anatomical characteristics of the leaves after water stress vary between species. Craven et al. (2010) reported no changes in the thickness of the abaxial and adaxial epidermis or in the spongy and palisade parenchyma in *Acacia koa*. In another study on four species of *Quercus*, anatomical characteristics were less responsive to water stress treatment than leaf physiological traits (QUERO et al., 2006).

Other studies reported anatomical changes after water stress; for example, in avocado, there was a reduction in epidermis thickness (CHARTZOULAKIS et al., 2002), while in two varieties of olive there was an increase in the thickness of the upper palisade and spongy mesophyll (ENNAJEH et al., 2010).

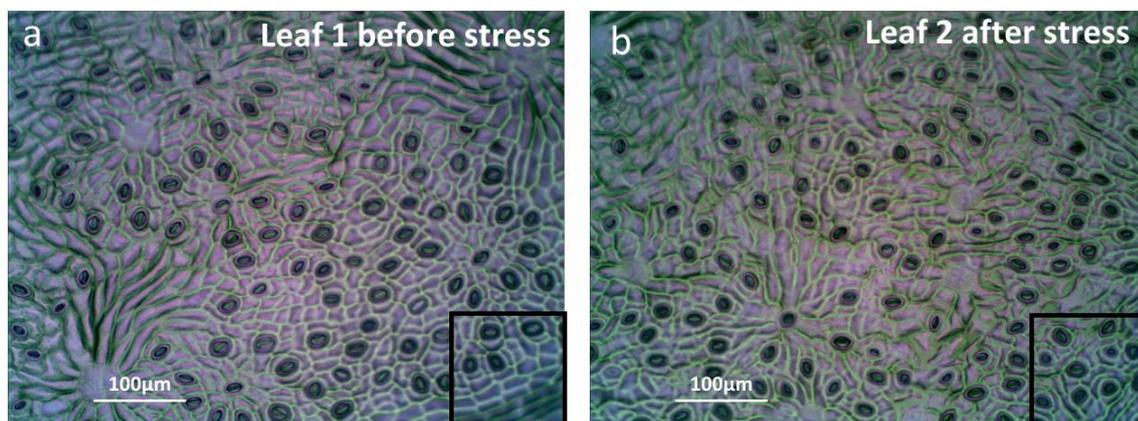


Figure 11 - Stomatal density on *Eucalyptus* leaves. Images of abaxial epidermis of leaf 1 before water stress (a) and other leaf 2 after water stress (b)

2.4. Conclusion

This experiment shows that eight *Eucalyptus* clones have similarities and differences in how they respond to water stress and rewatering:

- (1) The time required to recover photosynthesis did not differentiate clones, and all clones increased the photosynthetic rate until it exceeded or matched the control treatment.
- (2) All clones decreased g_s with increasing D in both treatments. All plastics and drought-tolerant clones (except GG) presented lower stomatal sensitivity to D under stress conditions than drought-sensitive clones.
- (3) GABA concentration was very sensitive to water stress conditions, showing that it is a signal that form an important link between environment and plant.
- (4) GABA concentration differ among all clones subjected to water stress, but there was no relation between these differences and tolerance to water stress.
- (5) Clonal variation in anatomical parameters was evident; COP (plastic) and SUZ (drought-tolerant) presented different anatomical characteristics such as homogeneous mesophyll and amphi-hipostomatic leaves.
- (6) All clones increased the number of stomata and reduced leaf thickness after water stress, but there was no response to water stress for the other parameters.

According to our results, response of g_s to D was the best physiological variable that can differentiate drought-tolerant and drought-sensitive clones, and GABA is an indicator of the beginning and the end of critical periods reached after the implementation of management strategies that can minimize drought situations.

References

- ALVARES, C.A.; STAPE, J.L.; SENTELHAS, P.C.; GONÇALVES, J.L.M.; SPAROVEK, G. Köppen's climate classification map for Brazil. **Meteorologische Zeitschrift**, Stuttgart, v. 22, p. 711-728, 2013.
- ADDINGTON, R.N.; MITCHELL, R.J.; OREN, R.; DONOVAN, L.A. Stomatal sensitivity to vapor pressure deficit and its relationship to hydraulic conductance in *Pinus palustris*. **Tree Physiology**, Durham, v. 24, p. 561-569, 2004.
- ASKAR, A.; RUBACH, K.; SCHORMULLER, J. Dunnschichtchromatographische Trennung der in Bananen vorkommenden Amin-Fraktion. **Chemische Microbiologie und Technologie Lebenshem**, Nürnberg, v. 1, p. 187-190, 1972.
- BARGER, G.; DALE, H.H. A third active principle in ergot extracts. **Proceedings of Chemical Society**, London, v. 26, p. 128-129, 1910.

BARBAGALLO, M.G.; COLLESANO, G.; SOTTILE, I. Ricerche sulla densità e sulle caratteristiche biometriche degli stomi nella vite. In: *GIORNATE SCIENTIFICHE S.O.I.*, 3., 1996, Erice. Erice: SOI, 1996. p. 65-66.

BAKER, N.R.; BOWYER, J.R. **Photoinhibition of photosynthesis from molecular mechanism to the field.** Oxford: Bios Scientific, 1994. 471 p.

BEERLING, D.J.; DE MICCO, V. Seasonal dimorphism in the Mediterranean *Cistus incanus* L. subsp. *incanus*. **Annals of Botany**, Oxford, v. 87, p. 789-794, 2001.

BOLARIN, M.C.; SANTA-CRUZ, A.; CAYUELA, E.; PEREZ-ALFOCEA, F. Short-term solute changes in leaves and roots of cultivated and wild tomato seedlings under salinity. **Journal of Plant Physiology**, Leipzig, v. 147, p. 463-468, 1995.

BOR, M.; SECKIN, B.; OZGUR, R.; YILMAZ, O.; OZDEMIR, F.; TURKAN, I. Comparative effects of drought, salt, heavy metal and heat stresses on gamma-aminobutyric acid levels of sesame (*Sesamum indicum* L.). **Acta Physiologia Plantarum**, Heidelberg, v. 31, p. 655-659, 2009.

BOUCHÉ, N.; FROMM, H. GABA in plants: just a metabolite? **Trends in Plant Science**, Cambridge, v. 9, n. 3, p. 110-115, 2004.

BOUCHÉ, N.; FAIT, A.; BOUCHEZ, D.; MOLLER, S.; FROMM, H. Mitochondrial succinic-semialdehyde dehydrogenase of the γ -aminobutyrate shunt is required to restrict levels of reactive oxygen intermediates in plants. **Proceedings of the National Academy of Sciences of the USA**, Washington, v. 100, n. 11, p. 6843-6848, 2003.

BOWN, A.W.; SHELP, B.J. The metabolism and functions of γ -aminobutyric acid. **Plant Physiology**, Lancaster, v. 115, p. 1-5, 1997.

BOWN, A.W.; HALL, D.E.; MACGREGOR, K.B. Insect footsteps on leaves stimulate the accumulation of 4-aminobutyrate and can be visualized through increased chlorophyll fluorescence and superoxide production. **Plant Physiology**, Lancaster, v. 129, p. 1430-1434, 2002.

BOWDEN, K.; BROWN, B.G.; BATTY, J.E. 5-hydroxytryptamine: its occurrence in cowhage. **Nature**, London, v. 174, p. 925-926, 1954.

BOSABALIDIS, A.M.; KOFIDIS, G. Comparative effects of drought stress on leaf anatomy of two olive cultivars. **Plant Science**, Davis, v. 163, p. 375-379, 2002.

BROOKER, I. Botany of the eucalypts, In: COPPEN, J.W. **Eucalyptus: the genus Eucalyptus**. London: Taylor & Francis, 2002. p. 3-35.

BREDA, N.; HUC, R.; GRANIER, A.; DREYER, E. Temperate forests trees and stands under severe drought: a review of ecophysiological responses, adaptation process and long-term consequences. **Annals of Forest Science**, Dordrecht, v. 63, p. 625-644, 2006.

BUELOW, W.; GISVOLD, O. A photochemical investigations of *Hermidium alipes*. **Journal of the American Pharmaceutical Association**, New Jersey, v. 33, p. 270-274, 1944.

BUSSOTTI, F.; BETTINI, D.; GROSSONI, P.; MANSUINO, S.; NIBBI, R.; SODA, C.; TANI, C. Structural and functional traits of *Quercus ilex* in response to water availability. **Environmental and Experimental Botany**, Paris, v. 47, p. 11-23, 2002.

BUVE, N.; RISPAIL, N.; LAINE, P.; CLIQUET, J.B.; OURRY, A.; LE DEUNFF, E. Putative role of GABA as a long distance signal in up-regulation of nitrate uptake in *Brassica napus* L. **Plant Cell Environmental**, Malden, v. 27, p. 1035-1046, 2004.

CAMPOSEO, S.; PALASCIANO, M.; VIVALDI, G.A.; GODINI, A. Effect of increasing climatic water deficit on some leaf and stomatal parameters of wild and cultivated almonds under Mediterranean conditions. **Scientia Horticulturae**, British Columbia, v. 127, p. 234-241, 2011.

CARROLL, A.D.; FOX, G.G.; LAURIE, S.; PHILLIPS R.; RATCLIFFE, R.G.; STEWART, G.R. Ammonium assimilation and the role of gamma-aminobutyric acid in pH homeostasis in carrot cell suspensions. **Plant Physiology**, Lancaster, v. 106, p. 513-520, 1994.

CASSON, S.A.; HETHERINGTON, A.M. Environmental regulation of stomatal development. **Current Opinion of Plant Biology**, Missouri, v. 13, p. 90-95, 2010.

CATSKY, J.; SOLÁROVÁ, J.; POSPOŠILOVÁ, J.; TICHÁ, I. Conductances for carbon dioxide transfer in the leaf. In: SESTÁK, Z. (Ed.). **Photosynthesis during leaf development**. Dorbrecht: Dr. W. Junk Publ., 1985. p. 217-249.

CHARTZOULAKIS, K.; PATAKAS, A.; KOFIDIS, G.; BOSABALIDIS, A.; NASTOU, A. Water stress affects leaf anatomy, gas exchange, water relations and growth of two avocado cultivars. **Scientia Horticulturae**, British Columbia, v. 95, p. 39-50, 2002.

CHRISTMAN, M.A.; RICHARDS, J.H.; MCKAY, J.K.; STAHL, E.A.; JUENGER, T.E.; DONOVAN, L.L.A. Genetic variation in *Arabidopsis thaliana* for night-time leaf conductance. **Plant, Cell and Environment**, Malden, v. 31, p. 1170-1178, 2008.

CLIFFORD, S.V.; BLACK C.R.; ROBERTS, J.A.; STRONACH I.M.; SINGLETON-JONES, P.R.; MOHAMED A.D.; AZAM-ALI, S.N. The effect of elevated atmospheric CO₂ and drought on stomatal frequency in groundnut (*Arachis hypogaea* L.). **Journal of Experimental Botany**, Lancaster, v. 46, p. 847-852, 1995.

CORREIA, B.; PINTÓ-MARIJUAN, M.; NEVES, L.; BROSSA, R.; DIAS, M.C.; COSTA, A.; CASTRO, B.B.; ARAÚJO, C.; SANTOS, C.; CHAVES, M.M.; PINTO, G. Water stress and recovery in the performance of two *Eucalyptus globulus* clones: physiological and biochemical profiles. **Physiologia Plantarum**, Malden, v. 150, p. 580-592, 2014.

CRAVEN, D.; GULAMHUSSEIN, S.; BERLYN, G.P. Physiological and anatomical responses of *Acacia koa* (Gray) seedlings to varying light and drought conditions. **Environmental and Experimental Botany**, Paris, v. 69, p. 205-213, 2010.

CUTLER, D.F.; BOTHA, T.; STEVENSON, D.W. A folha. In: CUTLER, D.F. **Anatomia vegetal: uma abordagem aplicada**. Porto Alegre: Artmed, 2011. p. 85-133.

- CUTLER, J.M.; RAINS, D.W.; LOOMIS, R.S. The importance of cell size in the water relations of plants. **Physiologia Plantarum**, Malden, v. 40, p. 255-260, 1977.
- DE DIEGO, N.; PEREZ-ALFOCEA, F.; CANTERO, E.; LACUESTA, M.; MONCALEAN, P. Physiological response to drought in radiata pine: phytohormone implication at leaf level. **Tree Physiology**, Durham, v. 32, p. 435-449, 2012.
- DE DIEGO, N.; SAMPEDRO, M.C.; BARRIO, R.J.; SAIZ-FERNANDEZ, I.; MONCALEAN, P.; LACUESTA, M. Solute accumulation and elastic modulus changes in six radiata pine breeds exposed to drought. **Tree Physiology**, Durham, v. 33, p. 69-80, 2013.
- DEEWATTHANAWONG, R.; NOCK J.F.; WATKINS C.B. γ -Aminobutyric acid (GABA) accumulation in four strawberry cultivars in response to elevated CO₂ storage. **Postharvest Biology and Technology**, Washington, v. 57, p. 92-96, 2010.
- DE LUCIA, E.H.; WHITEHEAD, D.; CLEARWATER, M.J. The relative limitation of photosynthesis by mesophyll conductance in co-occurring species in a temperate rainforest dominated by conifer *Dacrydium cupressinum*. **Functional Plant Biology**, Clayton South, v. 30, p. 1197-1204, 2003.
- DICKISON, W.C. **Integrative plant anatomy**. San Diego: Academic Press, 2000. 534 p.
- DONALD, C.M. The breeding of crop ideotypes. **Euphytica**, Dordrecht, v. 17, p. 385-403, 1968.
- DYE, P.J. Response of *Eucalyptus grandis* trees to soil water deficits. **Tree Physiology**, Durham, v. 16, p. 233-238, 1996.
- EKSTEEN, A.B.; GRZESKOWIAK, V.; JONES, N.B.; PAMMENTER, N.W. Stomatal characteristics of *Eucalyptus grandis* clonal hybrids in response to water stress, **Southern Forests**, Menlo Park, v. 75, n. 3, p. 105-111, 2013.
- ELIAS, P. Stomata density and size of apple trees growing in irrigated and non irrigated conditions. **Biologia**, Bratislava, v. 50, p. 115-118, 1995.
- ENGLAND, J.R.; ATTIWILL, P.M. Changes in stomatal frequency, stomatal conductance and cuticle thickness during leaf expansion in the broad-leaved evergreen species, *Eucalyptus regnans*, **Trees**, Heidelberg, v. 25, p. 987-996, 2011.
- ENNAJEH, M.; VADEL, A.M.; COCHARD, H.; KHEMIRA, H. Comparative impacts of water stress on the leaf anatomy of a drought-resistant and a drought-sensitive olive cultivar, **Journal of Horticultural Science**, Bangalore, v. 85, n. 4, p. 289-294, 2010.
- EWERS, B.E.; OREN, R.; JOHNSEN, K.H.; LANDSBERG, J.J. Estimating maximum mean canopy stomatal conductance for use in models. **Canadian Journal of Forest Research**, Ottawa, v. 31, p. 198-207, 2011.
- EWIS, A.J. Acetylcholine, a new active principle of ergot. **Biochemical Journal**, London, v. 8, p. 44-49, 1914.

FAHN, A.; CUTLER, D.F. Xerophytes. In BRAUN, H.J.; CARLQUIST, S.; OZENDA, P.; ROTH, I. (Ed.). **Encyclopedia of plant anatomy**. Berlin; Stuttgart: Gebrüder Borntraeger, 1992. p. 28-30.

FAIT, A.; YELLIN, A.; FROMM, H. GABA and GHB neurotransmitters in plants and animals. In: BALUSKA, F.; MANCUSO, S.; VOLKMANN, D. **Communication in plants**. Rehovot; Berlin; Heidelberg: Springer 2006. p. 171-185.

FAO. **The state of food and agriculture**, 2010-2011, Rome, 2011. 160 p.

FLEXAS, J.; BARON, M.; BOTA, J. Photosynthesis limitations during water stress acclimation and recovery in the drought-adapted Vitis hybrid Richter-110 (*V. berlandieri* x *V. rupestris*). **Journal of Experimental Botany**, Lancaster, v. 60, p. 2361-2377, 2009.

FRASER, L.H.; GRENALL, A.; CARLYLE, C.; TURKINGTON, R.; FRIEDMAN, C.R. Adaptive phenotypic plasticity of *Pseudoroegneria spicata*: response of stomata density, leaf area and biomass to changes in water supply and increased temperature. **Annals of Botany**, Oxford, v. 103, p. 769-775, 2009.

GALLE, A.; FLOREZ-SARASA, I.; THAMEUR, A.; DE PAEPE, R.; FLEXAS, J.; RIBAS-CARBO, M. Effects of drought stress and subsequent rewatering on photosynthetic and respiratory pathways in *Nicotiana sylvestris* wild type and the mitochondrial complex I-deficient CMSII mutante. **Journal of Experimental Botany**, Lancaster, v. 61, p. 765-775, 2009.

GALMÉS, J.; MEDRANO, H.; FLEXAS, J. Photosynthetic limitations in response to water stress and recovery Mediterranean plants with different growth forms. **New Phytologist**, Lancaster, v. 175, p. 81-93, 2007.

GHARUN, M.; TURNBULL, T.L.; PFAUTSCH, S.; ADAMS, M.A. Stomatal structure and physiology do not explain differences in water use among montane eucalyptus. **Oecologia**, Buenos Aires, v. 177, p. 1171-1181, 2015.

GUCCI, R.; GRUMELLI, A.; COSTAGLI, G.; TOGNETTI, R.; MONNOCCI, A.; VITAGLIANO, C. Stomatal characteristics of two olive cultivars “Frantoio” and “Leccino”. **Acta Horticulturae**, Leuven, v. 586, p. 541-544, 2002.

GÜLCAN, R.; MISIRLI, A. Importance of stomata in evaluating the vigour of *Prunus mahaleb* rootstocks. In: XXIII Int. Hort. Congr., Firenze (Italy), 27 August - 1 September 1990, No. 4030, 1990

HAMAMISHI, E.T.; THOMAS, B.R.; CAMPBELL, M.M. Drought induces alterations in the stomatal development program in *Populus*, **Journal of Experimental Botany**, Lancaster, v. 63, p. 4959-4971, 2012.

HANOWER, P.; BRZOZOWSKA, J. Influence d'un choc osmotique sur la composition des feuilles de cotonnier em acides amines libres. **Phytochemistry**, London, v. 14, p. 1691-1694, 1975.

- HATMI, S.; GRUAU, C.; TROTEL-AZIZ, P.; VILLAUME, S.; RABENOELINA, F.; BAILLIEUL, F.; EULLAFFROY, P.; CLÉMENT, C.; FERCHICHI, A.; AZIZ, A. Drought stress tolerance in grapevine involves activation of polyamine oxidation contributing to improved immune response and low susceptibility to *Botrytis cinerea*. **Journal of Experimental Botany**, Lancaster, v. 66, n. 3, p. 775-787, 2015.
- HUAMAN, C.A.M. **Impacto do estresse abiótico em plantas no contexto das mudanças climáticas**. 2010. 62 p. Tese (Livre-docência) – Faculdade de Filosofia, Ciências e Letras, Universidade de São Paulo, Ribeirão Preto, 2010.
- JAMES, S.A.; BELL, T. Morphology and anatomy of leaves of *Eucalyptus camaldulensis* clones: variation between geographically separated locations. **Australian Journal of Botany**, New York, v. 43, p. 415– 433, 1995.
- JOHNSON, J.D.; FERRELL, W.K. Stomatal response to vapour pressure deficit and the effect of plant water stress. **Plant, Cell and Environmental**, Malden, v. 6, p. 451–456, 1983.
- KINNERSLEY, A.M. Bioactivity of AuxiGro™ plant metabolic primer, a formulation containing GABA and glutamic acid. In: ANNUAL MEETING PLANT GROWTH REGULATION SOCIETY OF AMERICA, 25, 1998, Chicago. **Proceedings...** Chicago: PGRSA, 1998. p. 89-94.
- KINNERSLEY, A.M.; TURANO, F.J. Gamma aminobutyric acid (GABA) and plant responses to stress. **Critical Reviews in Plant Sciences**, Davis, v. 19, n. 6, p. 479-509, 2000.
- LARCHER, W. **Ecofisiologia vegetal**. São Carlos: RiMa, 2006. 550 p.
- LAWLOR, D.W.; CORNIG, G. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. **Plant, Cell and Environment**, Malden, v. 25, p. 275-294, 2002.
- LOURO, R.P.; SANTIAGO, L.J.; SANTOS, A.V.; MACHADO, R.D. Ultrastructure of *Eucalyptus grandis* x *E. urophylla* plants cultivated ex vitro in greenhouse and field conditions. **Trees**, Heidelberg, v. 17, p. 11-22, 2003.
- MAC GREGOR, K.B.; SHELP, B.J.; PEIRIS, S.; BOWN, A.W. Overexpression of glutamate decarboxylase in transgenic tobacco plants deters feeding by phytophagous insect larvae. **Journal of Chemical Ecology**, Tampa, v. 29, p. 2177-2182, 2003.
- MAHERALI, H.; JOHNSON, H.B.; JACKSON, R.B. Stomatal sensitivity to vapour pressure difference over a subambient to elevated CO₂ gradient in a C₃/C₄ grassland. **Plant, Cell and Environment**, Malden, v. 26, p. 1297-1306, 2003.
- MARRICHI, A.H.C. **Caracterização da capacidade fotossintética e da condutância estomática em sete clones comerciais de *Eucalyptus* e seus padrões de resposta ao déficit de pressão de vapor**. 2009. 104p. Dissertação (Mestrado em Recursos Florestais) - Escola Superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo, Piracicaba, 2009.

- MARTIN, T.A.; JOHNSEN, K.H.; WHITE, T.L. Ideotype development in southern pines: rationale and strategies for overcoming scale-related obstacles, **Forest Science**, Bethesda, v. 47, p. 21-28, 2001.
- MASCLAUX-DAUBRESSE, C. Diurnal changes in the expression of glutamate dehydrogenase and nitrate reductase are involved in the C/N balance of tobacco source leaves. **Plant, Cell and Environment**, Malden, v. 25, p. 1451-1462, 2002.
- MASSMAN, W.J.; KAUFMANN, M.R. Stomatal response to certain environmental factors: a comparison of models for subalpine trees in the Rocky Mountains. **Agricultural and Forest Meteorology**, Connecticut, v. 54, p. 155-167, 1991.
- MAYER, R.R.; CHERRY, J.L.; RHODES, D. Effects of heat shock on amino acid metabolism of cowpea cells. **Phytochemistry**, London, v. 94, p. 796–810, 1990.
- MAZZUCOTELLI, E.; TARTARI, A.; CATTIVELLI, L.; FORIANI, G. Metabolism of GABA during cold acclimation and freezing and its relationship to frost tolerance in barley and wheat. **Journal of Experimental Botany**, Lancaster, v. 57, p. 3755-3766, 2006.
- MC LEAN, M.D.; YEVTUSHENKO, D.P.; DESCHENE, A.; VAN CAUWENBERGHE, O.R.; MAKHMOUDOVA, A.; POTTER, J.W.; BOWN, A.W.; SHELP, B.J. Overexpression of glutamate decarboxylase in transgenic tobacco plants confers resistance to the northern root knot nematode. **Molecular Breeding**, Lleida, v. 11, p. 277-285, 2003.
- MCCAUGHEY, J.H.; IACOBELLI, A. Modelling stomatal conductance in a northern deciduous forest, Chalk River, Ontario. **Canadian Journal of Forestry Research**, Ottawa, v. 24, p. 904-910, 1994.
- MEDIAVILLA, S.; ESCUDEIRO, A. Stomatal responses to drought of mature trees and seedlings of two co-occurring Mediterranean oaks. **Forest Ecology and Management**, Amsterdam, v. 187, p. 281-294, 2004.
- MEIDNER H.; MANSFIELD T.A. **Physiology of stomata**. Nova York, McGraw-Hill, 1968, 176 p.
- MICHAELI, S.; LAGOR, K. A mitochondrial GABA permease connects the GABA shunt and the TCA cycle, and is essential for normal carbon metabolism. **The Plant Journal**, Michigan, v. 67, p. 485-498, 2011.
- MIELKE, M.S.; OLIVA, M.A.; BARROS, N.F.; PENCHEL, R.M.; MARTINEZ, C.A.; ALMEIDA, A.C. Stomatal control of transpiration in the canopy of a clonal *Eucalyptus grandis* plantation. **Trees**, Heidelberg, v. 13, p. 152-160, 1998.
- MIRANDA, V.; BAKER, N.R.; LONG, S.P. Anatomical variation along the length of the *Zea mays* leaf in relation to photosynthesis. **New Phytologist**, Lancaster, v. 88, p. 595-605, 1981.
- MOKOTEDI, M.E.O. Water relations of *Eucalyptus nitens* x *Eucalyptus grandis*: is there interclonal variation in response to experimentally imposed water stress? **Southern Forests**, Menlo Park, v. 75, n. 4, p. 213-220, 2013.

- MONTEITH, J.L. A reinterpretation of stomatal response to humidity. **Plant, Cell and Environmental**, Malden, v. 18, p. 357-364, 1995.
- MOORE, R.; CLARK, W.D.; VODOPICH, D.S. **Botany**. Boston: MsGraw Hill, 1995. 919 p.
- MOTT, K.A.; PARKHURST, D.F. Stomatal responses to humidity in air and helox. **Plant, Cell and Environment**, Malden, v. 14, p. 509-515, 1991.
- MOTT, K.A.; GIBSON, A.C.; O'LEARY, J.W. The adaptative significance of amphistomatic leaves. **Plant, Cell and Environmental**, Malden, v. 5, p. 455-460, 1982.
- NAYYAR, H.; KAUR, R. γ -aminobutyric acid (GABA) imparts partial protection from heat stress injury to rice seedlings by improving leaf turgor and upregulating osmoprotectants and antioxidants. **Journal of Plant Growth and Regulation**, Dordrecht, v. 33, p. 408-419, 2014.
- NAUTIYAL, S.; BADOLA, H.K.; PAL, M.; NEGI, D.S. Plant responses to water stress: changes in growth, dry matter production, stomatal frequency and leaf anatomy. **Biologia Plantarum**, Dordrecht, v. 36, n. 1, p. 91-97, 1994.
- NEJAD, A.R.; HARBINSON, J.; VAN MEETEREN, U. Dynamics of spatial heterogeneity of stomatal closure in *Tradescantia virginiana* altered by growth at high relative air humidity, **Journal of Experimental Botany**, Lancaster, v. 57, p. 3669-3678, 2006.
- OCHELTREE, T.W.; NIPPERT, J.B.; PRASAD, P.V.V. Stomatal responses to changes in vapor pressure deficit tissue-specific differences in hydraulic conductance, **Plant, Cell and Environmental**, Malden, v. 37, p. 132-139, 2013.
- OGLE, K.; REYNOLDS, F.F. Desert dogma revisited: coupling of stomatal conductance and photosynthesis in the desert shrub, *Larrea tridentata*. **Plant, Cell and Environment**, Malden, v. 25, p. 909-921, 2002.
- OREN, R.; SPERRY, J.S.; KATUL, G.G.; PATAKI, D.E.; EWERS, B.E.; PHILLIPS, N.; SCHAFER, K.V.R. Survey and synthesis of intra and interspecific variation in stomata sensitivity to vapour pressure deficit. **Plant, Cell and Environmental**, Malden, v. 22, p. 1515-1526, 1999.
- PALMA, F.; CARVAJAL, F.; JAMILENA, M.; GARRIDO, D. Contribution of polyamines and other related metabolites to the maintenance of zucchini fruit quality during cold storage. **Plant Physiology and Biochemistry**, Bari, v. 82, p. 161-171, 2014.
- PARKHURST, D.F. Diffusion of CO₂ and other gases inside leaves. **New Phytologist**, Lancaster, v. 126, p. 449-479, 1994.
- PARKHURST, D.F.; MOTT, K.A. Intercellular diffusion limits to CO₂ uptake in leaves. **Plant Physiology**, Rockville, v. 94, p. 1024-1032, 1990.
- PIDGEON, J.D.; OBER, S.E.; QI, A.; CLARK, J.A.C.; ROYAL, A.; JAGGARD, K.W. Using multi-environment sugar beet variety traits to screen for drought tolerance. **Field Crops Research**, Bonn, v. 95, p. 268-279, 2006.

POHJONEN, V. Establishment of fuelwood plantations in Ethiopian forestry. **Forest, Ecology and Management**, Amsterdam, v. 36, p. 19-31, 1989.

QUERO, J.L.; VILLAR, R.; MARANÓN, T.; ZAMORA, R. Interactions of drought and shade effects on seedlings of four *Quercus* species: physiological and structural leaf responses. **New Phytologist**, Lancaster, v. 170, p. 819-834, 2006.

R CORE TEAM. **R: a language and environment for statistical computing**. Vienna: R Foundation for Statistical Computing, 2014. Disponível em: <<http://www.R-project.org/>>. Acesso em: 20 jun 2015.

RAGGI, V. Changes in free amino acids and osmotic adjustment in leaves of water-stressed bean. **Plant Physiology**, Davis, v. 91, p. 427-434, 1994.

ROLIN, D.; BALDET, P.; JUST, D.; CHEVALIER, C.; BIRAN, M.; RAYMOND, P. NMR study of low subcellular pH during the development of cherry tomato fruit. **Australian Journal Plant Physiology**, Clayton South, v. 27, p. 61-69, 2000.

ROSHCHINA, V.V. **Neurotransmitters in plant life**. Moscow: Science Publishers, 2001. 283 p.

SALIENDRA, N.Z.; SPERRY, J.S.; COMSTOCK, J.P. Influence of leaf water status on stomatal response to humidity, hydraulic conductance and soil drought in *Betula occidentalis*. **Planta**, Heidelberg, v. 196, p. 357-366, 1995.

SALISBURY, E.J. On the causes and ecological significance of stomatal frequency, with special reference to the woodland flora. **Philosophical Transactions of the Royal Society of London**, London, v. 216, p. 1-65, 1927.

SCHABERG, P.G.; MINOCHA, R.; LONG, S.; HALMAN, J.M.; HAWLEY, G.J.; EAGAR, C. Calcium addition at the Hubbard Brook Experimental Forest increases the capacity for stress tolerance and carbon capture in red spruce (*Picea rubens*) trees during the cold season. **Trees**, Heidelberg, v. 25, p. 1053-1061, 2011.

SCHOLANDER, P.F.; HAMMEL, H.T.; BRADSTREET E.D.; HEMMINGSEN, E.A. Sap pressure in vascular plants. **Science**, Washington, v. 48, p. 339-346, 1965.

SCHWACKE, R.; GRALLATH, S.; BREITKREUZ, K.E.; STRANSKY, E.; STRANSKY, H.; FROMMER, W.B.; RENTSCH, D. LeProT1, a transporter for proline, glycine betaine and gamma aminobutyric acid in tomato pollen. **Plant Cell**, Los Angeles, v. 11, p. 377-392, 1999.

SCHULZE, E.D.; BECK, E.; MULLER-HOHENSTEIN, K. **Plant ecology**. Berlin: Springer, 1982. 702 p.

SHANG, H.; CAO, S.; YANG, Z.; CAI, Y.; ZHENG, Y.; Effect of exogenous gamma-aminobutyric acid treatment on proline accumulation and chilling injury in peach fruit after long-term cold storage. **Journal of Agriculture and Food Chemistry**, Freising, v. 59, p. 1264-1268, 2011.

SHELP, B.J.; BOWN, A.W.; MCLEAN, M.D. Metabolism and functions of γ -aminobutyric acid. **Trends in Plants Science**, Cambridge, v. 41, p. 446-452, 1999.

SHELP, B.J.; MULLEN, R.T.; WALLER, J.C. Compartmentation of GABA metabolism raises intriguing questions. **Trends in Plant Science**, Cambridge, v. 17, n. 2, p. 57-59, 2012.

SHTEIN, I.; SHIMON, M.; RIOV, J.; PHILOSOPH-HADAS, S. Interconnection of seasonal temperature, vascular traits, leaf anatomy and hydraulic performance in cut *Dodonaea* "Dana" branches. **Postharvest Biology and Technology**, Washington, v. 61, p. 184-192, 2011.

SILVA, E.C.; NOGUEIRAL, R.J.M.C.; VALE, F.H.A.; ARAUJO, F.P.D.; PIMENTA, M.A. Stomatal changes induced by intermitente drought in four umbu tree genotyped. **Brazilian Journal of Plant Physiology**, Campos dos Goytacazes, v. 121, p. 33-42, 2009.

SLACK, E.M. Studies of stomatal distribution on the leaves of four apple varieties. **Journal of Horticultural Sciences**, Bangalore, v. 49, p. 95-103, 1974.

SOFO, A.; DICHIO, B.; XILOYANNIS, C.; MASIA, A. Effects of different irradiance levels on some antioxidant enzymes and on malondialdehyde content during rewatering in olive tree. **Plant Science**, Davis, v. 166, p. 293-302, 2004.

SOLÁROVÁ, J.; POSPISILOVÁ, J. Photosynthetic characteristics during ontogenesis of leaves. 8. Stomatal diffusive conductance and stomata reactivity. **Photosynthetica**, Dordrecht, v. 17, p. 101-151, 1983.

SPERRY, J.S.; ADLER, F.R.; CAMPBELL, G.S.; COMSTOCK J.P. Limitation of plant water use by rhizosphere and xylem conductance: results from a model. **Plant Cell and Environment**, Malden, v. 21, p. 347-359, 1998.

SNEDDEN, W.A.; ARAZI, T.; FROMM H.; SHELP, B.J. Calcium/Calmodulin activation of soybean glutamate decarboxylase. **Plant Physiology**, Davis, v. 108, p. 543-549, 1995.

STEWART, F.C.; THOMPSON, J.F.; AND DENT, C.E. γ -aminobutyric acid: a constituent of the potato tuber? **Science**, Washington, v.110, p. 439-440, 1949.

STEUDLE, E.; ZIMMERMANN, U.; LUTTGE, U. Effect of turgor pressure and cell size on the elasticity of plant cells. **Plant Physiology**, Rockville, v. 59, p. 285-289, 1977.

TERASHIMA, I. Anatomy of non-uniform leaf photosynthesis. **Photosynthesis Research**, Dordrecht, v. 31, p. 195-212, 1992.

THOMPSON, J.F.; STEWART, C.R.; MORRIS, C.J. Changes in amino acid content of excised leaves during incubation. I. The effect of water content of leaves and atmospheric oxygen level. **Plant Physiology**, Davis, v. 41, p. 1578-1582, 1996.

TURNER, N.C. Stomatal behavior and water status of maize, sorghum and tobacco under field conditions. II. At low soil water potential. **Plant Physiology**, Rockville, v. 53, p. 360-365, 1974.

UPHOF, J.C.; HUMMEL, K. Plant hairs. In: FINK, S.; ZIEGLER, H.; CUTLER, D.F.; ROTH, I. **Encyclopedia of plant anatomy**. Berlin: Gebruder Borntraeger, 1962. p. 280-292. (Band 4. Teil. 5)

VOGELMANN, T.C.; MARTIN, G. The functional significance of palisade tissue: penetration of directional versus diffuse light. **Plant, Cell and Environmental**, Malden, v. 16, p. 65-72, 1993.

WALLACE, W.; SECOR, J.; SCHRADER, L.E.; Rapid accumulation of γ -aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness or mechanical manipulation. **Plant Physiology**, Davis, v. 75, p. 170-175, 1984.

WANG, Y.; LUO, Z.; HUANG, X.; YANG, K.; GAO, S.; DU, R. Effect of exogenous γ -aminobutyric acid (GABA) treatment on chilling injury and antioxidant capacity in banana peel. **Scientia Horticulturae**, British Columbia, v. 168, p. 132-137, 2014.

WARREN, C.R.; ARANDA, I.; CANO, F.J. Responses to water stress of gas exchange and metabolites in *Eucalyptus* and *Acacia spp.* **Plant, Cell and Environment**, Malden, v. 34, p. 1609-1629, 2011.

WARREN, C.R.; ARANDA, I.; CANO, F.J. Metabolomics demonstrates divergent responses of two *Eucalyptus* species to water stress. **Metabolomics**, New York, v. 8, p. 186-200, 2012.

WARREN, C.R.; LIVINGSTON, N.J.; TURPIN, D.H. Water stress decreases the transfer conductance of Douglas-fir (*Pseudotsuga menziesii*) seedlings. **Tree Physiology**, Durham, v. 24, p. 971-979, 2004.

_____. Metabolomics demonstrates divergent responses of two *Eucalyptus* species to water stress. **Metabolomics**, New York, v. 8, p. 186-200, 2012.

WHITEHEAD, D.; BEADLE, C.L. Physiological regulation of productivity and water use in *Eucalyptus*: a review. **Forest Ecology and Management**, Amsterdam, v. 193, p. 113-140, 2004.

WOOD, S.N. **Generalized additive models**: an introduction with R. Boca Raton: Chapman and Hall; CRC Press, 2006. 410 p.

XU, Z.; ZHOU, G. Responses of leaf stomata density to water status and its relationship with photosynthesis in a grass. **Journal of Experimental Botany**, Lancaster, v. 59, p. 3317-3325, 2008.

YANG, J.; ORDIZ I.; JAWORSKI J.G.; BEACHY R.N. Induced accumulation of cuticular waxes enhances drought tolerance in Arabidopsis by changes in development of stomata. **Plant Physiology and Biochemistry**, Bari, v. 49, p. 1448-1455, 2011.

YANG, A.P.; CAO, S.F.; YANG, Z.F.; CAI, Y.T.; ZHENG, Y.H. Gamma-aminobutyric acid treatment reduces chilling injury and activates the defense response of peach fruit. **Food Chemistry**, Reading, v. 129, p. 1619-1622, 2011.

YONG, J.W.H.; WONG, S.C.; FARQUHAR, G.D. Stomatal response to changes in vapour pressure difference between leaf and air. **Plant, Cell and Environmental**, Malden, v. 20, p. 1213-1216, 1997.

YU, C.; ZENG, L.; SHENG, K.; CHEN, F.; ZHOU, T.; ZHENG, X.; YU, T. γ -aminobutyric acid induces resistance against *Penicillium expansum* by priming of defense responses in pear fruit, **Food Chemistry**, Reading, v. 159, p. 29-37, 2014.

3 XYLEM VULNERABILITY TO CAVITATION IN *Pinus flexilis*: ARE THERE DIFFERENCES BETWEEN WHITE PINE BLISTER RUST SUSCEPTIBLE VERSUS RESISTANT FAMILIES?

Abstract

The devastating impacts of the white pine blister rust disease (WPBR) and the unknown outcomes of climate change suggest that the urgency to understand the physiological characteristics of different pine families are fundamental to help on the selection of rust-resistance families. The objective of this study is to investigate the variability in cavitation and xylem anatomy of *Pinus flexilis*. We will test two hypotheses: (1) there is no difference in 50% loss of hydraulic conductivity (P50) or mean cavitation pressure (MCP) between WPBR resistant and susceptible limber pine families; (2) families from higher altitudes will be more resistant to cavitation than families from lower altitudes. We studied seedlings from six families previously shown to contain the dominant Cr4 allele (Resistant families) and six families' without the Cr4 allele (Susceptible families) were grown from seed collected at sites differing in altitude (three families from high altitude and three families from low altitude). Hydraulic conductivity of each seedling was measured using the methods of Sperry, Donnelly, 1988 and Tyree, 1989. A vulnerability curve was determined using the centrifugal force method (ALDER et al., 1997). We calculated the MCP from Weibull curve and P50 for each stem (LENS et al., 2011). Our studies reveal that although there was a significant difference in the MCP among all families of the Limber Pine (varying between -3.63 to -4.84 MPa); this was not related to WPBR. Overall, no consistent trend of vulnerability properties was observed across families when low and high altitudes were compared ($p=0.61$). Area of conduits showed a relatively narrow range (varied between 154.7 to 208 μm^2), and showed no consistent variation with altitude ($p=0.35$) and no relation with resistance to WPBR ($p=0.67$). The conduit length showed considerable variation (varied from 144.1 to 743.7 μm) and no clear pattern was found regarding variation between conduits length and altitude ($p=0.51$). We observed that rust-susceptible families presented higher length than resistant in low and high altitudes. The wall thickness showed slight variation (ranged between 3.4 and 4.6 μm) and we observed that there was no variation with altitude ($p=0.66$) and resistance to WPBR. Anatomy data of all families were pooled with MCP data and we observed no correlation between these variables. The strong directional selection pressures on native population will increase rust-resistant individuals, and our research highlights that there was a significant difference in the mean cavitation pressure among all families of the Limber Pine but this was not related to WPBR resistance and altitude origin, this indicates that rust-resistance will not affect the distribution of resulting population.

Keywords: Mean cavitation pressure; WPBR; Hydraulic conductivity; Limber pine; Anatomy xylem

3.1 Introduction

Since 1910, the introduction of the non-native fungal pathogen (*Cronartium ribicola* J.C Fisch) from Eurasia responsible for the lethal white pine blister rust disease (WPBR) has resulted in a devastating impact on North American white pine species. *Pinus flexilis* (Limber Pine) is one of the nine white pine species that are highly susceptible to WPBR. Limber pine is a long-lived tree species that has a broad range, extending from Rocky Mountains to the eastern

Sierra Nevada and eastern Oregon over a large elevational gradient ranging from 870 to 3500m (SCHOETTLE et al., 2014).

At high elevations, limber pine is a keystone species, and is often the only tree that can survive in these extreme environments. This species plays several important ecological roles, such as: being one of the first species that colonize a site after a fire, facilitating the establishment of high elevation late successional species, mediating snow capture and snowmelt, controlling erosion and providing food and habitat for diverse animals (SCHOETTLE, 2004; SCHOETTLE et al., 2014).

Some animals like Clark's nutcracker (*Nucifraga columbiana*) can help to enhance seed dispersal across the landscape because they can cache seeds many kilometers from the parent tree (VANDER WALL; BALDA, 1977). This interaction between animals and seeds dispersion of limber pine, increase the distribution of seedlings and facilitates successful establishment of this species (DONNEGEN; REBERTUS 1999). In addition, limber pine dominates dry sites because the conditions are not favorable for the growth of others species and the competition is minimal (LEPPER, 1974, SCHOETTLE; ROCHELLE, 2000).

The white pine bluster rust spores enter trees through the stomatal opening of young leaves, continues to develop between the cells of the inner bark, absorbing nutrients into the phloem cells and the hyphae can infect xylem causing cankers on the infected branch or stem killing the distal tissue (MCDONALD; HOFF, 2001). This disease also contributes to an increase in sensitivity of the tree to other abiotic (drought and climate change) and biotic (mountain pine beetle and dwarf mistletoe) which further limits limber pine's survival across its range (SCHOETTLE, 2004). Consequently, limber pine is considered a species that is seriously at risk of extinction in Alberta (ALBERTA GOVERNMENT, 2014).

Several reviews have addressed the projected impacts of climate change on forest ecosystem composition and productivity (PETERS, 1990; SAXE et al., 1998, 2001; WINNETT, 1998; HANSON; WELTZIN, 2000; KÖRNER, 2000; ABER et al., 2001; HANSEN et al., 2001; CIAIS et al., 2005; EASTERLING; APPS, 2005; BOISVENUE; MOHAN et al., 2009). Climate change will result in warmer temperatures and changed precipitation regimes, and these changes can decrease snowpack, summer evapotranspiration and increase the frequency and severity of droughts (CHMURA et al., 2011).

Since abiotic stress is a definitive agent to plants survival, the development of trees with genetic resistance is the main strategy that can provide potential success of restoration for this specie. Since the 1940s, breeding efforts are been developed to select families of white pine

species with heritable resistance to WPBR, as a potential pathway to restoring affected areas (KING et al., 2010).

Schoettle et al. (2014) identified an R gene for limber pine, named “Cr4” that confers complete resistance to WPBR. Families from trees containing this R gene had greater cold hardness and drought resistance than families without R gene, suggesting that plants with resistance to WPBR may have a different suit of stress tolerance traits (VOGAN; SCHOETTLE, 2015).

Water transport in woody plants is dependent on the hydraulic conductance of the soil to leaf pathway. Because water is pulled from soil to leaf through the plants xylem tissue, the water column is a metastable state and thus prone to cavitation if tensions exceed a specific water potential. A key physiological trait for understanding plant responses to cold and dry environments is the vulnerability of xylem tissue to cavitation. Embolism can occur in two ways: through freeze/thaw events and drought. Because limber pine grows in cold and dry environments, both mechanisms likely limit water transport in this species. Cavitation is an important parameter on the response of plants to water deficit, since it defines the plant negative pressure limit, and is determined by the quality of adhesion between the xylem wall and water (TYREE, et al. 1989). Xylem cavitation during drought has been considered one of the most causes of productivity loss in water stress conditions (PITA et al., 2003; LO GULLO et al., 2003; TOGNETTI et al., 1998).

Understanding the vulnerability of WPBR resistant and susceptible limber pine is key to successful restoration strategies especially if resistant versus susceptible families differ in their cavitation resistance.

Several studies show increasing body of evidence of variation in xylem vulnerability to cavitation among and within tree species (COCHARD 1992; TOGNETTI et al., 1998; PITA et al., 2003).

The mean of the distribution of incremental conductivity loss with xylem pressure (MCP) and the 50% loss of conductivity pressure (P50) are parameters that can be used to vulnerability of xylem tissue to cavitation of plants (HUBBARD et al.,2001; SPERRY et al., 2008; LENS et al., 2011).

Other important characteristic is the relation between cavitation resistance and xylem anatomy. Hacke et al. (2001) reported a trade-off between the resistance to drought-induced embolism and wood density and wall reinforcement, which relates the wall thickness to the span of conduits for the hydraulic mean diameter. Wood density and wall reinforcement tended

to be higher in embolism-resistant xylem of conifer twigs and the inverse tendency for the tracheid diameter (MAYR et al., 2006). However, is lacking case studies that have investigated integrative anatomical-physiological works (LENS et al., 2011).

It is unclear if selection for rust resistance will result in the loss of some physiological traits from these species, we do not know if accelerating the establishment of white pine blister rust resistant genotypes across the landscape can affect the conservation of the genetic diversity of this specie. This way, analyses of vulnerability of xylem cavitation related with anatomical parameters of rust-resistance and rust-susceptible families can provide important insights that can help our ability to rapidly develop and implement conservation programs.

The objective of this study is to investigate the variability in cavitation and xylem anatomy of *Pinus flexilis* families with different resistance to WPBR and from regions with different elevations. We tested two hypotheses about these patterns: (1) there is no difference in P50 or MCP between WPBR resistant and susceptible limber pine families; (2) families from higher altitudes will be more resistant to cavitation than families from lower altitudes.

3.2 Material and Methods

3.2.1 Plant material and seed sources

Seedlings from six families previously shown to contain the dominant Cr4 allele (Resistant families) and six families' without the Cr4 allele (Susceptible families) were grown from seed collected at sites differing in altitude (three families from high altitude and three families from low altitude) were selected for this study. Low elevations varied between 2450 to 2691m and high elevations were between 3289 to 3300m (Table 1).

Table 1 - Families phenotypes (resistance to rust) of limber pine and geographical characteristics of origin sites

Site ID	Family phenotype	Elevation	Latitude	Longitude	Precipitation	Mean Temperature
– # –	– # –	– m –	– ° –	– ° –	– mm –	– °C –
21	Resistant	High	40,2945	105,571	610	-0,2
313	Resistant	High	39,9337	-105,659	628	-0,8
147	Resistant	High			630	-0,6
301	Resistant	Low	40,9694	-105,528	369	4,2
37	Resistant	Low			350	3,0
321	Resistant	Low	41,2678	-105,434	348	2,9
105	Susceptible	High	40,3983	-105,669	602	-0,5
112	Susceptible	High	40,0085	-105,569	586	0,9
145	Susceptible	High			600	-0,3
31	Susceptible	Low	40,3304	-105,4081	507	3,9
38	Susceptible	Low			505	4,0
126	Susceptible	Low	40,3384	-105,618	517	2,5

Seeds were cold stratified for 60 days, and then sown into plastic boxes in a growth chamber (25°C, 100% RH) in April, 2010 at USDA Forest Service Dorena Genetic Resource Center (DGRC; Cotage Grove, OR). Upon radicle emergence, seedlings were transplanted into Ray Leach cone-tainers (164cm³; Stuewe and Sons, Inc. Tangent, OR) and moved to the greenhouse. In early spring 2012, seedlings were transplanted to 2310 cm³ “Shot One” treepots (Stuewe and Sons, Tangent, OR) and moved outside at DGRC.

In March 2014, seedlings were sent to a greenhouse at Colorado State University and plants were irrigated regularly every day. We selected five limber pine seedlings with similar branching patterns from each family. The seedlings were between 15 and 20 cm tall and with 4 years old.

3.2.2 Resin Removal

As limber pine seedlings have resin ducts in their stem tissue that can inhibit flow from cut stems, we established the following protocol to eliminate influences of the resin on our measurements. We cut all seedlings one day before measurements, left them overnight on the bench inside of plastics bags with wet paper towels to prevent dehydration. The next day, we shaved off a small amount of tissue from cut ends to remove any accumulated resin, and spun

the stem segment on a centrifuge rotor for sixteen minutes at a speed that corresponding - 0.25MPa (POCKMAN et al., 1995). The experimental protocol to remove resin out of the stems is summarized in Figure 1. Tests on two separate families (5 reps each) revealed that this method successfully removed excess resin and did not influence maximum hydraulic conductance (Figure 2).

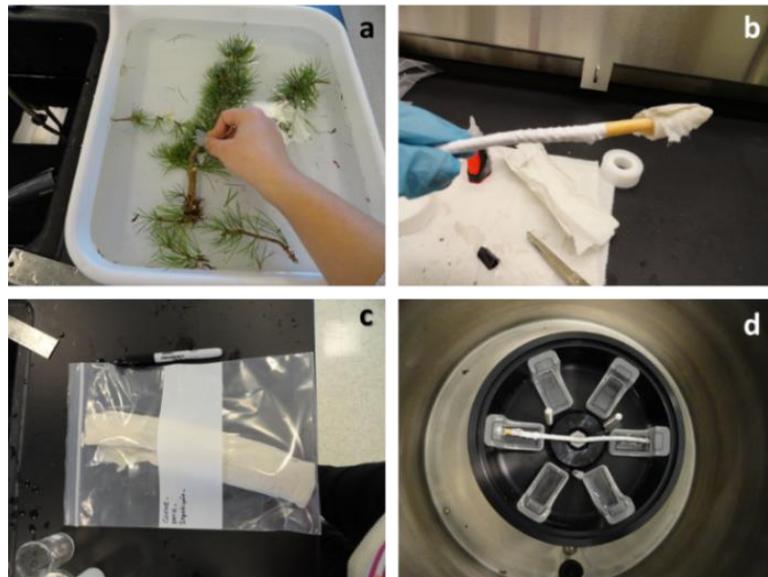


Figure 1 - Cutting the stem underwater and removing lateral branches and needles (a), sealing the cut surfaces using superglue and tape to prevent leaks (b), segment on the bench overnight inside of plastics bags and covered with wet paper (c) and spinning the segment centered on a centrifuge rotor (d)

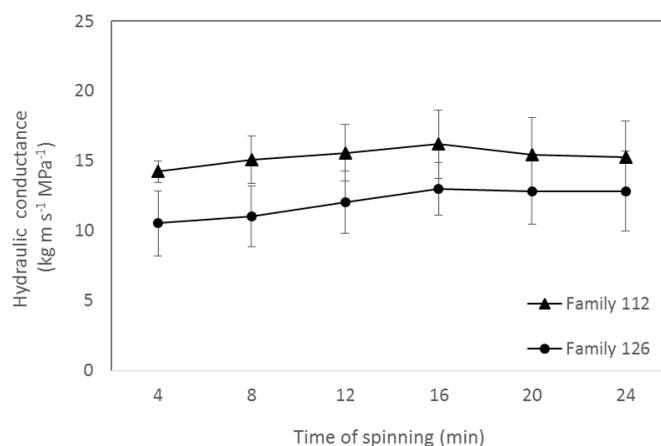


Figure 2 - Effects of time of spinning on the hydraulic conductance of two families of *Pinus flexilis* (average of five repetitions per family) to remove the stem resin. The hydraulic conductance increased until 16 minutes, an evidence of the positive effect of the rotation to remove the resin stem, after this period the hydraulic conductance started to decrease, indicating the beginning of the negative effect of the rotation to the stem

3.2.3 Hydraulic conductivity

Hydraulic conductivity of each seedling was measured using the methods of Sperry, Donnelly, 1988 and Tyree, 1989. Plants stems were cut under water at the root collar, close to the soil to relieve tension in the xylem and a 140 mm segment was removed from the seedlings to measure hydraulic conductivity (WHEELER et al., 2013; MARTORELL et al., 2014). Lateral branches, leaves and all bark of the segment were removed, the cut surfaces was sealed using superglue (Loctite; Henkel North America, Rocky Hill, CT, USA) and tape (TaegaSeal PTFE Tape, USA) to prevent leaks.

Before each measurement, we flushed each stem to remove any native embolism applying 100kPa (Figure 3).

The initial hydraulic conductance (k_i) was measured using degassed, purified water, filtered to 18.2 megahm-cm (Thermo Scientific, Barnstead E-Pure). Flow was estimated using a 10^{-5} resolution balance (Sartorius, LE225D).

We measured the diameter for the stem tops (two measurements for each top), using a caliper to calculate stem cross area. We calculated hydraulic conductance (K_L) as:

$$K_L = F/\Delta P/A$$

where: F is the net flow rate, ΔP is the pressure gradient (measured through the ruler) and A is the stem area (Figure 3).

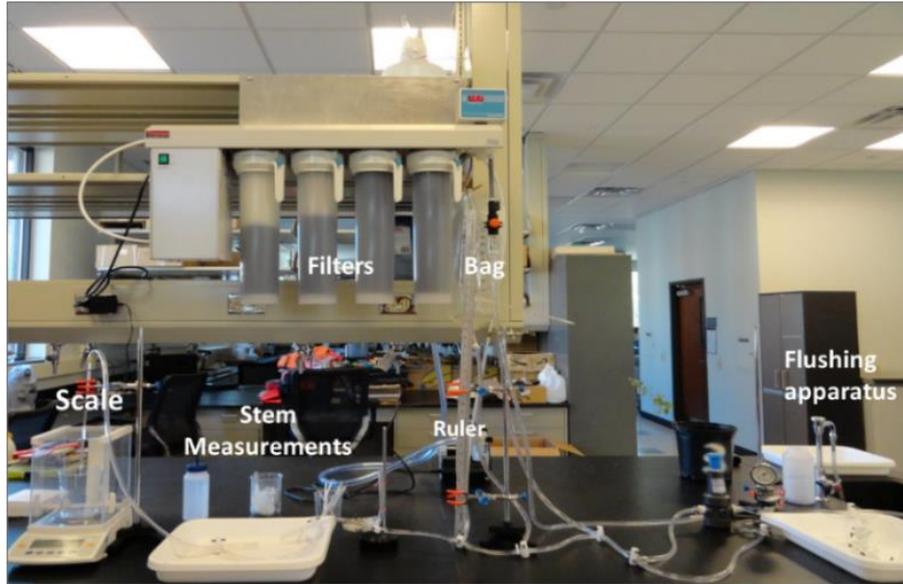


Figure 3 - Hydraulic conductivity apparatus

3.2.4 Vulnerability to cavitation

A vulnerability curve was determined using the centrifugal force method (ALDER et al., 1997). The segment was centered on a centrifuge rotor (Du Pont Instruments, Sorvall RC-5B) and spun during 4 minutes along its long axis. Spin rates corresponded to xylem pressures beginning at -0,25 MPa and were increased by 0.5 MPa increments until complete cavitation.

We fitted a function with the relationship between percentage loss of hydraulic conductivity (k_{loss}) and xylem pressure (Ψ_{xylem}) to determine the vulnerability curve, which show the decrease in hydraulic conductivity with increasing negative xylem pressure (Figure 4). We calculated the mean cavitation pressure (MCP) for each stem from Weibull curve and the xylem pressure corresponding 50% loss of hydraulic conductivity (P_{50}) (LENS et al., 2011).

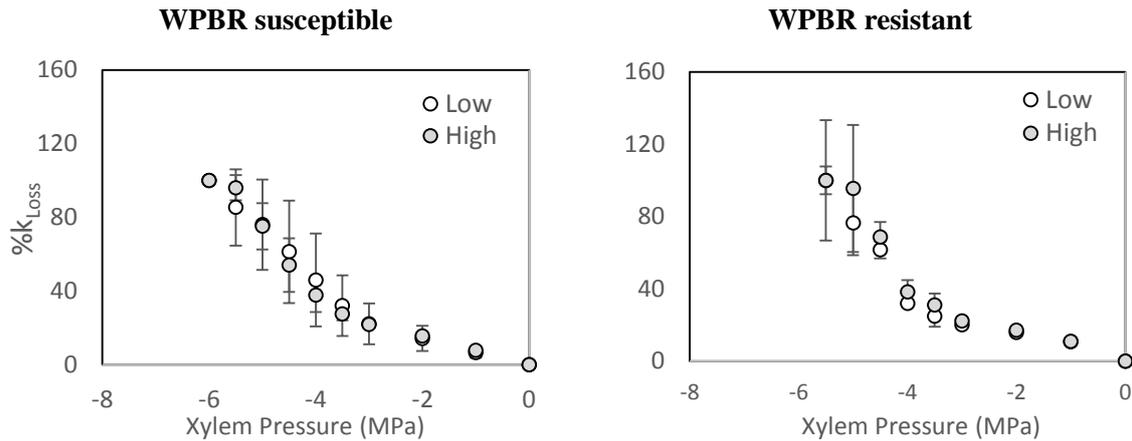


Figure 4 - Vulnerability curve for stems of 12 families of *Pinus flexilis* seedlings showing mean percentage loss of stem hydraulic conductivity (% k_{loss}) versus xylem pressure to WPBR susceptible and resistant at low and high altitude (n=60)

3.2.5 Anatomical measurements

Small tissue samples of stem (2 cm of length) were sectioned, fixed in Karnovsky solution and stored refrigerated at 4 °C (KARNOVSKY, 1965). Subsequently, stem samples were sectioned in longitudinal and transversal direction in manual sliding microtome (Leica SM2000R). Histological sections were colored with Astra Blue (1%) and Basic Fuchsin (0.0125%) using the protocol described by Roeser (1972). In addition, solution of Glycerin (50%) was used to hydrate and to make histological slides.

Transverse sections were used to measure individual conduit areas (A_c) and thickness of the vessels wall (T_w) and longitudinal sections were used to measure vessel element length (L_s). We evaluated recent growth rings, incorporating just the late-wood, a total of 75 conduits per family (5 stems x 3 photographic x 5 measurements) were analyzed using a light microscope (ZEISS®-JENEMED2) interfaced with a digital camera (Premiere® MA88-300). All images were analyzed using the ImageJ (1.46r, 2012) software.

3.2.6 Data analysis

The Weibull function was fitted to our vulnerability curve data to describe relationship between K_{Loss} and ψ_{xylem} . The described function is given as the equation $\%k_{\text{Loss}} = 100 - 100 \times \exp(-(-P_{\text{total}}/b))^c$, where b and c are constants generated by the curve fitting procedure and P_{total} is the pressure difference causing cavitation (SPERRY et al., 1997).

Analysis of variance was performed to assess the effects of resistance, altitude and its interaction over the stress (MCP and P50) and the anatomical variables (length, area and thickness). As no significant effect was found for the stress variables, we used the F test ($p < 0.10$) to compare families, followed by Tukey's multiple comparison test at 5% level of significance (package ExpDes).

The same was done for area, length and thickness. We also tested the significance of Pearson's correlation between anatomical variables against MCP. All analysis were performed using the software R (R CORE TEAM, 2014).

3.3 Results

3.3.1 Mean cavitation pressure

Although there was a significant difference in the mean cavitation pressure among all families of the Limber Pine, this was not related to WPBR resistance. The families studied showed a range of MCP varying between -3.63 to -4.84 MPa. The highest resistance to drought-induced embolism was found in 38 family (MCP at -4.84MPa) and it was statically similar with 301, 112, 37, 145 and 21 families. Families 105, 313, 147 and 321 showed an intermediate resistance to cavitation (MCP ranged between -3.92 and 3.82MPa). The most susceptible to drought-induced were found in 131 and 126 families, with -3.63 and -3.64MPa of MCP respectively. MCP averaged 2.9% more negative than P50 and the two measures were highly correlated ($r^2 = 0.91$; Table 2)

Overall, no consistent trend of vulnerability properties was observed across families when low and high altitudes were compared ($p=0.613$). A comparison between families from high and low-altitudes revealed lowest resistance to drought-induced at the low altitude in four families (31, 126, 321 and 37). In the others families from low altitude (301 and 38), the trend was reversed and more negative were found at lower altitude.

No clear pattern was found regarding variation of MCP with rust-resistance ($p=0.464$). In four out of six families (31, 126, 105, and 145) that are susceptible to WPBR, MCP showed lower resistance to drought-induced, and it was statistically similar with five out of six resistant families (321, 147, 313, 21 and 37).

Table 2 - Mean cavitation pressure (MCP), 50% loss of hydraulic conductivity (P_{50}) and correlation between MCP and P_{50} (r^2) in 12 families of *Pinus flexilis* with differences in resistant to white pine blister rust and altitude origin region

Families	Resistance to WPBR	Altitude	MCP	P_{50}	r^2
— # —	— # —	— # —	— % —	— % —	— # —
31	Susceptible	Low	-3.63 a	-3.63 a	0.95
126	Susceptible	Low	-3.64 a	-3.67 a	0.90
321	Resistant	Low	-3.82 ab	-3.72 a	0.92
147	Resistant	High	-3.85 ab	-3.84 a	0.84
313	Resistant	High	-3.91 abc	-3.96 ab	0.93
105	Susceptible	High	-3.92 abc	-4.01 ab	0.95
21	Resistant	High	-4.09 abcd	-4.18 abc	0.93
145	Susceptible	High	-4.10 abcd	-4.15 abc	0.93
37	Resistant	Low	-4.17 abcd	-4.18 abc	0.91
112	Susceptible	High	-4.56 bcd	-4.44 abc	0.85
301	Resistant	Low	-4.66 cd	-4.73 bc	0.92
38	Susceptible	Low	-4.84 d	-4.92 c	0.94
Average			-4.14	-4.02	0.91
$P_{\text{resistance}}$			0.613	0.488	
P_{altitude}			0.464	0.608	
$P_{\text{resistance} \times \text{elevation}}$			0.166	0.334	

*P is the probability calculated by Test F, when $P < 0,1$ that are significant difference

3.3.2 Xylem anatomy

Area of conduits showed a relatively narrow range (varied between 154.7 to 208 μm^2 and average of 181.1 μm^2), and showed no consistent variation with elevation ($p=0.355$) and no relation with resistance to WPBR ($p=0.673$).

The conduit length showed considerable variation (varied from 144.1 to 743.7 μm and average of 378.6 μm) and no clear pattern was found regarding variation between conduits length and altitude ($p=0.51$). We observed an effect of resistance to WPBR in conduit length ($p=0.07$) and generally rust-susceptible families presented higher length than resistant in low and high altitudes (Figure 5).

The wall thickness showed slight variation (ranged between 3.4 and 4.6 μm , with average of 4.0 μm) and we observed that there was no variation with altitude ($p=0.666$) and resistance to WPBR ($p=0.360$).

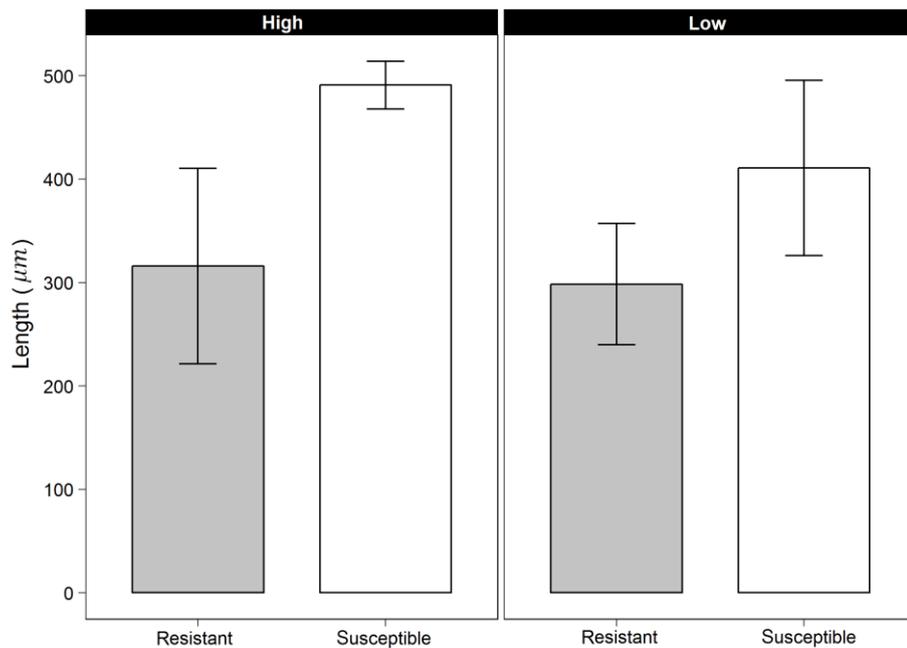


Figure 5 - Length of conduits to families of rust-resistant and rust-susceptible to white pine blister rust from low and high altitude regions

3.3.3 Correlations between xylem anatomy and MCP

Anatomy data of all families were pooled with MCP data, which did not have spanned a much broader range in embolism resistance (-3.63 to -4.84MPa). For the resistant families, area of conduits was significantly negative correlated ($r = -0.98$) with MCP, no correlation between length and MCP ($r=-0.12$) was founded and there was a marginally positive tendency, but not significant, correlation between thickness and MCP ($r=0.28$).

For the susceptible families we found no correlation between MCP and area ($r=0.04$), negative correlation (not significant) between MCP and length ($r=-0.52$) and positive correlation (not significant) between thickness and MCP ($r=0.57$) (Figure 6).

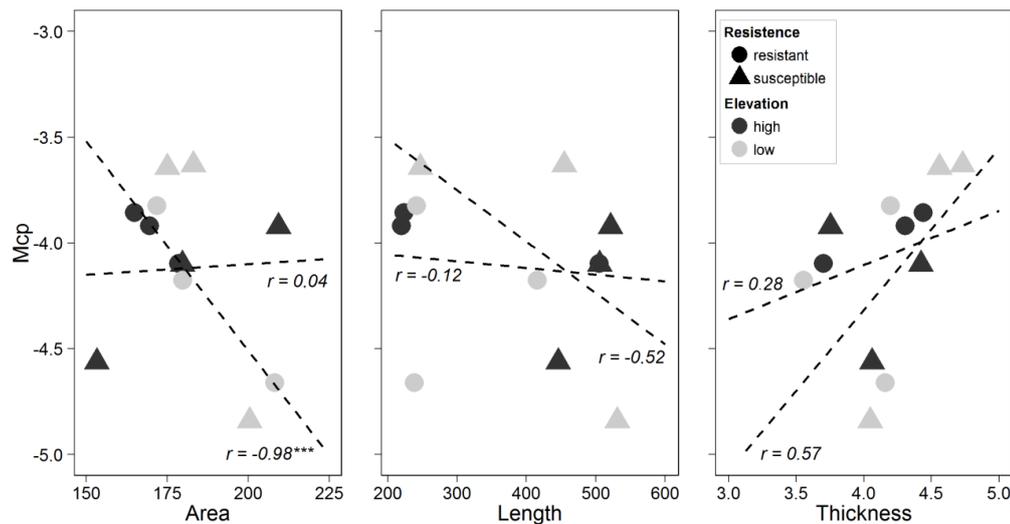


Figure 6 - Correlation between anatomy variables (area, length and thickness of conduits) and mean cavitation pressure (MCP) of *Pinus flexilis*

3.4 Discussion

The devastating impacts of the WPBR and the unknown outcomes of climate change suggest that the urgency to understand these ecosystems is high, and the key to successful restoration is facilitate the increase in rust-resistance families on the landscape, whether it is through natural selection or planting rust-resistance pine seedlings. Therefore, to understand the physiological characteristics of different pine families are fundamental to help on the selection of genetic material and to recommend where they need to be planted on the landscape (KEANE; SCHOETTLE, 2011).

Studies of physiological properties related to water stress, anatomical parameters and embolism vulnerability can provide important insights about adaptations to support water transport through xylem over long distances. Xylem vulnerabilities of our study families were similar to values published by others authors with *Pinus* species with a P50 from -2.3 to -7.0 MPa (HACKE et al., 2004; MARTINEZ-VILALTA et al., 2004; PITTERMANN et al., 2006; MAYR et al., 2006).

Here we report that there was a significant difference in the mean cavitation pressure among all families of the Limber Pine, but it was not related to WPBR resistance.

Four of the six families in the present study showed a less negative MCP at a low altitude, but the other two families from low altitude were characterized by particularly drought-resistant xylem (Table 2). Some studies dealing with vulnerability embolism in conifers from upper elevation and low altitude revealed higher resistance to embolism in species from the

lower altitude (SPARKS; BLACK, 1999; MAYR et al., 2006). In contrast, Mayr et al. (2002) showed that *Picea abies* had a consistent increase in resistance to drought-induced embolism with increasing altitude, and Maherali and DeLucia (2000) found no difference between embolism vulnerability comparing desert and montane population of *Pinus ponderosa*.

Our results indicate that there is not a large variation (-3.63 to -4.84MPa of MCP) in vulnerability to embolism in *Pinus flexilis* shoots, and families from high elevations showed a similar or even greater xylem vulnerability than low altitude families. Despite extreme cold conditions founded at high altitude, that although present higher total precipitation (604 mm) than low altitude regions (449 mm), the mean temperature annual average is very low (0.2°C) compared to low altitude regions (3.3°C) (Table 2). Mayr et al. (2006) studying three species of *Pinus* observed the same occurrence too, no consistent trend of vulnerability properties was observed across species from low and high altitudes were compared.

This apparent discrepancy can be explained, because besides vulnerability properties to cavitation, plants have different physiological and ecological strategies that can be used in stress situations. For example, by changing root:shoot ratios (FAY et al., 2003); altering specific leaf area (WRIGHT et al., 2002); adjusting permeability of the cell membranes (JAVOT; MAUREL, 2002); by controlling stomatal conductance (MASEDA; FERNANDEZ, 2006); by changing stem anatomy (VON ARX, et al., 2012).

Plants that survive in high elevations often are protected from frost-drought (and freeze-thaw events) by the snow pack, once snow is a very good insulator against chilling temperatures (MAYR et al., 2003), which can persist during the whole winter season at the high elevations but not a low altitude. Trees may increase their energetic or structural investments to cope with increased stress factors at high altitude. Generally, *Pinus flexilis* tends to exhibit a conservative strategy of growth, stomatal behavior and resource-use (PATAKI et al., 2000; LETTS et al., 2009), as well as a high drought sensitivity across a range of elevations and vegetation cover (MOYES et al., 2013).

Roots are generally more vulnerable to cavitation than shoots in conifers (SPERRY; IKEDA, 1997; KAVANAGH et al., 1999) and may influence water transport in different ways than shoots. Vulnerability to cavitation may be a more phenotypically plastic trait in roots than in shoots (SPERRY; IKEDA, 1997), raising the possibility that roots from high-altitude trees were more resistant to xylem cavitation than roots from low-altitude trees.

Possible correlations between disease resistance and plant tolerance to abiotic stress have been shown in other species (STHULTZ et al., 2009), for example, some proteins that are involved in resistance to *C. ribicola* are known to be expressed in plants in response to abiotic

stress (DAVIDSON; EKRAMODDOULLAH, 1997; EKRAMODDOULLAH; TAN, 1998). Vogan and Schoettle (2015) observed that rust-resistant families present higher cold tolerance than susceptible families and the presence of Cr4 gene in limber pine can be strongly related with cold tolerance.

However, this relation between biotic resistance and plant tolerance to abiotic stress is complex because other studies have been found different results. Stultz et al. (2009), for example, showed that under drought conditions, resistant seedlings of *Pinus edulis* to the shoot-boring moth (*Dioryctria albobittella*) died sooner than seedlings from susceptible mothers. The authors suggest that there is a metabolic cost associated with herbivore resistance because seedlings reallocated resources from stem growth to defense against herbivore attack (STEVENS et al., 2007).

In our study we did not find a significant relationship between MCP and rust-resistance or rust-susceptible families (Table 2). These results are in agreement with Vogan and Schoettle (2015) who showed no significant difference in drought induced damage between rust-resistant and rust-susceptible of six families of *Pinus flexilis*. However, they found that resistant families presented lower stomatal conductance compared to rust-susceptible under drought conditions.

Regarding the variation between anatomy and rust-resistance and elevation, we just found significant higher conduits length in rust-susceptible families compared to rust-resistance families (Figure 4).

Generally, mechanical safety is a clear component of this constraint and it is more important for angiosperms because they rely on fibres for providing wood strength, whereas conifer tracheids function in both transport and support. In this element of the efficiency versus safety tradeoff, conifer xylem is superior to angiosperm xylem (SPERRY et al., 2008).

Some previous studies have shown a relationship between anatomy characteristics and resistance to drought embolism (HACKE et al., 2001, 2004; MAYR et al., 2002; WOO et al., 2001, 2004; PITTERMAN et al., 2006; MAYR et al., 2006; SPERRY et al., 2008; VON ARX et al., 2012).

One possible explanation for our not observing any relationship between anatomical characteristics and MCP, is because in this study we found only a small variation of MCP values (-3.63 to -4.84MPa), such that all families studied presented a relatively high resistance to cavitation. Other studies have found a larger range of MCP values. For example in *Pinus ponderosa*, P_{50} ranged from -2.6 to -3.6Mpa (MAHERALI et al., 1999), for two resistant species of genus *Acer*, the MCP varied between 3.33 and 3.06 MPa (LENS et al., 2011), in a study

comparing different species of angiosperms, the most resistant species showed maximum values of MCP -3.4Mpa (SPERRY et al., 2007).

Other characteristic that we need to consider is that although there is a genetic component to vessel-size distribution in most species (CARLQUIST, 2001; CHRISTENSEN-DALSGAARD et al., 2008), phenotypic plasticity in this trait would enable individuals and species to dynamically adjust to the wide range climate conditions (VON ARX et al., 2012). In this study, we evaluated the anatomical characteristics of seedlings grown under the same climatic conditions. Further study of stem anatomical variables of these families grown in their natural environment might generate information about phenotypic plasticity acquired for these genetic materials to adapt to the environment during their lifetimes.

With the continued spread of WPBR, extensive mortality will occur in trees without genetic resistance to this disease. Our results show that genetic variation related with rust-resistance is not related with tolerance to drought embolism, and this occurrence could be important in determining the response of a plant species to climate change and to recommend these different rust-resistant families to be planted in different regions.

As was presented in previous studies (SCHOETTLE et al., 2014; VOGAN; SCHOETTLE, 2015), the inclusion of genetic-based resistance to WPBR can play an important role in determining tree survival to the disease. Studies examining physiological strategies, like resistant to drought embolism, is an important tool that can be used to complementary the strategies of the genotype selection, and avoid the increasing of mortality events during a series of drought episodes such as that reported in limber pine from 1985 to 1995 in Sierra Nevada (MILLAR et al., 2007).

The strong directional selection pressures on native population will increase rust-resistant individuals, and our research highlights that there was a significant difference in the mean cavitation pressure among all families of the Limber Pine, but this was not related to WPBR resistance and altitude origin, this indicates that rust-resistance will not affect the distribution of resulting population.

References

ABER, J.; NEILSON, R.P.; MCNULTY, S.; LENIHAN, J.M.; BACHEL, N.D.; DRAPEK, R.J. Forest processes and global environmental change: predicting the effects of individual and multiple stressors, **BioScience**, Oxford, v. 51, p. 735-751, 2001.

ALDER, N.N.; POCKMAN, W.T.; SPERRY, J.S.; NUISMER, S. Use of centrifugal force in the study of xylem cavitation. **Journal of Experimental Botany**, Lancaster, v. 48, p. 665-674, 1997.

BOISVENUE, C.; RUNNING, S.W. Impacts of climate change on natural forest productivity - evidence since the middle of the 20th century. **Global Change Biology**, Malden, v. 12, p. 862-882, 2006.

CARLQUIST, S. **Comparative wood anatomy systematic, ecological and evolutionary aspects of dicotyledon wood**. Berlin: Springer, 2001. 448 p.

CHMURA, D.J.; ANDERSON, P.D.; HOWE, G.T.; HARRINGTON, C.A.; HALOFSKY, J.E.; PETERSON, D.L.; SHAW, D.C.; CLAIR, J.B.S. Forest responses to climate change in the northwestern United States: ecophysiological foundations for adaptive management. **Forest, Ecology and Management**, Amsterdam, v. 261, p. 1121-1142, 2011.

CHRISTENSEN-DALSGAARD, K.K.; ENNOS, A.R.; FOURNIER, M. Are radial changes in vascular anatomy mechanically induced or an ageing process? Evidence from observations on buttressed tree root systems. **Trees**, Heidelberg, v. 22, p. 543-550, 2008.

CIAIS, P.; REICHSTEIN, M.; VIOVY, N.; GRANIER, A.; OGEE, J.; ALLARD, V.; AUBINET, M.; BUCHMANN, N.; BERNHOFER, C.; CARRARA, A.; CHEVALLIER, F.; DE NOBLET, N.; FRIEND, A.D.; FRIEDLINGSTEIN, P.; GRUNWALD, T.; HEINESCH, B.; KERONEN, P.; KNOHL, A.; KRINNER, G.; LOUSTAU, D.; MANCA, G.; MATTEUCCI, G.; MIGLIETTA, F.; OURCIVAL, J.M.; PAPALE, D.; PILEGAARD, K.; RAMBAL, S.; SEUFERT, G.; SOUSSANA, J.F.; SANZ, M.J. Europe-wide reduction in primary productivity caused by the heat and drought in 2003. **Nature**, London, v. 437, n. 7058, p. 529-533, 2005.

COCHARD, H.; CRUIZIAT, P.; TYREE, M.T. Use of positive pressures to establish vulnerability curves: further support for the air-seeding hypothesis and implications for pressure-volume analysis. **Plant Physiology**, Davis, v. 100, p. 205-209, 1992.

DAVIDSON, J.J.; EKRAMODDOULLAH, A.K.M. Analysis of bark protein in blister rust-resistant and susceptible western white pine (*Pinus monticola*). **Tree Physiology**, Durham, v. 17, p. 663-669, 1997.

DONNEGEN, J.A.; REBERTUN, A.J. Rates and mechanisms of subalpine forest succession along an environmental gradient. **Ecology**, Ithaca, v. 80, p. 1370-1384, 1999.

EASTERLING, W.; APPS, M. Assessing the consequences of climate change for food and forest resources: a view from the IPCC. **Climatic Change**, Dordrecht, v. 70, p. 165-189, 2005.

EKRAMODDOULLAH, A.K.M.; TAN, Y. Differential accumulation of proteins in resistant and susceptible sugar pine (*Pinus lambertiana*) seedlings inoculated with white pine blister rust fungus (*Cronartium ribicola*). **Canadian Journal Plant Pathology**, Burnaby, v. 20, p. 308-318, 1998.

FAY, P.A.; CARLISLE, J.D.; KNAPP, A.K.; BLAIR, J.M.; COLLINS, S.L. Productivity responses to altered rainfall patterns in a C-4-dominated grassland. **Oecologia**, Buenos Aires, v. 137, p. 245-251, 2003.

GOVERNMENT OF ALBERTA. **Species assessed by Alberta's endangered species conservation committee**: short list on line. <<http://esrd.alberta.ca/fish-wildlife/species-at-risk/documents/SpeciesAssessed-Endangered-Jul18-2014.pdf>>. Acesso em: 25 jun. 2015.

HACKE, U.G.; SPERRY, J.S.; PITTERMANN, J. Analysis of circular bordered pit function II. Gymnosperm tracheids with torus-margo pit membranes. **American Journal of Botany**, St. Louis, v. 91, p. 386-400, 2004.

HACKE, U.G.; SPERRY, J.S.; POCKMAN, W.P.; DAVIS, S.D; MCCULLOH, K.A; Trends in wood density and structure are linked to prevention of xylem implosion by negative pressure. **Oecologia**, Buenos Aires, v. 126, p. 457-461, 2001.

HANSEN, A.J.; NEILSON, R.R.; DALE, V.H.; FLATHER, CH.; IVERSON, L.R.; CURRIE, D.J.; SHAFER, S.; COOK, R.; BARTLEIN, P.J. Global change in forests: responses of species, communities, and biomes. **BioScience**, Oxford, v. 51, p. 765-779, 2001.

HANSON, P.J.; WELTZIN, J.F. Drought disturbance from climate change: response of United States forests. **Science of the Total Environment**, Barcelona, v. 262, p. 205-220, 2000.

HUBBARD, R.M.; RYAN, M.G.; STILLER V.; SPERRY, J.S. Stomatal conductance and photosynthesis vary linearly with plant hydraulic conductance in ponderosa pine. **Plant, Cell and Environment**, Malden, v. 24, p. 113-121, 2001.

JAVOT, H.; MAUREL, C. The role of aquaporins in root water uptake. **Annals of Botany**, Oxford, v. 90, p. 301-313, 2002.

KARNOVSKY, M.J.A. A formaldehyde – glutaraldehyde fixative of high osmolality for use in electron microscopy. **Journal of Cell Biology**, New York, v. 27, n. 2, p. 137-138, 1965.

KAVANAGH, K.L.; BOND, B.J.; AITKEN, S.N.; GARTNER, B.L.; KNOWE, S. Shoot and root vulnerability to xylem cavitation in four populations of Douglas-fir seedlings. **Tree Physiology**, Durham, v. 19, p. 31-37, 1999.

KEANE, R.E.; SCHOETLE, A.W. Strategies, tools, and challenges for sustaining and restoring high elevation five-needle white pine forests in western North America. In: KEANE, R.E.; TOMBACK, D.F.; MURRAY, M.P.; SMITH, C.M. (Ed.). **The future of high-elevation, five-needle white pines in western North America**. Fort Collins: United States Station, 2011. p. 276-294.

KING, J.N.; DAVID, A.; NOSHAD, D.; SMITH, J. A review of genetic approaches to the management of blister rust in white pines. **Forest Pathology**, Malden, v. 40, p. 292-313, 2010.

KORNER, C. Biosphere responses to CO₂ enrichment. **Ecological Applications**, Ithaca, v. 10, p. 1590-1619, 2000.

LO GULLO, M.A.; SALLEO, S. Different vulnerabilities of *Quercus ilex L.* to freeze and summer drought-induced xylem embolism: an ecological interpretation. **Plant, Cell and Environment**, Malden, v. 16, p. 511-519, 1993.

LEPPER, M.G. *Pinus flexilis* James and its environmental relationships. 1974. 197 p. Thesis (Ph.D) - University of California, Davis, 1974.

LETTS, M.G.; NAKONECHNY, K.N.; VAN GAALEN, K.E.; SMITH, C.M. Physiological acclimation of *Pinus flexilis* to drought stress on contrasting slope aspects in Waterton Lakes National Park, Alberta, Canada. **Canadian Journal Forest Research**, Ottawa, v. 39, p. 629-641, 2009.

LENS, F.; SPERRY, J.S.; CHRISTMAN, M.A.; CHOAT, B.; RABAEY, D.; JANSEN, S. Testing hypothesis that link wood anatomy to cavitation resistance and hydraulic conductivity in the genus *Acer*. **New Phytologist**, Lancaster, v. 190, p. 709-723, 2011.

MAHERALI, H. **Water relations of ponderosa pine in contrasting environments: implications for global climate change**. 1999. 146 p. Thesis (Ph.D) - University of Illinois, Urbana, 1999.

MASEDA, P.H.; FERNANDEZ, R.J. Stay wet or else: three ways in which plants can adjust hydraulically to their environment. **Journal of Experimental Botany**, Oxford, v. 57, p. 3963–3977, 2006.

MARTINEZ-VILALTA, J.; SALA, A.; PINOL, J. The hydraulic architecture of Pinaceae: a review. **Plant Ecology**, Berlin, v. 171, p. 3-13, 2004.

MARTORELL, S.; DIAZ-ESPEJO, A.; MEDRANO, H.; BALL, M.C.; CHOAT, B.; Rapid hydraulic recovery in *Eucalyptus pauciflora* after drought: linkages between stem hydraulics and leaf gas exchange. **Plant, Cell and Environment**, Malden, v. 37, p. 617-626, 2014.

MAYR, S; WOLFSCHWENGER, M; BAUER, H. Winter-drought induced embolism in Norway spruce (*Picea abies*) at the Alpine timberline. **Physiologia Plantarum**, Malden, v. 115, p. 74-80, 2002.

MAYR, S.; GRUBER, A.; SCHWIENBACHER, S.; DAMON, B. Winter embolism in a “krummholz” shrub (*Pinus mugo*) growing at the alpine timberline. **Austrian Journal of Forest Science**, Vienna, v. 120, p. 29-38, 2003.

MAYR, S.; HACKE, U.; SCHMID, P.; SCHWIENBACHER, F.; GRUBER, A. Frost drought in conifers at the alpine timberline: xylem dysfunction and adaptations. **Ecology**, Ithaca, v. 87, n. 12, p. 3175-3185, 2006.

MCDONALD, G.I.; HOFF, R.J. Blister rust: an introduced plaque. In: TOMBACK, D.F.; ARNO, S.F.; KEANE, R.E. (Ed.). **White pine communities**. Washington: Island Press, 2001. p. 193-220.

MILLAR, C.I.; WESTFALL, E.D.; DELANY, D.L. Response of high-elevation limber pine (*Pinus flexilis*) to multiyear drought and 20th-century warming, Sierra Nevada, California, USA. **Canadian Journal of Forest Research**, Ottawa, v. 37, p. 2508-2520, 2007.

MOHAN, J.E.; COX, R.M.; IVERSON, L.R. Composition and carbon dynamics of forests in northeastern North America in a future, warmer world. **Canadian Journal of Forest Research**, Ottawa, v. 39, p. 213-230, 2009.

MOYES, A.B.; CASTANHA, C.; GERMINO, M.J.; KUEPPERS, L.M. Warming and the dependence of limber pine (*Pinus flexilis*) establishment on summer soil moisture within and above its current elevation range. **Oecologia**, Buenos Aires, v. 171, p. 271-283, 2013.

PATAKI, D.E.; OREN, R.; SMITH, W.K. Sap flux of co-occurring species in a western subalpine forest during seasonal soil drought. **Ecology**, Ithaca, v. 81, p. 2557-2566, 2000.

PETERS, R.L. Effects of global warming on forests. **Forest Ecology and Management**, Amsterdam, v. 35, p. 13-33, 1990.

PITA, P.; GASCO, A.; PARDOS J.A. Xylem cavitation, leaf growth and leaf water potential in *Eucalyptus globulus* clones under well-watered and drought conditions. **Functional Plant Biology**, Clayton South, v. 30, p. 891-199, 2003.

PITTERMANN, J.; SPERRY, J.S. Analysis of freeze-thaw embolism in conifers. The interaction between cavitation pressure and tracheid size. **Plant Physiology**, Davis, v. 140, p. 374-382, 2006.

POCKMAN, W.; SPERRY, J.S.; O'LEARY, J.W. Sustained and significant negative water pressure in xylem. **Nature**, London, v. 378, p. 715-716, 1995.

SAXE, H.; ELLSWORTH, D.S.; HEATH, J. Tree and forest functioning in an enriched CO₂ atmosphere. **New Phytologist**, Lancaster, v. 139, p. 395-436, 1998.

SCHLARBAUM, S.E.; KRIEBEL, H.B. (Ed.). **Breeding and genetic resources of five-needle pines: growth, adaptability and pest resistance**. Fort Collins: USDA, Forest Service, Rocky Mountain Research Station, 2004. 276 p.

SCHOETTLE, A.E.; ROCHELLE, S.G.; Morphological variation of *Pinus flexilis* (Pinaceae), a bird-dispersed pine, across a range of elevation. **American Journal of Botany**, St. Louis, v. 87, p. 1797-1806, 2000.

SCHOETTLE, A.W. Ecological roles of five-needle pines in Colorado: potential consequences of their loss. In: SNIEZKO, R.A.; SAMMAN, S.; SCHLARBAUM, S.E.; KRIEBEL, H.B. (Ed.). **Breeding and genetic resources of five-needle pines: growth, adaptability and pest resistance**. Fort Collins: USDA, Forest Service, Rocky Mountain Research Station, 2004. p. 124-135.

SCHOETTLE, A.W.; SNIEZKO, R.A.; KEGLEY, A.; BURNS, K.S. White pine blister rust resistance in limber pine: evidence for a major gene. **Phytopathology**, St. Paul, v. 104, n. 2, p. 163-173, 2014.

SCHULZE, E.D.; VESALA, T.; VALENTINI, R. Europe-wide reduction in primary productivity caused by the heat and drought in 2003. **Nature**, London, v. 437, p. 529-533, 2005.

SPARKS, J.P.; BLACK R.A. Regulation of water loss in populations of *Populus trichocarpa*: the role of stomatal control in preventing xylem cavitation. **Tree Physiology**, Durham, v. 19, p. 453-459, 1999.

- SPERRY, J.S.; IKEDA, T. Xylem cavitation in roots and stems of Douglas-fir and white fir. **Tree Physiology**, Durham, v. 17, p. 275-280, 1997.
- SPERRY, J.S.; TYREE, M.T. Mechanism of water stress-induced xylem embolism. **Plant Physiology**, Davis, v. 88, p. 581-587, 1988.
- SPERRY, J.S.; MEINZER, F.C.; MCCULLOH, K.A.; Safety and efficiency conflicts in hydraulic architecture: scaling from tissue to trees. **Plant, Cell and Environment**, Malden, v. 31, p. 632-645, 2008.
- SPERRY, J.S.; HACKE, U.G.; FIELD, T.S.; SANO, Y.; SIKKEMA, E.H. Hydraulic consequences of vessel evolution in angiosperms. **International Journal of Plant Science**, Chicago, v. 168, n. 8, p. 1127-1139, 2007.
- STEVENS, M.T.; WALLER, D.M.; LINDROTH, R.L. Resistance and tolerance in *Populus tremuloides*: genetic variation, costs, and environmental dependency. **Evolutionary Ecology**, Dordrecht, v. 21, p. 829-847, 2007.
- STHULTZ, C.M.; GEHRING, C.A.; WHITHAM, T.G. Deadly combination of genes and drought: increased mortality of herbivore-resistant trees in a foundation species. **Global Change Biology**, Malden, v. 15, p. 1949-1961, 2009.
- TOGNETTI, R.; LONGOBUCCO, A.; RASCHI, A.; Vulnerability of xylem to embolism in relation to plant hydraulic resistance in *Quercus pubescens* and *Quercus ilex* co-occurring in a Mediterranean coppice stand in central Italy. **New Phytologist**, Lancaster, v. 139, p. 437-447, 1998.
- TYREE, M.Y.; SPERRY, J.S. Vulnerability of xylem to cavitation and embolism. **Annual Review of Plant Physiology and Molecular Biology**, Palo Alto, v. 40, p. 19-38, 1989.
- VALDER WALL, S.B.; BALDA, R.P. Coadaptation of the Clark's nutcracker and the pinon pine for efficient seed harvest and dispersal. **Ecology Monographs**, Ithaca, v. 47, p. 89-111, 1977.
- VOGAN, P.J.; SCHOETTLE, A.W. Selection for resistance to white pine bluster rust affects the abiotic stress tolerances of limber pine. **Forest Ecology and Management**, Amsterdam, v. 344, p. 110-119, 2015.
- VON ARX, G.; ARCHER, S.R.; HUGHES, M.K. Long-term functional plasticity in plant hydraulic architecture in response to supplemental moisture. **Annals of Botany**, Oxford, v. 109, p. 1091-1100, 2012.
- WHEELER, J.K.; HUGGET, B.A.; TOFTE, A.N.; ROCKWELL, F.E.; HOLBROOK, M. Cutting xylem under tension or supersaturated with gas can generate PLC and the appearance of rapid recovery from embolism. **Plant, Cell and Environment**, Malden, v. 36, n. 11, p. 1938-1949, 2013.
- WINNETT, S.M. Potential effects of climate change on US forests: a review. **Climate Research**, Oldendorf, v. 11, p. 39-49, 1998.

WOO, K.S.; MCDONALD, G.I.; FINS, L. USDA Forest Proceedings. In. SNIEZKO, R.A., SAMMAN, S. Influence of seedling physiology on expression of blister rust resistance in needles of western white pine. Fort Collins: USDA, Forest Service, Rocky Mountain Research Station, 2004. 32p.

WOO, K.S.; FINS, L.; MCDONALD, G.I.; WIESE, M.V. Differences in needle morphology between blister rust resistant and susceptible western white pine stocks. **Canadian Journal Forest Research**, Ottawa, v. 31, p. 1880-1886, 2001.

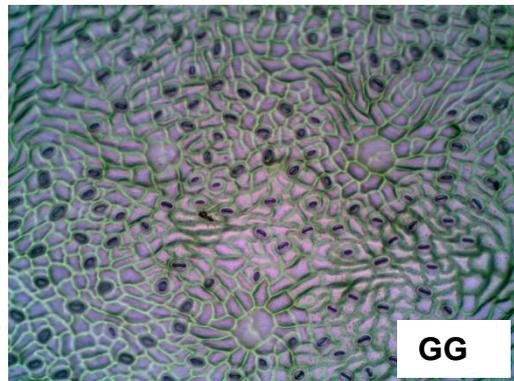
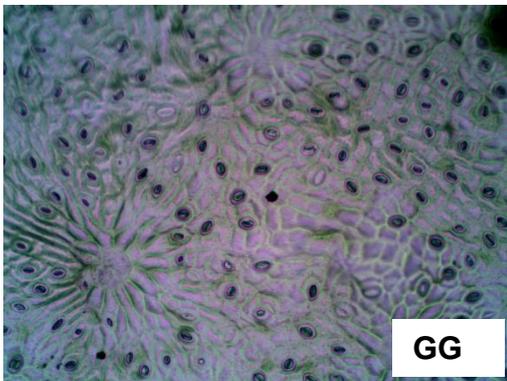
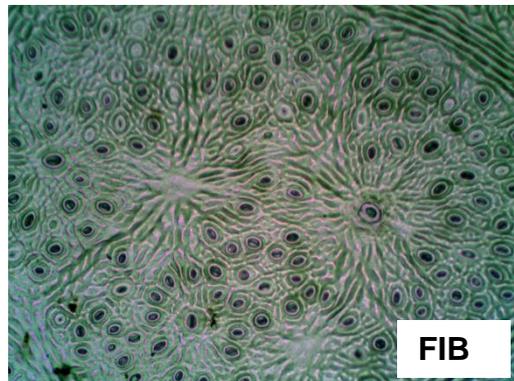
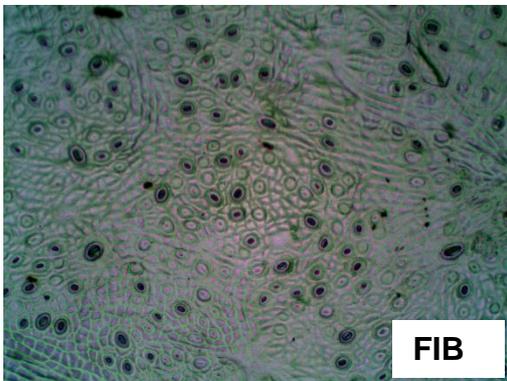
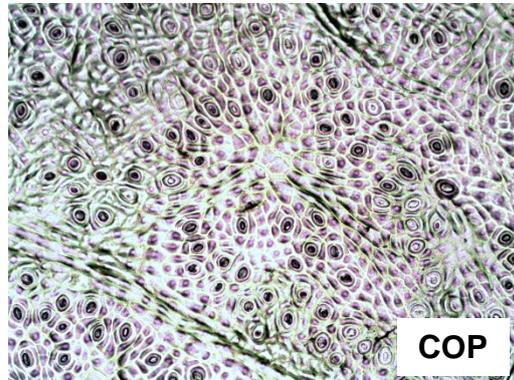
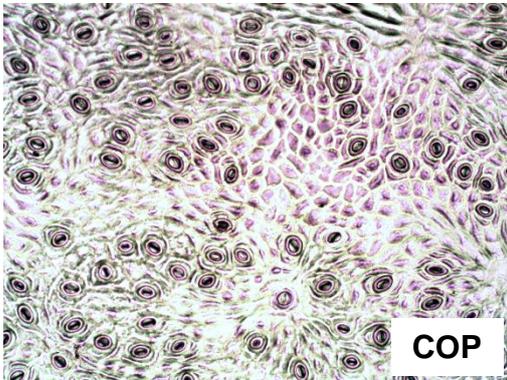
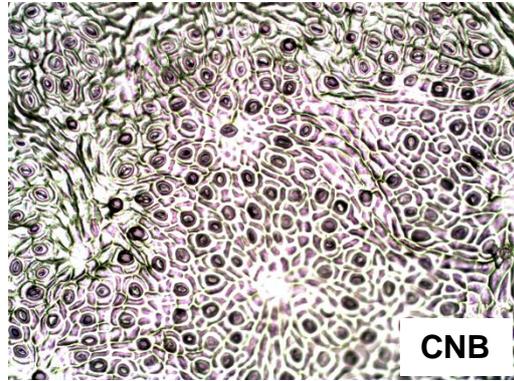
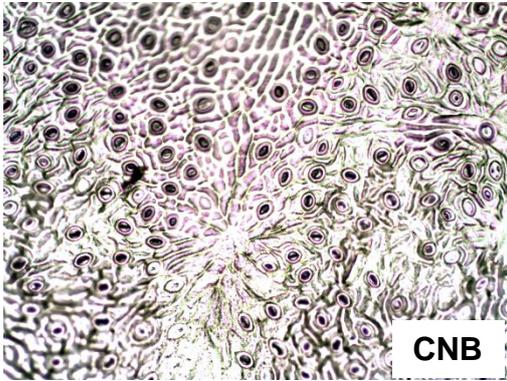
WRIGHT, I.J.; WESTOBY, M.; REICH, P.B. Convergence towards higher leaf mass per area in dry and nutrient-poor habitats has different consequences for leaf life span. **Journal of Ecology**, London, v. 90, p. 534–543, 2002.

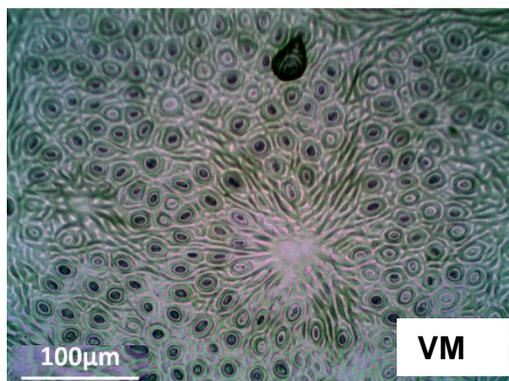
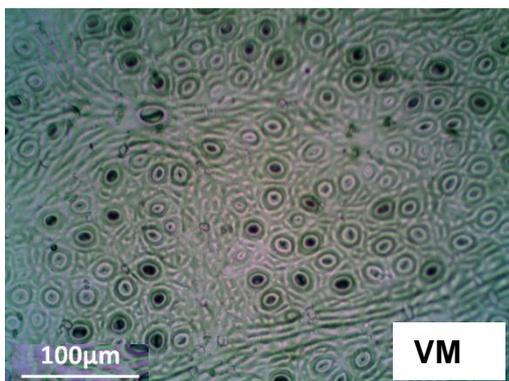
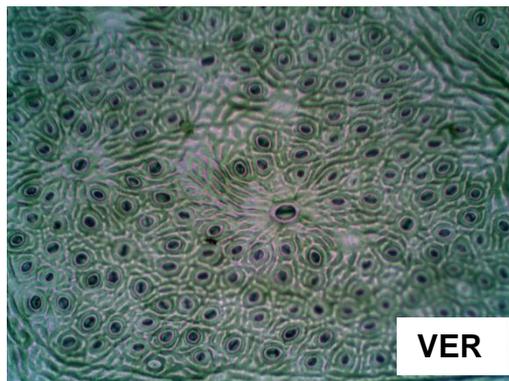
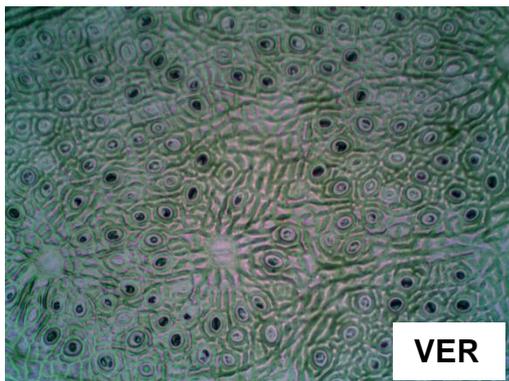
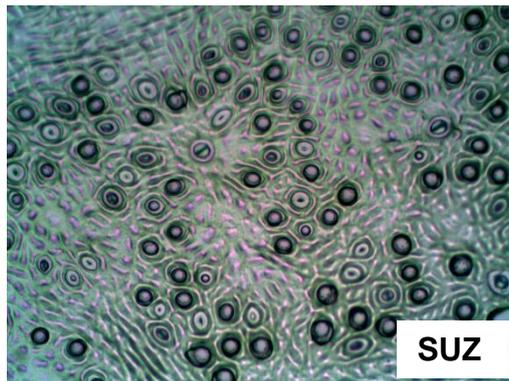
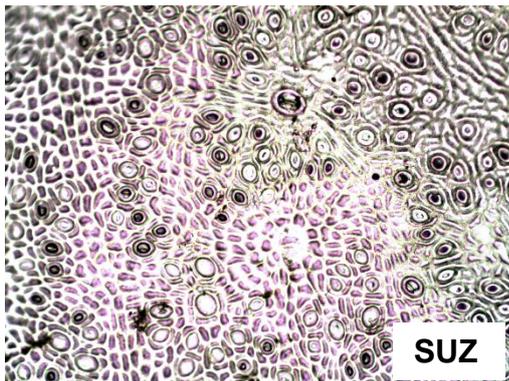
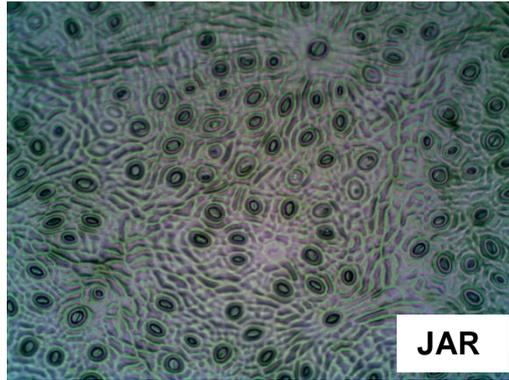
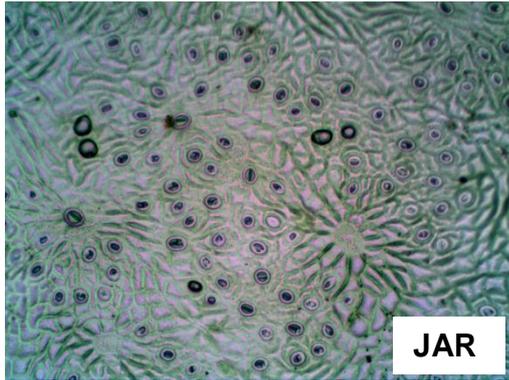
APPENDICES

Appendix A

Leaf before stress

Leaf after stress





Appendix B

Water stress treatment



