

Article

Responses of Contrasting Tree Functional Types to Air Warming and Drought

Elisabet Martínez-Sancho ^{1,*} , Lizeth K. Vásconez Navas ¹, Hannes Seidel ¹, Isabel Dorado-Liñán ² and Annette Menzel ^{1,3}

¹ Ecoclimatology, Department of Ecology and Ecosystem Management, Technische Universität München, Hans-Carl-von-Carlowitz-Platz 2, 85354 Freising, Germany; lizvasconez@gmail.com (L.K.V.N.); hseidel@wzw.tum.de (H.S.); amenzel@wzw.tum.de (A.M.)

² Departamento de Silvicultura y Gestión de los Sistemas Forestales, CIFOR-INIA, Carretera de la Coruña Km 7.5, 28040 Madrid, Spain; dorado.isabel@inia.es

³ Institute for Advanced Study, Technische Universität München, Lichtenbergstraße 2a, 85748 Garching, Germany

* Correspondence: martinez@wzw.tum.de; Tel.: +49-8161-714-748

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Abstract: Climate change-induced rise of air temperatures and the increase of extreme climatic events, such as droughts, will largely affect plant growth and hydraulics, leading to mortality events all over the globe. In this study, we investigated the growth and hydraulic responses of seedlings of contrasting functional types. *Pinus sylvestris*, *Quercus* spp. and *Castanea sativa* seedlings were grown in a common garden experiment under four treatments: control, air warming, drought and their combination during two consecutive growing periods. Height and diameter increments, stomatal conductance and stem water potentials were measured during both growing seasons. Additionally, hydraulic parameters such as xylem-specific native and maximum hydraulic conductivities, and native percentage of loss of conductivity were measured at the end of the entire experiment. Our results clearly pointed to different adaptive strategies of the studied species. Scots pine displayed a relatively isohydric behavior with a strict stomata control prohibiting native embolism whereas sweet chestnut and oak as relatively anisohydric species displayed an increased loss of native conductivity as a results of low water potentials. Seasonal timing of shoot and diameter growth also differed among functional types influencing drought impacts. Additionally, the possibility of embolism reversal seemed to be limited under the study conditions.

Keywords: climate change; drought; hydraulic conductivity; stem water potential; manipulation experiment; plant functional types; tree growth; stomatal conductance; warming

1. Introduction

Climate change is certainly affecting forests at different spatial and temporal scales [1]. In fact, forests are especially sensitive to climate change because trees have a long life-span reducing their ability to adapt to environmental changes [2]. In the last decades, several mortality events have been observed across different ecosystems, species and continents triggered by drought and heat spells; pointing to a global vulnerability of forests to the ongoing climate change [3]. Among the climate changes forecasted by the last Intergovernmental Panel on Climate Change (IPCC) report [4], not only a rise of mean temperatures and an irregular rainfall distribution, but also an increase in frequency and intensity of extreme climate events such as drought are predicted by the end of 21th century for Europe. Thus, the risk of forest mortality may increase as a consequence of future detrimental climatic conditions. Since forest ecosystems play a major role in different natural cycles, climate change-induced mortality would imply, for instance, changes in ecological communities leading to a loss of ecosystem services [5]. Nonetheless, many questions remain unanswered and require a deeper

understanding of climate change-induced impacts, such as species-specific physiological thresholds of drought severity and duration [6].

Forests acclimation comprises molecular, physiological, and structural adjustments [7]. In plants, the water movement is initiated by transpiration through the stomata pulling water from the roots to the leaves through the xylem. This pathway connects soil and atmosphere through the plant and is well known as the soil-plant-atmosphere-continuum (SPAC) [8]. Plants have developed different strategies to cope with drought by adjusting different traits along the SPAC such as a reduction of leaf area [9] or an increase of the sapwood area-to-leaf area ratio [10]. Therefore, the study of plant hydraulic traits has lately become the main approach to understand plant vulnerability to fast changing conditions [11]. Plants have different physiological strategies to cope with drought. During dry spells, stomatal closure is the main plant mechanism to limit water loss, and thus, to maintain water potentials within the safety margins. Therefore, species have been classified in two groups based on the degree of stomatal closure in response to drought [12,13], although recent studies claim that there is a continuum between isohydric and anisohydric behaviors [14,15]. On the one side, relatively isohydric species show moderate constant maximum values of water potentials which means that stomatal closure occurs faster, avoiding water loss through transpiration. These species have been described as more prone to suffer carbon starvation during prolonged dry spells [6], since the early stomatal closure also stops assimilation, and thus, plants are obliged to rely on carbon reserves [16]. On the other side, relatively anisohydric species perform a more relaxed stomatal regulation allowing water potentials to reach more negative values during drought conditions. As a consequence, carbon assimilation is not interrupted, but during intense drought events cavitation may occur inside the xylem conduits. Cavitation breaks the continuity of water columns and hence the water supply to transpiring leaves. Xylem embolism reduces hydraulic conductance and may ultimately result in the hydraulic failure of the plant hydraulic system [17]. These two different strategies include several trade-offs between the level of xylem tension and carbon uptake through photosynthesis. Thus, the strategy followed by a given tree species and the intensity and duration of the dry spells are crucial to determine tree performance and survival.

Several studies have claimed the possibility of refilling gas-filled conduits driven by over-atmospheric root/stem pressures originating during night or specific periods of the year [18,19] or even when the xylem is still under tension driven by phloem unloading [20]. Although the refilling of the gas-filled conduits has been better explained in angiosperms, it also seems to occur in conifers [21], but the mechanisms behind might differ from those proposed for angiosperms due to xylem structural differences. However, there is some controversy about conduit refilling since it could be affected by measurement artifacts [22]. Nevertheless, the study of hydraulic traits such as hydraulic conductivity, stomatal conductance and water potentials of different functional tree types (isohydric vs. anisohydric, conifer vs. angiosperm), and the species-specific ability of conduits refilling under controlled conditions is a prerequisite to assess species resilience under future climatic conditions.

Plants may have benefited from the rise in temperature, for instance, due to an extension of the growing season [23,24]. Although tree responses are usually species-specific, the increase of temperature alone can also enhance plant growth [25] through higher carbon assimilation if water availability is not restricted [26], but can promote drought stress by raising the water vapor deficit [27]. Drought impacts on tree physiology describe a circle that starts by affecting water transport and assimilation and continues with allocation processes such as growth [28]. Several studies have associated the drought-induced growth reduction to physiological tree decline and with overall tree mortality [29,30]. In fact, tissue formation is inhibited by drought long before carbon supply falls short because of drought-induced limitations of gas exchange [31]. Moreover, plant-plant interactions seem to have an interspecific effect on the drought tolerance of tree species to cope with future climate scenarios [32–34], since competition decreases individual radial growth as a result of reduced nutrient and water availability [35]. Previous studies have suggested that tree-to-tree competition, as a long-term predisposing stressor, may be an additional risk factor for drought-induced mortality in

water-stressed areas [36]. Therefore, additional parameters such as stand density need to be included to evaluate the physiological response of different tree species to drought.

The seedling stage is considered to be a very drought sensitive and mortality susceptible period of plant development, given the lower rooting volume and depth, as well as lower carbohydrate reserves [37,38]. Seedling establishment is directly related to sustainable forest cover and production [39]; thus, the influence of climate on trees at this stage has been recalled to probably influence regeneration negatively inducing alternate forest communities [40]. Although most of the studies analyzing seedling responses under controlled conditions are conducted in pots, the se may produce some artifacts most likely due to restrictions of the natural root system [41].

The present study aims to investigate the response of seedlings of three European tree species with contrasting functional types (*Castanea sativa* Mill., *Pinus sylvestris* L., and *Quercus* spp.) under predicted future climatic conditions in an experimental field. A factorial combination of warming and drought was achieved using an open air-heating system and an automatic rain-out shelter, respectively. This experimental design allowed natural root development of the seedlings contrary to pot experiments. Moreover, high and low competition levels were taken into consideration. We assessed plant responses as changes in ecophysiological parameters (e.g., stomatal conductance, water potentials and plant hydraulics) and growth performance. We focused on the following questions: (i) Are there inter-specific differences in the response to prolonged drought and warming? (ii) Is competition enhancing drought stress? (iii) Is the relation between stomatal conductance and water potential equal among species? (iv) Do the studied species recover their hydraulic conductivity after experiencing very low water potentials?

2. Materials and Methods

2.1. Study Species

Sweet chestnut (*Castanea sativa* Mill.) is a medium sized deciduous tree from the *Fagaceae* family. As a ring-porous species, its hydraulic system is made up of vessels. It is considered to be a drought tolerant species. Thus, being anisohydric, it keeps stomata open under drought conditions [42]. Sessile and pedunculate oak (*Quercus petraea* (Matt.) Liebl. and *Quercus robur* L., respectively) are large deciduous trees that, similar to sweet chestnut, also belong to the *Fagaceae* family and have a ring-porous wood anatomy. Both species share most of their functional characteristics and are usually regarded in the literature as a unique cluster *Quercus* spp. due to frequent hybridization. *Quercus* spp. are also anisohydric, tolerating hydraulic tension in their xylem by opened stomata and therefore, higher rate of photosynthesis under water restricted conditions [43]. Scots pine (*Pinus sylvestris* L.) from the *Pinaceae* family is an evergreen conifer with a wide distribution range across Eurasia. Its xylem is made of non-specialized tracheids that perform conductive and structural functions. Scots pine is an isohydric species since its stomatal closure occurs faster under drought conditions [44], thus maintaining steady water potentials.

2.2. Experimental Design

The experiment was set up at an open field site located in the Gewächshauslaborzentrum Dürnast (GHL) of the TUM School of Life Sciences Weihenstephan (48°24' N, 11°41' E, 445 m a.s.l.). According to the German Meteorological Service, the climate conditions at this location are characterized by a mean annual temperature of 7.9 °C and 785 mm of mean annual precipitation for the period 1971–2000.

The soil composition of the 87 m² parcel was carefully prepared to ensure a good drainage and thus reduced water availability for the plants. Three different layers of soil, reaching 120 cm in total, comprised deep down, 35 cm of gravel, followed by 50 cm of pure sand and a 35-cm top layer of sand with 30% humus. This soil composition is similar to the soils from the Franconian plateau [45], one of the driest and warmest regions in Germany [46], having a 32.5% of maximum water capacity and a pH of 7.2. The experiment was set up following a split-plot design (Figure S1) with four treatments during

the growing season: *control*, temperature increase (*temp*), rainfall exclusion (*dry*) and the combination of temperature increase and rainfall exclusion (*drytemp*). However, due to technical reasons, it was not possible the randomization of treatments and species within the plots, which may lead to potential limitations in the interpretation of the results. The temperature increase was accomplished by using a water heating system, which was connected to corrugated pipelines mounted 2 cm over the ground gridding each seedling. This structure located at the treatments *temp* and *drytemp* fulfilled the objective of heating the air by 1–2 °C compared to ambient temperature in the surrounding of the seedlings. Drought conditions were achieved with an automated transparent rainout shelter, which covered the plots of the *dry* and *drytemp* treatments during precipitation events. Lateral water movement was prohibited by concrete foundations of the rainout shelter as well as vertical plastic sheets in the soil between the treatments. Each treatment plot had a total area of 18.85 m².

A total of 384 seedlings of *Castanea sativa*, *Quercus* spp. and *Pinus sylvestris* (128 per species) were planted on 21 November 2013. Each treatment had 96 seedlings (32 per species). The seedlings were one-year-old in the case of sweet chestnut and oak and two-year-old in the case of Scots pine. In order to study the effect of density, each treatment plot consisted of three subplots: two subplots with a high density of seedlings (7.57 seedlings m⁻², 12 per species) and one subplot with a low density of seedlings (3.69 seedlings m⁻², eight per species; Figure S1). The plant material was obtained from a nursery. After planting, the seedlings had an acclimation period of seven months (from November 2013 to June 2014) before the treatments started. The first treatment period in 2014 started on 23 June and ended on 14 October. During winter 2014–2015 the treatments were stopped and seedlings experienced normal site conditions. The second treatment period in 2015 ran from 30 June until 15 September.

The temperature in all treatment plots was continuously monitored with a temperature sensor (FF-10V-INT-TE, B+B Thermo-Technik GmbH, Donaueschingen, Germany). Soil moisture was recorded twice a week during the treatment periods at 20 and 60 cm depth using a profile soil moisture sensor and readout unit (PR2 Profile Probe and HH2 Moisture Meter, Delta-T Devices Ltd., Cambridge, UK). However, due to technical problems, there were some gaps in both time series (air temperature and soil moisture).

2.3. Growing Conditions

During the treatment periods, marked differences of air temperature and soil moisture were observed among the plots (Figure 1). Mean recorded temperatures were higher in *temp* and *drytemp* compared to *dry* and *control*, thus evidencing the effect of the heating system (Figure S2). However, higher absolute mean temperatures were recorded in 2015 indicating an extraordinary warm summer in the region (Figure S3). In fact, in 2015 the highest temperatures since 1880 were recorded in Germany (German Meteorological Service).

Soil moisture was in general higher in the *control* treatment and declined along the *temp*, *dry* and *drytemp* treatments. The seasonal soil moisture evolution differed between the years 2014 and 2015. In 2014 the *control* and *temp* treatments displayed a higher content of water at both depths compared to *dry* and *drytemp* which additionally displayed a decreasing trend at 20 cm depth. In 2015 the soil moisture in all treatments showed nearly the same values at 20 cm depth pointing to a clear impact of the natural drought in 2015. In contrast to the 20 cm depth, clear differences were still detectable at 60 cm depth among the treatments.

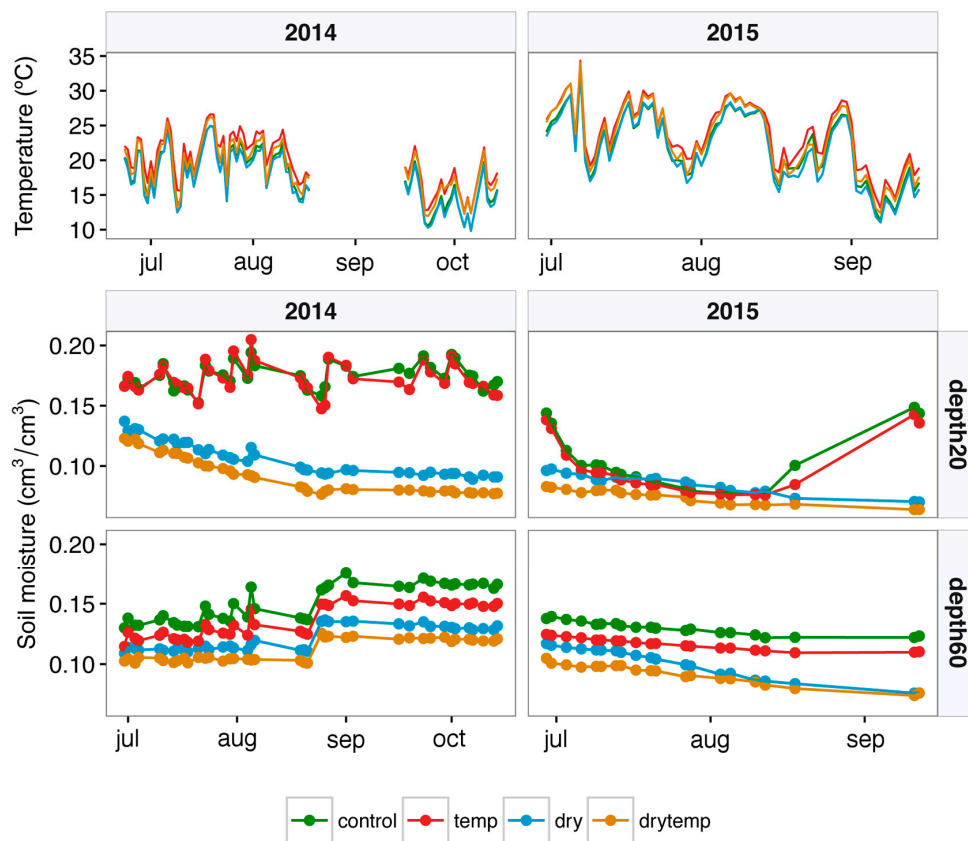


Figure 1. Climate conditions during the two treatment periods in 2014 and 2015. Mean daily temperatures are shown in the upper panels. Middle and lower panels display soil moisture at 20 and 60 cm depth, respectively. Abscissas labels are placed at the beginning of the month.

2.4. Field Measurements

Height and diameter of six seedlings per species, treatment and subplot were measured once a month during the treatment periods. Stem diameter was recorded 1.5 cm above the ground level with a pocket measuring slide caliper of 0.01 mm of resolution (0220 DIGI-MET IP65, Helios-Preisser, Gammertingen, Germany). The height of the seedlings was measured from the base of the seedling to the terminal tip using a folding rule.

Stem water potential (Ψ_{STEM}) and stomatal conductance (g_s) were only measured in the high density plots due to time restrictions. In 2014 Ψ_{STEM} and g_s were measured every week or every second week depending on the weather conditions avoiding rainy conditions from 23 June to 14 October and 5 June to 26 September, respectively. In 2015, Ψ_{STEM} and g_s were measured simultaneously on 7 July, 5 and 21 August, and 11 September.

Ψ_{STEM} was measured on six seedlings per species and treatment using a Scholander type pressure chamber, (PMS Instrument Company, Albany, NY, USA). A fully developed leaf of each seedling was covered with aluminum foil and was enclosed in a plastic bag in the evening before the day of harvesting. This procedure stops leaf transpiration and enables the leaf water potential to equilibrate with the xylem water potential at the petiole [47]. On the next day (11:00–15:00) each leaf was removed with a razor blade and immediately placed into the pressure chamber. Since the water potential is strongly influenced by time of the day, the measurements were completely randomized each time. The stomatal conductance (g_s) was measured on fully developed leaves avoiding main veins with a porometer (SC-1 Leaf Porometer, Decagon Devices, Pullman, WA, USA).

2.5. Laboratory Measurements

The plant material for hydraulic analyses was harvested at the end of the second treatment period (September–October 2015). The harvest schedule was randomized in order to avoid possible time interactions. The identical six seedlings per species, treatment and subplot measured for growth traits were analyzed. Entire seedlings including the root system were collected, immediately sprayed with water, placed inside plastic bags and transported to the laboratory. At the same day and once in the laboratory, the whole seedling was submerged under water and successively cut by trimming the ends of each segment with a fresh razor blade. This procedure was applied in order to avoid any potential artificial embolism [48,49]. The 3-year-old internode of each seedling was cut, labeled and kept under water to avoid dehydration. Diameter and length of the resulting segments were recorded for each sample (Table S1). The stem sections were quickly connected to the Sperry hydraulic conductivity apparatus [50]. The set-up consists mainly of tubes and containers filled up with a degassed and deionized solution of 20 mM KCl and 1 mM CaCl₂ which allows controlling probable ionic effects on pit membrane conductance. The gravity-induced water flow rate through the segments was measured every 10 s with a high precision electronic balance (Mettler-Toledo XS204R, Mettler-Toledo AG, Greifensee, Switzerland) interfaced with a computer.

The native hydraulic conductivity (K_H , kg m MPa⁻¹ s⁻¹) was calculated as:

$$K_H = (F \cdot L) / \Delta P \quad (1)$$

where F is the flow rate (kg s⁻¹), L is the length of the segment (m) and ΔP the driving force (MPa). The xylem-specific native hydraulic conductivity ($K_{S-NATIVE}$, kg m⁻¹ MPa⁻¹ s⁻¹) was then calculated as K_H normalized by the total cross-sectional xylem area (m²). Cross sections were prepared from the center of each stem. The total cross-sectional xylem area was captured using a digital camera (Canon Rebel T2i, Canon, Krefeld, Germany) connected to a binocular microscope (Leica S6D, Leica Camera AG, Wetzlar, Germany) and afterwards measured with the software ImageJ (IJ 1.46r, National Institutes of Health, Bethesda, MD, USA). Areas corresponding to the pith and the bark of the stems were excluded from the measurements.

Native xylem embolism was reversed by applying two different methods for conifers and angiosperms due to their contrasting physiologies [51]. In the case of Scots pine, the stem portions were submerged in a degassed solution of 20 mM KCl and 1 mM CaCl₂ and vacuum infiltrated for 1 h. In the case of chestnut and oak, the segments were connected to a tubing apparatus and flushed, with the same measuring solution, for 1 h at 100 kPa [51]. Afterwards, the xylem-specific maximum hydraulic conductivity (K_{S-MAX}) was measured following the same procedure as for the $K_{S-NATIVE}$. The native percentage of loss of hydraulic conductivity (PLC) was calculated as the percentage of $K_{S-NATIVE}$ relative to K_{S-MAX} in order to determine the degree to which the maximum hydraulic conductivity had been reduced.

2.6. Statistical Analyses

Due to their non-normal distribution, the inter- and intraspecific differences in growth and hydraulic variables were analyzed by Kruskal-Wallis tests. To test pairwise differences, a Dunn's test with a Bonferroni correction was performed. Additionally, the effect of seedling density on the different response variables (height, diameter, and hydraulic measurements) was determined with pairwise Wilcoxon signed-rank tests.

The influence of species, treatment and year on stem water potential and stomatal conductance was analyzed for the whole study period from end of June 2014 until beginning of September 2015 using linear mixed effects models (R package nlme; [52]). The starting model contained the three-way interaction of the factorial dummy variables species, treatment and year and single individuals were used as a random variable to account for repeated measurements. Variance function structure classes were applied to assure homoscedasticity of residuals. These models were then simplified by removing

the terms that did not improve the model fit based on the Akaike information criterion (AIC) using the stepAIC function with backward and forward selection (R package MASS, [53]). The final models of stem water potential and stomatal conductance contained all possible two-way interactions of species, treatment and year and the three-way interaction of species, treatment and year, respectively.

The relation between simultaneously measured stem water potential and stomatal conductance in 2015 was assessed by fitting exponential functions. In order to determine which xylem tension drives the loss of conductivity, the minimum and the last recorded stem water potentials were related to PLC. This relation was assessed fitting a Weibull function as commonly used for vulnerability curves [54]:

$$\text{PLC}/100 = 1 - \exp[-(-\Psi_{\text{STEM}}/b)^c] \quad (2)$$

where b and c are fitting constants. Fitting was carried out using the nls function (R package stats). The resulting models per species were compared by AIC values.

All the statistical analyses were performed using the different mentioned packages implemented in the R statistical software (R Development Core Team 2008).

3. Results

3.1. Density Effect

In general, seedling density had little effects on the studied parameters (diameter, height, $K_{S-NATIVE}$, K_{S-MAX} and PLC) (Table S2). Among the 84 possible pairwise comparisons, only nine were significant ($p < 0.05$), six of them for sweet chestnut, one for oak and two for Scots pine. Sweet chestnuts from the low-density *control* showed higher diameter increases than those at the high-density *control* whereas the opposite pattern was true for *dry*. Sweet chestnut from low-density subplots of *drytemp* displayed higher height increments than those from the low-density. The opposite pattern was found for Scots pine at *control*. Regarding hydraulics, sweet chestnut from high-density *dry* showed higher $K_{S-NATIVE}$ values, which translated into lower PLC values compared to low-density. However, seedlings from high-density *drytemp* also showed higher K_{S-MAX} than those from low-density but this did not affect PLC. Contrary to sweet chestnut, oak from the low-density *dry* showed higher values of $K_{S-NATIVE}$ than those from high-density. PLC from Scots pine at low-density *temp* were significantly higher than those from high-density.

Therefore, since there were only marginal and even not congruent density effects, they were not further considered in the analyses.

3.2. Growth Effects

In 2014 and 2015, the diameter increments of Scots pine in both *control* and *dry* were significantly higher than those of the other two species (Figure 2a,b). In 2014, oak displayed significantly lower diameter increments than the other two species in *drytemp* and *temp* with no significant differences between sweet chestnut and Scots pine. At the intraspecific level, seedlings of all species growing without water restrictions in 2014 (*control* and *temp*) showed significant larger diameter increments than those from *dry* and *drytemp*. In contrast, in 2015, diameter increments of Scots pine did not show any significant differences among treatments, whereas sweet chestnut showed the same pattern as in 2014 and oak of *control* and *dry* displayed significantly larger diameter increments compared to *drytemp* and *temp*.

Height increments 2014 of oak and sweet chestnut in *dry* were significantly lower than those of Scots pine, whereas in *temp* and *drytemp* oak displayed significantly lower height increments than the other two species (Figure 2c). No significant differences in height increments 2014 were found among species in *control*. While there were no significant intraspecific differences among oak and Scots pine, sweet chestnut from *dry* and *drytemp* grew significantly less in height than those from *control*. Additionally, sweet chestnut from *dry* displayed lower height growth than those from *control*.

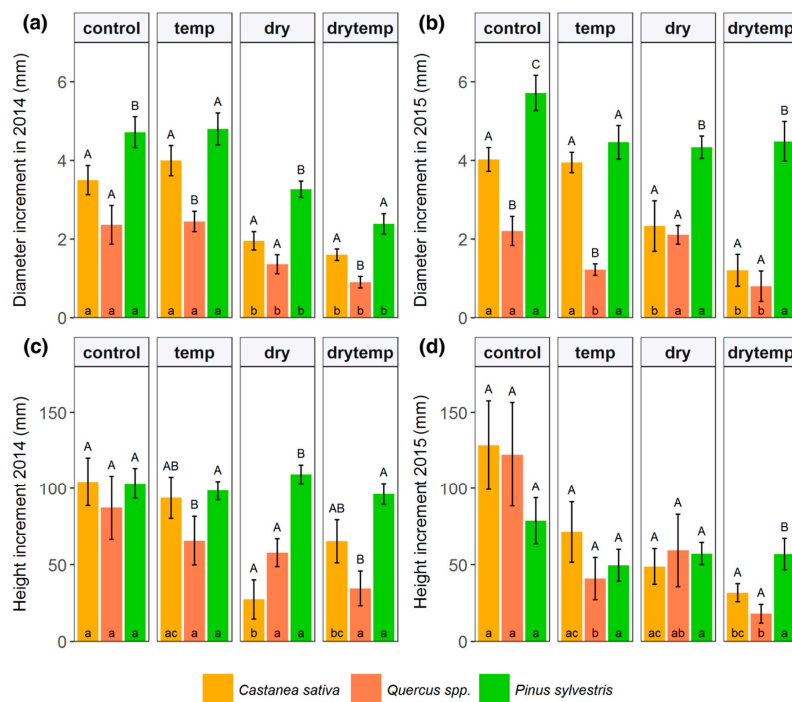


Figure 2. Diameter (a,b) and height (c,d) increments for both treatment periods 2014 (a,c) and 2015 (b,d). Bars represent mean \pm SE. Upper and lowercase letters represent inter- and intraspecific significant differences ($p < 0.05$) within and between treatments, respectively.

No interspecific differences in height increments 2015 were found in *control*, *temp* and *dry* (Figure 2d), whereas Scots pine had significantly larger height growth than the other two species in *drytemp*. At intraspecific level, no significant differences were found among Scots pine treatments, while oak seedlings from *control* showed significantly higher height increments than those from *temp* and *drytemp*. Sweet chestnut seedlings from *drytemp* grew significantly less in height than those from *control*.

3.3. Stem Water Potential and Stomatal Conductance

The variation in stem water potential (Ψ_{STEM}) was explained by all possible two-way interactions of species, treatment and year. In general, Scots pine had the highest Ψ_{STEM} per treatment and year, followed by sweet chestnut and oak (Figure 3b). Furthermore, Scots pine exhibited a smaller reduction of Ψ_{STEM} (<0.3 MPa) across treatments and years than the other two species (Figure 3a). Within each species and treatment, Ψ_{STEM} was lower in 2015 than in 2014 (Figure 3b,c and see Figure S4 for temporal evolution).

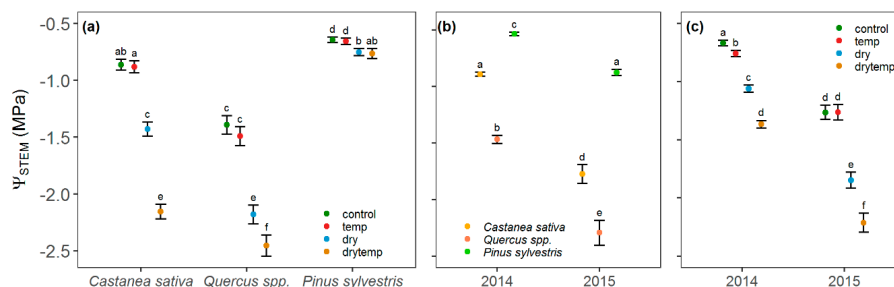


Figure 3. Variation of mean estimated stem water potential (Ψ_{STEM}) and their standard errors induced by (a) species and treatment; (b) species and year, as well as (c) treatment and year. Estimates within each panel that do not share the same letter are significantly different at $p < 0.05$.

More specifically, compared to *control*, *temp* did not have an effect on Ψ_{STEM} for all species, whereas Ψ_{STEM} of *dry* and *drytemp* was pronouncedly decreased for sweet chestnut and oak (Figure 3a). In 2014, Ψ_{STEM} declined along the *control*, *temp*, *dry* treatments towards the *drytemp* (Figure 3c). In 2015, Ψ_{STEM} in *control* and *temp* was as low as in *dry* during 2014 with an even stronger reduction *dry* and *drytemp* (Figure 3c).

The best model for stomatal conductance (g_s) included the tree-way interaction of species, treatment and year (Figure 4). Similar to Ψ_{STEM} , *temp* did not affect g_s . Values of 2015 were generally lower within treatments for sweet chestnut and oak but not for Scots pine. Scots pine showed lower g_s per treatment than sweet chestnut and oak in 2014, but no differences were found in 2015. In general, g_s in *dry* and *drytemp* was significantly lower than in *control* except for Scots pine in 2014. For sweet chestnut in 2014 and 2015 and for Scots pine in 2014, g_s did not significantly decrease in *dry* and *drytemp* compared to *temp*, although it did for oak. However, *drytemp* amplified the reduction of g_s compared to *dry* in sweet chestnut and in Scots pine in 2014. g_s in oak did not differ between *dry* and *drytemp*.

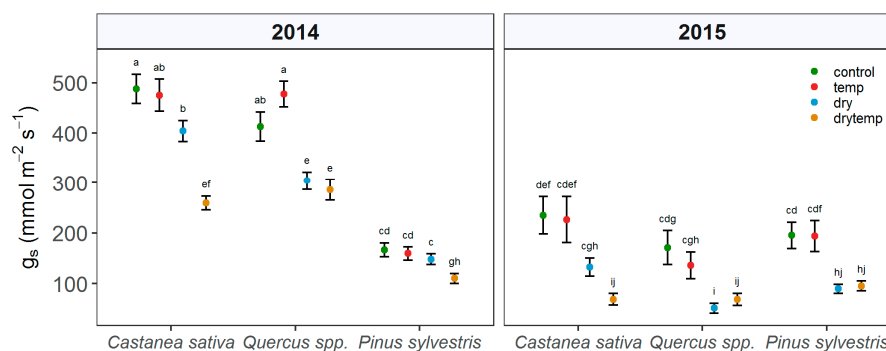


Figure 4. Variation of mean estimated stomatal conductance (g_s) and their standard errors induced by species treatment and year. Estimates within and across panels that do not share the same letter are significantly different at $p < 0.05$.

In all three species, g_s decreased as a function of declining Ψ_{STEM} (Figure 5). The relationship was significantly fit by an exponential function in all cases. Although sweet chestnut and oak showed a more relaxed stomatal closure, oak reached 90% of stomatal closure at lower Ψ_{STEM} than sweet chestnut (-4.61 MPa and -3.59 MPa, respectively). In contrast, g_s in Scots pine sharply responded to decreasing water potential and underwent 90% of stomatal closure at around $\Psi_{STEM} -1.43$ MPa.

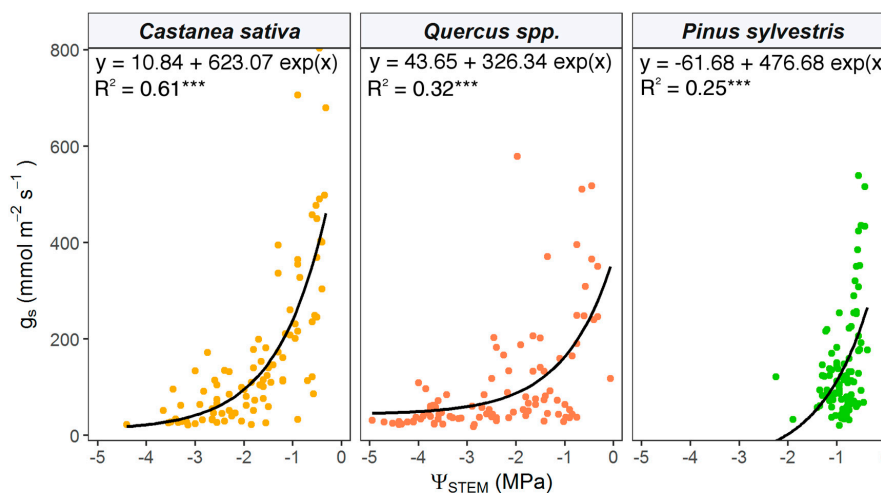


Figure 5. Relationships and their exponential fit between stomatal conductance g_s and stem water potential Ψ_{STEM} in 2015. *** $p < 0.001$.

3.4. Hydraulic Measurements

Sweet chestnut and Scots pine displayed significantly higher $K_{S-NATIVE}$ values than oak in the *control*, whereas in the other treatments Scots pine showed significantly higher values than the broadleaf species (Figure 6). Sweet chestnut $K_{S-NATIVE}$ values decreased significantly with increasing stress conditions, reaching the lowest values in *drytemp*. $K_{S-NATIVE}$ values of oak in *drytemp* were significantly lower than those of *temp*, but did not significantly differ from *control* and *dry*. Scots pine seedlings of *drytemp* displayed significantly lower $K_{S-NATIVE}$ values than *dry* and *temp*.

Sweet chestnut displayed significantly higher K_{S-MAX} than Scots pine and oak in the *control* treatment (Figure 6), but no interspecific differences were found for the other treatments. Similarly to $K_{S-NATIVE}$, sweet chestnut showed a significant decrease in K_{S-MAX} towards increasing aridity: K_{S-MAX} in *drytemp* was 75% lower than in the *control* treatment. No significant differences were found among treatments in K_{S-MAX} of oak and, in the case of Scots pine, K_{S-MAX} followed the same pattern as $K_{S-NATIVE}$.

In general, the percent loss of conductivity (PLC) was significantly higher for the broadleaf species than for Scots pine, regardless of the treatment (Figure 6). The highest PLC values of the broadleaf species were recorded in *drytemp* reaching approximately 75% and 90% for oak and sweet chestnut, respectively. Non-significant differences in PLC were found between oak and sweet chestnut in any of the treatments. PLC of Scots pine and oak did not differ among treatments. In contrast, PLC of sweet chestnut increased towards aridity, i.e., seedlings from *dry* and *drytemp* displayed significantly higher PLC values than those in the other treatments.

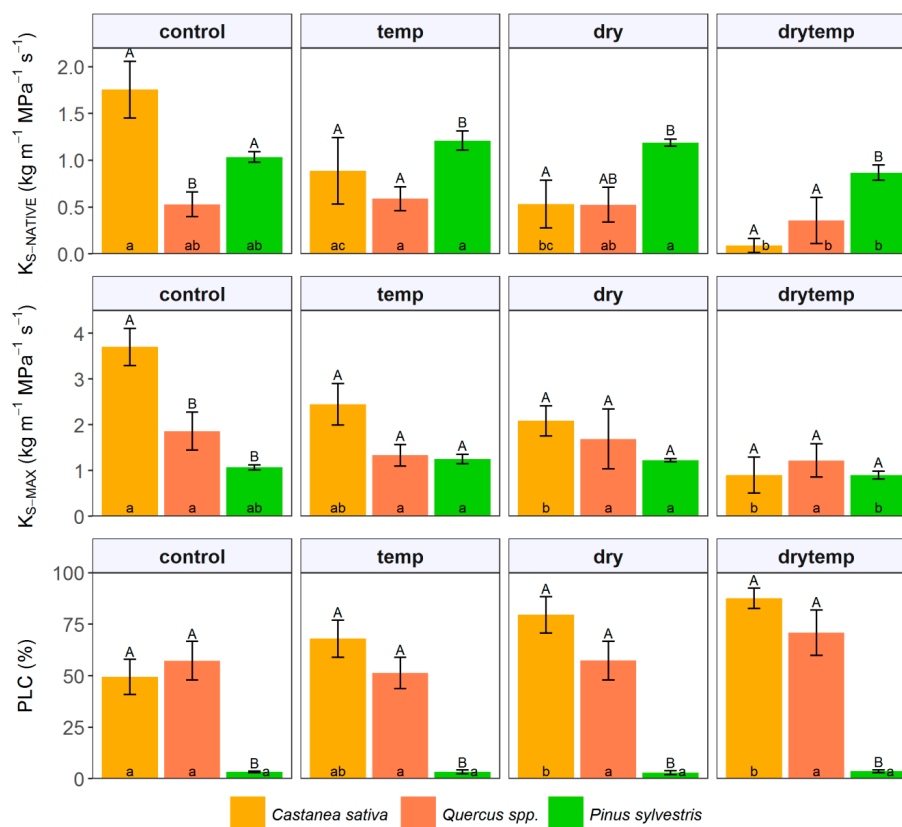


Figure 6. Native specific-hydraulic conductivity $K_{S-NATIVE}$, maximum specific-hydraulic conductivity K_{S-MAX} and percentage of loss of hydraulic conductivity PLC of the study species under the four treatments. Bars represent means and their standard errors. Upper and lowercase letters represent inter- and intraspecific significant differences ($p < 0.05$) within and between treatments, respectively.

3.5. PLC versus Minimum and Last Recorded Ψ_{STEM}

Weibull functions were fitted to explained PLC based on the minimum and the last recorded stem water potentials for each species (Figure 7). In the case of sweet chestnut, PLC data were better fitted by the minimum than the last recorded water potential, which can also be seen in the lower AIC value of the model based on the minimum stem water potential (Table 1). The relationship followed a sigmoidal curve typically reported for vulnerability curves. However, PLC data did not cover the whole possible range of Ψ_{STEM} .

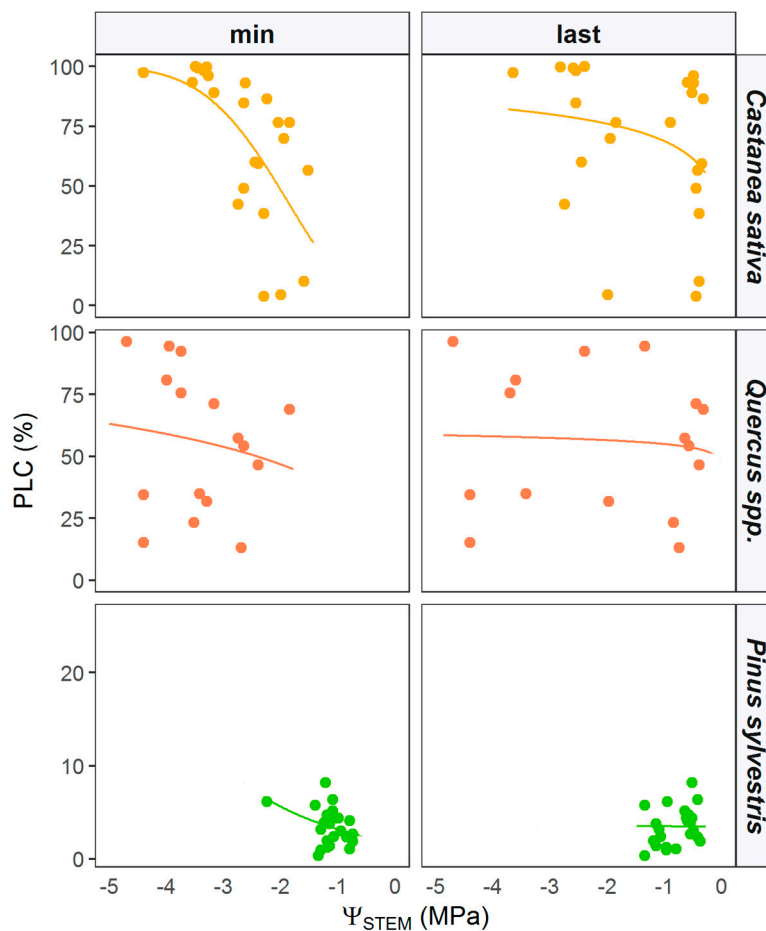


Figure 7. Relationships and corresponding Weibull function fits between the minimum recorded during the study period 2015 (**left** panels) as well as the last recorded (**right** panels) Ψ_{STEM} and the percentage of loss of conductivity (PLC).

In case of oak, there was no substantial difference between both models. Similarly to sweet chestnut, PLC of Scots pine was better explained by the minimum Ψ_{STEM} . However, Scots pine generally displayed only low values of PLC less than 10% and relative high Ψ_{STEM} above -1.5 MPa. Consequently, the vulnerability curves are very restricted.

Table 1. AIC values of the Weibull models using the minimum recorded (min) and the last measured (last) stem water potential Ψ_{STEM} .

AIC	<i>Castanea sativa</i>	<i>Quercus spp.</i>	<i>Pinus sylvestris</i>
min	216.53	156.90	32.63
last	226.65	157.19	38.37

4. Discussion

4.1. Effect of Seedling Density

In general, our results showed little effects of density on growth and hydraulics. An increase in radial growth would be expected at the low-density subplots regardless of the species due to a reduced competition for resources, e.g., light, water and nutrients [32,55,56]. This effect was only found in sweet chestnut (*control* 2015), but an opposite pattern was found for *dry*. However, a higher sensitivity to competition displayed by sweet chestnut is in agreement with [32] who found a slightly higher effect of competition on the response to aridity in broadleaf species (but see [56]).

Scots pine as a pioneer and light-demanding species [57] might have grown more in height in the high-density subplots in order to be more competitive, as pointed in our results. However, this effect was only clear in 2014 when the weather conditions were rather milder compared to 2015 (Figure S3) when the differences due to competition disappeared. On the contrary, both broadleaf species, as relatively shade tolerant species [58], did not show significant differences in height increment except for sweet chestnut in *drytemp*. However, it should be considered that height increment was measured only in the main shoots, neglecting the growth of secondary shoots, which can vary in different competition levels, especially in broadleaf species as reported by [59].

Just a few studies have assessed the effect of density on plant hydraulics. Trees growing in high-density stands displayed lower water potentials and sap flow, particularly during drought [60]. Our results did not show clear patterns in the effect of density on hydraulics, although sweet chestnut and oak showed some differences in $K_{S-NATIVE}$ and K_{S-MAX} between high- and low-densities in *dry*. Moreover, the se differences in $K_{S-NATIVE}$ and K_{S-MAX} were not translated into significant differences in PLC.

Our results are based on a two-year experiment and thus, we cannot exclude the possibility that long-term competition could lead to further challenges in overcoming drought events, as found by [33,34].

4.2. Effects of Warming and Drought on Growth

In contrast to the general assumption that temperature facilitates carbon allocation to growth if water availability is not limiting [61], especially among deciduous species [62], warming conditions did not lead to significantly larger height or diameter increments, regardless of the species. Even more, during 2015, when the conditions were more stressful, oak seedlings growing at *temp* displayed lower increments pointing to a high sensitivity to warming, especially for young individuals as reported by [63]. This finding contradicts [64] who found a temperature-induced stem shoot elongation but a diameter reduction in different species of the genus *Quercus*. According to our results, Scots pine seedlings did not increase their height increments under warming as reported by [65]. It should be considered that positive warming effects have mostly been reported in long-term studies [66] and our experiment might have not been long enough to observe those positive temperature effects.

Drought had a higher impact on growth, and we found species-specific responses. In general, our results pointed to a growth reduction under restricted water availability and thus this effect was clearly enhanced by warming, as also reported by [67]. Scots pine shoot length was not affected at all, most likely due to the timing of shoot growth. The elongation of the main stem shoot starts early in the growing season and achieves its maximum length in June [68]. Thus, most shoot growth took place before the treatment period (i.e., summer), avoiding the applied stress conditions. On the contrary, oak and sweet chestnut height increments were largely affected by the drought treatment in 2014 and 2015. Their duration of shoot elongation is longer and thus the drought treatment may reduce or delay the number of flushes [69,70] since these species are able to flush several times during a growing season [71]. In 2015 Scots pine shoot length was considerably shorter than in 2014. We hypothesize that the isohydric strategy of Scots pine reduced carbon uptake and reserves during the first experimental drought period [72], which might be a limiting factor at the beginning of the following growing

season. However, a detailed analysis would be needed to confirm this hypothesis since contradictory results were found regarding the carbohydrate storage of Scots pine under drought [73]. In contrast, the anisohydric species oak and sweet chestnut exhibited similar height increments in 2014 and 2015 due to a more relaxed stomatal control as indicated by the higher g_s . The period of diameter increment of all species lasted longer during the growing season and, consequently, was clearly affected by the experimental droughts. In 2014 Scots pine growing under drought conditions grew less in diameter than in the *control*, whereas this effect completely disappeared in 2015. However, broadleaf species showed this pattern in both years. Unexpectedly, Scots pine was the species showing the highest diameter increments even under drought conditions most likely due to morphological differences of the species (main stem/shoot, multilateral stems). It should be taken into considerations that the artificial poor soil composition in our experimental field could have intensified the stress conditions. Besides, our experimental design with a limited randomization of the species and treatments, may have induced limitations in our results.

4.3. Contrasting Hydraulic Strategies among the Study Species

Our results are in agreement with previous studies regarding the strategies of the selected species to cope with drought conditions. Scots pine displayed a relatively isohydric strategy avoiding possible drought damages through a strict stomata control as also previously reported by [44,74]. Stomatal conductance was drastically reduced in seedlings growing at *dry* and *drytemp* treatments, and these differences were even higher during the more severe treatment period of 2015. The early stomatal closure allowed maintaining relatively high stem water potentials, with significant but small differences between treatments which translated into a small percent loss of conductivity (PLC). These low values of native loss of conductivity found in our study are in disagreement with the ones reported by [72], who found a strong decline in native PLC in a Scots pine population located in the NE Iberian Peninsula. However, a more extensive study assessing the native loss of conductivity across Scots pine stands, located at the southern limit of the species distribution during one of the driest summers in the area, found similar values as ours [51]. Nevertheless, this conservative strategy allows keeping the xylem functional in water transport during several years, since the annual build-up of new xylem tissue just accounts for 15–20% of the total hydraulic conductivity [75]. Moreover, such a strategy intends to maintain the potential hydraulic conductivity even under extreme conditions, which can be clearly seen in our results since Scots pine displayed higher $K_{S-NATIVE}$ values at increasing aridity than the rest of the study species.

In contrast to Scots pine, sweet chestnut and oak displayed relatively anisohydric behaviors with a relaxed stomatal control. However, the hard conditions that the seedlings experienced during 2015 might have surpassed their limits (as noticed by visual observations of decaying oak seedlings), and stomatal conductance was exponentially reduced to similar levels than Scots pine, especially in the *dry* and *drytemp* treatments. Stem water potentials tracked the fluctuations in soil water availability in both years. The low water potentials displayed by both species, especially by oak which could be related to decay processes, turned into a generalized native loss of hydraulic conductivity (PLC). Such a less strict stomatal strategy and the high native PLC values have been commonly reported by other authors [75]. In fact, this strategy is linked to a renovation of the xylem, since all hydraulic conductivity is recovered by new earlywood vessels that are formed in the next growing season [76]. Interestingly, the increase in PLC was related to an increase in aridity only in sweet chestnut, with low PLC in *control* and maximum PLC in *drytemp*. Our results also agree with global studies that reported wider safety margins in conifers than in angiosperms [17], operating closer to their critical water potentials that may cause catastrophic xylem dysfunctions. The threshold of the critical water potential is typically reported as the water potential that causes 50% of loss of conductivity in conifers, whereas new studies highlighted that the critical water potential in angiosperms is closer to the 80% as a consequence of the narrower safety margins (P88) [75]. We hypothesize that oak seedlings may have surpassed the P88 value in all treatments and, as a consequence of its looser stomatal control, may have

experienced similar PLC values in all treatments, leading to decay processes as visually observed. This was also observed in the *control* treatment: as a consequence of the harsher conditions in 2015, some oak seedlings reached low water potentials (-3 MPa, Figure S4). The PLC values reported for oak in relation to the observed water potentials are in agreement with previous studies [77].

According to the Hagen-Poiseuille law [8], the maximum hydraulic conductivity is directly related to the conduit size. Therefore, the two ring-porous species recorded higher values of K_{S-MAX} than Scots pine since their xylem architecture is better designed for an efficient water transport. Similar results were found in other studies comparing angiosperms and conifers [51]. However, with increasing aridity, sweet chestnut, Scots pine and to a lesser degree oak displayed lower K_{S-MAX} suggesting an acclimation of the xylem. This acclimation can be explained by the xylem plasticity and by the height differences among treatments, in the case of sweet chestnut and oak. Recent studies highlighted the importance of the widening trend of the xylem conduits towards the stem base on hydraulic conductivity measurements [78]. Such a universal configuration of the xylem architecture [79,80] predicts that conduits should widen from stem/branch tip to the base, and ultimately result in an increase of the hydraulic conductivity. On the other hand, xylem-conduit adjustments in number and size of the conduits have been reported for angiosperms [81] and conifers [82] in order to avoid possible damages at the expenses of modifying the potential conductivity. This may be particularly important for Scots pine in our study since no differences in height were reported among treatments. Therefore, further analyses in the wood anatomy of the selected species should be done to confirm our hypotheses. Potential artifacts due to seedling harvest and handling have been discarded, since the entire sapling was removed from the soil, kept moist, cut under water and measured the same day.

In order to indirectly assess the possibility of conduit refilling, the minimum and the latest measured water potentials experienced by the seedlings were related to the native loss of conductivity. In the case of sweet chestnut and Scots pine, the minimum stem water potential better explained the native amount of embolisms, which points to a reduced capability to recover the hydraulic conductivity. In Scots pine, low PLC values suggested that the hydraulic safety margins were far from the point causing a hydraulic dysfunction. In the case of oak, the models did not statistically differ. However, as previously stated, oak showed decay processes (visual observations), which could have masked the results. In the case of the ring-porous species, this finding would be supported by the annual regeneration of the xylem as described in [76]. Although conduit refilling has been proved in conifers [83] and might be a plausible explanation for our low PLC values, little is known about the required conditions and how this process occurs under negative xylem tension [84]. Our result may indirectly point to a reduced possibility of the conduits refilling during the growing season after experiencing the lowest water potential. However, we cannot discard that such a refilling could occur during non-vegetation periods as reported by [83]. Further studies measuring the evolution of the percentage of embolism during the growing season, would be helpful to understand such processes.

5. Conclusions

The contrasting strategies displayed by the studied species translated into divergent responses to drought and/or warming. The relatively isohydric strategy of Scots pine preserved its hydraulic conductivity but may have reduced tree height growth during the second year. However, Scots pine seemed to be able to mobilize reserves even with closed stomata. In contrast, the relatively anisohydric species sweet chestnut and oak lost most of their hydraulic conductivity particularly during the most severe dry spell of 2015, and their growth was also adjusted to the climatic conditions. The decay processes observed among oak seedlings could indicate that the species-specific threshold of stress was surpassed. Therefore, the intensity and length of future climate change-induced dry spells will be decisive for the performance and survival of the seedlings. More studies are needed in order to disentangle whether the capacity and the conditions of the gas-filled xylem conduits in saplings differ from the adult trees.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4907/8/11/450/s1. Figure S1: Climatic treatments and density arrangement and dimensions, Figure S2. Difference in maximum, mean and minimum temperatures between the control and each treatment for the years 2014 and 2015, Table S1: Dimensions of the 3-year old internode segments as mean value \pm standard deviation ($n = 12$) per treatment, Table S2. Influence of the density of plantation in the studied variables.

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