

UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR

DE INGENIEROS DE MONTES

**RESPUESTAS ANATÓMICO FISIOLÓGICAS
FRENTE A ESTRÉS HÍDRICO EN
PLANTACIONES DE ESPECIES DE
CRECIMIENTO RÁPIDO PARA LA
PRODUCCIÓN DE BIOMASA.**

TESIS DOCTORAL

MARÍA JOSÉ HERNÁNDEZ GARASA

Ingeniera de Montes

Madrid, 2015

DEPARTAMENTO DE SILVOPASCICULTURA
ESCUELA TÉCNICA SUPERIOR DE INGENIEROS DE MONTES
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AUTOR: MARÍA JOSÉ HERNÁNDEZ GARASA

Ingeniera de Montes

DIRECTORA: PILAR PITA ANDREU

Dr Ingeniero de Montes

Madrid, noviembre de 2015

Tribunal nombrado por el Mgfco. Y Excmo. Sr. Rector de la
Universidad Politécnica de Madrid, el día

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Realizado el acto de defensa y lectura de la Tesis el día

.....de.....de 2015

Calificación.....

El Presidente

Los Vocales

El Secretario

A FERNANDO

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RESUMEN

El objetivo general de la presente Tesis es identificar algunas de las características anatómico-fisiológicas que confieren la capacidad de alcanzar una mejor productividad bajo clima mediterráneo a plantas de diversos genotipos de los géneros *Populus* y *Eucalyptus*, caracterizados por su carácter pionero, elevado crecimiento y vulnerabilidad a la cavitación.

En los dos primeros capítulos se hace un seguimiento de la conductancia estomática a una selección de clones de eucalipto cultivados en invernadero, sometidos a diferentes dotaciones hídricas. Se realizaron además mediciones periódicas del pH de la savia del tallo y de la pérdida de conductividad hidráulica para investigar su implicación en la regulación química e hidráulica del cierre estomático. Las variaciones en el pH de la savia obtenidas parecen responder a cambios en el déficit de presión de vapor de agua atmosférico y no a diferencias en la disponibilidad de agua en el suelo. La conductancia estomática presentó una correlación positiva significativa con el pH de la savia, pero no con la conductividad hidráulica. La variabilidad de la conductividad hidráulica máxima se discute a la luz de recientes investigaciones sobre los materiales constituyentes de las membranas de las punteaduras.

Los clones que mostraron mayores conductancias hidráulicas y estomáticas presentaron valores más altos de producción y supervivencia, poniendo de manifiesto la utilidad del estudio de estas variables. Por el contrario, los valores más bajos de conductancia estomática e hidráulica se encontraron en clones que han resultado un fracaso en plantaciones comerciales, en particular, fue destacable el mal resultado de un clon procedente de autocruzamiento respecto de otros híbridos.

En el tercer capítulo de la tesis se estudian características anatómicas y funcionales del xilema relacionadas con la eficiencia en el transporte de agua a las hojas, y que pueden afectar directa o indirectamente a la transpiración y al crecimiento. Los estudios anatómicos fueron realizados sobre brotes anuales de chopo en una plantación situada en Granada, en condiciones de riego limitante. La combinación de rasgos anatómicos más favorable de cara a la producción de biomasa fue una densidad alta de vasos de diámetro intermedio. Los clones más productivos figuraron entre los más resistentes a la cavitación.

Para estudiar el crecimiento de masas arboladas se utilizan frecuentemente parámetros fisiológicos como el índice de área foliar (LAI). La estimación del LAI a partir de fotografías hemisféricas aplicada a tallares de chopo plantados a alta densidad y a turno corto para biomasa se lleva a cabo mediante una metodología reciente empleada y discutida en el cuarto capítulo de la Tesis. Los resultados muestran que las diferencias de producción existentes entre genotipos, localidades de medición con diferentes dosis de riego, y años, pueden predecirse a partir de la determinación del índice de área foliar tanto por métodos directos como indirectos de estimación.

Tanto los estudios realizados en eucalipto como en chopo han mostrado que los genotipos con menores producciones de biomasa en campo alcanzaron los menores valores de conductancia estomática en las condiciones más favorables así como el menor número de vasos en el xilema. La estrecha relación entre crecimiento y LAI confirma una vez más la importancia del desarrollo de la copa para sostener un buen crecimiento. El mayor desarrollo de la copa y rendimiento en biomasa se midieron en uno de los clones con un número de vasos más elevado, y menor vulnerabilidad a la cavitación en condiciones de estrés. Estos resultados ponen de manifiesto la importancia de las características anatómicas y funcionales del xilema como condicionantes del patrón de crecimiento de las plantas y el comportamiento de los estomas.

ABSTRACT

A number of anatomical xylem traits and physiological variables were analyzed in genotypes of both the *Populus* and *Eucalyptus* genera with the main aim of identifying traits in the genotypes which confer the ability to produce an acceptable biomass yield under Mediterranean climatic conditions.

In the first two chapters of this PhD, the results of two experiments carried out on several clones of the species *Eucalyptus globulus* Labill. are presented. Chapters three and four include the results of another two trials on four poplar hybrid genotypes.

One of the initial plant responses to water stress is stomatal closure, which can be triggered by hydraulic and/or chemical signals. The two first chapters of this PhD deal with trials in which stomatal conductance and percentage loss of hydraulic conductivity were monitored on a set of eucalyptus clones supplied by ENCE (former National Cellulose Company) and currently used in the company's own commercial plantings. The experimental trials were carried out in greenhouses and the plants were submitted to two different watering regimes. The pH of the stem sap was periodically measured as the greenhouse temperature and humidity changed. The aim of these measurements was to investigate the role of both sap pH and percentage loss of hydraulic conductivity on stomatal regulation. The results obtained suggest that changes in sap pH are a response to vapor pressure deficit changes rather than to differences in soil water availability. We found significant correlation between stomatal conductance and sap pH, although no significant relationship was found between stomatal conductance and hydraulic conductivity. Variability in maximum hydraulic conductivity is discussed based on recent pit membrane constituent research.

The study of hydraulic conductivity proved helpful in order to detect the clones with both higher growth and greater chance of survival, since clones displaying the lowest hydraulic conductivities were those that failed in commercial plantings.

Anatomical xylem traits define the water transport efficiency to leaves and can therefore limit transpiration and growth. The third chapter of this PhD addresses anatomical xylem traits in poplar. One year old stem samples were taken from a water-stressed trial in Granada. The anatomical xylem study proved useful for detecting the lowest yielding genotypes. Clones with intermediate vessel size and high vessel densities were found to be those with the highest biomass yield. Differences in cavitation resistance depending on the clone tested and the water treatment applied were also found. The clones with the highest biomass yield were found to be among the most cavitation resistant clones in each watering regime.

Xylem and physiological traits along with stomatal behavior are useful tools to determine plant growth. In order to study plantings or forests, it is more common to employ other physiological variables such as leaf area index (LAI). LAI estimation from hemispherical photographs applied to short rotation woody crops is a recently developed method that still requires fine tuning through further investigation. In the fourth chapter, data from LAI monitoring over two consecutive years were analyzed in two different locations where different irrigation treatments were applied. The results showed that differences in yield between genotypes, different irrigation regimes and years could be predicted by using the LAI estimates, either through direct or indirect estimation methods.

Our studies of poplar and eucalyptus have shown that the field-grown genotypes with the lowest biomass yield displayed the lowest values of stomatal conductance under the most favorable environmental conditions and also had a low number of xylem conduits. The close relationship between LAI and growth highlights the importance of crown development in

biomass growth. The highest LAI and biomass yield were recorded in one of the clones with higher vessel density and the lowest vulnerability to cavitation under stress conditions. These results underline the importance of research into anatomical and functional traits as factors influencing plant growth patterns and stomatal behavior.

INTRODUCCIÓN



INTRODUCCIÓN

De los aproximadamente 15 millones de metros cúbicos de madera que se cortan anualmente en España, el 68% se obtiene de plantaciones forestales intensivas de crecimiento rápido, principalmente localizadas en el Norte de España, que posee un clima atlántico, suave y lluvioso, favorable para la producción forestal. Parte de esas plantaciones forestales de crecimiento rápido se encuentran en clima mediterráneo como son las plantaciones de eucalipto situadas en el Suroeste de España, las choperas situadas en las vegas de toda la Península o los tallares de chopo a turno corto con fines energéticos, experimentales en la actualidad y cultivados en regadío. *Eucalyptus globulus* es la frondosa que produce el mayor volumen anual de madera en España, seguida por los cultivos de híbridos de chopo (AEF 2013).

La Directiva 2009/28/CE del Parlamento Europeo y del Consejo de Europa relativa al fomento del uso de energía procedente de fuentes renovables establece objetivos mínimos vinculantes para el conjunto de la Unión Europea y para cada uno de los Estados miembros. Concretamente, la directiva establece como objetivo conseguir una cuota mínima del 20% de energía procedente de fuentes renovables en el consumo final bruto de energía de la Unión Europea, y el mismo objetivo se ha establecido para España. Esta responsabilidad ha revitalizado la investigación en materia de plantaciones de alta densidad y turno corto, que se realiza en España principalmente con híbridos de chopo y ha conducido al cambio de uso de algunas de las plantaciones existentes de *Eucalyptus globulus* Labill que han pasado a destinar sus productos a centrales eléctricas. La principal característica de las plantaciones de crecimiento rápido con fines energéticos estriba en los bajos requerimientos de calidad exigidos a la madera.

Las plantaciones de crecimiento rápido localizadas en ambientes mediterráneos, debido a la irregularidad inherente a este clima, están sometidas a un ambiente cambiante, a fuertes dosis de irradiancia solar coincidentes con la temporada de sequía, altas temperaturas y a otros tipos de estrés entre los que el más importante en nuestras latitudes es la falta de agua, la sequía.

La sequía es uno de los factores que más inciden en la disminución de la producción de todos los cultivos en el mundo (UNFCCC 94). Las plantas presentan diferentes estrategias para afrontar la sequía (Larcher 95, Lewitt 80): desde las que eluden la sequía muriendo y dejando sus semillas para que germinen en la siguiente estación hasta las que la soportan (resistentes), como es el caso de los géneros y especies con los que tratamos en este trabajo: chopos y eucaliptos.

En condiciones de estrés hídrico el suministro hídrico es menor o/y el déficit de presión de vapor atmosférico es mayor, y las plantas presentan adaptaciones a la sequía orientadas a controlar el gasto de agua, como disminuir la superficie foliar, y con ella el crecimiento, aumentar la eficiencia en el uso del agua, para que al abrir los estomas, se pierda la menor cantidad posible de vapor de agua por molécula de CO₂ absorbida por la planta, modificar la densidad estomática (David et al. 2005), incluso tirar las hojas para evitar la transpiración, o incrementar la proporción de biomasa radical (absorbe agua) frente a biomasa aérea (pierde agua).

El agua en la planta se mueve siguiendo un gradiente de potencial hídrico. Para modelizar el transporte de agua se utiliza frecuentemente una analogía a la ley de Ohm o a la ley de Darcy o de Fick (Meinzer 2002, Vilagrosa et al. 2012, Noblin et al. 2007), pues todas ellas relacionan un flujo con un gradiente, ya sea de potencial hídrico, de energía potencial o de concentración. En la ley de Ohm: $V=IR^{-1}$, V es la diferencia de potencial eléctrico, R es la resistencia al paso de la corriente e I es la intensidad de corriente, y para emplearla como modelo de transporte de agua en las plantas se considera que V es el gradiente de potencial hídrico ($\Delta\Psi$) entre el suelo y la atmósfera que rodea a las hojas, R es la resistencia al paso del flujo de savia desde el suelo hasta las hojas, cuya inversa es la conductancia hidráulica (kh) y por último I se asimila al flujo de agua transportado desde el suelo hasta las hojas donde es transpirado en su mayor parte (95%) (Kramer and Boyer 1995), de modo que la ley puede expresarse como:

$$\text{FLUJO DE SAVIA}=\Delta\Psi*Kh \quad (\text{eq 1})$$

Del mismo modo, el flujo de agua que difunde del mesófilo cuando se abren los estomas, es decir, la transpiración, se puede modelizar como un flujo proporcional al déficit de presión de vapor atmosférico:

$$\text{TRANSPIRACIÓN}=g_w*GPV \quad (\text{eq2})$$

Donde g_w es la conductancia al vapor de agua, que engloba a las conductancias estomáticas del mesófilo y de la capa límite; aunque en situación de estomas abiertos la conductancia estomática adquiere la mayor importancia. Por último GPV es el gradiente de presión de vapor entre el mesófilo y la atmósfera.

La teoría de la cohesión-tensión de Dixon, aunque controvertida, es actualmente la teoría más aceptada para explicar el movimiento de agua en las plantas. Según ella, las fuerzas de cohesión entre moléculas de agua y de adhesión a las paredes celulares hacen que cuando una molécula de agua sale del mesófilo de la hoja, arrastre a otras moléculas unidas a ella. De este modo se puede considerar que el agua forma un continuo desde la atmósfera hasta el suelo, y el agua transpirada crea una succión en el xilema que se propaga hasta el suelo. Podría hacerse la aproximación de que el 95% del agua absorbida por la planta es devuelta a la atmósfera en forma de vapor de agua mediante la transpiración. Por lo tanto, y sin tener en cuenta el agua que pasa a formar parte del cuerpo de la planta y la que se acumula en ella, en estado estacionario podrían igualarse las dos ecuaciones anteriores, expresando previamente la ecuación uno por unidad de área foliar:

$$\Delta\Psi*LSC= g_w*GPV \quad (\text{eq 3})$$

Donde LSC es la conductancia específica referida al área foliar:

Aunque los modelos son simplificaciones de la realidad, podría esperarse en sistemas sencillos como es el caso de las plantas pequeñas, una coordinación entre la conductancia estomática y la conductancia hidráulica.

La conductancia estomática, es la inversa de la resistencia que oponen los estomas a la difusión de gases. Un oportuno cierre estomático permite disminuir el flujo de agua que sale de

la planta, disminuyendo la transpiración. Es una de las variables clave a estudiar cuando se analizan las respuestas de las plantas frente al estrés. La apertura estomática permite la captación del CO₂, y la refrigeración de la hoja cuando las temperaturas son altas. La diferencia en la presión de vapor de agua existente entre el interior y exterior de la planta determina la máxima tasa de transpiración. Transpiración y disponibilidad de agua en el suelo a su vez determinan la magnitud del potencial hídrico, que si es muy negativo puede dar lugar a la ruptura de la columna de agua y a la formación de embolismos que interrumpen el transporte de la savia, y que si son muy numerosos pueden comprometer el suministro de agua dentro de la planta. La existencia de un control estomático que permita cerrar estomas antes de que se produzca un nivel de embolismo que colapse el xilema es fundamental para la supervivencia de las plantas.

Los estomas responden a diferentes estímulos: luz, concentración de CO₂, a mayor o menor contenido de humedad en el suelo o en la atmósfera, a veces a través de posibles señales químicas, como cambios en el pH de la savia en el xilema, cambios en la concentración de ácido abscísico, (ABA), implicado en el cierre estomático (Israelsson et al. 2006) y a veces mediante señales hidráulicas (descenso del potencial hídrico, pérdida de conductividad hidráulica) (Tombesi et al. 2015, Holtä et al. 2012). La utilización de cultivos split pot, en los que la raíz de una planta quedaba dividida en varios volúmenes de suelo sometidos a diferentes contenidos de humedad, fortaleció la idea de la existencia de señales químicas, pues tras someter a una de las partes de la raíz a estrés hídrico, se producía cierre estomático en las hojas de la planta sin que ésta experimentara una caída importante en el potencial hídrico (Blackman & Davies 1985, Comstock 2001). En la savia que fluye por el xilema se han medido incrementos en la concentración de ácido abscísico (ABA) producido en raíces cuando las plantas se someten a estrés, y se han propuesto mecanismos para explicar la relación entre el incremento de ABA en la savia y el cierre estomático, basados en el papel que el ácido abscísico tiene sobre el tráfico de iones en el transporte de solutos hacia las células de guarda y su cierre (Schroeder et al. 2001). El estudio de las señales implicadas en el cierre estomático puede conducirnos a detectar diferencias clonales en la sensibilidad a las mismas que pueda suponer una ventaja para la supervivencia en ambientes cambiantes.

La respuesta estomática de las plantas frente al estrés hídrico dista mucho de ser homogénea. Mientras algunas plantas cierran estomas muy rápidamente ante pequeños decrementos del potencial hídrico, y no sufren oscilaciones importantes del mismo (plantas isohídricas), hay otras que no cierran estomas hasta haber disminuido notablemente su potencial hídrico (anisohídricas). Las especies utilizadas en esta tesis pertenecen a ambos grupos: *Eucalyptus globulus* muestra un comportamiento anisohídrico, y de entre los híbridos empleados del género *Populus*, I-214 es considerado como isohídrico (Tardieu & Simmoneau 98), si bien, esta clasificación es relativa e incluso dentro de una misma especie se pueden encontrar comportamientos diferentes en lo referente a la respuesta estomática. Damour et al. (2010) en un artículo de revisión de modelos de conductancia estomática afirman que la sensibilidad de la respuesta estomática al ácido abscísico depende del comportamiento isohídrico o anisohídrico de la especie. En plantas isohídricas la sensibilidad es inversamente proporcional al potencial hídrico y en plantas anisohídricas la respuesta estomática está regulada fundamentalmente por el ácido abscísico (Tardieu et al. 1996).

En el segundo capítulo de la tesis se estudia el pH de la savia como posible señal de cierre estomático en cinco clones de eucalipto

Cuando la sequía pasa de ser un fenómeno esporádico a convertirse en crónico, puede que el control estomático no sea suficiente para evitar el colapso del xilema y aparezcan respuestas adicionales. Numerosos estudios demuestran una disminución de la conductancia hidráulica del xilema en condiciones de déficit hídrico (Lauri et al. 2014, Pangle et al. 2015, Anderegg et al. 2014). Algunos autores han sugerido que esta respuesta puede contribuir a un uso más gradual del agua en el suelo (Eamus et al. 2000, Hutley et al. 2001, Do et al. 2008). La planta puede incrementar la resistencia al paso del agua incrementando el número de ramas o de brotes, haciéndose arbustiva, acortando la longitud de los vasos, impermeabilizando las punteaduras, o disminuyendo el diámetro de los vasos, de modo que se incremente la pérdida de carga en los mismos durante el transporte. Estos cambios anatómicos suelen ir acompañados de una disminución del tamaño de las hojas y del crecimiento, y no son reversibles; suponen una vez finalizado el periodo de limitación del recurso, una limitación al crecimiento durante el tiempo necesario para crear nuevo tejido conductor adaptado a las nuevas circunstancias. Estas posibles respuestas al estrés se traducen generalmente en disminuciones de la vulnerabilidad a la cavitación. (Awad et al. 2012, Zolfaghar et al. 2015)

La vulnerabilidad a la cavitación es otro de los parámetros más ampliamente utilizados para estudiar la capacidad de las plantas de resistir situaciones de estrés. Cuando la diferencia en el gradiente de presión de vapor existente entre la atmósfera y la planta es muy elevado, se alcanzan potenciales hídricos muy negativos en el xilema que pueden dar lugar a la rotura de las columnas de savia en el interior de los vasos xilemáticos. Si la planta no responde, no cierra estomas o la respuesta no es suficiente y se mantienen presiones muy negativas en el xilema en una superficie conductora cada vez menor, el embolismo puede propagarse por la planta a través de las punteaduras.

La vulnerabilidad a la cavitación es una medida de la facilidad del xilema para embolizarse y suele expresarse como la tensión xilemática a la que la planta pierde el 50% de su conductividad máxima por entrada de aire en el xilema. Es una característica que cambia en función del medio en que la planta crece, es una de las características que hacen que las plantas se aclimaten a cambios en el ambiente. Pammenter (1998) o Zolfaghar (2015) en eucalipto y Awad (2010) en chopo entre otros muchos trabajos han mostrado cómo las plantas de estas especies sometidas a estrés incrementan su resistencia a la cavitación respecto a las plantas bien regadas.

En los capítulos 1, 2 y 3 se estudia la conductividad hidráulica y la vulnerabilidad a la cavitación de eucaliptos y chopos y los cambios que el estrés provoca en estas variables.

La vulnerabilidad a la cavitación es una propiedad intrínseca de la anatomía del xilema (Fichot 2015), y del mismo modo que lo hace la vulnerabilidad a la cavitación, las células xilemáticas pueden cambiar para adaptarse a las condiciones del medio. Hay especies más y menos plásticas, incluso dentro de la misma especie hay genotipos más y menos plásticos. En el caso del estrés hídrico, es común observar en especies con bajo suministro hídrico una

disminución en el tamaño de las células en general (Lombardini 2006) y en las que componen el xilema en particular; lo que en términos hidráulicos se traduce en un incremento de la resistencia al paso del agua, o dicho de otro modo, en una disminución de la conductividad hidráulica (Lovisol y Schubert 98 en Vitis, Fichot et al. 2010 en chopo, Nijse et al 2001 en Crisantemo, etc). El compromiso entre eficiencia hidráulica y seguridad frente a embolismos es conocido como el *trade-off efficiency safety*: cuando las plantas aumentan la resistencia al paso de la savia, ello supone un coste en términos de crecimiento pues disminuye la capacidad de transporte, que está ligada a la capacidad de absorber los recursos necesarios para el crecimiento de la planta. La existencia de este compromiso está muy documentada en numerosas especies, aunque hay trabajos en los que la relación entre eficiencia y seguridad no está clara. Por ejemplo, Maherali *et al.* (2004), revisando datos de 150 especies encuentran claro el *trade-off* en coníferas pero no en angiospermas. Igualmente hay otros trabajos como el de Burgess et al (2014) en secuías o Fichot et al (2010) y Plavcová & Hacke (2012) en chopo en los que se pone en duda la existencia de este *trade off*, dejando abierta la puerta a la posibilidad de encontrar genotipos productivos y resistentes a la cavitación entre la variabilidad genética existente. Es digno de mención el caso de *Acacia harpophylla*, (Van der Driessche 1971), una especie australiana de regiones semiáridas altamente resistente al estrés que presenta tasas fotosintéticas equivalentes a las de una especie de clima templado cuando crece en condiciones de agua no limitantes y temperaturas suaves.

La relación entre la anatomía del xilema y la conductividad hidráulica, es una relación que liga estructura y función. Los vasos de grandes luces, largos, con punteaduras permeables, serán capaces de transportar un flujo mayor que vasos delgados, vasos con punteaduras poco permeables o vasos cortos que obligan a la savia a atravesar mayor número de punteaduras.

Basándose en estudios florísticos como los del botánico Carlquist (1988), en los que se observa que en las zonas tropicales la evolución ha conducido a la existencia de plantas con tejido xilemático muy eficiente desde el punto de vista conductivo y muy vulnerables a la cavitación, y que las plantas de zonas desérticas presentan xilemas de numerosos vasos de pequeño diámetro y muy resistentes a la cavitación, se han realizado numerosos estudios anatómicos del xilema buscando las causas de las diferencias en la vulnerabilidad a la cavitación en las plantas, pensando que el diámetro de los elementos de los vasos podría ser un elemento de gran importancia a la hora de determinar la vulnerabilidad a la cavitación y la capacidad de crecimiento de la planta, pues dentro de una planta sí se ha comprobado que se embolizan en primer lugar los vasos de mayor tamaño (Hacke & Sperry 2001). Hay muchos resultados apoyando la relación entre el diámetro del vaso y la mayor vulnerabilidad de éstos a la cavitación; por ejemplo, en climas fríos, hay una selección natural de especies con vasos de diámetros pequeños (Schreiber et al.2011), pues los vasos de grandes diámetros son embolizados fácilmente por la formación de burbujas que tiene lugar durante el deshielo. Sin embargo, en el caso de las plantas sometidas a estrés hídrico, no está claro que las plantas cuyos vasos presentan mayor diámetro sean más vulnerables a la cavitación, y es frecuente encontrar falta de correlación entre vulnerabilidad a la cavitación y el diámetro de los vasos (Tyree et al., 1994, Pockman & Sperry, 2000; Hacke et al., 2006, Burges et al 2006, Vynia et al 2013).

La longitud de los vasos debería tener una importancia crucial en la vulnerabilidad del xilema a la cavitación, ya que la conducción de la savia de un vaso a otro ha de producirse por punteaduras, que suponen una resistencia al paso del flujo muchísimo más elevada que la que supone pasar de un elemento de vaso a otro. Por lo tanto, cuanto más largos sean los vasos, menos punteaduras habrá de atravesar el flujo de savia, y la eficiencia en el transporte será mayor; aunque el embolismo de un vaso sería más dañino en un vaso largo que en un vaso corto (Comstock & Sperry 2000, Jacobsen et al 2012). Los estudios de longitud de vasos son escasos, dado lo laborioso de la tarea. La longitud de un vaso en micrómetros, medida con un microscopio, es de dimensiones enormes comparada con la magnitud del diámetro, y los trabajos existentes sobre longitudes de vasos suelen basarse en estimaciones indirectas haciendo una serie de cortes a lo largo de un segmento de tallo inyectado con alguna sustancia coloreada y observando al microscopio el número de vasos rellenos en cada sección muestreada (Zimmermann 1981, Ewers & Fisher 1989, Nijsee J 2004). Según la hipótesis de “*air-seeding*” la cavitación de elementos conductores del xilema se inicia en las punteaduras (Shen et al. 2012).

Wheeler et al en 2005 no encontraron relación entre la resistencia de las punteaduras y la vulnerabilidad a la cavitación en 15 especies de angiospermas, por lo que propusieron “the rare pit hypothesis”, hipótesis en la que se propone que las burbujas de aire, cuando las tensiones son muy negativas, pasan de un vaso a otro lateralmente por la punteadura más débil del vaso. Supuestamente, cuantas más punteaduras tiene un vaso, mayor sería la probabilidad de que una fallara y se produjera el paso del aire a través de la punteadura. Sin embargo, el hecho de que las especies que vegetan en sitios áridos presenten vasos de diámetros menores que las especies de sitios húmedos hace pensar que pudiera existir una relación entre el diámetro del vaso y las características de las punteaduras, de forma que los vasos de mayor tamaño tuvieran punteaduras más débiles que las especies de vaso de menor diámetro. Esto estaría justificado desde un punto de vista del desarrollo de la planta, pues cuando existe un suministro de agua abundante la división celular es rápida, la demanda de carbohidratos también, y la pared primaria se deposita con menos medios materiales y mayores presiones debido a una mayor presión de turgencia de los tejidos que en el caso de suministro limitante de agua, caso en el que la demanda de hidratos de carbono es menor y las paredes primarias que forman las membranas de las punteaduras pueden percibir una mayor cantidad de materia prima y presentar una menor porosidad (Tyree & Sperry 1989).

Si no existe relación entre el diámetro del vaso y las características de las punteaduras es posible que no haya relación entre la vulnerabilidad a la cavitación y la eficiencia conductiva. Vasos largos y de gran diámetro podrían presentar punteaduras muy resistentes al paso de aire. Esto supondría un xilema en parte eficiente, por las magnitudes diámetro y longitud, y en parte resistente, por las características de las punteaduras, aunque la parte eficiente aporta vulnerabilidad, ya que la inutilización por embolia de un vaso voluminoso supone un porcentaje de conductividad hidráulica no despreciable. La relación entre diámetro y longitud de vasos no es clara: aunque algunos trabajos apuntan a que están relacionados (Martínez-Vilalta et al. (2002), Cai et al. 2010, Zimmermann & Jeje 1981, Ewers & Fisher 1989), otros como el de Jacobsen et al (2012) no encontraron relación lineal significativa entre diámetro y longitud medios de vasos de diferentes especies de árboles y lianas y sí encontraron relación entre ambas variables para arbustos.

Todas estas consideraciones han conducido a la publicación de trabajos en los que se pone en duda la existencia en determinadas especies como en Sequoia (Burges et al 2006) o en particular en chopo (Plavcová & Hacke 2012, Fichot et al 2010) del conocido como *trade-off efficiency safety*, compromiso entre eficiencia y seguridad, por el cual se considera que si la planta construye un xilema eficiente éste será vulnerable y si invierte en xilema seguro será ineficiente, y supondrá un mayor costo de creación del xilema y probablemente se traducirá en un menor crecimiento. El hallazgo de casos en los que los genotipos más productivos no son los más vulnerables indica la posible existencia, dentro de la variabilidad genética que ofrecen especies como el chopo y el eucalipto, de genotipos que puedan soportar condiciones de sequía y ofrecer crecimientos comerciales aceptables.

En el capítulo 3 se discute sobre la existencia del compromiso entre eficiencia y seguridad en cuatro genotipos de Populus empleados en cultivos energéticos experimentales en condiciones de estrés.

Del mismo modo que el xilema de la planta lleva impresas características funcionales del mismo, el desarrollo de la superficie foliar se ve afectado por los cambios que se han ido sucediendo en suministro hídrico, incrementos de déficit de presión de vapor, variaciones de intensidad lumínica, etc. La sequía induce en los organismos vegetales respuestas encaminadas generalmente a reducir la superficie foliar e incrementar el grosor del mesófilo; se producen ajustes en la conductancia estomática y en la conductividad hidráulica y por tanto en la tasa fotosintética y en último término en el crecimiento de las hojas y de la planta entera (Marron et al 2005, Fichot et al 2009) que es lo que, también en último término, nos interesa.

Los trabajos de laboratorio e invernadero en condiciones semicontroladas aportan información muy valiosa, pero también es conveniente realizar trabajos en el campo, que tengan en cuenta todas las posibles causas de variación en las condiciones ambientales, y un horizonte temporal más amplio al considerado en los ensayos de invernadero. La superficie foliar capta la radiación fotosintéticamente activa y es la encargada de absorber CO₂ y perder agua, por lo que la determinación del índice de área foliar (LAI) aporta información no sólo de la productividad de la plantación (Pellis et al. 2004, Dunlap & Stettler 1998, Ridge *et al.*, 1986, Zavitkovski *et al.*, 1976), sino también de su consumo de agua. Distintas tasas de conductancia estomática entre clones podrían equilibrarse con diferentes índices de área foliar.

La medición de la superficie foliar es una tarea tediosa, por lo que se han desarrollado métodos indirectos que permitan estimarlo a partir de otras variables fáciles de medir, como la transmisión de la luz a través de la copa, empleada por ejemplo por el dispositivo LAI-2000. Con el mismo fundamento científico puede estimarse el LAI a partir de fotografías hemisféricas.

En el cuarto capítulo de la tesis se ha realizado el seguimiento del LAI en dos plantaciones de demostración de chopo en turno corto y alta densidad en dos localidades del norte y centro peninsular durante dos años, empleando los mismos clones estudiados en el capítulo 3.

En estas plantaciones de crecimiento rápido es habitual utilizar material genético mejorado, generalmente seleccionado para presentar elevadas producciones y resistencia a enfermedades. Uno de los principales problemas de las especies comerciales: eucalipto, chopo, pino radiata, picea abies....es que experimentan una marcada interacción genotipo x ambiente en la producción de biomasa. Existe interacción genotipo-ambiente cuando un mismo genotipo muestra diferentes producciones sometido a diferentes ambientes. Es un fenómeno extraordinariamente común y dificulta la comparación general de genotipos, pues el genotipo más productivo en una estación ecológica puede pasar a ser uno de los menos productivos en otra y obliga en ocasiones a los mejoradores a seleccionar genotipos para un determinado ambiente o a optar por genotipos intermedios, sacrificando productividad.

En todos los capítulos se estudian rasgos anatómicos y/o fisiológicos de plantas sometidas a más de un ambiente o tratamiento con el objetivo final de encontrar diferencias entre clones y entre diferentes ambientes que permitan identificar genotipos más y menos adaptables así como qué rasgos puedan ser más importantes de cara a la producción de biomasa y supervivencia de la masa.

A continuación se presenta un breve resumen del contenido de los cuatro capítulos que componen la tesis doctoral:

En el primer capítulo se somete a seis clones de eucalipto a dos diferentes tratamientos hídricos durante un mes, uno de los regímenes consiste en una reducción del 20% respecto a un tratamiento control (regado a 95% de la capacidad de campo). Posteriormente, durante tres semanas más se somete a la totalidad de las plantas a un único régimen hídrico, no limitante y se observan las respuestas de las plantas sometidas a cada tratamiento al medioambiente del invernadero durante el mes de junio. Se estudiaron variables anatómicas del xilema (número de vasos, diámetro de vasos, superficie conductora, distribuciones diamétricas) y variables fisiológicas: de intercambio gaseoso y de conductividad hidráulica. Se observó cómo se aclimataron las plantas sometidas a los dos tratamientos a las nuevas condiciones. Durante el mes de junio se produjo una disminución de la conductividad hidráulica en las plantas sometidas a ambos tratamientos, siendo ésta mucho más notable en las plantas que habían pertenecido al régimen de riego más favorable. No solamente disminuyó la conductividad hidráulica, sino que disminuyó la conductividad hidráulica máxima, también en ambos tratamientos. La disminución de la conductividad máxima no pudo ser atribuida a la generación de un nuevo xilema menos conductivo, pues los análisis del xilema indicaron precisamente lo contrario.

Las diferencias entre clones no fueron tan marcadas como las diferencias entre tratamientos. Los clones que llegaron al final del ensayo con mayor y menor conductividad hidráulica máxima coincidieron con los clones con menor tamaño de los vasos y con menor número de vasos respectivamente.

Se propone como hipótesis que la disminución de la conductividad máxima sea una consecuencia de la impermeabilización transitoria de las punteaduras de los vasos embolizados con el objeto de aislarlos del resto del xilema para poder ser rellenados.

La conductancia estomática se coordinó con la conductividad hidráulica y ambas disminuyeron hacia la tercera semana y última del experimento. La conductividad hidráulica comenzó a disminuir antes que la conductancia estomática. Los resultados obtenidos sugieren que la disminución del flujo de savia podría ser el desencadenante del cierre parcial estomático detectado, sin descartar que pudiese existir alguna señal química implicada.

En el segundo capítulo se estudia cómo varían la conductancia estomática y la conductividad hidráulica en cinco clones de eucalipto a medida que cambian las condiciones atmosféricas en plantas sometidas a diferentes dosis de riego. También se midió la variación del pH de la savia y el potencial hídrico con el objeto de determinar si el cierre estomático provocado por el estrés al bajar el potencial hídrico puede ser debido a señales hidráulicas, químicas o a ambas. Los resultados mostraron que a raíz de una subida continuada en el déficit de vapor de presión, el potencial hídrico bajó coincidiendo con una disminución tanto en el pH de la savia como en la conductancia estomática. No hubo diferencias entre clones en el valor del pH alcanzado, pero sí en los valores de conductancia estomática. Algunos clones cerraron estomas más eficientemente que otros. La pérdida de conductividad hidráulica se mantuvo sin cambios significativos a lo largo de todo el experimento, mientras que el pH de la savia mostró una relación lineal con el potencial hídrico, sugiriendo la posible existencia de una señal química como responsable del cierre estomático. Los cambios en el pH de la savia se produjeron en ambos tratamientos de riego, indicando que la señal de cierre estomático pueda no ser desencadenada por falta de agua en el suelo y por tanto, en caso de existir una señal química desencadenante de cierre estomático, esta no parece que sea una señal emitida desde la raíz a las hojas.

En el tercer capítulo, se lleva a cabo un estudio anatómico del xilema en plantas pertenecientes a cuatro genotipos de chopo situados en una plantación experimental cuyo objetivo era evaluar la producción de biomasa en densidades de 20.000 pies/ha. La plantación fue sometida a restricciones hídricas que produjeron una defoliación total.

Se midieron número y diámetro de todos los vasos de las secciones normales (a 130 cm de altura) de una muestra de 52 elementos. El estudio tenía como fin encontrar diferencias anatómicas entre clones relacionadas con el crecimiento. Para complementar este estudio anatómico se construyeron unas curvas de vulnerabilidad con los mismos cuatro genotipos sometidos a dos regímenes hídricos diferentes empleando dos métodos: centrífuga y deshidratación. Se analizaron las diferencias en la vulnerabilidad a la cavitación entre clones. Actualmente el método de la centrífuga para obtener curvas de vulnerabilidad es controvertido, pues se sospecha que pueda generar curvas desplazadas hacia valores menos negativos de potencial hídrico, es decir, incrementaría la vulnerabilidad. Los resultados, efectivamente, mostraron que las curvas obtenidas a partir de muestras centrifugadas están desplazadas hacia valores menos negativos, pero el ranking de clones por vulnerabilidad fue parecido empleando ambos métodos.

Los resultados revelaron la aclimatación de las plantas a los distintos regímenes hídricos y la diferente plasticidad de los clones estudiados frente a la vulnerabilidad a la cavitación. Las diferencias en vulnerabilidad a la cavitación no explicaron las diferencias clonales de crecimiento observadas en el campo. Se han encontrado diferencias significativas

en el xilema de los cuatro clones ensayados, desde clones con pocos vasos de gran diámetro a clones con vasos muy pequeños y no demasiado numerosos. Esta información es de utilidad para interpretar el comportamiento productivo de los clones en el campo y en parte para relacionar la vulnerabilidad a la cavitación con la anatomía. Los clones más productivos presentaron el mayor número de vasos de tamaño intermedio en condiciones de estrés.

Se discute sobre la posible no existencia del compromiso entre eficiencia y seguridad pues los clones más productivos figuraron entre los más resistentes a la cavitación, aunque algunos clones poco productivos resultaron tan resistentes o más que los que presentaron mayores producciones.

El último y cuarto capítulo compara el índice de área foliar en dos plantaciones de demostración de chopo con fines energéticos situadas en Almazán (Soria) y en Valtierra (Navarra) empleando los mismos genotipos estudiados en el capítulo 3 durante dos años consecutivos. Ambas localizaciones presentan una marcada diferencia climática y de dosis de riego. En Navarra la dosis de riego fue aproximadamente el doble que la de Almazán. El LAI se estimó por dos métodos: se tomaron fotografías hemisféricas de la cubierta de ambas plantaciones para calcular el LAI a partir de modelos que relacionan el índice de huecos en la cubierta con el índice de área foliar y se comparó con el LAI medido por métodos directos, consistente en tomar una muestra de árboles representativa, pesar todas las hojas de la muestra y multiplicar este peso por la inversa del área foliar específica (SLA) (g/m^2) obtenido en una submuestra menor.

Los resultados muestran una elevada correlación entre biomasa y LAI, ya sea estimado a partir de métodos directos o indirectos. Los sitios y clones más productivos presentaron mayores índices de área foliar y lo contrario sucedió con los sitios y clones menos productivos.

El LAI obtenido por medios indirectos no siempre infraestimó el LAI obtenido por medios directos, como suele ser habitual al no cumplirse las hipótesis de partida para aplicar la ley de Beer Lambert. La violación del supuesto de distribución aleatoria de hojas se produjo en Almazán porque el diseño de la plantación, consistente en surcos dobles separados 3.5 m entre sí, unido al escaso desarrollo de la plantación dio lugar a un espacio vacío regular entre filas. Aunque los modelos empleados para estimar el LAI en Almazán arrojaron valores más bajos que los obtenidos a partir del LAI directo, la infraestimación fue la misma para todos los clones, de modo que las diferencias entre clones se conservaron iguales que en la estimación directa. La plantación de Valtierra en el segundo año de medición fue afectada severamente por la roya (*Melampsora spp*) y no se observó correlación entre la producción y el LAI, debido a defoliación prematura. De un año a otro se observan cambios en el ranking clonal de producción de biomasa. El clon I-214 durante el primer año figura entre los clones con menor producción, mientras que el segundo año, después de un recepe pasa a ser el clon más productivo en Almazán y equiparable a los de mayor crecimiento en Valtierra. Estos cambios podrían tener su origen en la diferente afección de la roya y en la diferente distribución de los recursos durante el primer año por parte de los clones. El recepe podría haber favorecido a los clones que invierten más recursos en las raíces y no desarrollan la enfermedad.

OBJETIVOS



OBJETIVOS

El objetivo general de esta tesis es identificar algunas de las características anatómico-fisiológicas que confieren la capacidad de alcanzar una mejor productividad bajo clima mediterráneo a plantas de diversos genotipos de los géneros *Populus* y *Eucalyptus*, caracterizados por su carácter pionero, elevado crecimiento y vulnerabilidad a la cavitación. Se definen los siguientes objetivos concretos:

En eucalipto:

1)- Analizar las diferencias entre clones en la respuesta estomática a déficit hídrico y déficit de presión de vapor de la atmósfera

2)- Investigar el papel del pH de la savia y de la conductancia hidráulica del tallo en la regulación de la conductancia estomática y las posibles diferencias entre clones.

En chopo:

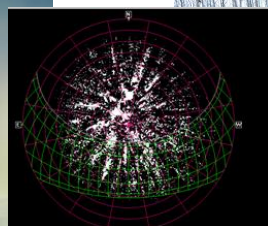
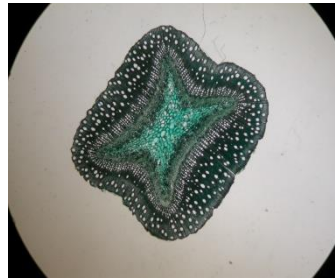
3)-Investigar la relación del crecimiento con los parámetros anatómicos: “número de vasos por sección transversal de tallo”, “superficie conductora”, “area transversal media de los vasos”, “densidad de vasos”, “ratio área-densidad” y “conductividad hidráulica teórica” e investigar las diferencias entre clones

4)-Analizar si la vulnerabilidad a la cavitación de cuatro genotipos empleados en la *Red de Parcelas de Cultivos Leñosos en Alta Densidad y Turno Corto del INIA*, está relacionada con la producción.

5)-Analizar si los métodos indirectos de estimación del índice de área foliar son fiables para hacer un seguimiento de la producción de las plantaciones y detectar diferencias entre clones y años.

6)-Comprobar si el trabajo realizado contribuye a explicar las diferencias de producción encontradas en los diferentes sitios de ensayo (interacción genotipo x ambiente) dentro de la ya mencionada *Red de Parcelas de Cultivos leñosos*.

RESUMEN DE MATERIAL Y MÉTODOS



RESUMEN DE MATERIAL Y MÉTODOS

Se presenta a continuación un escueto resumen de los materiales y métodos empleados. El contenido detallado se encuentra dentro de cada uno de los capítulos.

MATERIAL VEGETAL:

Capítulo uno: *Eucalyptus globulus*: dos clones F_0 : C13 y C14 y cuatro clones F_1 : H231, H354, H456, y H491, éste último es un híbrido C14xC14.

Capítulo dos: *Eucalyptus globulus*: un clon F_0 : C14 y cuatro F_1 , OD, SA y PI, T, híbridos de C14 y dos clones más: uno común para OD y SA y otro para PI y T

Capítulos tres y cuatro: se emplearon cuatro genotipos híbridos: dos clones *Populus x euramericana* (AF2 e I-214) y dos híbridos *Populus x interamericana x nigra* (Monviso y Pegaso).

MÉTODOS:

En los dos primeros capítulos se sometieron las plantas a dos tratamientos con diferente dotación de riego en invernadero. En el capítulo uno, una vez finalizada la aplicación de los tratamientos, se realizaron mediciones de potencial hídrico, conductividad hidráulica, e intercambio gaseoso; en el capítulo dos, las mediciones se llevaron a cabo mientras las plantas seguían sometidas a los tratamientos de riego. En el capítulo dos además de lo anterior se llevaron a cabo mediciones periódicas del pH de la savia. En ambos capítulos se midió el crecimiento en superficie foliar y en biomasa.

En el capítulo tres se realizó un estudio de la anatomía del xilema de cuatro genotipos híbridos de chopo en una plantación sometida a estrés hídrico severo situada en Granada. En esta plantación se llevaron a cabo mediciones de intercambio gaseoso y de variables de crecimiento. El estudio de la vulnerabilidad a la cavitación se realizó en un ensayo establecido para tal fin en los campos de ensayo del CIFOR-INIA en Madrid sobre plantas de un año de los mismos cuatro clones sometidas a dos regímenes de riego, uno de los cuales supuso la interrupción del riego durante dos meses.

En el último capítulo se realizó un seguimiento del índice de área foliar de dos plantaciones durante dos años consecutivos correspondientes a los dos primeros periodos vegetativos de las plantaciones. El índice de área foliar (LAI) se estimó a partir del índice de huecos medido en fotografías hemisféricas a partir de la ley de Beer Lambert y se comparó posteriormente con el LAI obtenido directamente a partir de pesada de las hojas de los árboles. Ambos se correlacionaron con la producción de biomasa de ambas plantaciones, obtenida a partir de mediciones destructivas.

Listado de variables a las que se hace referencia en los capítulos y la discusión y unidades:

Variables de crecimiento:

Biomasa leñosa (g, o $\text{Mg ha}^{-1} \text{ año}^{-1}$): pesada en estufa con 0% de humedad.

Área foliar (cm^2): superficie de todas las hojas de la planta medida con Winfolia®

Área foliar apical (Lap) (m^2): superficie de las hojas distales a la sección más pequeña de la muestra empleada para realizar las mediciones de conductancia hidráulica.

SLA: Área foliar específica (cm^2/g): área foliar dividida por el peso seco (0% humedad) de la misma

Altura tallo (cm)

D_{10} (mm), d_{130} (mm): diámetros basales (d_{10}) y normales (d_{130}) medidos a 10 y 130 cm de altura respectivamente, medidos con calibre desde el suelo.

Volumen (cm^3): volumen de madera en verde calculado a partir de diámetros (d_{10} , d_{130}) y alturas utilizando las fórmulas del cono y el tronco de cono.

Variables anatómicas:

Número de vasos: número de vasos en cada sección transversal.

Área media del vaso (μm^2): promedio del área de todos los vasos por sección transversal

Superficie conductora (μm^2): suma del área de todos los vasos por sección transversal

Área de la sección transversal (mm^2): incluye xilema, corteza y médula.

Densidad de vasos (mm^{-2})=número de vasos/ superficie del xilema (mm^2)

AD-RATIO ($\mu\text{m}^2 \text{mm}^2$)= Área media del vaso/densidad de vasos

Conductividad hidráulica teórica $K_{ht} = \Sigma \pi dh^4 / 128 \eta$ (dh =diámetro hidráulico de cada vaso= $4\text{área}/\text{perímetro}$; η : coeficiente de viscosidad dinámica del agua)

Variables fisiológicas:

-De intercambio gaseoso, medidas con un IRGA (infrared gas analyzer):

Tasa de fotosíntesis (A): $\mu\text{mol m}^{-2} \text{s}^{-1}$

Conductancia estomática (gs): $\text{mol m}^{-2} \text{s}^{-1}$

Transpiración (E): $\text{mmol m}^{-2} \text{s}^{-1}$

Eficiencia intrínseca en el uso del agua (IWUE): $\mu\text{mol mol}^{-1}$

-Hidráulicas:

Conductancia hidráulica (k): ($\text{Kg s}^{-1} \text{MPa}^{-1}$). Flujo de agua que pasa por una sección transversal de tallo sometida a una presión dada de magnitud tal que no permite el desplazamiento de las posibles burbujas de agua que pudieran existir en el xilema. Se obtiene mediante pesadas (método de Sperry) o mediante lectura directa en el dispositivo Xyl'em®

Conductancia hidráulica nativa (k_i): conductancia hidráulica medida sobre un espécimen que no ha sido sometido a ninguna intervención previa.

Conductancia hidráulica máxima (k_{max}): ($\text{Kg s}^{-1} \text{MPa}^{-1}$). Es la conductancia hidráulica medida después de haber sometido a la muestra durante un periodo de tiempo al paso de agua a una presión lo suficientemente alta como para permitir eliminar los posibles embolismos existentes en el xilema.

Conductancia hidráulica específica relativa al área foliar (LSC): ($\text{Kg s}^{-1} \text{MPa}^{-1} \text{m}^{-2}$). Es la conductancia hidráulica relativa al área foliar distal a la sección mínima de la muestra. $LSC=k_i/Lap$; donde k_i es la conductancia hidráulica nativa y Lap es el área foliar apical alimentada de savia a través de la sección considerada.

Conductividad hidráulica Q, K : ($\text{Kg s}^{-1} \text{MPa}^{-1} \text{m}^{-1}$) . Es la conductancia hidráulica multiplicada por la longitud de la muestra (L): $Q=k*L$

Conductividad hidráulica máxima Q_{max}, K_{max} : ($\text{Kg s}^{-1} \text{MPa}^{-1} \text{m}^{-1}$). Es la conductancia hidráulica máxima multiplicada por la longitud de la muestra (L): $Q_{max}=k_{max}*L$

Conductividad hidráulica específica relativa al área foliar (Q_L): ($\text{Kg s}^{-1} \text{MPa}^{-1} \text{m}^{-1}$) Es la conductancia específica foliar relativa al área foliar multiplicada por la longitud de la muestra (L): $Q_L=LSC*L$

Conductividad hidráulica específica máxima relativa al área foliar (Q_{LMAX}): ($\text{Kg s}^{-1} \text{MPa}^{-1} \text{m}^{-1}$) Es la conductividad hidráulica máxima dividida entre el área foliar distal a la sección mínima de la muestra: $Q_{LMAX}=Q_{max}/Lap$

Conductividad hidráulica específica relativa a la sección transversal (K_{XS}): ($\text{Kg s}^{-1} \text{MPa}^{-1} \text{m}^{-1}$). Es la conductividad hidráulica dividida entre el área de la sección transversal (A_s). $K_{XS}=K/A_s$

Pérdida de conductancia o conductividad hidráulica (PLC):(%) . Es la diferencia entre conductancia máxima y nativa relativa a la conductividad máxima:

$$PLC (\%) = 100(k_{max}-k_i)/k_{max}$$

-Otras:

Evapotranspiración: g/planta. Obtenida mediante medición del peso de la planta y su envase antes y después de cada riego.

Potencial hídrico (Ψ) (MPa): Medido con una cámara de Scholander.

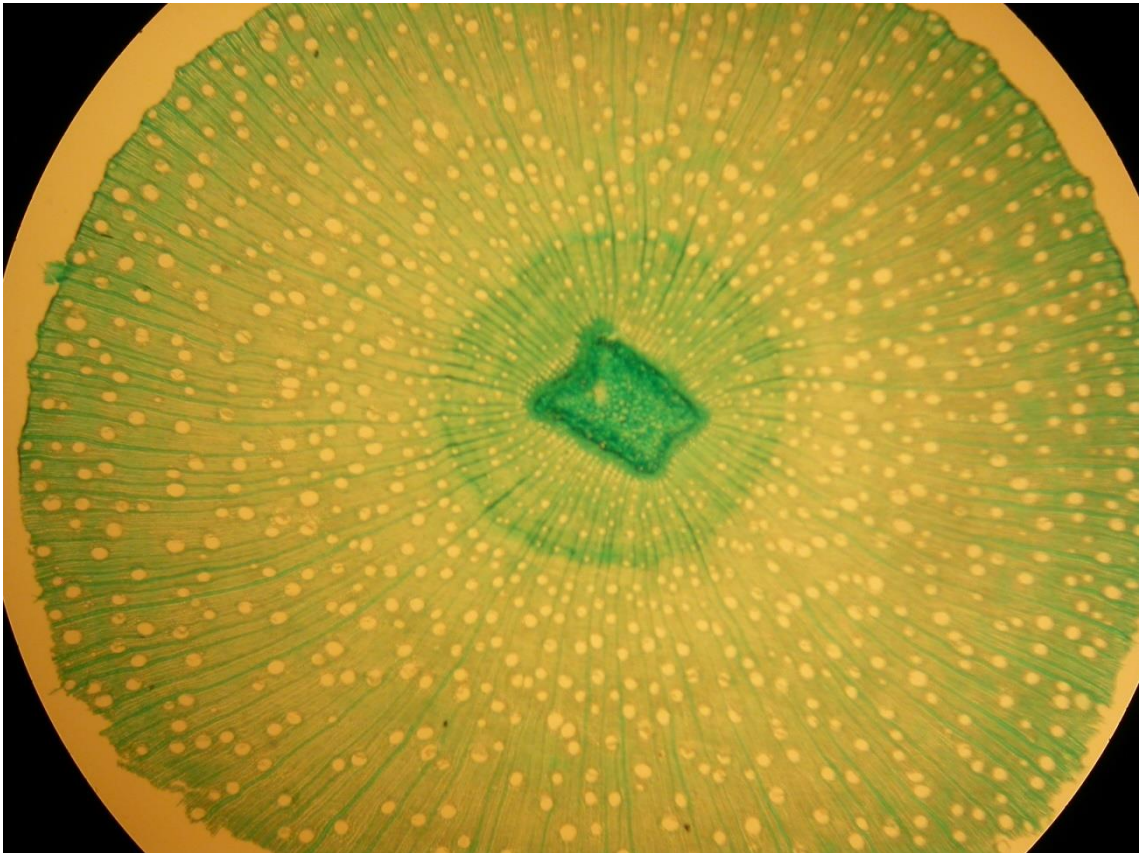
Déficit de presión de vapor (DPV) (kPa): obtenido a partir del porcentaje de humedad relativa y la temperatura en el invernadero proporcionados por un termohigrógrafo a partir de la fórmula $VPD=Psat(1-RH)$; donde $Psat$ es la presión de vapor en saturación del agua a una temperatura dada y RH es la humedad relativa del invernadero en tanto por uno.

pH: valor negativo del logaritmo de la concentración de protones en la savia del xilema medidos con un microelectrodo en savia extraída de la planta.

CAPÍTULOS



CAPÍTULO 1



CAPÍTULO 1

HYDRAULIC CONSTRAINTS TO GAS EXCHANGE IN F_0 AND F_1 *Eucalyptus globulus* Labill. CLONES

RESUMEN

Se realizaron mediciones de intercambio gaseoso, conductividad hidráulica del xilema y crecimiento en plantas jóvenes de seis clones de *Eucalyptus globulus* Labill.: dos F_0 y cuatro F_1 entre los que se incluyó un clon procedente de autocruzamiento. Las plantas se cultivaron en invernadero y fueron sometidas a dos regímenes de riego. Las mediciones de conductividad hidráulica se llevaron a cabo en porciones de tallo sometidas a deshidratación natural a lo largo de tres semanas consecutivas. El clon híbrido producto de autocruzamiento figuró entre los clones que mostraron menores valores en las variables de crecimiento. La vulnerabilidad a la cavitación se incrementó y la conductividad hidráulica específica relativa al área foliar, tanto nativa (Q_L) como máxima ($Q_{L,MAX}$) disminuyeron de la primera a la tercera semana de mediciones, sin que se observaran disminuciones en el diámetro o la superficie de los vasos del xilema, sugiriendo la existencia de cambios en la permeabilidad de las membranas de las punteaduras. Además se observó una disminución significativa de la conductancia estomática máxima en la tercera semana de mediciones.

Los valores más elevados de $Q_{L,MAX}$ se registraron en el clon que alcanzó el mayor crecimiento, y los más bajos en el clon H491 procedente de autocruzamiento. Los máximos valores de conductancia estomática obtenidos en H491 apenas llegaron a un tercio de los valores medidos en el resto de los clones, indicando una reducida capacidad de refrigeración de la hoja y una pobre adaptación a ambientes mediterráneos.

El decrecimiento de la conductancia estomática sucedió simultáneamente a la coincidencia en el tiempo de los valores de conductividad hidráulica específica referida al área foliar alcanzados por plantas de los dos regímenes de riego. Este resultado sugiere que el cierre estomático podría haberse producido para preservar la capacidad hidráulica del tallo.

HYDRAULIC CONSTRAINTS TO GAS EXCHANGE IN F₀ AND F₁ *Eucalyptus globulus* Labill. CLONES

MARIA JOSE HERNANDEZ², SVEN MUTKE², FERNANDO MONTES² and PILAR PITA^{1,3}.

(1) Unidad de Anatomía, Fisiología y Genética Forestal. Escuela Técnica Superior de Ingenieros de Montes. Ciudad Universitaria, 28040 Madrid, Spain.

(2) Dep. Sistemas y Recursos Forestales, CIFOR-INIA, Carretera La Coruña km 7.5, 28040 Madrid, Spain.

ABSTRACT

Growth, gas exchange, and xylem hydraulic conductivity were measured in young plants from two F₀ and four F₁ *Eucalyptus globulus* Labill. clones (including one inbred clone). Plants were grown under two different watering regimes in a greenhouse experiment. Hydraulic measurements were carried out on stem portions that had undergone natural drying, over three consecutive weeks. The inbred clone was always among those displaying the lowest values for growth variables. Xylem vulnerability to cavitation increased and both native and maximum leaf specific hydraulic conductivity (Q_L and $Q_{L,MAX}$) decreased from the first to the third week, with no changes in the vessel area to leaf area ratio, suggesting the existence of changes in the permeability of intervessel pit membranes. Accordingly, a significant decrease in maximum stomatal conductance was observed in the third week of measurements. The highest values of $Q_{L,MAX}$ were measured in the clone that attained the highest growth, and the lowest in the inbred clone. Maximum values of stomatal conductance measured in the inbred clone were a third of those measured in the rest of the clones, indicating a reduced evaporative cooling capacity and poor adaptation to Mediterranean environments. A decline in stomatal conductance was found to concur with the convergence of leaf specific hydraulic conductivity values for plants belonging to both watering treatments. This result suggests that stomatal closure may act to preserve the hydraulic capacity of the stem.

Keywords: Drought resistance, growth, hydraulic conductance, inbreeding, stomatal conductance, xylem cavitation.

INTRODUCTION

Increasing xylem tensions due to high evaporative demand or soil water deficit lead to vessel embolism through air seeding (Tyree and Zimmermann 2002), xylem cavitation and thus xylem dysfunction, and a decrease in water transport capacity in trees. This may be particularly relevant in Mediterranean environments, where high temperature and low relative humidity may cause xylem water potential to drop below the cavitation threshold even when soil water is abundant. High cavitation resistance is considered a key component of drought tolerance (Maherali et al. 2004). Woody plants from dry habitats usually show greater resistance to water stress induced cavitation than plants from more mesic habitats (Brodribb and Hill 1999, Kavanagh et al. 1999, Sperry 2000, Froux et al. 2002). However, species showing relatively low xylem resistance to cavitation might be able to survive and grow in drought-prone environments, developing alternative mechanisms to maintain a favourable water

status, such as deep rooting, early leaf shedding or early stomatal closure (Vilagrosa et al. 2003, Piñol and Sala 2000, Machado and Tyree 1994). Results from several studies suggest that xylem conduits, in some species at least, undergo frequent cycles of cavitation and embolism repair (refilling) (Holbrook et al. 2001, Domec et al. 2006, Martorell et al. 2013). Though the underlying mechanism still remains unclear, refilling may be concurrent with transpiration (McCully 1999, Tyree et al. 1999, Hacke and Sperry 2003), causing hydraulic conductance to vary diurnally as a result of both processes. In this context, not only xylem vulnerability to cavitation, but also the capability of refilling embolized vessels should be considered in ecological studies (Zwieniecki and Holbrook 1998). In the present study, native embolism was measured directly on portions detached from plants that had undergone natural drying. This was done not only to account for both cavitation and refilling but also to avoid undesirable effects of artificial drying such as the flow rate increases over the course of the measurement period reported by Prior and Eamus (2000) or the changes in the vulnerability profile reported by Wickberg and Ögren (2004).

Decreases in stomatal conductance have been found to prevent xylem cavitation in several woody species (Vilagrosa et al. 2003, Cochard et al. 2002, Lemoine et al. 2002, Salleo et al. 2000). However, stomatal responses to drought stress may only help to avoid cavitation in the short term. Long-term responses such as early leaf shedding or decreasing maximum hydraulic conductance have been found in several tree species, including eucalypts (Vander Willigen and Pammenter 1998, Vilagrosa et al. 2003, Pita et al. 2003).

Species with high hydraulic conductance may achieve higher diurnal stomatal conductance, and thus higher photosynthetic rates and growth (Nardini and Salleo 2000). However, increased hydraulic conductance has been related to higher vulnerability to cavitation (Nardini and Pitt 1999, Nardini and Salleo 2000, Himrane et al. 2004) and may threaten survival, especially in exceptionally hot and dry years, a fact that must be taken into account in a scenario of global climate change. Hydraulic conductance has been reported to decrease under drought conditions, limiting tree water use even after the conclusion of a drought period (Eamus et al. 2000). Moreover, high resistance to cavitation has been related to wood density (Wikberg and Ögren 2004) and may be costly in mesic environments (Maherali et al. 2004). Wikberg and Ögren (2004, 2007) suggested that increasing drought tolerance may lead to decreased growth in willow (*Salix* sp.). Similar results have been reported for *Salix* and *Populus* by Cochard et al. (2007). Conversely, Wang et al. (2003) found that selecting for both fast growth and drought tolerance may be possible in the case of the Lodgepole pine (*Pinus contorta* Dougl. ex Loud).

Fast-growing pioneer species are often susceptible to water deficits in the early stages of growth due to increased biomass allocation to the shoot (Brodribb and Hill 2000). *Eucalyptus globulus* Labill. plantations established in SW Spain are frequently submitted to severe water stress. In a previous study, lower values were measured for long-term water use efficiency in those *E. globulus* genotypes that attained higher growth and survival rates under field conditions (Pita et al. 2001). This suggests that the ability to support high transpiration rates may be crucial in terms of successfully competing for available water. The main objectives of our study were (1) to identify hydraulic differences between six *E. globulus* clones displaying contrasting growth and survival rates under a Dry Mediterranean climate

and (2) to assess the relevance of hydraulic constraints on gas exchange in young plants of the species.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWING CONDITIONS

Rooted cuttings from two F_0 and four F_1 clones were obtained from Grupo Empresarial ENCE S.A., Spain. Both C13 and C14 clones are representative of the commercial F_0 clones, and are currently used in eucalypt plantations in Spain. The F_1 clones (H231, H354, H463 and H491) were derived from crosses between C14 and other commercial clones, except H491, which is an inbred C14 x C14 clone. Results from two field trials revealed that three years after plantation, the survival rate, height and diameter at breast height (DBH) were severely limited in the inbred clone (Table 1). Survival was similar or slightly lower in F_1 compared to F_0 clones (excluding H491), whereas growth was higher in F_1 clones (again excluding the inbred H491) at both study sites (Table 1). Clone H231 displayed both high growth and survival rates and is considered to be the best of the six clones tested. Differences between clones in growth and survival were greater in the trial established on deep, sandy soil compared with that established on more shallow, slate soil, as might be expected (Table 1).

Table 1: Survival, diameter at breast height (DBH) and height at age three years, from field trials established on slate soil (1) and deep, sandy soil (2) in SW Spain. Both trials were established following a complete random block design, $n=20$ plants clone⁻¹ trial⁻¹, >147 clones trial⁻¹.

Clone	C13		C14		H231		H354		H463		H491(inbred)	
Trial	1	2	1	2	1	2	1	2	1	2	1	2
Survival (%)	75% - 73%		75% - 80%		70% - 95%		60% - 55%		55% - 80%		55% - 50%	
Height(m)	6.4 – 12.4		5.7 – 12.4		6.7 – 14.0		7.6 – 13.8		7.4 – 13.1		4.8 – 8.4	
DBH(cm)	6.4 – 11.0		5.4 – 10.9		6.8 – 13.7		6.9 – 13.5		7.1 – 12.9		4.5 – 6.0	

At the 10-leaf pair stage, twenty plants per clone were transplanted to 3-l pots filled with 1180 g (dry weight) of a 3:1 (v:v) peat:sand mixture. Plants were placed in a greenhouse in such a way that the number of border plants was the same for all the clones. Temperatures ranged from 16-32°C and maximum photosynthetically active radiation was 1700 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After a six-week acclimation period in the greenhouse, the plants were divided into two groups (10 plants per clone in each group) and two watering treatments were established (Day 0, April 29th). From then until Day 35, all plants were watered to constant weight, once a week at the beginning and three times a week at the end of this period. On average, plants belonging to the low-watering treatment (LW) received 80% of the water supplied to those in the high-watering treatment (HW). From Day 35 onwards, plants were watered every one, two or three days, with no differences between watering treatments, whilst at the same time carrying out gas exchange and hydraulic measurements.

On Day 9, 48 plants (4 plants per clone and watering treatment) were moved to a growth chamber (22°C-20°C day-night, 60% RH). After a 15 h night, predawn leaf water

potential was measured on a single fully-expanded leaf of the seventh node using a pressure chamber (PMS Instruments Co., Corvallis, OR). The plants in their containers were weighed and volumetric soil water content was measured with a TDR device (Trime-FM, IMKO Micromodultechnik GmbH, Ettlingen, Germany). Once these measurements had been taken, the plants were returned to the greenhouse. On Day 14, the whole procedure was carried out on 48 drought-treated plants (8 plants per clone).

Volumetric soil water content (SWC) was measured on Days 9 and 14 as well as on days 35-55 when hydraulic conductivity measurements were also taken. Volumetric soil water content was positively correlated with the weight of each plant plus its container (W_T) throughout $SWC = 0.0362W_T - 56.195$ ($R^2 = 0.84$, $n = 199$) (Eqn.1). No significant differences were found among regression lines fitted to data from different dates, indicating a negligible effect of time (and thus plant size) on the relationship between both variables. A single equation was thus obtained for all data combined. This equation was used to calculate soil water content values from W_T following the imposition of the watering treatments (Figure 1a), avoiding an excessive use of the TDR probe, which could damage the roots.

GROWTH, ANATOMICAL AND MORPHOLOGICAL TRAITS

On Day 0, non-destructive measurements of leaf area were obtained by drawing all the leaves of two plants per clone on tracing paper. An Image Analyzer (LI-3000, Li-Cor Inc) was used to estimate leaf area from leaf drawings. Leaf area (L_A) was related to maximum leaf blade length (L_L) and width (L_W) throughout $L_A = a + b L_W + c L_L$ (one equation for each clone, $R^2 > 0.90$ for all the clones). Mean plant leaf area was calculated from leaf blade length and width, measured on all the leaves of six randomly chosen plants per clone on Day 0.

When the hydraulic conductivity measurements were carried out (Days 35-55), the whole plant leaf area was also measured, separating those leaves distal to the segment used for hydraulic measurements from the rest of the leaves, and leaves on lateral shoots (auxiliary leaves) from leaves directly attached to the main stem (main leaves). Leaves were oven-dried to constant weight at 70°C and the dry weight of the main and auxiliary leaves measured. Specific leaf area (SLA) of the main and auxiliary leaves was calculated as the quotient of leaf area by leaf dry weight. Total height (cm) and dry biomass shoot weight (g) were also recorded.

Distal portions of half the stems used in hydraulic measurements were kept in FAA (ethanol, glacial acetic acid and formaldehyde, 90:5:5) and later cut with a sliding microtome (Leica SM2400, Leica Microsystems GmbH) and stained with fast green. Unfortunately, some of the samples were damaged and only 20 LW plants (3-4 per clone) and 19 HW plants (2-5 per clone) could be used to determine the number of vessels, vessel lumen area distribution, total vessel lumen area per cross section (VLA) and cross section area using WinCell Regular® software (Regent Instruments Inc., Canada).

GAS EXCHANGE

On Days 35, 43 and 50, stomatal conductance, transpiration rate and net photosynthetic rate were measured on one single leaf of the sixth node (counted from the apex), using a LCA4 IRGA (Analytical Development Co, Hoddesdon UK). Measurements were carried out inside the greenhouse, under natural light ($PAR > 1100 \mu\text{mol m}^{-2} \text{s}^{-1}$), between 9:30 and 11:30 (solar time). Vapour pressure deficit ranged from 3.2-4.0 kPa on Day 35 to 2.5-3.3 kPa on Days 43 and 50. Gas exchange parameters were measured on 4-9 LW plants per clone each day. Measurements were carried out in batches (one plant per clone in each batch). Gas exchange measurements were carried out only on LW plants to avoid increasing the time of measurement and the effect of midday stomatal closure.

HYDRAULIC TRAITS

Xylem embolism in plant stems was quantified by determining the hydraulic conductance ($\text{Kg H}_2\text{O MPa}^{-1} \text{s}^{-1}$) of the xylem before and after removing embolism using the flushing method (Sperry et al. 1988). Hydraulic measurements were carried out at mid-morning (8:00-9:30, solar time), over three weeks (Days 35-55). Environmental conditions remained stable throughout the sampling days, with minimum temperature ranging from 18-20°C, a maximum temperature of 32°C inside the greenhouse and sunny weather. In order to minimize the effect of time lapse, one or two plants per clone from one single watering treatment were measured each day. Each day, 6-10 plants were taken to the laboratory (less than 5 minutes from the greenhouse) in groups of two. Leaf water potential was measured immediately on a single leaf of the sixth node (counted from the apex) with a pressure chamber. Each plant was weighed with its container and volumetric soil water content was measured. The stem was then cut under water (at the base and just below the sixth node). All the leaves were removed under water. The stems were left soaking for at least 20 minutes before placing them in the rubber tubes for the hydraulic conductance measurements. Hydraulic conductance was measured at a pressure drop of 0.0064 MPa before (K_i) and after (K_m) pressurizing the stems at 0.08 MPa for 30 min, which was found to be enough to remove embolism since further perfusion did not result in increased conductance. The perfusing solution was 1‰ HCl in distilled water, degassed by agitating under vacuum and filtered to 0.2 μm . The percentage loss of hydraulic conductance (PLC) was calculated from: $\text{PLC} = 100 (K_m - K_i) K_m^{-1}$. The stem length and cross-section stem diameters were then measured. The hydraulic conductivity (Q_h) was calculated as the hydraulic conductance multiplied by the length of the stem segment, and leaf specific hydraulic conductivity (Q_L) was obtained by dividing Q_h by the amount of leaf area distal to the cut end.

The maximum hydraulic conductivity ($Q_{h, \text{MAX}}$) was calculated as the maximum hydraulic conductance multiplied by the length of the stem segment, and maximum leaf specific hydraulic conductivity ($Q_{L, \text{MAX}}$) was obtained by dividing $Q_{h, \text{MAX}}$ by the amount of leaf area distal to the cut end.

STATISTICAL ANALYSES

The existence of significant differences between clones and watering treatments in morphological or physiological variables was assessed by means of ANCOVA analysis using time, leaf area or cross section area as covariables. Differences between clones and watering treatments in specific leaf area (SLA) were assessed by means of ANCOVA. Differences between clones, watering treatments and week of measurement in variables standardized as $Q_{L,MAX}$ or VLA to apical leaf area ratio were evaluated with ANOVA. Interactions between factors were taken into account in both ANOVA and ANCOVA. The validity of the basic assumptions, especially those of linearity, independence, normality and homocedasticity of residuals, was checked graphically and data were log-transformed where necessary. Tukey tests were used to compare means when interactions were not significant. The percentage of plants that had a PLC higher than 70% was analysed by a logistic regression model (generalised linear model), taking into account the clone, watering treatment and time interval (first two weeks versus the third one) as categorical predictor variables and the leaf water potential as covariate. The relationship between SWC and W_T was analyzed by simple linear regression. The relationships between leaf area, maximum leaf length and maximum leaf width were analyzed with a multiple linear regression model. All statistical comparisons were considered significantly different at $P < 0.05$. Analyses were performed using the version 9.1 of SAS software.

RESULTS

SOIL WATER CONTENT AND PREDAWN LEAF WATER POTENTIAL

Average soil water content remained between 27% and 4% throughout the experiment (Figure 1a). Transpiration rates were as high as $175 \text{ g plant}^{-1} \text{ day}^{-1}$ (measured in HW plants from clone H463, data not shown). Despite frequent irrigation, several plants from both watering treatments showed reversible turgor loss at midday at the end of each watering cycle. The tight relationship between soil water content and predawn leaf water potential (Figure 1b) shows that the time lapse between transplantation and the beginning of watering treatments was enough to allow root expansion and a complete colonization of the container. Predawn leaf water potential values remained above -0.8 MPa when measured at soil water contents higher than 5% and decreased sharply as SWC fell below 5% (Figure 1b). It must be pointed out that the values of SWC in Figure 1a are mean values. Soil water content fell below the critical level of 5% in several plants from both watering treatments throughout the study.

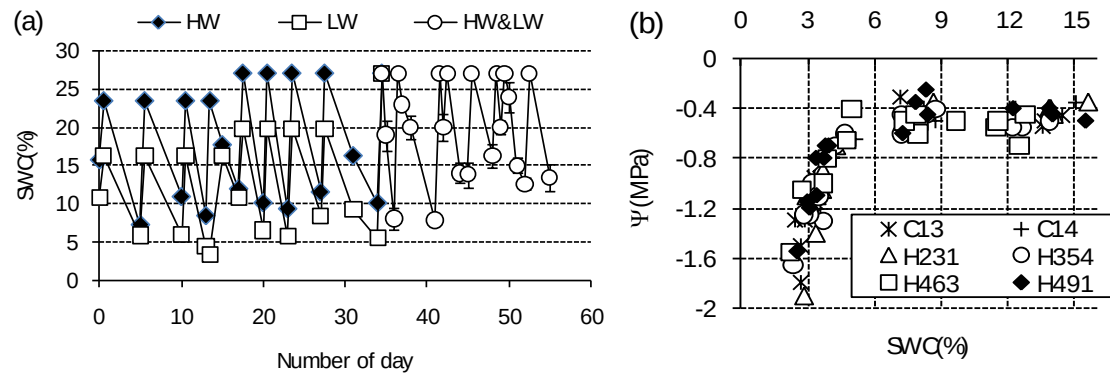


Figure 1 (a): Average values of volumetric soil water content (SWC) measured on ‘high-watered’ (HW) and ‘low-watered’ (LW) plants during watering treatment imposition (Days 0-34) and hydraulic measurements (Days 35-55). Error bars not visible indicate SE smaller than the symbol. (b): Relationship between volumetric soil water content and predawn leaf water potential, measured in six *E. globulus* clones on Day 9 (SWC>6%) and Day 14 (SWC<6%)

GROWTH, MORPHOLOGICAL AND ANATOMICAL TRAITS

There were no significant differences in leaf area between clones at the beginning of the watering treatments (Day 0). At the time the hydraulic conductance measurements were taken, there were significant differences in leaf area and in height both between clones ($P=0.0014$, $P=0.0001$) respectively and between watering treatments ($P<0.001$) (Table 2). The maximum growth in leaf area was measured in clones C13, H354 and H463, whereas the lowest leaf area growth was measured in the F_0 clone C14 (Table 3). Significant differences between clones were not found for biomass using time as covariate, despite of the fact that clone and watering treatment were significant factors in the ANCOVA analysis. Water shortage decreased biomass significantly (tables 2, 3). The comparison of shoot biomass per unit of leaf area across the three clones with higher leaf area (C13, H463 and H354), showed that the F_0 clone C13 yielded less biomass than F_1 clones per unit of leaf area. (Table 4)

Water shortage significantly decreased specific leaf area (SLA), both in main ($P=0.002$) and auxiliary leaves ($P=0.007$). (Table 5) Specific leaf area measured on auxiliary leaves was higher than that measured on main leaves (Table 4) as might be expected given that most auxiliary leaves were growing leaves. Clones C13 and C14 showed the highest and lowest SLA in main leaves respectively (Fig 2).

Table 2: ANCOVA table of dry shoot biomass (g), height and leaf area. Biomass was analysed using two different covariates: time (model 1) and leaf area (model 2)

Dependent variable (y)	→	biomass (g)			height (cm)		leaf area (cm ²)		biomass (g)		
<i>model (1): y=day +day*clone+day*clone*watering treatment (wt)</i>											
<i>model (2): biomass=leaf area +leaf area*clone+leaf area*clone*watering treatment (wt)</i>											
MODEL		1			1		1		2		
r ²		0.51			0.55		0.61		0.75		
Source		DF			DF		DF		Source		
Model	MS	12	8.972	12	800.022	12	1154143.33	Model	MS	13.33	
Error	MSE	69	1.475	91	84.526	90	97712.61	Error	MSE	0.77	
	pvalue	<.0001			<.0001		<.0001		pvalue	<.0001	
FACTORS											
day	MS	1	51.618	1	3485.993	1	10305130.0	leaf area	MS	46.33	
	pvalue	<.0001			<.0001		<.0001		pvalue	<.0001	
day*clone	MS	5	5.530	5	895.965	5	421661.82	leaf area *clone	MS	7.16	
	pvalue	0.0046			<.0001		0.0014		pvalue	0.0006	
day*wt	MS	1	21.564	1	2323.146	1	1870843.87	leaf area *wt	MS	3.49	
	pvalue	0.0003			<.0001		<.0001		pvalue	0.0410	
day*wt*clone	MS	5	1.716	5	70.132	5	122293.99	leaf area *wt*clone	MS	1.21	
	pvalue	0.3363			0.5318		0.2919		pvalue	0.2228	

Table 3: Parameter estimates of the model: $y = \text{day} + \text{day} * \text{clone} + \text{day} * \text{clone} * \text{watering treatment (wt)}$ using indicator variables to compare factor levels: clones and watering treatments. Estimates are calculated in relation to a reference factor level. The reference clone was C14 for height and leaf area and H491 for shoot biomass. In the case of watering treatment (wt) factor, low watered regime was the reference level.

height (model1)				leaf area (model1)			biomass (model1)			
Parameter	Estimate	SE	Pr > t	Estimate	SE	Pr > t	Parameter	Estimate	SE	Pr > t
Intercept	55.71	6.42	<.0001	244.04	222.7	0.2762	Intercept	-2.93	1.31	0.03
day	0.57	0.15	0.0002	41.18	5.12	<.0001	day	0.14	0.03	<.0001
day*clone C13	0.27	0.09	0.0044	12.01	3.11	0.0002	day*clone C13	0.010	0.01	0.37
day*clone H231	0.21	0.10	0.0322	4.69	3.32	0.1612	day*clone C14	0.018	0.01	0.16
day*clone H354	0.39	0.09	<.0001	7.88	3.18	0.0149	day*clone H231	0.007	0.01	0.60
day*clone H463	0.41	0.10	<.0001	8.00	3.31	0.0177	day*clone H354	0.022	0.01	0.06
day*clone H491	0.08	0.09	0.3792	3.68	3.11	0.2395	day*clone H463	0.021	0.01	0.08
day*clone C14	0.00	.	.	0.00	.	.	day*clone H491	0.00	.	.
day*wt HW	0.22	0.10	0.0241	7.64	3.32	0.0238	day*wt HW	0.03	0.01	0.03
day*wt LW	0.00	.	.	0.00	.	.	daynumber*wt LW	0.00	.	.

SE=standard error LW= low watered HW=high watered

Table 4: Parameter estimates of the model $biomass = leaf\ area + leaf\ area * clone + leaf\ area * clone * watering\ treatment$ using indicator variables to compare clones and watering treatments. Estimates represent the differences between the corresponding clone and the reference clone (H463). Only the three clones displaying higher and not significantly different leaf area were included in the analysis. Estimates represent the differences between the corresponding clone estimate and the reference clone (H463).

biomass =leaf area+leaf area*clone+leaf area*clone*watering treatment (wt)				
Parameter	Estimate	Standar Error	t Value	Pr > t
Intercept	-2.474802	0.99980	-2.48	0.0183
leaf area	-0.000389	0.00038	7.39	<.0001
Leaf area*clone C13	-0.000002	0.00016	-2.35	0.0243
Leaf area*clone H354	0.000000	0.00016	-0.01	0.9895
Leaf area*clone H463				

Reference group: H463

Table 5. Mean and standard error of specific leaf area (SLA) of auxiliary (a) and main (m) leaves, shoot height and shoot biomass measured in plants from six *E. globulus* clones.

	SLAa cm ² /g		SLAm cm ² /g	
	mean	stderr	mean	stderr
clone (p-value)	0.1254		<0.0001	
C14	242.73	6.04	190.19 c	3.5
H354	233.73	4.87	197.52bc	5.80
H491	248.88	5.65	198.01bc	4.30
H231	241.19	7.92	204.12bc	4.25
H463	247.27	6.27	217.78ab	6.29
C13	259.59	8.91	229.75a	6.04
watering regime (p-value)	0.0073		0.0021	
HW	252.97a	4.62	213.2a	4.14
LW	238.57b	3.10	200.51b	2.63
watering*clone	0.9565		0.4299	

Means with the same letter are not significantly different within a column;
HW=high watered plants, LW=low watered plants

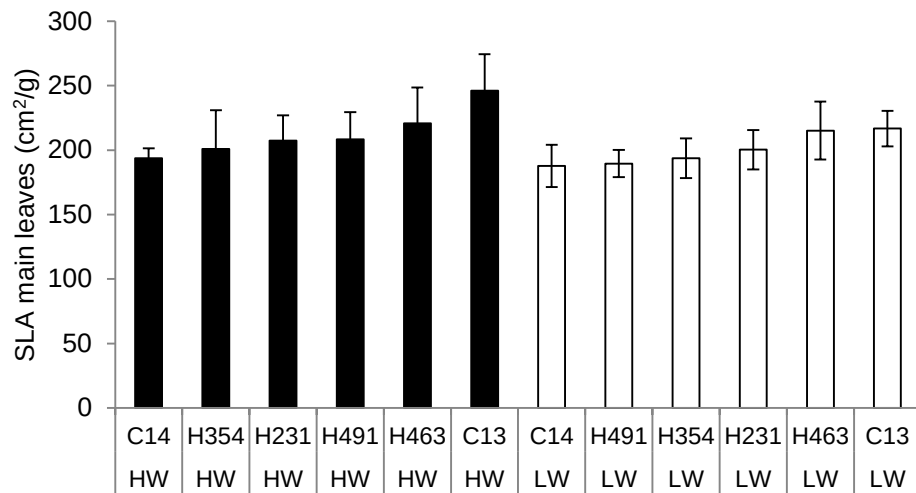


Figure 2: SLA specific leaf area of main shoot leaves per watering treatment and clone. Black and white bars represent high watered (HW) and low watered (LW) plants respectively.

Results of an ANCOVA analysis carried out on anatomical variables with stem cross section as covariate are presented in table 6. We found no watering treatment effect in any of the three anatomical variables tested. There were significant differences between clones in the total vessel lumen area per cross section unit (VLA). The highest values for VLA per unit of cross section were measured in clones C14 and C13 followed by F1 clones H463 and H354 and the lowest in the H231 and H491 clone. H231 proved the clone with the smallest vessels. H491 displayed the smallest number of vessel per cross section, although differences with the rest of the clones were not significant. Significant differences between clones were found in the vessel cross sectional area (xylem conducting area) to leaf area ratio ($P=0.01$), with no significant effect of time or treatment. The highest values for the vessel area to leaf area ratio were measured in clones C14, H463 and H354 and the lowest in clone H231 (Figure 3).

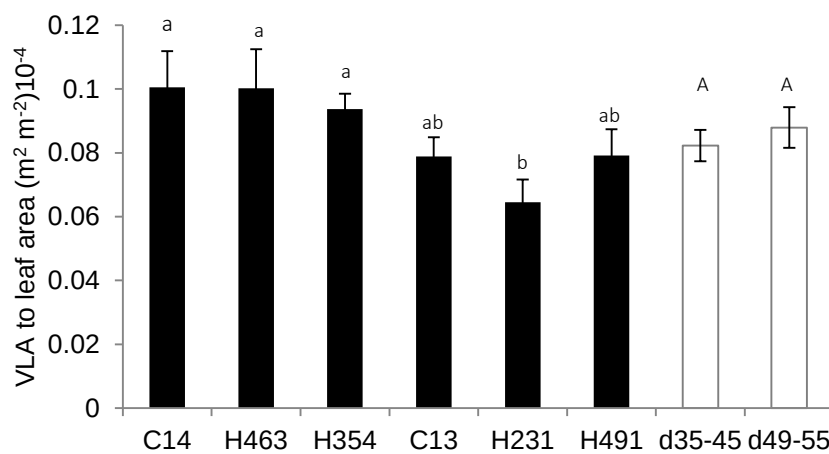


Figure. 3 Mean $\pm$ SE total vessel lumen area (VLA) to leaf area ratio measured in six *E. globulus* clones on Days 35-55 ($n=6-8$ plants clone⁻¹). Mean values across clones measured on Days 35-45 and Days 49-55 are also given. There were no significant differences between watering treatments and thus data for both watering regimes were pooled together

Table 6: ANCOVA results for three anatomical variables tested in five *E. globulus* clones using stem cross section as covariable . Different letters denote significant differences at $p < 0.05$ using Tukey mean test.

Dependent variable		Vessel number per cross section	mean vessel area (μm^2)	VLA (mm^2) (TOTAL VESSEL LUMEN AREA)
Source of variation	r ²	0.64	0.67	0.73
MODEL (DF)	MS	21837.75(8)	138504.46(8)	0.04046(8)
ERROR	MSE	3473.90(28)	19481.341(28)	0.00388(30)
	p-value	0.0001	<0.0001	<0.0001
FACTORS				
Covariate (cov)=stem cross section area (mm^2)	MSE	113539.92(1)	316584.6(1)	0.44015(1)
	p-value	<0.0001	<0.0001	<0.0001
Clone	MSE	4661.55(5)	699906.91(5)	0.08401(5)
	p-value	0.2759	0.0002	0.0044
Watering (wt)	MSE	6332.39(1)	4172.4435(1)	0.00062(1)
	p-value	0.1878	0.6471	0.6899
Clone*wt	MSE	2024.96(1)	7342.87(1)	0.000323(1)
	p-value	0.4516	0.5442	0.7747
Adjusted means (*) $\pm$ standard error				
C13		300.7 $\pm$ 27.4a	963.1$\pm$64.8a	0.299 $\pm$ 0.025 ab
C14		332.4 $\pm$ 25.4a	1081.8$\pm$60.2a	0.359 $\pm$0.027a
H 231		317.3 $\pm$ 22.8a	630.1$\pm$53.9b	0.211$\pm$ 0.023 b
H354		278.0 $\pm$ 24.7a	955.5$\pm$58.4a	0.276 $\pm$ 0.026 ab
H463		267.9 $\pm$ 28.6a	917.8$\pm$67.8a	0.254 $\pm$ 0.026 ab
H491		258.78 $\pm$ 22.7a	867.20 $\pm$ 53.85ab	0.235 $\pm$ 0.024ab

(*)Adjusted means $\bar{Y}_{adjusti} = \bar{Y}_i - \beta * (\bar{X}_i - \bar{X})$ where $\bar{Y}_i$ is the dependent variable mean of the i^{th} treatment, $\bar{X}_i$ is the covariate mean of the i^{th} -treatment, and $\bar{X}$ is the covariate overall mean value. β is a weighted average of the slopes of the linear regressions for all treatment groups.

HYDRAULIC TRAITS

On Days 35-45, a percentage loss of hydraulic conductivity (PLC) higher than 70% was measured in 36% of the plants (Figure 4a), whereas 74% of the plants measured on Days 49-55 exceeded this value (Figure 4b). There was a very significant ($P < 0.001$) effect of time and leaf water potential and no significant effect of clone or watering treatment on the percentage of plants with a PLC higher than 70%. The percentage of deviance explained by the model was 27%. Despite of the fact that the effect of clone was no significant it is interesting to note that clone H491 exhibited the highest increase in the percentage of plants that exceeded a PLC of 70% from Days 35-45 (37.5%) to Days 49-55 (100%). All the plants from clone H491 measured on Days 49-55 had a PLC higher than 70%. Moreover, more than half these plants had a leaf water potential higher than -1MPa at the time of measuring (Figure 4b), in contrast with the other clones, in which less than 25% of the plants had a water potential higher than 1MPa (Fig. 4b).

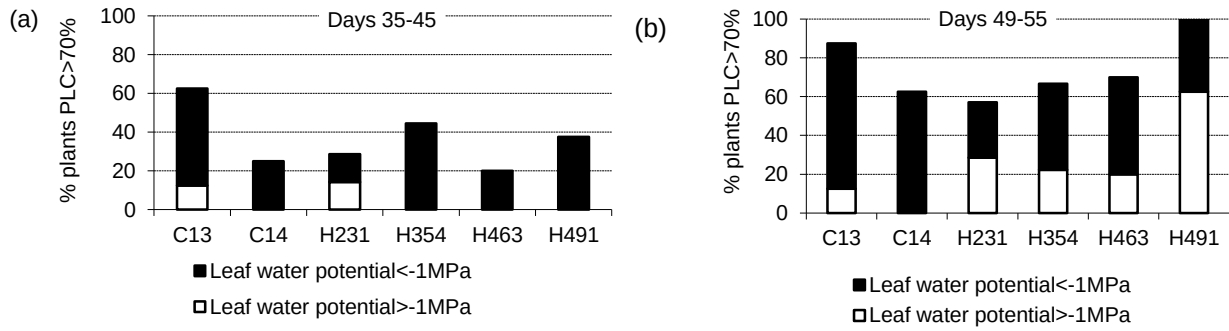


Figure 4 (a): Percentage of plants in which PLC was higher than 70% in Days 35-45 (n=54) and (b) Days 49-55 (n=48)

The maximum leaf specific hydraulic conductivity ($Q_{L,MAX}$) significantly decreased with time in both watering treatments ($p < 0.001$) (Table 7, Figure 5). Differences between watering treatments in $Q_{L,MAX}$ were only significant ($P = 0.03$) on Days 35-38, just after the end of watering treatments. There were significant differences between clones in $Q_{L,MAX}$ and a significant clone $\times$ time interaction. Maximum values of $Q_{L,MAX}$ were measured in plants from clone H463 on Days 35-38, Fig 5a. The lowest values of $Q_{L,MAX}$ were measured in the inbred clone (H491) on Days 49-55 (Figure 6). The smallest decrease in $Q_{L,MAX}$ from the second (Days 42-45) to the third week of measurements (Days 49-55) was measured in clone H231 (Figure 6).

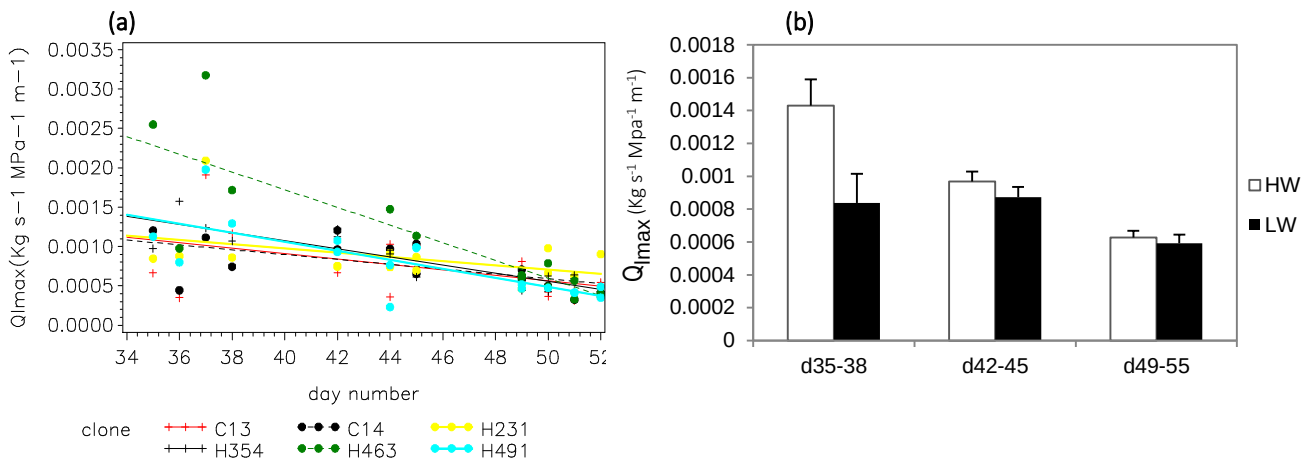


Fig. 5: (a) Plot of $Q_{L,MAX}$ values measured each day on six *Eucalyptus globulus* clones. Each data was measured on a single plant belonging to either the HW or LW watering regime. (b) Values of maximum leaf specific hydraulic conductivity ($Q_{L,MAX}$) averaged by week and watering treatment (wt)

Table 7: ANOVA table of maximum and native leaf specific conductivity (Q_{LMAX}) and Q_L respectively , considering watering treatment (wt), clone, time as categorical predictor (weeks) and interactions.

Q_{LMAX}				Q_L		
Source	DF	MS	Pr > F	DF	MS	Pr > F
<i>clone</i>	5	2.62E-7	0.0391	5	1.71E-7	0.0007
<i>wt</i>	1	1.58E-6	0.0002	1	6.7E-7	<0.001
<i>week</i>	2	3.44E-6	<.0001	2	1.45E-7	<.0001
<i>clone*wt</i>	5	1.36E-7	0.2762	5	6.8E-8	0.085
<i>clone*week</i>	10	2.59E-7	0.0134	10	1.14E-7	0.059
<i>wt*week</i>	2	5.67E-7	0.0066	2	3.74E-7	<0.0001

Df=degree freedom, MS: mean square, wt=watering treatment.

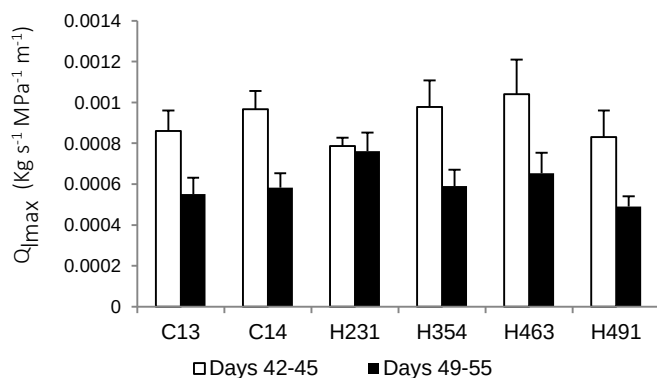


Figure 6: Average values of maximum leaf specific hydraulic conductivity (Q_{LMAX}) measured on six *E. globulus* clones on Days 42-45 and Days 49-55. As differences between watering treatments were not significant during this period of time, data from both treatments are pooled together

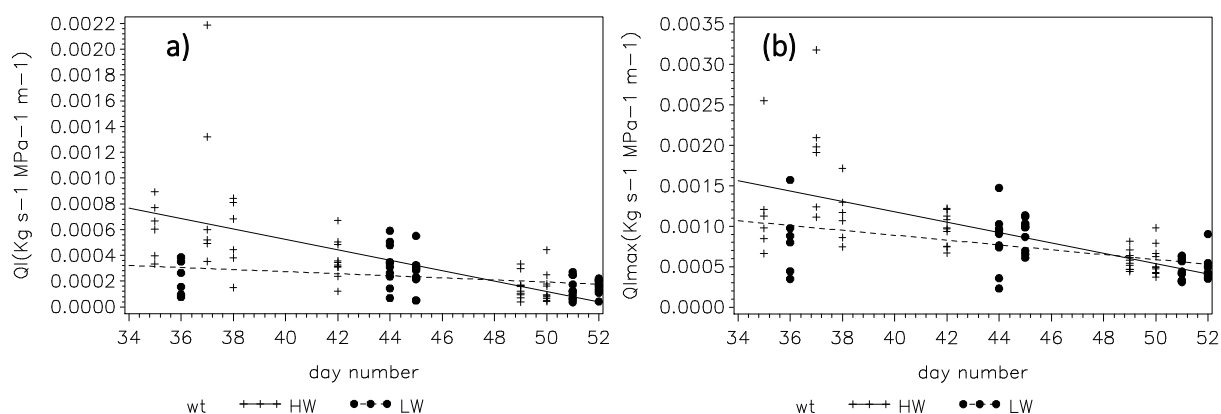


Figure 7: (a) Time course of native hydraulic conductivity (Q_L) and (b) maximum hydraulic conductivity (Q_{Lmax}) in both watering treatments (wt): LW=low watered plants (plus symbols and dashed line) and HW=high watered plants (black points and continuous line).

Native leaf specific hydraulic conductivity (Q_L) also decreased with time and water shortage. (Fig 7a). Values of native conductivity for both treatments converged on day 48. Q_L values for plants belonging to the more favourable watering treatment exhibitet a steepest decline over time than less watered plants, which kept their Q_L within a smaller range of values. The same was observed in Q_{Lmax} (Fig 7b).

PLC increased steadily over time from day 35 onwards in high watered plants whereas it remained almost constant or decreased slightly in low watered plants. Water potential remained between -0.9 and -1.2 MPa except for LW plants measured in the first week (Fig. 8b)

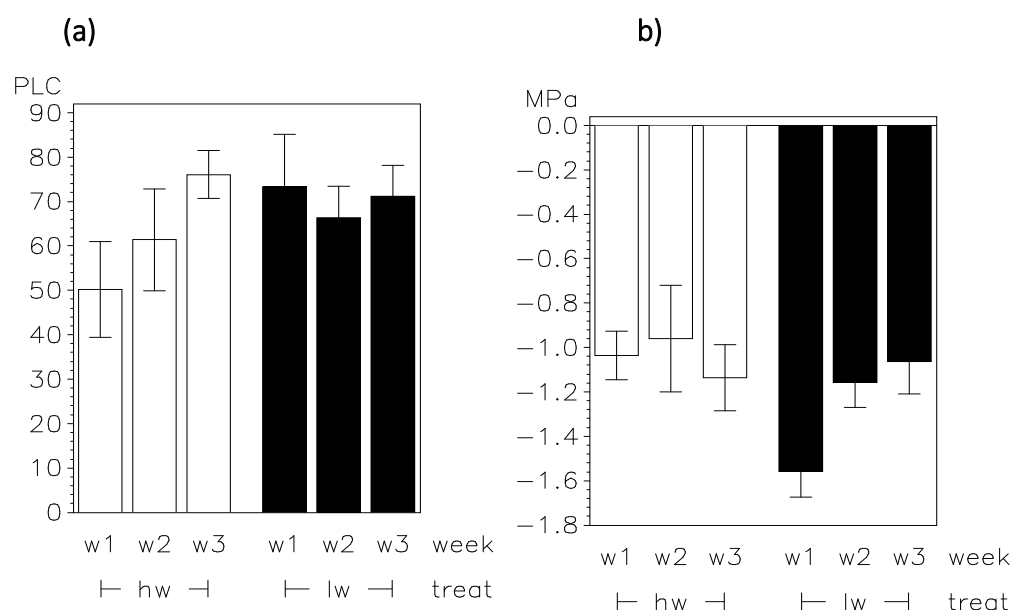


Figure 8: (a) PLC time course per watering treatment. (b) Evolution of xylem water potential in plants from both watering treatments (white bars correspond to high watered (hw) plants, whereas black bars correspond to low watered (lw) plants).

To investigate the possible reasons for the decrease in Q_{LMAX} over time we analyzed the differences in the distal stem cross-sectional area, vessel lumen area (VLA) and the diametric distribution of xylem vessels, between low watered plants measured on Days 35-45 and Days 49-55. Both the distal stem cross-sectional area and VLA were significantly larger in plants measured on Days 49-55 ($6.9 \text{ mm}^2 \pm 0.20$, $0.369 \text{ mm}^2 \pm 0.027$, mean $\pm$ SE) in comparison to those measured on Days 35-45 ($5.9 \text{ mm}^2 \pm 0.22$, $0.227 \text{ mm}^2 \pm 0.023$), as might be expected due to plants growth. Accordingly, the distribution of xylem vessels showed a shift towards larger vessels in plants measured on Days 49-55 in relation to plants measured on 42-45 Days (Figure 9). Given these results, an increase in hydraulic conductance would be expected according to the Hagen-Poiseuille equation (Hacke and Sperry 2001) in LW plants. However, the values of hydraulic conductance measured before and after embolism removal Q_L and Q_{Lmax} were lower in plants measured on Days 49-55 than in those measured on Days 35-45. These results suggest that decreases in Q_{LMAX} over time were due to factors affecting the path of water flow through the stem, such as, for example, the permeability of the pit pore membranes.

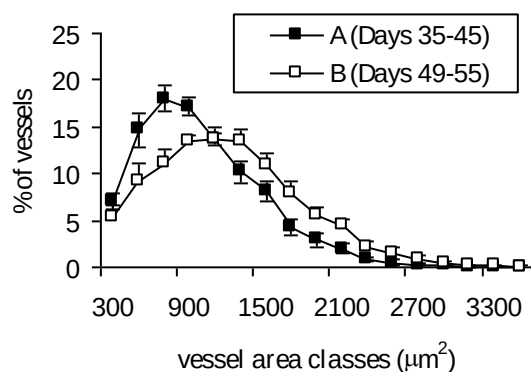


Figure 9 Vessel area distribution in the distal stem section of 20 'low-watered' plants (3-4 per clone), half of them used in hydraulic measurements carried out on Days 35-45 and the other half used in hydraulic measurements carried out on Days 49-55.

GAS EXCHANGE

Maximum values of stomatal conductance measured in F_0 and outcrossed F_1 clones were almost threefold the maximum value measured in the inbred clone (Fig 10), however, significant differences between clones were not found for any of the gas exchange variable tested. The maximum values of stomatal conductance were measured during the first two weeks of measurements (Days 35 and 43) and were significantly higher than those measured on Day 50 ($P=0.014$) (Figure 11).

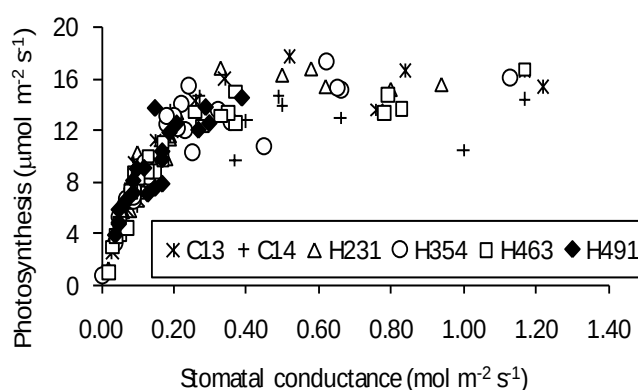


Figure 10 Relationship between net photosynthetic rate and stomatal conductance in six *E. globulus* clones. Data were measured on Days 35, 43, and 50

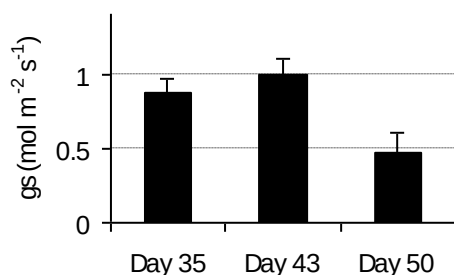


Figure 11 The average maximum values of stomatal conductance (gs) measured in all clones on Day 35, Day 43 and Day 50

DISCUSSION

The maximum growth in biomass and height was measured in two F_1 clones (H463 and H354) (Table 3) in the greenhouse, and the minimum growth was recorded in both F_0 clones C13 and C14 and in H491. Under field conditions clones H231, H463 and H354 proved to be those which yielded the highest volume of biomass whereas the lowest growth was recorded in the inbred H491, the survival and growth of which were strongly limited (Table 1). Such differences in growth between natural and semi-controlled conditions are not surprising. The use of potted plants implies that irrigation must be frequent and root growth limited, thus it is not possible to detect variations in terms of the ability of the different clones to explore the soil in search of water, which may be relevant in a deep rooting species such as *E. globulus*. The lowest leaf area growth rates were measured in clones C14 and H491 and the highest in clones C13 and H463. The lowest and highest values of specific leaf area (SLA) were measured in the same clones. Investment in leaf area is by far the most important factor in promoting growth during establishment (Pereira et al. 1995 in Pita et al. 2005), but it is at the expense of investment in other structures, for example wood density and roots. Decreasing SLA under drought conditions may improve water use efficiency, but is costly in terms of growth (Lambers et al. 2008), in fact SLA is usually correlated with relative growth rate (Shipley 2006). Both a high relative growth rate and high SLA are traits of invasive species, as has been reported in woody angiosperms (Grotkopp and Rejmanek 2007) and pinus species (Grotkopp et al. 2002), because a high specific leaf area enhances the opportunistic capture of light.

The values of maximum leaf specific hydraulic conductivity ($Q_{L,MAX}$) measured in the present study are similar to those reported for several *Eucalyptus* species (Vander Willigen and Pammenter 1998, Prior and Eamus 2000) or *Populus* species (Harvey and Van Den Driessche 1997, Sparks and Black 1999), but much higher than those reported for Mediterranean sclerophyllous shrubs (Vilagrosa et al. 2003) or Mediterranean evergreen trees (Tognetti et al. 1998). Species with a high $Q_{L,MAX}$ can afford to lose a relatively high percentage of hydraulic conductance (presumably due to the loss of the wider vessels), because it is not the percentage loss of conductance that limits gas exchange and growth but the remaining conductance (Vander Willigen et al. 2000). The highest values of $Q_{L,MAX}$ and Q_L were measured in plants from clone H463. Interestingly, the leaf area of this clone was one of the highest measured. It is worth noting that clone H463 has been rejected as a commercial clone; because of its tendency to produce epicormic shoots under harsh conditions. Therefore, a high Q_L or $Q_{L,MAX}$ alone may not be a sufficiently reliable selection criterion for improved growth in water-limiting environments.

A significant time x clone interaction was found for $Q_{L,MAX}$, e.g., the clonal ranking for this parameter changed over time (Table 7, Figures 5a and 6). During the last measurement period (Days 49-55) the highest and the lowest values of $Q_{L,MAX}$ were measured in the best (H231) and the worst (H491) clones respectively, according to field trials. Moreover H231 was the clone with narrowest vessels and H491 (inbred) the clone with lowest number of vessels. The disablement of a fixed number of vessels would involve a higher percentage of Q_{max} lost in the clone with less vessels and a smaller percentage in the clone with smaller vessels.

Immediately following the watering treatments (Days 35-38), values of Q_L and $Q_{L,MAX}$ measured in LW plants were significantly lower than those measured in HW plants (Figure 5b, 7). Similar results have previously been reported for this and other eucalypt species (Vander Willigen and Pammenter 1998, Prior and Eamus 2000, Pita et al. 2003). Decreases in hydraulic conductance are considered advantageous, since they can lead to a more gradual use of soil water (Alder et al. 1996, Sperry 2000) and prevent runaway cavitation.

Leaf specific hydraulic conductivity (Q_L) decreased (Figure 7a) and native PLC increased in high watered plants with no significant change in Ψ from the first to the last set of measurements (Figs 8a,b). Xylem vulnerability to cavitation may increase after cycles of cavitation and refilling that weaken pit pore resistance to cavitation, leading to higher values of PLC at moderate values of Ψ , a phenomenon that has been termed cavitation fatigue (Hacke et al. 2001, Stiller and Sperry 2002). Cavitation fatigue is related to the strength and durability of pit membranes. Both a “weakened response” and a “resilient response” have been found among woody species after a single cycle of drought and recovery (Alder et al. 1997, Hacke et al 2001), leading to a clear change in the shape of the vulnerability curve in weakened plants. Zwieniecki and Holbrook (1998), or Hacke et al. (2001) showed that cavitation and refilling can be considered current events in the xylem of at least some species, and Martorell et al 2013 reported refilling in *Eucalyptus pauciflora*. In our study the high PLC values found on the third week after treatments at values for potential of about -1 MPa could indicate the existence of fatigue, particularly in LW plants submitted to higher water stress. The increase in Q_L observed in LW plants from the first to the second week of measurements (Fig 7) may also suggest the existence of a certain degree of refilling.

Increases in PLC with time may also result from embolism accumulation throughout the experiment, under a rate of embolism generation higher than that of the potential of recovery one. LW plants were able to maintain g_s values over $0.8 \text{ mol m}^{-2} \text{ s}^{-1}$ with 80% of conductivity lost until the last week, when g_s decreased, what concurred with the convergence of the decreasing hydraulic conductivity for plants of both watering treatments, suggesting that the decrease of hydraulic conductance below a threshold value could have triggered the decline in stomatal conductance. Decreasing stomatal conductance with increasing PLC may allow leaf water potential to be maintained within a safe range, avoiding a catastrophic xylem failure and leading to a more gradual use of soil water (Jones and Sutherland 1991). This response seems particularly relevant under high vapour pressure deficits where leaf water potential may fall; even in the presence of abundant soil water and thus stomatal closure would not be triggered by ‘soil-drying signals’.

There were no apparent anatomical reasons for a decrease in $Q_{L,MAX}$ between Days 35-45 and Days 49-55, since both xylem conducting area and mean vessel size were larger in plants measured on Days 49-55 in relation to those measured previously. Previous studies (Van Ieperen et al 2000, Hacke and Sperry 2001, Domec et al. 2007, Van Ieperen 2007) show the importance of pit membranes in determining flow rates in the xylem. Moreover, pit pores in the vessel wall connect not only to other vessels but also to parenchyma cells (paratracheal parenchyma is abundant in *E. globulus*), which may be relevant to xylem refilling (Holbrook and Zwieniecki 1999, McCully 1999). Melcher et al. (2003), studying vulnerability to cavitation of individual vessels, reported a significant increase in vulnerability with vessel age and a decrease in hydraulic conductance in older annual rings in comparison to much more resistant

younger vessels. Our results support these findings, suggesting that changes in the sap flow path that increase xylem vulnerability to cavitation may also decrease maximum hydraulic conductance even over short periods of time. Recent studies concerning hydrated pit membranes suggest that the microfibrill network of pit membranes could be covered by a gel phase (Pesacreta 2005, Lee et al 2012 in Rockwell et al 2014). This led us to hypothesize in order to explain changes in Q_{Lmax} that plants could have suffered chemical changes in some pit membrane components that would have lead to a transient impermeabilization of pits that would confine air in embolized vessels and would keep sap from being driven through the impermeabilized pits, diminishing the connectivity of the xylem vessel network and thus the maximum conductivity, since many hydrogel sealed pits with pectin as components would have resisted the flushing of 80 Pa applied to remove embolism. Hong et al (2008) reported that the pore pressure in a hydrogel swelling in equilibrium is less negative than in solid microfibrills because the stress on the pit membrane fibril network covered by hidrogel is absorbed mainly by the molecules (solvent) that can migrate within the polymeric gel causing changes in shape and volume. The transient pit impermeabilization process would have progressed in our study with the rise in PLC, as did the decrease in Q_{Lmax} .

Clone H231 exhibited the lowest decrease in Q_{LMAX} between Days 42-45 and Days 49-55 (Figure 6) and the lowest Q_{LMAX} under the more favourable conditions of Days 42-45, when higher values of stomatal conductance were measured compared to Days 49-55. These results could be related with the fact that H231 displayed the narrowest vessels. According to the Hagen-Poiseuille equation, flow in xylem vessels is proportional to the fourth power of vessel radius. Therefore, the loss (due to embolism) or hindrance (due to a decrease in the permeability of the pit pore membranes) of flow in a high-conducting, wide xylem vessel should result in a greater decrease in the hydraulic conductance of the stem if compared to narrow vessels. Clone H491 had the second narrowest vessels, but in contrast to clone H231 it did exhibit a decrease in Q_{LMAX} between the second and third week of measurements. This result could be explained by the fact that H491 also had the lowest number of vessels per stem section. Although caution should be exercised when interpreting the results, given the reduced size of the sample, it is worth noting that in the present study only six plants had less than 200 vessels. Four of these plants belonged to the inbred clone (H491), which displayed the lowest survival rates under field conditions. Ewers et al. in a study of 2007 examined the adaptive value of vessel redundancy (number of vessels per organ) in order to increase the resistance of plants to water stress. They concluded that a high number of vessels would improve water stress tolerance. The frequency of vessels has been found to increase along a mesic-xeric gradient in several Mediterranean trees and shrubs (De Micco et al 2008), whereas decreased water availability resulted in a significant increase in vessel density in *Populus deltoides* x *Populus nigra* hybrids (Fichot et al 2009).

The maximum values of stomatal conductance measured in clone H491 were one third of those measured in the rest of the clones. Increased stomatal conductance may improve evaporative cooling capacity and heat tolerance. Therefore, reduced stomatal conductance and transpiration rate in the inbred clone (H491) may explain the lower values for growth and survival measured in field trials (Table 1) not only resulting from a decrease in photosynthesis but also because of a decrease in heat tolerance. It has been reported that inbreeding has a negative effect on survival and growth rates in tree species although no differences were detected in gas exchange or stable carbon isotope discrimination among

inbred and out-crossed black spruce (*Picea mariana* (Mill) B.S.P) trees (Johnsen et al. 1999, Johnsen et al. 2003). The lower values of stomatal conductance measured in the inbred clone in the present study lead to a slightly lower maximum value of photosynthesis in relation to other F₁ clones and, perhaps more importantly, a narrower margin for adjusting water use without a significant drop in photosynthesis (Figure 10), displaying less plasticity than the other five clones tested.

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CAPÍTULO 2



CAPÍTULO 2

THE EFFECT OF VAPOUR PRESSURE DEFICIT ON STOMATAL CONDUCTANCE, SAP PH AND LEAF-SPECIFIC HYDRAULIC CONDUCTANCE IN *Eucalyptus globulus* CLONES GROWN UNDER TWO WATERING REGIMES

RESUMEN

La conductancia estomática es considerada un factor clave en el estudio de la adaptación de las plantas al estrés hídrico. Las predicciones de cambio climático auguran un incremento de fenómenos meteorológicos extremos, que podrían comprometer la supervivencia de las plantaciones de *Eucalyptus globulus* situadas en el Suroeste de España. En este capítulo se investiga en qué medida las variaciones observadas en la conductancia estomática en respuesta al déficit de presión de vapor atmosférico se deben a la intermediación de señales hidráulicas o químicas en cinco clones de *Eucalyptus globulus* cultivados en invernadero.

Se plantaron bajo invernadero estaquillas ya enraizadas en envases de 5 l. a las que se sometió a dos regímenes de riego. En cada planta se tomaron mediciones consecutivas de conductancia estomática, potencial hídrico del tallo, pH de la savia y conductancia hidráulica. Estas mediciones se llevaron a cabo durante cuatro semanas en las que el rango de valores de déficit de presión de vapor osciló entre 0.42 y 2.25 kPa. También se midieron la evapotranspiración, el crecimiento del área foliar y la biomasa del tallo.

Se observaron diferencias significativas entre clones y diferentes tratamientos de riego en la conductancia estomática y en la conductancia hidráulica referida al área foliar pero no en el pH de la savia. El pH de la savia, el potencial hídrico y la conductancia estomática sufrieron un descenso durante unos días consecutivos en los que se incrementó el déficit de presión de vapor. No se encontró correlación significativa entre la conductancia estomática y la conductividad específica referida al área foliar. El cierre estomático evitó que el potencial hídrico bajara de -1.8 MPa. La pérdida de conductividad hidráulica se mantuvo entre valores de un 40% y un 85%. Los valores máximos y mínimos de conductividad hidráulica referida al área foliar se midieron en clones pertenecientes a las mismas familias. La escasez de agua produjo una reducción tanto en el crecimiento como en la evapotranspiración. La disminución de la transpiración osciló entre el 14 y el 32% en los cinco clones ensayados.

La alcalinización de la savia parece ser un mecanismo de respuesta a cambios en las condiciones atmosféricas más que a cambios en el contenido de agua en el suelo. El cierre estomático se produjo tras una pérdida importante de conductancia hidráulica. Se han encontrado diferencias intraespecíficas que sugieren la posibilidad de mejora de la producción mediante la selección en condiciones limitantes de agua combinadas con elevadas temperaturas en las primeras etapas del crecimiento.

THE EFFECT OF VAPOUR PRESSURE DEFICIT ON STOMATAL CONDUCTANCE, SAP PH AND LEAF-SPECIFIC HYDRAULIC CONDUCTANCE IN *Eucalyptus globulus* CLONES GROWN UNDER TWO WATERING REGIMES

Maria Jose HERNANDEZ², Fernando MONTES², Federico RUIZ³, Gustavo LOPEZ^{3,4}, Pilar PITA¹

(1) E.T.S.I. Montes, Universidad Politécnica de Madrid. Ciudad Universitaria s/n. 28040 Madrid, Spain.

(2) CIFOR-INIA, Ctra de la Coruña km 7.5, 28040 Madrid, Spain.

(3) Grupo Empresarial ENCE SA. Ctra A-5000 km 7.5. Apartado 223. 21007 Huelva, Spain.

(4) Current address: R&D Arara Abadi, Sinarmas Forestry, Riau, Indonesia

ABSTRACT

Stomatal conductance has long been considered of key interest in the study of plant adaptation to water stress. The expected increase in extreme meteorological events under a climate change scenario may compromise survival in *Eucalyptus globulus* plantations established in southwestern Spain. We investigated to what extent changes in stomatal conductance in response to high vapour pressure deficits and water shortage are mediated by hydraulic and chemical signals in greenhouse grown *Eucalyptus globulus* clones.

Rooted cuttings were grown in pots and submitted to two watering regimes. Stomatal conductance, shoot water potential, sap pH and hydraulic conductance were measured consecutively in each plant over four weeks under vapour pressure deficits ranging 0.42 to 2.25 kPa. Evapotranspiration, growth in leaf area and shoot biomass were also determined. We found a significant effect of both clone and watering regime in stomatal conductance and leaf-specific hydraulic conductance, but not in sap pH. Sap pH decreased as water potential and stomatal conductance decreased under increasing vapour pressure deficit. There was no significant relationship between stomatal conductance and leaf specific hydraulic conductance.

Stomata closure precluded shoot water potential from falling below -1.8MPa. The percentage loss of hydraulic conductance ranged from 40% to 85%. The highest and lowest leaf-specific hydraulic conductances were measured in clones from the same half-sib families. Water shortage reduced growth and evapotranspiration, decreases in evapotranspiration ranging 14%-32% in the five clones tested.

Sap alkalization appears to be a mechanism of response to changes in atmospheric conditions rather than soil water in the species. Stomata closed after a considerable amount of hydraulic conductance was lost, although intra-specific differences in leaf specific hydraulic conductance suggest the possibility of selection for improved productivity under water limiting conditions combined with high temperatures in the early stages of growth.

INTRODUCTION

Eucalyptus globulus Labill. is widely used for pulp production around the world and can be considered one of the most important eucalypt species given its high growth rate and pulping properties. *E. globulus* plantations established in SW Spain are submitted to both high temperatures and severe summer drought (Pita et al. 2001). The use of selected clones has improved both growth and survival under such limiting conditions. However, some of these

clones might fail under extreme meteorological conditions, as shown by the effects of the exceptional drought of 2005.

Stomatal conductance has long been considered of key interest in the study of plant adaptation to drought and high temperatures (Damour et al 2010, Grossnickle and Russel 2010, Pearce et al. 2005). This is particularly true for *E. globulus* plantations established under Mediterranean climates. Firstly, because high vapour pressure deficits may result in water stress even when soil water is abundant. Secondly, because *E. globulus* was found to reach its highest productivities through lower water-use efficiency in field trials established in southwestern Spain (Pita et al. 2001a). After analyzing the strong dependence of a wide range of photosynthetic parameters on stomatal conductance, Medrano et al. (2002) proposed the use of mid-morning, light-saturated stomatal conductance as a reference parameter to reflect the intensity of water stress. Stomata control several trade-offs that determine growth under water limiting conditions. Minimizing water loss by stomatal closure under drought conditions reduces CO₂ uptake and leaf cooling via transpiration but increases water use efficiency while allowing the plant to avoid low shoot water potentials. Stomata have long been recognized as an efficient means of controlling the risk of xylem embolism (Jones and Sutherland 1991), at least under non-extreme soil water deficits (Meinzer et al. 2009). In some species, stomata may close at the incipience of xylem embolism, as in walnut (*Juglans regia* x *nigra*) (Cochard et al. 2002). In other species, stomatal conductance and transpiration are maximized at the expense of a certain degree of embolism (Manzoni et al. 2013).

Both hydraulic and chemical signals participate in the regulation of stomatal conductance. Among others, stomata have been found to respond to cavitation-induced changes in stem hydraulic conductance (Tombasi et al 2015, Ripullone et al. 2007), the abscisic acid (ABA) concentration in the xylem sap (Heilmeyer et al. 2007, McAdam & Brodribb 2015) and changes in xylem sap pH: xylem sap alkalization (Sobeih et al. 2004, Wan et al. 2004).

Root-to-shoot signalling is often considered to be important in regulating shoot growth and water use when soil conditions change. Identifying signal molecules and their roles is seen as a potential way to modify crop water use (Dodd 2005). In contrast, root signalling has been considered less effective for very tall species, in which signal transmission may be too slow for a feed-forward model of short-term stomatal response and thus other factors, such as ABA production or release within the leaves may be more important (Heilmeyer et al. 2007). However, it must be considered that changes in xylem sap may arise from root export of signalling substances but also from changes in sap composition during long-distance transport in the stem (Dodd 2005). The vessel-associated cells from axial xylem parenchyma are those which are best suited to play a major role in the control of sap composition (Alves et al. 2004). In this sense, working with a species like *E. globulus* would seem particularly interesting, not only because it is capable of reaching both great heights and high transpiration rates, but also because it has abundant axial parenchyma in the xylem. The use of juvenile plant material seems appropriate since juvenile size classes tend to suffer more extreme stress because of their relatively shallow root systems (Matzner et al. 2003). Moreover, the highest values of stomatal conductance were found to be reached at early stages of growth in several tree species (Matzner et al. 2003, Mediavilla and Escudero 2003).

The objective of this study was to investigate the combined effects of high vapour pressure deficit (VPD) and water shortage on stomatal conductance in *E. globulus* clones. More precisely, we aimed to (a) investigate the extent to which changes in stomatal conductance are mediated by changes in hydraulic conductance and/or xylem sap pH in the species and (b) attempt to identify differences between clones in the response to water shortage and high VPD. We hypothesized that (1) xylem sap pH may increase with decreasing soil water; (2) xylem sap pH may respond to changes in VPD and (3) Hydraulic traits may differ between clones.

To test these hypotheses, a greenhouse experiment was carried out with closely related *E. globulus* genotypes of contrasting drought resistance.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWING CONDITIONS

The experiment was carried out in a greenhouse (15°C -35°C), from May to the end of June. Maximum photosynthetically active radiation (PAR) was $1600 \mu\text{mol m}^{-2} \text{s}^{-1}$. Air temperature and relative humidity (RH) were recorded with a Lambrecht thermo-hygrograph. Saturation vapour pressure (P_{sat}) was calculated at 2h intervals from air temperature (Nobel 2009) and VPD was derived from:

$$VPD = P_{\text{sat}} \left(1 - \frac{RH(\%)}{100}\right)$$

Vapour pressure deficit was highest at the end of the experiment (Fig.1).

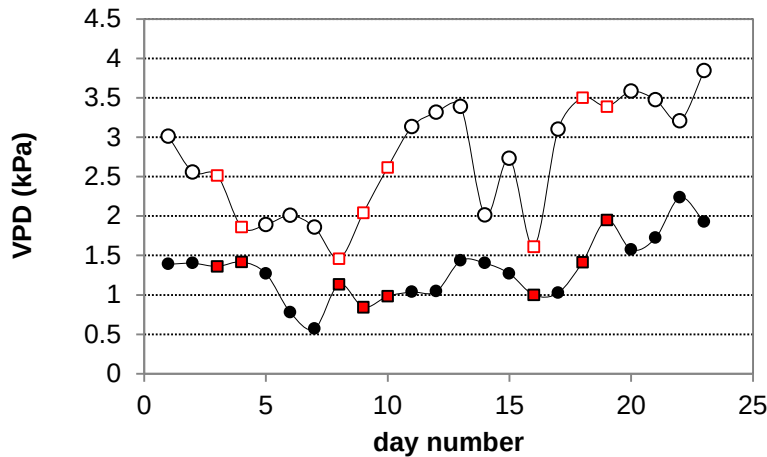


Figure 1: Time course of vapour pressure deficit inside the greenhouse. Empty symbols correspond to daily maxima and solid symbols to mid-morning values. Squares denote the days on which synchronous measurements of stomatal conductance, Ψ , sap pH and hydraulic conductance were carried out (VPD data not available for day d26)

Eighty *E. globulus* rooted cuttings grown from scions less than one-year-old from five different clones were transplanted to 5 l pots filled with the same weight of lightly fertilized peat (KEKKILÄ B6 white 420, Finland) mixed with perlite (1:1 v:v). Five extra pots were used to draw the relationship between the volumetric soil water content (H_{vol}) measured with a TDR

probe (Trime IMKO, Germany) and pot weight, covering the range of weight values in the experiment. The relationship between both variables ($H_{vol} = 0.0162W - 16.45$, $r^2 = 0.96$ $n=25$) was used to determine volumetric soil water content from the weight of the potted plants. Plant weight was considered negligible, since it was much lower than the pot weight (Figure 2). Clones T and OD are F_1 clones that had been widely used in commercial plantations in SW Spain. Interestingly, both clones differed in their response to the exceptional drought of 2005 (worst drought since 1947, Aemet 2005). Clone T was most affected and therefore withdrawn from production from then on. Clone C14 is a F_0 clone that shows an enhanced survival rate but lower growth rates than clones OD and T. Clones PI and SA are F_1 clones that belong to the same half-sib families as OD and T respectively, and were chosen for this study simply because of their shared affiliation with the others, clone C14 being the common progenitor.

After transplanting, plants were allowed to grow and acclimate for a period of three weeks. Plants were watered two to three times a week and fertilized twice with 1g plant⁻¹ of soluble Peters® (20:20:20) during this period. On May 31 (d0), two watering regimes (R1 and R2) were established. In the case of R1, the plants were watered until a weight of 2600 g was reached, while for R2 plants the figure was 2300 g. These values corresponded to 90% and 73% of volumetric soil water content at field capacity for R1 and R2 respectively. Plants from both watering regimes were watered up to these weights throughout the experiment. All plants were watered on Mondays, Wednesdays and Fridays except during the third week of measurements in which R2 plants were not watered on Wednesday to increase the level of water stress.

MEASUREMENTS

The amount of water lost by evapotranspiration was calculated between irrigations from the weight of each plant before and after watering.

Synchronous measurement of stomatal conductance, sap pH, Ψ and hydraulic conductance (physiological parameters hereafter) were carried out plant by plant on days d3, d4, d8, d9, d10, d16, d18 d19, d23 and d26. From day d3 to day d10, six plants per day (three from each watering regime, from the same clones) were measured and harvested. On days d16-d19 the sample size was increased up to ten plants per day (one per clone and watering regime). Ten plants from one single watering regime (two per clone) were measured on d23 (R1) and d26 (R2) in order to establish whether the moment at which the measurements were taken had a significant effect on the physiological parameters.

Stomatal conductance to water vapour and net photosynthetic rate were measured in the youngest fully expanded leaf using a portable gas exchange chamber Li-Cor 6400XT (Li-Cor; Lincoln, USA). All measurements were made between 11:00 and 13:00 hours (local time), under 300 W metal halide lamps to ensure a PAR above 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. We later verified that there was no significant relationship between the rate of photosynthesis and PAR values in the range 1000-1600 $\mu\text{mol m}^{-2} \text{s}^{-1}$, meaning that light intensity could be considered saturating for all measurements.

Immediately after measuring gas exchange, each plant was taken to the laboratory (less than five minutes from the greenhouse), weighed with its container and cut under water just below the 6th-7th node. Time of harvesting was annotated for each plant. Water potential (Ψ) was determined in the shoot apex using a Scholander type pressure chamber (Plant Moisture

Systems, Santa Barbara, CA, USA). Prior to this, about 3 cm of bark was removed from the cut end of the apical portion of the stem. After recording Ψ , an over pressure of 0.2-0.4 MPa was applied to leaves in order to collect xylem sap. Xylem sap pH was measured immediately afterwards using a microelectrode (Model 5208, CRISON Instruments) interfaced with a pHmeter (CRISON microPH 2002). A similar procedure has been used previously in shoots (Dodd et al. 2003) and leaves (Rodrigues et al. 2008).

At the same time as the water potential and sap pH were measured, the basal portion of the plant was prepared for hydraulic conductance measurements: all leaves were cut off under water and the stem was fixed to a tubing system connected to a low-pressure water reservoir. Hydraulic conductance was determined before (k_i) and after (k_M) removing xylem embolism as explained elsewhere (Pita et al. 2003). The percentage loss of hydraulic conductance (PLC) was calculated from:

$$PLC = 100 \frac{k_M - k_i}{k_M}$$

To determine plant leaf area (WinFolia, Regent Instruments, Canada), the leaves in the apical portion (Lap) were separated from the basal leaves. Leaf specific hydraulic conductance (LSC) was calculated from:

$$LSC = \frac{k_i}{L_{ap}}$$

Stems and leaves were oven dried at 60°C and weighed.

STATISTICAL ANALYSES

Analysis of covariance (ANCOVA) was used to analyze the effect of clone and watering regime on all variables. Either time (day number) or leaf area were used as continuous predictors when analyzing differences in growth and evapotranspiration, whereas vapour pressure deficit was used as a continuous predictor when analyzing differences in the physiological parameters. Multiple linear regression analysis using indicator variables was employed to assess differences between clones (Seltman 2015) when interactions between factors were found, and adjusted means were used otherwise.

ANCOVA was also used to verify that there was no significant effect of the time of harvesting on the physiological parameters, using the time of harvesting (HH:MM) as continuous predictor. The significance of the relationships between stomatal conductance and other physiological parameters was also analyzed with an ANCOVA analysis. Relationships between variables were analyzed through simple linear regression. The effect of the clone or watering regime was also tested on a daily basis through one-way ANOVA.

Percentage data were arcsin transformed prior to analyses. All variables were tested for normality and homogeneity of variance. Differences were considered statistically significant at

$P \leq 0.05$. Tukey's and LSD method was used to separate the means. The 9.2 software version of SAS (SAS Institute Inc. 1989) was used for all tests.

RESULTS

GROWTH AND EVAPOTRANSPIRATION

Shoot biomass, leaf area and the total amount of water lost by evapotranspiration increased linearly with time ($P < 0.0001$ for all three variables)(Table 1). Water shortage significantly decreased growth in leaf area, shoot biomass and evapotranspiration (Table 1). Figure 2 shows the time course of average shoot biomass for both watering regimes. Similar results were obtained for leaf area or evapotranspiration.

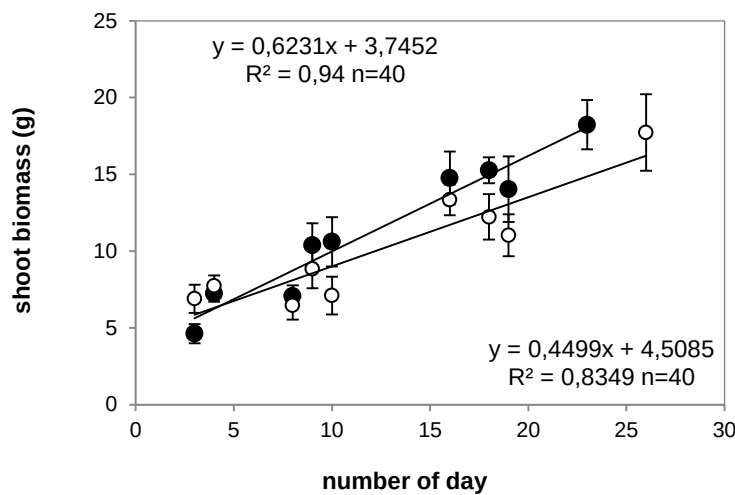


Figure 2: Time course of average shoot biomass for both watering regimes. Open symbols stands for R2 watering treatment and solid symbols correspond to treatment R1.

The mean rate of evapotranspiration measured between irrigations in R1 plants increased from $212 \pm 7.5 \text{ g day}^{-1}$ at the beginning of the experiment up to $397 \pm 20.8 \text{ g day}^{-1}$ at the end. The total amount of water lost by evapotranspiration increased linearly with time.

Significant interactions time x watering treatment and time x watering treatment x clone were found in the three growth variables (table 1, fig 3), thus clone effect was analyzed separately per treatment (table 2). We found significant differences in evapotranspiration between clones under the less favourable water regime ($p=0.0018$), the lowest evaporation values corresponded to PI and the highest to T (Table 3). Although no significant global clone effect was identified for evapotranspiration under the R1 treatment (p -value 0.08), differences between C14, with the lower evapotranspiration and the rest of the clones were significant (table 3). We also found significant clone effect in biomass and leaf area in well-watered plants ($p=0.0026$ and 0.0147) respectively, but not in stressed plants (table 2). Significant differences were found between the clone C14 and the rest of the clones in leaf area and biomass yield, C14 being the clone with the lowest biomass yield and the smallest leaf area. (Table 3

Table 1: Results of the ANCOVA for growth variables. Time was used as covariate to analyze all variables and leaf area was also used as covariate to analyze ETP. Different letters denote significant differences at $p < 0.05$.

Dependent variable (Y)	DF	ETP* (g/plant)	ETP* (g/plant)	leaf area (mm ² /plant)	dry biomass (g/plant)
ANCOVA MODEL	(1)	$Y = day + day * trat + day * trat + day * trat * clone + error$			
	(2)	$Y = covariable + clone + wt + clone * wt + error$			
Model used		(1)	(2)	(2)	(2)
r ²		0.87	0.9	0.71	0.71
Source of variation					
MODEL	MS	10	388893727.6	403374757.9	35107945.78
ERROR	MSE	66	59478670.8	43610749.8	14527000.22
	p-value		<0.0001	<0.0001	<0.0001
FACTORS					
Covariate	MSE	1	327333222.8	28298879.90	1217.39
(cov)=time	p-value		<0.0001	<0.0001	<0.0001
cov=leaf area	MSE	1	343115067.8		
	p-value		<0.0001		
Clone	MSE		7411236.5		
	p-value		0.0326		
Watering (wt)	MSE		1665372.5		
	p-value		0.1172		
Clone*wt	MSE		1066376.0		
	p-value		0.8055		
cov*treatment	MSE	1	40367347.9	3178125.71	90.58
	p-value		<0.0001	0.0003	0.0028
Cov*wt*clone	MSE	8	24384639.4	4735354.85	235.51
	p-value		<0.0023	0.0127	0.0046
CLONE/Watering treatment		Adjusted means ± standard error**			
C14		4598.29 ± 212.55a			
OD		4386.65 ± 204.9ab			
PI		4337.54 ± 210.67ab			
SA		3696.7 ± 203.9b			
T		4042.71 ± 211.59ab			
R1		4365.18 ± 136.35a			
R2		4059.60 ± 131.20a			

Adjusted means $\bar{Y}_{adjusti} = \bar{Y}_i - \beta * (\bar{X}_i - \bar{X})$ where $\bar{Y}_i$ is the dependent variable mean of the i^{th} treatment, $\bar{X}_i$ is the covariate mean of the i^{th} -treatment, and $\bar{X}$ is the covariate overall mean value. β is a weighted average of the slopes of the linear regressions for all treatment groups. R1: watering treatment (90% of field capacity), R2 (73% of field capacity)

Table 2: ANCOVA table of growth variables analyzed separately per treatment using the model
 $Y = \text{day} + \text{clone} \cdot \text{day}$

Variable	Source	df	Sum of Squares	Mean Square	F-value	Pr>F
Model variable=day+clone*day						
WATERING TREATMENT=R1 (90 % FIELD CAPACITY)						
ETP	Model ($r^2=0.84$)	5	1093523857	218704771	193.4	<.0001
	Error	2	36173577	1130424		
	Corrected Total	7	1129697434			
Leaf area	Model ($r^2=0.65$)	5	183731322.8	36746264.6	135.5	<.0001
	Error	32	8675199.3	271100		
	Corrected Total	37	192406522.1			
Biomass	Model ($r^2=0.69$)	5	6952.3	1390.5	147.6	<.0001
	Error	32	301.4	9.45		
	Corrected Total	37	7253.8			
WATERING TREATMENT=R2 (73% FIELD CAPACITY)						
ETP	Model ($r^2=0.87$)	5	162869043	32573808.6	49.7	<.0001
	Error	35	22940974.9	655456.4		
	Uncorrected Total	40	185810018			
Leaf area	Model ($r^2=0.65$)	5	125577775.7	25115555.1	62.41	<.0001
	Error	35	14085334	402438.1		
	Corrected Total	40	139663109.7			
Biomass	Model ($r^2=0.69$)	5	183731322.8	36746264.6	135.5	<.0001
	Error	32	8675199.3	271100		
	Corrected Total	37	192406522.1			
FACTORS						
WATERING TREATMENT=R1 (90% FIELD CAPACITY)						
ETP	day	1	1077532322	1077532322	953.2	<.0001
	day*clone	4	10566312	2641578	2.34	0.0765
Leaf area	day	1	181023856.4	181023856.4	667.7	<.0001
	day*clone	4	3440014.5	860003.6	3.17	0.0265
Biomass	day	1	6867.09	6867.09	728.9	<.0001
	day*clone	4	170.1	42.5	4.52	0.0053
WATERING TREATMENT=R2						
ETP	day	1	623829488.1	623829488.1	946.1	<.0001
	day*clone	4	13758074.7	3439518.7	5.22	0.002
Leaf area	day	1	119881854.5	119881854.5	297.8	<.0001
	day*clone	4	1906598.3	476649.6	1.18	0.3347
Biomass	day	1	4841.3	4841.3	293.8	<.0001
	day*clone	4	75.13	18.8	1.14	0.3538

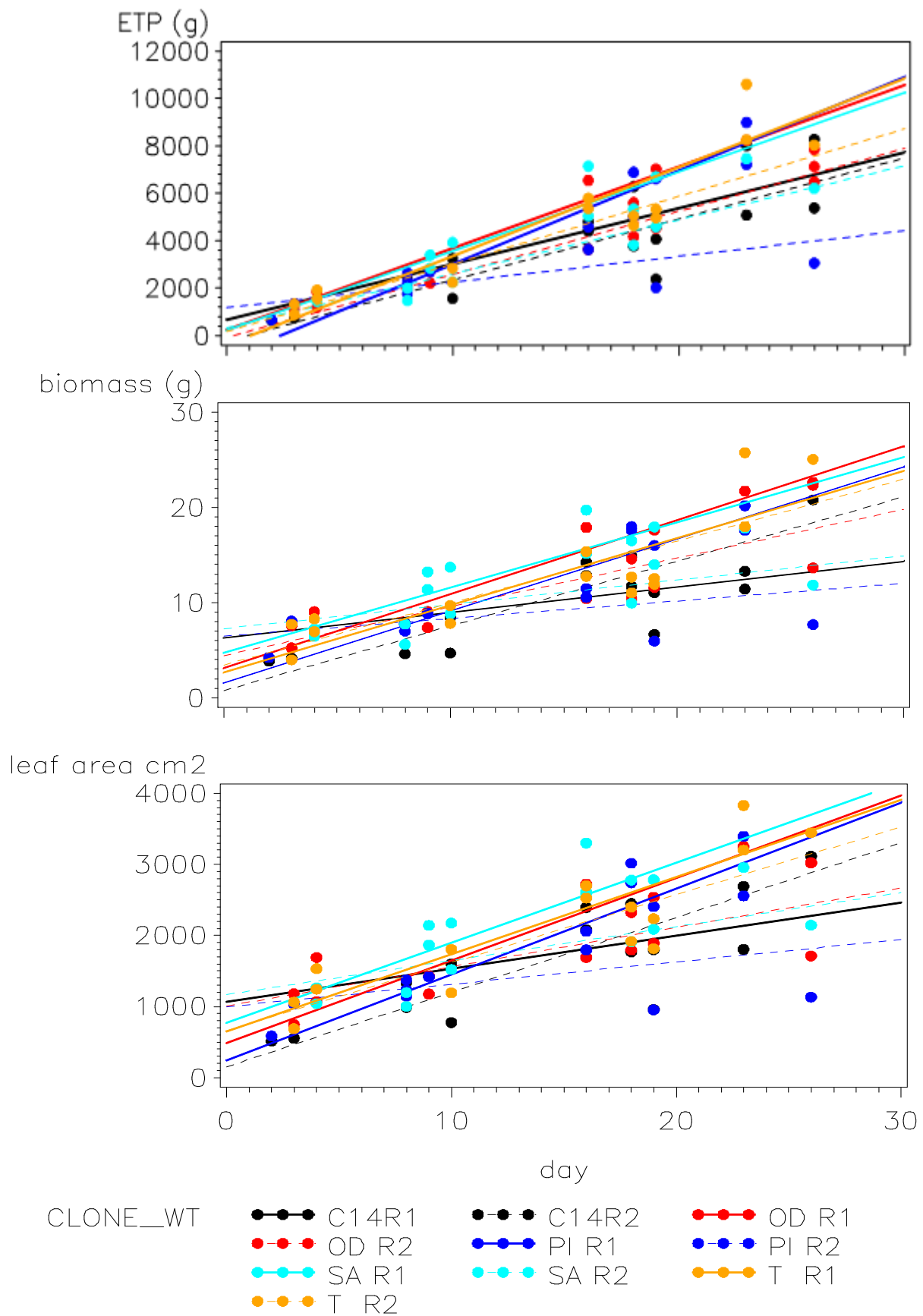


Figure 3: Time course of evapotranspiration (ETP) (above), biomass per plant (g) (in the middle), and leaf area by clone and watering treatment (clone_wt) (down). R1 (90% field capacity), R2 (73% field capacity). Dashed lines represent plants undergoing R2 water regime.

Table 3: Parameter estimates obtained from multiple regression analysis using indicator variables to test differences between clones. The model used was: $\text{variable} = \text{day} + \sum (\text{day} * \text{clone}_i * I_{j-1})$, where j =levels of factor clone, I =indicator variable $I_i=1$ when $\text{clone}=\text{clone}_i$, otherwise $I_i=0$. Reference groups were clone C14 for treatment R1 and clone T for R2, such that estimates represent the difference in model estimates between the corresponding clone and the reference clone

Variable	Parameter	Estimate	Standard Error	t Value	Pr > t	Parameter	Estimate	Standard Error	t Value	Pr > t
WATERING TREATMENT =R1 (90% FIELD CAPACITY)						WATERING TREATMENT =R2 (73% FIELD CAPACITY)				
ETP	day	272.4	22.8	11.9	<.0001	day	294.51	19.46	15.14	<.0001
	day*clone OD	86.2	35.2	2.4	0.02	day*clone OD	-32.09	24.35	-1.32	0.1958
	day*clone T	81.3	32.4	2.5	0.0176	day*clone C14	-48.88	25.41	-1.92	0.0623
	day*clone PI	72.3	32.4	2.2	0.0326	day*clone PI	-119.42	27.45	-4.35	0.0001
	day*clone SA	75.3	34.2	2.2	0.0353	day*clone SA	-47.12	27.01	-1.75	0.0894
	day*clone C14	0 (reference clone)				day*clone T	0 (reference clone)			
Leaf area	day	104.3	11.1	9.3	<.0001	day	132.09	15.20	8.69	<.0001
	day*clone OD	40.2	17.2	2.33	0.026	day*clone OD	-28.61	19.02	-1.50	0.1416
	day*clone T	39.8	15.9	2.5	0.0176	day*clone C14	-18.45	21.34	-0.86	0.3931
	day*clone PI	29.8	15.8	1.88	0.0688	day*clone PI	-43.79	21.45	-2.04	0.0487
	day*clone SA	55.7	16.7	3.32	0.0022	day*clone SA	-15.81	21.10	-0.75	0.4587
	day*clone C14	0 (reference clone)				day*clone T	0 (reference clone)			
Biomass	day	0.61	0.06	9.22	<.0001	day	0.84	0.10	8.64	<.0001
	day*clone OD	0.35	0.1	3.43	0.0017	day*clone OD	-0.12	0.12	-0.96	0.3456
	day*clone T	0.24	0.09	2.59	0.0145	day*clone C14	-0.12	0.14	-0.87	0.3899
	day*clone PI	0.23	0.09	2.47	0.0189	day*clone PI	-0.29	0.14	-2.09	0.0437
	day*clone SA	0.37	0.09	3.73	0.0008	day*clone SA	-0.16	0.14	-1.19	0.2439
	day*clone C14	0 (reference clone)				day*clone T	0 (reference clone)			

PHYSIOLOGICAL PARAMETERS

Increasing maximum VPD inside the greenhouse (Fig. 1) led to a decrease in both Ψ and mid-morning stomatal conductance in plants from both watering regimes the last days of the study. Stomatal conductance and Ψ remained above $0.5 \text{ mol m}^{-2} \text{ s}^{-1}$ and -1.4 MPa respectively from d3 to d16 and fell below these threshold values from d18 onwards (Fig. 4a,b). Xylem sap pH tended to decrease as stomatal conductance decreased (Fig. 4a,c), whereas PLC remained high and stable throughout the experiment (except for plants from treatment R2 on d4).

No significant effect of the watering regime on xylem sap pH was found on any of the measurement occasions (Fig. 4c, Table 4). This result was particularly striking for d18 and d19, when R2 plants were submitted to a four-day drought cycle, while R1 plants were watered every two days. Stomatal conductance was significantly higher in R1 plants compared to R2 plants on d18 and d19 (Fig.4a). Despite the differences in soil water and stomatal conductance, xylem sap pH values were almost identical for both watering regimes on both days (Fig. 4c).

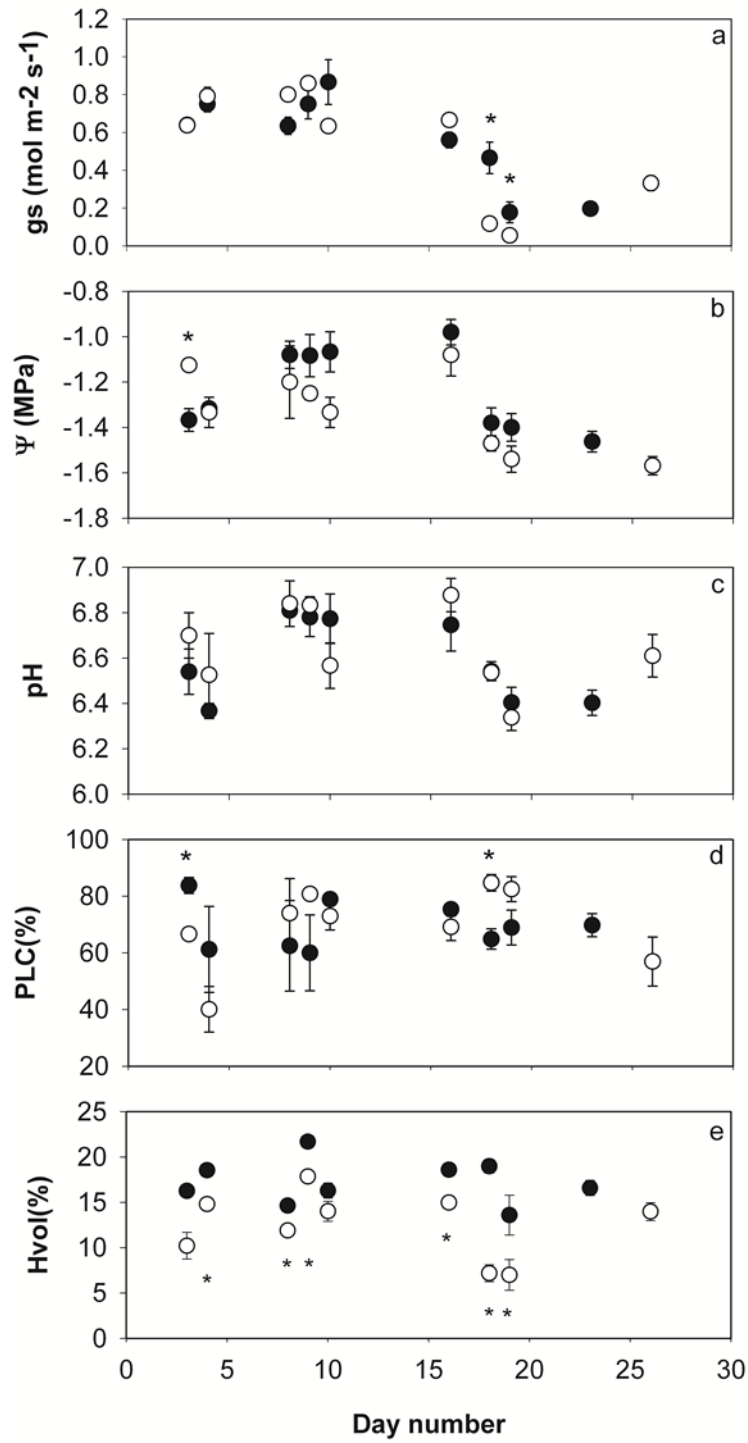


Figure 4: Daily mean $\pm$ SE values of (a) Light-saturated mid-morning stomatal conductance, (b) water potential, (c) xylem sap pH (d) percentage loss of stem hydraulic conductance (e) Mean volumetric soil water content for the plants measured each day. Data are means of 3-10 observations for two watering regimes, R1 (filled symbols) or R2 (empty symbols). The asterisk denotes significant differences between watering regimes.

Results from the ANCOVA revealed a significant effect of VPD on all variables tested except PLC (Table 4). There was a tight coordination in the response of stomatal conductance, xylem sap pH and Ψ to VPD (Fig. 5). Stomata closure precluded Ψ from falling below -1.8MPa throughout the experiment (Fig 5b,c). Xylem sap pH decreased as VPD increased (Fig 5a) but there were no significant differences between either clones or watering regimes in xylem sap pH, nor was there a significant clone x watering regime interaction (Table 4). There was a significant effect of the watering regime on stomatal conductance, water potential and LSC. We found significant differences between clones in stomatal conductance and LSC. The effect of clone x watering regime was not significant for any of the physiological variables (table 4).

Table 4: ANCOVA results used for physiological variables. Different letters denote significant differences at $p < 0.05$ (*) ETP= evapotranspiration per plant

Dependent variable (Y)		Ψ (MPa)	pH	gs* (mol m ⁻² s ⁻¹)	LSC* (Kg s ⁻¹ m ⁻² MPa ⁻¹)	PLC* (%)
Model	Y=covariable+clone+wt+clone*wt+error					
Source of variation						
	r2	0.48	0.46	0.62	0.35	0.116
MODEL	MS	0.169	0.1815	0.3514	4.77E-08	2265.8
ERROR	MSE	0.028	0.0333	0.0346	1.73E-08	226.58
	p-value	<.0001	<.0001	<.0001	0.0083	0.6003
FACTORS						
cov= DPV	MSE	1.3929	1.495	3.096	9.02E-08	26.89
	p-value	<.0001	<0.0001	<0.0001	0.0265	0.7545
Clone (α_i)	MSE	0.0065	0.026	0.108	6.72E-08	279.46
	p-value	0.9209	0.5427	0.021	0.0077	0.4012
Watering (wt)	MSE	0.3784	0.006	0.223	9.27E-08	71.25
	p-value	0.0005	0.6733	0.0135	0.0246	0.6109
Clone*wt	MSE	0.0328	0.024	0.002	7.48E-09	247.66
	p-value	0.3364	0.5832	0.9935	0.7841	0.4644
Clone/watering treatment		Adjusted means				
C14		-1.328±0.045 a	6.59±0.048a	0.338±0.051b	(35.18±3.67)E-05ab	70.94±4.39a
OD		-1.333±0.042 a	6.54±0.046a	0.501±0.047ab	(40.18±3.85) E-05a	65.90±3.94a
PI		-1.352±0.045 a	6.64±0.049a	0.414±0.050ab	(40.91±3.66) E-05a	67.64±4.24a
SA		-1.322±0.042 a	6.61±0.045a	0.515±0.046ab	(23.75±3.54) E-05b	77.76±3.93a
T		-1.293±0.043 a	6.56±0.047a	0.559±0.048a	(29.93±3.98) E-05ab	70.29±4.05a
R1		-1.253±0.027a	6.58±0.030a	0.522±0.030a	(37.93 ±2.4)E-05a	70.34±2.55a
R2		-1.398±0.028b	6.59±0.030a	0.409±0.032b	(30.05±2.4) E-05b	70.67±2.66a

Adjusted means $\bar{Y}_{adjusti} = \bar{Y}_i - \beta * (\bar{X}_i - \bar{X})$ where $\bar{Y}_i$ is the dependent variable mean of the i^{th} treatment, $\bar{X}_i$ is the covariate mean of the i^{th} -treatment, and $\bar{X}$ is the covariate overall mean value. β is a weighted average of the slopes of the linear regressions for all treatment groups

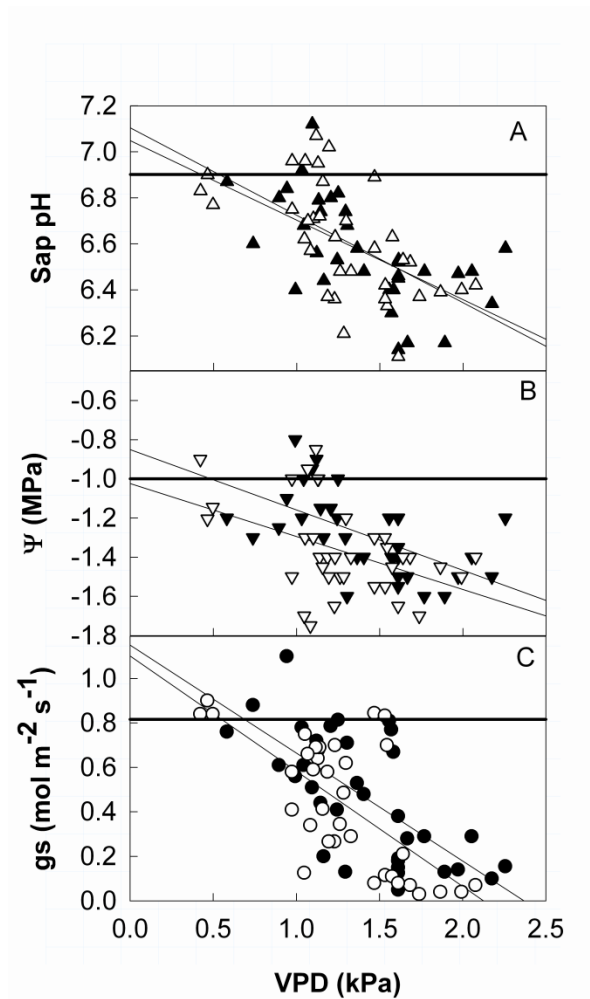


Figure 5: Relationships between air vapour pressure deficit inside the greenhouse at the time of measuring and (a) sap pH, (b) shoot water potential and (c) stomatal conductance. Every point within each graph corresponds to data measured on one single plant. Filled symbols correspond to plants under the more favourable watering regime (R1) and empty symbols to the less favourable R2 watering. Regression lines were fitted to both watering regimes. The horizontal thick line in each graph signals the value for the 90th percentile calculated for each parameter, pooling together data from both watering regimes (n=70).

Qualitative analysis of stomatal conductance showed that both the lowest median and lowest 75% percentile were measured in clone C14 (Fig. 6d). The highest stomatal conductance for the 25% percentile was measured in clone T (Fig. 6d). The latter result shows that under the most stressful conditions plants from clone T did not close stomata as efficiently as the others. In accordance with these results, Tukey's test only showed significant differences in stomatal conductance between clone C14 and clone T (Table 1). These results are also in accordance with evapotranspiration data, since mean evapotranspiration in clone T was significantly higher than evapotranspiration in C14 (table 3). The lowest pH value for the 75% percentile was measured in clone T, for which a pH higher than 6.6 was measured only in 25% of the plants (Fig. 6c). Median PLC values were between 70% and 80% for all the clones (Fig 6b). The lowest LSC values for the 25% percentile were measured in clones T and SA. The smallest interquartile range corresponded to this later clone (Fig. 6a). The highest LSC median was measured in clone OD. Interestingly, LSC values measured in clones OD and PI were significantly different from the rest of the clones (Table 4). These clones belong to the same half-sib family.

For all clones and treatments combined, there was a highly significant relationship between stomatal conductance and values of Ψ ($r^2=0.34$, $P<0.0001$) or sap pH ($r^2=0.21$, $P=0.0004$) and no relationship with either PLC ($r^2=0.04$, $P=0.08$) or LSC ($r^2=0.007$, $P=0.51$) (table 5).

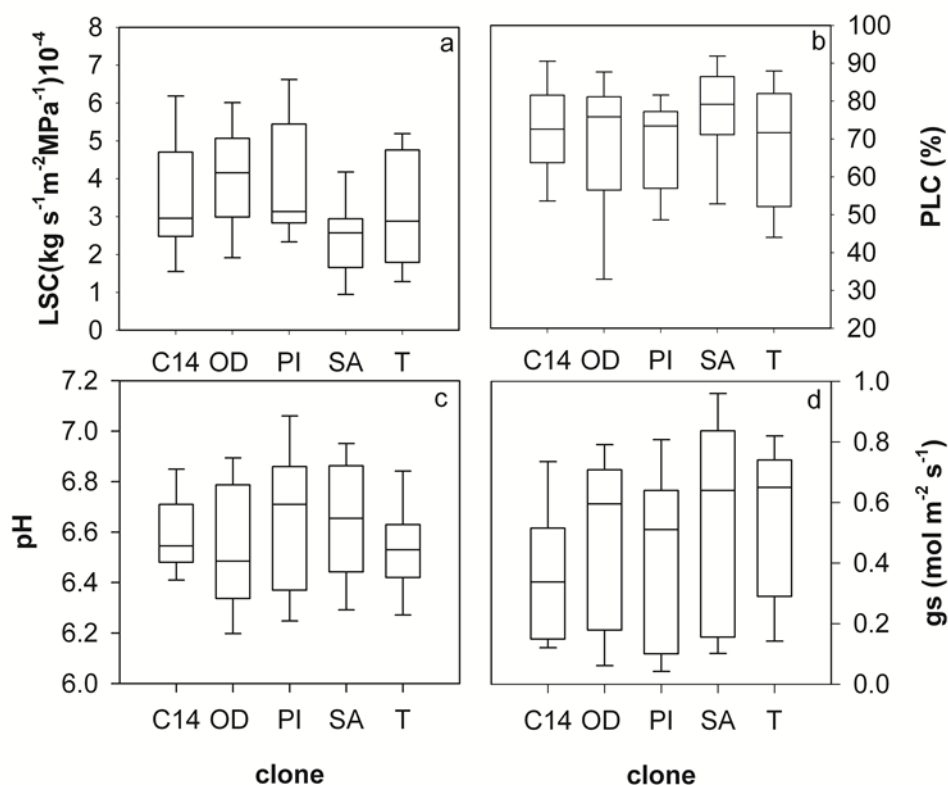


Figure 6: Box and whisker plots of leaf specific hydraulic conductance (a), the percentage loss of hydraulic conductance (b), xylem sap pH (c) and stomatal conductance for the five clones tested. The boundaries of the box represent the 25th and 75th percentiles, mid-line within the box indicates the median and whisker caps show the 10th and 90th percentiles.

Table 5: regression analysis of percentage loss of conductivity (PLC), and leaf specific hydraulic conductivity (LSC) *versus* stomatal conductance (gs)

MODEL: PLC=gs ($r^2=0.03$)						MODEL: LSC=gs ($r^2=0.01$)					
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F	DF	Sum of Squares	Mean Square	F Value	Pr > F	
Model	1	837.44	837.44	3.16	0.0798	1	1.77E-08	1.77E-08	0.8	0.3748	
Error	72	19100	265.28			60	1.33E-06	2.21E-08			
Parameter Estimates											
Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > t	DF	Parameter Estimate	Standard Error	t Value	Pr > t	
Intercept	1	75.91	3.80	19.98	<.0001	1	3.10E-04	3.52E-05	8.79	<.0001	
gs	1	-12.26	6.90	-1.78	0.0798	1	6.13E-05	6.86E-05	0.89	0.3748	

Stomatal conductance significantly decreased as Ψ became more negative (Fig.7). The highest pH values occurred mainly under concurrent comparatively high stomatal conductance ($g_s > 0.4 \text{ mol m}^{-2} \text{ s}^{-1}$) and high Ψ (Fig 7) and were recorded just before the beginning of pH declines on days 8 and 16 (Fig 4). Alkalization occurred in plants from both watering regimes between d4 and d8 (Fig 4). Sap pH also increased in R2 plants between d10 and d16 and between d19 and d26 (Fig 4). Sap acidification was found under concurrent both stomatal conductance and Ψ decline (Fig. 7).

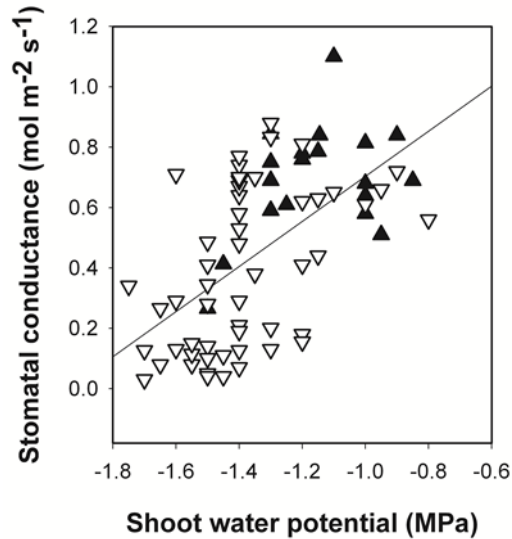


Figure 7: Relationship between stomatal conductance and shoot water potential. Each pair of values was measured consecutively on a single plant. Filled symbols correspond to data measured in plants with a xylem sap pH higher than 6.77, which was the average 75% percentile for pH data. Data from all clones and treatments are pooled together. See text for further details.

DISCUSSION

STOMATAL CONDUCTANCE AND HYDRAULIC CONDUCTANCE OF THE STEM

High values of stomatal conductance, such as those measured in the present study (Fig. 5) have been associated with low water-use efficiency in fast-growing pioneer species (Pearce et al. 2005) and may be advantageous to compete for available soil water. The adaptive advantage of comparatively high stomatal conductance for eucalypt plantations will depend on the ability to keep a tight control of stomata, in order to avoid a catastrophic xylem failure. Previous studies have reported high values of native embolism in this (Pita et al. 2003) and other woody species. For example, a PLC as high as 76.7% was measured in less than two-year old twigs from field grown *Eucalyptus crebra* and *Eucalyptus xanthoclada* (Rice et al. 2004). Native root xylem embolism was found to increase in *Pinus ponderosa* from 45% to 75% as the dry season progressed (Domec et al. 2004). In a recent study Trifilò et al. (2014) concluded that PLC values as high as 50-60% were still compatible with a relatively high stomatal conductance in *Ceratonia siliqua*, *Laurus nobilis* and *Olea europea* trees. It has been suggested that the formation of embolism may be common in some woody species (Tyree and Sperry 1988) and may even have some positive side effects, such as increasing the hydraulic capacitance (Vergeynst et al 2014). Though common occurrence of xylem cavitation remains controversial (Cochard and Delzon 2013), the differences in stomatal regulation between coexisting ferns and angiosperms reported by Brodribb and Holbrook (2004) suggest that the evolution of a more specialized stomatal physiology may allow gas exchange to be maximized by forcing the xylem to achieve its highest flow rate, in a riskier but more successful water-use strategy (Sperry 2004). Tolerance of a certain degree of embolism may depend, although not exclusively, on the ability of some species to refill embolized vessels (Trifilò et al. 2014). The suggestion that the hydraulic conductance of the stem is overbuilt (Sterck et al 2011) must be taken into consideration. In addition, growth of xylem tissue may allow a significant recovery of lost hydraulic function in some tree species (Urli et al. 2013). Furthermore, tree species such as *Populus tremula* or *Quercus robur* may lose all their leaves when reaching a PLC higher than 80%, but still be capable of resprouting after watering (Urli et al 2013). Therefore, not only the loss of hydraulic conductance but also the amount of remaining hydraulic conductance must be considered when analyzing stem hydraulic constraints to gas exchange.

Leaf specific hydraulic conductance (LSC) is a measure of the hydraulic sufficiency of the stem to supply water to the leaves (Tyree and Zimmermann 2002). Values of LSC may vary between plants showing similar PLC values, due to differences in the maximum hydraulic conductance, which is strongly dependent on vessel size (Tyree and Zimmermann 2002) or differences in the surface of leaves fed by the stem. This was the case for plants belonging to different watering regimes in the present study. Although water shortage significantly decreased LSC, it had no significant effect on PLC. Decreases in LSC under drought conditions have been previously reported in other tree species (Domec et al. 2009, Sellin et al. 2014) and are considered a plastic response to water stress that may compromise water use, and therefore growth, even after the water supply is restored (Eamus et al. 2000). The effect of decreasing LSC on growth was inconclusive in our study, since both LSC and growth decreased in R2 in relation to R1 plants but the significant effect of the clone on LSC values did not result in significant differences in growth between clones.

We found no significant relationship between values of stomatal conductance and either PLC or LSC. Moreover, values of stomatal conductance as high as $0.81 \text{ mol m}^{-2} \text{ s}^{-1}$ were measured in plants with a PLC close to 80% in the present study (Fig.4). These results suggest that loss of hydraulic conductance had scarce effect on limiting stomatal conductance under our experimental conditions. On the other hand, stomata closure precluded shoot water potential to drop below -1.8 MPa (Fig 5) while average PLC remained below 80% throughout the experiment (Fig 4). Therefore, stomata closure does not seem to be a consequence of decreases in hydraulic conductance as previously documented in other species (Hubbard et al. 2001, Ocheltree et al. 2013), but rather, is an attempt to prevent stem hydraulic conductance from decreasing any further. In accordance with these results, Urli et al. (2013) found that the embolism threshold leading to irreversible drought damage was close to 88% in five angiosperm tree species. Moreover, it was found that four out of the five tree species reached water potentials close to their minimum recoverable potential under drought conditions.

XYLEM SAP PH

Water shortage reduced shoot growth, water potential, stomatal conductance and LSC, but had no significant effect on sap pH. Under our experimental conditions, the values for xylem sap pH ranged from 6.1 to 7. These values are similar to those reported for *Populus deltoides* (Aubrey et al. 2011) and *Populus nigra* (Secchi et al. 2012) but are higher than those measured in stems of field grown *E. globulus* by Cerasoli et al (2009). Despite this wide range of values, we found no significant variation in xylem sap pH between watering regimes. This result suggests that the variation in sap pH did not act as a mechanism of root to shoot signalling of soil water deficit under our experimental conditions. This is particularly interesting given the short size of the plants used, since the effect of path length on signal transmission is negligible in seedlings compared to tall trees.

Changes in sap pH can be triggered by environmental conditions that stimulate transpiration, such as VPD (Chaves and Oliveira 2004). In a recent study, Aubrey et al (2011) observed that xylem sap pH derived from stems and twigs of *Populus deltoides* L. increased when VPD was lowest, and concluded that sap pH may increase under environmental conditions that result in low transpiration rates. In eucalyptus we found a negative correlation between sap pH and VPD (Fig.5), and measured the highest values of sap pH in plants that showed no strong stomatal limitations to transpiration.

Whereas xylem sap alkalization has been reported as a common effect of several kinds of stress, the response pattern of stomatal conductance to elevated xylem pH remains unclear. Sharp and Davies (2009) showed that increasing sap pH was not a universal response to water stress; they tested 22 perennial species and found concurrent sap alkalization and stomatal closure in 4 cases, acidification in 4 cases and no correlation between sap pH and stomatal conductance in the rest of the species. Secchi et al. (2012) observed that severe water stress resulted in a sudden drop of xylem sap pH in *Populus nigra* that could be linked to the chemistry of the energy demanding refilling process in which proton pumps are involved (De Boer & Volkov 2003). In accordance with this, we measured a decrease of about 0.4 pH units from d16 to d19 (Fig 4). After the pH decline, alkalization occurred (Fig 4). This alkalization may be the natural recovery of sap pH level once the process that yields protons ceases. In fact PLC

decreased on day 26 for R2 plants. The values of sap pH found were high enough to allow the anionic form of abscisic acid to reach the leaf apoplast in a percentage higher than 50% without being trapped in the symplast (Wilkinson & Davies 98, Boursiac et al. 2013).

The lowest values of stomatal conductance were measured on d19 in plants from both watering regimes, which indicates that plants measured on d19 were suffering a high level of water stress according to Medrano et al. (2002). Stem sap alkalization occurred mainly under concurrent high stomatal conductances and high Ψ under our experimental conditions (Fig 7). Interestingly, sap pH values in the 90th percentile were measured at VPD ranging from 0.97 to 1.2 kPa, whereas the VPD range for stomatal conductance values in the 90th percentile was 0.42 to 1.53 kPa (Fig 5). These results suggest that sap alkalization may have preceded stomata closure in response to increasing VPD.

Unfortunately, it is not possible to confirm whether mid-morning sap alkalization in the stem could have resulted in stomata closure at midday or in the afternoon. Therefore, the role of stem sap pH in stomata regulation in the species awaits further research.

CLONAL EFFECTS

Since there were no significant differences in evapotranspiration at the beginning, the differences found for the whole period of time reflect the combined effect of the increase in leaf area and variations in stomatal conductance between plants belonging to different watering regimes or clones. In accordance with these results, we found a significant clone effect for stomatal conductance (Table 4). The lowest evapotranspiration rate was measured under the more favourable watering regime in the F_0 clone (C14) (Table 3, Fig.3). Stomatal conductance was also lowest in this clone (Fig.6). Therefore, F_1 clones were able to profit from extra soil water to a greater extent than the F_0 clone.

The highest rate of evapotranspiration was measured in clone T under the less favourable R2 treatment (Table 3). Accordingly, values of stomatal conductance measured under the most stressful conditions were higher in clone T than in the rest of the clones (Table 4). These results can contribute to explain why clone T was severely affected by the exceptional drought of 2005, particularly if we consider that the lowest values of LSC were measured in clones T and SA (Fig. 6a). In a previous study, Vilagrosa et al. (2003) suggested the existence of a LSC threshold for early leaf shedding. This is a common response to drought in *E. globulus* plantations established in SW Spain. It is also a costly response in terms of growth and productivity. Early leaf shedding can also be considered the last line of plant defense against the effects of drought. Clones with a low LSC seem therefore less capable of achieving a reasonable growth and survival under Mediterranean climates. This seems particularly relevant under a climate change scenario with a predicted increase in extreme meteorological events.

In conclusion: Changes in xylem sap did not show a clear relationship with soil water status, contrary to our first hypothesis, but sap pH decreased significantly as VPD increased, in agreement with our second hypothesis. Stomata closed after a considerable amount of hydraulic conductance was lost, although the clone effect for leaf specific hydraulic conductance was significant, suggesting the possibility of selection for improved productivity under water limiting conditions combined with high temperatures in the early stages of growth.

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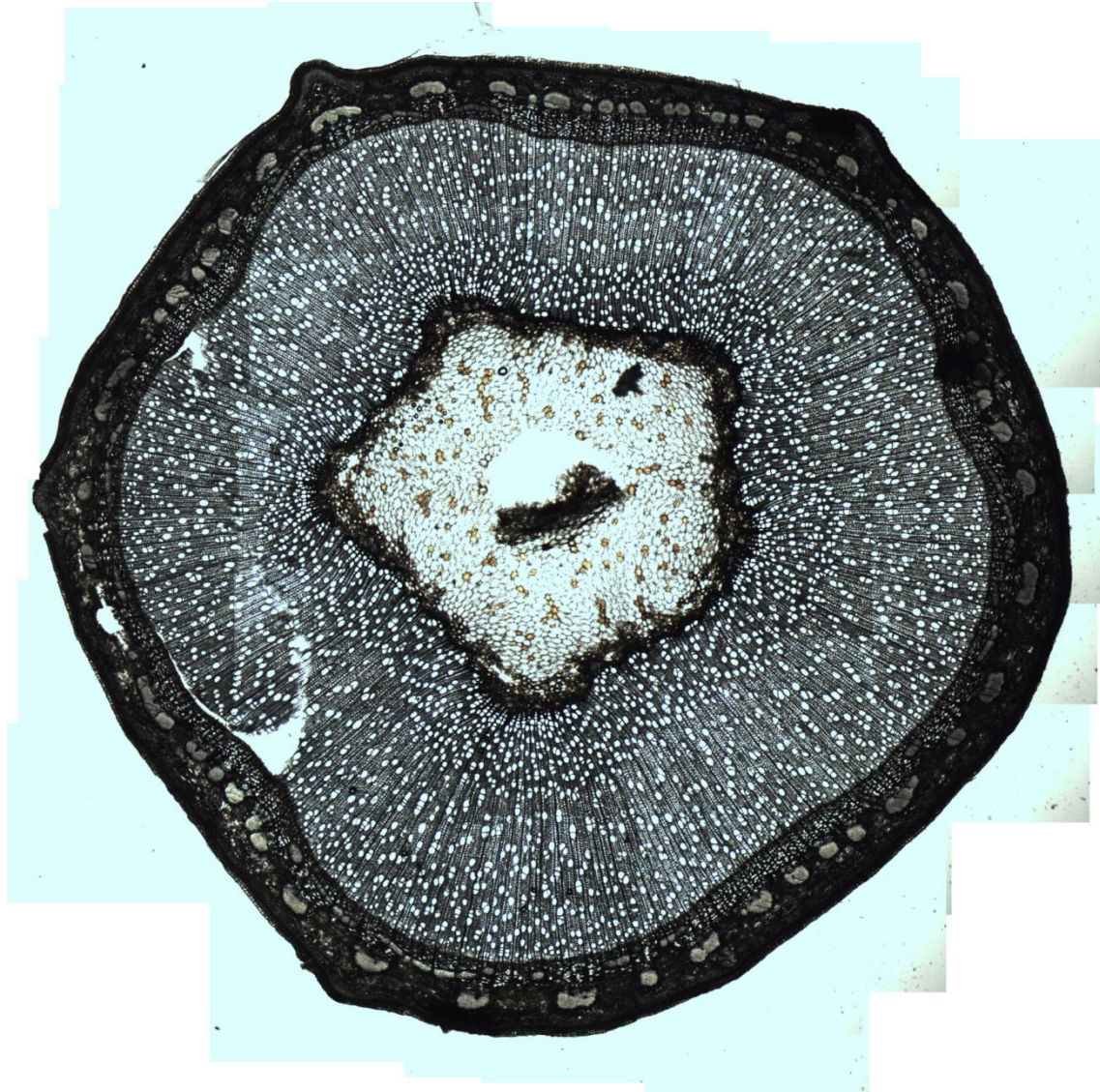
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CAPÍTULO 3

GROWTH, VULNERABILITY TO CAVITATION AND ANATOMICAL TRAITS OF FOUR HYBRID POPLAR CLONES USED AS SHORT ROTATION ENERGY CROPS IN SPAIN

RESUMEN

En 2005 se creó la Red de Parcelas de Cultivos Leñosos en Alta Densidad y Turno Corto, con el objeto de evaluar la viabilidad de cultivos intensivos de alta densidad y turno corto con fines energéticos en localidades con clima mediterráneo. Entender las respuestas de una selección de genotipos híbridos de chopo al estrés hídrico tiene interés para ayudar a los propietarios de plantaciones de este tipo a seleccionar los mejores clones en términos de producción y resistencia a la sequía.

La conductividad hidráulica máxima es la máxima capacidad que tiene la planta de transportar agua a través del tallo y está relacionada con el crecimiento. La conductancia hidráulica puede limitar el crecimiento imponiendo límites físicos al transporte de agua y por ende a la transpiración y a la fotosíntesis.

Con el objeto de investigar si las diferencias de producción encontradas entre clones pueden explicarse a partir de diferencias anatómicas en el xilema, se midieron una serie de variables de crecimiento y anatómicas en cuatro genotipos híbridos de chopo en una parcela de demostración de la producción de cuatro hectáreas situada en Granada, en el sur de España. La plantación de Granada sufrió restricciones hídricas y no fue regada en dos meses.

En Madrid se instaló un ensayo con los mismos cuatro clones sometidos a dos regímenes de riego para construir las curvas de vulnerabilidad empleando dos métodos: centrífuga y deshidratación.

Los cuatro clones presentaron diferencias en sus características xilemáticas. Los clones más productivos presentaron un mayor número de vasos de tamaño intermedio. Esta combinación de elementos xilemáticos parece haber optimizado la eficiencia conductiva y la seguridad. Monviso, uno de los clones que alcanzó mayores producciones, emitió el mayor número de ramas silépticas. Los clones menos productivos fueron Pegaso, que exhibió la menor superficie conductora, y el clon I-214 con el menor número de ramas silépticas y el mayor valor para el ratio entre área media del vaso y densidad de vasos.

Las curvas de vulnerabilidad construidas a partir de muestras centrifugadas no presentaron diferencias significativas entre clones, mientras que sí las presentaron las curvas generadas mediante el método de deshidratación. Las curvas correspondientes a las plantas regadas mostraron una vulnerabilidad (P_{50}) significativamente mayor que las de las plantas estresadas. Los clones que acumularon más biomasa figuraron entre los más resistentes a la cavitación, lo que sugiere que existe la posibilidad de conseguir mejorar la producción mediante selección clonal en ambientes con episodios de sequía.

La vulnerabilidad a la cavitación de los clones ensayados ha presentado diferentes grados de plasticidad. El clon I-214 fue el más plástico. Las plantas de este clon sometidas a estrés fueron las más vulnerables, mientras que las estresadas mostraron la mayor resistencia.

GROWTH, VULNERABILITY TO CAVITATION AND ANATOMICAL TRAITS OF FOUR HYBRID POPLAR CLONES USED AS SHORT ROTATION ENERGY CROPS IN SPAIN

HERNÁNDEZ GARASA M.J.¹, SIXTO BLANCO H.¹, CAÑELLAS REY DE VIÑAS I.¹, PITA ANDREU P.²,

(1) CIFOR-INIA, Ctra de la Coruña km 7.5, 28040 Madrid, Spain.

(2) E.T.S.I. Montes, Universidad Politécnica de Madrid. Ciudad Universitaria s/n. 28040 Madrid, Spain.

ABSTRACT

In 2005, a network of experimental short-rotation Poplar plots was established across Spain in order to assess the viability of this type of crops in Mediterranean environments. Understanding the responses to water stress in certain poplar genotypes used for short rotation forestry is of particular interest to help tree breeders select the most suitable genotypes in terms of yield and drought resistance.

Maximum hydraulic conductivity in a plant stem implies maximum water transportation capacity through the stem and is related to growth. Hydraulic conductance may constrain plant growth by imposing physical limits to water transport and therefore transpiration and photosynthesis.

In order to investigate the extent to which differences in yield between poplar clones can be explained by differences in xylem anatomy, xylem anatomical traits and growth variables were measured in four poplar clones in a four hectare demonstration trial in the South of Spain (Granada), where plants had experienced a severe summer drought. With the aim of detecting possible vulnerability to cavitation differences between clones, an experimental trial was established in Madrid, testing the same four poplar clones previously tested in Granada but with two contrasting water regimes. This trial provided samples for building vulnerability curves for both centrifuge and dehydration methods

Anatomical differences were found between clones growing under drought conditions. The clones with the highest biomass yield, Monviso and AF2, had a higher number of intermediate size vessels. This anatomical combination of xylem elements (number and vessel area) seems to optimize efficiency and safety in the more productive clones. Monviso, one of the highest yielding clones, developed the largest number of sylleptic branches. In contrast, the clones with the lowest yield displayed the smallest conducting area, in the case of Pegaso, and the highest AD-ratio along with the lowest number of sylleptic branches in the case of I-214.

Centrifuge based vulnerability curves did not reveal significant differences between clones, whereas the bench dehydration method did. Results from the dehydration method showed significantly higher vulnerability (P_{50}) for well-watered plants than for plants subjected to water stress. The clones which displayed the highest biomass yield were among the most cavitation resistant, which suggests that through appropriate clone selection yields could be improved in drought-prone environments.

Different clones showed different degrees of plasticity as regards vulnerability to cavitation when submitted to different watering regimes. Clone I-214 showed the greatest plasticity for P_{50} , being the most vulnerable clone under the well-watered regime and the most resistant under drought conditions.

INTRODUCTION

The European Union (EU) energy policy has to address a number of challenges such as greenhouse gas emissions, which should be reduced by more than 80% by 2050, and the dependency on imported energy source (UE 2012 Energy Roadmap 2050). Two essential elements of the EU energy policy are the increase in reliance on renewable energy and the diversification of energy sources. As a renewable energy source, biomass provides an interesting alternative due to the null carbon balance resulting from its production and use as well as the opportunity to use abandoned agricultural land (Sixto et al. 2010).

Short rotation poplar crops are widespread throughout Europe both for biomass production and research purposes. However, most of the existing plantations are used for experimental purposes, with the exceptions of the UK, Sweden, and Italy (Eppler U& Petersen J.E. 2007). Poplar trees of the gender *Populus* are among the fastest growing temperate trees, consistent with their role as vegetational pioneers (Eckenwalder 1996). Besides their fast growth, it is known that poplars are very drought sensitive trees (Cochard et al. 2007). In Mediterranean environments, where drought lasts at least 2 months, it is necessary to irrigate short rotation crops. Maintaining irrigation in large areas can be problematic and water restrictions may be imposed due to drought. As a result, low irrigation doses could lead to high stress and even death of the poplar plantation.

In 2005, a network of experimental short-rotation Poplar plots was established across Spain in order to assess the viability of this type of crop on irrigated land in Mediterranean environments. Understanding the responses to water stress in certain poplar genotypes used for short rotation forestry is of particular interest to help tree breeders select the most suitable genotypes in terms of yield and drought resistance.

Given that xylem hydraulic conductivity can limit plant productivity (Manzoni 2013) and even cause plant death (Barigah et al. 2013), it is particularly important in drought prone environments to choose genotypes capable of withstanding drought events. When xylem is exposed to very high negative pressures, water is thought to be transported in a metastable state, and cavitation occurs in some vessels due to the sudden change of state of sap to gas. Air can then pass through the intervessel pit membrane pores from embolized vessels to adjacent, intact vessels or through cracks in the vessel walls. This is known as the air seeding hypothesis (Tyree and Zimmermann 2002).

Differences among species as regards cavitation resistance are partially explained by the 'rare pit' hypothesis. This hypothesis suggests that cavitation resistance depends on the leakiest pit of a vessel, such that the larger the pit vessel area the greater the probability of pit failure (Wheeler et al. 2005, Christman et al. 2009). Recent studies (Lens et al. 2011, Plavcova & Hacke 2013, Capron et al. 2014,) have shown the importance not only of the quantity of pits and their probability of failure, but also the importance of quality. It is thought that the

membrane deflects as a result of the difference in pressure across vessels, and that pores become larger by deformation. This was confirmed by Capron et al. (2014) who characterized the microstructure of the vessel of poplar through optical, electronic transmission and atomic force microscopy. The thickness, porosity, elasticity, and recovery capacity of the membrane pit constituents should play a key role in the cavitation resistance of plants. Taking all this into account, the cavitation resistance of a plant depends mainly on the quality and quantity of pits rather than on the vessel diameter (Lens et al. 2011). However, a relationship may exist between vessel diameter and both pit number and diameter or between vessel growth speed and quality of pits. A number of authors such as Martinez-Vilalta et al. (2002), when comparing several species, have obtained results which are consistent with a positive relationship between vessel diameter and the extent of intervessel pitting. However, the relationship between intervessel pitting and vessel size is not clear. Some authors have reported either no correlation or only a very weak correlation between vessel diameter and cavitation resistance in poplar (Cochard et al. 2007, Fichot et al. 2010) whereas others, in contrast, have pointed to the existence of a relationship (Awad et al. 2010). The fact that no correlation was found between vessel diameter and cavitation resistance could mean that vessel diameter and pit properties are unrelated and therefore it should be possible find clones with relatively high conductive efficiency which are both drought resistant and potentially high yielding.

Vulnerability curves are an accepted tool for assessing the cavitation resistance of plants, which is an intrinsic property of the xylem tissue (Fichot et al. 2015). Thus, vulnerability curves indirectly provide information with regard to the intrinsic properties of xylem.

The plasticity of poplar species has been widely documented. Many authors have found different phenotypic responses to different treatments. For example, Harvey & Van Den Driessche (1996) found different pit pore diameters in plants submitted to different phosphorus fertilization treatments while Plavcová et al. (2012) found differences in growth, xylem anatomy and vulnerability to cavitation in plants submitted to different irrigation and fertilization treatments. Plavcová et al. (2010) also reported differences in pit membrane and middle compound lamella thickness between plants subjected to different irradiance treatments and Fichot et al. (2010) found differences in cell size between plants subjected to different irrigation regimes, etc. This plasticity contributes to the known genotype x environment interaction, further complicating the choice of the most appropriate genotype for each environment.

In this study, certain xylem traits, among them the vessel area, were studied in four poplar clones, three of which were selected specifically for short rotation crops in Italy. The fourth clone was I-214, commonly used in Spain and in the Mediterranean poplar sector. The vulnerability curves of these clones were built using two methods: centrifuge and dehydration, and the results were compared.

Bearing in mind the general aim which is to select the highest yielding clone capable of withstanding the environmental conditions at each site, the main focus of this study was to investigate the relationships between yield and both cavitation resistance and xylem anatomy in four poplar clones. We hypothesized that (1) clones with larger vessels may not attain the highest productivity under drought conditions, (2) that clones attaining the highest yield under

well-watered conditions would be more vulnerable to cavitation than low yielding clones,(3) that under stress conditions the highest yielding clone may be one of the most cavitation resistant clones.

MATERIALS AND METHODS

EXPERIMENTAL SITES, PLANT MATERIAL, TREATMENT IMPOSITION

A four-complete randomized block design, each of a hectare in size, was established in Granada (37° 12'N; 3° 42'W) in March 2006. 20.000 unrooted cuttings per hectare of four hybrid poplar clones were planted in rows 1m apart. The plantation was basin irrigated every 15 days in 2006. In January 2007 the plantation was cut back and multiple shoots resprouted from the stumps in spring. Watering of the Granada crop was performed only twice in 2007, at the end of June and at the beginning of September, due to water restrictions imposed by the lack of rain during that year. The plants suffered an almost complete defoliation due to water stress. In each block, a representative assessment plot of 4x4 plants was established per clone and measurements were taken in January 2008 on one year old shoots.

The four hybrid poplar clones tested were: two *Populus x euramericana* clones AF2, and I-214, and two *Populus x interamericana x Populus nigra* clones Monviso and Pegaso.

In the Spring of 2013, an experimental trial was established in Madrid in a nursery field belonging to the CIFOR-INIA (40° 27' N; 3° 45'W). Unrooted cuttings of the same four poplar clones used in Granada were planted. The trial was 12x6 m in size, the plants being distributed in 12 rows 1m apart, each row including 12 randomly placed plants (3 per clone) with a spacing of 0.5m. In Madrid, 14l day⁻¹ were applied to all plants by means of a drip irrigation system. All the plants were fertilized in mid-June with the same amount (25 ml) of NPK 21:8:11 (ENTEC®Nitrofoska®21 Eurochem agro) placed close to each plant next to the drip line. In August, the first two water regimes were imposed in order to compare growth and vulnerability curves between well-watered and stressed plants. Well-watered plants (The six most northerly rows) were kept watered with 14l day⁻¹, and the stressed plants were watered with 14l every other day until August the 10th and after this date irrigation was suppressed. Buds began to close in the last week of September.

GROWTH MEASUREMENTS

Measurements in Granada were carried out in January 2008 on 16 plants (without leaves) per clone and block (assessment plots). All basal and breast height shoot diameters (dbh), were measured with digital calipers and shoot height was measured using a graduated pole. Branches per plant were counted in all plots. The plants were then cut, weighed and the humidity content determined in the lab by oven drying a subsample to constant weight. Volume per plant was calculated by adding the volumes of all single shoots assuming the shape of a shoot to be a cone. Shoot biomass was estimated from plant biomass by assigning to each shoot a biomass ratio equal to the shoot to plant volume ratio.

In Madrid, the basal and breast height shoot diameters and height were recorded for all shoots per plant except those located in the four central rows, which were not measured to avoid the border effect. Dry biomass yield was estimated from a shoot weight-volume regression obtained from data collected during the first vegetative period in Granada. A

comparison of this regression model with another shoot weight volume regression built from data obtained in the first vegetative period in Soria (located 200 Km from Madrid) using the same genotypes and planting density, revealed no significant differences between both lines. Although the plantation located in Granada was subjected to a drought environment while the plantation in Soria was well-watered, no significant differences in the weight-volume regressions were found despite differences in the biomass yield (0.435 Mg ha⁻¹ año⁻¹ in Soria and 4.11 Mg ha⁻¹ año⁻¹ in Granada) and all the data were pooled together to obtain the abovementioned regression equation.

ANATOMICAL MEASUREMENTS

Sampling of plant material for anatomical measurements was performed once shedding was finished in January 2008 on one year old poplar shoots taken from the Granada trial. In the first three blocks, the measured shoots of two plants per clone were marked with a white tape at breast height, and 2 cm long stem samples were taken at that height and kept in 50% alcohol until lab storage, where the alcohol was changed for FAA (20% formaldehyde, glacial acetic acid, 50% alcohol and distilled water at a ratio of 7,1: 3,6: 64,3: 25). Stem cross sections with a thickness of 40 µm were taken from the stem samples by means of a sledge microtome, mounted with glycerin and observed using a light microscope (MEIJI ML 5000 Techno Co LTd). The images were captured at a magnification of 40x using a digital camera (Moticam 2300) and joined together using Adobe Photoshop CS2 to compose a single image of the whole cross section.

The area corresponding to pith, xylem and bark were measured in each cross section using the cell[^]D (Olympus) software, and within the xylem, the number of vessels, area, diameter and perimeter of each vessel were also recorded. For each cross section, the following variables were calculated from these data: sum of vessel hydraulic diameters calculated as four times the vessel area divided by the perimeter in order to take into account the vessel shape (López & Mintegui 83), conducting area as the sum of the individual vessel areas, average vessel diameter and area, vessel density as the number of vessels per xylem area in mm², AD ratio defined as the average vessel diameter divided by vessel density, and the theoretical hydraulic conductivity (Kht) calculated by means of the Hagen Poiseuille law (Equation 1), where Dh_i is the hydraulic diameter of each vessel in one cross section and η is the dynamic viscosity index of water considered at lab temperature.

$$Kht = \frac{\sum \pi Dh_i^4}{128\eta} \quad \text{equation 1}$$

PHYSIOLOGICAL MEASUREMENTS

Gas exchange measurements

On the morning of June 27th2007, the day after the first watering, between 10:30 and 12:30 a.m., net CO₂ assimilation rate, (A, µmol m⁻² s⁻¹), stomatal conductance to water vapour (gs, mol m⁻² s⁻¹), and transpiration rate (E, mmol m⁻² s⁻¹) were measured in Granada on five

plants per block and clone using an IRGA LCA-4 (ADC BioScientific Ltd). Intrinsic water use efficiency (IWUE, $\mu\text{mol}/\text{mol}$) was then calculated as the ratio of A to gs.

Hydraulic measurements

Samples for hydraulic measurements were taken in October 2013 from a one year old experimental plot established in Madrid. Since this trial was located about 15 minutes from the lab, undesirable effects arising during transport such as gel formation, or wound responses were minimized.

Shoots were cut in early morning at their base and under water by placing a plastic funnel full of water around the base of the plant. The cut plant was then wrapped in wet paper towel, placed in a black bag and carried to the lab where the plants were taken out of the bag one-by-one to measure the hydraulic conductance using a XYL'EM (Xylem Embolism Meter, Bronkhorst, Montigny les Cormeilles, France).

Firstly, progressive negative tensions were achieved in the xylem via centrifugation, using a centrifuge (Sorvall RC 5C PLUS). Segments used for hydraulic measurements were taken from each shoot just under the first mature leaf, and trimmed with a razor blade underwater to a final length of 14 cm. Before the samples were inserted in the centrifuge, stem segments were fitted to the xyl'em manifold, immersed under water. A flow of water was made to pass through the samples at a pressure of 2kPa in order to obtain the native hydraulic conductance (k_{native}). This water head was selected so as not to exceed the $2T/r_c$ threshold, where T is the surface tension of water (0.0728 N m^{-1} at 20°C) and r_c is the radius of the widest conduit in the sample (Van Ieperen *et al.* 2001 in Melcher *et al.* 2012). To obtain the maximum hydraulic conductance (k_{max}), an additional flush lasting 5 minutes at 180 kPa was applied to the samples in order to eliminate xylem embolisms. Conductance measurements were performed using degassed 10mMKCl and 1mM CaCl_2 solution $0.2\mu\text{m}$ filtered to avoid slow clogging in vessels.

Stem segments were then inserted in the centrifuge and spun repeatedly to progressively more negative pressures ranging from -0.12 to -2.5 MPa. Hydraulic conductance (k_i) was measured after each pressure increment until it dropped below 95% of the k_{max} value. Percentage loss of hydraulic conductance was calculated using equation 2.

$$PLC = 100 \frac{k_{\text{max}} - k_i}{k_{\text{max}}} \quad \text{equation 2}$$

The initial plan was to induce cavitation by centrifugation but only part of the plants could be measured prior to the irreparable centrifuge breakdown. Cavitation was induced in the rest of the plants by bench drying. The total number of stem segments used per clone and watering treatment to measure hydraulic conductance at the moment of centrifuge breakdown varied between 6 and 16, and only some of the watering regime-clone combinations had been measured.

Cavitation was induced by bench dehydration in an additional sample of 160 stem segments in order to complete the PLC measurements. Plants were allowed to dehydrate

gradually on the lab bench, and the xylem tension was measured with a Scholander chamber on a leaf separated from the plant under water. Stem samples of around 4 cm in length located under the first mature leaf were cut under water, placed in the manifold of the xylem apparatus and the conductance measured k_i . A flush lasting 4 minutes at 180 kPa was then applied and the maximum conductance (k_{max}) and PLC (eq2) were measured again.

Percentage loss of hydraulic conductivity (PLC) was plotted against the corresponding xylem pressure to generate vulnerability curves (VCs), fitted to the Pammenter and Vander Willigen equation for each clone and water regime combination (Equation 3.), where ψ is the negative tension in the xylem and a and b are parameters. Parameter b in the Pammenter equation represents the xylem pressure at which percentage loss of conductivity reaches 50% (P_{50}). It is commonly used as a measure of vulnerability to cavitation. Parameter a is related to the slope of the VC. The higher the slope, the greater the vulnerability and it is thought to be related to the variation in pit pore diameters (Pammenter & Vander Willigen 98).

$$PLC = \frac{100}{1 + e^{a(\psi - b)}} + \varepsilon$$

equation3

The cross section areas at both ends of each sample were impregnated with ink and printed on paper. These impressions were then photographed with a digital camera and measured using image analysis software (cell[^]D) to determine the stem cross section surface. The specific hydraulic conductance (k_{xs}) of the stem cross section was calculated as the ratio of hydraulic conductance (k_i) to the largest stem cross section A_s (eq4). Specific hydraulic conductivity K_{xs} was calculated as the product of k_{xs} by the stem segment length (eq 5).

$$K_{xs} = \frac{k_i}{A_s} \quad \text{equation 4}$$

$$K_{xs} = k_{xs} * \text{length} \quad \text{equation 5}$$

STATISTICS

All the analyses were carried out using the GLM and NLIN procedures of the SAS software version 9.2 (SAS Institute Inc. 1989).

Growth variables

In order to evaluate the clone effect on plant growth variables, a one way analysis of variance (ANOVA) was carried out in Granada. In Madrid, a two way ANOVA was performed to evaluate the effect of clone and watering treatment on growth. The General Linear Model procedure of the SAS/STAT software version 9.2 was used for this purpose. The general linear model used to fit the measured data was:

$$GW = \text{water regime} + \text{clone} + (\text{water regime} * \text{clone}) + \epsilon$$

Where GW represents the growth variable tested, water regime and clone are the fixed effect factors and (water regime* clone) represents the interaction between factors. ϵ stands for the random error.

Differences between means were tested using the Newman Keuls mean test with a significance level of 0.05.

Anatomical features

Given the high correlation found between the anatomical features of xylem and the shoot size, in order to evaluate differences between clones, a covariance analysis (ANCOVA) was carried out using the shoot volume distal to breast height as covariate, since the cross sections were taken at this height. Distal volume is the volume that receives the sap from the analyzed section. Significance level was 5% and variables were log transformed in order to ensure linearity.

When significant interaction between factors was not found, adjusted means were calculated by means of the Tukey test.

Physiological variables

Gas exchange:

A high correlation was found between gas exchange measurements and the time at which measurements were recorded, therefore, in order to assess the differences between clones in terms of gas exchange variables an ANCOVA was carried out using time as covariate, clone as categorical predictor and the interaction between the two of them was also included in the model. Clonal differences between intercept and slope values of the regression lines fitted to each clone were assessed by comparing parameters of the models by means of F tests (option solution in SAS GLM procedure).

Hydraulic measurements:

Pairs of observations of xylem water potential and PLC were fitted to the Pammenter & Vander Willigen equation by means of the SAS nlin procedure. Mean square reduction tests were used to compare the VCs obtained by different methods (www.ats.ucla.edu). Since they proved to be significantly different, well-watered to stressed plants along with the different possible combinations of clone-treatment were also compared using mean square reduction tests separately per method.

Having determined that there were also significant differences between watering treatments, clones and clone-water treatment interaction, the significance of the differences between parameters (*a*, *b*) corresponding to the different clone-water regime combinations was evaluated by comparing confidence intervals, setting the appropriate significance level in accordance with the number of comparisons carried out. Parameters were considered significantly different when their confidence intervals did not overlap.

Finally, differences in specific hydraulic conductivity K_{xs} for both clone and treatment were assessed by means of an ANCOVA, using xylem water potential as covariate. The model used was:

$$K_{xs} \sim \text{xylem water potential} + \text{clone} + \text{watering-treatment} + \text{clone} * \text{watering-treatment}$$

RESULTS

GROWTH

In Granada, clone I-214 yielded the lowest amount of dry biomass per hectare and showed significantly lower values than the rest of the clones for all measured growth variables except for the number of shoots per plant (I-124 having the highest number). In contrast, the clones with the highest biomass yield were Monviso and AF2, the former also presenting significantly higher values than the rest of the clones for dominant shoot volume and number of branches per plant (Table 1).

In Madrid, significant differences in growth between watering treatments were not found for any of the variables. The growth increment in *Populus xeuramericana* hybrids from stressed to watered treatments was greater than for *Populus xinteramericanaxnigra*, the Monviso clone even registering a decrease. The clone effect, volume per plant, main shoot volume per plant and estimated dry biomass were significantly higher for AF2 than for the rest of the clones. Interaction between factors was only detected for estimated dry biomass. Clone AF2 and Monviso had the highest biomass yield in the well-watered and water-deficit treatments respectively, their means differing from those of the rest of the clones. The Pegaso clone exhibited the lowest biomass yield in both treatments and was significantly different from AF2 in the well-watered regime and from Monviso in the water-deficit treatment.

Differences in biomass yield between stressed plants in Madrid and Granada are attributed to the differences in root age in each plantation.

The growth variables in Madrid were transformed by standardizing their variance function in order to avoid heterocedasticity.

Table 1: growth variable means in Granada and Madrid. Results of the ANOVA and Newman Keuls mean test applied to the variables at both sites.

Site	Mortality (%)	Shoots per plant		d ₁₀ (mm)		d ₁₃₀ (mm)		ht max (cm)		Branches per plant		Volume per plant (cm ³)		Main shoot volume (cm ³)		Dry biomass yield (Mg. ha ⁻¹ yr ⁻¹)	
		<i>m</i>	<i>std</i>	<i>m</i>	<i>std</i>	<i>m</i>	<i>std</i>	<i>m</i>	<i>std</i>	<i>m</i>	<i>std</i>	<i>m</i>	<i>std</i>	<i>m</i>	<i>std</i>	<i>m</i>	<i>std</i>
GRANADA																	
Clone effect (<i>p-value</i>)		0.058		0.006		0.0006		0.002		<0.001		0.02		<0.0001		<0.002	
AF2	10.9	6.00 b	2.12	17.14ab	3.65	10.32ab	4.22	230.51a	56.66	9.7 b	13.83	581.18a	361.3	246.32 b	148.60	4.06ab	2.03
I-214	7.8	7.51 a	4.00	15.74b	2.11	9.65b	1.91	228.14b	33.25	3.3 c	5.47	463.96a	199.4	192.09 c	67.82	3.16c	0.20
Monviso	6.2	6.42 ab	2.11	17.91ab	3.78	12.26a	2.99	262.31a	53.23	22 .0a	18.75	576.5 a	280.7	304.27 a	150.41	4.64a	1.50
Pegaso	21.9	6.78 ab	2.48	18.20ab	4.26	11.77a	3.40	233.34a	44.75	10.6 b	18.71	570.17a	336.3	286.55ab	160.93	3.64bc	1.02
MADRID																	
Watering effect (<i>pvalue</i>)		0.4835		0.3534		0.1814		0.2713				0.1155		0.1295		0.1108	
Stressed	16.7	2.35a	0.95	9.44a	4.05	4.75a	3.18	94.90a	50.19			42.01		39.26a	57.18	0.56b	0.46
Well watered	13.9	2.47a	0.90	11.41a	7.92	9.72a	5.84	119.87a	88.54			120.49		112.06a	216.09	1.23a	1.96
Clone effect (<i>p-value</i>)		0.255		0.0281		0.8003		0.171				0.0145		0.0147		0.003	
AF2	16.7	2.07a	1.03	14.04a	9.56	10.00a	6.73	137.20a	95.19			194.39a		183.19a	286.88	1.94a	2.58
I-214	27.7	2.38a	0.96	9.11ab	4.77	5.79 a	5.71	101.85a	74.09			37.9b		35.67b	58.67	0.53b	0.28
Monviso	5.5	2.41a	0.94	10.78ab	4.51	6.42a	2.70	110.88a	59.83			62.0b		58.82b	71.24	0.82b	0.57
Pegaso	11.1	2.75a	0.68	7.66b	3.18	6.82a	.	79.44a	48.96			28.0b		22.95b	31.46	0.26b	0.25
Watering x clone (<i>p-value</i>)		0.8655		0.2656		0.6804		0.5712				0.1269		0.1476		0.03	
Stressed																	
AF2	33	2a	1.1	10.19a	4.73	6.01a	4.33	104.33a	58.27			56.79a		56.23a	76.78	0.58 ab	0.63
I-214	44	2.14a	1.07	8.25a	2.97	2.08a	0.32	82.86a	38.98			17.62a		16.99a	17.26	0.44 ab	0.25
Monviso	0	2.33a	1	11.62a	4.84	6.16a	3.2	114.67a	57.47			69.97a		66.77a	76.57	0.93a	0.58
Pegaso	0	2.78a	0.67	7.68a	2.65	.	.	78.22a	43.98			23.15a		17.77a	21.7	0.25b	0.22
Well watered																	
AF2	0	2.11a	1.05	16.61a	11.28	11.6a	7.21	159.11a	111.29			286.1 a		267.83a	346.68	2.84a	3.04
I-214	33	2.67a	0.82	10.12a	6.46	9.5a	6.54	124a	101.28			61.5ab		57.47a	82.73	0.62ab	0.53
Monviso	11	2.5a	0.93	9.85a	4.22	6.68a	3.37	106.63a	66.1			53.0 b		49.89a	68.76	0.71ab	0.65
Pegaso	44	2.71a	0.76	7.63a	3.99	6.82a	.	81a	58.38			35.2b		29.61a	41.89	0.27b	0.39

Figures followed by different letters in the same column refer to significant differences at a 0.05% significance level; d₁₀ (mm): basal diameter of the dominant (highest) shoot of the stool; d₁₃₀ (mm): diameter at breast height of the dominant stem in a shoot; ht max (cm): height of the highest shoot in the stool branches per plant: number of branches per stool; main shoot volume is the mean value of the dominant shoot's volume, Dry biomass yield: oven dry tons per hectare/year of dry biomass m=mean; std=standard deviation.

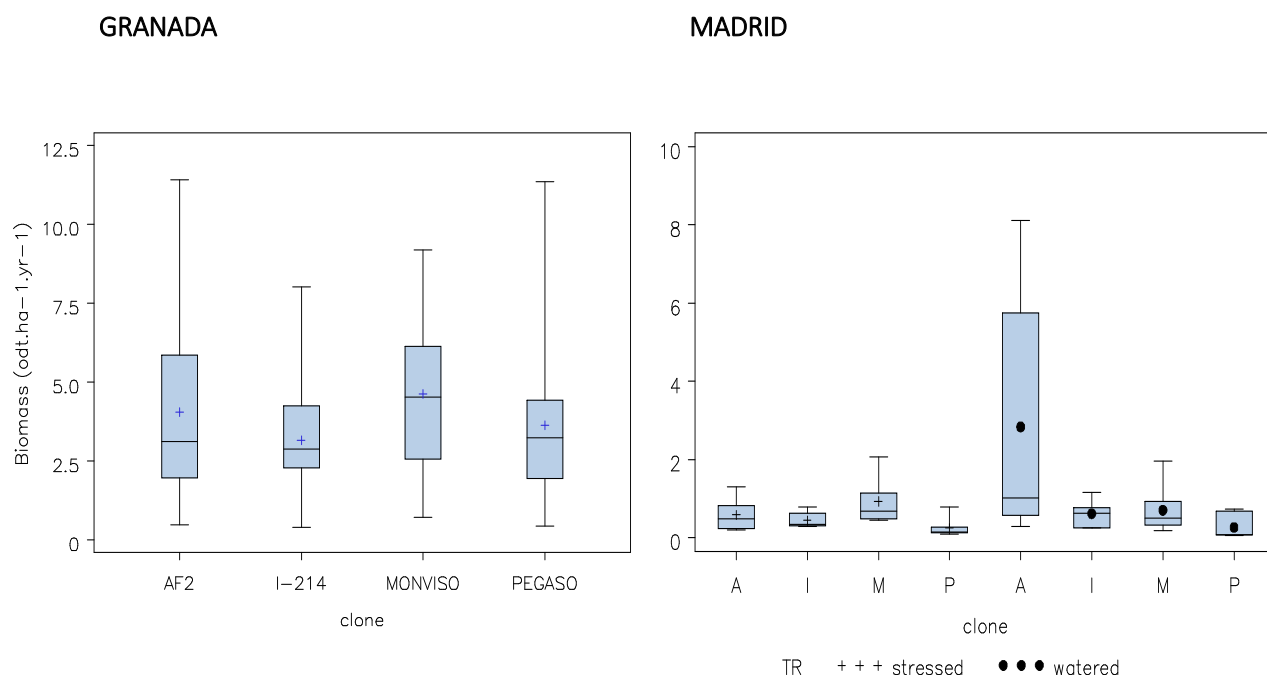


Figure 1: Dry biomass (Mg.ha⁻¹.yr⁻¹) yielded in Granada (left) under drought regime and in Madrid (right) under two contrasting water regimes. Letters on the horizontal axis on the right are the initials of the clone. The boundaries of the box represent the 25th and 75th percentiles; mid-line within the box indicates the median, + and black dot symbols indicate the mean and endpoints of upper and lower whiskers are maximum and minimum values.

ANATOMICAL TRAITS

Xylem anatomical traits were positively related with distal stem volume. The more voluminous the stem, the larger the corresponding mean vessel area, the smaller the vessel density and the larger the absolute number of vessels and the xylem conducting area. However, these relationships between volume and xylem traits were not equal for all genotypes. In this regard, the significant differences between genotypes are summarized in the ancova table (table2).

Mean vessel diameter: The analysis of covariance showed a highly significant clone effect ($p < 0.0001$), and non-significant interaction between the clone factor and covariate (volume distal to breast height). The mean vessel diameter of the I-214 clone was greater than the rest of the clones.

Number of vessels per cross section: The covariance analysis of the mean number of vessels per volume unit revealed neither genotype effect ($p = 0.058$) nor interaction between covariate and genotype. Since the descriptive statistical plots appeared to show that AF2 had more vessels than I-214 and Pegaso, two planned contrasts were carried out to compare the AF2 clone with both the Pegaso and I-214 clones. Significant differences were found between their means, with a p-value of 0.0092 for the first comparison (AF2-Pegaso) and $p = 0.01$ for the comparison between AF2 and I-214. The I-214 clone was found to have less vessels than AF2 although they were larger.

Xylem conducting area and shoot volume showed a highly significant lineal correlation ($p < 0.0001$) and the genotype effect was also highly significant ($p = 0.0014$). The I-214 clone had

the highest xylem conducting area, significantly different from that of Pegaso, which displayed the lowest xylem conducting area per unit of distal to breast height volume.

Table 2. results of the ANCOVA and Tukey adjusted mean test applied to the xylem anatomical variables

ANCOVA model $y = \text{covariate} + \text{clone} + \text{covariate} * \text{clon} + \epsilon$							
Dependent variable log transformed log(y) →		df	Mean vessel area (μm^2)	Count of vessels at breast height cross section	Theoretical xylem conducting area (μm^2)	AD-RATIO (vessel area to vessel density ratio)	Kht index*
Source of variation ↓	r^2		0.77	0.91	0.91	0.76	0.86
MODEL	SS	7	12.728	39.066	87.681	42.861	161.57 8
ERROR	SSE	41	3.654	3.951	8.371	13.173	26.24
	p-value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
FACTORS							
Covariate Log (volume, cm^3)	SS	1	8.376	26.064	64.737	30.501	113.01 2
	p-value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Covariate log (kht index)		1					
Clone	SS	3	2.736	0.7785	3.788	9.04	18.048
	p-value		<0.0001	0.058	0.0014	<0.0001	<0.0001
Covariate*clone	SS	3	0.3946	0.2078	1.306	0.6399	5.4564
	p-value		0.3946	0.5464	0.1109	0.5890	0.0495
Adjusted means ± standard error							
AF2	Log(y)		6.69±0.09b	8.058±0.098a	14.748±0.143ab	1.3858±0.182b	
	Exp(log(y))		804.32±1.1	3158.97±1.10	2540826.55±1.15	4.0±1.20	
I-214	Log(y)		7.11±0.08a	7.750±0.084ab	14.864±0.123a	2.4766±0.168a	
	Exp(log(y))		1224.15±1.1	2321.57±1.09	2853337.73±1.13	11.9±1.18	
MONVISO	Log(y)		6.69±0.097b	8.0011±0.10ab	14.692±0.147ab	1.514±0.187b	
	Exp(log(y))		804.32±1.10	2984.24±1.11	2402450.94±1.16	4.54±1.21	
PEGASO	Log(y)		6.49±0.096b	7.66±0.099b	14.306±0.145b	1.375±0.184b	
	Exp(log(y))		658.52±1.10	2121.76±1.10	1633115.34±1.16	3.96±1.20	

df= degrees of freedom, (*) Khtindex is the theoretical hydraulic conductance divided by the constant $128\eta/\pi$

Adjusted means : $\bar{Y}_{\text{adjust}i} = \bar{Y}_i - \beta * (\bar{X}_i - \bar{X})$ where $\bar{Y}_i$ is the dependent variable mean of the i^{th} treatment, $\bar{X}_i$ is the covariate mean of the i^{th} -treatment, and $\bar{X}$ is the covariate overall mean value. β is a weighted average of the slopes of the linear regressions for both treatment groups. Tukey test at 0.05 significance level was applied to assess mean differences. Means in the same column followed by the same letter are considered equal. Means are not displayed when there is significant covariate*clone interaction.

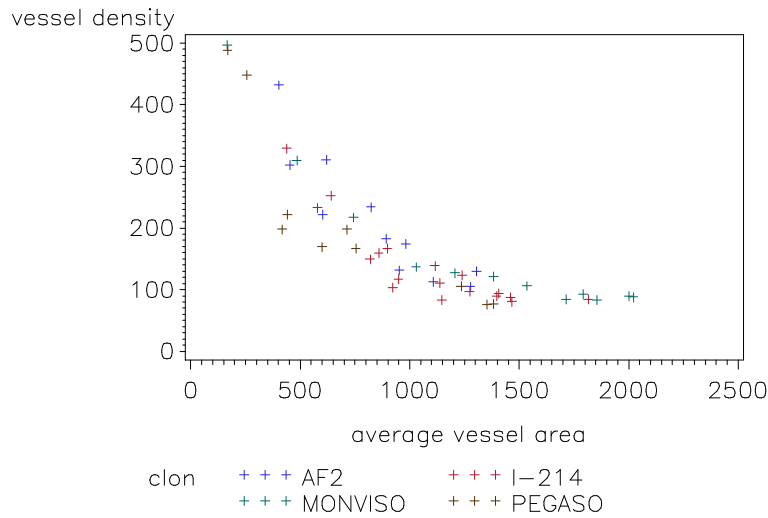


Figure 2: Decrease in vessel density (mm^{-2}) with increasing mean vessel area (μm^2)

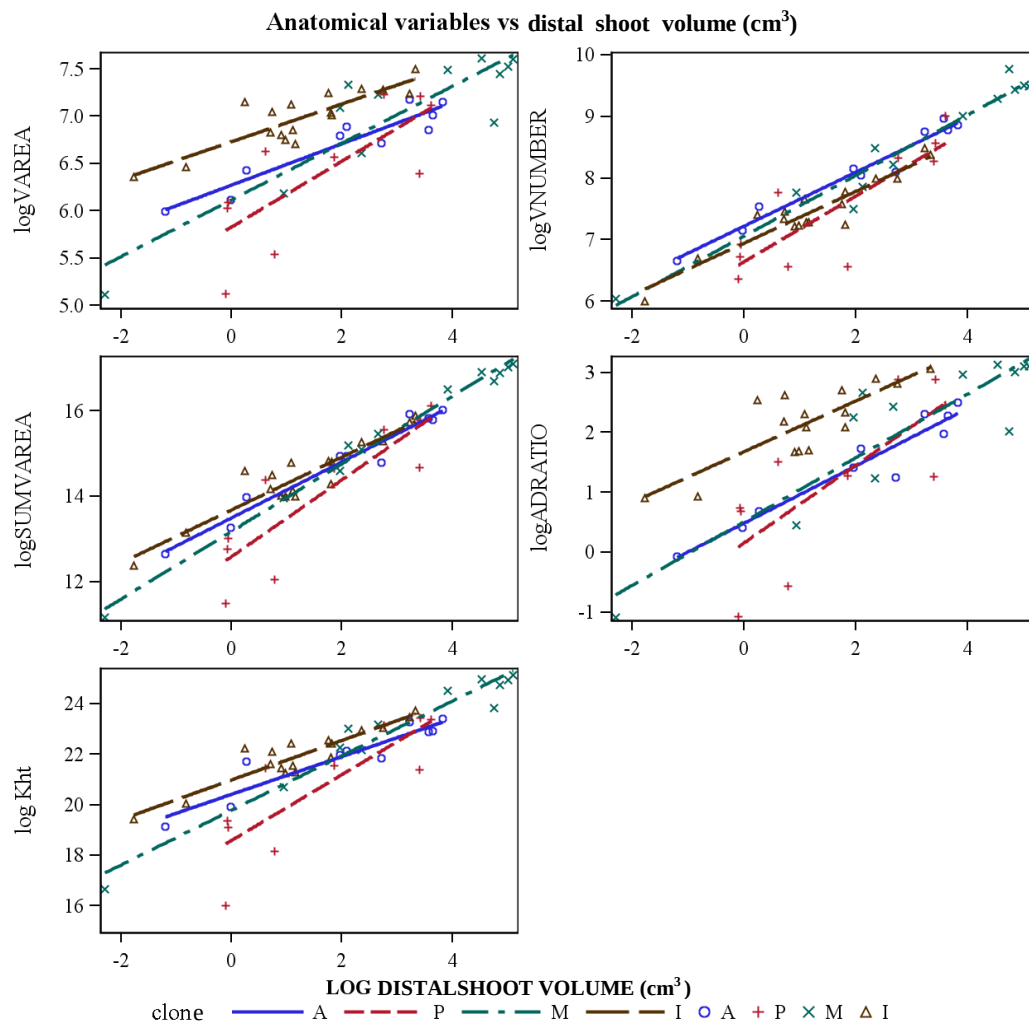


Figure 3: log-log transformed plot of anatomical variables against distal volume measured in four hybrid poplar clones. Varea =vessel area, vnumber=vessel number, sumarea=conductive area by cross section, AD ratio=vessel area to vessel density ratio, and kht=theoretical hydraulic conductivity calculated by the Poiseuille law. Legend: A=AF2, P=Pegaso, M=Monviso, I=I-214

AD-Ratio: There was a significant genotype effect for this variable, clone I-214 having the highest value. Given that radial variation in wood properties is higher in juvenile than in mature wood (Tsuchiya & Furukawa 2009, Luostarinen & Möttönen 2010) and that, according to our observations, vessel density decreases while mean vessel diameter increases with plant size (Fig 2), the AD-ratio shows that as I-214 grows, the mean vessel area increases more than in the rest of the clones per unit of decreased vessel density. If the average vessel area grows, it means that the more external vessels have to grow much more in order to attain a significant increment in the mean, taking into account the high number of vessels per cross section.

Theoretical hydraulic conductivity (Kht): Since there was a weak but significant interaction between genotype and covariate, planned contrasts were carried out to compare the intercept and slopes of the regression lines of AF2, I-214 and Monviso with those of Pegaso, which was used as reference clone. Both *P. x euramericana* hybrids AF2 and I-214 displayed significantly higher values for the intercept (p-values 0.0017 and <0.0001 respectively) and significantly smaller values for the slope than Pegaso (p-values 0.018 and 0.027 respectively). The Monviso intercept was significantly higher than that of Pegaso. As can be observed in figure 3, the kht regression lines of euramerican clones were above those of the interamericana x nigra hybrids and all lines tend to converge when it came to more voluminous samples, although the slope of the Monviso regression line was not significantly different from any other clone.

PHYSIOLOGICAL RESULTS

Gas exchange measurements

The analysis of covariance of the photosynthetic rate, stomatal conductance and transpiration with time (hour of measurement) as the covariate revealed significant clone effect and interaction between clone and covariate ($p < 0.0002$). (Table 3)

The photosynthetic rates of Monviso and AF2 showed significantly higher slope and intercept than Pegaso (Table 3), which displayed an evolution of the photosynthetic rate over time following an almost horizontal line (Fig 4). In contrast, the photosynthetic rates of Monviso and AF2 dropped as temperature increased from midmorning onwards. The Monviso, AF2 and I-214 clones also displayed significantly different values from those of Pegaso as regards stomatal conductance and transpiration (intercept and slope) (Table 3). As can be seen in figures 4b, and 4c, the stomatal conductance and transpiration regression lines of Pegaso before 11:30 a.m are below the analogous regression lines of the other three clones. However, from about 11:30 onwards, stomatal conductance and transpiration remained almost constant for Pegaso while they declined in the rest of the clones.

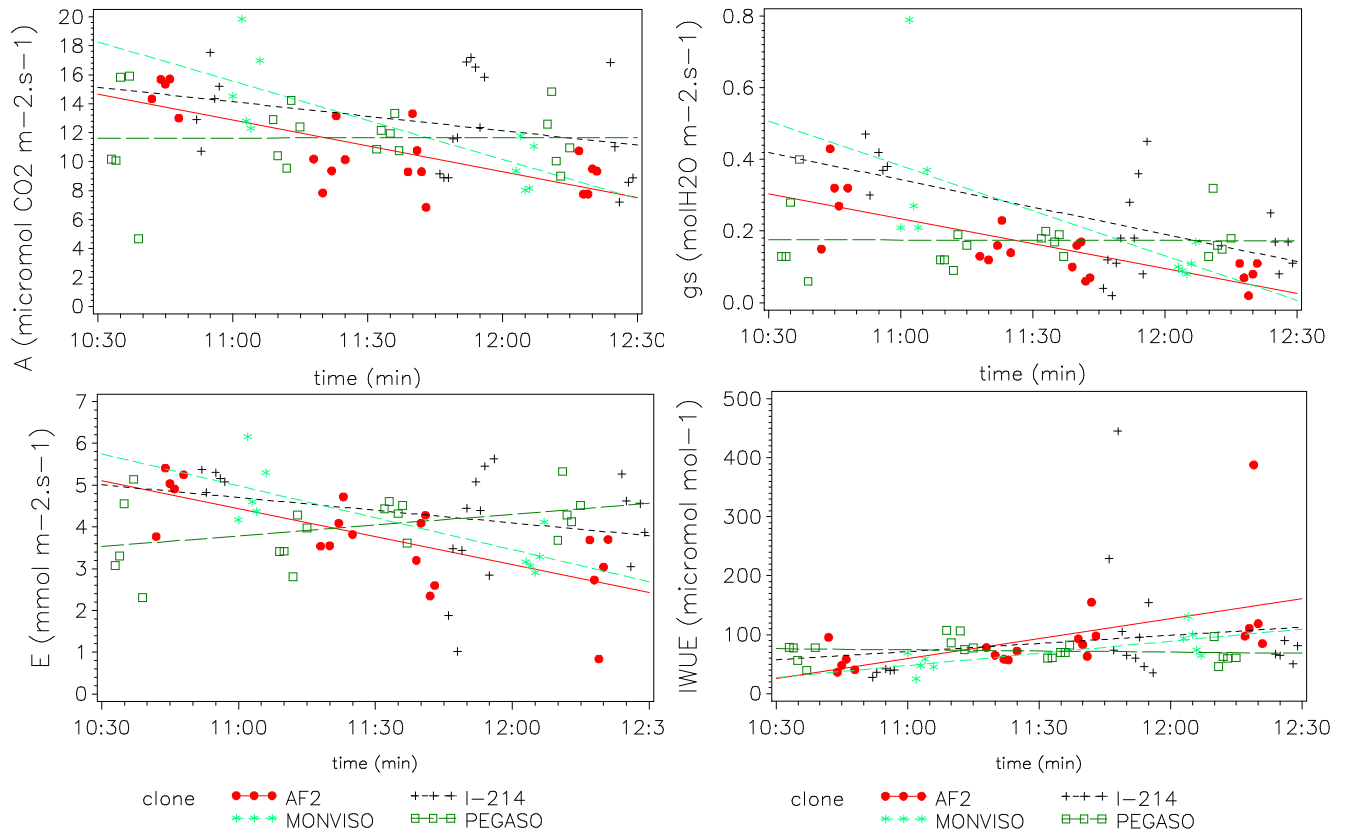


Figure 4: Photosynthetic rate (a), Stomatal conductance (b), Transpiration (c) and Intrinsic water use efficiency (IWUE) represented over the course of the time.

Table 3. Results of the ANCOVA and Tukey adjusted mean test applied to the gas exchange variables measured in four hybrid poplar clones.

Dependent variable (y)		df	Photosynthesis rate ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Stomatal conductance ($\text{mol m}^{-2}\text{s}^{-1}$)	Transpiration rate ($\text{mol m}^{-2}.\text{s}^{-1}$)	WUIE (A gs^{-1})				
ANCOVA MODEL Y=TIME+CLONE +TIME*CLONE+ERROR										
Source of variation		r ²	0.34	0.41	0.29	0.15				
MODEL	SS (DF)	7	245.69	0.07	3.34	6693.30				
ERROR	SSE(DF)	67	480.42	0.01	0.87	3941.49				
	p-value		0.0002	<0.0001	<.0001	0.12				
FACTORS										
Covariate time (horas)	SS (DF)	1	152.68	0.36	8.8	24019.90				
	p-value		<.0001	<.0001	<.0001	0.02				
Clone	SS (DF)	3	24.04	0.05	4.21	16261.95				
	p-value		0.02	0.01	<.0001	0.26				
time*clone	SS (DF)	3	71.59	0.04	4.84	5671.46				
	p-value		0.02	0.01	<.0001	0.24				
Comparison of parameter estimates of the ANCOVA model using Pegaso as reference group.										
Parameters			ESTIMATE	PR<F	ESTIMATE	PR<F	ESTIMATE	PR<F		
Intercept			11.3439	0.3341	0.1900	0.6768	-1.9044	0.6413	116.6573	0.6709
clone	AF2		40.7188	0.0186	1.5650	0.0200	21.0241	0.0007	-801.2418	0.0468
clone	I-214		24.7911	0.1573	1.8282	0.0085	13.3021	0.0313	-349.2467	0.3932
clone	MONVISO		51.0310	0.0047	2.1813	0.0020	16.9716	0.0069	-416.6278	0.3123
clone	PEGASO		0.0000	.	0.0000	.	0.000	.	0.0000	.
time			0.000007	0.9799	0.0000	0.9729	0.00014	0.1517	-0.0011	0.8727
time*clone	AF2		-0.0010	0.0173	0.0000	0.0195	-0.0005	0.0006	0.0199	0.0419
time*clone	I-214		-0.0006	0.1795	0.0000	0.0116	-0.0003	0.0344	0.0087	0.3720
time*clone	MONVISO		-0.0012	0.0052	-0.0001	0.0027	-0.0004	0.0070	0.0099	0.3117
time*clone	PEGASO		0.0000	.	0.0000	.	0.0000	.	0.0000	.

Hydraulic measurements

Table 4 shows both a and b parameter estimates of the Pammenter and Vander Willigen curves, mean square errors, and confidence intervals for each method, clone and water treatment. Figure 5 shows the plotted means and confidence intervals of b coefficients to facilitate understanding of the text.

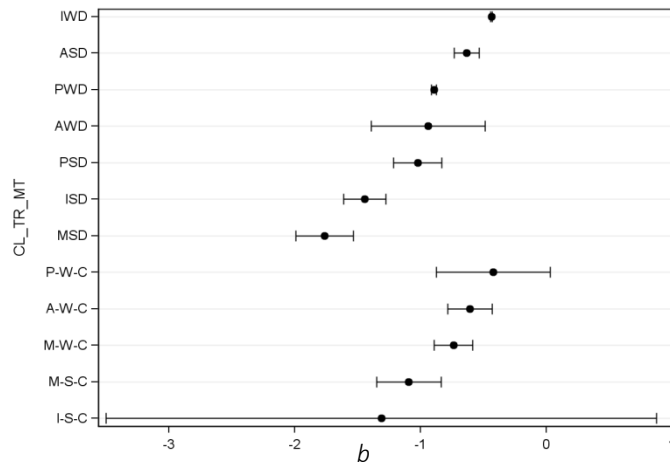


Figure 5: means (black dots) and confidence intervals of b coefficients per clone, treatment and method (CL_TR_MT) ordered by method and magnitude. The first of the three letters on the vertical axis denotes the clone (A=AF2, I=I-214, M=Monviso and P=pegaso), the second letter corresponds to the watering regime (W) or stressed (S), and the third letter is the method employed to induce embolism: D (dehydration) and C (centrifuge)

Comparison of vulnerability curves (VCs) built using different methods:

The results of the mean square reduction test applied to data obtained from different methods revealed a significantly higher b value for those obtained through centrifuge (Table 4, Fig.5). The VCs obtained using both methods were s-shaped as well as r-shaped. For instance, the dehydration-based VC for well-watered I-214 as well as the centrifuge based curve for stressed AF2 were r-shaped, whilst the centrifuge-based VC for stressed Monviso and dehydration-based VC for stressed I-214 were s-shaped (Fig. 6).

Vulnerability curves obtained using the dehydration method:

Differences between treatments:

Dehydration-based VC corresponding to stressed plants was shifted towards more negative xylem tensions than the well-watered one. Differences between their corresponding b -coefficient values were significant (their confidence intervals did not overlap, (Table 4). The coefficient a of the stressed curve was also significantly lower than that of the well-watered vulnerability curve. (Table 4)

Table 4: Parameter estimates of the Pammenter and Vander Willigen model, mean standard error and 99.7 % confidence intervals by method and for each watering regime-clone combination. When the confidence intervals of two compared parameters do not overlap, it is considered that these parameters are significantly different at 99.7% level.

Bench dehydration method									
Clone		Estimate	std	(99.7%)		Estimate	std	(99.7%)	
			error	confidence limits			error	confidence limits	
Watering treatment			Coefficient b (Ψ_{50})				Coefficient a		
WS		-1.2345	0.0527	-1.3381	-1.1309	1.9845	0.1517	1.6859	2.2831
WW		-0.7109	0.0485	-0.8063	-0.6156	3.5731	0.4169	2.7528	4.3934
Watering treatment-clone combinations									
WS	AF2	-0.631	0.045	-0.771	-0.492	5.269	1.325	1.210	9.328
	I-214	-1.445	0.086	-1.710	-1.180	2.053	0.279	1.197	2.909
	MONVISO	-1.762	0.141	-2.193	-1.331	1.820	0.431	0.501	3.139
	PEGASO	-1.022	0.074	-1.250	-0.794	2.196	0.267	1.380	3.013
WW	AF2	-0.940	0.141	-1.373	-0.508	2.110	0.479	0.642	3.578
	I-214	-0.437	0.0047	-0.451	-0.422	106.0	70.307	-109.3	321.3
	PEGASO	-0.8938b	0.0164	-0.9439	-0.8436	27.500	15.607	-20.29	75.29
Centrifuge method									
Clone		Estimate	stderror	(99.7%)		Estimate	stderror	(99.7%)	
			confidence limits				confidence limits		
Watering treatment			Coefficient b (Ψ_{50})				Coefficient a		
WS		-1.0938	0.1842	-1.4578	-0.7299	1.4943	0.4684	0.5685	2.4200
WW		-0.5554	0.0659	-0.6855	-0.4252	1.8384	0.2487	1.3468	2.3300
Watering treatment-clone combinations									
WS	I-214	-1.3123	1.1437	-4.7284	2.1038	1.0388	1.2199	-2.6050	4.6825
	MONVISO	-1.0930	0.1727	-1.6090	-0.5771	1.6280	0.5662	-0.0631	3.3192
WW	AF2	-0.6071	0.0999	-0.9056	-0.3085	2.3319	0.4850	0.8832	3.7805
	MONVISO	-0.7381	0.1029	-1.0454	-0.4307	2.8431	0.6174	0.9992	4.6870
	PEGASO	-0.4216	0.1673	-0.9212	0.0781	2.2244	1.1241	-1.1329	5.5818

WS: water stressed, WW: well-watered

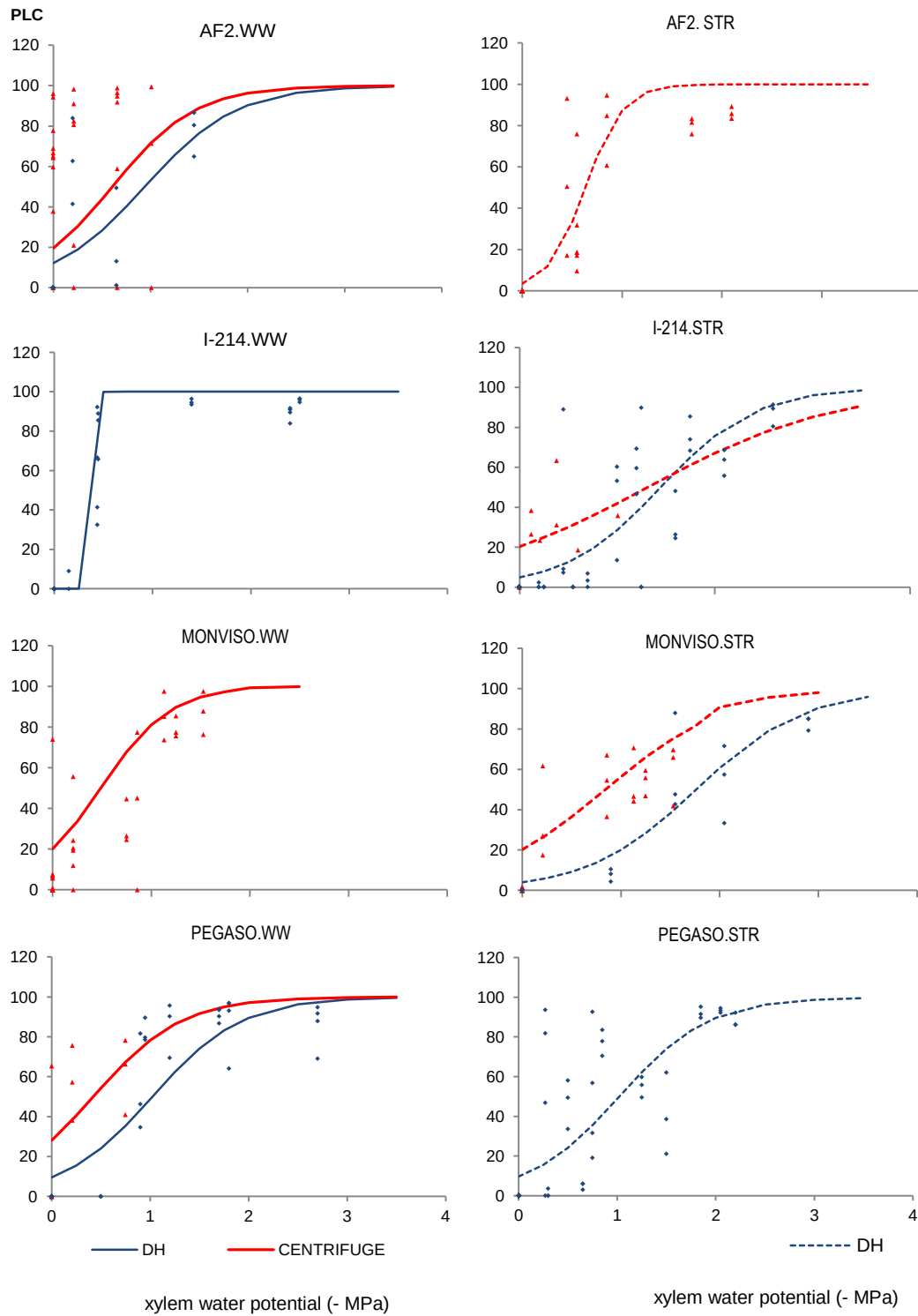


Figure 6: PLC values measured after induce xylem tension through dehydration (blue) method or centrifuge (red). Lines are the Pammenter and Vander Willigen curves fitted to the PLC measurements grouped by method. Absolute values of xylem water potential are plotted.

Differences between clone-watering regime combinations:

-Parameter a :

We found no significant differences between the slopes (parameter a) of the VCs corresponding to the eight clone-watering treatment combinations.

-Parameter b :

Only clone I-214 displayed significant differences in the b parameters of the VCs corresponding to the two watering regimes, indicating that I-214 was the most vulnerable clone under well-watered conditions (b value of I-214 was significantly higher (less negative) than the rest of the clone-treatment combinations) (Table 4, Fig 5, Fig 7) as well as being, together with the Monviso clone, one of the most cavitation resistant when submitted to a water stress regime.

The AF2 clone subjected to the well-watered regime was found to be as vulnerable as both Pegaso (under either watering regime) and stressed AF2. (Table 4, Fig 5)

Under stress, the VC of AF2 showed a significantly higher b value than the rest of the clones (Fig5, table 4). The next clone in the ranking of vulnerability under stress was Pegaso, and the lowest values of b were obtained in VCs of clones I-214 and Monviso, which were the most resistant to cavitation under water stress (differences between I-214 and Monviso were not significant). Stressed AF2 proved even more vulnerable (less negative b value) than Pegaso under the well-watered regime (Fig 5).

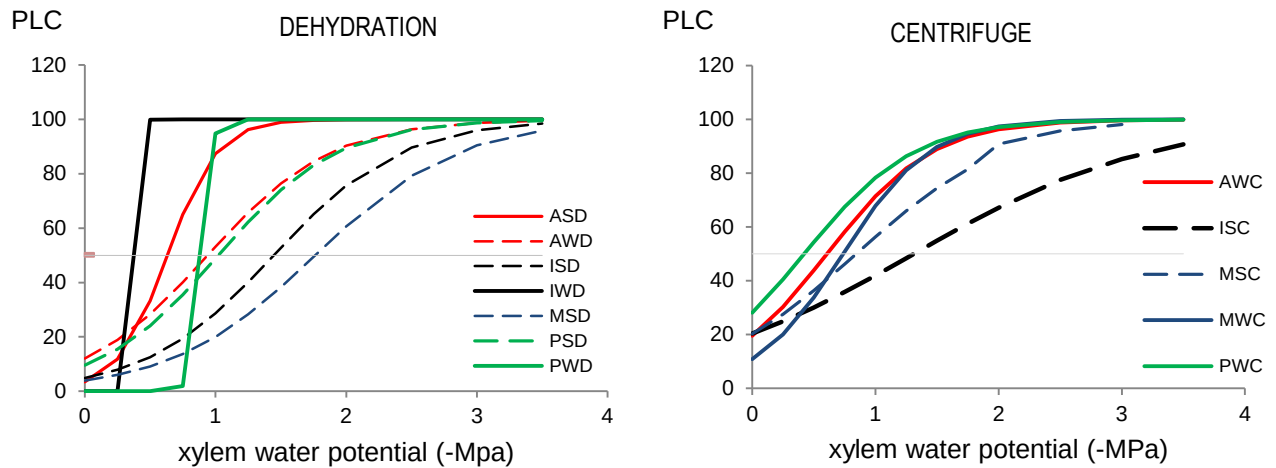


Figure 7: Vulnerability curves obtained from dehydration (left) and centrifuge (right) . Dotted lines correspond to the stressed treatment. Letters in the legend stand for the clone (A=AF2, I=I-214, M=Monviso, P=Pegaso), followed by watering regime (W=well watererd, S=Stressed) and method (D=dehydration, C=Centrifuge)

Vulnerability curves obtained from centrifuge:

The coefficients a and b of the centrifuge-based VC corresponding to the stressed watering treatment were significantly different from the coefficients of the corresponding well-watered vulnerability curve. Coefficients of the stressed curve were lower than those of

the well-watered curve (Table 4). We found no differences in the vulnerability curves of the different clone-watering regime combinations. As in the dehydration-based VCs, well-watered Monviso was as vulnerable as AF2 and Pegaso under the more favourable watering regime.

Clonal differences in specific conductivity:

The analysis of covariance carried out on specific hydraulic conductivity (K_{xs}), calculated as the ratio of hydraulic conductivity (K_i) to maximum cross-section sample, considering xylem tension as covariate, showed that under the well-watered regime, AF2 was the clone with highest conductivity in relation to the rest of the clones (Tables 5, 6, Fig 8). Under the stressed water regime no clone effect was found. Since a significant watering treatment x clone interaction was identified, data from both treatments were analyzed separately (table 5).

Table 5: ANCOVA table of specific hydraulic conductivity (K_{xs}), using xylem water potential (XWP) (MPa) as covariate

Model K _{xs} ~XWP +clone+XWP*clone								
WELL WATERED					STRESSED			
Source	DF	Mean Square	F value	Pr > F	DF	Mean Square	F value	Pr > F
MODEL	5	2422.87	9.88	<.0001	7	1347.22	2.05	0.0574
ERROR	46	245.13			93	658.69		
Factors								
XWP	1	4904.61	20.01	<.0001	1	3316.37	5.03	0.0272
CLONE	2	2563.05	10.46	0.0002	3	408.93	0.62	0.6033
XWP*CLONE	2	1029.14	4.2	0.0211	3	366.75	0.56	0.6449

Table 6: Estimates and standard errors of the ANCOVA model K_{xs}=intercept+ XWP+clone_i* (dummy) +XWP* clone_i. Pegaso was used as reference group. Solution of t tests. The significance level is 0.05

Parameter	k _{xs} estimates	Standard Error	t Value	Pr > t
Intercept (reference group)	36.163	6.004	6.02	<.0001
(XWP)	-15.941	5.720	-2.79	0.0063
CLONE AF2	60.078	12.369	4.86	<.0001
CLONE I-214	36.020	9.492	3.79	0.0002
CLONE PEGASO	0.000	.	.	.
Slope				
XWP*CLONE AF2	-45.093	17.501	-2.58	0.0113
XWP *CLONE I-214	-16.199	8.6344	-1.88	0.0633
XWP *CLONE PEGASO	0.000	.	.	.

XWP= xylem water potential (MPa)

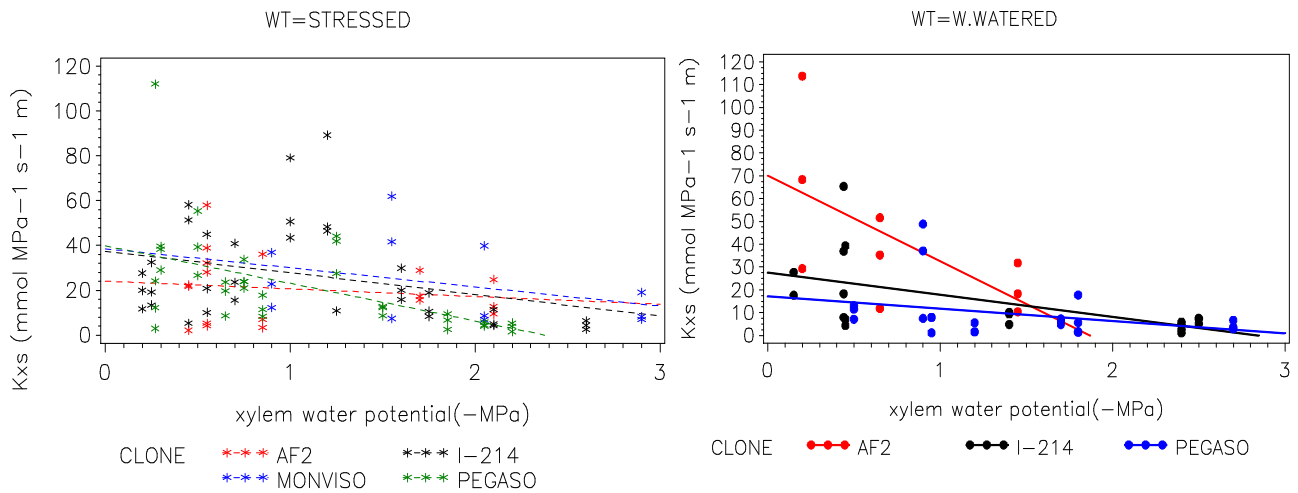


Figure 8: Specific hydraulic conductivity ($K_{xs}=k_i/A_s$) versus xylem water potential in stressed (left) and well watered (right) plants. (wt=watering treatment, K_i : native hydraulic conductivity A_s = cross section area). All data plotted were obtained through the bench dehydration method.

DISCUSSION:

ANATOMICAL AND PHYSIOLOGICAL TRAITS RELATED TO YIELD (GRANADA)

Both a high number of vessels and an intermediate vessel area were common anatomical xylem traits observed in the clones with the highest biomass yield (Monviso and AF2). A high number of vessels may make them better able to withstand stress, since the loss of functionality in a vessel would involve a lower percentage loss in conductivity. Hacke et al. (2006) argued that if vessel vulnerability remained constant, increasing the number of vessels would increase conductivity, but not at the expense of safety. It may be that Monviso and AF2, by distributing their xylem conducting elements in this way, optimize conductivity as well as safety under stressed environments.

Furthermore, Monviso displayed a significantly higher number of branches than the rest of the clones, the number being twice that of AF2 and Pegaso and seven times that of I-214. In Granada, all the branches developed on newly formed shoots which did not exhibit a dormant or rest period, therefore all branches were sylleptic (Wu & Hinkley 2015). Ceulemanns et al. 1990 reported the vital role of sylleptic branches in maximizing the carbon productivity of the tree crown in *P trichocarpa x deltoides* (*Pxinteramericana*) hybrids, since they are involved in the increase of the plant leaf area.

The limited biomass yield of Pegaso can be attributed to the lower theoretical hydraulic conductance (k_{ht}) caused by the lower number of vessels and xylem conducting area. This theoretical high hydraulic resistance to sap flow is in accordance with the lower stomatal conductivity also exhibited by Pegaso. The xylem conducting area of the Pegaso clone comprised a low number of vessels of intermediate-low size. These anatomical xylem traits, in relation to those of the other clones, can be considered appropriate traits for withstanding drought, although they are not considered favorable traits for growth (Sperry et

al. 2007). Poplars are considered pioneers trees (Eckenwalder 96). High hydraulic efficiency and stomatal conductance are traits of these species that reflect an opportunistic behavior (Tyree et al., 98).

The AD-ratio is similar to the Carlquist (1977) vulnerability index (VI), with mean vessel area instead of vessel diameter to vessel density ratio. Higher VI values would provide a rough indication of the ability of the plant to withstand drought (Scholz et al. 2013). According to these indexes, the I-214 clone was significantly less able than the others to withstand drought. As the shoot stem grows, I-214 develops wider vessels, which may lead to higher vulnerability and therefore low hydraulic conductivity due to dysfunction if a positive link between vessel diameter and pit properties is considered. Given the high number of shoots developed by I-214, it would appear that the strategy of this clone is to avoid having excessively wide vessels by sprouting a greater number of thinner shoots with smaller vessels instead of a few wider shoots. The low number of branches growing on the shoots of the I-214 clone may have contributed to a lower leaf area development, which in turn may have reduced plant growth.

Returning to anatomy, if pit properties and vessel diameter were unrelated, wide vessels might be more cavitation resistant than narrower vessels (Lens et al. 2010). If this was the case, as suggested by the results from the vulnerability curves, the low biomass yield of I-214 could be attributed to low conductivity caused by pit impermeability instead of by vessel embolism dysfunction. This lack of trade-off between growth and vessel diameter was also found by other authors such as Hajek et al. (2014), who calculated Kht in certain demes of *Populus tremula* and *P.tremuloides*, and found no correlation between growth rate and theoretical hydraulic conductivity. However, they did find correlation between xylem specific hydraulic conductivity and growth, suggesting that this could be due to differences in pit properties between different genotypes.

YIELD-CAVITATION RESISTANCE (MADRID)

The Values of P_{50} obtained in this work were of the same magnitude as those obtained by Lenka Plavcová et al. (2012) for a hybrid clone (*P. trichocarpa x deltoides*).

In addition, in order to relate growth with vulnerability to cavitation, the Madrid trial was directed towards obtaining a rough estimation of the relationship between cavitation resistance and xylem anatomy. We use the term 'rough estimation' because we are conscious of the fact that the environmental conditions and soil properties are not identical in the stressed treatment plots in Madrid and Granada. However both locations have similarly hot, dry summers without rain or irrigation for two whole months, a high level of irradiance and maximum temperatures at midday around 35 °C; hence high evaporative demand and dry soil conditions. Furthermore, in line with our observations in the present study, Weitz et al. (2006) reported that average vessel area (in each clone) is determined mainly by stem diameter rather than height or age. Therefore, there is no reason to believe that stressed plants in Madrid might have a very different xylem anatomy to those in Granada. The plant diameters in Madrid fall within the range of plant diameters recorded in Granada. Given that in Granada I-214 was the clone with the highest vessel diameter and AD-ratio, it was expected to be the most vulnerable clone under water stress conditions in Madrid. However, the results revealed the opposite. I-214 proved to be the most resistant clone under stressed conditions along with

Monviso, whereas Pegaso and AF2 were more vulnerable than both Monviso and I-214. In accordance with these results, Beikircher et al. (2013) reported the highest vulnerability to cavitation in the *Malus* cultivar with the narrowest vessels. Moreover, AF2, with a significantly higher number of vessels, was more vulnerable under the stressed water regime than Pegaso, suggesting that differences in pit properties carry more weight as regards vulnerability to cavitation than number and diameter of vessels.

Drought-induced vulnerability to cavitation depends on many factors such as number and diameter of vessels, vessel length (Jacobsen et al. 2012), xylem networking or pit permeability, which includes mechanical and chemical pit properties (Lens et al. 2010, Awad et al. 2010, Loepfe et al. 2007, Rockwell et al. 2014). The interaction of all these factors makes it difficult predict vulnerability from vessel number and diameter and while some authors, such as Martínez-Vilalta (2002) in different species or Awad et al. (2010) in a hybrid poplar clone, have found a positive relationship between vessel diameter and vulnerability to cavitation, others have reported only a weak relationship (Gleason et al. 2015) or no relationship at all.

In the experimental trial in Madrid, the clones with the highest yield were among the most cavitation resistant of the clones analyzed separately according to the watering regime applied. AF2, under the well-watered regime, was more cavitation resistant than I-214, and Monviso was more resistant than both AF2 and Pegaso under stressed conditions. Pegaso and I-214 were the clones which yielded the least biomass regardless of the watering regime they were subjected to and they were grouped as either resistant or vulnerable clones depending on the watering regime to which they were subjected. Therefore, the clones classified as resistant do not necessarily exhibit high growth, although it should be noted that within the genotype range tested it is possible to select the clone with the highest biomass yield and greatest resistance to cavitation. These results are in accordance with those obtained by Fichot et al. in 2010, who compared eight unrelated euramerican poplar genotypes and obtained the highest growth for the most cavitation resistant clonal copies. Guet et al. (2015) also found no correlation between resistance to cavitation and growth in 33 genotypes of *Populus nigra*, although they found relationship between vulnerability to cavitation and hydraulic efficiency. However, in a study of willows and poplars, Cochard et al. (2007) did find a correlation between xylem cavitation and yield. This disparity of results is not surprising considering the above mentioned factors and interactions affecting cavitation resistance in addition to the known phenotypic plasticity and acclimation ability of poplar (Awad et al. 2010, Wu & Hincley 2010, Plavcová et al. 2012)

Under the well-watered regime, clone AF2 displayed higher (cross-section) specific hydraulic conductivity and growth (Fig 3), whereas the most vulnerable to cavitation was the I-214 clone. Among the stressed plants, no clonal differences were found as regards specific conductivity but there were differences in vulnerability to cavitation. These results do not point to an efficiency/safety trade-off, which may mean that it would be possible to find high yielding clones which are resistant to cavitation. Many authors report that the efficiency/safety trade-off is far from universal. For example, Maherali *et al.* (2004), in an analysis of data from 150 species, found a clear efficiency/safety *trade-off* in conifers and evergreen angiosperm but not in deciduous angiosperms. In the case of the latter, no

correlation between Ψ_{50} and hydraulic conductivity was found. Gleason et al. (2015) found a weak trade-off in a study of 355 angiosperm species. Similarly, research conducted by Burgess et al. (2014), working with *Sequoia sempervirens*. Don, as well as studies undertaken by Fichot et al. (2010) and Plavcová & Hacke (2012) with poplar, all obtained results that questioned the universal occurrence of the efficiency/safety trade-off. Of the 355 species tested by Gleason et al. (2015) some were found to be vulnerable to cavitation and exhibited low efficiency, but none of them were found to display resistance as well as efficiency.

Clone I-214 had the greatest plasticity as regards vulnerability to cavitation, becoming the most or the least vulnerable depending on the water treatment, which is supported by some studies reporting high plasticity in poplar vulnerability to cavitation. Plavcová et al. (2011, 2012), found differences in pit membrane thickness and porosity when the same poplar clone was subjected to different irradiance treatments, such that shaded plants were less cavitation resistant and had a thinner pit membrane. Choat et al. (2008) studied the pit properties of 14 hardwood species and found that the more vulnerable species had thinner pit membranes. The new xylem grown by I-214 under stress may have incorporated certain characteristics that provide it with high cavitation resistance such as less permeable pit membranes, or shorter vessels, or both, whereas well-watered plants may have suffered pit fatigue, a well-known occurrence in poplar (Hacke et al. 2001, Feng et al. 2015).

In summary, we found clonal differences in xylem anatomical traits and, as expected, the clone with the widest vessels, I-214, did not exhibit the highest biomass yield under water stress conditions in Granada, nor was it the highest yielding clone under the well-watered regime in Madrid. Xylem anatomical characteristics along with the number of sylleptic branches were useful traits for detecting differences between clones in terms of biomass yield. Resistance to cavitation is one the traits common to the clones with the highest biomass yield in Madrid regardless of the water regime to which they were subjected, although it is also possible to find cavitation resistant but poor yielding clones. There is no clear trade-off between efficiency and safety.

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CAPÍTULO 4



CAPÍTULO 4

EVOLUTION OF LEAF AREA INDEX IN TWO POPLAR SHORT ROTATION ENERGY CROPS. COMPARISON BETWEEN DIRECT AND INDIRECT LAI ESTIMATION METHODS

RESUMEN

En dos plantaciones de chopo destinadas a producción de biomasa con fines energéticos (plantaciones de alta densidad y turno corto), se han medido durante dos años consecutivos la producción de biomasa seca y el índice de área foliar, este último por dos métodos: (i) semidirecto, mediante pesada de todas las hojas de una muestra de árboles, e (ii), indirectamente mediante toma de fotos hemisféricas y aplicación de modelos de ajuste de la fracción de huecos de la cubierta.

Se han utilizado tres modelos diferentes para estimar el índice de área foliar a partir de la fracción de huecos obtenida por digitalización de las fotografías hemisféricas. Estos modelos son el modelo de Poisson y dos modelos más propuestos recientemente para pino silvestre y que aportan al modelo de Poisson un factor de corrección para el agrupamiento de las hojas: el primero contempla independientemente el efecto de agrupamiento de las hojas y el efecto de inclinación de las mismas, y el segundo pondera el efecto de ambos factores en función del ángulo que forma el rayo de luz incidente con el cénit.

Los resultados muestran una buena correlación entre la producción y el índice de área foliar ya sea medido de forma semi-directa o indirectamente. El modelo para estimar el índice de área foliar que mejor se ajusta a la fracción de huecos medida en las fotografías hemisféricas es el tercer modelo presentado propuesto para pino silvestre. Se propone el estudio de la evolución del índice de área foliar en estas plantaciones estimándolo indirectamente a partir de fotografías hemisféricas y utilizando los modelos que aportan modificaciones al modelo de Poisson así como probar nuevos modelos en las masas plantadas con diseños más irregulares.

Palabras clave

Índice de área foliar, biomasa, cultivos energéticos, fotografías hemisféricas

EVOLUTION OF LEAF AREA INDEX IN TWO POPLAR SHORT ROTATION ENERGY CROPS. COMPARISON BETWEEN DIRECT AND INDIRECT LAI ESTIMATION METHODS

HERNÁNDEZ GARASA, M.J.¹, MONTES PITA F.¹, PITA ANDREU P.², VISCASILLAS GÓMEZ, A.¹, SIXTO BLANCO, H.¹, CAÑELLAS REY DE VIÑAS, I.¹

¹ Dpto. Sistemas y Recursos Forestales. CIFOR-INIA. Ctra La Coruña km 7,5. Madrid 28040.

² Dpto. de Silvopascicultura, Universidad Politécnica de Madrid, Ciudad Universitaria s/n, 28040, Madrid.

ABSTRACT

In 2006 and 2007, biomass production and leaf area index (LAI) were measured at two short rotation poplar plantations located around 250 Km apart. LAI was measured by means of both (i) direct measurements, which involved weighing all the leaves of some trees and measuring their leaf areas, and (ii), indirect measurements, through the application of models that relate LAI to the skygap distribution recorded in hemispherical photographs.

Three different models were used in order to estimate LAI from the gap fraction obtained by digitalizing hemispherical photographs. The first model employed was the Poisson distribution model, and then two other models initially proposed for *Pinus sylvestris* L., both of which add a correction factor to the Poisson model for clumping of leaves and branches. The difference between the two latter models was that one of them includes the clumping effect of leaves and the foliage inclination effect as independent effects, whereas the second model considers both effects weighted by the angle formed by the beam direction with the zenith.

The LAI results and biomass production were highly correlated regardless of the LAI method considered.

The model which considered clumping and foliage inclination effects weighted by the angle of beam direction and the zenith was the one that provided the best fit to the gap fraction distribution measured on hemispherical photographs. The use of the two latter models is proposed for the indirect LAI estimation method in order to assess the evolution of LAI over time in this type of plantation. Further modifications are recommended in order to solve the problem of infraestimation of the LAI where planting designs are uneven.

INTRODUCTION

Recurrent increases in oil cost, along with the challenges posed by climate change have led to the rise in investment by governments in the development of renewable energy sources in order to contribute to a clean, sustainable energy supply and maintain a net neutral carbon balance.

As part of the current Renewable Energy promotion plan (PANER in Spanish) (2011-2012) the Spanish government has applied the main European guidelines on renewable energy (directive 2009/28/CEE) in accordance with the particular circumstances of the country in an attempt to create the conditions needed to increase the use and development of biomass as an energy source. The creation of a biomass trade is an important objective in the push to increase the contribution of renewable energy sources to the gross Energy consumption in Spain to 20% by 2020.

Among the woody energy crops, poplar is one of the most widespread in the Mediterranean area because of its high plasticity, high growth and ease of propagation. These pioneer species trees are competition tolerant, which allows them to become established and survive safely in crowded plantings and they are also suited to regular harvesting every few years. These are common silvicultural practices in short rotation woody crops.

The rotation that maximizes volume production (optimum rotation) is unknown at the beginning of the plantation. Strong & Hansen (1993) hypothesized that the time needed to reach maximum mean annual biomass productivity (optimum rotation) is linearly related to the time required for canopy closure. In the field, the moment at which canopy closure is reached is not evident, and determining the optimum rotation requires the measurement of current annual biomass increment and the calculation of mean annual increment.

Many studies have reported a highly significant correlation between leaf area index (LAI) and biomass production (Verlinden M.S. et al. 2014, Pellis *et al.*, 2004, Fang et al. 1999 Linder., 1985, Zavitovski *et al.*, 1976, Ridge *et al.*, 1986, Dunlap & Stettler, 1998). Therefore the LAI estimation in this type of plantations could provide useful information with regard to the moment at which canopy closure and optimum rotation length is reached.

Apart from identifying the rotation length that maximizes biomass or volume, it is also important to determine which genotypes are capable of yielding a greater amount of biomass and whether any of them yield more biomass with a lower LAI, that is, genotypes with more efficient foliage. Pellis et al (2004) tested different poplar clones in a short rotation coppice culture and reported that LAI was the main genetically controlled foliar variable underlying biomass productivity.

The overall objective of this study is to use indirect approaches to estimate future LAI, in turn determining the moment of canopy closure, and to compare the LAI of different genotypes. With this objective in mind we attempt to determine whether LAI estimation from hemispherical photographs is a sufficiently reliable approach for use in the comparison of different genotypes, different years and at different sites. The specific objectives are (1) to compare LAI obtained from direct estimations with LAI obtained using indirect (hemispherical

photograph) measurements over two consecutive years and (2) to assess whether there is a good correspondence between LAI estimations and biomass yield.

MATERIAL AND METHODS

SITES AND EXPERIMENTAL DESIGN

The LAI measurements were carried out at two plantations located in Almazán (Soria) and Valtierra (Navarra). Soil characteristics, altitude and coordinates of both sites are summarized in table 1. The Almazán site is at a higher altitude and has colder, more continental conditions than the Valtierra site. The vegetative period at Almazán is therefore shorter and late frosts are not infrequent, so it is reasonable to expect a lower biomass yield than at Valtierra where the climate is milder, the vegetative period is longer and freezing temperatures are usually limited to mid-winter, when plants no longer have leaves and frosts do not damage the plants.

Table 1: Site coordinates and soil parameters

Site	pH	clay (%)	silt (%)	altitude (%)	latitude (%)	longitude (%)
Almazán	8.7	32.5	56.5	827	40° 48'	15° 17'W
Valtierra	8.6	20.0	20.0	263	42° 10'	15°17' W

The different irrigation dose applied at each site also contributes to the expectation of a lower biomass production at Almazán than at Valtierra. Whereas Valtierra was basin irrigated, with an annual dose of about 5000 m³/ha-año, the Almazán planting was sprinkler (first year) and surface (second year) irrigated and the annual dose was about 1800 m³ha⁻¹ year⁻¹.

In the framework of the nationwide project “On Cultivos”, the objective of which was to test the viability of biomass as a renewable energy source, a Short Rotation Poplar Crop Network was established which comprised a large number of biomass yield demonstration trials throughout the traditional poplar growing areas of Spain. The genetic material employed at the two study sites included a set of four clones, two of which, I-214 and AF2, are *Populus x euramericana* hybrids while the other two, Monviso and Pegaso, are *Populus x interamericana x nigra* hybrids. The clones AF2, Monviso and Pegaso, were selected in Italy specifically for use in high density short rotation biomass plantations, whereas the I-214 clone, widely used in Spain in intensive popluculture, acts as a control and its behavior in this type of plantations is also tested.

The planting density was 20.000 trees per hectare at both sites and the minimum area occupied by each trial was 3 hectares. A size of 4 hectares was employed at Almazán and 3 hectares at Valtierra. The four clones were distributed in a random block design at both sites, and planting was carried out in March 2006.

Planting designs were different at each site. At Valtierra, 20 cm long unrooted cuttings were planted at 0.5 m spacing in rows 1 m apart, whereas at Almazán planting followed a double row design with alternate rows spaced 0.5 and 3 meters apart and a spacing of about 33cm between plants in each row.

In 2006, weed control and fertilization were carried out at both sites. Weed control was done before and after planting by applying herbicides; Oxyfluorfen (4l ha⁻¹) prior to planting and Glufosinate Ammonium at the same dose after planting. Mechanical weeding procedures were also used. A basic dressing was applied to the soil at Almazán using 400 Kg ha⁻¹ of N:P:K 12:22:22 and at Valtierra 1790 Kg ha⁻¹ of 9:23:30 NPK complex was applied. Fertilization was carried out at Almazán by applying 250Kg ha⁻¹ of Calcium Amonium Nitrate and at Valtierra by adding 520 Kg ha⁻¹ of the same fertilizer. In the winter of 2006-2007, the plantations were cut back in order to promote resprouting at both sites.

In 2006 at Almazán, both the Monviso and Pegaso clones suffered high mortality (more than 20%) and a decision was taken to remove the remaining plants of these two clones and re-plant them in March 2007. The only exceptions were Monviso plants in block1, where mortality was not as high so the plants were not removed. Hence, in the 2007 vegetative, the Monviso and Pegaso plants (except the Monviso block 1) had younger root systems than plants belonging to the I-214 and AF2 clones.

In the second year of the plantations, top dressing was only carried out in Almazán, where 600Kg ha⁻¹ of a NPK 12:24:12complex fertilizer was applied.

LAI measurements were taken in September 2006 and 2007 prior to shedding and after bud closure, once the growing period was over. Biomass measurements were recorded after shedding in winter 2006 and 2007.

INDIRECT LAI MEASUREMENTS

Every year in September, for each site, clone and block, a representative plot was selected in order to estimate LAI from hemispherical photographs. Six evenly spaced photographs were taken on the diagonal of a square with sides of equal length to the repeat unit of the planting design: e.g. 1m in Valtierra and 3.5 m in the case of Almazán, and positioned with one of its sides on a plantation row.

Positioning the camera to take photographs right at the center and at the extremes of the diagonal was avoided so as not to include the most favorable and least favorable cases of LAI estimation.

A Nikon ® FM camera with a Sigma 8mm fisheye lens was used on a 20 cm high adjustable level tripod. The LAI was estimated from this height upwards. In the two first years of these plantations the trees had leaves from the base to the top.

The photographs were analyzed using the Hemiview 2.1, Canopy Analysis Software (Delta-T Devices Ltd.). This program provides the gap fraction (P0) for each azimuth and zenith combination selected in all the photographs. Three different models were fitted to the P0 data obtained from Hemiview in order to obtain LAI.

1.-The Poisson gap frequency model:

The Poisson gap frequency model gives the probability of no contact of the sunbeam with the canopy as a function of the angular distribution of the foliage elements, of the sunbeam angle with the zenith (θ), and of the LAI. Its mathematical expression is as follows:

$$P_0 = e^{\frac{-G(\theta, \theta') \cdot LAI_{eff}}{\cos(\theta)}} \quad (\text{eq1})$$

Where P_0 is the probability of no contact of a sunbeam on the canopy, $G(\theta, \theta')$ is the function that gives the projection of the leaves on the plane perpendicular to the beam direction, which takes into consideration the sunbeam angle with the zenith (θ) and the foliage angle (θ') e.g. the angle formed by the normal to the leaf with the zenith. LAI_{eff} takes into account not only the projection of leaf area on a plane parallel to the soil, but also the projection of branches and stems (Nilson and Kuusk, 2004).

The $G(\theta, \theta')$ function used was that proposed by Warren-Wilson & Revé (1959) (Eq 2).

$$G(\theta, \theta') = \begin{cases} \cos \theta \cdot \cos \theta' & \text{si } \theta + \theta' \leq 90^\circ \\ \frac{2}{\pi} \cdot \text{seno } \theta \cdot \text{seno } \theta' \cdot \text{seno}(\arccos(\cot \theta \cdot \cot \theta')) + (1 - \frac{2}{\pi} \arccos(\cot \theta \cdot \cot \theta')) \cdot (\cos \theta \cdot \cos \theta') & \text{si } \theta + \theta' > 90^\circ \end{cases} \quad (\text{eq 2})$$

2.-The second model fitted to the gap fraction distribution obtained from Hemiview processing was a modification of the Poisson model that considers the clumping of foliage elements in the canopy. The effect of this correction factor is to increase LAI for a determined gap fraction in relation to eq1. Given that the foliage clumping effect is more apparent in small angles with the zenith than in larger angles, the correction factor has the following expression (Montes et al 2007) (eq3):

$$\Omega(\theta) = \Omega_0 \cdot \cos^2(\theta) + \text{sen}^2(\theta) \quad (\text{eq3})$$

When θ is close to the zenith (small angles), $\Omega(\theta)$ adopts a value close to Ω_0 and when the angles with zenith are about 90° , $\Omega(\theta)$ takes values close to 1, which means that the correction factor, since it is a multiplicative factor, has no effect.

The second model used to estimate LAI, taking into account the clumping effect is as follows (eq 4):

$$P_0 = e^{\frac{-G(\theta, \theta') \cdot LAI \cdot \Omega(\theta)}{\cos(\theta)}} \quad (\text{eq 4})$$

3.-The third model employed was also proposed by Montes et al (2007) and also modifies the Poisson model but in a different way: this third model includes a correction

function $\Omega(\theta)$ that weights the effects of foliage element clumping and foliage inclination by means of the zenith angle, in such a way that when angles are close to the zenith, the more pronounced effect is going to be the clumping effect whereas when angles are close to 90° the foliage inclination effect will be stronger. The model equation is the same as eq 4 but differs in the equation of the correction function, which is expressed as follows (eq5):

$$\Omega(\theta) = \Omega_0 \cdot \cos^2(\theta) + G(\theta, \theta') \cdot \sin^2(\theta) \quad (\text{eq5})$$

DIRECT LAI MEASUREMENTS

Every year in September, in the same places where hemispherical photographs had been taken, all the leaves from six trees were removed and weighed. The trees selected were located as close as possible to the diagonal where the photographs were taken. Once the leaves had been collected, the green weight was recorded and a subsample of about 500g was sent to the lab to determine the specific leaf area (SLA) (cm^2g^{-1}). The SLA was obtained by cutting foliar discs of known area from a subsample of 50 green leaves per clone and placing them in an oven at 100 °C to obtain the dry weight. The SLA (cm^2g^{-1}) was calculated as the ratio of the disc area to the mean dry weight of the disc. The rest of the leaves in the subsample were oven dried to determine the dry weight of the leaves. Direct LAI was obtained for each year, site, clone, and block as the product of the dry leaf weight of a tree by the SLA obtained in the lab.

BIOMASS ESTIMATION

In December 2006 and 2007, an assessment plot with 16 plants each was set up for each clone in all the blocks at both sites. All the plants in these plots were cut and weighed. A subsample of 1Kg of wood from each clone was taken to the lab, oven dried at 100°C until constant weight and the humidity percentage determined.

Clonal differences in biomass yield were assessed through a two way ANOVA with block and clone as factors. The Student-Newman Keuls mean test with a 5% confidence level was applied to separate means.

Linear correlation analysis was also carried out between LAI (from direct and indirect measurements) and biomass estimations. An ANCOVA was carried out in order to test if there were clonal differences in direct LAI per biomass unit, using biomass as covariate.

Analyses were carried out with the statistical analysis software SAS (SAS Institute., 2004).

RESULTS

Table 2 shows the results of the dry biomass production estimates obtained in Almazán and Valtierra in 2006 and 2007 and the LAI estimates obtained by means of direct and indirect methods using the three above mentioned models.

Table 2: Dry biomass ($\text{Mg ha}^{-1}\text{yr}^{-1}$) and LAI estimates from direct and indirect measurements

SITE	DATE	VARIABLE	CLONE			
			AF2	I-214	MONVISO	PEGASO
ALMAZÁN	Dec-06	Biomass ($\text{Mg ha}^{-1}\text{yr}^{-1}$)	0,83 a	0,39 b	0,36 b	0,13 b
		LAI direct	1,69a	1,15b	0,92 b	0,75b
		LAI (model1)	0,34	0,26	0,22	0,16
		LAI (model2)	0,43	0,33	0,26	0,18
		LAI (model3)	0,42	0,28	0,26	0,17
	Dec-07	Biomass ($\text{Mg ha}^{-1}\text{yr}^{-1}$)	6,97 b	8,68 a	3,77 c	0,74 d
		LAI (direct)	6,45b	8,42a	2,73c	0,72d
		LAI (model1)	2,06	2,37	0,77	0,25
		LAI (model2)	2,52	3,12	0,98	0,327
		LAI (model3)	2,19	2,87	1,33	0,27
VALTIERRA	Dec-06	Biomass ($\text{Mg ha}^{-1}\text{yr}^{-1}$)	7,79 a	6,37 b	8,02 a	4,27 c
		LAI direct	5,26a	4,26b	6,28a	3,33c
		LAI (model1)	2,32	2,11	3,04	1,87
		LAI (model2)	3,19	2,67	3,85	2,35
		LAI (model3)	6,7	5,6	8,2	4,9
	Dec-07	Biomass ($\text{Mg ha}^{-1}\text{yr}^{-1}$)	12,9 a	13,3 a	12,6 a	9,6 b
		LAI direct	0,9	3	1,9	1,8
		LAI (model1)	0,65	0,89	0,54	0,78
		LAI (model2)	0,76	1,01	0,56	0,68
		LAI (model3)	0,66	0,81	0,45	0,53

Means in the same row followed by the same letter are significantly different ($p < 0.05$) Mean test used was Snedecor- Newman-Keuls mean test

The ranking of clones by biomass yield not only differed between the sites but even within the same site, as occurred at Almazán for biomass yield in different years. In 2006, AF2 was the highest yielding clone at Almazán whereas in the second year (2007), after cutting back, I-214 reached significantly higher biomass yield figures than AF2 and both the P. x interamericana x P.nigra hybrids, since the roots of these latter clones were a year behind those of the P.x euramerican clones.

At Valtierra, Monviso and AF2 yielded the highest amount of biomass per hectare in the first year, followed by I-214 and Pegaso. The year after coppicing, I-214 yielded as much biomass as AF2 and Monviso. As occurred at Almazán, it seems that I-214 performs better the second year than in the establishment year. The lower production of Pegaso was also due to high mortality at Valtierra.

The ranking of clones by LAI follows the same order as the biomass yield ranking except at Valtierra in 2007 due to early defoliation caused by a rust infection that severely affected AF2 and Monviso.

DIRECT LAI AND BIOMASS YIELD

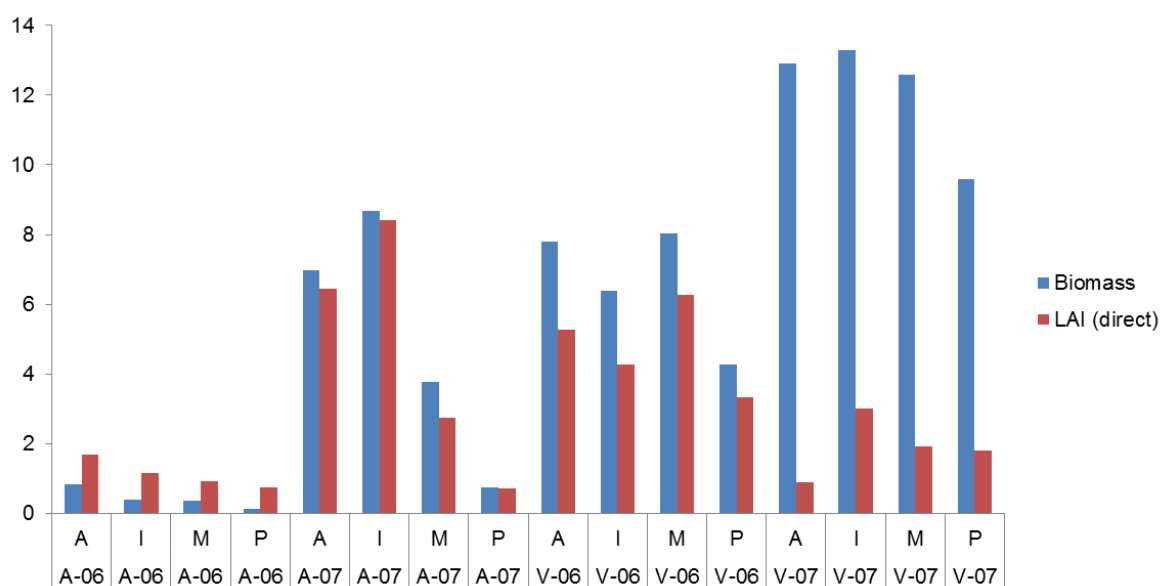


Figure 1: Biomass in Mg ha⁻¹ yr⁻¹ (blue bars) and LAI (brown bars) obtained from direct measurements of four clones in Almazán (A) and Valtierra (V) in 2006 (06) and 2007 (07). The first letter of the clone name is written on the line below the horizontal axis. On the second line below the horizontal axis are the initials of the plantation (A=Almazán and V=Valtierra) followed by two digits corresponding to the year of measurement.

Table 3: Results of linear correlation analysis between LAI obtained by direct measurements and biomass yield.

	Almazán 2006	Almazán 2007	Valtierra 2006	Valtierra 2007
LAI (mean)	1.16	4.58	4.78	12.1
r^2	0.51	0.97	0.67	0.06
p-value	0.0042	0.0009	<0.0001	0.4123

There was a significant positive correlation between direct LAI measurements and biomass yield in Almazán and in Valtierra in 2006, the clones with greater LAI obtaining higher biomass yields (Table 3, Fig 1). In 2007, no linear correlation was found between LAI and biomass yield at Valtierra. The rust infection (*Melampsora* sp.) usually affects this type of crop at the end of vegetative period, towards the end of August. This causes the trees to begin shedding in early September. The LAI measurements were supposed to be taken when buds were closed in order to capture the total development of the LAI. However, by September 15th, when temperatures are still mild and days are long, it was already too late at Valtierra as the trees belonging to the AF2 and Monviso clones had lost a considerable amount of their leaves. Measurements should have been taken at the end of August instead of in the middle of September. As shedding was different in intensity for each clone, AF2 and Monviso being the most affected, no linear correlation was found between biomass yield and LAI.

The ANCOVA carried out on direct LAI measurements using biomass yield as covariate did not reveal a clone effect for any of the sites or years analyzed, suggesting equal productive efficiency of LAI in all clones. However, performing a one way ANOVA on direct LAI estimations in all blocks and sites using clone as factor, a clone effect was found and the Snedecor-Newman Keuls test separated means in the same way as for biomass, except in Valtierra 2007.

DIRECT AND INDIRECT LAI MEASUREMENTS

Direct and indirect LAI measurements showed highly significant linear correlation. Indirect LAI measurements were also correlated with biomass yield (table 4). Again, the biomass clonal ranking coincides with clonal LAI ranking regardless of the estimation method employed.

LAI values obtained using the direct method were higher than those obtained by indirect measurements. The smallest differences between direct and indirect estimations were found for Valtierra in 2006, where the third indirect model employed displayed LAI estimations of the same magnitude, or even slightly higher than the direct LAI. This can be explained by the fact that the LAI obtained from indirect measurements includes the projected surface of branches and stems. The largest differences between direct and indirect methods (not including the 2007 Valtierra estimates), were found for Almazán in 2006.

Table 4: Correlation matrix between the four LAI estimation methods employed and biomass yield. Pearson coefficients and p-values. Estimates obtained in 2007 in Valtierra were not included in the correlation analysis.

Pearson Correlation Coefficients					
	p-values				
	direct	indirect1	indirect2	indirect3	Biomass
direct	1.0000	0.90881	0.90980	0.65544	0.96061
		<.0001	<.0001	0.0207	<.0001
indirect1	0.9088	1.00000	0.99827	0.90145	0.96386
	<.0001		<.0001	<.0001	<.0001
indirect2	0.9098	0.99827	1.00000	0.90618	0.96837
	<.0001	<.0001		<.0001	<.0001
indirect3	0.6554	0.90145	0.90618	1.00000	0.80236
	0.0207	<.0001	<.0001		0.0017
Biomass	0.9606	0.96386	0.96837	0.80236	1.00000
	<.0001	<.0001	<.0001	0.0017	

The estimation error obtained using the Poisson model (indirect 1), as can be seen in table 5, is higher than that obtained with the other two indirect models proposed, both of which are modifications of the first. Errors found in the indirect models 2 and 3 were similar. Figure 2 shows how indirect model 3 (the third model mentioned in the material and methods section), is closer to the line 1:1, which means that estimation by this model is closer to the direct LAI estimation

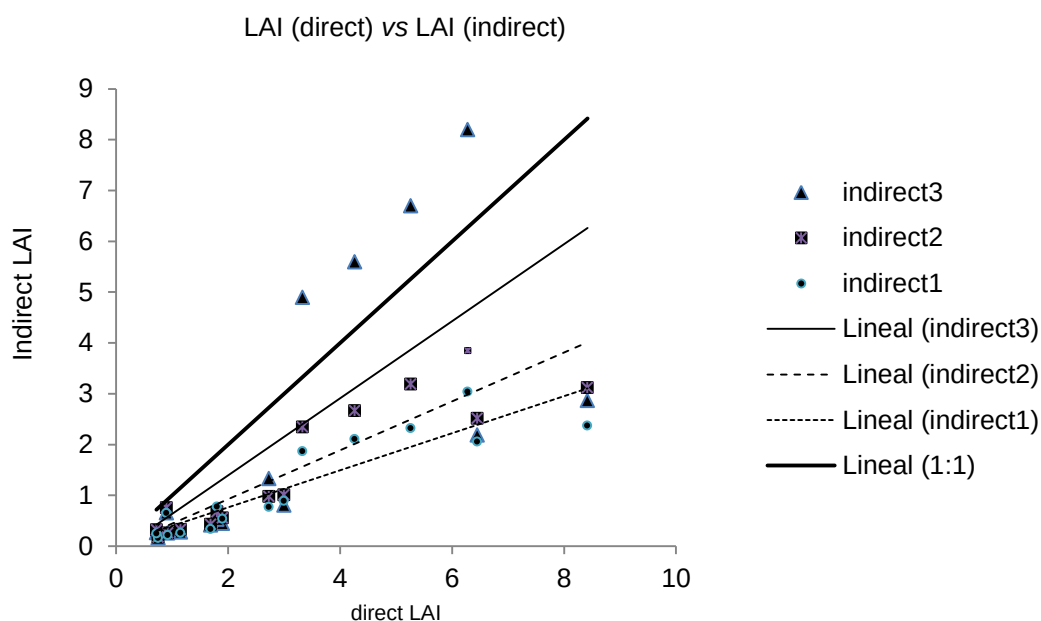


Figure 2: relationship between indirect and direct LAI measurements. The names ‘indirect1’, ‘indirect2’ and ‘indirect3’ refer to the models employed to estimate LAI with the correlative number corresponding to the order of presentation in the material and methods. 1:1 line is the x=y function.

Tabla 5: Estimation errors (sum of squares of differences between gap fraction measurements on hemispherical photographs and predicted model values).

SITE	DATE	MODEL	CLONE			
			AF2	I-214	MONVISO	PEGASO
ALMAZÁN	2006	1	0,0101	0,0150	0,0668	0,0620
		2	0,0039	0,0035	0,0017	0,00810
		3	0,0360	0,0003	0,0015	0,0071
	2007	1	0.0019	0.0050	0.0020	0.0650
		2	0.0016	0.0003	0.0005	0.0027
		3	0,0013	0,0003	0,1150	0,0025
	2006	1	0,0100	0,0380	0,0099	0,0142
		2	0,0032	0,0004	0,0019	0,0017
		3	0,0023	0,0021	0,0011	0,0009
VALTIERRA	2007	1	0,0318	0,0134	0,0102	0,0290
		2	0,0298	0,0137	0,0094	0,029
		3	0,029	0,0129	0,0099	0,035

Smallest error values are displayed in bold.

DISCUSSION

The LAI estimation of the probability of no contact of a sunbeam with the canopy is based on the Beer-Lambert empirical law. This law relates the transmittance of a light beam that goes through a medium with homogeneous turbidity with the properties of that medium and with the properties of the light (wavelength).

Monsi & Saeki (1953) adapted the Beer Lambert equation to allow LAI to be estimated from it by assuming that transmittance is equivalent to gap fraction and that leaves are randomly distributed and infinitely small compared with the totality of the canopy. In 2006 and 2007 at Almazán, the LAI estimates obtained from the second and third indirect models underestimated the LAI in relation to direct LAI estimation, whereas in the case of Valtierra in 2006, the LAI estimation provided values which were similar to the direct LAI estimates. The better behavior of models 2 and 3 in Valtierra may be due to the fact that the initial hypothesis was fulfilled more precisely. In Valtierra (2006), the trees were larger than in Almazán (2006) and almost filled the whole hemispherical photograph, from the soil to the zenith, with the gaps adopting a random distribution. However, at Almazán in the same year, the trees were much shorter and consequently there was a large central gap without leaves, corresponding to the space between rows, which violates the hypothesis of randomness of leaf distribution in the canopy. Breda (2003), in a review of different methods for estimating LAI, confirmed the fact that indirect LAI estimation methods based on hemispherical photographs using the Poisson distribution (indirect model 1) always infraestimated LAI due to the violation of the hypothesis of leaf distribution randomness within the canopy (this is equivalent to assimilating the canopy to a homogeneously turbid medium). The corrections proposed for *Pinus sylvestris* by Montes et al (2007) attempted to mitigate the non-satisfaction of that hypothesis. At both sites, models 2 and 3 fitted more accurately than the Poisson model and yielded higher LAI estimates.

At Almazán in 2007, the trees were as large as in Valtierra (2006) but again the LAI was infraestimated in comparison to direct LAI estimates. In this case, the plantation design was not regular, and the spacing between rows was 3m instead of the 1m spacing used in Valtierra. Hence, at Almazán in 2007 there continued to be a central space without trees so the hypothesis of randomness was still being violated.

In any case, the LAI ranking by clone was the same, regardless of the estimation method employed. The same was true of the LAI ranking by year or site, thus allowing comparison of LAI and biomass yields between clones, years and locations despite the infraestimations.

Bearing in mind that the end goal of this work is to determine whether indirect LAI measurements can be reliably used in the future in this type of plantations, and that in the future trees will be higher and therefore will fill all the hemispherical photograph, the errors will diminish and the reliability is expected to increase with tree size.

CHASON et al. (1991), using the negative binomial distribution instead the Poisson distribution, reported an increase of LAI estimates obtained using indirect methods.

The negative binomial distribution is less restrictive as regards the fulfillment of initial conditions. For example, leaves may be considered of negligible size in relation to the canopy.

Clonal differences in biomass and in LAI were found at both Almazán and Valtierra. Pegaso exhibited the lowest LAI and biomass yield in all cases. AF2 was the highest yielding clone at both sites along with Monviso. However, AF2, and to a lesser extent Monviso, were also the most affected by rust infection. Verlinden et al. (2013) in a study including 12 poplar genotypes reported that rust affection was negatively correlated with biomass growth. This rust infection could be the reason underlying the changes in the clonal ranking between 2006 and 2007 at both sites. I-214 became the highest yielding clone in 2007 at Almazán, and in 2007 at Valtierra, even surpassing the biomass yield of AF2 and Monviso (although the differences were not significant). Another factor that could have influenced the changes in the clonal ranking was the coppicing carried out in winter 2006/2007, which favored the clones with higher below-ground to above-ground biomass ratio. Scarazzia-Mugnozza et al (1997), compared the allocation of biomass in different *P.trichocarpa* x *P.deltoides* hybrids and found that the clones which apportioned a higher amount of biomass to the roots exhibited faster leaf and stem growth early in the following growth season. They also found that the more branchy clones also had more branchy roots, although the roots did not accumulate more biomass. This was also reported by Friend et al. (1991). It may be that I-214, the least branchy clone at both sites in 2006 (data not shown), apportioned more biomass to the roots than AF2 and Monviso, which displayed greater development of the aerial part, perhaps at the expense of root development. Thus, the cutting back of the plants, carried out at the end of the first growing season, could have been particularly advantageous to I-214 and may have allowed this clone to 'catch up' with the Monviso and AF2 clones. In fact, I-214 has hardly ever been among the highest biomass yielding clones in the Short Rotation Poplar Network, (Sixto et al 2013), except where the plants have been cut back.

CONCLUSIONS

Indirect methods of LAI estimation are useful for comparing LAI between clones, sites and years. The modifications to the Poisson distribution proposed by Montes et al (2007) resulted in less error in the LAI estimation. Biomass was well correlated with LAI regardless of the method employed. More research is still needed in order to obtain a more accurate LAI estimation from models which can be applied to all initial conditions.

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DISCUSIÓN



DISCUSIÓN

SEÑALIZACIÓN Y “REFILLING”

A pesar de lo profusamente estudiada y modelizada que ha sido la conductancia estomática (Damour et al. 2010), todavía no se conoce completamente el mecanismo que desencadena el cierre estomático. Es posible que no suceda del mismo modo para todas las especies, ya que hay publicados resultados que apuntan hacia una regulación hidráulica predominante (Salleo et al 2000) mientras otros indican que la regulación química por medio de la hormona ABA (ácido abscísico) es la que determina el cierre estomático (Xie et al. 2006, Bauer et al. 2013). También hay trabajos en los que se han encontrado ambos tipos de señales (Tombesi et al.2013).

En los casos en los que la regulación hídrica del cierre estomático es considerada predominante, las plantas detectarían el embolismo, y cerrarían los estomas antes de alcanzar el potencial hídrico que indujera un nivel de embolismo peligroso para la supervivencia de la planta. Salleo et al. (2000) observaron en plantas de *Laurus nobilis* L. que se iniciaba un cierre parcial de estomas cuando los valores de potencial hídrico se acercaban al umbral a partir del cual se había detectado la generación de embolismo. Este umbral se determinó midiendo simultáneamente valores de potencial y detectando embolia a partir de ultrasonidos en tallos y hojas. Estos resultados sugieren que la planta está detectando de algún modo el umbral de embolia que no se debe rebasar (señal hidráulica).

Las plantas de eucalipto ensayadas en esta tesis han presentado valores de conductancia estomática altos (en torno a $0.8 \text{ mol m}^{-2} \text{ s}^{-1}$) con niveles de embolia muy elevados, en torno al 70%. La disminución de la conductancia estomática coincidió en el tiempo con la convergencia de los valores de LSC en plantas sometidas a distintos regímenes hídricos, siendo LSC la conductividad hidráulica específica relativa al área foliar. Este resultado podría indicar la existencia de un umbral mínimo de conductividad hidráulica específica a partir del cual las hojas ya no recibirían flujo suficiente y se cerrarían los estomas. Vilagrosa et al. (2003) encontraron cierre estomático en dos especies mediterráneas cuando se igualaron sus valores de LSC. Estos resultados sugieren que la señal hidráulica desencadenante del cierre estomático depende de la conductividad hidráulica remanente y no tanto del porcentaje de conductividad perdida por embolia.

Pero un nivel muy bajo de conductividad hidráulica tendría que implicar un elevado porcentaje de pérdida de conductividad hidráulica¹ (PLC), si la conductividad hidráulica máxima fuese un valor de referencia fijo, que como tal se considera cuando se elaboran las curvas de vulnerabilidad a la cavitación. Sin embargo, nuestros resultados muestran que la conductividad hidráulica máxima (k_{max}) no es un valor estático, y puede variar en cortos espacios de tiempo. Observamos que k_{max} disminuía durante el tiempo en que se realizaron las mediciones (tres semanas), por lo tanto una disminución de conductividad nativa relativa a una conductividad máxima decreciente, no se traduce en una pérdida de conductividad

¹ $\text{PLC} = \frac{k_{\text{max}} - k_{\text{nativa}}}{k_{\text{max}}}$

significativa, por lo que es más difícil utilizar los valores de PLC como umbral de señalización. Tombesi et al. (2013), en dos variedades de *Vitis vinifera* L. mostraron que el cierre estomático se produjo inicialmente como consecuencia de un incremento en el porcentaje de pérdida de conductividad hidráulica. Es posible que en función de la estática que sea la conductividad máxima hidráulica, sea más o menos sencillo detectar las señales hidráulicas atendiendo a los valores de PLC.

Tombesi et al. (2013) no solamente observaron una señal hidráulica desencadenando el cierre estomático en vid, sino que también encontraron una señal química: midieron un incremento en la concentración de ácido abscísico (ABA) en la savia que según suponen los autores tendría el objetivo de mantener cerrados los estomas (aunque las condiciones ambientales fuesen favorables) para permitir la recuperación de la embolia mediante un proceso de *refilling*.

En el segundo capítulo se midió la variación del pH de la savia a lo largo del tiempo con el objeto de relacionarla con la variación en la conductancia estomática, por su posible vinculación con la acción del ácido abscísico en respuesta al estrés hídrico (cierre estomático). Wilkinson & Davies (1997), Wilkinson et al. (1998), o Bahrún et al. (2002) son algunos de los trabajos en los que se muestra un incremento en el pH de la savia de plantas sometidas a estrés hídrico. Tombesi (2013) y Mc Adam & Brodribb (2015), entre otros muchos observaron incrementos en la concentración de ABA en la savia de plantas sometidas a estrés hídrico. Wilkinson y Davies (1997) justificaron la posible relación entre el incremento en el pH y la mayor concentración de ABA de la savia del siguiente modo: un pH básico favorece que el ABA, que es un ácido débil con constante ácida $pK_a=4.7$, esté mayoritariamente en su forma aniónica desprotonada, caracterizada por su lipofobia, por lo tanto no tendría afinidad por las membranas celulares y llegaría a los estomas circulando por el apoplasto en medio de la corriente transpiratoria sin quedar atrapado por el simplasto. Una disminución en el pH de la savia supondría un incremento en la forma protonada lipófila del ABA, que tendría afinidad por las membranas celulares y una vez atravesadas quedaría confinado en el citoplasma, cuyo pH es cercano a la neutralidad y donde la mayor parte del ácido quedaría en forma aniónica y no podría atravesar de nuevo las membranas celulares para llegar a los estomas.

El resultado del seguimiento del pH de la savia reveló una marcada disminución del mismo (de aproximadamente 0.5 puntos) entre los días 15 y 20 de medición. Esta disminución se produjo simultáneamente a un cierre estomático casi total en ambos regímenes de riego, pasando los valores de conductancia estomática de 0.8 a valores inferiores a $0.2 \text{ mol m}^{-2} \text{ s}^{-1}$ entre los dos días mencionados. Estos resultados sugieren que no es la diferencia en el contenido de agua del suelo lo que ha desencadenado el cierre estomático, y por tanto no ha habido una señal desde la raíz hasta las hojas.

Thomas & Eamus (2002), estudiando en el campo las variaciones estacionales de pH de la savia, concentración de ácido abscísico y potencial hídrico encontraron que disminuciones en el potencial hídrico sucedían simultáneamente a incrementos en las concentraciones de ácido abscísico, y en algunas especies observaron alcalinización del pH y en otras acidificación (en caducifolias). Sharp & Davies (2009) mostraron que la alcalinización del pH de la savia como respuesta al estrés hídrico procedente de escasez de agua en el suelo

no era un fenómeno universal, ni tampoco la acidificación. Tras someter 22 especies diferentes a condiciones limitantes de agua en el suelo, con valores de PAR en torno a los 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, no observaron cambios en el pH de la savia como respuesta a la falta de agua en el suelo en la mayor parte de las especies ensayadas. Encontraron alcalinización de la savia en las cuatro especies más isohídricas de todas, en las que no se observaron valores bajos de potencial hídrico. Ninguna anisohídrica presentó alcalinización del pH. Los autores argumentan que es posible que la disminución del pH se deba a otros procesos químicos gobernando la química de la savia, en particular se mencionan procesos relacionados con la nutrición de las plantas.

Las especies anisohídricas como el eucalipto antes de cerrar estomas alcanzan potenciales hídricos más negativos que las isohídricas, y por tanto exponen el xilema a un mayor riesgo de embolismo. La reparación de ese embolismo (*"refilling"*) es lo que podría estar produciendo una disminución del pH de la savia. Diversos estudios utilizando modernas técnicas de visualización como tomografías computerizadas de rayos X (Brodersen et al 2010) han demostrado la existencia de *refilling*, aunque el mecanismo de acción no se conoce por completo. Los resultados de las investigaciones realizadas al respecto indican que el proceso consiste en una descarga por parte de las células parenquimáticas de solutos en los vasos embolizados con el objeto de incrementar el potencial osmótico y atraer agua hacia ellos. Secchi & Zwieniecki (2012) comparando la savia de vasos embolizados con la de vasos sin embolizar en plantas de *Populus trichocarpa* (Torr & Gray) observaron que la savia en vasos embolizados poseía una concentración mucho mayor de azúcares e iones y un potencial osmótico más elevado que la savia de plantas sin estresar además de un pH claramente más ácido. El transporte de azúcares y agua a través de las membranas de las células de parénquima es un proceso energéticamente dependiente en el que están implicadas ATP-ases cuyos protones pueden estar vinculados a las variaciones de pH de la savia del xilema.

La consideración de esta información conjuntamente nos conduce a interpretar la acidificación observada en nuestro trabajo como la posible consecuencia de un proceso de refilling. Analizando la evolución de pH, potencial hídrico, conductancia estomática y déficit de presión de vapor (DPV) se aprecia que después de un incremento continuado de los valores máximos diarios de déficit de presión de vapor se produjo un descenso en el pH de la savia que coincidió con una fuerte disminución de la conductancia estomática en ambos tratamientos. Tras la disminución de pH, éste se recupera. El día 26 ya se observa en plantas estresadas una recuperación de la conductancia estomática y de PLC. Los resultados podrían estar reflejando los ajustes estomáticos e hidráulicos que las plantas están realizando como respuesta a riegos y cambios atmosféricos para asegurar el suministro hídrico a las hojas: las bajadas de pH coincidirían con recuperaciones de embolia (*refilling*), tras las cuales, y una vez todos o parte de los vasos rellenados, se producen recuperaciones de los valores de pH (alcalinizaciones) ya con potenciales hídricos medios o altos (poco negativos).

Los valores de pH medidos en el capítulo 2 fueron altos (mayores de 6.2) durante todo el ensayo. A pH=4.7 las concentraciones de ácido abscísico protonada y aniónica están en equilibrio. Por encima de ese valor, a medida que se incrementa el pH, aumenta la proporción de forma ABA-(aniónica) en detrimento de la forma protonada, que puede ser secuestrada en el simplasto. Dado que los valores de pH medidos a lo largo del ensayo fueron mayores que el

pKa del ácido, si se hubiese producido un incremento de ABA en cualquier momento, el pH de la savia habría sido favorable para enviarlo hasta los estomas sin que fuese atrapado en el simplasto de las hojas.

Los resultados que hemos obtenido no permiten descartar la existencia de señales hidráulicas o químicas como reguladoras del cierre estomático. Es posible, que convivan ambas.

DISMINUCIÓN DE LA CONDUCTIVIDAD MÁXIMA

Hay un punto en el que parece que coinciden varios autores entre los que se encuentran Secchi & Zwieniecki (2012) y Brodersen et al. (2010) y es en que los vasos en los que se produce refilling han de estar hidráulicamente aislados del xilema en tensión.

En los dos primeros capítulos de eucalipto se ha observado una disminución de la conductividad máxima durante el lapso de tiempo en el que se realizaron las mediciones. ¿Cómo es posible que esto suceda si el tamaño de los vasos se incrementó y con él la conductividad máxima potencial, o teórica calculada con la fórmula de Poiseuille? Una posible explicación al fenómeno observado estaría relacionada con lo mencionado en el párrafo anterior. Si la planta trata de aislar los vasos embolizados para poder rellenarlos impermeabilizando transitoriamente las punteaduras, este vaso “impermeabilizado” quedaría inutilizado y aislado del resto de la red de vasos xilemáticos durante el tiempo que tarda en rellenarse entorpeciendo la circulación de la savia, lo que se reflejaría tanto en las mediciones de conductividad nativa como en las de conductividad máxima, pues con la aplicación de agua a presión realizada en los tallos para eliminar embolia y determinar conductividad máxima no se eliminarían los efectos del tratamiento impermeabilizante o aislante y los vasos embolizados sometidos a tratamiento aislante no podrían ser desembolizados. Estudios como los de Pesacreta et al. (2005) o Lee et al. (2012), sugieren que las microfibrillas de celulosa de las membranas de las punteaduras están inmersas en una densa capa de hidrogel que les confiere mayor resistencia debido a que las moléculas del solvente al recibir las tensiones pueden cambiar de posición, evitando que afecten a la fracción sólida del hidrogel (Hong et al. 2008). La membrana de las punteaduras se forma a partir de la pared primaria con la que puede compartir componentes, entre los que están presentes las pectinas (Rockwell et al. 2014, Plavcová et al 2011). De hecho, se ha experimentado tratando tallos con pectoliasa y el efecto conseguido fue incrementar (hacer menos negativo) el potencial al que se emboliza el 50% del xilema, es decir, reducir la resistencia a la cavitación. (Dusotoit Coucaud et al., 2014 in Rockwell et al. 2014). Además, diferencias en la composición de la savia podrían afectar a las propiedades del hidrogel y provocar cambios en la permeabilidad de las punteaduras.

En el capítulo 2 se observó una elevada variabilidad en la conductividad hidráulica máxima y también en la nativa. Entre el primer y el último día de medición, la conductividad hidráulica nativa en plantas regadas experimentó oscilaciones pero alcanzó de nuevo el valor de partida, se recuperó y las plantas exhibieron incrementos en biomasa del 278%. En plantas estresadas, la conductividad hidráulica en el último día de ensayo fue un 11% inferior al valor registrado el primer día de medición, porcentaje que coincide exactamente con la reducción del riego en plantas estresadas respecto a regadas. Las plantas estresadas entre la primera y la última medición de conductividad hidráulica incrementaron la biomasa en un 262%.

COMPARACIÓN CLONAL: CRECIMIENTO, CONDUCTANCIA ESTOMÁTICA Y CONDUCTIVIDAD HIDRÁULICA

El estrés hídrico produjo una disminución del crecimiento. Las diferencias más significativas entre clones se hallaron en los regímenes hídricos mejor dotados, mientras que en los regímenes de riego con mayor limitación de agua, generalmente no se encontraron diferencias estadísticamente significativas.

Se observó que el incremento porcentual de biomasa producido en el tratamiento más regado respecto al tratamiento con mayor limitación hídrica es igual a la diferencia en porcentaje entre dosis de riego. Los clones mejor regados en el capítulo uno produjeron un 20% más de biomasa que las plantas de los clones menos regados, y las dosis de riego fueron un 73% y un 90% de la capacidad de campo en volumen, y en el segundo ensayo la diferencia de biomasa fue del 12% entre ambos tratamientos (las plantas se regaron por pesada hasta 2600 y 2900 g). Los dos ensayos de eucalipto se realizaron en invernadero en la misma época del año, pero se utilizaron envases de distinto tamaño: 2 litros y 5 litros. La producción de biomasa de un ensayo a otro se multiplicó por la relación de peso entre envases, es decir, por 2.5. Estos resultados muestran cuantitativamente cómo afecta la falta de agua a la producción en condiciones controladas. Sería interesante poder comprobar si esta proporcionalidad se conserva a lo largo de la vida de la planta por su utilidad en la gestión de plantaciones comerciales. En las plantaciones experimentales de chopo instaladas en la Red de Parcelas de Cultivos Leñosos en Corta Rotación, en condiciones de campo no se ha observado esta proporcionalidad, pues el desarrollo libre de la raíz en campo puede permitir a las plantas acceder a fuentes de agua no controladas.

Los clones de eucalipto que presentaron mayores crecimientos en superficie foliar, tasas de evapotranspiración y conductancias estomáticas fueron híbridos F_1 , que aprovecharon más eficientemente los recursos que los clones F_0 . Esto no se cumplió para el caso del clon H491, un F_1 procedente del autocruzamiento de C14, cuyo crecimiento estuvo al nivel de los clones F_0 en el invernadero, y presentó altos niveles de mortalidad y escaso crecimiento en plantaciones comerciales, poniendo de manifiesto la elevada mortalidad, el escaso crecimiento y menor resistencia al estrés que normalmente se asocia a especímenes procedentes de autocruzamiento (Johnsen et al. 1999, 2003, Fox & Reed 2011). Los valores máximos de conductancia estomática medidos en H491 fueron del orden de una tercera parte de los medidos en otros cinco clones, lo que implica una menor capacidad de refrigeración de la hoja y por tanto una menor adaptación al estrés además de un menor crecimiento asociado a la menor absorción de nutrientes que tiene lugar cuando la tasa de transpiración es menor (Scholz et al 2007). Estos resultados ayudan a explicar las bajas producciones y elevada mortalidad obtenidas por H491 en plantaciones comerciales y confirman la importancia de la selección e hibridación de genotipos como herramienta para la mejora de la producción.

En general los clones que resultaron más productivos en el campo también lo fueron en el invernadero con algunas variaciones que pueden achacarse a las limitaciones que impone el crecimiento en una maceta (Poorter et al. 2012); aunque también el hecho de estar sometidos a las mismas condiciones de suelo permite averiguar qué genotipos pueden desarrollarse mejor partiendo de las mismas condiciones. La diferencia más notable entre

condiciones de campo y controladas se halló en dos clones: en el clon H231 que en el campo presentó una de las mayores producciones mientras que en invernadero mostró un desarrollo intermedio, y en el clon H491, que en invernadero no presentó un desarrollo tan reducido como en el campo.

Se encontraron algunas diferencias interesantes entre clones en variables como la conductancia estomática y la conductividad hidráulica: se detectaron los clones con un comportamiento más arriesgado, es decir, que transpiraron más y alcanzaron valores de conductividad hidráulica más bajos. En particular, el clon T presentó los valores más altos de conductancia estomática en condiciones de riego más desfavorables. Este clon mantuvo niveles de transpiración altos en condiciones de estrés hídrico y valores de conductividad hidráulica significativamente menores que otros clones estudiados, por lo que sería un buen candidato a sufrir defoliación precoz bajo estrés severo. Es el clon que arrojó los mejores datos de producción en el campo dada su eficiencia en el uso de los recursos, sin embargo, ese *modus operandi* arriesgado en la sequía de 2005, que fue la sequía más acentuada en 40 años, avocó al fracaso a las plantaciones de este clon, que fue retirado de producción.

Los clones que presentaron mayores crecimientos en el invernadero también alcanzaron los mayores valores de conductancia hidráulica. Sin embargo también fue retirado de producción el clon H463, uno de los clones con mejores resultados en campo e invernadero, por proliferación de brotes epicórmicos, indicando que estar en posesión de una conductividad hidráulica elevada por sí solo no constituye motivo de selección clonal.

ANATOMÍA

Las características anatómicas estudiadas en eucalipto y en chopo han sido de utilidad para comprender los resultados fisiológicos obtenidos, mostrando la relación existente entre estructura y función.

Los clones H231 de eucalipto y Pegaso de chopo presentan las menores superficies conductoras por unidad de sección transversal de tallo comparados con el resto de clones de sus respectivos ensayos. La presencia de vasos de pequeño diámetro en el xilema impidió a ambos clones aprovechar eficientemente los recursos del medio cuando las condiciones fueron más favorables. Pegaso presentó la menor conductancia estomática máxima y H231 la menor conductividad hidráulica máxima en las mejores condiciones comparadas con las del resto de clones de sus correspondientes ensayos. Estos resultados muestran la importancia de seleccionar clones con cualidades como tener valores altos de conductividad hidráulica y conductancia estomática que les permitan crecer lo máximo posible cuando las condiciones del medio son favorables, es decir, clones con un xilema eficiente. La cuestión es si un xilema eficiente puede ser además seguro. Recientes estudios como los de Gleason et al. (2015) revelan en ensayos testando más de 400 especies que la relación entre eficiencia y seguridad es débil, y encuentran especies poco eficientes y vulnerables como Pegaso, pero no encuentran entre el gran número de especies estudiadas ninguna especie eficiente y resistente, por lo que se concluye al final, que de algún modo, sí debe existir ese compromiso.

El estudio del xilema realizado en el tercer capítulo de la tesis tenía como objetivo averiguar si los clones que alcanzaban mayores crecimientos poseían alguna característica en

el xilema que les atribuyera alguna ventaja competitiva desde el punto de vista productivo. La plantación de chopo situada en Granada en la que se tomaron las muestras sufrió restricciones hídricas en verano que condujeron a la defoliación total de la plantación, tras la cual se aplicó un riego y las plantas rebrotaron. Se partía de la hipótesis de que los clones con los vasos más grandes serían más vulnerables y habrían producido menos biomasa en Granada. Y efectivamente, el clon que presentó el menor crecimiento fue el de vasos de mayor tamaño. Los clones que mostraron los mejores crecimientos en situaciones de estrés hídrico en la plantación de Granada exhibieron mayor número de vasos y de un tamaño intermedio. Los clones con características anatómicas que adoptaron valores extremos ofrecieron peores resultados: el clon que presentó los vasos más grandes pero menor número de ellos y aquel que presentó los vasos más pequeños fueron los menos productivos. Una eficiencia hidráulica intermedia resultó el óptimo en condiciones de estrés severo.

Los clones de chopo más productivos en cada uno de los tratamientos figuraron entre los más resistentes a la cavitación. Aunque la sequía hace disminuir el crecimiento, este resultado puede indicar que es posible encontrar clones adaptados a las condiciones ambientales mediterráneas y con un crecimiento aceptable. Si asumimos que existe una débil relación entre eficiencia y seguridad, nuestros resultados indicarían que en ambientes mediterráneos el óptimo productivo se encontraría en una eficiencia hidráulica intermedia y una elevada resistencia a la cavitación.

El análisis de la vulnerabilidad a partir de la construcción de curvas mostró que la vulnerabilidad a la cavitación es una característica que experimenta aclimatación en chopo. Nuestros resultados corroboran los obtenidos por Plavcová et al. (2011); las plantas sometidas a tratamiento de riego fueron más vulnerables que aquellas sometidas a déficit hídrico. No todos los clones presentaron la misma plasticidad: el que exhibió la mayor plasticidad en lo relativo a la vulnerabilidad a la cavitación fue I-214. Este clon figuró entre los clones más resistentes en condiciones de estrés y fue el más vulnerable de todos cuando fue sometido al tratamiento de riego más favorable. AF2, sin embargo resultó el menos plástico, y no presentó diferencias en vulnerabilidad cuando se le sometió a diferentes regímenes de riego. Esta variabilidad fenotípica por un lado es un rasgo que puede ser considerado una ventaja adaptativa frente a cambios en el medio, y tiene el inconveniente de dificultar la predicción *a priori* sobre los fenotipos que se generarán en cada situación.

En dos especies vulnerables a la cavitación como son eucalipto y chopo, no solamente es importante evitar la generación de embolia en los vasos, sino repararla una vez ha sucedido (Zwieniecki and Holbrook 1998). Tanto en chopo como en eucalipto se ha documentado el fenómeno de *refilling* (Hacke et al 2001, Martorell et al. 2013), si bien puede haber variaciones entre genotipos. Es probable que en situaciones de déficit hídrico sea más sencillo rellenar un xilema compuesto por muchos vasos medianos, con mayores posibilidades de interconexión, que rellenar un xilema de pocos vasos de gran tamaño como el de I-214. De hecho, es un fenómeno común encontrar en las maderas de ejemplares sometidos a estrés un mayor número de vasos de menor tamaño que cuando han vegetado en terrenos con mayor disponibilidad de agua. (Fichot et al. 2009, Coccozza et al 2011).

La diferencia más llamativa a primera vista entre la anatomía del xilema de chopo y del eucalipto en los tallos de un periodo vegetativo estudiados es que en eucalipto los vasos son solitarios, mientras que en chopo son muy numerosos los vasos múltiples en todos los clones ensayados. No hay consenso en la interpretación del significado de la existencia de vasos múltiples: Loepfe et al. (2007) observaron que un incremento en la interconectividad de la red de vasos suponía un mayor riesgo de dispersión de embolismo, pero también mayores valores de conductividad, mientras por el contrario, Carlquist (1984) propuso la hipótesis contraria: que el mayor agrupamiento de vasos favorece a las especies de lugares más áridos, que presentan mayor número de vasos agrupados que especies que habitan en lugares más húmedos. Lens et al (2010) estudiando varias especies del género *Acer*, observaron que los clones más resistentes a la cavitación mostraban mayor número de vasos agrupados que los más vulnerables. En nuestro trabajo el rango de valores de potencial medidos es similar en ambas especies, un poco más amplio por ambos extremos en chopo por estar cultivado en suelo, sin embargo, *Eucalyptus globulus* alcanza elevadas producciones en terrenos de secano, mientras que las plantaciones de chopos están ligadas siempre o a la capa freática o a la aportación de riegos para obtener un rendimiento aceptable.

CRECIMIENTO Y SUPERFICIE TOTAL FOTOSINTETIZADORA

El área foliar está muy relacionada con la producción forestal (Verlinden et al. 2014, Pellis et al. 2004, Fang et al. 1999, Dunlap & Stettler 1998, Ridge et al. 1986, Linder. 1985, Zavitovski et al., 1976). Los clones de eucalipto y chopo que han alcanzado mayores valores de biomasa presentaron mayores desarrollos de área foliar, con la excepción del clon de eucalipto C13, que mostró una de las mayores áreas foliares en invernadero pero ésta no se tradujo en una mayor producción de biomasa. Este fue el clon con menor área foliar distribuída en ramas. En las plantaciones de chopo de Granada, el clon con mayor número y peso de ramas fue el clon más productivo. Y lo mismo se ha observado en las plantaciones de chopo del capítulo 4. Las ramas emitidas por todas las plantas empleadas en esta tesis son ramas silépticas, no proceden de una yema que haya experimentado un periodo de durmancia. La emisión de este tipo de ramas está muy condicionada por las circunstancias ambientales, como afirman Wu & Hinckley (2001) y juegan un papel muy importante en la formación de la copa y en la captación de carbono en los primeros años de las plantaciones (Ceulemanns et al. 1990).

Los valores de índice de área foliar (LAI) estimados indirectamente a partir de fotografías hemisféricas presentaron correlaciones muy elevadas con los valores de LAI estimados directamente a partir de la superficie y peso de las hojas así como con la producción de biomasa. Los resultados mostraron, por tanto, una elevada fiabilidad para comparar la producción de biomasa entre sitios, años y clones a partir de las fotografías a pesar de la infraestimación ocasionada por la violación de las hipótesis de partida (considerar la cubiera como un medio homogéneamente turbio). Los modelos propuestos por Montes et al. (2007) considerando el efecto de agrupación de las hojas y y el ángulo medio de inclinación de las mismas no infraestimaron el índice de área foliar en masas cerradas. El modelo de Poisson, empleado por el software de algunos dispositivos comerciales, infraestimó el LAI en masas abiertas y cerradas en nuestras parcelas de ensayo.

En conclusión, la información obtenida de las mediciones anatómicas, morfológicas y fisiológicas realizadas contribuye a explicar la elevada interacción genotipo x ambiente encontrada en las plantaciones de campo.

CONCLUSIONES



CONCLUSIONES

1.-El conjunto de variables anatómicas y fisiológicas estudiadas ha sido de gran utilidad para comprender las diferencias de producción existentes entre diferentes genotipos en plantaciones tanto comerciales como experimentales en el mismo o en diferentes sitios de ensayo.

2.-Los valores de conductividad hidráulica y conductancia estomática de los genotipos más productivos figuraron entre los valores más altos medidos, mientras que los genotipos menos productivos presentaron los valores más bajos de conductividad y conductancia.

3.-Estar en posesión de un xilema con una baja superficie conductora compuesta por vasos pequeños ha sido la característica común de los clones que han sido retirados de plantaciones comerciales o experimentales por su elevada mortalidad en condiciones de estrés tanto en eucalipto como en chopo. En los dos casos encontrados (clones H-231 y Pegaso) la conductancia estomática en condiciones de estrés fue superior a la del resto de los clones.

4.-Los clones que han presentado producciones más elevadas en condiciones de estrés exhibieron mayor número de vasos y de tamaño intermedio. Los clones con valores medios extremos de superficie de lumen de vasos o con un número muy pequeño de vasos han resultado menos productivos.

5.-No se ha detectado relación entre crecimiento y vulnerabilidad a la cavitación en los genotipos ensayados. Los clones más productivos figuraron entre los más resistentes a la cavitación. Este resultado abre la puerta a la búsqueda de genotipos productivos entre los más resistentes.

6.- Los valores de conductividad hidráulica máxima pueden variar en cortos espacios de tiempo. Esto se debe tener en cuenta cuando se utiliza el parámetro pérdida de conductividad hidráulica para evaluar la respuesta a estrés hídrico.

7.-La disminución de la conductividad máxima hidráulica en eucalipto puede ser consecuencia de una impermeabilización de las membranas de las punteaduras y podría formar parte de un mecanismo de *refilling* del xilema de eucalipto.

8.-El cierre estomático parcial o total detectado en los dos primeros ensayos de eucalipto ha sucedido simultáneamente a la coincidencia de los valores de conductividad máxima en las plantas pertenecientes a los dos regímenes hídricos ensayados. Esto indica una posible señal hidráulica que marca la necesidad de cierre

estomático por haberse alcanzado el umbral mínimo de conductividad necesario para abastecer de savia a las hojas.

9.-El pH de la savia disminuyó coincidiendo con un incremento en el déficit de presión de vapor durante varios días consecutivos, justo después de haber coincidido los valores de conductividad máxima de las plantas correspondientes a diferentes regímenes de riego. La disminución del pH podría ser consecuencia de un proceso de *refilling*.

10.-Los modelos empleados para estimar el LAI a partir de fotografías hemisféricas que tienen en cuenta el agrupamiento de las hojas y su inclinación han arrojado resultados muy bien correlacionados con el LAI obtenido a partir de métodos directos y con la biomasa.

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