

Xylem anatomical and growth responses of the dwarf shrub *Vaccinium myrtillus* to experimental CO₂ enrichment and soil warming at treeline

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Abstract

Plant growth responses to environmental changes may be linked to xylem anatomical adjustments. The study of such links is essential for improving our understanding of plant functioning under global change. We investigated the xylem anatomy and above-ground growth of the dwarf shrub *Vaccinium myrtillus* in the understorey of *Larix decidua* and *Pinus uncinata* at the Swiss treeline after 9 years of free-air CO₂ enrichment (+200 ppm) and 6 years of soil warming (+4 °C). We aimed to determine the responses of xylem anatomical traits and growth to these treatments, and to analyse xylem anatomy–growth relationships. We quantified anatomical characteristics of vessels and ray parenchyma and measured xylem ring width (RW), above-ground biomass and shoot elongation as growth parameters. Our results showed strong positive correlations between theoretical hydraulic conductivity (Kh) and shoot increment length or total biomass across all treatments. However, while soil warming stimulated shoot elongation and RW, it reduced vessel size (Dh) by 14%. Elevated CO₂ had smaller effects than soil warming: it increased Dh (5%) in the last experimental years and only influenced growth by increasing basal stem size. The abundance of ray parenchyma, representing storage capacity, did not change under any treatment. Our results demonstrate a link between growth and stem Kh in *V. myrtillus*, but its growth responses to warming were not explained by the observed xylem anatomical changes. Smaller Dh under warming may increase resistance to freezing events frequently occurring at treeline and suggests that hydraulic efficiency is not limiting for *V. myrtillus* growing on moist soils at treeline. Our findings suggest that future higher atmospheric CO₂ concentrations will have smaller effects on *V. myrtillus* growth and functioning than rising temperatures at high elevations; further, growth stimulation of this species under future warmer conditions may not be synchronized with xylem adjustments favouring hydraulic efficiency.

Keywords: global change, hydraulic conductivity, hydraulically weighted mean vessel diameter (Dh), ray parenchyma, xylem ring

1. Introduction

Global atmospheric CO₂ concentration and air temperature are predicted to continue to increase in the next decades. Climate models forecast a terrestrial air temperature increase of 1.2–4.8 °C by the end of the 21st century (IPCC 2014), especially in high-elevation and high-latitude ecosystems (Collins et al. 2013; Rangwala et al. 2013; Mountain Research Initiative EDW Working Group 2015). These ecosystems are particularly sensitive to global warming because they experience low temperatures during the growing season, with frequent freezing events, and long snow cover durations. In addition, high-elevation ecosystems may be particularly responsive to increasing CO₂ concentrations because of the lower CO₂ partial pressure associated with reduced atmospheric pressure at higher altitudes (Körner 2003). Indeed, some studies at high-elevation sites have shown evidence of growth stimulation of some plant species under CO₂ enrichment (Dawes et al. 2011a, b, 2015), although other studies have not found such positive growth responses (Körner et al. 1997; Hättenschwiler and Körner 1998; Inauen et al. 2012). Nevertheless, while numerous studies have focused on plant responses to warming in alpine and Arctic ecosystems (e.g., Kudo and Suzuki 2003; Klanderud 2008; Campioli et al. 2013), CO₂ enrichment experiments in these areas are scarce (Tissue and Oechel 1987; Gwynn-Jones et al. 1997; Körner et al. 1997; Inauen et al. 2012; Dawes et al. 2015).

Dwarf shrubs cover extensive areas in many alpine and Arctic ecosystems. At the alpine treeline, warmer temperatures and the abandonment of extensive grazing and agricultural practices have led to a re-colonization of pastures by shrub species (Rundqvist et al. 2011; Ropars and Bordreau 2012). This shrub expansion can cause drastic changes in the ecosystem C balance, with potential feedbacks to climate warming (Myers-Smith et al. 2011; D'Odorico et al. 2013). Therefore, an in-depth understanding of the ecology and physiology of alpine shrubs and how these species respond to global change is essential for predicting future community and ecosystem changes.

Plant physiological responses and structural adjustments are key to coping with short and long-term environmental variability. One of the most important plant physiological processes is axial water transport which, in angiosperms, is carried out by a network of dead lignified tubular cells, i.e. vessels. Plants optimize water transport efficiency and safety by regulating the structural characteristics of the water conducting system (Hacke and Sperry 2001). According to the Hagen-Poiseuille law, hydraulic

efficiency increases with vessel lumen diameter to the fourth power (Tyree and Ewers 1991). However, the wider vessels are the higher their vulnerability to water transport failures due to frost-induced cavitation in cold environments (Hacke and Sperry 2001). This phenomenon takes place during freeze-thaw events when air bubbles are gassed out as xylem sap freezes, interrupting water transport in the vessels. In this situation, a strong connectivity or grouping among vessels might provide alternative pathways for water to bypass cavitated vessels (Tyree et al. 1994, von Arx et al. 2013). Nevertheless, such anatomical features might enhance the risk of cavitation spreading from one vessel to the next by aspiration of air through the pit pores (Sperry and Tyree 1988; Loepfe et al. 2007). Other important xylem functions, i.e. storage and radial transport of water, nutrients and carbon, are performed by ray parenchyma tissue (Plavcová and Jansen 2015). In fact, ray and axial parenchyma represent the greatest stores of non-structural carbohydrates (NSC) in wood. Therefore, the amount and size of ray parenchyma tissue might reflect variations in the amount of NSC in the plant (von Arx et al. 2012; Plavcová et al. 2016; but see von Arx et al. 2017). Despite the important physiological function of ray parenchyma, this tissue has been underrepresented in studies on the relationship between wood anatomy and the environment (but see Lev-Yadun 1998; Olano et al. 2013; Fonti et al. 2015; von Arx et al. 2015; Prendin et al. 2018). Quantitative assessments of the structures responsible for all these functions may provide knowledge on plant conductive and storage capacity, as well as facilitating retrospective reconstruction of plant structural and functional responses to environmental variability (Fonti et al. 2010; Fonti and Jansen 2012; Schweingruber et al. 2013; von Arx et al. 2016).

Most of the existing warming and CO₂ enrichment studies on xylem anatomy are based on trees (mostly tree seedlings or saplings; but see Gorsuch and Oberbauer 2002 and Rico et al. 2013 for studies on other growth forms). Some of these studies showed that experimental warming led to larger water conducting cells (Maherali and DeLucia 2000; Gorsuch and Oberbauer 2002; Petit et al. 2011; Balducci et al. 2016, McCulloh et al. 2016). However, other studies showed the opposite response (Thomas et al. 2004; Kilpeläinen et al. 2007). CO₂ enrichment was found to induce wider vessel lumina (Atkinson and Taylor 1996; Kilpeläinen et al. 2007; Kostianinen et al. 2014), while no response (Maherali and DeLucia 2000; Rico et al. 2013; Watanabe et al. 2016) or even a negative response (Overdieck et al. 2007) was observed in other experiments. A number of CO₂ enrichment experiments have observed greater contents

of NSC in plant tissues (e.g. Asshoff and Hättenschwiler 2005; Handa et al. 2005), but it remains unclear whether the enhanced availability in carbohydrates also induces a change in wood anatomy (e.g. in ray parenchyma) allowing to store more carbohydrates. Many questions clearly remain unanswered regarding plant functional adaptations to warming and CO₂ enrichment, especially for tundra shrubs. Furthermore, the scarcity of studies combining both CO₂ and warming treatments highlights the need to explore interactive effects of these two factors on plant growth and functioning (but see Maherali and DeLucia 2000; Ziche and Overdieck 2004; Kilpeläinen et al. 2007; Dieleman et al. 2012).

Here, we studied xylem anatomical responses of the dwarf shrub *Vaccinium myrtillus* to a combined 9-year CO₂ enrichment and 6-year soil warming experiment at treeline in the Swiss Alps. Previous studies within this experiment demonstrated that *V. myrtillus* benefited from both treatments, with increased shoot increment length, above-ground biomass and xylem growth-ring widths (RW) (Dawes et al. 2011a, 2015; Anadon-Rosell et al. 2014). In this study, we aimed to investigate adjustments at the xylem anatomical level and determine whether they are related to growth responses to these treatments. Such a link is expected because xylem anatomical structures define key functions for plant survival and performance (Hacke and Sperry 2001; Plavcová and Jansen 2015), and small changes in xylem anatomical characteristics (e.g. in the lumen diameter of vessels) can considerably improve xylem functioning, yielding better plant performance and a potential advantage over competitors. Specifically, we hypothesized that (i) growth and xylem anatomical parameters would be positively related because a higher hydraulic efficiency could promote growth, and that (ii) increased CO₂ concentrations and warmer soil temperatures would enhance growth, associated with (iii) improved hydraulic efficiency and increased storage capacity.

2. Materials and Methods

2.1 Study site and experimental design

This study was conducted at the end of a long-term CO₂-enrichment (2001–2009) and soil warming (2007–2012) experiment (Dawes et al. 2015). The experimental site was located at Stillberg, Davos, in the Central Alps, Switzerland (9° 52' E, 46° 46' N). The site lies within a 5-ha long-term afforestation area where tree seedlings were planted into the intact dwarf shrub community in 1975 (Barbeito et al. 2012).

The CO₂ enrichment and soil warming experiment was located on a NE-exposed 25–30° slope at 2180 m a.s.l., slightly above the present treeline (Barbeito et al. 2012) and covered an area of 2500 m². From 1975 to 2012, the mean air temperature and mean precipitation during the main growing season months (June–August) were 9.2 °C and 444 mm, respectively (data from a nearby WSL climate station located at 2090 m a.s.l.). Detailed climate information for the study period 2001–2012 is provided in Table A1. Soil types are sandy Ranker and Podzols (Lithic Cryumbrepts and Typic Cryorthods), derived from siliceous paragneis parent material, and have a 5–20-cm-thick organic layer (Hagedorn et al. 2010).

In 2001, a free-air CO₂ enrichment (FACE) experiment was started (Hättenschwiler et al. 2002). A total of 40 hexagonal 1.1 m² plots were created, 20 with one *Pinus mugo* ssp. *uncinata* (DC.) Domin individual in the centre and 20 with one *Larix decidua* Mill. individual in the centre. The trees were sparsely distributed, not forming a closed canopy, and therefore each plot consisted of a single tree surrounded by a dense cover of understorey vegetation dominated by the dwarf shrub species *Vaccinium myrtillus* L., *Vaccinium gaultherioides* Bigelow (group *V. uliginosum* agg.) and *Empetrum nigrum* L. subsp. *hermaphroditum* (Hagerup) Böcher. The plots were assigned to 10 groups of 4 neighbouring plots, half of which were randomly assigned to an elevated CO₂ treatment (+ 200 ppm) and half of which did not receive additional CO₂. The system released pure CO₂ gas through laser-punched drip irrigation tubes hung vertically around a hexagonal frame surrounding each plot. Enrichment was supplied during daytime hours throughout the snow-free seasons from 2001 to 2009. For more details on the FACE setup and performance see Hättenschwiler et al. (2002) and Dawes et al. (2013).

In spring 2007, a soil warming treatment was added to the experiment, and it was applied during each snow-free season until August 2012. Within each of the 10 CO₂ treatment groups, one plot of each tree species was randomly assigned to a soil warming treatment, yielding a balanced split-split plot design with a replication of five individual plots for each combination of CO₂ concentration, soil warming and plot tree species. The soil warming treatment was applied using 420-W heating cables laid on the ground surface with a distance of 5 cm between neighbouring cables. Each year, the heating was turned on directly after snowmelt and turned off before the site was covered with snow during wintertime (see Hagedorn et al. 2010 for more details). Soil warming increased the growing season mean soil temperature at 5 cm depth by 3.1 to 4.4 °C over the six seasons of heating (Dawes et al. 2015). Air temperature within

the dwarf shrub canopy was increased by 0.9 °C at 20 cm above ground (Hagedorn et al. 2010). The warming treatment had a slight drying effect on the soil organic layer during the first three years of treatment but the soil matric water potential at 5 cm depth was always above -300 hPa in all plots, indicating high soil moisture throughout all the study years (Dawes et al. 2014).

2.2 Plant sampling and sample preparation

During early August 2012, we harvested five *V. myrtillus* ramets per plot, clipping them at ground level. We made cross-sections of 20 µm thickness from the basal 1.5 cm of each ramet using a WSL-lab-microtome (Gärtner, Lucchinetti and Schweingruber 2015) and stained the sections with a mixture of safranin and astrablue. The sections were dehydrated for preservation by rinsing with ethanol solutions of increasing concentration (75%, 96% and 100%). Then they were immersed in xylol, imbedded in Canada-Balsam and dried at 60 °C for 24 hours (Gärtner and Schweingruber 2013). We also measured leaf, new shoot and total above-ground biomass of each ramet after drying them at 60 °C for 48h. In addition, we measured new shoot increment length (i.e. average of the length of three shoots formed in 2012). More details on plant harvesting, above-ground biomass and shoot increment length measurements, and sample preparation are given in Anadon-Rosell et al. (2014). In this study, we carried out all measurements on the oldest available ramet per plot, ranging from 5 to 21 years old. For the quantification of xylem anatomical parameters we followed the protocol detailed in von Arx et al. (2016). In short, we photographed the sections at ×40 magnification through a microscope with a digital camera (Canon EOS 650D mounted on an Olympus BX41 microscope featuring distortion-free lenses), took several overlapping photographs per cross-section, and stitched them with the software PTGui (New House Internet Services BV, Rotterdam, NL) to obtain a high-resolution image (0.94 pixels/µm) of each full cross-section (Fig. 1). We used the software ROXAS v2.1 (von Arx et al. 2013; Wegner et al. 2013) to quantify the vessel lumen area and spatial arrangement, as well as ring area and width. Only vessel lumina > 50 µm² in area were considered to avoid confounding the very smallest vessels with fibre cells. The borders of the xylem growth rings were drawn manually, accounting for missing rings based on a cross-dating procedure previously performed on the same samples (Anadon-Rosell et al. 2014).

2.3 Quantification of anatomical traits

For each annual ring, we quantified xylem anatomical parameters related to hydraulic efficiency, spatial vessel arrangement, storage and radial transport of water, nutrients and carbon, and growth (Table 1). As parameters related to hydraulic efficiency, we considered cross-sectional vessel lumen area at the 95th percentile (CA 95%, i.e. the widest and thus most conductive vessels; Petrucco et al. 2017), hydraulically weighted mean vessel diameter (D_h , calculated as $\Sigma d^5 / \Sigma d^4$, where d is the cross-sectional mean lumen diameter of each vessel; Kolb and Sperry 1999), theoretical hydraulic conductivity (K_h) based on Hagen-Poiseuille's law (Tyree and Zimmermann 2002) and theoretical xylem-specific hydraulic conductivity (K_s) calculated as $K_h / \text{ring area}$. The spatial vessel arrangement was expressed as vessel density (CD), vessel grouping index (V_G), mean group size of grouped cells (V_M) and vessel solitary fraction (V_S) (von Arx et al. 2013). Storage and radial transport capacity were assessed by ray density and ray seriality, i.e. the number of parallel cell lines per ray (Carlquist 2001). We considered biseriate and multiseriate rays relatively more important for storage than uniseriate rays because the need to provide sufficient connectivity between vessels and rays (Fonti et al. 2015) is already fulfilled by uniseriate rays. Both ray metrics were assessed for the outmost ring (formed in 2012). Ray density was quantified as the number of rays per millimetre of circumference in the stem cross-section. Ray seriality was obtained by counting the number of parallel cell lines forming each ray. Finally, we used the number of vessels (CNo), mean ring width (RW) and ring area (RA), as well as above-ground biomass and new shoot increment length measurements as parameters related to growth.

2.4 Statistical analysis

We assessed the relationship among the xylem anatomical parameters and checked the coherence within groups of parameters with similar functions using Pearson's correlations. To evaluate effects of the treatments on the xylem anatomical parameters, we used linear mixed-effects models fitted with the restricted maximum likelihood estimation method (REML). We fitted two separate models for the two key periods of the experiment, one for the CO₂-treatment period (2001-2009) and one for the warming-treatment period (2007-2012). The CO₂-treatment period model included CO₂, plot tree species (larch or pine), study year and their interactions as fixed effects. The warming-treatment period model additionally

included warming and the interactions between all factors as fixed effects (thus, also accounting for the CO₂ and soil warming interactive effects). Both models included cambial age as a covariate when it was significant to account for ontogenetic effects on response variables. For the analysis of the treatment effects on ray density, we included CO₂, warming, plot tree species and their interactions as fixed effects. For all models, we tested 3- and 4-way interactions with likelihood ratio tests and excluded these terms when they did not contribute significantly to the model fit. The random effects structure reflected the hierarchy of the split-split plot design, with plot (i.e. one ramet) nested within soil warming treatment group, nested within CO₂ treatment group. We applied a residual auto-correlation structure, corAR1 (Pinheiro et al. 2016), to account for violation of independence of residuals from measurements made on the same ramet in different years. The year 2012 was excluded from the analyses of xylem ring widths, ring area and number of vessels, as ring growth was not completely finished at the sampling time. For all analyses, we visually checked the assumptions of normality and homoscedasticity of residuals and we log- or square root-transformed response variables when necessary to meet these assumptions. In addition, we used the varIdent structure (Zuur et al. 2009) when the residual variance differed between levels of treatments.

We tested Pearson's correlations between the xylem anatomical parameters and biomass and new shoot increment length measurements. We further explored the relationship between Kh and above-ground growth to determine the contribution of xylem rings to the hydraulic conductivity of *V. myrtillus*, i.e. the sapwood area. For this, we calculated the relative ring position from bark (outermost rings) to pith (innermost rings) by dividing the absolute bark-to-pith position of each ring by the total number of rings in that sample; we then grouped relative ring positions into 0.1-unit classes. We assessed the variation between Kh and above-ground growth correlations additively (adding Kh of one more ring each time) from the outermost rings to the innermost rings. Finally, we analysed the effects of CO₂, warming and plot tree species on ray seriality with the non-parametric Kolmogorov-Smirnov test.

We considered effects significant at $P < 0.05$ and marginally significant at $0.05 \leq P < 0.1$ to account for the relatively low replication of the study. We performed all the analyses with R 3.1.2 (R Core Team, 2015). For linear mixed effects models we used the nlme package (Pinheiro et al. 2016).

3. Results

3.1 Age trends and inter-annual variability of anatomical parameters

The xylem anatomical traits showed, with a few exceptions, very strong age trends, and traits related to hydraulic efficiency and growth were also influenced by climate conditions in specific calendar years (Table 2). However, the relatively small number of study years did not allow a more detailed analysis of potential effects of inter-annual climate variability.

3.2 Correlations among anatomical parameters and growth-anatomy links

Results of the correlation analyses generally supported our grouping of anatomical parameters with similar functions (Table 1, Fig. 2). Amongst parameters related to hydraulic efficiency, Dh and CA95% were strongly positively correlated ($r = 0.99$). However, Kh was slightly better correlated with growth-related parameters ($r \geq 0.65$) than with hydraulic-efficiency parameters ($|r| \leq 0.62$). The link between Kh and Ks was quite weak ($r = 0.43$), showing a certain independence between per-area (Ks) and overall (Kh) conductivity. The correlations between parameters describing spatial vessel arrangement ranged between $|r| = 0.45$ and 0.96 , with CD showing a comparably weak association with the three grouping traits ($|r| \leq 0.52$). Growth-related parameters (CNo, RW, RA) were correlated, with $r \geq 0.74$, and Kh was strongly correlated with them, especially with RA and CNo ($r \geq 0.90$). Across the functionally-related groups defined, the hydraulic-efficiency parameters were positively correlated with vessel grouping (V_G , $r \geq 0.30$) and negatively linked to the proportion of solitary vessels (V_S , $r \leq -0.62$). To simplify the study of such a large number of parameters, we selected one parameter (i.e. the most widely used in xylem anatomy studies) for each of the functionally-related groups aforementioned. Thus, hereafter we focus on Dh, V_G and RW, as well as on Kh due to its functional importance and its strong link to growth, and CD for its lack of correlation with the rest of the parameters.

We did not find correlations between the majority of the xylem anatomical parameters and above-ground biomass or new shoot increment length, except for stem Kh (i.e. theoretical potential hydraulic conductivity for the whole cross-section), which was significantly correlated with all the biomass parameters and with new shoot increment length (Fig. 3a, Table A2). Moreover, new shoot increment

length was positively correlated with RW. All the biomass parameters and new shoot increment length were positively correlated with stem diameter (Table A2).

The correlation coefficient (r) between Kh and biomass increased with an increasing number of rings considered, from the outermost rings to the innermost rings (Fig. 3b, upper panel). However, the curve of correlation coefficients levelled off when considering the outmost c. 70% of all rings, suggesting that rings closer to the pith hardly contributed to the whole stem conductivity and conferred less support to above-ground biomass than outermost rings. Correlation coefficients calculated for RA vs. above-ground biomass provided similar results (data not shown), confirming that rings contributed to water transport proportionally to their area. Correlations between Kh and new shoot increment length were more variable (Fig. 3b, lower panel), and the highest correlations were found for the smaller class of relative ring position (up to 0.1), probably indicating that the elongation of new shoots mostly relies on the outermost part of the xylem.

3.3 Xylem anatomical and growth responses to CO₂ enrichment

The responses to CO₂ enrichment of the considered functional xylem anatomical traits were small, delayed, or occurring in interaction with other factors. Within the group of hydraulic efficiency traits, Dh responded positively to CO₂ enrichment during the last few years of the CO₂-treatment period (2006–2009), especially for *V. myrtillus* growing under pine (significant CO₂ × tree × year interaction), and it was marginally significantly enhanced by CO₂ enrichment during the warming-treatment period (2007–2012; Table 2, Fig. 4; and see Dh raw means for the different treatment periods in Table A3). In contrast, Kh did not respond to CO₂ enrichment in any of the treatment periods. There was, though, a marginally significant increase in V_G to CO₂ enrichment during the warming-treatment period (Tables 2, 3). The analyses of storage and radial transport capacity through ray traits showed that ray absolute density was greater under elevated CO₂ (207.8 ± 10.3 rays/cross-section) than under ambient CO₂ (170.4 ± 11.3 rays/cross-section) ($F_{1,8} = 7.10$, $P = 0.029$). However, these differences were linked to the perimeter of the stem, which was also larger under elevated CO₂ (7.37 ± 0.30 mm vs. 6.13 ± 0.42 mm; $F_{1,8} = 10.08$, $P = 0.013$). Thus, standardised ray density did not differ between treatments ($F_{1,8} = 0.07$, $P = 0.805$). Ray seriality was also unaffected by CO₂ enrichment (Fig. 5). RW did not differ significantly between CO₂

treatments for either of the treatment periods (Tables 2, 3, Fig. A2). The CO₂ treatment did not affect new shoot increment length, nor leaf, new shoot or total above-ground biomass (all measured in 2012), which was expected because the treatment ended in 2009, three years before sampling. Since the CO₂ treatment did not contribute to the model fits (tested with likelihood ratio methods), it was excluded from the models for these variables.

3.4 Xylem anatomical and growth responses to soil warming

Soil warming generally had larger effects than CO₂ enrichment on the anatomical traits of *V. myrtillus*. Among the traits related to hydraulic efficiency, Dh decreased with warming, especially in the first years of the treatment (2007-2009), when Dh of ramets in warmed plots was, on average, 14% smaller than that of ramets in unwarmed plots (significant warming and warming × year effects) (Table 2, Fig. 4). Kh showed a significant warming × year interaction, with an initial decrease followed by a slight non-significant increase compared to in unwarmed conditions. V_G decreased in the first treatment years (significant warming × year interaction; Tables 2, 3). Regarding storage and radial transport traits (i.e. ray parenchyma parameters), warming did not influence absolute ($F_{1,8} = 0.12$, $P = 0.743$) or per unit distance ($F_{1,8} = 0.12$, $P = 0.740$) ray density, but it reduced the proportion of biseriate rays (3.8% vs. 4.7% of the total rays) ($D = 0.45$, $P = 0.035$; Fig. 5). RW decreased in the first treatment years and showed a relative increase in the last treatment years under warming compared to unwarmed conditions (significant warming × year interaction; Table 2, Fig. A2). At the end of the experiment (2012), there was a significant effect of warming on *V. myrtillus* new shoot increment length, irrespective of plot tree species and CO₂ treatment. Shoots were 22% longer in warmed plots than in unwarmed plots ($F_{1,9} = 5.53$, $P = 0.043$, Fig. A1). In contrast, we did not find significant differences in leaf ($F_{1,9} = 1.69$, $P = 0.226$), new shoot ($F_{1,9} = 2.02$, $P = 0.189$) or total above-ground biomass ($F_{1,9} = 1.59$, $P = 0.239$) between warming treatments. Finally, ramets were slightly older in unwarmed than warmed plots ($F_{1,8} = 5.93$, $P = 0.041$). We did not find any significant warming × CO₂ interactions for the xylem anatomical traits (Table 2) or for growth.

3.5 Influence of tree species on *V. myrtillus* xylem anatomical traits and growth

The tree species present in the plot played an important role in *V. myrtillus* anatomy and its response to the treatments. Dh was 7% larger in *V. myrtillus* ramets growing under pine than under larch when pooled across CO₂ treatments and years. We found a similar pattern for the warming-treatment years, with a 9% (Dh) difference (Table 2, Fig. 4). In contrast, RW was marginally significantly reduced under pine compared to larch (Tables 2, 3, Fig. A2). Plot tree species did not have a significant effect on absolute ray density ($F_{1,15} = 1.51$, $P = 0.237$), ray density per unit distance ($F_{1,15} = 1.72$, $P = 0.209$) or ray seriality ($P > 0.832$), and it did not affect spatial vessel arrangement parameters (Tables 2, 3). Ramets were slightly older under pine than under larch ($F_{1,16} = 5.11$, $P = 0.038$). However, overstorey tree species identity did not have an effect on *V. myrtillus* total above-ground biomass ($F_{1,17} = 0.14$, $P = 0.712$), leaf biomass ($F_{1,18} = 0.04$, $P = 0.845$), new shoot biomass ($F_{1,18} = 0.02$, $P = 0.895$) or new shoot increment length ($F_{1,18} = 0.26$, $P = 0.618$; but marginally significant warming \times tree species interaction, $F_{1,18} = 3.93$, $P = 0.063$).

4. Discussion

Our results showed strong links between *V. myrtillus* xylem anatomical traits and growth across treatments. We found plastic responses in xylem anatomy and growth to warming and, to a lesser extent, to CO₂ enrichment. Warming induced a decrease in vessel size, which may improve safety at our treeline study site where freezing events are frequent. In addition, the tree species present in the plot had a significant influence on *V. myrtillus* xylem anatomical traits and their response to the treatments.

4.1 Links between growth and xylem anatomy

As we hypothesized, our results showed a link between xylem anatomical features and plant growth, independent of the treatments. This was apparent in the strong correlation between plant-level conductance (Kh) and aboveground growth. Kh was the only hydraulic-efficiency parameter to show this relationship, likely because above-ground biomass is also a cumulative trait. The consideration of an increasing number of growth rings in the calculation of Kh significantly improved its relationship with above-ground biomass, probably because it better reflected the true number of conducting sapwood rings. However, the relationship between new shoot increment length and Kh did not change when more than the outermost rings were included, indicating that the largest contribution of water for new shoots comes

from the outermost sapwood rings only. This would imply a more effective strategy for water transport because crossing ring boundaries with narrower latewood vessels increases pathway length resistance (Petit et al. 2016). The link between xylem anatomy and growth was also evident in the strong correlation between Kh and RW, independent of the treatments (Fig. 2).

4.2 CO₂ enrichment has little effect on anatomy, growth and storage

We found a small and lagged positive response of anatomical features to the 9-year CO₂ enrichment treatment. RW did not respond to the CO₂ enrichment, but we found an increase in basal stem perimeter in CO₂-elevated plots, indicative of growth stimulation. This is in agreement with growth increases under CO₂ enrichment reported for this and other treeline plant species (e.g. Dawes et al. 2011a, 2015).

Growth stimulation at elevated CO₂ concentrations may be associated with wood anatomical adjustments to support the increasing demand of water and nutrients and to allow a greater storage capacity for carbohydrates. Indeed, some studies have found positive effects of CO₂ enrichment on vessel size. Atkinson and Taylor (1996) reported an increase in vessel size of *Quercus robur* seedlings with higher CO₂ concentrations, and an experiment on aspen and birch seedlings found that radial growth and vessel size of aspen were increased under elevated CO₂ concentrations throughout the experimental years (Kostiainen et al. 2014). Conversely, an improved CO₂ supply may also reduce water loss during photosynthesis (Bunce 2004, Leuzinger and Körner 2007), which may allow the plants to invest more in hydraulic safety by forming narrower vessels. In our study, CO₂ enrichment had both small positive effects on growth and delayed positive effects on hydraulic efficiency, indicating slight cumulative effects of elevated CO₂ concentrations on *V. myrtillus* performance. However, based on a previous study by Dawes et al. (2011a), where the authors found increased shoot increment lengths in *V. myrtillus* under CO₂ enrichment, we expected to find a clearer growth stimulation in our study ramets, reflected on parameters such as RW. In fact, Anadon-Rosell et al. (2014) showed a strong correlation between shoot elongation and RW in this species. We suggest that the use of different study ramets and a smaller dataset, which comprises higher inter-ramet variability than larger datasets, might partly explain the different results obtained. In any case, our results point to a low responsiveness of *V. myrtillus* growth and xylem anatomy to CO₂ enrichment.

The lack of CO₂ effects on the ray parenchyma might be because cross-sections were made at the stem base. Belowground organs (i.e. roots and rhizomes) store large amounts of carbohydrates, especially in clonal tundra plant species (Billings and Mooney 1968), and ray responses in belowground tissues of *V. myrtillus* may have occurred. In fact, Handa et al. (2008) showed increased starch contents in tree roots under elevated CO₂ at the same experimental site. Nevertheless, some studies have shown that the amount of ray parenchyma can be unresponsive to environmental conditions (Overdieck et al. 2007; Fonti et al. 2015) or respond with a lag of several years (von Arx et al. 2017). This could be related to the fact that, once initiated, rays grow and extend to maintain the connection with the cambium and phloem (Spicer 2014). Overall, our results show that CO₂ enrichment did not promote above-ground non-structural carbohydrate storage capacity in *V. myrtillus*, suggesting a low or slow plasticity of ray parenchyma tissue to environmental conditions and implying that enhanced accumulation of NSC in plant tissues observed in our experiment (Asshoff and Hättenschwiler 2005) did not lead to wood anatomical adjustments that enhanced carbohydrate storage in stems.

4.3 Warming effects on hydraulic efficiency and safety

Xylem anatomical traits of *V. myrtillus* showed high plasticity in response to the warming treatment but, contrary to our hypothesis, vessel size decreased under warming. This response also contrasts with results from previous studies in cold regions of positive warming effects on the vessel size of woody species. In a study near Toolik Lake, Alaska, Gorsuch and Oberbauer (2002) showed that the shrub *Salix pulchra* had larger vessel diameters and densities under an increase of 5 °C inside growth chambers. Other warming experiments on tree species have also reported increases in conduit sizes with warming (Maherali and DeLucia 2000; Petit et al. 2011; McCulloh et al. 2016). In contrast, like in our study, Kilpeläinen et al. (2007) found narrower lumina in earlywood tracheids of Scots Pine (*Pinus sylvestris*) under warming. Thomas et al. (2004) reported similar effects of temperature on the vessels of *Eucalyptus camaldulensis* in growth chambers, where plants were grown at 20, 25 and 37 °C; and Prendin et al. (2018) also found narrower tracheids in the roots of *Larix decidua* under warming at our same experimental site. A plausible explanation for these results is that higher temperatures caused faster cell differentiation rates through an increase in auxin levels (Franklin et al. 2011), leading to a shortened cell expansion phase, which resulted

in narrower vessels (Aloni and Zimmermann 1983). Moreover, all these authors related their findings to a decreased viscosity of water with warming (Roderick and Berry 2001) and suggested that equal amounts of water could be transported by smaller vessel lumina. In a high-elevation site such as Stillberg, where early-season frosts are frequent (Rixen et al. 2012), smaller vessel sizes may be advantageous, as this reduces the risk of cavitation induced by freeze-thaw events (Tyree and Sperry 1989). The plastic behaviour of *V. myrtillus* xylem may be particularly relevant because this species has a relatively low freezing resistance (Martin et al. 2010; Wheeler et al. 2014), and a potentially faster phenological development under warming may enhance the risk of freezing damage in leaf tissue (Martin et al. 2010). Thus, the negative effects of the soil warming treatment on vessel size and xylem-specific hydraulic conductivity in *V. myrtillus* might have improved hydraulic safety without compromising water transport. At our experimental site, snow melted rapidly from warmed plots after an early summer snow event in the first warming year (2007) and left plants without an insulating snow cover when a freezing event occurred (Rixen et al. 2012), which might have also induced the observed formation of smaller vessels. In line with this, the decrease in vessel grouping with warming in the first treatment years may also reflect an adjustment to cope with freeze-thaw cycles, as freezing-induced cavitation would spread with more difficulty through solitary than through grouped vessels. The relative increase in vessel size after the first three years of warming could reflect a progressive recovery from the 2007 frost or the need to supply water to an increasing biomass (the warming-induced increase in above-ground growth, reflected in RW and new shoot increment length stimulation, did not stop after three years of warming). The decrease in biseriate and multiseriate rays with warming (although only significant for biseriate rays) may indicate that higher temperatures promoted above-ground growth at the expense of investments into storage capacity (von Arx et al. 2012).

Responses to CO₂ enrichment and soil warming were largely independent, as indicated by the lack of significant CO₂ × warming interactions for the study parameters. In a review on plant and soil responses to experimental combinations of CO₂ enrichment and warming, Dieleman et al. (2012) showed that additive and synergistic effects of the two treatments have rarely been reported, whereas antagonistic effects are more common. This is clearly supported by our findings, as the effects of the two treatments on *V. myrtillus* hydraulics were opposing. Nevertheless, the lack of interactive CO₂ × warming effects on

xylem anatomical traits does not exclude the possibility that the combination of these treatments influenced the dynamics of wood formation (i.e. cell production). Compensatory effects between the rate and duration of cell differentiation processes could have led to the lack treatment effects on xylem anatomical traits (Balducci et al. 2016).

The fact that growth responses to warming in this study only partly support previous findings, where all biomass parameters significantly increased under warming (Anadon-Rosell et al. 2014), could be explained by the use of a subset of *V. myrtillus* plants in this study, which includes a single ramet per plot, compared to the use of five or more ramets per plot in the previous study.

4.4 Importance of overstorey tree species on V. myrtillus xylem anatomy and growth

The xylem anatomy of *V. myrtillus* showed clear differences between plots with different tree species, regardless of the treatment applied, and its response to the treatments also differed depending on the tree species. Although the wider vessels and the larger response to CO₂ under pine than under larch could be due to ontogenetic effects (ramets under pine were older), Dh-age relationships showed that, for the same cambial age, vessel size was also larger under pine (Fig. A3). In a previous study in the same experiment, *V. myrtillus* phenology was advanced under larch and above-ground biomass was greater under pine (Anadon-Rosell et al. 2014). Overall, growing conditions under pine seem more favourable than under larch, probably related to later snowmelt in spring and a perennial canopy, and thus greater protection from frost damage and freezing-induced cavitation during early-season freezing events. This could also explain why ramets growing under pine showed greater increases in Dh and Kh under CO₂ enrichment than those growing under larch. Alternatively, *V. myrtillus* may find more suitable conditions under pine as a result of greater light infiltration under its canopy during the growing season (Dawes et al. 2011a) or a lower nitrogen availability under pine trees, as indicated by lower nitrogen concentrations and $\delta^{15}\text{N}$ values in *V. myrtillus* leaves (Dawes et al. 2017), which may have led to a competitive advantage over more N-demanding graminoids.

5. Conclusions

Our results show that, although growth and xylem anatomy of *V. myrtillus* are linked in terms of stem hydraulic conductivity, they may not respond in the same way to environmental changes. *Vaccinium myrtillus* growth and hydraulic efficiency were slightly stimulated under CO₂ enrichment, although increases in vessel size occurred only at the end of the experimental years and growth responses were only evident through an increase in the basal stem perimeter. The soil warming treatment had larger effects than the CO₂ enrichment treatment, although they were in opposite directions. While soil warming stimulated *V. myrtillus* growth, it decreased vessel sizes. Thus, growth stimulation under warming in this species was not explained by accompanying changes in the xylem anatomical structure, indicating that hydraulic efficiency is not limiting for *V. myrtillus* at our alpine treeline site. In addition, since freezing events occur frequently at treeline, smaller vessel sizes may help avoid damage from frost-induced cavitation. The lack of response in above-ground storage tissues suggests that growth is prioritised over investments on storage capacity under soil warming and CO₂ enrichment. Future warmer conditions at high elevations will have larger effects on *V. myrtillus* than increasing atmospheric CO₂ concentrations, and growth stimulation under higher temperatures can be expected to occur before adjustments of the xylem anatomical structures favouring increased hydraulic efficiency.

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Tables

Table 1. Parameters included in the study with their descriptions and units.

Function	Parameter	Units	Description
Hydraulic efficiency	CA95%	μm^2	Cross-sectional vessel lumen area at 95 th percentile
	Dh	μm	Hydraulically weighted mean vessel diameter
	Kh *	$\text{kg m MPa}^{-1} \text{s}^{-1}$	Theoretical hydraulic conductivity
	Ks *	$\text{kg m}^{-1} \text{MPa}^{-1} \text{s}^{-1}$	Theoretical xylem-specific hydraulic conductivity
Growth	CNo	-	Number of vessels
	RW	μm	Ring width
	RA	mm^2	Ring area
Spatial vessel distribution	CD	no./mm^2	Vessel density
	V _G	-	Vessel grouping index
	V _M	-	Mean group size of grouped vessels
	V _S	-	Vessel solitary fraction
Storage and radial transport	Ray density	no./mm	Number of rays per millimetre of stem circumference
	Ray seriality	-	Number of parallel cell lines per ray

* based on Hagen-Poiseuille law (Tyree and Ewers 1991)

Table 2. Results of the linear mixed-effects model testing the effects of CO₂ enrichment (CO₂), soil warming (W), plot tree species (Tree) and treatment year (Year) on the studied xylem anatomical parameters of *V. myrtillus*. Three- and four-way interactions are only shown when included in the model. Significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.1$) effects are given in bold. For abbreviation explanations see Table 1.

	Dh			Kh			CD			V _G			CNo			RW		
	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>
a) CO₂ years																		
Cambial age	1, 201	110.42	<0.001	1, 209	41.93	<0.001	1, 209	5.80	0.017	1, 208	108.90	<0.001	-	-	-	1, 211	13.73	0.000
CO ₂	1, 8	1.82	0.215	1, 8	1.40	0.270	1, 8	0.08	0.789	1, 8	2.50	0.153	1, 8	3.66	0.092	1, 8	0.07	0.802
Tree	1, 28	7.74	0.010	1, 28	1.32	0.261	1, 28	1.07	0.311	1, 28	0.76	0.392	1, 28	0.01	0.914	1, 28	1.99	0.170
Year	8, 201	3.06	0.003	8, 209	5.96	<0.001	8, 209	1.91	0.061	8, 208	1.10	0.368	8, 182	5.38	<0.001	8, 211	5.97	<0.001
CO ₂ × Tree	1, 28	0.42	0.523	1, 28	0.05	0.823	1, 28	1.01	0.323	1, 28	0.00	0.990	1, 28	0.16	0.689	1, 28	0.02	0.886
CO ₂ × Year	8, 201	1.21	0.296	8, 209	0.54	0.825	8, 209	0.82	0.588	8, 208	0.93	0.497	8, 182	1.00	0.440	8, 211	0.69	0.698
Tree × Year	8, 201	0.72	0.673	8, 209	2.02	0.046	8, 209	0.46	0.883	8, 208	0.23	0.986	8, 182	0.77	0.630	8, 211	1.97	0.052
CO ₂ × Tree × Year	8, 201	1.89	0.064	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
b) Warming years																		
Cambial age	1, 167	30.82	<0.001	1, 167	4.81	0.030	-	-	-	1, 166	40.01	<0.001	-	-	-	1, 125	6.18	0.014
CO ₂	1, 8	5.17	0.053	1, 8	1.84	0.212	1, 8	0.15	0.712	1, 8	3.51	0.098	1, 8	4.32	0.071	1, 8	0.06	0.817
W	1, 8	9.56	0.015	1, 8	0.01	0.906	1, 8	0.86	0.380	1, 8	0.93	0.363	1, 8	0.40	0.543	1, 8	1.09	0.328
Tree	1, 17	11.19	0.004	1, 17	0.59	0.453	1, 17	0.55	0.467	1, 17	2.01	0.175	1, 17	0.23	0.637	1, 16	4.50	0.050
Year	5, 167	7.56	<0.001	5, 167	4.93	0.000	5, 168	4.02	0.002	5, 166	0.60	0.700	4, 134	3.23	0.015	4, 125	3.53	0.009
CO ₂ × W	1, 8	0.00	0.960	1, 8	0.41	0.541	1, 8	0.90	0.371	1, 8	1.03	0.341	1, 8	1.75	0.222	1, 8	1.18	0.310
CO ₂ × Tree	1, 17	1.08	0.313	1, 17	0.91	0.353	1, 17	1.56	0.229	1, 17	0.48	0.498	1, 17	0.00	0.958	1, 16	0.16	0.695
CO ₂ × Year	5, 167	0.52	0.759	5, 167	0.67	0.646	5, 168	0.32	0.901	5, 166	0.51	0.769	4, 134	0.49	0.741	4, 125	0.39	0.813
W × Tree	1, 17	1.64	0.218	1, 17	0.83	0.376	1, 17	2.28	0.149	1, 17	0.02	0.900	1, 17	0.23	0.640	1, 16	0.36	0.559
W × Year	5, 167	3.91	0.002	5, 167	4.12	0.002	5, 168	1.57	0.172	5, 166	2.91	0.015	4, 134	3.19	0.015	4, 125	3.27	0.014
Tree × Yr	5, 167	0.83	0.532	5, 167	0.53	0.751	5, 168	0.92	0.467	5, 166	0.56	0.729	4, 134	0.39	0.818	4, 125	0.35	0.845
CO ₂ × W × Year	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4, 125	1.29	0.276
CO ₂ × W × Tree	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1, 16	0.12	0.733
CO ₂ × Tree × Year	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4, 125	1.29	0.279
W × Tree × Year	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4, 125	1.62	0.173

Table 3. Mean values (± 1 SE) of the xylem anatomical parameters studied for the CO₂-treatment years 2001–2009 (a) and for the warming-treatment years 2007–2012 (b). Mean values of original data are given for each combination of CO₂ treatment (ambient or elevated) and plot tree species (larch or pine) averaged over the study years during the CO₂-treatment years ($n = 10$) and for each combination of CO₂ treatment, soil warming treatment and plot tree species across the study years during the warming-treatment years ($n = 5$). For Dh, more detailed data are provided in Fig. 4 and Table A3.

a) CO₂-treatment years										
CO ₂ treatment	Tree species	Kh (kg MPa ⁻¹ s ⁻¹)		CD (no./mm ²)		V _G		RW (μm)		
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	
Ambient	Larch	4.51E-14	5.35E-15	1009.90	21.54	1.35	0.02	112.02	9.96	
	Pine	5.57E-14	6.96E-15	1009.89	18.92	1.35	0.02	98.85	9.88	
Enhanced	Larch	5.95E-14	7.39E-15	991.56	25.15	1.37	0.02	128.77	13.11	
	Pine	7.34E-14	6.20E-15	1060.10	17.86	1.41	0.01	95.86	7.02	
b) Warming-treatment years										
Warming treatment	CO ₂ treatment	Tree species	Kh (kg MPa ⁻¹ s ⁻¹)		CD (no./mm ²)		V _G		RW (μm)	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE
Unwarmed	Ambient	Larch	5.46E-14	5.92E-15	1046.20	38.10	1.42	0.03	101.80	10.29
		Pine	8.54E-14	1.37E-14	984.46	32.42	1.46	0.02	100.60	21.29
	Enhanced	Larch	6.48E-14	9.18E-15	1021.94	32.98	1.44	0.02	86.04	9.48
		Pine	9.51E-14	8.58E-15	1024.86	23.70	1.50	0.02	68.96	6.59
Warmed	Ambient	Larch	8.34E-14	1.55E-14	1042.38	29.93	1.38	0.02	115.96	15.27
		Pine	5.74E-14	1.02E-14	1070.38	26.74	1.36	0.02	78.29	8.03
	Enhanced	Larch	8.06E-14	1.19E-14	962.46	31.73	1.36	0.02	164.33	21.39
		Pine	1.03E-13	1.08E-14	1090.39	20.30	1.50	0.02	101.16	11.41

Figure legends

Figure 1. (a) *Vaccinium myrtillus* ramet; (b) cross-section cut at ground level and stained with safranin and astrablue. Details of wood anatomical parameters are shown on the right side.

Figure 2. Pearson's correlation matrix for the considered xylem anatomical parameters (see Table 1 for explanations of the abbreviations). Values for the CO₂ treatments, warming treatments, plot tree species and study years are plotted together. Positive correlations are green and negative correlations are red. Correlation coefficients (r) are shown and significant correlations are indicated by asterisks at $0.05 < P < 0.10$ (marginally significant, *), $0.01 < P < 0.05$ (**) and $P \leq 0.01$ (***). Non-significant correlations are indicated by n.s.

Figure 3. (a) Relationship between stem Kh and total above-ground biomass (top) and new shoot increment length (bottom), both measured in 2012. Coefficients of determination (r^2) and error probabilities of the regression slope (P values) are shown ($n = 40$). (b) Variation in the correlation coefficient (r) between Kh and above-ground biomass (top) and new shoot increment length (bottom) with increasing number of rings considered. X-axis units are accumulated relative ring position from bark (outermost rings, left) to pith (innermost rings, right). Individual relative ring positions were calculated by dividing the absolute bark-to-pith position of each ring by the total number of rings in the respective sample. Relative ring positions were then grouped into discrete 0.1-unit classes. All the correlations between biomass or shoot increment length and Kh were significant at $P < 0.05$ except for those indicated by ns (non-significant, $P > 0.1$) or by ms (marginally significant at $0.1 > P \geq 0.05$).

Figure 4. Variation of *Vaccinium myrtillus* hydraulically weighted mean diameter (Dh) through time during the CO₂-treatment years 2001–2009 (left panels) and during the warming-treatment years 2007–2012 (right panels) for each plot tree species (larch in top panels, pine in bottom panels). Mean values ± 1 SE are shown, estimated from statistical models for a ramet of average cambial age. For the CO₂-treatment years, each combination of CO₂ treatment (ambient or elevated) and plot tree species is

shown ($n = 10$). For the warming-treatment years, each combination of CO₂ treatment (A = ambient, E = elevated), soil warming treatment (C = unwarmed, W = warmed) and plot tree species is shown ($n = 5$). Vertical dashed lines indicate the end of the CO₂ treatment; the shaded area indicates pre-warming values from 2006. Significant effects included CO₂ treatment (CO₂), warming treatment (W), plot tree species (T) and study year (Y) at $0.05 < P < 0.10$ (marginally significant, *), $0.01 < P < 0.05$ (**) and $P \leq 0.01$ (***), and are shown in the right bottom corner of the lower panels. See Table 3 for detailed statistical results.

Figure 5. Percentage of uniseriate (lefthand y-axis) and biseriate and multiseriate (righthand y-axis) rays for each combination of CO₂ level (A = ambient, E = elevated) and soil warming treatment (C = unwarmed, W = warmed). Mean values ± 1 SE are shown. Measurements were made on the last ring (year 2012). Asterisks (**) indicate a significant effect of the warming treatment (W) on the percentage of biseriate rays at $0.01 < P < 0.05$.

Fig. 1

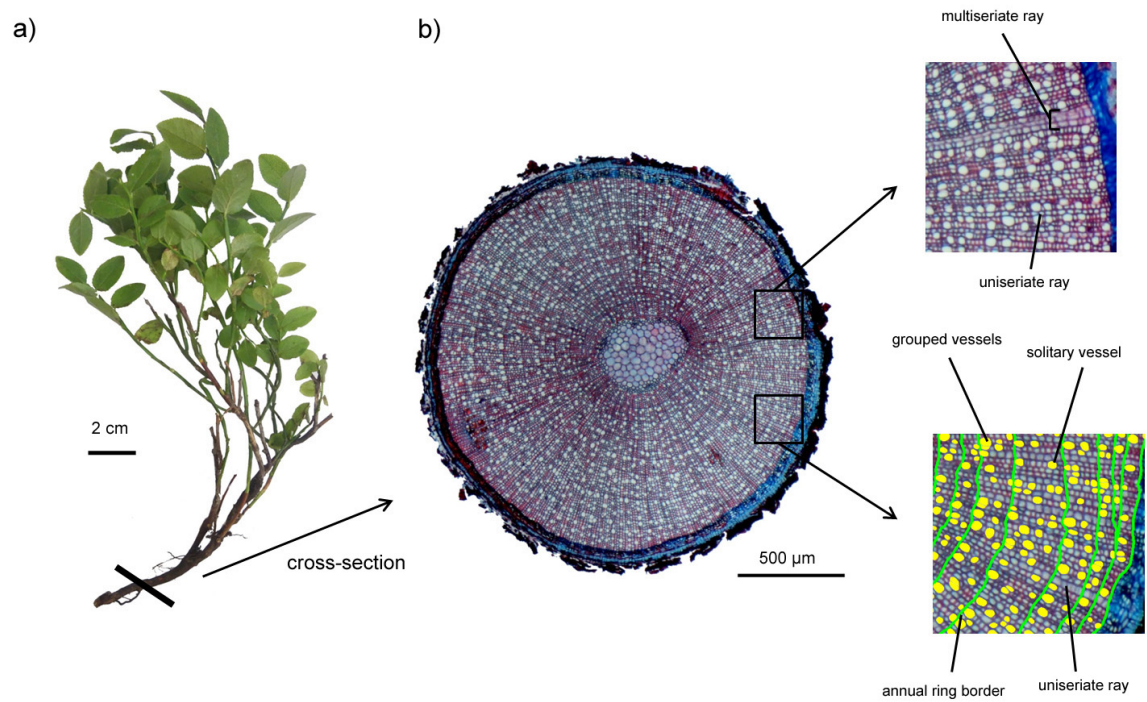


Fig. 2

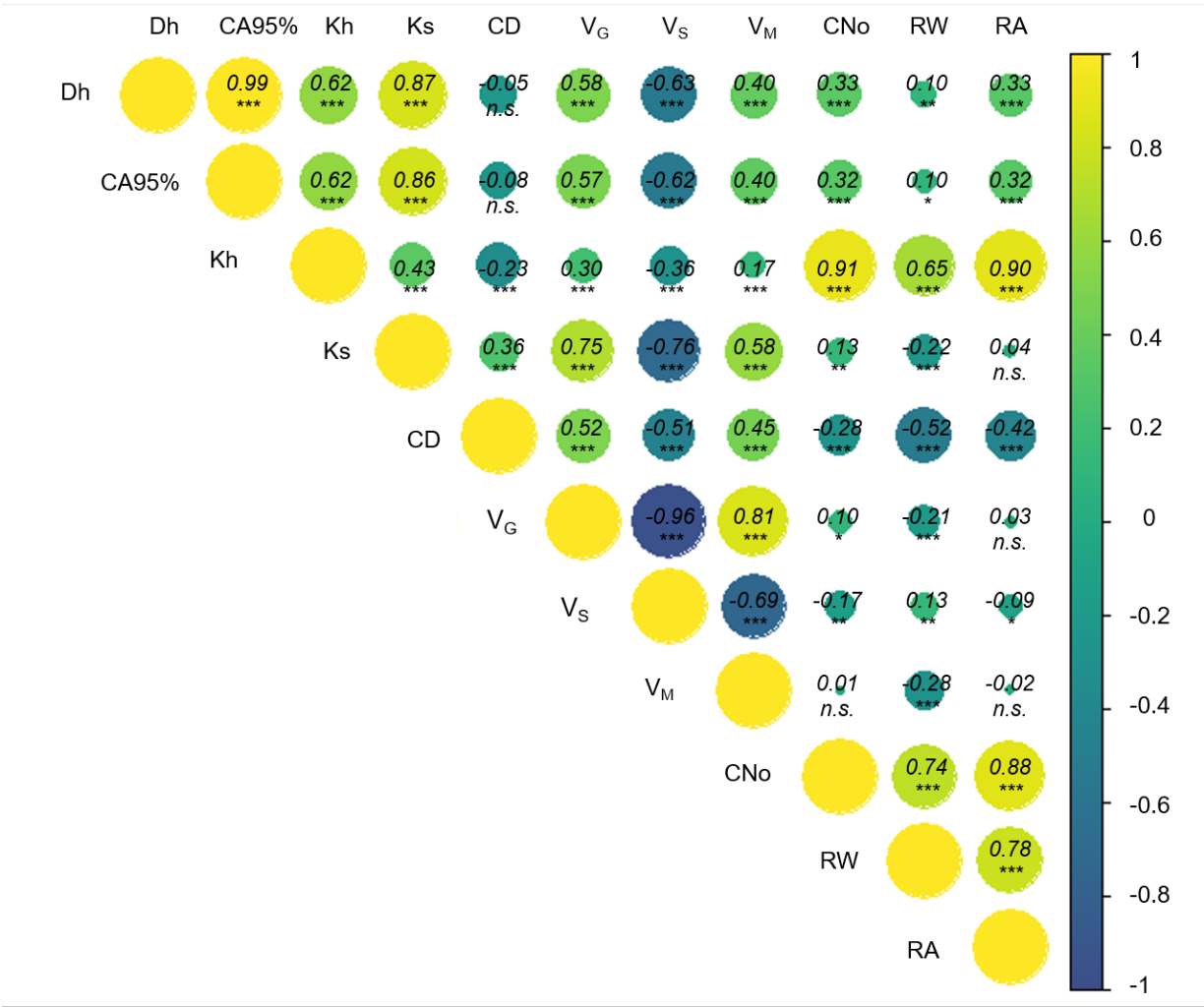


Fig. 3

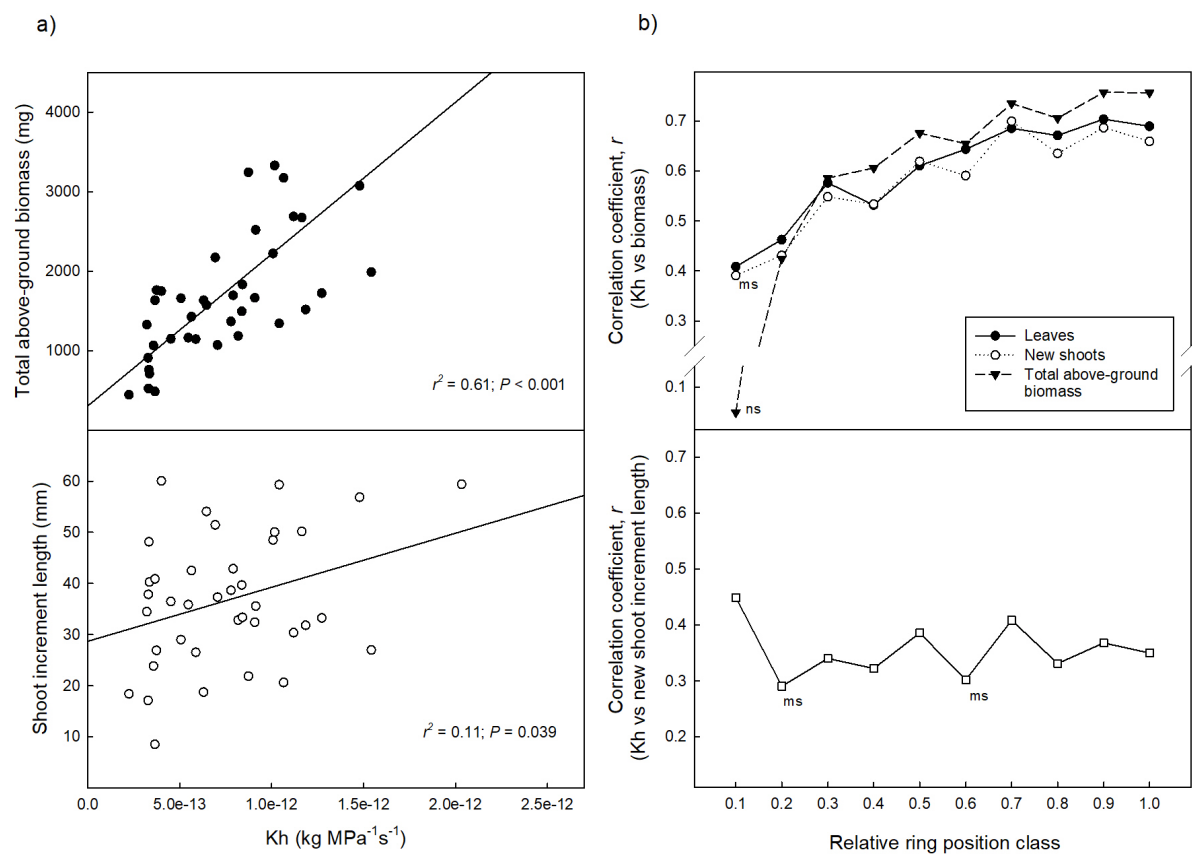


Fig. 4

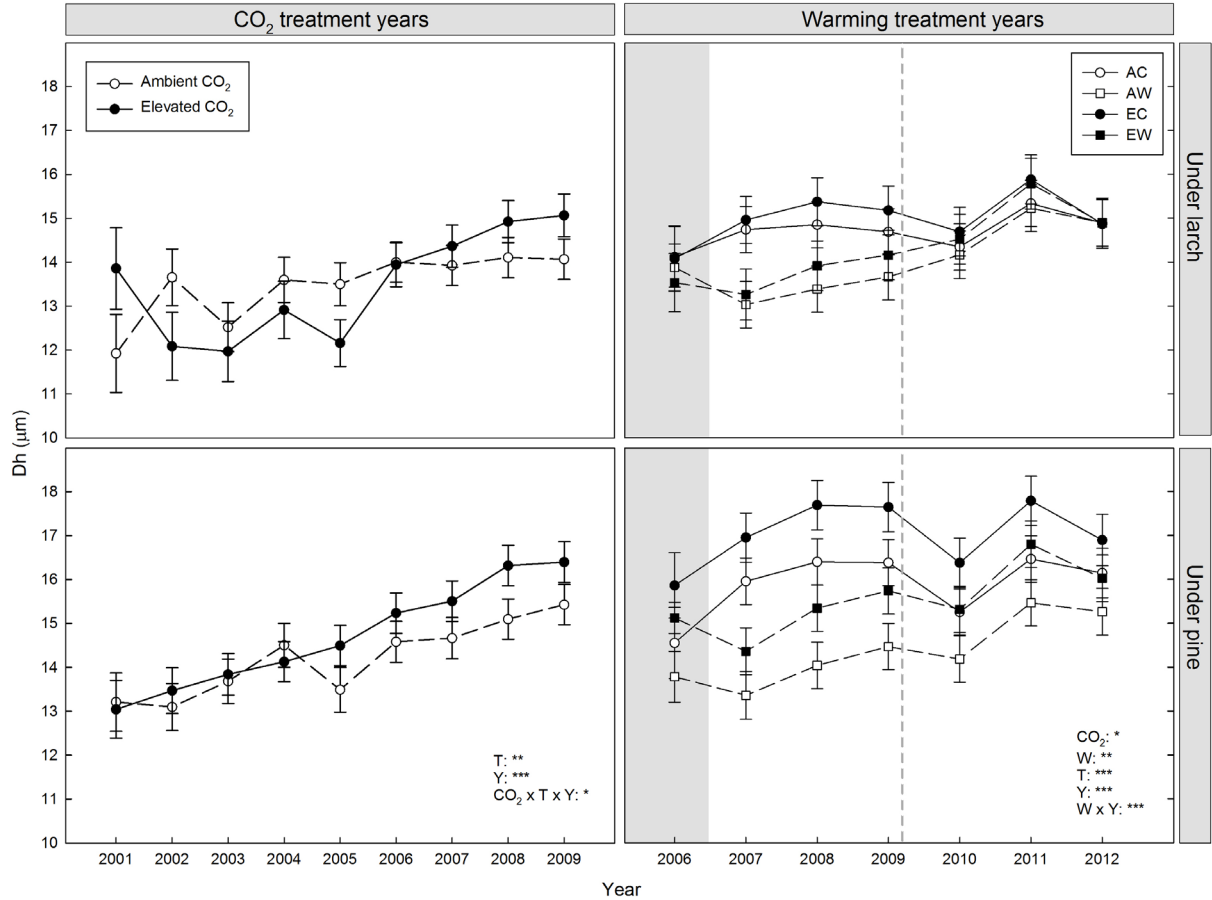


Fig. 5

