

UNIVERSIDAD POLITÉCNICA DE MADRID
ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA DE MONTES,
FORESTAL Y DEL MEDIO NATURAL



**MECHANISMS OF ACCLIMATION IN
FAGUS SYLVATICA L. TO
COMBINED ABIOTIC STRESS
UNDER A HIGH-END CLIMATE
SCENARIO**

DOCTORAL THESIS

Submitted for the degree of Doctor by:

FAUSTINO RUBIO PÉREZ

Forestry Engineer
&
Licentiate in Chemical Sciences

Madrid, 2025



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Doctoral Degree in Advanced Forest Research

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Under the supervision of:

Dra. Rosa Ana López Rodríguez

Dr. Francisco Javier Cano Martín

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Abstract

Climate warming is imposing unprecedented abiotic stresses (heat, drought, high VPD) on temperate forests, threatening sensitive species such as European beech (*Fagus sylvatica*). This thesis investigates how *Fagus sylvatica* seedlings acclimate to elevated growth temperature alone and in combination with drought and shade – conditions expected under a high-end climate scenario. Using controlled-environment experiments with constant vapor pressure deficit (VPD), seedlings were grown at optimal ($\approx 25^\circ\text{C}$) or elevated ($+7^\circ\text{C}$) temperatures under factorial light (high vs. low) and water (well-watered vs. moderate drought) treatments. Leaf gas exchange (net CO_2 assimilation A_n , stomatal conductance g_s , mesophyll conductance g_m), chlorophyll fluorescence (Φ_{PSII} , NPQ), and derived photosynthetic parameters (V_{cmax} , J_{max} , T_{opt} , respiration R_d/R_l) were measured, alongside growth, leaf morphology (leaf area, LMA), water relations (osmotic potential, leaf maximum Young's modulus of elasticity), xylem hydraulic traits (safety margins, conductivity, Hubert value), and morphological traits (height, diameter, biomass, xylem anatomy and wood density).

Warmed seedlings exhibited pronounced photosynthetic acclimation: net assimilation was $\sim 30\%$ lower across leaf temperatures, yet the thermal optimum (T_{opt}) remained unchanged. This reflected biochemical downregulation (reduced V_{cmax} and J_{max}) rather than stomatal or mesophyll limitations. High-temperature growth also lowered mitochondrial and photorespiratory carbon losses and greatly enhanced non-photochemical quenching (NPQ), indicating a “min–max” strategy of conserving carbon (via reduced respiration) while boosting photoprotective dissipation. Morphologically, warm-grown seedlings produced smaller, thinner leaves (lower LMA), aiding heat dissipation but increasing water demand. Under ample water, elevated temperature caused stomata to open more widely (higher g_s), raising transpiration (E) and lowering intrinsic water-use efficiency (iWUE).

Although the lowest overall growth was observed in seedlings under low light—due primarily to light limitation rather than stress—the most severe physiological stress occurred under combined drought and high temperature. In this “hot drought” scenario, carbon losses were greatest: net assimilation dropped by $\sim 60\%$, and dark respiration more than doubled compared to ambient conditions. This shift reflected a strong prioritization of photoprotection (via rapid stomatal closure and increased NPQ) over carbon gain. The result was a marked impairment in osmotic adjustment and significant reductions in leaf water safety margins,

highlighting drought—especially in combination with warming—as the dominant constraint on hydraulic safety.

Even when light availability limited growth more than drought, it still modulated these stress responses in meaningful ways. Shade-grown seedlings, despite their reduced biomass, maintained similar light-saturated net assimilation (A_n) to high-light plants, but with lower respiration rates—evidence of efficient shade acclimation. Under warming, these seedlings adopted a resource-acquisitive strategy: producing many low-LMA leaves to maximize light capture and potential carbon gain, though this came at the cost of elevated water use. In contrast, under high light, warming triggered a conservative response: sharp reductions in leaf area and total biomass, alongside increased transpiration, indicating a shift toward drought avoidance. Ultimately, shade-grown seedlings prioritized carbon gain at the expense of hydraulic safety, whereas sun-grown seedlings curtailed growth to preserve water and protect hydraulic function.

Altogether, these findings reveal that while beech seedlings exhibit significant physiological plasticity, their acclimation to warming—particularly under compound stress—is only partial. Elevated growth temperature triggers a trade-off: it conserves leaf function in warmth by downregulating photosynthetic capacity and respiration and by enhancing thermal protection (NPQ), yet this “water-spending” strategy narrows safety margins in carbon and water balance. Crucially, multi-stressor experiments uncovered synergies absent in single-factor studies: simultaneous heat, drought, and shade caused unique response syndromes (e.g. combined leaf area loss, hydraulic narrowing) that severely suppressed growth and assimilation.

In conclusion, *Fagus sylvatica* demonstrates notable physiological plasticity under moderate warming, but combined heat and drought impose severe carbon limitations that may constrain regeneration. These results suggest that future beech forest resilience will depend on the frequency of heat–drought extremes and the availability of cool, moist microsites (e.g. shaded understory) to buffer seedlings. By elucidating the integrated acclimation mechanisms across photosynthesis, stomatal behavior, morphology, and hydraulics, this thesis provides a cohesive picture of beech seedling responses to high-end climate change, with implications for predicting temperate forest dynamics under multiple interacting stresses.

Resumen

El calentamiento global está imponiendo un estrés abiótico sin precedentes (calor, sequía y alto déficit de presión de vapor – VPD) en los bosques templados, amenazando especies vulnerables como el haya europea (*Fagus sylvatica* L.). Esta tesis busca investigar como responden plántulas de *Fagus sylvatica* a distintas temperaturas de crecimiento en combinación con sequía y sombra. Para ello, se emplearon condiciones controladas manteniendo constante el VPD. Las plántulas se desarrollaron a temperaturas óptimas (25°C) o elevadas (32°C) bajo un diseño factorial de tratamientos: irradiación (alta vs. baja) e irrigación (bien regadas vs. sequía moderada). A lo largo de los experimentos, se midieron parámetros de intercambio gaseoso (asimilación neta A_n , conductancia estomática g_s , y del mesófilo g_m), fluorescencia de la clorofila (Φ_{PSII} , NPQ), y parámetros fotosintéticos derivados de los anteriores (V_{cmax} , J_{max} , T_{opt} , respiration R_d/R_i), junto a crecimiento y morfología foliar (area foliar, peso específico foliar - LMA), relaciones hídricas (potencial osmótico, módulo de elasticidad de la hoja), parámetros de transporte de agua del xilema (márgenes de seguridad, conductividad hidráulica y Hubert value), y parámetros morfológicos (altura, diámetro, biomasa, anatomía del xilema y densidad de la madera).

Las plántulas crecidas a mayor temperatura mostraron una pronunciada aclimatación fotosintética: su asimilación neta fue un 30% menor a lo largo de un amplio rango de temperaturas foliares, aunque su temperatura óptima (T_{opt}) se mantuvo. Este fenómeno es indicativo de una regulación bioquímica (reduciendo V_{cmax} and J_{max}) más que de una limitación estomática o de la conductancia del mesófilo. Las altas temperaturas de crecimiento también contribuyeron a disminuir las pérdidas de carbono ocasionadas por la respiración y fotorrespiración, al mismo tiempo que promovieron un incremento del “non-photochemical quenching” (NPQ), indicando una estrategia “min-max” enfocada a la conservación de carbono (a través de reducir los costes asociados a la respiración) al tiempo que promovían mecanismos de disipación energética y de fotoprotección. Morfológicamente, las plántulas crecidas a alta temperatura generaron hojas más pequeñas y finas (menor LMA), que contribuyen a una mejor disipación térmica a coste de una mayor demanda evaporativa. Sin restricciones hídricas, la temperatura promovió una mayor apertura estomática (mayor g_s), contribuyendo a una mayor transpiración (E) y una menor eficiencia intrínseca en el uso del agua (iWUE).

En caso de déficit hídrico, las altas temperaturas promovieron un mayor estrés: la combinación de estos factores promovió unas mayores pérdidas de carbono. La A_n mostró una fuerte disminución (~60% al compararla con el control), mientras que la respiración un fuerte incremento. En este escenario, los estomas se cerraron rápidamente, lo que promovió el incremento del NPQ, translocando energía a la fotoprotección y disipación de energía térmica a expensas de un peor balance de carbono. Estas plántulas mostraron además una falta de ajuste osmótico, y, por tanto, menores márgenes de seguridad de la hoja, indicando una mayor vulnerabilidad hidráulica como consecuencia de este estrés combinado.

La disponibilidad de luz amortiguó estas respuestas, especialmente en aquellos tratamientos bien regados. Las plántulas crecidas en sombra alcanzaron A_n similares que las plantas en luz bajo luz saturante. Al mismo tiempo, mostraron a una menor respiración, lo que sugiere una aclimatación eficiente a condiciones de baja irradiancia. No obstante, en términos de crecimiento la luz mostró ser más limitante que la sequía a pesar de mostrar esta elevada capacidad de adaptación. La falta de energía lumínica restringió más el crecimiento que la sequía a expensas de una mayor resiliencia. Sorprendentemente, las altas temperaturas en combinación con la sombra promovieron una estrategia adquisitiva: las plántulas desarrollaron más hojas de menor LMA, maximizando la captura de luz, y, por lo tanto, un mejor balance de carbono en condiciones de luz saturante a expensas de menor eficiencia en el uso del agua. En contraste, bajo alta irradiancia, las altas temperaturas promovieron una estrategia más conservativa: el área foliar y la biomasa total disminuyeron considerablemente, al mismo tiempo que la transpiración se vio fuertemente incrementada. Por tanto, las plántulas crecidas a la sombra sacrificaron la seguridad de sus sistemas hidráulicos buscando una mayor ganancia de carbono, mientras que las plantas crecidas a alta irradiancia sacrificaron un mayor consumo hídrico para ser capaces de mantener su crecimiento.

Juntos, estos hechos muestran que, aunque las plántulas de haya mostraron una elevada plasticidad fisiológica a su temperatura óptima de crecimiento, solo mostraron una aclimatación parcial en un escenario simulado de calentamiento global, especialmente bajo condiciones de multi-estrés. Las altas temperaturas de crecimiento promovieron mecanismos de compensación: las funciones de la hoja se mantuvieron a estas altas temperaturas mediante una regulación a la baja de su capacidad fotosintética y de la respiración, al tiempo que se producía un incremento de la disipación de energía térmica (NPQ), aún si esta estrategia de un

mayor “gasto de agua” promovía una disminución de los márgenes de seguridad en términos de carbono y agua. Además, estos experimentos simulando estreses multifactoriales cubren sinergias que de otra manera no pueden ser detectadas o cuantificadas en experimentos con un único estrés o factor. Por ejemplo, las altas temperaturas, la sequía o la sombra aisladas no habrían reflejado sus efectos conjuntos (e.j. disminución del área foliar y de los márgenes de seguridad) que además inhibieron en gran medida el crecimiento y la fotosíntesis.

En conclusión, aunque las plántulas de *Fagus sylvatica* mostraron una considerable plasticidad fisiológica, en un escenario de cambio climático, la combinación de altas temperaturas y sequía impusieron fuertes limitaciones en el balance de carbono que pueden comprometer la regeneración de esta especie. Estos resultados sugieren que, en el futuro, la resiliencia de los hayedos dependerá de la frecuencia de las olas de calor, las sequías y la disponibilidad de micrositios más fríos y húmedos (e.j. sotobosque) para amortiguar estos efectos. Mediante el escrutinio de los mecanismos de aclimatación a través de la fotosíntesis, dinámicas estomáticas, morfología y conductividad hidráulica, esta tesis proporciona una perspectiva global sobre cómo se desenvolverán las plántulas de *Fagus sylvatica* en un escenario de cambio climático, con fuertes implicaciones en la predicción de cómo serán las dinámicas de los bosques templados bajo múltiples estreses simultáneos.

Al integrar procesos de aclimatación que abarcan desde la fotosíntesis y el comportamiento estomático hasta la morfología foliar, la funcionalidad hidráulica y el reparto de biomasa, esta tesis ofrece una visión comprensiva del desempeño de las plántulas bajo escenarios climáticos extremos, y contribuye a anticipar el devenir de los bosques templados ante estreses múltiples e interconectados.

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Abbreviations and Acronyms

Morphological and Growth Traits

Symbol	Definition	Values (units)
h	Plant height	(cm)
d	Plant diameter	(mm)
leaves	Number of fully developed leaves	(n ^o)
TLA	Total plant leaf area	(cm ²)
LA	Average leaf area	(cm ²)
RB	Root biomass (absolute or relative)	(g or %)
SB	Shoot biomass (absolute or relative)	(g or %)
LB	Leaf biomass (absolute or relative)	(g or %)
ρ (rho)	Wood density	(g cm ⁻³)

Gas Exchange and Photosynthetic Parameters

Symbol	Definition (exact wording from document)	Values (units)
A_n or P_N	Leaf net assimilation rate (net CO ₂ assimilation)	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
C_c	Chloroplastic CO ₂ concentration	($\mu\text{mol CO}_2 \text{ mol}^{-1}$)
C_i	Intercellular CO ₂ concentration	($\mu\text{mol CO}_2 \text{ mol}^{-1}$)
E	Leaf transpiration	($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)
R_a or R_D	Respiration rate in darkness (leaf respiration)	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
R_l	Mitochondrial respiration rate in the light	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
g_m	Mesophyll conductance to CO ₂	($\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
g_s	Stomatal conductance to CO ₂	($\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
g_{sw}	Leaf stomatal conductance to water vapor	($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)
$g_{\min}$	Leaf minimum conductance to water vapor	($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)
R_{PR}	Rate of photorespiratory CO ₂ release	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
V_c	Rubisco carboxylation rate	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
$V_{c\max}$	Maximum Rubisco carboxylation rate	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
V_o	Rubisco oxygenation rate	($\mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$)
WUE	Water use efficiency	($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$)
iWUE or WUE _i	Intrinsic water use efficiency	($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$)
$\delta^{13}\text{C}$	Carbon isotope composition	(‰)
N_a	Leaf nitrogen per area	($\mu\text{mol N mm}^{-2}$)
LMA	Leaf mass per area	(g m^{-2})

Chlorophyll Fluorescence Parameters

Symbol	Definition (exact wording from document)	Values (units)
F_v/F_m	Maximum quantum efficiency of PSII	(dimensionless)
F_v'/F_m'	Operating quantum efficiency of PSII	(dimensionless)
NPQ	Non-photochemical quenching	(dimensionless)
Φ_{PSII}	Effective quantum yield of photosystem II	(dimensionless)
qL	Fraction of open PSII centres	(dimensionless)

*(PSII = Photosystem II. F_0 , F_m , F_0' , F_m' are used in text for minimal and maximal fluorescence yields, but are not separately listed in the abbreviations table.)

Stomatal and Leaf Anatomy

Symbol	Definition (exact wording from document)	Values (units)
SD	Stomatal density	(n° stomata per mm ²)
L_s	Stomatal length	(µm)
W_s	Stomatal complex width	(µm)
Pl_s	Stomatal pore length	(µm)
GW_s	Guard cell width	(µm)
PCI	Potential conductance index	(µm ² mm ⁻²)

Water Relations and Hydraulic Parameters

Symbol	Definition (exact wording from document)	Values (units)
$C\Pi_{100}$	Leaf capacitance at full turgor	(MPa ⁻¹)
$C\Pi_0$	Leaf capacitance at turgor loss point	(MPa ⁻¹)
Π_{100}	Leaf osmotic potential at full turgor	(MPa)
Π_0	Leaf turgor loss point	(MPa)
ϵ_{leaf}	Leaf maximum Young's modulus of elasticity	(MPa)
RWC_{Π_0}	Relative water content at the turgor loss point	(%)
SWC	Leaf-saturated water content	(g g ⁻¹)
Ψ_{leaf}	Leaf midday water potential	(MPa)
Ψ_{stem}	Stem midday water potential	(MPa)
Ψ_{pd}	Predawn water potential	(MPa)
SM_{leaf}	Leaf safety margin	(MPa)
K_s	Hydraulic specific conductivity	(kg m ⁻¹ s ⁻¹ Mpa ⁻¹)
K_l	Leaf hydraulic conductivity	(mmol m ⁻¹ s ⁻¹ Mpa ⁻¹)
H_v	Huber value	(m ² m ⁻²)
k_{plant}	Plant hydraulic conductance	(mmol m ⁻² s ⁻¹ Mpa ⁻¹)
VA	Average vessel lumen area	(µm ²)
VD	Vessel density	(n° vessels per mm ²)
RP	Xylem area occupied by radial parenchyma	(%)
D_h	Hydraulic diameter	(µm)

General and Experimental Abbreviations

Symbol	Definition (exact wording from document)	Values (units)
VPD	vapor pressure deficit	(kPa)
RH	relative humidity	(%)
PPFD	photosynthetic photon flux density	($\mu\text{mol m}^{-2} \text{s}^{-1}$)
GE	gas exchange	(dimensionless)
CF	chlorophyll α fluorescence	(dimensionless)
IRGA	infrared gas analyzer	(n/a)
MPF	multiphase flash (method)	(n/a)
ANOVA	analysis of variance	(n/a)
LME	linear mixed-effects models	(n/a)
PCA	Principal Component Analysis	(n/a)
T_{growth}	growing temperature	(°C)
T25	25/15 $\pm$ 0.5 °C (day/night) temperature regime (named T25)	(°C)
T32	32.5/22.5 $\pm$ 0.5 °C (day/night) temperature regime (named T32)	(°C)
W	well-watered (water availability)	(n/a)
S	water-stressed (water availability)	(n/a)
H	high (light intensity)	(n/a)
L	low (light intensity)	(n/a)

1. General Introduction

1.1. Climate change and new stresses on temperate forests

Rising global temperatures and shifting precipitation patterns are imposing unprecedented stresses on temperate forest ecosystems. Higher air temperatures intensify atmospheric dryness (vapor-pressure deficit) and increase the frequency and severity of heatwaves (Dai, 2013; IPCC, 2023; Schönbeck et al., 2022). Since the late 19th century, Europe has experienced a 1.5°C increase in average temperature (IPCC, 2023), a trend projected to continue, though rates of warming vary by region and season. Northern Europe is warming more rapidly in winter, with a noticeable decline in extreme cold events (EEA, 2017). By contrast, the Mediterranean basin—especially the Iberian Peninsula—is undergoing more pronounced summer warming, characterized by an increase in extreme heat events (Pereira et al., 2021).

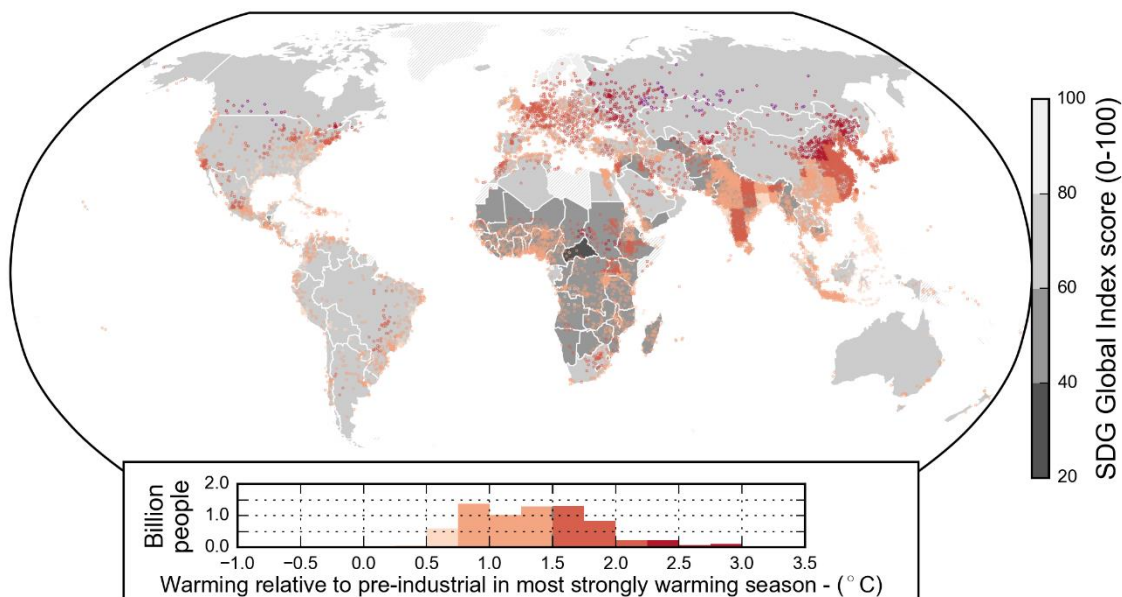


Figure 1. Human experience of present-day warming. Different shades of pink to purple indicated by the inset histogram show estimated warming for the season that has warmed the most at a given location between the periods 1850–1900 and 2006–2015, during which global average temperatures rose by 0.91°C in this dataset (Cowtan and Way, 2014) and 0.87°C in the multi-dataset average. The density of dots indicates the population (in 2010) in any 1° × 1° grid box. The underlay shows national Sustainable Development Goal (SDG) Global Index Scores indicating performance across the 17 SDGs. Hatching indicates missing SDG index data (e.g., Greenland). The histogram shows the population living in regions experiencing different levels of warming (at 0.25°C increments). Source: (IPCC, 2023).

While projections for future precipitation remain less consistent, a general trend of increasing rainfall in northern Europe and decreasing rainfall in southern regions has been reported (Kjellström et al., 2011). At the same time, extreme climatic events such as heatwaves, droughts, and intense rainfall episodes are expected to become more common and severe (Beniston et al., 2007; Goodess, 2013; Lenderink & van Meijgaard, 2008). Notably, even areas with projected increases in precipitation may still experience intensified summer droughts due to higher evaporation rates (Wong et al., 2011).

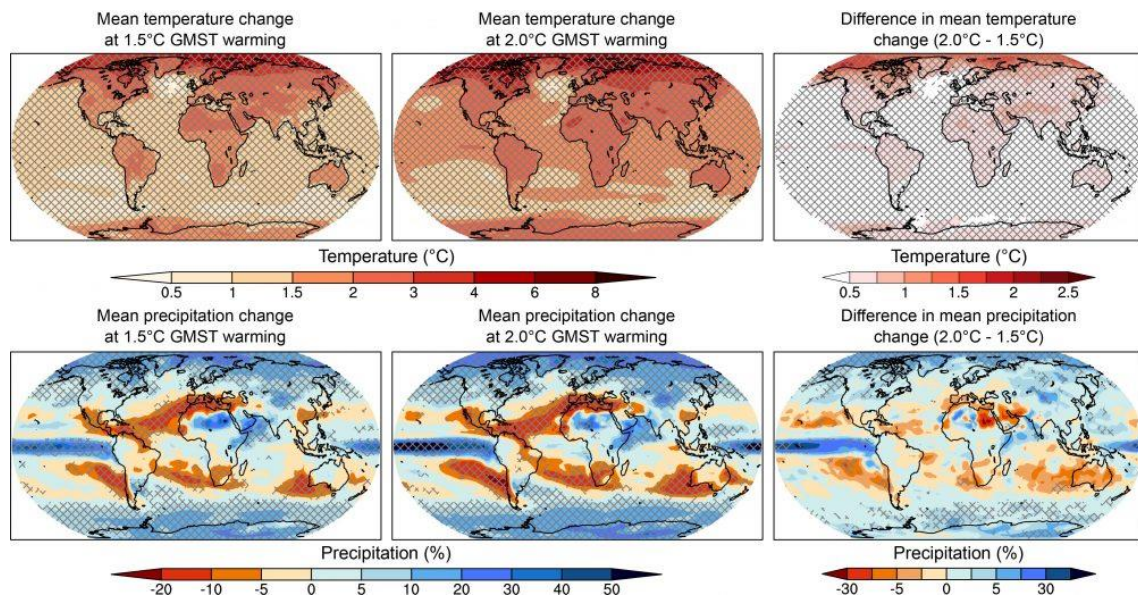


Figure 2. Projected changes in mean temperature (top) and mean precipitation (bottom) at 1.5°C (left) and 2°C (middle) of global warming compared to the pre-industrial period (1861–1880), and the difference between 1.5°C and 2°C of global warming (right). Source: (IPCC, 2023).

These climatic changes exacerbate thermal and water stresses on plants, disrupting their energy and water balances, constraining growth (Feller, 2016; Jumrani & Bhatia, 2019), regeneration (Cochrane et al., 2015), and in extreme cases, lead to widespread vegetation mortality (Adams et al., 2009; Flexas & Medrano, 2002; Hammond et al., 2022).

Tree species are particularly vulnerable due to their long-life cycles and limited migration capacity, which prevent them from easily escaping or genetically adapting to the rapid onset of hotter, drier conditions. Consequently, rear-edge populations are increasingly exposed to climatic conditions near the limits of species tolerance, raising concerns about forest health and persistence under climate change (Dorado-Liñán et al., 2017; Jump et al., 2006; Valladares et al., 2006).

Recent projections underscore the growing vulnerability of temperate European forest trees to ongoing warming. Species distribution models (SDMs) forecast substantial range contractions for many key species by 2061–2080, especially under scenarios involving intense temperature increases. For instance, Dyderski et al. (2018) found that native pioneer and coniferous species—such as *Betula pendula*, *Picea abies*, and *Pinus sylvestris*—could lose over half of their current range, with the greatest losses expected in southern and eastern Europe, where warming and drying trends are most pronounced. Even for potentially expanding species like *Fagus sylvatica*, southern rear-edge populations remain highly vulnerable (Jump et al., 2006).

Because temperature is a key determinant of physiological functioning and ecological viability, it plays a central role in defining species' geographic limits. Moreover, temperature is closely tied to atmospheric vapor pressure deficit (VPD), a driver of plant water stress. Yet, many distribution models emphasize average conditions rather than capturing the full range of thermal extremes, potentially underestimating the impact of warming on species persistence (Beniston et al., 2007; Kjellström et al., 2011; Woodin et al., 2013).

Notably, most species distribution models that forecast range shifts under climate change rely on the climatic requirements of adult trees (Benito Garzón et al., 2019), often overlooking reproductive and regeneration phases, which are critical to overall fitness and typically highly sensitive to climatic conditions (Verdú & Traveset, 2005). Woodin et al. (2013) highlight that physiological performance metrics—such as the transient event margin (TEM), which represents the gap between energy balance failure and lethal thresholds—are essential for improving forecasts. Species with narrow TEMs are especially prone to collapse under even minor warming near their thermal limits, suggesting that conventional models may overestimate the resilience of many temperate tree species under future climates.

Compounding these concerns is the fact that climate stresses often occur simultaneously rather than in isolation. Heat and drought frequently coincide during summer months, and in forest understories these stressors are further exacerbated with low light availability. Natural regenerated seedlings commonly experience this triple interaction of sustained warming, episodic water deficit, and shade from the overstory.

Such multifaceted stress scenarios greatly complicate plant responses (Zandalinas et al., 2024). During soil water deficits, plants typically close their stomata to reduce water loss, but this simultaneously restricts CO₂ uptake and impairs photosynthesis. Stomatal closure also limits evaporative cooling, compromising the plant's ability to regulate leaf temperature (Gates, 1968). Under intense light, this can lead to uncontrolled leaf heating. When elevated temperatures coincide with drought, the effects are not merely additive; rather, synergistic feedbacks amplify water loss and thermal stress (Zandalinas et al., 2021). This accelerates leaf heating, disrupts metabolic processes, and leads to a steeper decline in photosynthetic efficiency and overall plant vitality (Cano et al., 2013; Deluigi et al., 2025; Feller, 2016; Rubio et al., 2025).

Phenotypic plasticity enables plants to modulate physiological and morphological traits in response to changing conditions, potentially enhancing performance under stress (Sultan, 2000; Van Buskirk & Steiner, 2009). For instance, elevated temperatures can stimulate enzymatic activity and promote higher metabolic rates (Moore et al., 2021), while also increasing stomatal conductance and transpiration, aiding in leaf cooling (Gates, 1968). Under drought, plants may engage in osmotic adjustment, accumulating solutes to maintain cell turgor and sustain physiological functions even as water availability declines (Bartlett et al., 2012; Feller, 2016). In shaded environments, plastic responses often involve morphological shifts such as larger, thinner leaves, increased chlorophyll b content, and expanded photosystem II (PSII) antennae, all aimed at maximizing light capture (Aranda et al., 2005; Ruban, 2016).

However, these adjustments involve inherent trade-offs. Traits that are advantageous under one stress may become maladaptive when multiple stresses occur simultaneously (Zandalinas et al., 2021). For example, while high temperatures may promote beneficial transpiration, concurrent drought can lead to excessive water loss, escalating the risk of hydraulic failure and disrupting carbon balance (Didion-Gency et al., 2024; Feller, 2016). Similarly, shade-adapted traits—like reduced root investment, stem elongation, and expansive leaf area—can be detrimental when water is limited, as they compromise both water uptake and structural resilience (Aranda et al., 2005; Wang et al., 2021).

Understanding how plants acclimate to interacting stressors—and recognizing the limits of phenotypic plasticity—is crucial for predicting their responses under climate change. While plasticity allows plants to fine-tune key functional traits such as gas exchange, water use, and biomass allocation, the benefits often involve

trade-offs that may compromise performance under simultaneous or shifting stresses (Zandalinas et al., 2021). Traits advantageous under one stress may exacerbate vulnerability under another, especially when temperature, drought, and shade co-occur.

As forest ecosystems face increasingly complex environmental pressures, ecophysiological research must continue to unravel how these trait adjustments scale up to influence species survival and forest dynamics. This highlights why integrative, multi-stress frameworks are essential for advancing our understanding of plant resilience in a warming world (Mittler, 2006; Zandalinas & Mittler, 2022).

1.2. Effects of Warming on Photosynthetic Traits, Carbon Balance, and Water Relations

Increased temperatures affect plant growth and development, primarily due to alterations in carbon assimilation (Zahra et al., 2023). Empirical research has shown that trees respond differently to warming depending on their environment; for instance, trees in cold, wet areas generally experience increased growth, whereas trees in warm, dry conditions often face a decline in growth (Buechling et al., 2017; D'Orangeville et al., 2018; Hellmann et al., 2016; Mäkinen et al., 2002; Price et al., 2013). Ultimately, the effects of global warming on plant growth largely depend on whether plants are above or below their optimal temperature for growth (Drake et al., 2018; Reich et al., 2015; Yamori et al., 2014).

Temperature fundamentally regulates plant physiological processes, and most biochemical and growth functions exhibit a characteristic nonlinear response to temperature (Sharpe & DeMichele, 1977). At low to moderate temperatures, reaction rates increase exponentially with temperature (following an Arrhenius-type behavior) up to an optimal range, beyond which performance declines rapidly. In many temperate plants, net photosynthetic carbon assimilation (A_n) rises to a peak at an optimum leaf temperature (T_{opt}) and then falls sharply at supraoptimal temperatures as enzymes denature or become less efficient (Kumarathunge et al., 2019).

Observational studies have documented this pattern. For example, net photosynthesis typically rises as temperatures warm from spring to early summer, but once temperatures exceed a certain value, photosynthetic rates drop precipitously (Kumarathunge et al., 2020; Yamori et al., 2014). Growth trends

mirror this behaviour. Moderate warming can initially promote tree growth and leaf expansion — provided water and nutrients are abundant — because metabolic activity accelerates (Körner, 2006). However, once the temperature exceeds the thermal optimum, growth declines as carbon acquisition decreases (Way & Yamori, 2014). High temperatures constrain leaf area, through smaller or fewer leaves, and reduce A_n , directly limiting biomass production (Kumarathunge et al., 2020; Way & Oren, 2010). As occurs with growth, multiple lines of evidence indicate consistent interspecific differences in the temperature response of net photosynthesis, which are closely linked to the species' climatic origin (Scafaro et al., 2017). In general, plants originating from cooler climates tend to exhibit enhanced A_n at lower temperatures, while species from warmer regions perform better under higher temperatures (Berry & Björkman, 1980; Ledig & Korbobo, 1983), supporting the idea of a genetic adaptation of photosynthetic temperature responses to native climate conditions. In addition, plants possess the ability to modify their photosynthetic response to temperature in response to short-term changes in their thermal environment—a process known as thermal acclimation (Smith & Dukes, 2013; Yamori et al., 2014; Yamori et al., 2005). Consequently, the temperature sensitivity of photosynthesis in a given species can shift depending on the growing environment and may also fluctuate seasonally.

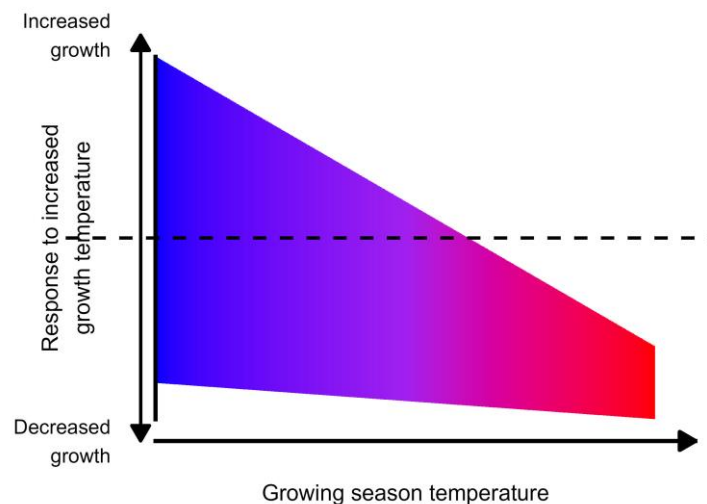


Figure 3. Hypothetical response of trees from different thermal environments to an increase in growth temperature. The coloured region indicates the possible range of responses. Species from colder environments (shown in blue) would generally experience positive growth in response to warming, although reduced growth could occur if water or nutrients were limiting. In contrast, species from warmer environments (in red) would always experience decreased growth, with less variation between individuals or species. Source: (Way & Oren, 2010)

A wide range of models have been developed to improve our understanding of the temperature response of A_n and its implications for plant carbon balance (Farquhar et al., 1980; Harley & Tenhunen, 1991; Leuning, 2002; Smith & Dukes, 2013). According to the widely accepted biochemical model, of C_3 photosynthesis developed by Farquhar, von Caemmerer, and Berry (1980), net CO_2 assimilation (A_n) is determined by the minimum of three biochemical limitations: the rate of carboxylation (V_c) and oxygenation (V_o) of the Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), the rate of RuBP regeneration (linked to electron transport), and the rate of triose phosphate utilization. Additionally, a term for CO_2 release in the light from non-photorespiratory processes (R_l) is subtracted to estimate A_n .

While these processes are strongly influenced by temperature, their thermal behaviour is often investigated through the analysis of the maximum carboxylation rate of Rubisco (V_{cmax}), which is commonly used as a proxy for the enzymatic capacity for CO_2 fixation (Bermudez et al., 2021; Dreyer et al., 2001; Scafaro et al., 2017). Numerous studies have shown that the activity of fully activated Rubisco—and hence V_{cmax} —remains relatively stable across a wide temperature range, including both low and high extremes (Bernacchi et al., 2001; Crafts-Brandner et al., 1997; M. E. Salvucci & S. J. Crafts-Brandner, 2004). However, other findings indicate that light-dependent Rubisco activation is inhibited under moderately high temperatures, leading to reductions in its activation state and a consequent decline in leaf CO_2 assimilation capacity (Crafts-Brandner & Salvucci, 2000; Feller et al., 1998; Kumar et al., 2009). A deeper understanding of this enzymatic response to temperature is crucial, as it is closely linked to plant growth and carbon balance, especially given that the dominant limitations to photosynthesis at supra-optimal temperatures remain unresolved.

Just as V_{cmax} serves as a proxy for assessing the enzymatic component of photosynthesis and its temperature sensitivity, the maximum rate of electron transport (J_{max}) is commonly used to evaluate the thermal response of the photosynthetic electron transport chain (PETC). Similar to Rubisco activity, PETC is closely linked to the overall leaf photosynthetic performance and may become a limiting factor under high-temperature stress (Bermudez et al., 2021; Falcioni et al., 2024; Wang et al., 2023).

Within the PETC, the thylakoid membrane system is particularly sensitive to heat. Among its components, Photosystem II (PSII) has been identified as one of the most thermolabile elements, in contrast to the more heat-tolerant Photosystem I

(PSI). Heat stress can reduce PSII activity by inactivating the oxygen-evolving complex, and disrupting electron flow within PSII reaction centers (Bukhov et al., 1990; Enami et al., 1994; Nash et al., 1985; Pospíšil & Tyystjärvi, 1999). These impairments, typically induced at relatively high temperatures and not easily reversible under light conditions, are believed to result from altered lipid–protein interactions. Specifically, elevated temperatures increase the fluidity of thylakoid membranes, compromising their structural integrity (Gounaris et al., 1984; Raison et al., 1982; Yordanov et al., 1986). In addition, moderate heat stress has been shown to increase membrane permeability, leading to proton leakage across the thylakoid membrane and impairing the proton gradient required for ATP synthase activity (Bukhov et al., 1999; Pastenes & Horton, 1996).

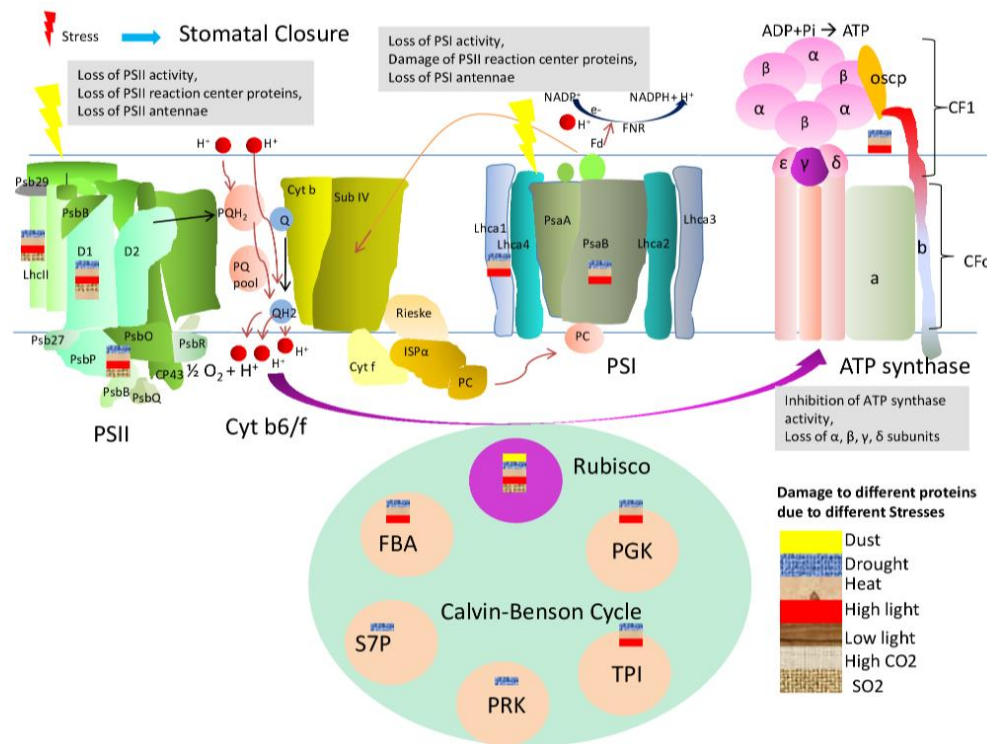


Figure 4. Schematic representation of the photosynthetic electron transport chain and the Calvin-Benson cycle under stress conditions. Key protein complexes of the light reactions (PSII, Cyt b6/f, PSI, and ATP synthase) and Calvin cycle enzymes are shown alongside their respective sensitivities to abiotic stresses, including drought, heat, high/low light, dust, elevated CO₂, and SO₂. Stresses induce stomatal closure and damage various protein subunits, impairing electron transport, photoprotection, ATP synthesis, and carbon fixation. Enzymes affected in the Calvin cycle include Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase), PGK (phosphoglycerate kinase), TPI (triose phosphate isomerase), PRK (phosphoribulokinase), S7P (sedoheptulose-7-phosphate), and FBA (fructose-1,6-bisphosphate aldolase). Color-coded bars represent the specific protein damage caused by different stresses. Source: (Dalal & Guest, 2021).

In addition to the enzymatic and electron transport limitations, mesophyll conductance (g_m) also plays a critical role in determining the photosynthetic

response to temperature. g_m reflects the internal diffusion of CO_2 from the intercellular airspaces to the sites of carboxylation within the chloroplast, and its temperature response—like V_{cmax} and J_{max} —can be also described using an Arrhenius-type function (Walker et al., 2013). To better understand its thermal sensitivity, g_m can be conceptualized as comprising two main components (Evans & Von Caemmerer, 2013): a liquid-phase resistance (through the cell wall, cytosol, and chloroplast stroma) and a membrane resistance (across the plasma membrane and chloroplast envelope). These two pathways respond differently to temperature. While the effects of temperature on liquid-phase CO_2 diffusion tend to be relatively stable, membrane permeability shows a much stronger temperature dependence. This suggests that membrane properties, including the activity of proteins such as aquaporins, may play a critical role in regulating g_m under thermal stress.

Variation in g_m 's temperature response across species—both C_3 and C_4 —supports the idea that membrane-related resistance contributes significantly to its thermal sensitivity. Ignoring this complexity in models may lead to misestimations of CO_2 assimilation, especially under fluctuating or high-temperature conditions (Evans, 2021).

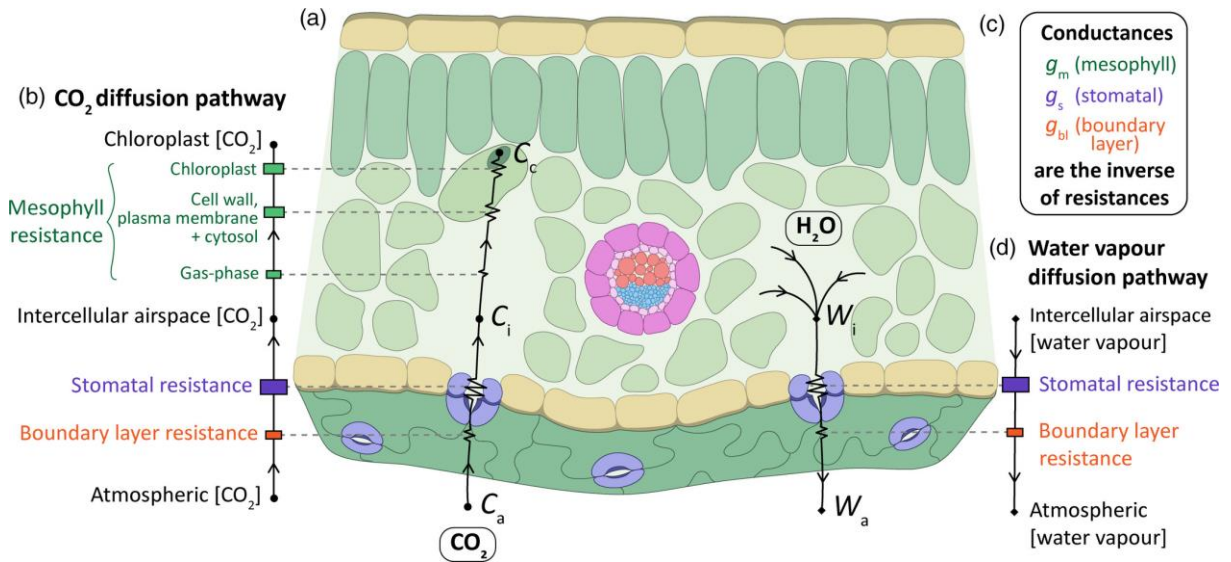


Figure 5. Pathways and resistances for CO_2 uptake and water vapor loss in a C_3 leaf. (a) Cross-section of a leaf showing gas exchange pathways. CO_2 diffuses from the atmosphere (C_a) into the intercellular airspaces (C_i), then through the mesophyll to the chloroplast (C_c), encountering a series of resistances. Conversely, water vapor diffuses out along a gradient from the leaf (W_i) to the atmosphere (W_a). (b) The CO_2 diffusion pathway includes boundary layer, stomatal, and mesophyll resistances (depicted by the size of colored boxes), corresponding to the conductances g_i (orange), g_s (purple), and g_m (green), respectively. These resistances span from the leaf surface through the stomatal pore and

into the mesophyll tissue and chloroplast. (c) Conductances (g_m , g_s , g_l) are the inverse of their respective resistances and are color-coded accordingly. (d) Water vapor exits the leaf through a parallel diffusion pathway, also encountering stomatal and boundary layer resistances. The diagram emphasizes the shared anatomical route and contrasting gradients for CO_2 influx and H_2O efflux.

Taken together, the thermal limitations affecting V_{cmax} , J_{max} , and g_m underscore the high vulnerability of photosynthetic capacity to rising temperatures. While these constraints reduce the capacity for carbon assimilation, respiratory processes including mitochondrial respiration in the light (R_l) and photorespiratory CO_2 release (R_{PR}) tend to increase with temperature, further aggravating the carbon imbalance under heat stress (Atkin & Tjoelker, 2003; Dusenge et al., 2019). This thermal decoupling between photosynthesis and respiration can result in net carbon deficits at the whole-plant level and help explain why tree growth often ceases during heat waves, even when light and nutrient conditions are otherwise favourable.

These temperature-induced changes in A_n are also reflected in leaf morphology and structure: leaves formed under heat stress tend to have lower mass per area (thinner, less dense leaves) and smaller size, traits interpreted as adaptive for heat dissipation but also indicative of carbon limitations during development (Leigh et al., 2017; Poorter et al., 2009). Together, these physiological and structural impairments underscore the challenge plants face under sustained thermal stress: reduced carbon gain, increased respiratory costs, and constrained leaf development.

Despite the detrimental effects of high temperatures, plants can adjust to warmer environments until certain limits. This capacity for thermal acclimation involves coordinated physiological adjustments that help mitigate the negative impacts of heat. For instance, many species down-regulate their basal metabolic activities (such as dark respiration and photosynthetic enzyme concentrations) when grown under elevated temperatures, thereby lowering maintenance carbon costs at the new thermal regime (Way & Yamori, 2014). In parallel, plants may shift the temperature optima of key processes upward: leaves developed in a warm environment often exhibit a higher T_{opt} for photosynthesis compared to those grown in cooler conditions (Crous et al., 2022; Kumarathunge et al., 2019). These acclimatory shifts can help stabilize carbon assimilation rates at warmer growth temperatures, conserving resources and preventing excessive respiratory losses. As a result, warming-acclimated plants sometimes maintain similar daily carbon gain to control plants, a pattern observed in several tree species (Oula Ghannoum

et al., 2010; Way & Oren, 2010) and is common in evergreen conifers, which often show strong photosynthetic homeostasis across temperatures (Yamori et al., 2014). However, in other species acclimation remains incomplete: despite biochemical adjustments (Way & Yamori, 2014). Chronic heat stress often still depresses photosynthetic performance and growth relative to optimal temperature conditions (Atkin & Tjoelker, 2003).

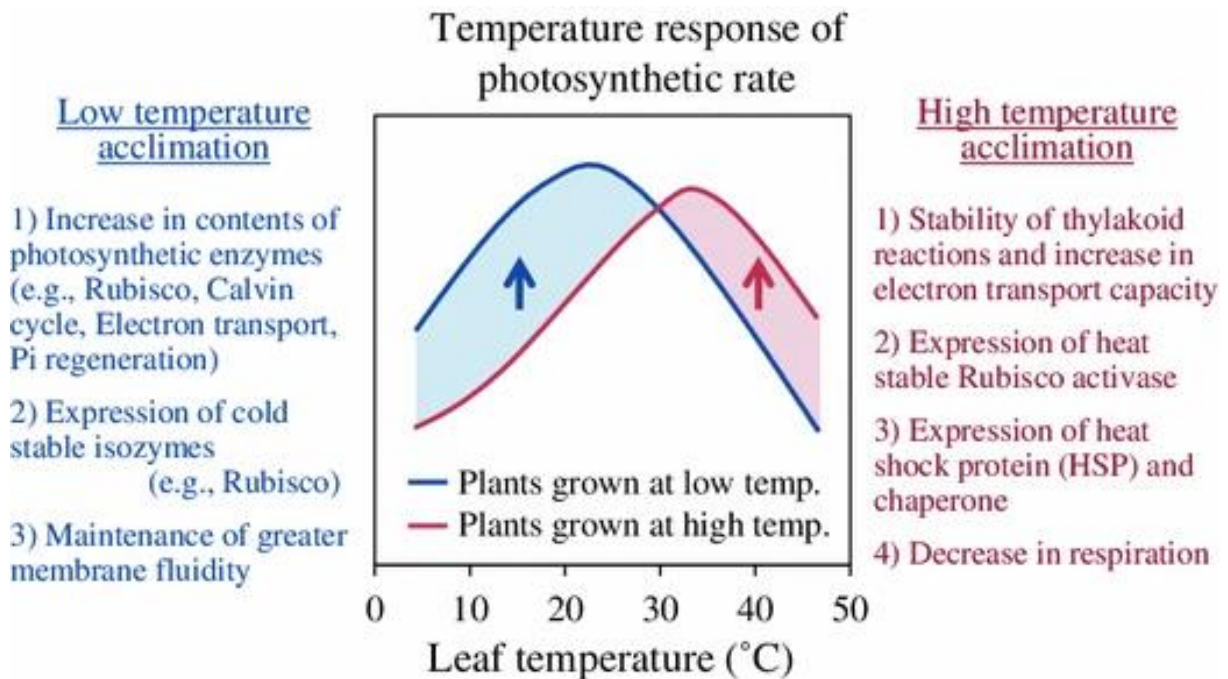


Figure 6. A classic, idealized diagram of temperature acclimation of the response of photosynthesis to temperature. The proposed mechanisms underlying the temperature acclimation of photosynthesis are summarized. Source: (Yamori et al., 2014).

Another critical aspect of heat stress in plants is its impact on water relations and stomatal behaviour. High temperatures and plant water status are closely interconnected, as warmer air can hold more water vapor, leading to an increased vapor pressure deficit (VPD) between the leaf interior and the atmosphere. Under a given stomatal aperture, this elevated VPD results in a substantial rise in transpiration rates as temperature increases (Grossiord et al., 2020).

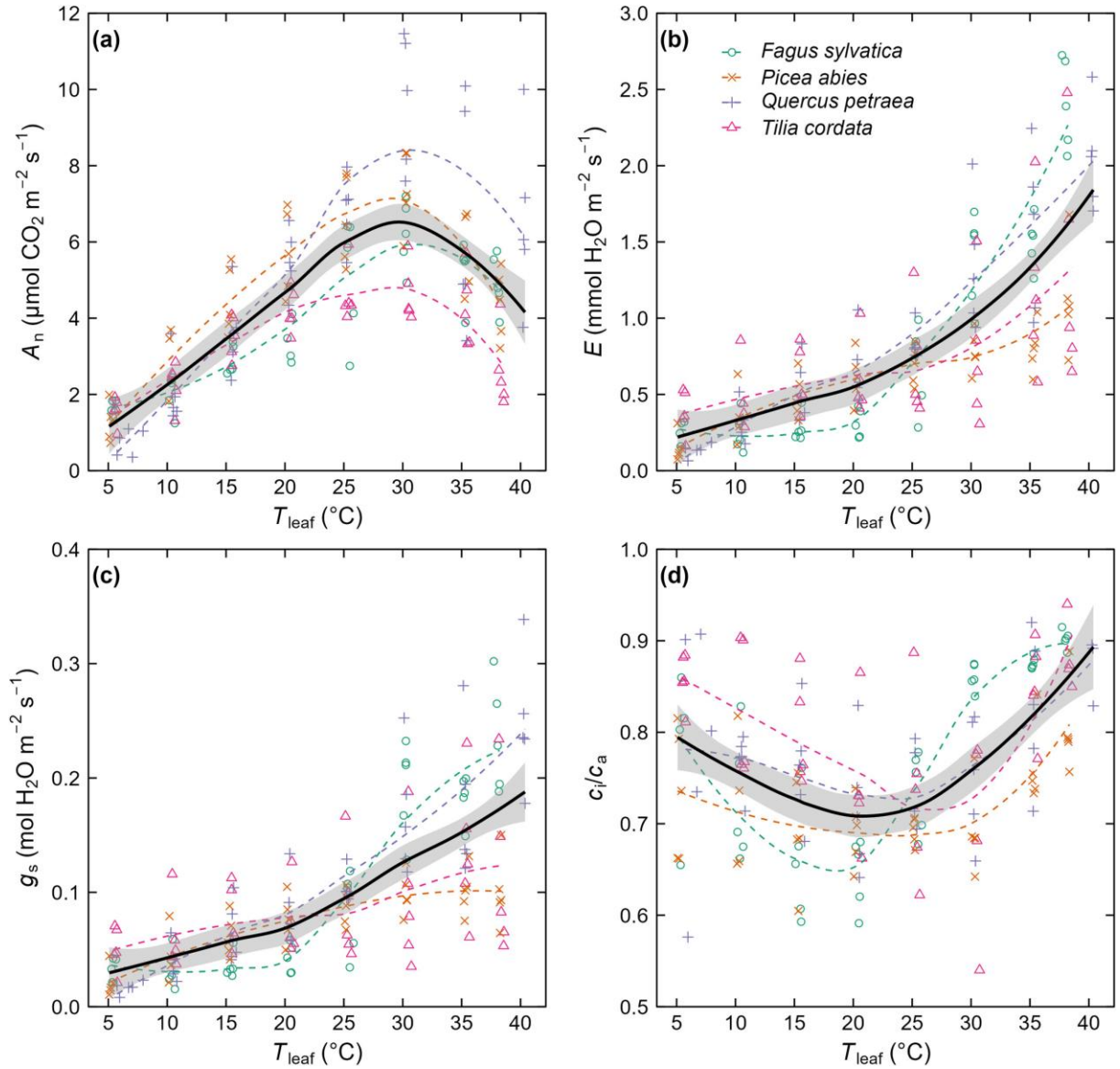


Figure 7. Gas exchange responses to increasing leaf temperature (T_{leaf}) in four temperate tree species. We show net photosynthesis rate, A_n (a); transpiration, E (b); stomatal conductance, g_s (c); and the ratio of intercellular to ambient CO_2 mole fraction, c_i/c_a (d). Each dashed line represents the smoothed trend for one species. The solid line and the grey band represent the smoothed trend averaged across all species and its SE, respectively. Different symbols and colours indicate different tree species ($n = 5$, per step of c. 5°C). Source: (Diao et al., 2024).

In response, plants often open their stomata to facilitate evaporative cooling and protect leaf tissues from overheating. However, this thermoregulatory strategy comes at a significant cost: substantial water loss (Drake et al., 2018; Sadok et al., 2021). Interestingly, many species exhibit a counterintuitive response beyond a certain thermal threshold. Even as photosynthetic capacity (A_n) declines at high temperatures, stomata may remain open or open further (De Kauwe et al., 2019; Marchin et al., 2023), prioritizing thermal regulation over carbon gain (evaporative cooling). This decoupling between photosynthesis and stomatal conductance has

been documented in warm-adapted species, where leaf gas exchange becomes increasingly unbalanced during thermal extremes (De Kauwe et al., 2019; Diao et al., 2024; Drake et al., 2018; Gauthey et al., 2024). Inconsequently, intrinsic water-use efficiency (iWUE, the ratio of carbon gained to water loss) declines, increasing the risk of dehydration at the whole-plant level (Schönbeck et al., 2022).

In essence, plants under heat stress face a fundamental trade-off: keeping stomata open to avoid thermal damage through evaporative cooling, at the cost of accelerated water loss, or closing stomata to conserve, thereby risking excessive leaf heating (Sadok et al., 2021). Both scenarios pose significant risks – continued transpiration under limited soil moisture can lead to hydraulic failure, whereas stomatal closure can result in leaf thermal damage (Gauthey et al., 2024) (Urban et al., 2017b). Thus, stomatal regulation under high temperatures entails a delicate balance among competing physiological demands: enabling CO₂ uptake for photosynthesis, minimizing water loss, and dissipating heat from the leaf surface (Drake et al., 2018; Grossiord et al., 2020; López et al., 2022).

Despite the known importance of stomatal behavior under thermal stress, the capacity of stomata to acclimate to higher evaporative demand (for example, by adjusting their sensitivity, kinetics, or developmental traits such as density) remains an open question (Damour et al., 2010; Martins et al., 2016). Nevertheless, there is growing evidence that heat-stressed plants operate closer to their safety margins and must maintain leaf water potentials above critical thresholds to prevent xylem embolism and irreversible hydraulic failure (Choat et al., 2018).

Some acclimatory responses in plant water relations have been observed under sustained exposure to elevated temperatures. For instance, seedlings grown in warmer environments frequently exhibit a lowered leaf turgor loss point (Π_0), reflecting osmotic adjustment that enhances their ability to retain water under low water potential conditions (Bartlett et al., 2012; Way et al., 2013). By maintaining cell turgor for longer, this adjustment contributes to improved dehydration tolerance during heat- and drought-induced stress episodes, thereby extending the functional range of gas exchange and delaying the onset of physiological failure (Marchin et al., 2022; Mas et al., 2024).

However, it is not yet fully understood to what extent the observed adjustments in plant water relations are driven directly by temperature itself versus indirectly by increase in evaporative demand or soil drying (Aranda et al., 2021; Urban et al., 2017a). Experimental setups where temperature is increased while holding VPD

constant provide valuable insights into the direct effects of heat on plants' water use and stomatal behaviour, independent of VPD- induced atmospheric drought (Day, 2000; Diao et al., 2024; Grossiord et al., 2020; Marchin et al., 2023). Such studies are essential for disentangling the physiological impacts of thermal stress per se from those arising from “atmospheric drought” effects (Garen & Michaletz, 2025; Novick et al., 2016).

In summary, elevated temperature alone can impose significant physiological stresses on forest species. Heat stress can impair photosynthetic function by exceeding thermal optima (Kumarathunge et al., 2019), increase respiratory carbon costs (Atkin & Tjoelker, 2003), alter leaf anatomy and enzyme activity (Crous et al., 2018), and generate conflicting demands on water use (Drake et al., 2018). Crucially, in natural ecosystems, heat stress rarely occurs in isolation. Periods of elevated temperature typically coincide with periods of low rainfall and/or high VPD, meaning that heat is frequently accompanied by drought (Allen et al., 2010). Even well-watered plants may experience functional water limitation during heatwaves due to excessive atmospheric drying. When soil moisture is also limited, the combined effects of heat and drought can be particularly severe, often surpassing the damage caused by either stressor alone (Zandalinas & Mittler, 2022). Thus, having examined the physiological responses of plants to high temperature, it is therefore essential to turn to the second major climate-related stressor impacting temperate forests: drought, and its interaction with heat.

1.3. Physiological responses to drought

Drought, defined as a deficit in soil water availability, exerts immediate and pervasive constraints on plant physiology, primarily by reducing the available water for transpiration and cell expansion. The first response of plants under drought is the closure of stomata to minimize water loss (Martin-StPaul et al., 2017). However, stomatal closure also limits CO₂ uptake for photosynthesis directly reducing carbon assimilation (Flexas & Medrano, 2002). Thus, a fundamental trade-off under drought is that water conservation strategies inevitably constrain the photosynthetic carbon gain. Prolonged or severe droughts lead to cumulative carbon starvation and can trigger metabolic imbalances and growth cessation (Adams et al., 2009). Over extended drought periods, trees may exhaust their stored carbohydrate reserves while maintenance respiration continues, resulting in negative carbon balances. If drought coincides with elevated temperatures, the combined stress can intensify respiratory demand and hydraulic

strain, significantly increasing the risk of mortality (Adams et al., 2009; McDowell et al., 2008). Empirical evidence from around the world (including temperate regions) has linked episodes of “global-change-type drought” – characterized by high heat and VPD along with low soil moisture – to regional forest die-off, underscoring how dangerous drought stress can become under climate change (Choat et al., 2018).

Even in the absence of outright mortality, drought can markedly impair growth. In response to declining water availability, trees reduce their transpirational surface by shedding leaves or producing smaller new foliage, and the growth of new tissues—both in height and diameter—slows or ceases altogether (Feller, 2016; Jin et al., 2011). While drought-stressed leaves often exhibit higher iWUE, this comes at the expense of total carbon uptake gain due to sustained stomatal limitation. Over time, the cumulative reduction in photosynthesis leads to diminished carbon allocation to wood formation, reproductive effort, and root turnover, thereby weakening the plant’s long-term vitality and resilience (McDowell, 2011; Sala et al., 2010). These changes suppose additional trade-offs. In response to drought, plants may alter their allocation patterns by investing more in roots relative to shoots, thereby exploring a larger soil volume (Markesteijn & Poorter, 2009).

There is also a hydraulic safety–efficiency trade-off at play: species with very drought-hardy xylem (high resistance to embolism) often have lower intrinsic hydraulic conductivity, which can slow growth even when water is abundant (McCulloh et al., 2016; Zanne et al., 2010).

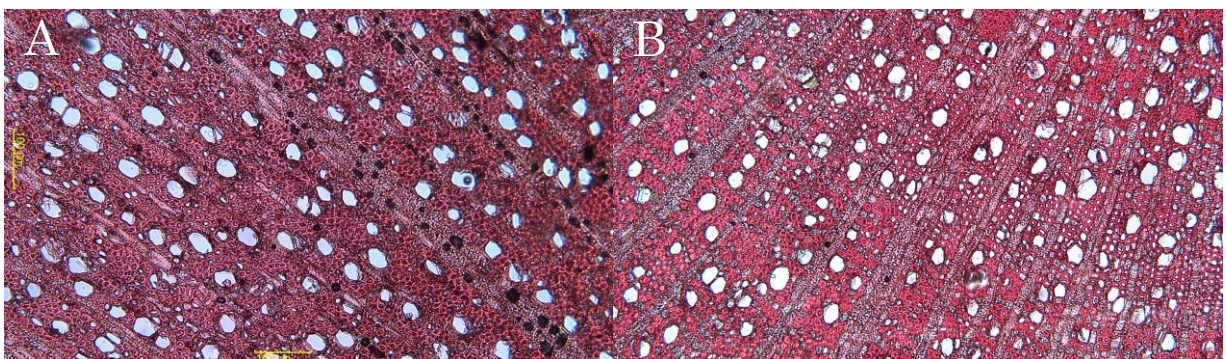


Figure 8. Transverse stem sections stained for xylem anatomy of Fagus sylvatica seedlings. (A) Under well-watered conditions, seedlings show fewer but larger vessels, favouring hydraulic efficiency. (B) Under drought stress, vessel density increases while vessel lumen area decreases, enhancing hydraulic safety by reducing the risk of embolism.

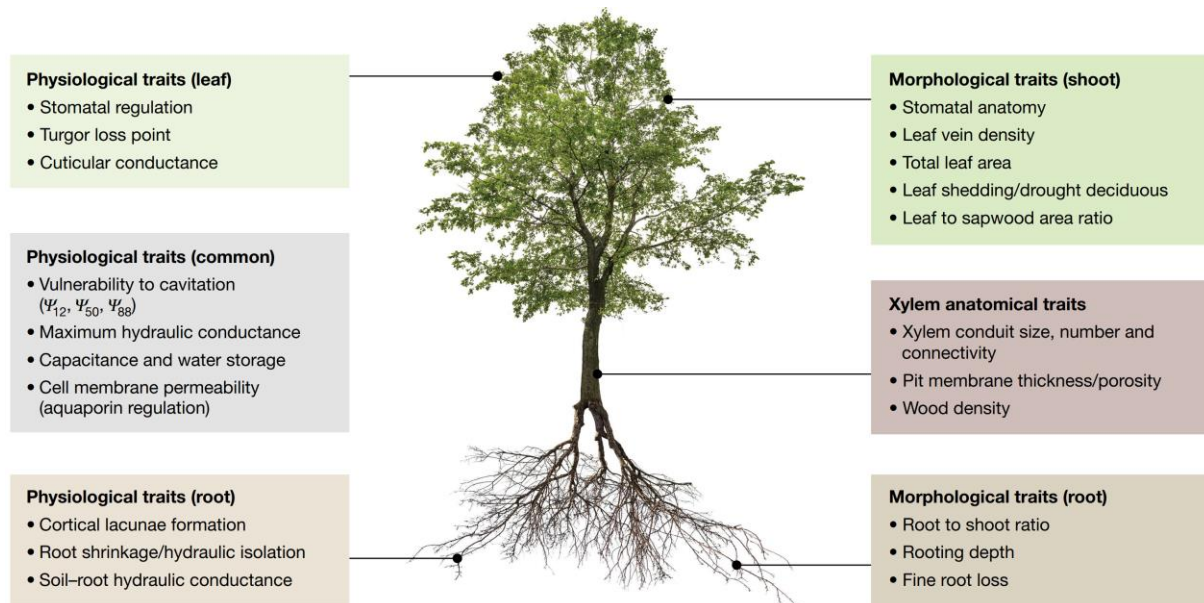


Figure 9. *Summary of key morphological, anatomical, and physiological traits involved in tree drought responses across organs. These traits underlie major trade-offs between hydraulic efficiency, safety, and water conservation strategies. Source: (Choat et al., 2018)*

Beyond these immediate effects, drought can also shift the plant's physiological operating range. The temperature optimum for photosynthesis tends to shift downward: that is, the peak of the photosynthetic temperature response curve occurs at lower temperature in droughted plants compared to well-watered ones (Kruse et al., 2019). This shift is attributed to a drought-induced reduction in internal CO_2 availability, caused by both stomatal closure and decreased mesophyll conductance, which in turn limits the efficiency of photosynthetic processes at high leaf temperatures (Cano et al., 2013; Scafaro et al., 2017).

In parallel to limiting gas exchange, drought also directly impairs the photosynthetic machinery. It reduces V_{cmax} and J_{max} (Bermudez et al., 2021). Under mild to moderate drought, limitations in RuBP regeneration and ATP synthesis primarily account for declines in J_{max} , occurring even before substantial stomatal closure (Flexas & Medrano, 2002; Jia & Gray, 2004; Peña-Rojas et al., 2004). As drought intensifies, these metabolic constraints also affect RuBP regeneration and reduced ATP availability subsequently diminish V_{cmax} (Portis, 2003). The severity of this inhibition varies considerably among species and stress levels (Pankovic et al., 1999; Parry et al., 2002; Pelloux et al., 2001). Ultimately, this biochemical impairment further compounds the limitation imposed by stomatal closure. Given that respiration continues consuming carbon resources, this exacerbates the

imbalance between carbon gain and loss, deepening the plant's carbon deficit under prolonged drought stress.

Drought also induces structural adjustments. For example, experiments have shown that drought lead to higher leaf mass per area (LMA) and reduced leaf area, compounding the reduction in whole plant carbon assimilation capacity (Kumarathunge et al., 2020).

To withstand drought episodes, plants many plants rely on physiological tolerance mechanisms. One key mechanism is osmotic adjustment, in which cells accumulate solutes such as sugars, amino acids, ions to lower their osmotic potential. This enables the maintenance of turgor pressure and physiological function at lower water potentials, allowing cells to remain active under dehydration stress (Abrams & Kubiske, 1990; Aranda et al., 2021; Aranda et al., 2005; Aranda et al., 1996).

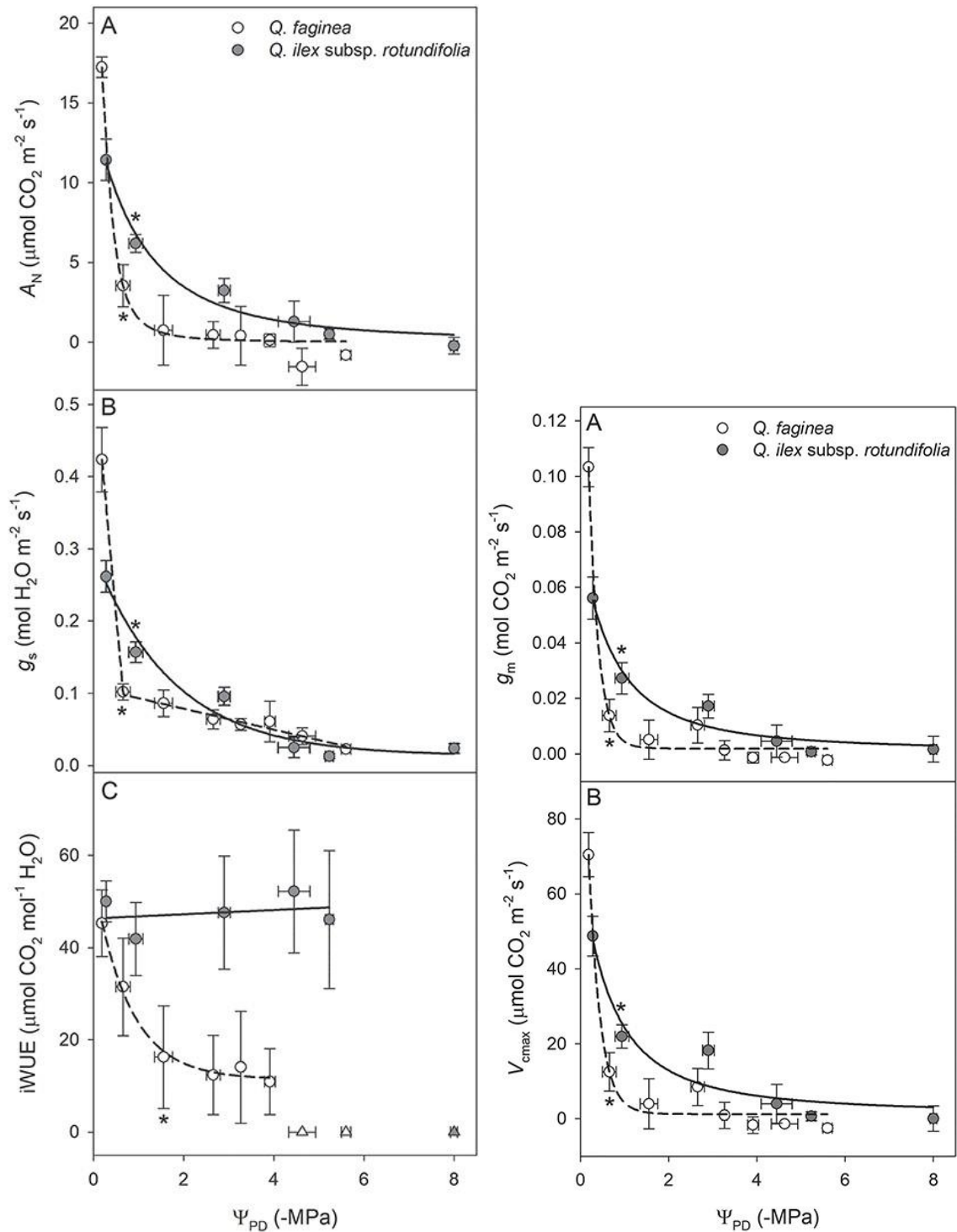


Figure 10. Responses of leaf-level physiological traits to drought in *Quercus faginea* (white symbols) and *Q. ilex subsp. rotundifolia* (grey symbols), plotted against predawn water potential (Ψ_{PD}). Left panels show changes in (A) net photosynthesis (A_N), (B) stomatal conductance (g_s), and (C) intrinsic water use efficiency (iWUE). Right panels show (A) mesophyll conductance (g_m) and (B) maximum carboxylation capacity (V_{cmax}). Data represent means $\pm$ SE across dates during the drought period. Measurements included green, yellow, and brown leaves, and weighted means were calculated per plant based on leaf color proportions. Asterisks indicate significant declines (pairwise t -test, $P < 0.05$)

relative to well-watered controls. Triangles in $iWUE$ indicate unreliable values due to negative A_N and extremely low g_s . Source: (Alonso-Forn et al., 2021).

This shift can be critical for avoiding wilting and sustaining minimal metabolic activity during prolonged dry spells. Additionally, plants may alter their allocation patterns in response to drought by investing more in root growth relative to shoots, thereby exploring a larger soil volume for water (Markesteijn & Poorter, 2009). Such plasticity in biomass allocation is well-documented: drought-stressed seedlings frequently prioritize root elongation at the expense of leaf area, which increases water uptake capacity and delays the onset of hydraulic failure (Magnani et al., 2002; Markesteijn & Poorter, 2009; Mencuccini et al., 2019). However, these adjustments have limits – they cannot fully compensate for extreme water shortage.

There is also a hydraulic safety–efficiency trade-off at play: species with very drought-hardy xylem (high resistance to embolism) often exhibit lower intrinsic hydraulic conductivity, which can limit growth even when water is abundant (Thomas et al., 2004). Conversely, species that prioritize high hydraulic efficiency to support rapid growth under favorable conditions tend to sacrifice hydraulic safety — a strategy that becomes maladaptive under extreme drought (Choat et al., 2018). Drought episodes can therefore push these species toward hydraulic failure if soil moisture drops below the threshold their xylem can tolerate without cavitation.

1.4. Physiological responses to simultaneous heat and drought stress

The combined effects of drought and heat are particularly detrimental to plants, often producing synergistic impacts that exceed the sum of each stress individually (Mittler, 2006; Suzuki et al., 2014). When elevated air temperatures coincide with low soil moisture, interactions between these stressors intensify both water loss and heat accumulation in plants (Zandalinas et al., 2018). Under water-limited conditions, plants are unable to dissipate heat effectively through transpiration, leading to increased leaf temperatures. This thermal buildup further disrupts metabolic processes, such as photosynthesis and respiration, compounding the physiological damage caused by each stressor alone (Teskey et al., 2015; Vile et al., 2012).

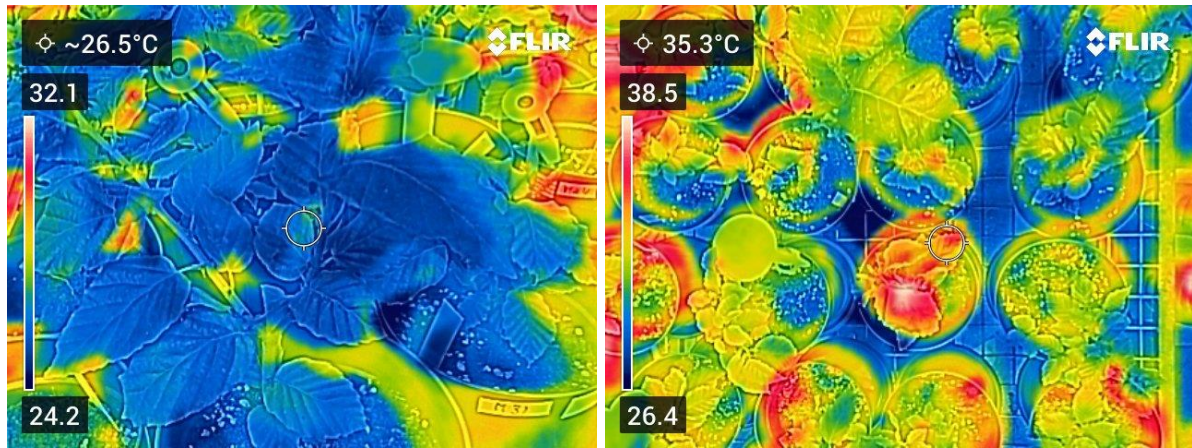


Figure 11. Thermal images of *Fagus sylvatica* seedlings under contrasting watering regimes. Infrared thermography of well-watered (left) and mildly drought-stressed (right) seedlings. The reduction in transpiration under drought conditions leads to diminished evaporative cooling, resulting in significantly elevated leaf temperatures compared to well-watered plants.

Experiments have shown that plants exposed to simultaneous heat and drought show sharper declines in photosynthetic performance and overall vigour than those subjected to either stress individually (Cano et al., 2013; Deluigi et al., 2025; Feller, 2016; Rubio et al., 2025). Under drought conditions, the primary limitation to A_n is diffusive driven by restricted CO_2 availability due to stomatal closure (Flexas & Medrano, 2002). However, when combined with elevated temperature, additional limitations at the biochemical and photochemical levels (e.g., Rubisco kinetics and electron transport) may arise (Flexas & Medrano, 2002). For example, under drought, V_{cmax} is constrained by impaired RuBP regeneration and reduced ATP availability (Kruse et al., 2019). When drought coincides with heat stress, this impairment may intensify, as elevated leaf temperatures—exacerbated by reduced evaporative cooling—further accelerate Rubisco degradation and deactivation (Crafts-Brandner & Salvucci, 2000; Sage & Kubien, 2007; Sage et al., 2008). Additionally, limited CO_2 availability further suppresses Rubisco activity, compounding the biochemical limitation (Flexas & Medrano, 2002). However, the specific mechanisms and severity of this combined limitation remain insufficiently characterized.

Similarly, J_{max} , is also vulnerable under concurrent heat and drought conditions. Elevated temperatures may disrupt the chloroplast redox balance and energy homeostasis, while drought limits the water needed for transpiration-based cooling. Together, these effects can damage the electron transport chain, particularly Photosystem II, and reduce ATP production (Allakhverdiev et al., 2008; Sharkey & Zhang, 2010).

The response of mesophyll conductance (g_m) to combined drought and heat is similarly complex. Under drought alone, g_m typically declines in parallel with A_n , although its relationship with stomatal conductance is variable across species (Niinemets et al., 2009). While stomatal closure predominantly limits A_n in some species, others show substantial reductions attributed specifically to decreased g_m . When drought coincides with elevated temperatures, g_m responses become even less predictable, sometimes increasing and potentially alleviating mesophyll limitations (Flexas et al., 2008; Scafaro et al., 2011).

Moreover, under hot and dry conditions, plants must activate energetically costly protective mechanisms due to the compromised capacity for evaporative cooling (Fahad et al., 2017). These include the induction of heat-shock proteins, antioxidants, and activate non-photochemical quenching pathways to dissipate excess light energy (Baker, 2008; Falcioni et al., 2024). Although these responses protect photosynthetic tissues from irreversible damage, they divert resources away from growth and reproduction (Feller, 2016). In foliage this combined heat and drought can lead to chronic photoinhibition (damage to the photosynthetic apparatus from excess light), prompting enhanced thermal energy dissipation and antioxidant activity (Feller, 2016). While such mechanisms help maintain leaf function, they also highlight the substantial metabolic cost plants incur under compounded environmental challenges (Fahad et al., 2017; Falcioni et al., 2024).

In essence, water deficit amplifies the negative effects of heat: a heat-stressed plant under dry conditions reaches its photosynthetic and growth limits at a lower temperature than a well-watered one. Moreover, recent research reinforces that the molecular and physiological pathways activated by combined drought and heat are complex and intertwined, sometimes creating new stress outcomes (Zandalinas & Mittler, 2022). This interaction helps clarify why drought and heat waves together are especially damaging to forests. When soils dry, even moderately warm days can become physiologically stressful, and extreme heat becomes devastating because the usual coping mechanism (transpiration cooling) is no longer available once stomata are closed (Adams et al., 2009; Park Williams et al., 2013). Under such conditions, leaf temperatures can soar, potentially causing thermal damage (scorching of leaf tissue, protein denaturation) even if the air temperature might not be lethal on its own.

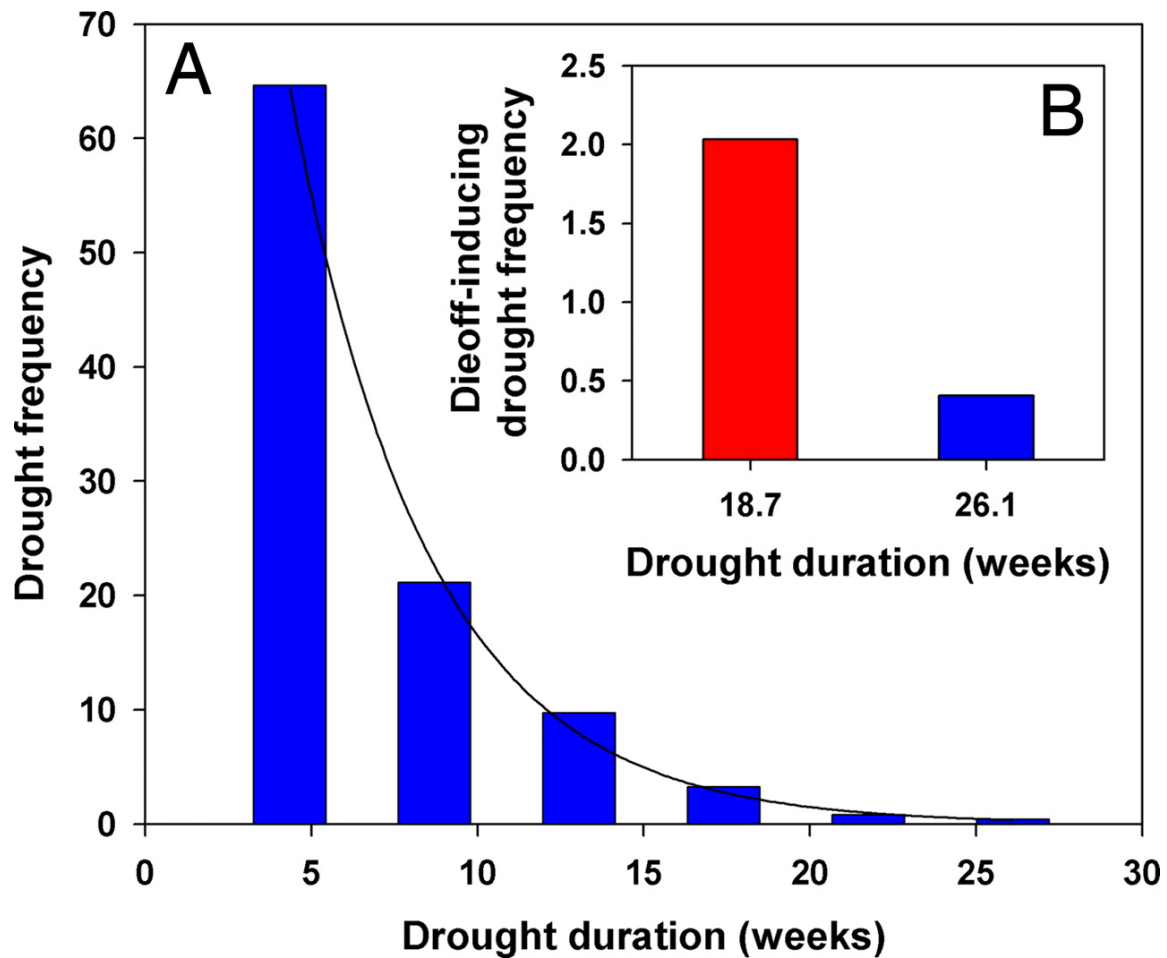


Figure 12. Drought frequency and die-off projections. (A) Duration and frequency of drought events from a 103-year record of regional climate. (B) A comparison of the frequency of a widespread die-off causing drought from the 103-year record (26.1-week regional drought, blue), and under a warmer-temperature, accelerated mortality scenario (18.7-week regional drought, red) for the Four Corners region. Source: extracted from (Adams et al., 2009).

1.5. Physiological responses to light availability

Plants exhibit a remarkable capacity to adjust leaf, shoot, and canopy traits in response to light availability (Niinemets, 2010b), leading to structural and physiological differentiation within the canopy (Larcher et al., 2012). Light is a critical resource, particularly in temperate forest understories, where its availability strongly limits the growth, survival, and competitive ability of juvenile and shade-adapted species (Beaudet & Messier, 1998; Kobe et al., 1995). Shade-tolerant species, such as *Fagus sylvatica*, display a suite of morphological and physiological traits that enhance their performance under low irradiance (Barigah et al., 2006; Cochard et al., 1999; Scartazza et al., 2016; Vega et al., 2020; Welander & Ottosson, 1998). Morphologically, these plants maximize their efficiency for

capturing limited light by developing larger, thinner leaves, with lower leaf mass area (LMA) (Poorter et al., 2009). Additionally, they may prioritize stem elongation to improve vertical light access (Welander & Ottosson, 1998). However, these structural adjustments are typically associated with reduced leaf nitrogen content per unit leaf area, thereby constraining photosynthetic capacity (Poorter et al., 2009).

At the physiological level, prolonged shade leads to a coordinated downregulation of A_n , primarily through decreases in V_{cmax} and J_{max} in a manner that closely tracks the overall decline in A_n (Legner et al., 2013). This proportional downregulation suggests an acclimatory adjustment to prevailing light conditions, wherein leaves optimize their photosynthetic machinery for efficiency at the irradiance level under which they developed (Givnish, 1988; Niinemets et al., 1998)

Under low light, photosynthesis is predominantly limited by energy supply. The reduced photon flux density constrains the electron transport rate, thereby limiting ATP and NADPH production, which in turn restricts RuBP regeneration (Laisk et al., 2005). Additionally, Rubisco activity declines under shade due to both a lower total enzyme content and reduced activation, the latter partly resulting from diminished activity of Rubisco activase, a light-dependent enzyme responsible for maintaining Rubisco in its active form (Portis, 2003; Michael E. Salvucci & Steven J. Crafts-Brandner, 2004). Despite these constraints, shade-adapted leaves exhibit compensatory photochemical responses. They often increase chlorophyll *b* and enlarged photosystem II (PSII) antennae, resulting in larger grana stacks and enhanced capture of diffuse radiation (Anderson, 1986; Lichtenthaler et al., 2007).

Additionally, shade-acclimated leaves often exhibit decreased mesophyll conductance (g_m) (Warren et al., 2007). This decline is primarily driven by anatomical modifications associated with shade conditions, such as expanded intercellular airspaces and reduced mesophyll cell surface area exposed to these spaces, both of which can affect CO₂ diffusion through both the gas-phase and liquid-phase pathways (Flexas et al., 2008; Tosens et al., 2012). The increased diffusion path length through the liquid phase (cell walls, cytosol, and chloroplast stroma), further constrains g_m (Nobel, 2009a). Although thinner cell walls in shaded leaves can partially offset this decline, the overall effect is usually a net reduction in g_m (Niinemets et al., 2009).

Under shaded conditions, both respiration and photorespiration are typically downregulated along with photosynthesis (Niinemets et al., 1998). Lower Rubisco content in shade-acclimated leaves reduces photorespiration, while lower nitrogen content and thinner structure contribute to decreased dark respiration rates (Noguchi et al., 2001; Villar et al., 1995). These coordinated reductions in respiratory processes help mitigate carbon losses under low irradiance and can be considered an adaptive strategy to maintain a favorable carbon balance. The diminished respiratory demand enhances net carbon gain, compensating, to some extent, for inherently low photosynthetic income in deep shade (Niinemets et al., 1998).

Despite these constraints imposed by limited light, shade-tolerant species such as *Fagus sylvatica* display a finely tuned photosynthetic machinery adapted to maximize performance in low-light environments (Cascio et al., 2010). While shade leaves generally exhibit lower absolute assimilation rates than sun leaves, they often reach near-optimal photosynthetic performance relative to their biochemical and structural configuration when exposed to saturating light (Legner et al., 2013; Valladares & Niinemets, 2008).

This suggests a high degree of specialization: leaf traits are optimized not only for survival in continuous low irradiance but also for exploiting transient light events—brief sunflecks that punctuate the forest understory and contribute disproportionately to daily carbon gain (Percy, 1990). Such efficiency reflects a strong evolutionary adaptation to shaded habitats. However, this specialization often comes with trade-offs. Traits that confer advantages under low light may reduce physiological plasticity or limit the ability to cope with other environmental stressors (Aranda et al., 2005; Valladares & Percy, 2002). In particular, the interplay between shade tolerance and vulnerability to drought or heat stress may constrain the resilience and regeneration potential of understory species under future climate scenarios (Niinemets, 2010a; Way & Yamori, 2014).

1.6. Physiological responses to concurring drought and shade

The morphological and physiological traits that enhance carbon acquisition under low light often entail trade-offs that reduce tolerance to other abiotic stresses, notably drought and heat (Bachofen et al., 2020; Valladares & Niinemets, 2008). As mentioned above, shade-grown leaves develop thinner leaves with low LMA and reduced investment in structural reinforcement (Poorter et al., 2009). While this

adjustment improves light interception efficiency, it also increases susceptibility to dehydration and physical damage under stress conditions (Poorter et al., 2009; Wright et al., 2004). Thinner leaves possess less thermal inertia, making them more prone to overheating, and they typically have a higher surface-to-volume ratio, which accelerates water loss.

Shade-acclimated plants also tend to exhibit reduced osmotic adjustment (I. Aranda et al., 2001). This limitation has been attributed to both lower concentrations of compatible solutes (osmolytes) and a reduced need for such adjustment under consistently humid, shaded conditions (I. Aranda et al., 2001; Valladares & Sánchez-Gómez, 2006). Consequently, when exposed to drought, these plants may fail to maintain cell turgor, leading to earlier stomatal closure and loss of hydraulic function. Additionally, low irradiance environments often reduce carbon availability for root development, and shade-acclimated individuals commonly allocate a smaller fraction of biomass to belowground organs (Rodríguez-Calcerrada et al., 2008). While this may be adaptive under stable, moist conditions where water uptake is not limiting, it leaves the plant vulnerable when soil moisture becomes scarce or when evaporative demand increases. A smaller root system limits water uptake capacity and the ability to access deeper soil layers, compromising drought resistance (Smith & Huston, 1989).

Together, these traits—low LMA, limited osmotic plasticity, and a reduced root-to-shoot ratio—reflect a resource-use strategy optimized for carbon gain under low light but constrained in robustness to concurrent stresses. This illustrates a central ecological trade-off: acclimation to shade often reduces resilience to drought or heat, particularly when these stresses occur simultaneously or sequentially (Niinemets, 2010a; Zandalinas et al., 2021).

1.7. Physiological Trade-offs Under Concurrent Shade and Heat

The combined impact of shade and warming imposes a distinct set of physiological challenges on forest seedlings—less acutely lethal than drought yet critically limiting for long-term carbon gain and growth (He et al., 2018). In shaded environments, light is already the most limiting resource. When warming occurs in these low-light conditions, the finely tuned balance of shade-acclimated physiology begins to unravel (Slot et al., 2019).

Shade-tolerant species display morphological and anatomical features which can compromise thermal resilience and reduce hydraulic safety margins (Rubio et al., 2025).

Warming reduces A_n in shaded leaves more than in sun-acclimated ones (Drake et al., 2016), likely because shade-developed leaves generally operate at temperatures several degrees below their optimal temperature (T_{opt}) for A_n (Kumarathunge et al., 2024). As ambient temperatures rise, Rubisco deactivation, ATP limitations, and enzymatic instability become prominent (Allakhverdiev et al., 2008; Feller et al., 1998; Mathur et al., 2014; Pospíšil & Tyystjärvi, 1999; Sharkey & Zhang, 2010). Unlike sun leaves which may benefit from higher thermal buffering and acclimation capacity, shade leaves lack sufficient energy input to counteract heat-induced metabolic constraints (Nie et al., 2025).

At the biochemical level, warming further impairs both Calvin cycle function and photochemical efficiency (Feller et al., 1998; Sharkey, 2005; Yan et al., 2011) (REF). Shade leaves contain less Rubisco and reduced levels of Rubisco activase, both of which are prone to heat-induced deactivation (Slot et al., 2019). Likewise, J_{max} is inherently lower in shade leaves and more vulnerable to heat-induced thylakoid damage (Bukhov et al., 1999).

Mesophyll conductance (g_m) in shaded leaves is already constrained by anatomical limitations (Flexas et al., 2008; Niinemets et al., 2009). Unlike in sun leaves, warming does not stimulate g_m under low PPFD, and although stomatal conductance (g_s) might increase in response to rising temperatures, internal CO_2 diffusion remains a limiting factor. As a result, elevated temperatures do not translate into improved carbon uptake in shaded leaves (He et al., 2018). Simultaneously, dark respiration (R_d) increases exponentially with temperature, while A_n does not scale accordingly in low light (Hernández et al., 2020). This imbalance can lead to negative carbon balance even at moderate warming. In parallel, photorespiration intensifies, further depleting scarce ATP/NADPH and aggravating the carbon deficit (Dusenge et al., 2019). These effects are especially critical in shaded environments where energy reserves are already marginal (Atkin & Tjoelker, 2003).

The energetic cost of photoprotection becomes another critical liability. Shade-acclimated leaves can activate non-photochemical quenching (NPQ) and produce heat-shock proteins (HSPs), in response to heat stress, but both mechanisms are ATP-dependent (Falcioni et al., 2024; Ruban, 2016). Under persistent low light,

energy production is insufficient to maintain these defences, resulting in impaired photoprotection (Ware et al., 2015). Consequently, thermal damage to PSII accumulates faster, and repair processes are slower than in sun leaves (Zivcak et al., 2014).

At the whole-plant level, shade acclimated seedlings exhibit additional vulnerabilities. Thin leaves with low LMA heat up quickly and are more susceptible to desiccation when VPD increases. In parallel, their shallow root systems provide limited capacity for water uptake, thus constraining transpirational cooling under warming (Rubio et al., 2025). Furthermore, a reduced osmotic capacity hampers turgor maintenance during thermal or water stress (I. Aranda et al., 2001), further exacerbating physiological decline under warming.

In summary, the concurrence of low irradiance and elevated temperature generates a compound physiological stress: reduced carbon gain, rising respiratory losses, and diminished capacity for photoprotection and recovery. Unlike the acute effects of drought or heat stress under high light, this interaction manifests as a gradual metabolic imbalance—one that progressively undermines carbon balance and growth. In some situations, though, shade provides some thermal buffering – the understory is cooler and less prone to extreme VPD at midday than open areas, which could help seedlings avoid the worst heat and evaporative demand (He et al., 2018).

For temperate forest regeneration, it is critical to understand these interactions: Seedlings germinating under the canopy will face different climate conditions than those in open clearings. As climate change progresses, altered disturbance regimes (like more frequent canopy gaps from storm damage or fire) might change the light environment of regenerating seedlings, thus altering how they experience heat and drought stresses.

The combined analysis of heat, drought, and shade clearly indicates that all three factors must be considered together to fully understand plant responses in a changing climate. These physiological interactions illustrate the complexity of plant acclimation to multiple, overlapping stresses and provide the foundation for the broader framework of co-limitation and vulnerability discussed in the following section.

1.8. Multiple Stress Effects and Co-limitation: From Leaf Physiology to Canopy Function

In natural ecosystems, plants seldom experience a single environmental constraint in isolation. Temperature, water availability, light, and nutrient supply vary concurrently, producing overlapping limitations that shape acclimation and survival (CITE). The classical *Law of the Minimum* (Liebig, 1840) describes growth as determined by the scarcest resource; however, this principle is rarely sufficient to explain plant behavior under realistic field conditions (CITE). A more comprehensive framework—the concept of *multiple resource limitation*—recognizes that several factors often interactively restrict performance, with their relative importance shifting across space, time, and organizational scale (Bloom et al., 1985; Chapin et al., 1987).

The mechanistic evidence reviewed in the preceding sections illustrates this principle across stress combinations. Under drought, water scarcity constrains evaporative cooling and amplifies thermal stress (CITE); when heat co-occurs, the biochemical machinery of photosynthesis and respiration becomes doubly impaired (CITE). In shaded conditions, limited energy input compromises the capacity to dissipate excess heat or repair damaged photosystems (CITE). Conversely, higher irradiance may alleviate carbon limitation but intensify hydraulic demand (CITE). These examples underscore that plant acclimation emerges not from tolerance to a single stressor but from a dynamic optimization among co-limiting processes that balance carbon gain, water conservation, and photoprotection (CITE).

At the canopy level, these leaf-scale mechanisms integrate into complex microclimatic patterns. Canopy thermoregulation can decouple leaf temperature from ambient air temperature, influencing heat-stress exposure and energy balance within forest stands (CITE). In dense canopies, microclimate heterogeneity—driven by shading, transpiration cooling, and crown structure—creates vertical gradients in temperature and vapor-pressure deficit (CITE). As a result, upper- and lower-canopy leaves often operate under distinct thermal regimes, and the extent of this decoupling can determine the magnitude of whole-canopy photosynthetic acclimation (CITE).

These canopy-scale processes are increasingly recognized as critical for modeling ecosystem carbon and water fluxes. Evidence from recent studies indicates that the thermal acclimation of canopy photosynthesis is widespread across biomes (CITE) and that accurate prediction of forest productivity requires coupling leaf-

level physiology with canopy energy-balance and hydrodynamic models (CITE). Incorporating mechanistic understanding of co-limitation—such as the limits of photosynthetic acclimation under combined heat and drought—into these frameworks will improve projections of forest resilience to climate change (CITE).

Understanding multiple limitation across scales therefore provides a conceptual bridge between mechanistic physiology and ecosystem-level resilience (CITE). It highlights that vulnerability to climate extremes arises when the trade-offs maintaining functional balance—between carbon assimilation, water use, and energy dissipation—can no longer be sustained (CITE). This integrative perspective sets the stage for examining how *Fagus sylvatica*, one of Europe's dominant temperate trees, embodies these principles of co-limitation and trade-off in its responses to environmental change.

1.9. *Fagus sylvatica* L. in a changing climate

European beech (*Fagus sylvatica*) is a foundational species of temperate forests in Europe, dominating much of Central Europe due to its high physiological amplitude and competitive ability (Leuschner, 1998; Leuschner et al., 2006). It occupies a broad range of environmental conditions—from moist Atlantic climates to drier continental interiors—across diverse temperate climate zones and soil types (Fang & Lechowicz, 2006).

Ecologically, beech is characterized by a high shade tolerance – it can successfully regenerate under a closed canopy and often outcompetes more light-demanding species in the understory. This shade tolerance has contributed to beech becoming the climax (late-successional) species in many Central European forests.

However, beech's dominance in mesic, shaded habitats comes with a trade-off of relatively low drought tolerance. This species shows a shallow rooting system and high stomatal sensitivity to water deficit, traits which make it susceptible to soil drying and atmospheric drought (Leuzinger et al., 2005; Schönbeck et al., 2022).

Despite its adaptability, *Fagus sylvatica* is notably climate-sensitive at the margins of its distribution, where drought limits its spread to the south and frost events constrain it to the north (Bolte et al., 2007; Giesecke et al., 2007; Jump et al., 2006). With ongoing climate change, its range is projected to shift northward and to higher elevations (Kramer et al., 2010; Saltré et al., 2015).

At El Hayedo de Montejo, a 125-ha relict forest in central Spain, *Fagus sylvatica* reaches one of its southwestern most distribution limits, growing under a Continental-Mediterranean climate with pronounced summer drought. Despite coexisting with species such as *Quercus pyrenaica* and *Q. petraea*, the local beech population originates predominantly from natural seedlings, underscoring its capacity to persist without vegetative reproduction (Rubio-Cuadrado et al., 2024). However, this provenance exhibits a distinctive ecological behavior, regenerating in a region where only a minority of years meet the climatic thresholds traditionally suitable for beech growth. Summer water stress, combined with increasing temperatures, raises concerns about the long-term viability of these edge populations (Aranda et al., 2002; Peuke et al., 2006; Rubio-Cuadrado et al., 2025).

The presence of mature individuals and ongoing natural regeneration suggest local adaptation, yet their persistence under future climate scenarios—especially with intensifying drought—remains uncertain (Rubio-Cuadrado et al., 2024). This makes the Montejo beech population both ecologically unique and potentially vulnerable to ongoing climatic shifts (Rubio-Cuadrado et al., 2025).

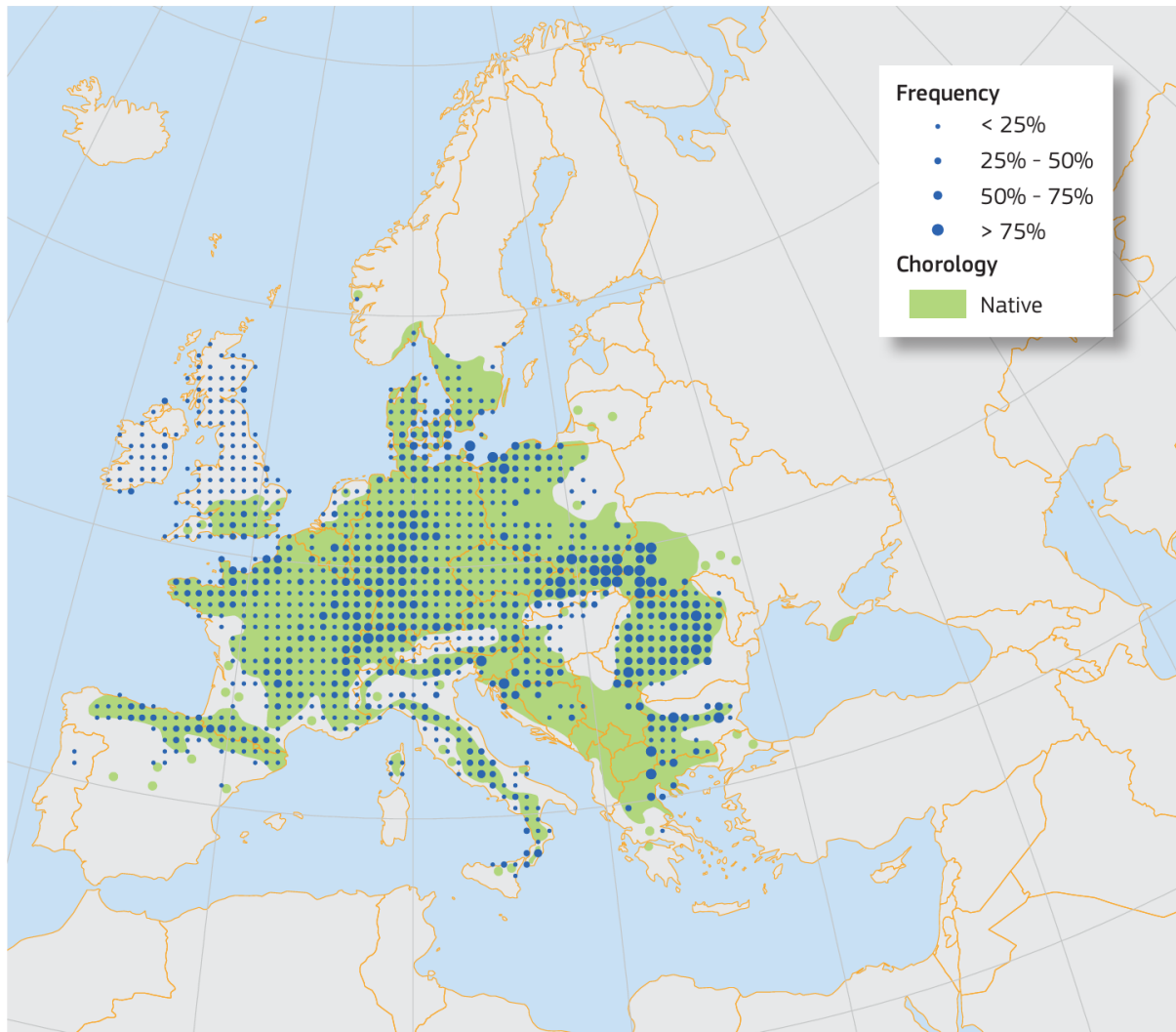


Figure 13. Distribution and chorology map for *Fagus sylvatica* (Houston Durrant et al., 2016). Map elaborated by the European Atlas of Forest Tree Species (San-Miguel-Ayanz et al., 2016).

In the Mediterranean region, at the southern edge of *Fagus sylvatica*'s distribution area, the species is already near its physiological limits for heat and drought stress. There, beech is typically confined to cooler, north-facing slopes or higher elevations, and even in such refuges, recent warming trends have led to regeneration failures (Muffler et al., 2021).

The species' inability to thrive under frequent severe droughts is evidenced by models projecting a substantial contraction of its suitable habitat under future climate scenarios (Geßler et al., 2007; Saltré et al., 2015; Zimmermann et al., 2015).

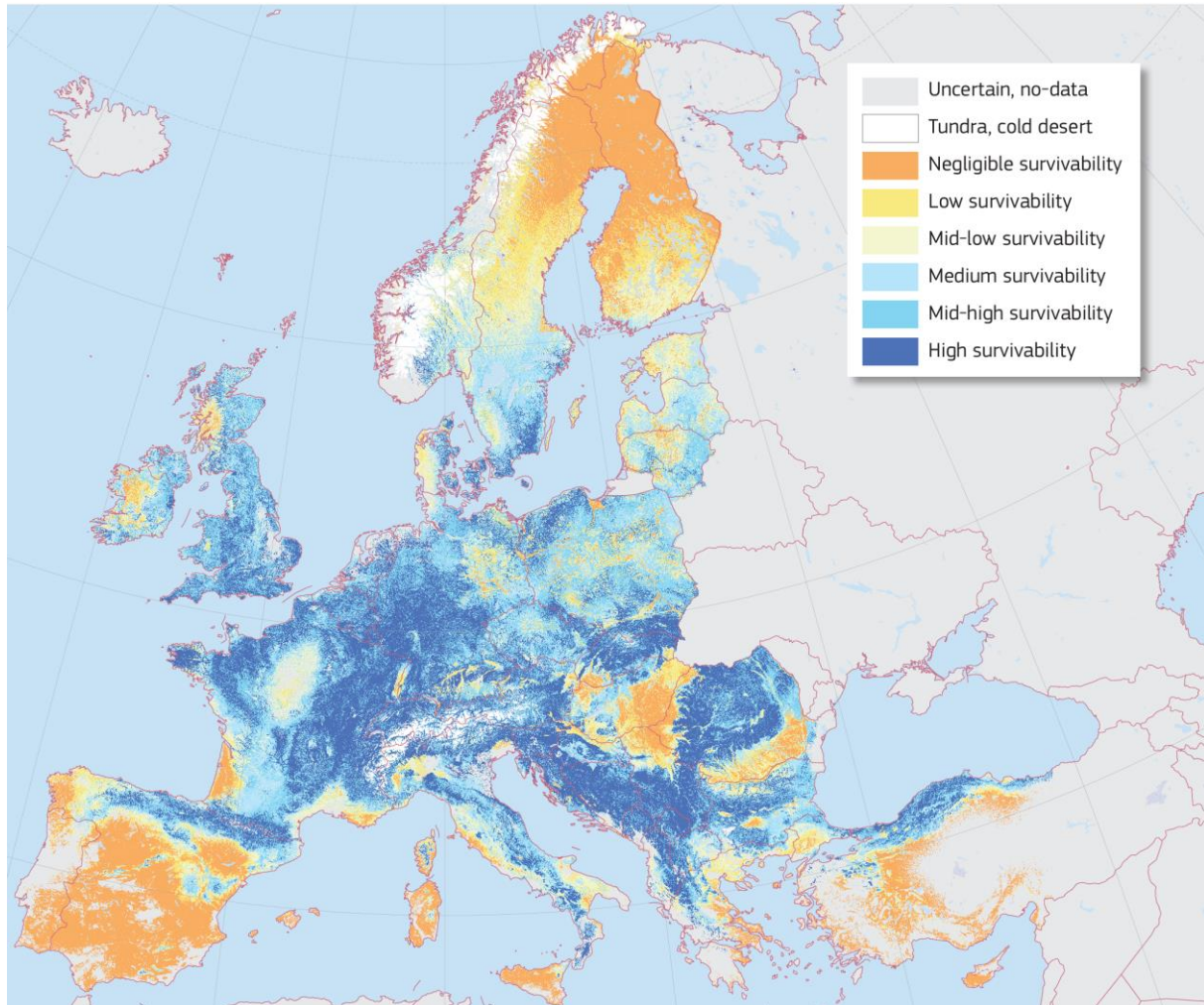


Figure 14. Maximum habitat suitability map for *Fagus sylvatica* (Houston Durrant et al., 2016). Map elaborated by the European Atlas of Forest Tree Species (San-Miguel-Ayanz et al., 2016).

For instance, climate envelope modelling for the Iberian Peninsula predicts major losses in beech forest area as temperatures rise and rainfall patterns change (del Río et al., 2018). Field observations align with these projections: foresters have documented declining growth and increased mortality in some low-elevation beech stands following the intense droughts and heatwaves of the past two decades (Leuschner, 2020; Mas et al., 2024). These observations underscore that *Fagus sylvatica* is among the temperate species most at risk from ongoing climate change in Europe's forests.

While beech is clearly stress-sensitive, its capacity for physiological adjustment at the leaf level is nonetheless notable (Vicente et al., 2022). Shade-acclimated individuals exhibit coordinated changes in morphology and biochemistry that support efficient light capture, although this typically involves a reduction in photosynthetic capacity and a tighter carbon budget (Scartazza et al., 2016). Under water stress, beech tends to limit transpiration early through stomatal regulation, yet this strategy also leads to marked declines in carbon assimilation and internal conductance, amplifying the trade-off between water conservation and carbon gain (Gallé & Feller, 2007). In multi-stress environments, such as hot and dry understories or exposed clearings, these constraints can overlap: warming increases respiratory costs while limiting photosynthesis, especially when light or water availability is suboptimal (Chauhan et al., 2023; Rubio et al., 2025). This imbalance between carbon acquisition and loss under simultaneous stress may reduce growth resilience, particularly in early developmental stages when metabolic margins are narrower (Aranda et al., 2015; Aranda et al., 2000; Aranda et al., 2018).

Although physiological responses to individual stresses like heat, drought or shade and their combinations have been described in detail, there is still limited understanding of how *Fagus sylvatica* integrates multiple simultaneous stressors. Combined effects—such as those involving heat, low light, and water deficit—may interact in non-additive ways that alter photosynthetic regulation, carbon balance, and tissue repair capacity (Deluigi et al., 2025; Wang et al., 2021). The lack of experimental data under such compound scenarios constitutes a critical knowledge gap, particularly with respect to the species' acclimation thresholds and potential trade-offs between resilience and efficiency.

Additionally, some evidence suggests there is intraspecific variability in how beech populations cope with stress: for example, populations from drier, warmer native ranges (e.g. southeast Europe) show slightly better drought resistance and different leaf trait syndromes compared to those from wetter regions, indicating a degree of local adaptation (Aranda et al., 2015). Pan-European summer droughts in 1976 and 2003 had a more pronounced negative impact on growth and canopy health in beech populations from central and northern Europe compared to those in southern regions (Jump et al., 2006, 2007; Peñuelas & Boada, 2003; Pretzsch et al., 2012).

Nevertheless, significant drought-induced growth declines have also been documented at the southern distribution limit of beech, particularly in Italy, Spain, and Croatia during anomalously warm and dry years regions (Jump et al., 2006, 2007; Peñuelas & Boada, 2003; Piovesan et al., 2008). In the Montseny Mountains of Spain, these climatic stresses, compounded by land use change, have led to increasing isolation and degradation of beech stands, resulting in an observed upward shift of the beech forest boundary, with Holm oak (*Quercus ilex*) increasingly replacing beech in lower elevations (Peñuelas & Boada, 2003).

At the Hayedo de Montejo, forest composition has shifted markedly over the past century, with *Fagus sylvatica* gradually regaining dominance following a mid-20th century expansion of *Quercus pyrenaica* after grazing was banned (Rubio-Cuadrado et al., 2024). The progressive closure of the canopy has favored shade-tolerant species, especially beech, which now exhibits strong regeneration and is expected to continue expanding in areas with deeper soils and gentler slopes (Rubio-Cuadrado et al., 2024). Although this rear-edge population of *Fagus sylvatica* has demonstrated unexpected vigor and resilience, especially under competitive light-limited conditions, its sensitivity to drought and late frosts poses a growing risk under intensifying climate stress (Rubio-Cuadrado et al., 2024; Rubio-Cuadrado et al., 2025). Future disturbances such as heatwaves could undermine this balance, particularly affecting older beech individuals already weakened by senescence and past land-use practices (Muffler et al., 2021; Rubio-Cuadrado et al., 2025; Zimmermann et al., 2015).

This genetic and phenotypic diversity offers a glimmer of hope that beech might harbour the capacity to adjust to new conditions, at least over the long term through natural selection. However, within the short time frame of the coming decades, beech forests will largely depend on phenotypic plasticity – the ability of individual trees and seedlings to adjust their physiology and morphology to buffer against climate stress – rather than on evolutionary change. The question remains: *How well can beech seedlings acclimate to the combination of increased heat, periodic drought, and varying light environments?* This question is at the heart of the present dissertation.

In this thesis, we focus on the mechanisms of acclimation in *Fagus sylvatica* under a high-end climate change scenario, examining how seedlings respond when exposed to multiple concurrent stresses. Building on the context outlined above, we recognize that future beech regeneration will likely occur under warmer

conditions, potentially drier soils, and either shaded understories or more open conditions following disturbances.

To address this, a series of controlled experiments were designed to simulate these factors and observe beech's responses. Specifically, we exposed one-year-old *Fagus sylvatica* seedlings to factorial combinations of elevated temperature, water deficit, and low light (shade) versus their optimal conditions, while carefully controlling atmospheric humidity to isolate the direct effects of warming. This approach allowed us to disentangle heat stress effects from drought-induced effects – a critical distinction, as highlighted earlier – and to assess any interactive effects with shade.

2. Objetives and hypothesis

This thesis is organized into six main sections: a general introduction, a comprehensive methodology, three experimental chapters —each addressing specific objectives and formatted as scientific articles—, a general discussion, and final conclusions. While drought and shade have received considerable attention as constraints on forest regeneration, the physiological impacts of warming — particularly in combination with co-occurring stresses — remain far less understood. This thesis addresses this knowledge gap by examining how elevated temperature alone, and in combination with water limitation and reduced light availability, influences the acclimation capacity of European beech (*Fagus sylvatica*) seedlings. The first experimental chapter investigates the thermal acclimation of leaf photosynthesis and stomatal regulation across a wide range of temperatures. The second chapter evaluates how leaf-level acclimation — specifically photosynthetic performance, respiratory costs, and stomatal dynamics — is shaped by the combined effects of heat, drought, and shade. The third chapter extends the analysis to whole-plant growth, water relations, and xylem hydraulic traits, thereby linking leaf-level responses to overall seedling performance and adaptive strategies.

The main objective of this doctoral thesis is to elucidate the physiological mechanisms underlying the acclimation capacity of *Fagus sylvatica* seedlings under a high-end climate change scenario characterized by warming, drought, and shade. In light of the increasing exposure of rear-edge beech populations to multiple abiotic stressors, this research seek to deliver an integrated assessment of photosynthetic, hydraulic, and morphological responses of juvenile individuals under controlled environmental conditions.

The specific objectives of the thesis are:

1. To evaluate the photosynthetic acclimation of beech seedlings to warming: how elevated growth temperature modifies photosynthetic capacity, thermal optima, and respiratory costs in beech seedlings, and whether acclimation involves biochemical, stomatal, or mesophyll adjustments..
2. To assess the interactive effects of warming and water limitation on carbon balance, photoprotective mechanisms, and hydraulic safety margins, identifying trade-offs between assimilation and stress tolerance..

3. To examine how light intensity modulates physiological and morphological acclimation, testing whether shade buffers or intensifies the effects of warming and drought on carbon and water economy..
4. To quantify how warming, drought, and shade, alone and in combination, affect xylem function, leaf morphology, and biomass allocation, and how these traits mediate the balance between acquisitive (growth-oriented) and conservative (stress-tolerant) strategies..
5. To synthesize responses across photosynthesis, water relations, and morphology, testing whether the combined effects of warming, drought, and shade are additive, synergistic, or antagonistic and to identify critical thresholds and trade-offs that may affect the resilience and regeneration capacity of beech forests under future climate conditions.

Based on this framework, we propose the following general hypotheses:

H1 (Photosynthetic acclimation to warming): Elevated growth temperature reduces photosynthetic capacity through biochemical downregulation ($V_{\max}$, $J_{\max}$), while maintaining thermal optima. This adjustment is accompanied by lower respiration and enhanced non-photochemical quenching, reflecting a shift toward carbon conservation and photoprotection.

H2 (Warming $\times$ drought): Under combined heat and drought, stomatal closure and impaired osmotic adjustment reduce carbon balance efficiency. Plants prioritize photoprotection over assimilation, leading to narrower hydraulic safety margins.

H3 (Role of shade): Shade-grown seedlings maintain photosynthetic efficiency with lower respiration rates, showing effective acclimation to low irradiance. However, under warming they shift toward acquisitive strategies (greater leaf deployment, lower LMA) at the expense of water-use efficiency.

H4 (High light responses): In high light, warming induces a conservative strategy characterized by reduced leaf area and biomass but elevated transpiration, thereby enhancing drought avoidance and hydraulic safety.

H5 (Morphological and hydraulic adjustments): Structural traits (xylem anatomy, LMA, biomass allocation) mediate trade-offs between acquisitive and conservative strategies, shaping seedling performance under different stress combinations.

H6 (Integration across stressors): The interaction of warming, drought, and shade generates non-additive effects, producing unique response syndromes (e.g.,

combined reductions in leaf area and hydraulic safety) that cannot be predicted from single-factor experiments.

3. General Materials and Methods

The common methodology used throughout this thesis is detailed below. A method is considered common if it is applied in at least two different studies. Study-specific methodologies or techniques are described separately in their respective sections.

3.1. Plant Material

Experiments were conducted on European beech (*Fagus sylvatica*) seedlings derived from seeds collected in a sub-Mediterranean beech forest at the southern limit of the species' range. Beech nuts were gathered in autumn 2021 from the Natural Reserve Hayedo de Montejo in central Spain (UTM ETRS89: 458,249 E; 4,551,615 N). This site is a montane mixed forest reserve where European beech is one of the dominant species, though *Quercus pyrenaica* is currently the most abundant. The area experiences a Continental-Mediterranean climate, with a mean annual temperature of 9.5 °C, approximately 900 mm of rainfall, and a marked summer dry period. The soil is classified as humic cambisol with an average depth of 50 cm, allowing for significant water storage during dry spells.

The collected seeds underwent cold stratification ($\approx 4^{\circ}\text{C}$, dark, ~ 3 months) to break dormancy before germination. After stratification, seeds were sown in February 2022. Each seed was planted in a 9 L plastic pot filled with a 75:25 (v:v) peat and sand mixture. A slow-release fertilizer was incorporated into the substrate of each pot (Nutricote® 16–4.4–8.3 N–P–K, at 2.5 g L^{-1}) to ensure baseline nutrient availability. Following germination, seedlings were grown under controlled environmental conditions as described below, until they reached the experimental size: height ($9.8 \pm 0.6\text{ cm}$), diameter ($4.2 \pm 0.2\text{ mm}$) and number of fully developed leaves (3 ± 0). All seedlings used in the experiments were from the same provenance and age cohort.

3.2. Growth Conditions and Experimental Design

Seedlings were grown in walk-in growth chambers under tightly controlled climate conditions designed to simulate present-day and future summer environments. Two identical growth chambers were used, each assigned a different temperature regime but kept at the same atmospheric moisture (vapor pressure deficit, VPD). One chamber was maintained at a day/night temperature of 25/15 °C (moderate, near-current ambient) with $\sim 40/68\%$ relative humidity (resulting in VPD $\sim 1.9/0.86$

kPa), and the other at 32.5/22.5 °C (elevated by $\sim +7$ °C) with $\sim 61/80\%$ relative humidity (also $\sim 1.9/0.86$ kPa VPD). Both chambers were on a 12 h light / 12 h dark photoperiod. Illumination during the light period was provided at a photosynthetic photon flux density (PPFD) of approximately $900 \mu\text{mol m}^{-2} \text{s}^{-1}$ at canopy height, simulating full sunlight conditions in the understory. Temperature and humidity were adjusted to simulate a high-end warming scenario for the region (approximately $+7.5$ °C), while maintaining equivalent vapor pressure deficit (VPD) across chambers. This control of VPD was not intended to replicate realistic climate projections—in which VPD would naturally increase with temperature—but rather to isolate the specific physiological effects of elevated temperature, independent of changes in atmospheric dryness.

All seedlings were initially kept well-watered to field capacity to promote healthy growth. Plants were irrigated daily to full soil water capacity throughout the initial acclimation period of 1 month. In the two multifactorial studies, additional stress treatments were imposed after this initial growth. A subset of 33 plants was subjected to soil drying to induce drought. In those experiments, watering was withheld until plant water status declined substantially – operationally when seedling stomatal conductance dropped to roughly one-third of that in well-watered controls. Thereafter, a minimal watering schedule was applied (every few days with a fixed volume corresponding to about one-third of normal daily evapotranspiration) to maintain a moderate drought condition over time. This approach ensured a controlled, steady level of water stress without causing acute wilting. Other environmental factors were held constant during drought imposition. For these multifactorial studies, a subset of seedlings was also grown under reduced light ($\sim 200 \mu\text{mol m}^{-2} \text{s}^{-1}$) using neutral shade nets to simulate understory shade. All seedlings were exposed to the same temperature and photoperiod regardless of light treatment.

To avoid positional biases and chamber effects, a randomized block design was employed in each chamber. Seedlings were randomly arranged within each growth chamber and re-positioned weekly to minimize any within-chamber light or temperature gradients. Moreover, chamber swapping was performed: every three weeks: plants were rotated between the two chambers while maintaining their assigned temperature treatment. In practice, a set of seedlings growing at 25 °C in one chamber would be moved to the other chamber (which was set to the same temperature regime) and vice versa. This rotation controlled for any slight differences between chambers (such as light distribution or airflow), effectively

distributing chamber effects evenly across treatments. Overall, the experimental design ensured that the only systematic differences between seedling groups were the imposed treatment factors (temperature regime, and when applicable, water availability and/or light level), thus allowing rigorous comparisons of physiological responses across studies.

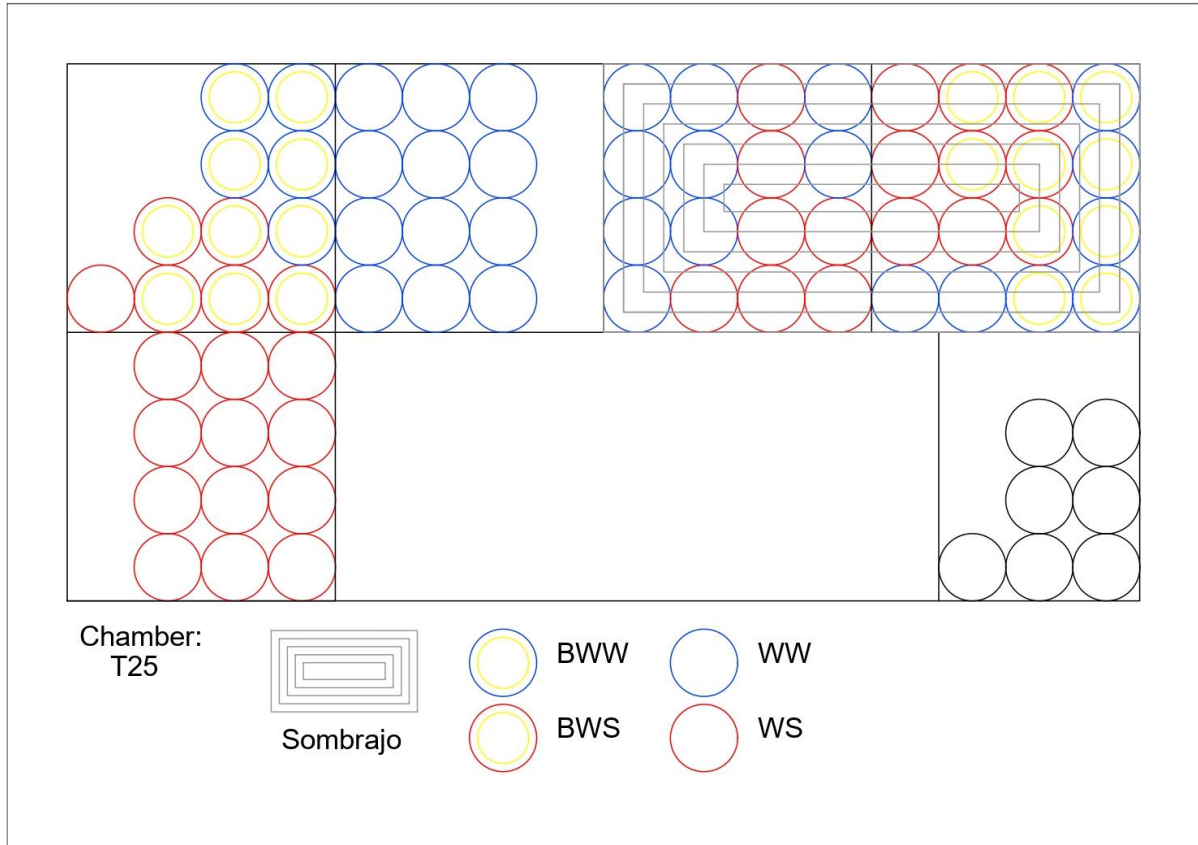


Figure 15. Schematic layout of pot distribution for the factorial stress treatments under the 25 °C growth chamber (T25). Blue circles represent well-watered treatments, while red circles indicate drought treatments. Yellow outlines correspond to beech seedlings destined to destructive measurements for biomass quantification (BWW and BWS), and solid-coloured circles indicate beech seedlings destined to non-destructive measurements. Grey rectangles ("Sombrajo") indicate low-light conditions applied to a subset of seedlings. The same layout was mirrored in a separate chamber simulating a warming scenario at 32 °C (T32).



Figure 16. *Fagus sylvatica* seedlings at the end of the acclimation period under factorial combinations of temperature, light, and water availability. Seedlings are organized in alternating horizontal rows, with **T32 (elevated temperature)** in the **first and third rows**, and **T25 (control temperature)** in the **second and fourth rows**. The **left side** of the chamber corresponds to **high light**, and the **right side** to **low light** conditions. Water treatments are color-coded: **blue labels** indicate **well-watered** seedlings and **red labels** indicate **water-stressed** ones. Treatment codes: HW (High light, Well-watered), HS (High light, Stressed), LW (Low light, Well-watered), LS (Low light, Stressed). Visual differences in leaf area, height, and senescence illustrate the cumulative effects of the treatments, with warming (T32) amplifying stress symptoms under drought and/or shade, while promoting greater evaporative demand under high light and ample water.

3.3. Gas Exchange and Chlorophyll Fluorescence Measurements

Gas exchange and chlorophyll fluorescence were measured on fully expanded, mature leaves to assess photosynthetic performance under each treatment. Measurements were conducted on intact leaves in situ using a portable open-path gas-exchange system (LI-6400XT, LI-COR Inc., USA) equipped with an integrated fluorescence leaf chamber (LI-6400-40). For each treatment group, a representative sample of seedlings (typically 4–6 individuals per chamber in each

experiment) was selected for detailed measurements. Prior to measurement campaigns, the instrument console was placed inside the respective growth chamber for at least one hour to allow all equipment to thermally equilibrate with the chamber environment. During measurements, the leaf cuvette conditions were set to mirror the seedling's growth conditions: for example, leaves of seedlings from the 25°C treatment were measured at ~25 °C leaf temperature and ~40% RH, whereas leaves from the 32.5°C treatment were measured at ~32°C and ~60% RH. In all cases, the reference CO₂ concentration in the cuvette was maintained at 400 μmol mol⁻¹ (near ambient CO₂) with a constant flow rate of 300 μmol s⁻¹. Cuvette leaf temperature was precisely regulated by adjusting the chamber block temperature to the desired set-point, and leaf temperature was monitored to ensure stability. Illumination in the cuvette was provided by built-in red/blue LED light sources. Actinic light intensity was set to saturating levels (approximately 1,200 μmol photons m⁻² s⁻¹, with ~10% blue light) for measurements under high-light conditions. This ensured that photosynthesis was light-saturated during gas exchange measurements. (For any low-light treatment plants, a proportionally lower actinic PPFD (~800 μmol m⁻² s⁻¹) was used to match their growth light level.) All gas-exchange measurements were made under near steady-state conditions, meaning each leaf was allowed to equilibrate at the set conditions until fluxes stabilized before data were recorded.



*Figure 17. LI-6400XT Portable Photosynthesis System with Leaf Chamber Fluorometer (LCF). This advanced gas exchange and fluorescence system is designed to quantify photosynthetic responses under controlled conditions. The labeled components represent the core functional architecture that defines how physiological data are acquired and interpreted. Key modules include the **console with integrated digital interface** (user input and data logging), **cable assembly** (signal and power transmission), **chemical tubes** (air scrubbing and conditioning), and the **optional CO₂ cartridge and regulator** (precise control of reference gas concentrations). The **6400-40 Leaf Chamber Fluorometer** (optional) and **sensor head/IRGA** form the analytical core, enabling simultaneous measurement of CO₂, H₂O, and chlorophyll fluorescence—providing a comprehensive readout of gas exchange and photochemical efficiency.*

Chlorophyll *a* fluorescence was recorded simultaneously with gas exchange for each leaf using the multiphase flash fluorescence technique. Steady-state fluorescence (F_s) was measured during light exposure, and a saturating flash was applied to determine light-adapted maximum fluorescence (F_m'). Immediately afterward, a brief (~3 s) far-red light was given to obtain the minimal fluorescence in light-adapted state (F_0'). These values allowed calculation of the effective

quantum yield of photosystem II ($\Phi_{\text{PSII}} = \Delta F/F_m'$, where $\Delta F = F_m' - F_s$) for each leaf under ambient light. Following gas exchange measurements, leaves were dark-acclimated (typically for 20 minutes) to measure dark-adapted fluorescence parameters: the minimal fluorescence F_0 (after dark acclimation) and the maximal fluorescence F_m (via a saturating flash in darkness). From these, the maximum quantum efficiency of PSII photochemistry was calculated as $F_v/F_m = (F_m - F_0)/F_m$, to check the photochemical integrity of leaves. Additional fluorescence-derived indicators were computed, including the fraction of open PSII reaction centers (qL) and the extent of non-photochemical quenching (NPQ), following standard formulas. All gas exchange and fluorescence readings were taken on the same portion of each leaf (avoiding major veins) to ensure consistency, and care was taken that leaves remained horizontal and unshaded during measurements.

To comprehensively characterize photosynthetic responses, a series of measurement protocols was applied uniformly across relevant studies. After initial steady-state gas exchange and fluorescence readings at the growth condition, leaves were subjected to a sequence of controlled assays: (i) a light-response curve under low O_2 , (ii) a measurement of dark respiration, and (iii) an assimilation–internal CO_2 (A_n-C_i) response curve. In practice, following the initial stabilized measurement at saturating light, the reference air was switched to a hypoxic gas mix (1% O_2 , balance N_2) to suppress photorespiration. Under this 1% O_2 atmosphere, a photosynthetic light-response curve (A_n –PPFD curve) was conducted by stepwise decreasing the light intensity from the saturating level down to low light (in ~8–10 steps) and logging net assimilation at each step once steady state was reached. This allowed determination of light-response parameters and facilitated calibration of electron transport measurements (see below). Afterwards, the cuvette was returned to ambient air (21% O_2), and the leaf was darkened to measure dark respiration (R_d). Leaves were kept in darkness for ~20 min and the net CO_2 efflux was then recorded as R_d , while simultaneously measuring the dark-level fluorescence to obtain F_0 and F_m in darkness. Following the dark period, the leaf was re-illuminated at saturating light and a CO_2 response curve (A_n-C_i curve) was performed under ambient O_2 . Starting from 400 ppm CO_2 , the reference CO_2 concentration was sequentially lowered (to 300, 220, 150, 100, 70 ppm) and then raised above ambient (350, 600, 900, 1000, 1200 ppm), with measurements taken at each step after stabilization. These A_n-C_i curves were later used to derive key photosynthetic parameters as described below. In some experiments, to avoid any potential effects of prolonged chamber exposure on the

leaves, certain response curves were repeated on a second day or with a second calibrated IRGA system to verify consistency.

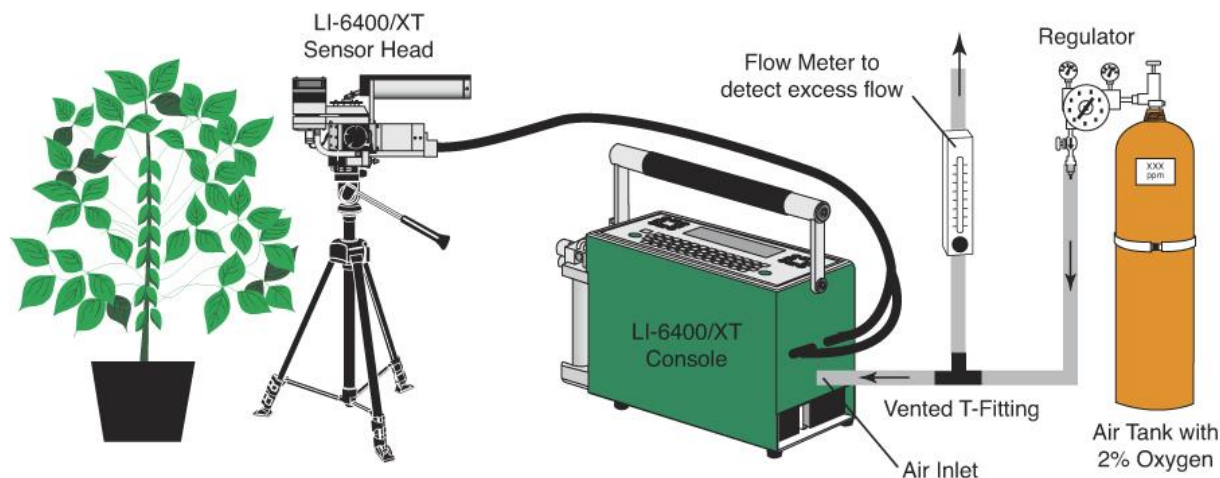


Figure 18. Experimental setup for gas exchange measurements under photorespiration-suppressing conditions using the LI-6400XT system. The system illustrates the use of a reference gas with low oxygen concentration (e.g., 2% O_2) to suppress photorespiration during measurements. Air from the **tank with 2% O_2** is regulated and directed to the **LI-6400XT console** via the **air inlet**, passing through a **vented T-fitting** to maintain appropriate flow and pressure. A **flow meter** detects excess flow to avoid overpressure. The modified air is then delivered to the **sensor head** enclosing the leaf, enabling accurate quantification of photosynthetic CO_2 assimilation under low photorespiratory conditions. This setup is commonly used to isolate Rubisco carboxylation activity and evaluate mesophyll conductance or chloroplastic CO_2 concentrations.

From the gas exchange and fluorescence data, we computed several derived parameters that were used consistently across studies. Apparent maximum carboxylation capacity of Rubisco (V_{cmax}) and the maximum rate of electron transport (J_{max}) were obtained by fitting the Farquhar biochemical model of photosynthesis to the A_n-C_i curves. The standard Farquhar model equations (Farquhar *et al.*, 1980) with appropriate temperature corrections and established modifications (Sharkey, 1985; Wullschleger, 1993) were used for this purpose. Model fitting yielded V_{cmax} and J_{max} at a reference temperature (often 25°C) for each treatment, as well as estimates of mitochondrial respiration in light (R_l) and other parameters, which were compared between warming treatments. Mesophyll conductance (g_m) was calculated using the combined gas exchange and fluorescence (variable J) method. In brief, the electron transport rate (J_f) inferred from chlorophyll fluorescence (using Φ_{PSII} and leaf absorbed light) was calibrated against the rate of CO_2 assimilation under low O_2 , allowing the estimation of g_m by accounting for the balance of electron transport and carbon assimilation. This method, based on Harley *et al.* (1992), provided an estimate of the internal CO_2 diffusional conductance from intercellular spaces to the chloroplast. Additionally,

water-use efficiency metrics were evaluated: intrinsic water-use efficiency (iWUE, the ratio of net CO₂ assimilation to stomatal conductance) and instantaneous water-use efficiency (WUE, the ratio of assimilation to transpiration) were calculated under light-saturated conditions. These indices helped compare how treatments affected the trade-off between carbon gain and water loss. All gas exchange data were corrected for any potential leakage of CO₂ in the IRGA system and for the small but non-zero cuticular conductance (using the independently measured $g_{\min}$) to ensure accuracy.

3.4. Leaf Morphological and Chemical Analyses

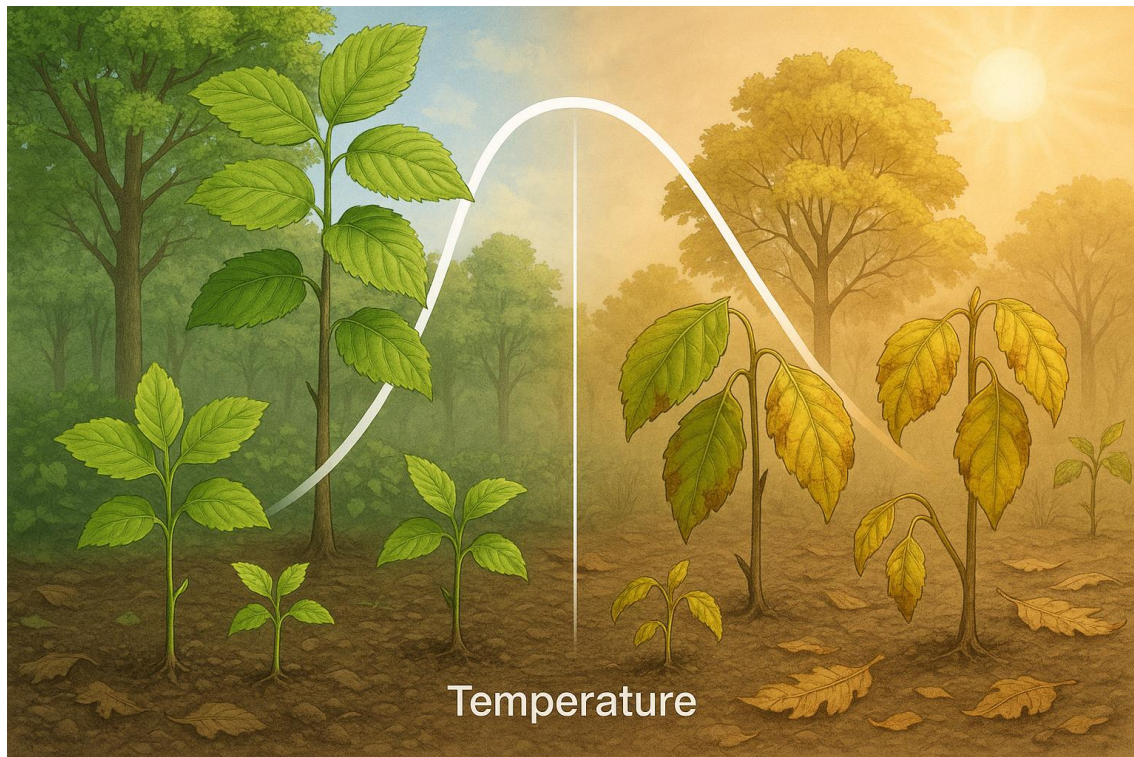
In each experiment, a set of leaf structural and chemical traits was measured to link physiological responses with plant functional characteristics. Specific leaf area (SLA) – often expressed as leaf mass per area (LMA, the inverse of SLA) – and leaf nitrogen content were determined on sampled leaves, and minimum leaf conductance ($g_{\min}$, cuticular conductance) was measured as an index of cuticular water loss. These measurements were made on healthy, fully expanded leaves (typically the same leaves or same plants used later for gas exchange, to ensure correspondence) and followed identical protocols across studies.

To measure minimum epidermal conductance ($g_{\min}$), an excised-leaf drying method was used. Approximately 1–2 days before gas exchange measurements, leaves were harvested from experimental plants (usually 4–6 leaves per treatment) and immediately sealed in plastic bags with moist paper to prevent water loss until analysis. In the laboratory, petioles of the excised leaves were rehydrated (kept in water in darkness, overnight) to ensure leaves were fully saturated. The next day, each leaf was exposed to dry air on a bench at controlled room conditions (~25°C, ~45% RH) and allowed to slowly desiccate with stomata closing naturally. The petiole was sealed with fast-drying adhesive to prevent xylem flow. Leaf weight was recorded at regular intervals on an analytical balance as the leaf dried. Once transpirational water loss reached a steady minimal rate (indicating stomatal closure), the residual evaporation was assumed to occur through the cuticle. This steady-state water loss ($E_{\min}$) was used to calculate $g_{\min}$ (minimum conductance) using the formula $g_{\min} = E_{\min} \cdot P / (2 \cdot \text{VPD})$, where P is atmospheric pressure and the factor 2 accounts for two leaf sides. The $g_{\min}$ values provide a measure of cuticular water permeability and were expressed on a one-sided leaf area basis.

After the $g_{\min}$ trials, those same leaves were used to determine leaf mass per area (LMA) and nitrogen content. Leaf area was measured (either by scanning and image analysis or using a leaf area meter) before drying. Leaves were then oven-dried at 70°C for at least 48 h to constant weight and weighed to obtain their dry mass. LMA was calculated as the dry mass divided by the leaf area (g m^{-2}). Subsequently, dried leaf samples were ground to a fine, homogeneous powder (using a ball mill grinder). Subsamples of the ground tissue were analyzed for total nitrogen (N) concentration by elemental analysis (combustion analysis) using an elemental analyzer coupled to an isotope-ratio mass spectrometer. The instrument was calibrated with standards to ensure accurate N quantification, and results were initially obtained on a mass percentage basis. Finally, leaf N content was expressed on an area basis (N_a , in mmol N cm^{-2}) by multiplying mass-based N concentration by LMA. This allowed comparison of leaf nitrogen investment per unit area across treatments. The procedures for LMA and N determination were identical for all relevant studies, providing a consistent set of leaf trait data (leaf thickness/density via LMA, and nutritional status via N) to interpret physiological differences. In some experiments, additional morphological measurements were recorded (e.g. plant height growth, leaf number, and biomass allocation) – these followed standard protocols (periodic measuring with a ruler or calipers for growth, and final harvest drying and weighing for biomass) but are not detailed here as they were specific to particular studies. Importantly, all studies shared the above core measurements of leaf functional traits for robust cross-comparison.

4. Results

4.1. Study 1: The Effect of Growth Temperature on Leaf Photosynthetic Capacity and Its Thermal Response in *Fagus sylvatica* L. Seedlings.



4.1.1. Introduction

Rising air temperatures are intensifying atmospheric vapour-pressure deficit (VPD) and driving more frequent and prolonged heatwaves (Dai, 2013; IPCC, 2023; Schönbeck et al., 2022). These changes in climate elevate thermal and water stress, disrupting plant energy balance, limiting growth and productivity, and, in extreme cases, leading to widespread vegetation mortality (Adams et al., 2009; Flexas & Medrano, 2002; Mooney, 1972). Yet, the physiological mechanisms underlying plant acclimation to thermal environments remain insufficiently characterized. Advancing this knowledge is crucial, as terrestrial vegetation plays a central role in climate regulation—accounting for ~61% of global evapotranspiration and absorbing nearly half of anthropogenic CO₂ emissions (Liu, 2013; Zhang et al., 2017). In this context, uncovering how plants adjust carbon balance in response to warming

is essential for predicting future ecosystem performance and feedbacks to the climate system.

Thermal acclimation capacity varies considerably across plant functional types. While evergreen species often maintain carbon gain by shifting—or flattening—the temperature optimum of photosynthesis, deciduous trees tend to exhibit weaker homeostasis (Yamori et al., 2014). These differences underpin our focus on one of Europe's most emblematic species, *Fagus sylvatica* L., a deciduous broadleaf species that is dominant in temperate forests but is limited climatically at its southern range margin, as is the case in the Mediterranean region (Jump et al., 2006). In Mediterranean environments, rising heat and drought already impair regeneration and carbon balance (del Río et al., 2018; Sabaté et al., 2002). Furthermore, recent findings further suggest that sudden heatwaves even in the absence of soil moisture deficit, can impair physiological function and threaten survival in *Fagus sylvatica*, emphasising the urgency of understanding its thermal response (Vander Mijnsbrugge et al., 2021). Mediterranean populations thus represent a natural model system for testing the limits of photosynthetic acclimation near the species' thermal threshold. To investigate these acclimation limits, it is necessary to focus on the physiological traits that regulate leaf-level carbon and water exchange under warming.

Photosynthesis is regulated by tightly coordinated biochemical and diffusive processes. To mechanistically model photosynthetic responses (Farquhar et al., 1980; Harley, Loreto, et al., 1992), researchers typically focus on traits such as the maximum Rubisco carboxylation rate (V_{cmax}), light saturated rate of electron transport (J_{max}), mesophyll conductance (g_m), mitochondrial respiration (R_l), and stomatal conductance to CO₂ (g_s). These parameters govern the balance between carbon uptake and water loss and are foundational to predictive models of leaf function.

Each of these traits generally increases with temperature up to an optimum (T_{opt} , yielding P_{opt}), followed by a sudden decline beyond that point (Kumarathunge et al., 2020; Kumarathunge et al., 2019). However, thermal acclimation responses remain inconsistent: some studies report upward shifts in T_{opt} with warming, while others find no change (Crous et al., 2018; Way & Yamori, 2014). Similarly, the relationship between warming, leaf nitrogen content, and photosynthetic capacity shows high variability and lacks clear

mechanistic explanation, warranting further investigation (Duarte et al., 2021).

Structural acclimation to warming can also occur, including shifts in leaf thickness and specific leaf area, with consequences for energy balance and evaporative cooling (Gates, 1968; Rubio et al., 2025). Stomatal responses to increased evaporative demand vary widely partial closure may conserve water but limit CO₂ uptake, while greater opening supports latent heat loss at the expense of water use efficiency. The balance between these strategies remains unresolved in Mediterranean *Fagus sylvatica*, where elevated temperatures may intensify water loss even without drought (Rubio et al., 2025). A further complication arises from uncontrolled VPD–temperature covariance, which can cascade through FvCB-modelled estimates of V_{cmax} , J_{max} , and g_{m} (Farquhar et al., 1980; Harley & Tenhunen, 1991). Without explicit VPD control, apparent thermal acclimation may be artefactual, underscoring the need to disentangle temperature effects on water relations and carbon flux.

A critical yet underexplored facet of thermal acclimation is the adjustment of α chlorophyll fluorescence (CF) traits, which reflect both photochemical efficiency and protective energy dissipation. Beyond *PSII* operating efficiency (Φ_{PSII}), metrics such as the fraction of open PSII centers (qL) and non-photochemical quenching (NPQ) provide mechanistic insight into how light energy is partitioned between electron transport and thermal dissipation (Murchie & Lawson, 2013). These traits are highly sensitive to temperature and are functionally integrated with stomatal behavior, mesophyll conductance, and electron transport. Together, they mediate the trade-off between CO₂ uptake, evaporative cooling, and photoprotection. Clarifying how CF traits acclimate under sustained warming is essential for predicting the resilience of photosynthesis under future climates (Alemu, 2020).

Surprisingly, data on supra-optimal (> 40 °C) responses in Mediterranean *Fagus sylvatica* are scarce, and almost no studies have separated growth-temperature acclimation from instantaneous thermal responses across a broad thermal gradient. To address this, we employed a dual experimental approach: i) Growth-temperature acclimation—seedlings were grown at 25 °C (current mean summer air temperature) or 32 °C (projected mid-century heatwave mean) under identical VPD, enabling clean physiological comparisons; ii) Full temperature-response curves—leaf gas exchange and

CF traits were measured across 20–45 °C with constant relative humidity (60%), yielding ecologically realistic VPD trajectories. These data offer mechanistic insight and provide robust inputs for next-generation photosynthesis and carbon-flux models, which are essential for simulating leaf-level processes under realistic, non-optimal thermal conditions.

We hypothesize that: (1) Growth temperature modulates photosynthetic performance in *Fagus sylvatica*, with moderate temperatures (25 °C) supporting higher net CO₂ assimilation due to lower respiratory costs and more effective nitrogen investment in photosynthetic function; (2) despite these absolute differences in photosynthetic performance, the temperature response of photosynthesis remains proportionally coordinated across growth treatments, reflecting a conserved balance among limiting processes (V_{cmax} , J_{max} , g_m); (3) however, higher growth temperatures enhance stomatal conductance and transpiration to support evaporative cooling, but also increase water loss and photoprotective demand due to excess excitation energy and elevated respiratory activity, potentially intensifying vulnerability to stress.

4.1.2. Material and Methods

4.1.2.1. Plant material and growth conditions

Beech seedlings were established from nuts collected in the Natural Reserve of ‘Hayedo de Montejo’ in 2021 (458,249; 4,551,615 UTM ETRS89). In February 2022, after three months at 4 °C in darkness and moist conditions, one seed per pot germinated within a few days of difference in 9 L pots, filled with a mixture of peat and sand (75–25% v:v). Each pot was fertilized with a release fertilizer (Nutricote®, 16N:4.4P:8.3K, 2.5 gr L⁻¹ of substrate). Pots were randomly arranged in two different walk-in growth chambers set at two different temperatures but with the same air *VPD*. One chamber was set with day/night temperatures and relative humidity of 25/15 ± 0.5 °C and relative humidity of 40/68 ± 1% (VPD of 1.91/0.86 ± 0.06 kPa) (named T25), close to the temperature optimum for photosynthesis for beech (Šigut et al., 2015), and the other to 32.5/22.5 ± 0.5 °C and 61/80 ± 1% (VPD of 1.91/0.86 ± 0.06 kPa) (named T32), both with a 12 h of photoperiod. A +7.5 °C increase in mean temperature is expected from June to August for Western and Central Europe and the Mediterranean region according to the scenario SSP5-8.5 for 34

models of AR6 CMIP6 (interactive-atlas.ipcc.ch) (IPCC, 2023). In each chamber, we established the following light intensity: high PPFD (ca. $600.0 \pm 2.9 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the canopy height), that was measured as the average of six different measurements using a quantum meter (MQ 200; Apogee Instruments Inc., Logan, UT, USA) in a horizontal position on leaves. Plants were watered daily to full capacity. Each week, plants were randomly repositioned within their respective chamber. Every three weeks, plants were rotated between chambers while maintaining their assigned warming treatment (i.e., identical conditions but a different growth chamber).

4.1.2.2. Minimum leaf conductance ($g_{\min}$), Specific Leaf Area and leaf Nitrogen content

Two days before starting gas exchange (GE) and α chlorophyll fluorescence (CF) measurements, on August 17th, we collected healthy, fully expanded leaves from the same plants that will be used for gas exchange measurements (c.a. four individuals per growth chamber). Each leaf was immediately stored in a plastic zip bag with moist tissue paper and kept in a portable cooler at 4 °C until it was taken to the laboratory. In the lab, leaves were stored at 4 °C in darkness with the petiole submerged in water overnight until the next day for measurements.

We estimated $g_{\min}$ through bench dehydration of the rehydrated leaves with petioles sealed with a high-strength adhesive (Loctite, Prism 401) (Araus et al., 1991). Briefly, the leaf area was measured at the beginning of dehydration, and leaves were placed on top of a perforated rack at constant air temperature and relative humidity (ca. 25 °C and 46%, respectively) without direct light. They were repeatedly weighed with an analytical balance to determine the steady-state rate of water lost by unit of leaf area once stomata were closed, i.e., epidermal transpiration ($E_{\min}$, $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$). To achieve the epidermal minimum conductance by one side of the leaf ($g_{\min}$, $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$), we followed the next approximation ($g_{\min} = E_{\min} P / (2 \text{ VPD})$), where P is the atmospheric pressure (94 kPa) and VPD is the air vapor pressure deficit (1.65 kPa), and the boundary layer conductance was assumed negligible.

Leaf mass area (LMA) was determined for these same leaves, that were oven-dried at 70°C until 48h and then weighed to estimate LMA as the ratio between the dry mass and its area. The same leaves were then ground to a

fine powder using a ball mixer mill (Retsch Mixer MM301, Leeds, UK). A subsample of the homogenized powder was weighed into tin foil capsules and sent to the Davies facilities, where absolute nitrogen content was quantified by combusting the material with an elemental analyzer interfaced to an isotope ratio mass spectrometer (PDZ Europa 20-20; Sercon Ltd., Cheshire, UK), yielding calibrated nitrogen concentrations. Finally, these nitrogen values were expressed as area basis using the *LMA* relationship.

4.1.2.3. Gas exchange and chlorophyll fluorescence measurements

Gas exchange (GE) and chlorophyll *a* fluorescence (CF) measurements were conducted between 19 August and 9 September on one fully expanded, horizontal, and unshaded leaf from four plants per growth chamber. Measurements were performed at six target temperatures (20 °C, 25 °C, 30 °C, 35 °C, 40 °C, and 45 °C) using a portable infrared gas analyzer (IRGA) system (LI-6400XT) equipped with a fluorescence chamber (LI-6400-40, Li-Cor, Lincoln, NE, USA). Prior to each measurement, environmental conditions within the growth chamber were adjusted and stabilized at the target temperature. Relative humidity (RH) was consistently maintained at 60% for both the growth chamber and the LI-6400XT. The IRGA system and plant were placed inside the chamber at least one hour in advance to ensure full thermal acclimation of the leaf and the equipment. LI-6400XT settings were kept constant across temperatures, allowing the VPD gradient to vary naturally with temperature. While constant VPD across temperatures was not technically feasible—due to potential condensation at higher temperatures—conditions were carefully stabilized to minimize artifacts. All measurements were conducted under controlled, near-steady-state conditions, allowing robust assessment of thermal and diffusive responses.

Leaf temperature was tightly controlled by adjusting the block temperature of the LI-6400XT to match each target temperature, minimizing thermal fluctuations and avoiding confounding effects on stomatal behavior. The reference CO₂ concentration was held constant at 400 μmol mol⁻¹, with an airflow rate of 300 μmol s⁻¹. Actinic irradiance was maintained at 1,200 μmol photons m⁻² s⁻¹, with 10% blue light.

During each light-acclimated gas exchange measurement, chlorophyll fluorescence was recorded simultaneously using the multiphase flash (MPF) method (Loriaux et al., 2013) to estimate light-adapted maximum

fluorescence (F_m'). Steady-state fluorescence (F_s) and minimal fluorescence in light (F_o') were also recorded, the latter following a short far-red pulse at the end of the light induction phase (Baker, 2008). These values enabled calculation of the effective PSII quantum yield as F_v'/F_m' .

Under dark conditions, maximum fluorescence (F_m) was obtained using a saturating pulse (0.8 s; 8,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), and minimal fluorescence (F_o) was recorded after a far-red pulse at the end of the dark induction period. These dark-acclimated values allowed calculation of maximum quantum efficiency (F_v/F_m).

Fluorescence-derived parameters such as the fraction of open PSII centres (qL), non-photochemical quenching (NPQ), and effective quantum yield of photosystem II (Φ_{PSII}) were also calculated according to (Kramer et al., 2004). To ensure consistency and enable reliable tracking of all measurements and their derived parameters, data were collected from the same leaf location at each target temperature.

After introducing the common settings for measurements, we detail this comprehensive measurement protocol, that was systematically applied to the same leaf at each target temperature and included the following: i) initial gas exchange and fluorescence measurements; ii) a low O_2 P_N -PPFD response curve; iii) dark respiration measurement; iv) an P_N - C_i response curve. Individual plants were revisited on subsequent days, ensuring each leaf was exposed to one temperature per day, with the full temperature sequence completed across six days.

Initially, gas exchange readings were monitored until stabilization, after which the first light-adapted measurements were recorded. Immediately following this, a light-response curve was conducted under a 1% O_2 atmosphere. The reference air was switched from ambient air to a premixed gas containing 1% O_2 balanced with N_2 , with total airflow unchanged. The LI-6400XT was accordingly reconfigured to reflect the switch from ambient (21%) to 1% O_2 . Actinic irradiance was sequentially reduced from the initial PPFD of 1,200 through stepwise PPFD levels of 1,000, 800, 500, 300, 200, 150, 100, 50, and 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. At each irradiance level, net photosynthesis (P_N) and fluorescence parameters were recorded once values stabilized, typically within 120–240 s.

Upon completing this step, the auxiliary gas supply providing the low O₂ mixture was disconnected, and the LI-6400XT configuration was reset to ambient oxygen (21%).

Next, dark respiration (R_D) and maximal fluorescence were assessed by switching off all light sources and maintaining complete darkness within both the growth chamber and LI-6400XT for 20 min. After this period, CO₂ exchange was recorded to quantify leaf dark respiration and chlorophyll fluorescence readings were simultaneously taken. Mitochondrial respiration in the light (R_l) was estimated as 0.5 R_D (Busch, 2013).

Subsequently, an ambient oxygen (21% O₂) P_N -C_i response curve was generated. Actinic irradiance was restored to 1,200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Once steady-state photosynthesis was re-established (approximately 20–30 min), reference CO₂ concentration ($[\text{CO}_2]_{\text{ref}}$) was systematically adjusted from the initial $[\text{CO}_2]_{\text{ref}}$ of 400 $\mu\text{mol mol}^{-1}$ through stepwise levels of 300, 220, 150, 100, 70, 350, 600, 900, 1,000, and 1,200 $\mu\text{mol mol}^{-1}$. Each step lasted 120–240 s.

Finally, CO₂ leakage was corrected (Flexas et al., 2007), and stomatal conductance was corrected for epidermal conductance (Araus et al., 1991) from all the GE measurements, including curves. The light-saturated intrinsic water-use efficiency (WUE_i) was calculated as the net photosynthesis divided by the stomatal conductance to water vapour ($\text{WUE}_i = P_N/g_{\text{sw}}$) while the light-saturated water-use efficiency (WUE) was calculated as the P_N divided by the leaf transpiration ($\text{WUE} = P_N/E$).

4.1.2.4. Calculation of mesophyll conductance (g_m)

Mesophyll conductance (g_m) was estimated using the **variable J method**, combining gas exchange and chlorophyll fluorescence measurements as described by Harley, Loreto, et al. (1992). To calibrate electron transport under non-photorespiratory conditions (1% O₂), we used light-response curves (P_N -PPFD) to establish a relationship between the theoretical electron transport rate ($J_a = 4(P_N + R_l)$)—assuming four electrons per CO₂ molecule fixed in the absence of photorespiration—and the electron transport rate derived from chlorophyll fluorescence (J_f). **J_f was calculated as ($J_f = (\alpha \beta) \text{PPFD } \Phi_{\text{PSII}}$)**, where Φ_{PSII} is the effective quantum yield of PSII, α is leaf absorptance, and β is the fraction of absorbed photons partitioned to PSII (Cano et al., 2014). The product $\alpha\beta$ was determined according to Valentini et al. (1995).

Mesophyll conductance (g_m , mol CO₂ m⁻² s⁻¹) was then calculated as:

$$g_m = \frac{P_N}{C_i - \frac{\Gamma^* [J_f + 8(P_N + R_l)]}{J_f - 4(P_N + R_l)}} \quad (1)$$

where P_N is the net CO₂ assimilation rate (μmol CO₂ m⁻² s⁻¹), C_i is the intercellular CO₂ concentration (μmol mol⁻¹), Γ^* is the CO₂ compensation point in the absence of mitochondrial respiration (μmol mol⁻¹) according to Bernacchi et al. (2001), and R_l is mitochondrial respiration in the light (μmol CO₂ m⁻² s⁻¹).

The chloroplastic CO₂ concentration (C_c , μmol mol⁻¹) was defined as:

$$C_c = C_i - \frac{A}{g_m} \quad (2)$$

Finally, when feasible, we incorporated our own estimations of R_l and g_m into subsequent calculations.

4.1.2.5. Calculation of maximum rate of carboxylation of Rubisco (V_{cmax}), apparent light saturated rate of electron transport (J_{high}), and light saturated maximum rate of electron transport (J_{max})

P_N - C_i response curves were fitted using the Farquhar model of photosynthesis (Farquhar et al., 1980) incorporating established modifications (Harley, Thomas, et al., 1992; Sharkey, 1985; Wullschlegel, 1993). Net assimilation (P_N , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) was modeled as:

$$P_N = V_c - 0.5V_o - R_l = \min \{P_c, P_r, P_p\} \quad (3)$$

P_N is also limited by one of three processes: Rubisco activity (P_c), RuBP regeneration via electron transport (P_r), or triose phosphate utilization (P_p), defined as:

$$P_c = \frac{(C - \Gamma^*)V_{cmax}}{C + K_c\left(1 + \frac{O}{K_o}\right)} - R_l \quad (4)$$

$$P_r = \frac{(C - \Gamma^*)J_{max}}{4C - 8\Gamma^*} - R_l \quad (5)$$

$$P_p = 3TPU - R_l \quad (6)$$

In these expressions, V_c and V_o are rates of carboxylation and oxygenation of RubisCO ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); R_l is the rate of CO_2 evolution in light resulting from processes other than photorespiration ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); $\min\{\}$ denotes the “the minimum of”; P_c , P_r and P_p are the net CO_2 assimilation rate limited by Rubisco, RuBP and TPU, respectively ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); TPU is the rate of phosphate release in triose phosphate utilization (starch and sucrose production); C and O are the partial pressures of CO_2 and O_2 at RubisCO (Pa); K_c and K_o are Michaelis-Menten constants for carboxylation and oxygenation respectively; C_i is the partial pressures of CO_2 in the intercellular air space (Pa); J_{max} is the maximum rate of electron transport ($\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}$) that is dependent upon incident light irradiance (I); the factor 4 in Eqn 5 indicates that for the regeneration of RuBP in the Calvin cycle, four electrons are necessary for produce enough ATP and NADPH (Farquhar et al., 1980).

The parameter J_{high} —the apparent saturation point of electron transport—was initially derived from P_N - C_i curve fitting. However, as noted by Buckley and Diaz-Espejo (2015), J_{high} underestimates the true asymptotic capacity for electron transport.

Therefore, to obtain $J_{\max}$ we applied the non-rectangular hyperbola model from von Caemmerer (2000), using the following equations:

$$I_2 = I \cdot \frac{\text{abs}(1-f_1)}{2} \quad (7)$$

$$J_{\text{high}} = \frac{I_2 + J_{\max} - \sqrt{(I_2 + J_{\max})^2 - 4\theta I_2 J_{\max}}}{2\theta} \quad (8)$$

Where I_2 it is the useful light absorbed by PSII, I it is the incident irradiance, abs it is the absorptance of leaves (0.85), f_1 corrects for spectral quality of light (0.15), θ is an empirical curvature factor and 0.7 is a good average value and J_{high} it is the apparent saturated rate of electron transport derived from P_N - C_i curve (von Caemmerer, 2000).

Solving for $J_{\max}$ yields:

$$J_{\max} = \frac{J_{\text{high}}(J_{\text{high}}\theta - I_2)}{J_{\text{high}} - I_2} \quad (9)$$

This correction ensures that $J_{\max}$ reflects the theoretical maximum capacity for electron transport under saturating light, rather than the empirical saturation point estimated from P_N - C_i fitting.

Finally, the rate of photorespiratory CO_2 release (R_{PR}) can be derived as half the oxygenation rate:

$$R_{\text{PR}} = 0.5V_o = \frac{P_N - R_l}{\frac{C_c}{\Gamma^*} - 1} \quad (10)$$

The factor 0.5 is added based on the assumption that one molecule of CO_2 is released for every two oxygenation reactions.

4.1.2.6. Temperature response models of P_N , V_{cmax} , J_f , $J_{\max}$, g_m , E , R_{PR} and R_l

Temperature response curves for P_N , V_{cmax} , J_f , $J_{\max}$, g_m and R_{PR} were fitted using an extended Arrhenius function that incorporates both activation and deactivation processes (Leuning, 1997; Sharpe & DeMichele, 1977):

$$P(T) = \frac{P(T_{\text{ref}}) e^{\left[\frac{E_a}{RT_{\text{ref}}}\left(1 - \frac{T_{\text{ref}}}{T}\right)\right]}}{\left(1 + e^{\left(\frac{\Delta ST_{\text{ref}} - E_d}{RT_{\text{ref}}}\right)}\right)} \left(1 + e^{\left(\frac{\Delta ST_{\text{ref}} - E_d}{RT_{\text{ref}}}\right)}\right) \quad (11)$$

In cases where no deactivation peak was observed within the measured temperature range (e.g., for R_l and E), a simplified Arrhenius function without deactivation terms was used:

$$P(T) = P(T_{ref}) e^{\left[\frac{E_a}{RT_{ref}} \left(1 - \frac{T_{ref}}{T} \right) \right]} \quad (12)$$

Where $P(T_{ref})$ it is the parameter value at a reference temperature in absence of deactivation (25 °C, 298K), E_a (J mol⁻¹) is the activation energy, R (8.3143 J K⁻¹ mol⁻¹) is the ideal gas constant, E_d (J mol⁻¹) is the deactivation energy, ΔS (J K⁻¹ mol⁻¹) is an entropy term, and T (K) is leaf temperature.

The theoretical temperature optimum (T_{opt}) for parameters modelled with deactivation was calculated as:

$$T_{opt} = \frac{-E_d}{R \ln \left(\frac{-E_a}{E_a - E_d} \right) - \Delta S} \quad (13)$$

4.1.2.7. Analysis of gas diffusional and biochemical limitations of the photosynthesis

The limitations to P_N imposed by the stomatal and mesophyll conductance, and biochemical processes, that could be attributed to V_{cmax} or J_{max} (eq. 6) were examined using the following equations as described in Grassi and Magnani (2005).

$$\frac{dP_N}{P_N} = L_S + L_M + L_B = l_s \frac{dg_s}{g_s} + l_m \frac{dg_m}{g_m} + l_b \frac{dV_{cmax}}{V_{cmax}} \text{ (or } l_b \frac{dJ_{max}}{J_{max}}) \quad (14)$$

$$l_s = \frac{g_{tot}/g_s \cdot \delta P_c \text{ (or } \delta P_j)/\delta C_c}{g_{tot} + \delta P_c \text{ (or } \delta P_j)/\delta C_c} \quad (15)$$

$$l_m = \frac{g_{tot}/g_m \cdot \delta P_c \text{ (or } \delta P_j)/\delta C_c}{g_{tot} + \delta P_c \text{ (or } \delta P_j)/\delta C_c} \quad (16)$$

$$l_b \text{ (by } V_{cmax} \text{ or } J_{max}) = \frac{g_{tot}}{g_{tot} + \delta P_c \text{ (or } \delta P_j)/\delta C_c} \quad (17)$$

Where g_s is stomatal conductance to CO_2 (g_s); g_{tot} is total conductance to CO_2 between the leaf surface and carboxylation sites ($1/g_{tot} = 1/g_s + 1/g_m$); L_S , L_M and L_B are the contributions of stomatal conductance, of mesophyll conductance and of maximum carboxylation rate (or maximum rate of electron transport) to dP_N/P_N , respectively; and l_s , l_m and l_b are the corresponding relative limitations ($0 < l_i < 1$, $i=s, m, b$). Additionally, $\partial P/\partial C_c$ and $\partial P_j/\partial C_c$, the partial differential of Equations 4 and 5 to C_c , were calculated using the following equations

$$\delta P_c/\delta C_c = \frac{V_{cmax} (\Gamma^* + K_c (1 + \frac{\Gamma^*}{K_c}))}{((C_c + K_c (1 + \frac{\Gamma^*}{K_c}))^2)} \quad (18)$$

$$\delta P_r/\delta C_c = \frac{3 \cdot J_{max} \cdot \Gamma^*}{4(C_c + \Gamma^*)^2} \quad (19)$$

4.1.2.8. Statistical analysis

After verifying that the data satisfied normality assumptions, one-way analyses of variance (ANOVA) were carried out in R v. 4.3.2 (R Development Core Team, 2024) using growth temperature as fixed factor for the leaf morphological variables. For all other traits, particularly the photosynthetic variables derived from GE and CF measurements, linear mixed-effects (LME) models were fitted via the “nlme” package in R v. 4.3.2, again after confirming normality. In these models, each measured photosynthetic trait was used as the response variable, with growth temperature, leaf temperature, and their interaction as fixed effects, and individuals as a random effect. All data met

assumptions of normality and homoscedasticity, and a significance level of $P \leq 0.05$ was applied. Model assumptions were assessed through standard diagnostic checks. Post hoc comparisons were performed using the Tukey HSD test (available in the “agricolae” package in R) to identify significant differences among factor levels.

4.1.3. Results

4.1.3.1. Temperature response of main photosynthetic traits

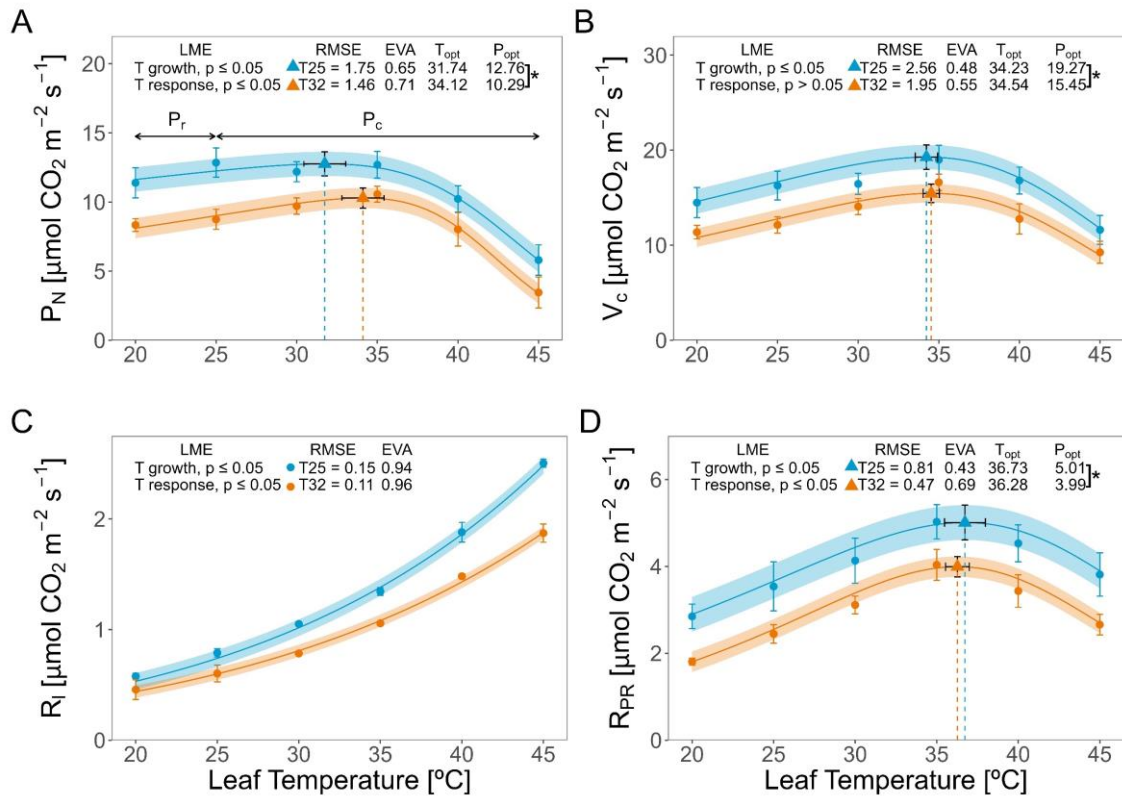


Figure 19. Temperature response curves of: A) net assimilation rate (P_N); B) RuBisCO carboxylation rate (V_c); C) mitochondrial respiration in the light (R_i); and D) rate of photorespiratory CO_2 release (R_{PR}) for plants grown at 25°C (T_{25} , blue) and 32°C (T_{32} , orange) growth temperatures, measured across six target leaf temperatures. Circular markers represent mean values $\pm$ standard error of measurements at each leaf temperature. Lines represent fits to the Arrhenius model, with shaded ribbons indicating 95% confidence intervals. Optimal temperatures (T_{opt}) and corresponding maximum rates (P_{opt}) obtained from the model fit are shown by triangular markers with horizontal and vertical error bars indicating their standard errors. Results of linear mixed-effects (LME) models testing the effects of growth temperature and temperature response, along with model fitting metrics (root mean squared error, RMSE; explained variance, EVA) and significant differences (P_{opt} or T_{opt} , marked with “*”) between growth temperatures, are provided at the top. Black arrows indicate the transition point from RuBP regeneration-limited assimilation (P_r) to Rubisco-limited assimilation (P_c) according to Farquhar et al. (1980).

Net CO₂ assimilation (P_N) exhibited a clear response to growth temperature across the measurement range (20–45 °C). T32 seedlings consistently showed lower assimilation rates than T25, with a 29% reduction in maximum P_N ($P_{\text{opt}} = 10.3 \pm 0.7$ vs $12.8 \pm 0.9 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), though the temperature of maximum assimilation remained stable between treatments (Fig. 19A). This decline in carbon gain was mirrored by lower Rubisco carboxylation rates (V_c) in T32 plants, although V_c responses shared similar T_{opt} (34.5 ± 0.6 °C) and curve shape across treatments, suggesting proportional biochemical scaling (Fig. 19B).

Mitochondrial respiration in the light (R_l) rose exponentially with measurement temperature and was consistently lower in T32 seedlings, indicating a possible downregulation of basal respiratory metabolism under warming (Fig. 19C). A similar trend was observed for rate of photorespiratory CO₂ release (R_{PR}), with significantly lower maximum rates in T32 plants ($P_{\text{opt}} = 4.0 \pm 0.3$ vs $5.0 \pm 0.4 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$; Fig. 19D).

4.1.3.2. Diffusive and Biochemical Responses to Temperature

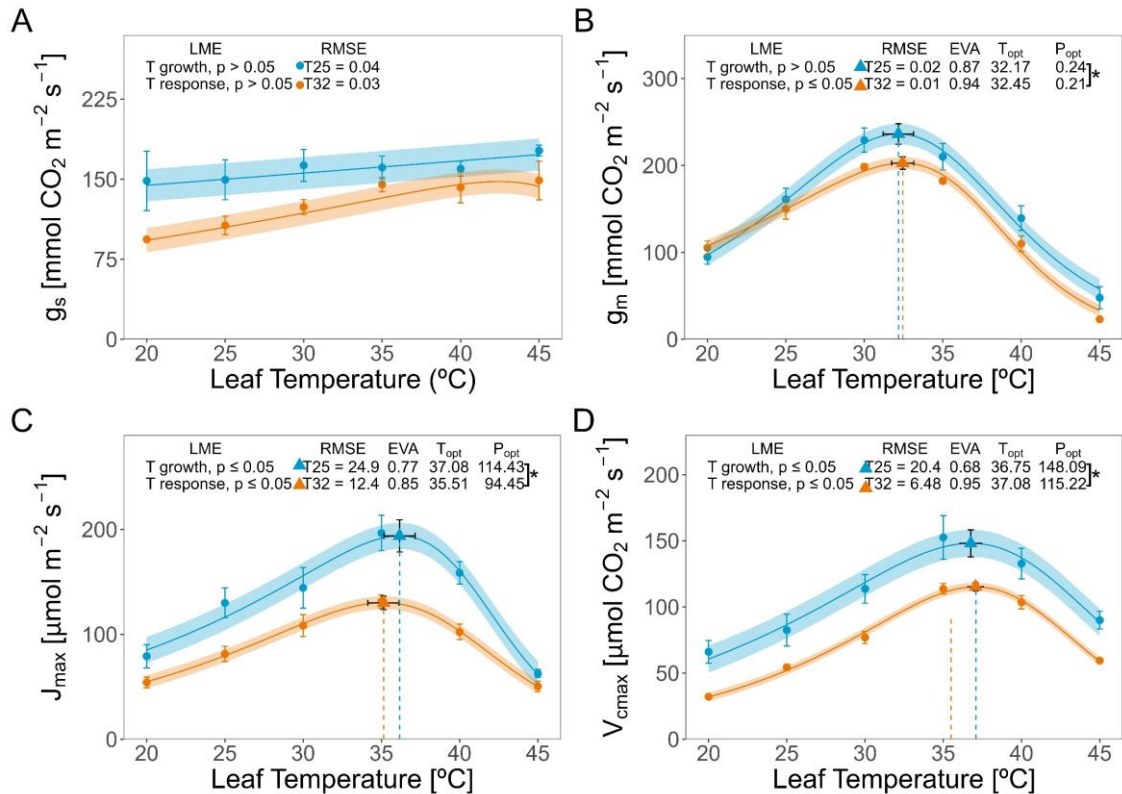


Figure 20. Temperature response curves of: A) stomatal conductance (g_s); B) mesophyll conductance (g_m); C) maximum rate of electron transport (J_{max}); and D) maximum rate of carboxylation of Rubisco (V_{cmax}) for plants grown at 25°C (T25, blue) and 32°C (T32, orange) growth temperatures, measured across six target leaf

*temperatures. Circular markers represent mean values $\pm$ standard error of measurements at each leaf temperature. Lines represent fits to the Arrhenius model, with shaded ribbons indicating 95% confidence intervals. Optimal temperatures (T_{opt}) and corresponding maximum rates (P_{opt}) obtained from the model fit are shown by triangular markers with horizontal and vertical error bars indicating their standard errors. Results of linear mixed-effects (LME) models testing the effects of growth temperature and temperature response, along with model fitting metrics (root mean squared error, RMSE; explained variance, EVA) and significant differences (P_{opt} or T_{opt} , marked with "**") between growth temperatures, are provided at the top.*

Stomatal conductance (g_s) showed no significant response to either growth temperature or measurement temperature, with overlapping values across the entire thermal range (Fig. 20A). This stability suggests that stomatal behavior in *Fagus sylvatica* remains relatively insensitive to moderate warming, maintaining similar diffusional capacity for CO₂ under both conditions.

In contrast, mesophyll conductance (g_m) exhibited a significant response to measurement temperature ($p \leq 0.05$), peaking around 32–34 °C in both T25 and T32 plants (Fig. 20B) but growth temperature did not significantly affect it.

Biochemical capacities, including the maximum rate of electron transport (J_{max}) and carboxylation (V_{cmax}), were significantly downregulated in T32 plants (Fig. 20C–D). Although the T_{opt} remained similar between treatments (~36–37 °C), P_{opt} values were ~20–30% lower in T32 seedlings (e.g., V_{cmax} : 148.2 ± 10.2 vs. 115 ± 3.2 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), indicating reduced investment in photosynthetic machinery under elevated growth temperature.

All of these photosynthetic parameters were fitted using an Arrhenius model (Table 1). Although no clear patterns emerged across activation energy (E_a), deactivation energy (E_d), or entropy (ΔS), differences in curve separation between growth temperatures confirmed distinct acclimation responses. Consistently lower peak values (P_{opt}) at elevated growth temperatures (T32) compared to optimal conditions (T25) highlight the acclimation effect. The explained variance (EVA) values remained high (0.65–0.98), indicating robust fits. These findings reflect a growth temperature-induced decrease in photosynthetic efficiency

Table 1. Summary table of mean values ($\pm$ standard error) of the main parameters describing temperature responses of leaf photosynthetic capacity in seedlings of *Fagus sylvatica* growing at two different temperatures for net assimilation rate (P_N), maximum rate of carboxylation (V_{cmax}), maximum rate of electron transport (J_{max}), mesophyll conductance (g_m) and mitochondrial respiration in the light (R_l). The fitting parameters are the following: value of each parameter at 25° (P at 25°C), activation energy (E_a), deactivation energy (E_d), entropy factor (ΔS), explained variance (EVA), optimal temperature (T_{opt}), modeled value of each parameter at the optimal temperature (P_{opt}), and the reduction in P_{opt} as one less the ratio of the P_{opt} for T32 divided by T25.

Parameter	Tgrowth [°C]	P at 25°C [Parameter units]	E_a [J mol ⁻¹]	E_d [J mol ⁻¹]	ΔS [J mol ⁻¹ K ⁻¹]	EVA -	T_{opt} [° C]	P_{opt} [Parameter units]	Reduction in P_{opt} -
P_N	25	12.3 $\pm$ 0.6	8073 $\pm$ 5147	230357 $\pm$ 39094	728 $\pm$ 123	0.65	31.7 $\pm$ 1.2	12.8 $\pm$ 0.9	0.29
	32	9 $\pm$ 0.5	15491 $\pm$ 2003	296462 $\pm$ 41844	941 $\pm$ 133	0.71	34.1 $\pm$ 1.4	10.3 $\pm$ 0.7	
V_{cmax}	25	86.6 $\pm$ 6.9	53053 $\pm$ 3088	211990 $\pm$ 17473	675 $\pm$ 56	0.68	36.8 $\pm$ 0.6	148.2 $\pm$ 10.2	0.22
	32	52 $\pm$ 1.9	71752 $\pm$ 4416	249382 $\pm$ 21121	797 $\pm$ 65	0.95	37.1 $\pm$ 0.5	115.2 $\pm$ 3.2	
J_{max}	25	116.3 $\pm$ 8.3	45836 $\pm$ 1483	312333 $\pm$ 36243	996 $\pm$ 114	0.77	36.2 $\pm$ 1	193.9 $\pm$ 12.4	0.33
	32	79.6 $\pm$ 4.2	55607 $\pm$ 6747	243091 $\pm$ 27614	779 $\pm$ 85	0.85	35.1 $\pm$ 1	130.1 $\pm$ 6.2	
g_m	25	0.16 $\pm$ 0.01	78994 $\pm$ 15209	232712 $\pm$ 21288	757 $\pm$ 67	0.87	32.2 $\pm$ 0.5	0.24 $\pm$ 0.01	0.17
	32	0.15 $\pm$ 0.01	50010 $\pm$ 2003	271055 $\pm$ 26316	875 $\pm$ 83	0.94	32.4 $\pm$ 1.4	0.20 $\pm$ 0.01	
R_l	25	0.74 $\pm$ 0.02	47707 $\pm$ 788			0.98			
	32	0.60 $\pm$ 0.02	45057 $\pm$ 599			0.96			

4.1.3.3. Leaf Morphological and Nitrogen Investment Responses to Growth Temperature

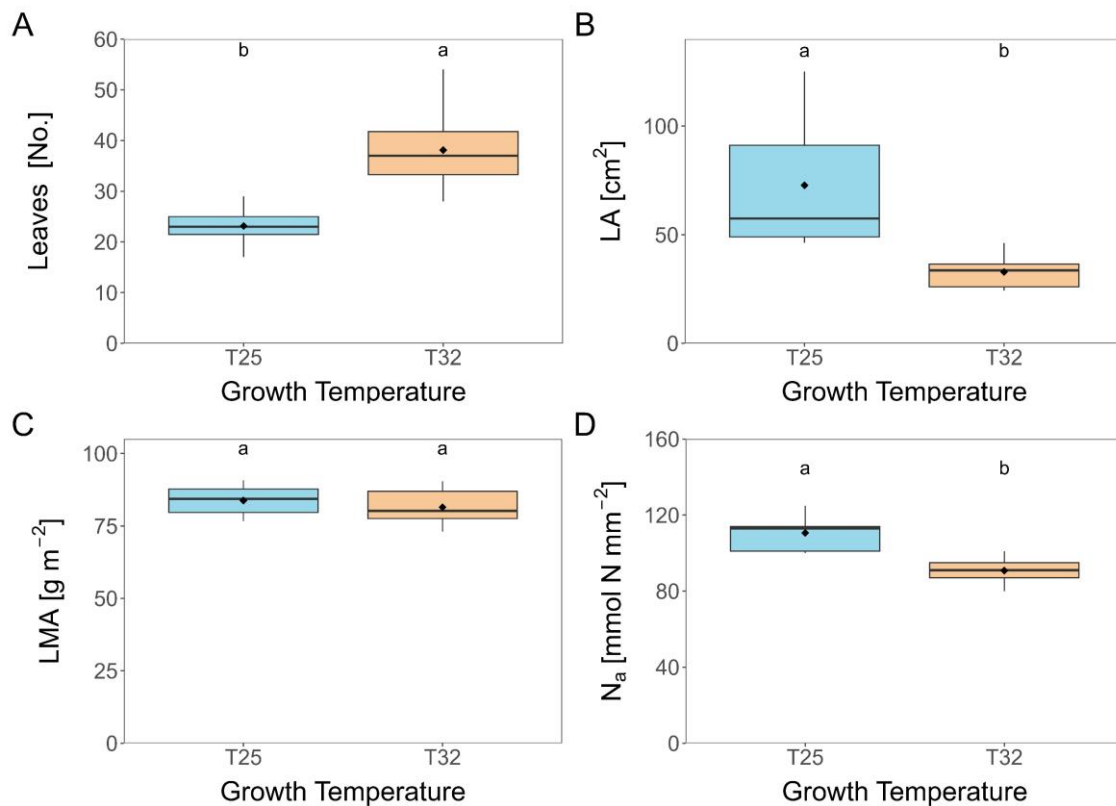


Figure 21. Plasticity of leaf morphology acclimation of beech seedlings growing at 25 °C (T25, blue) and 32 °C (T32, orange): A) nitrogen content by leaf area (N_a); B) leaf mass per area (LMA); C) number of developed leaves; and D) mean leaf area (LA). The straight horizontal black line within each boxplot indicates the median, while the mean value is represented as a solid black point. Different letters indicate significant differences among treatments.

Leaf morphological traits showed significant adjustments in response to elevated growth temperature. Plants grown at 32 °C (T32) produced significantly more leaves per plant (38.1 ± 2.3) compared to those at 25 °C (T25: 23.1 ± 1.4), representing a 65% increase (Fig. 21A). However, this increase was accompanied by a notable reduction in individual leaf area (LA), which declined by approximately 55%, from $72.7 \pm 12.4 \text{ cm}^2$ in T25 to $32.8 \pm 2.3 \text{ cm}^2$ in T32 (Fig. 21B). Despite this shift, leaf mass per area (LMA) remained statistically similar between treatments (T25: $83.8 \pm 2.1 \text{ g cm}^{-2}$; T32: $81.4 \pm 2.0 \text{ g cm}^{-2}$), suggesting no significant structural thickening or thinning (Fig. 21C). Meanwhile, leaf nitrogen per area (N_a) was reduced by nearly 26%, potentially limiting Rubisco content and contributing to the observed downregulation of photosynthesis (Fig. 21D).

4.1.3.4. Water Use, Stomatal Regulation, and Energy Dissipation under Warming

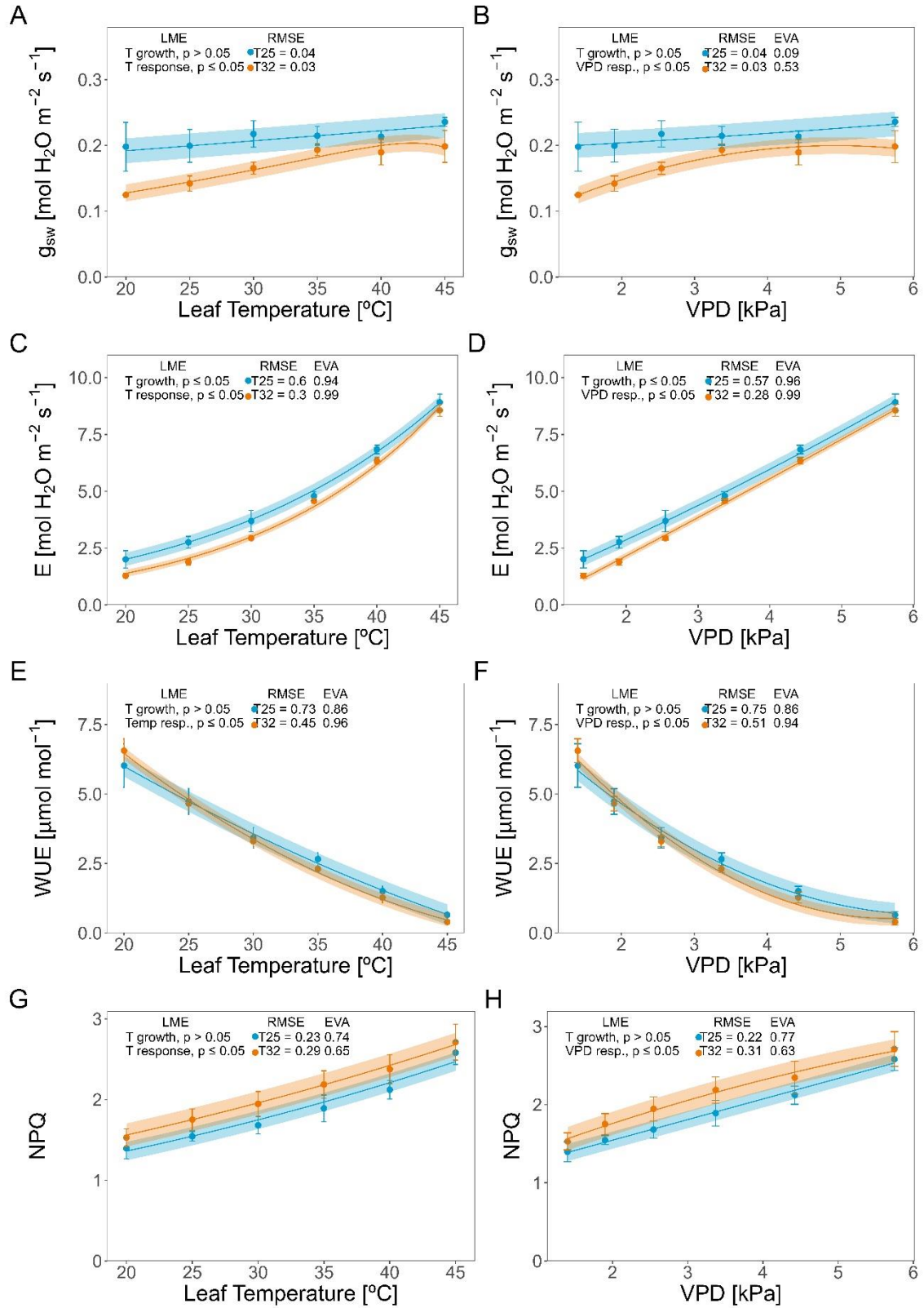


Figure 22. Temperature or VPD response curves of: A-B) stomatal conductance to water vapour (g_{sw}) vs leaf temperature; C-D) transpiration (E); E-F) water use efficiency

(WUE); and *G-H* non-photochemical quenching (NPQ) for plants grown at 25°C (T25, blue) and 32°C (T32, orange) growth temperatures, measured across six target leaf temperatures. Circular markers represent mean values $\pm$ standard error of measurements at each leaf temperature. Lines represent fits to the Arrhenius model, with shaded ribbons indicating 95% confidence intervals. Optimal temperatures (T_{opt}) and corresponding maximum rates (P_{opt}) obtained from the model fit are shown by triangular markers with horizontal and vertical error bars indicating their standard errors. Results of linear mixed-effects (LME) models testing the effects of growth temperature and temperature response, along with model fitting metrics (root mean squared error, RMSE; explained variance, EVA) and significant differences (P_{opt} or T_{opt} , marked with "**") between growth temperatures, are provided at the top.

Stomatal conductance to water vapor (g_{sw}) showed no significant differences across temperatures or between growth conditions (Fig. 22A), indicating limited stomatal sensitivity to either thermal stress or acclimation. In contrast, transpiration rate (E) increased exponentially with leaf temperature in both treatments (Fig. 21B), with consistently higher rates in T25 seedlings, despite similar g_{sw} values. This suggests enhanced evaporative cooling in T25 plants due to greater water loss per unit leaf area.

Intrinsic water use efficiency (WUE_i) followed a bell-shaped response, peaking around 30–35 °C and declining thereafter (Fig. 22C). T25 seedlings exhibited higher WUE_i across most temperatures, particularly at the optimum, where differences between treatments were statistically significant ($P_{opt} = 66.6 \pm 7.8 \mu\text{mol mol}^{-1}$). This reflects greater carbon assimilation per unit water lost in cooler-grown plants.

Non-photochemical quenching (NPQ) increased with temperature (Fig. 22D), with consistently higher values in T32 seedlings across the range.

4.1.3.5. Temperature Responses of PSII Photochemistry and Electron Transport

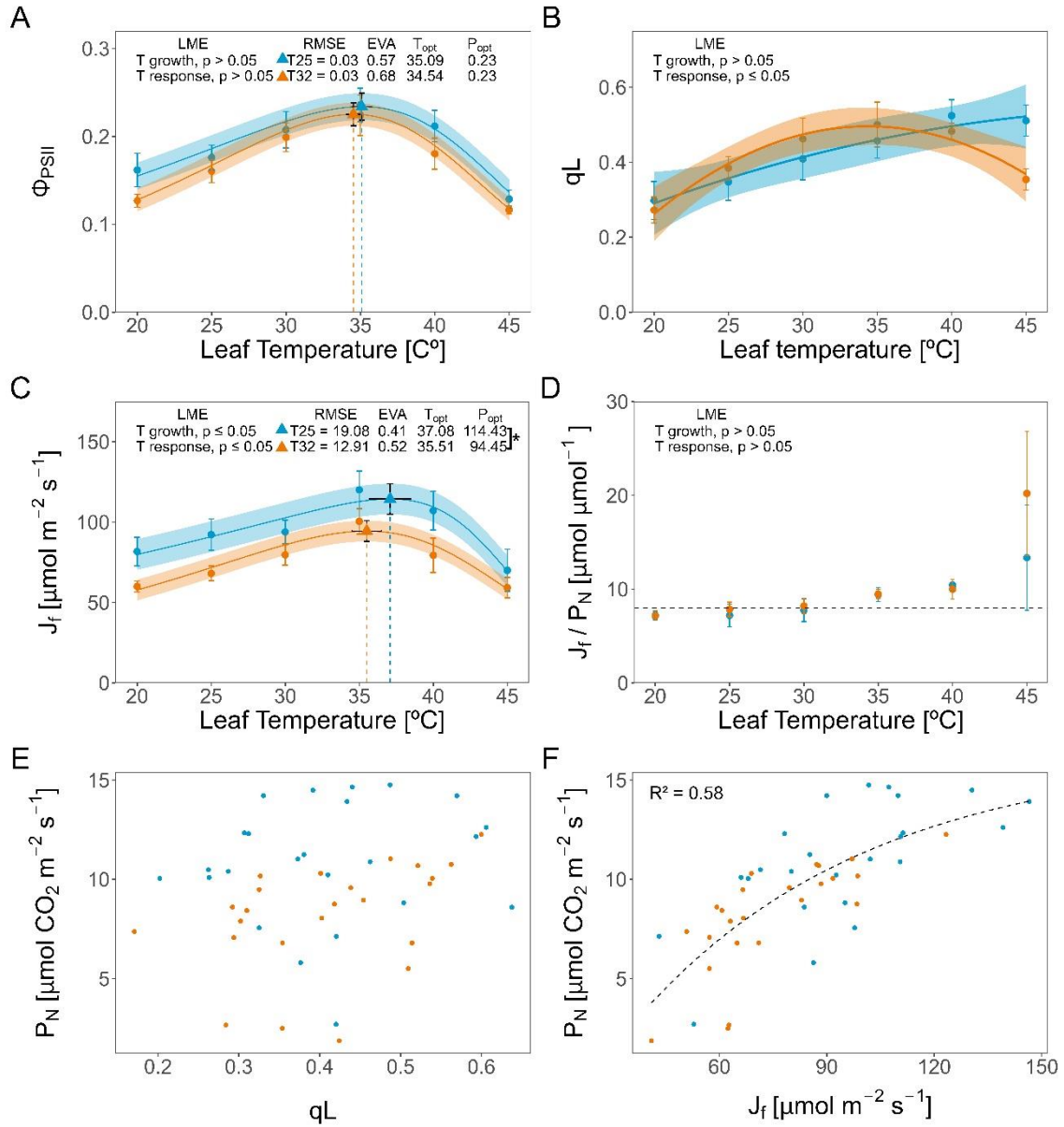


Figure 23. Temperature response curves of: A) effective quantum yield of photosystem II (Φ_{PSII}); B) fraction of open PSII centres (qL); C) electron transport rate (J_f); D) the ratio between electron transport rate and net assimilation rate (J_f/P_N); E) P_N vs qL ; and F) P_N vs J_f for plants grown at 25°C (T25, blue) and 32°C (T32, orange) growth temperatures, measured across six target leaf temperatures. Circular markers represent mean values $\pm$ standard error of measurements at each leaf temperature. Lines represent fits to the Arrhenius model, with shaded ribbons indicating 95% confidence intervals. Optimal temperatures (T_{opt}) and corresponding maximum rates (P_{opt}) obtained from the model fit are shown by triangular markers with horizontal and vertical error bars indicating their standard errors. Results of linear mixed-effects (LME) models testing the effects of growth temperature and temperature response, along with model fitting metrics (root mean squared error, RMSE; explained variance, EVA and coefficient of determination, R^2) and significant differences (P_{opt} or T_{opt} , marked with "**") between growth temperatures, are provided

at the top. In panel (D), the dashed horizontal line denotes the canonical stoichiometric threshold of ~8 electrons per CO_2 assimilated (Perera-Castro & Flexas, 2023). In panel (F), a second-order polynomial regression is used to describe the relationship between P_N and J_f .

Across both growth temperatures, effective quantum yield of photosystem II (Φ_{PSII} ; Fig. 23A) and the fraction of open PSII centres (qL; Fig. 23B) exhibited broadly similar temperature response curves, with no significant effect of growth temperature. While Φ_{PSII} peaked around 35 °C in both groups, temperature had a significant overall effect on both variables ($p \leq 0.05$), indicating thermal sensitivity independent of acclimation.

In contrast, the electron transport rate (J_f , Fig. 23C) showed significant effects of both growth temperature and measurement temperature ($p \leq 0.05$), with T25 seedlings maintaining consistently higher J_f values across the full thermal range. P_{opt} for J_f was significantly higher in T25 ($114.4 \pm 9.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) than in T32 ($94.5 \pm 6.9 \mu\text{mol m}^{-2} \text{s}^{-1}$), while optimal temperatures (T_{opt}) remained similar.

The ratio of J_f to P_N (Fig. 23D) increased with leaf temperature, particularly in T32 seedlings, exceeding the canonical threshold of ~8 electrons per CO_2 at high temperatures.

Table 2. Summary of F-values (numeric) and significance codes ($p < 0.05$, $*p < 0.01$, $**p < 0.001$, ns = not significant) obtained from linear mixed-effects models (lme) testing the effects of measurement temperature (Temp), growth chamber (Tgrowth), and their interaction on each evaluated trait, with random intercepts per plant. For abbreviations, check table 1.

Factor	Intercept	Temp	Tgrowth	Temp:Tgrowth
P_N	599.6435 ***	20.248 ***	12.2902 *	0.442 ns
g_m	855.3824 ***	108.7395 ***	4.0295 ns	1.6412 ns
V_{cmax}	326.2451 ***	13.3092 **	10.9641 *	0.0042 ns
J_{max}	478.9732 ***	40.42 ***	16.8117 **	2.4117 ns
R_i	2765.2697 ***	370.5947 ***	47.7526 **	7.5863 **
R_{PR}	427.4524 ***	12.5226 ***	8.5784 *	0.4199 ns
P_N/g_c	259.10912 ***	78.62098 ***	0.17658 ns	1.19703 ns
P_N/g_m	229.45405 ***	4.22791 *	0.79508 ns	2.25151 ns
P_N/V_{cmax}	448.2887 ***	153.3153 ***	1.957 ns	5.6649 ns
P_N/J_{max}	509.6716 ***	52.6507 ***	0.2021 ns	1.3948 ns
P_N/R_i	187.6507 ***	343.578 ***	0.0096 ns	0.0983 ns
P_N/R_{PR}	655.3306 ***	149.9635 ***	0.0314 ns	0.7415 ns
g_{sw}	434.147 ***	24.0411 ***	5.8281 ns	3.9733 ns
E	1533.0814 ***	182.763 ***	7.0102 *	0.6496 ns
WUE_i	259.10978 ***	78.62095 ***	0.17658 ns	1.19703 ns
WUE	278.3679 ***	407.8894 ***	0.3723 ns	0.166 ns
P_N/J_f	1285.1002 ***	62.3898 ***	1.9368 ns	0.8178 ns
P_N/NPQ	295.03258 ***	58.46349 ***	12.46311 *	3.54223 ns

Despite differences in absolute photosynthetic capacity, *Fagus sylvatica* seedlings exhibited highly consistent temperature-response patterns across growth temperatures. Linear mixed-effects (LME) models showed that leaf temperature had a significant effect on all traits analyzed ($p < 0.05$), while the interaction between temperature and growth temperature (Temp:Tgrowth) was not significant for most parameters. This suggests parallel thermal trajectories between T25 and T32 plants (Table 2).

Growth temperature had a significant main effect on a subset of traits, including P_N , V_{cmax} , J_{max} , R_l , R_{PR} , and E . However, the absence of significant interaction terms for P_N , g_m , V_{cmax} , and most ratio-based traits (e.g., P_N/V_{cmax} , P_N/J_{max}) suggest that the temperature response curves did not differ in shape between growth treatments (Figure 26).

Only R_l showed a significant Temp:Tgrowth interaction ($p < 0.01$), indicating some acclimation in respiratory sensitivity.

4.1.3.6. Limitations in net assimilation (P_N) in response to temperature

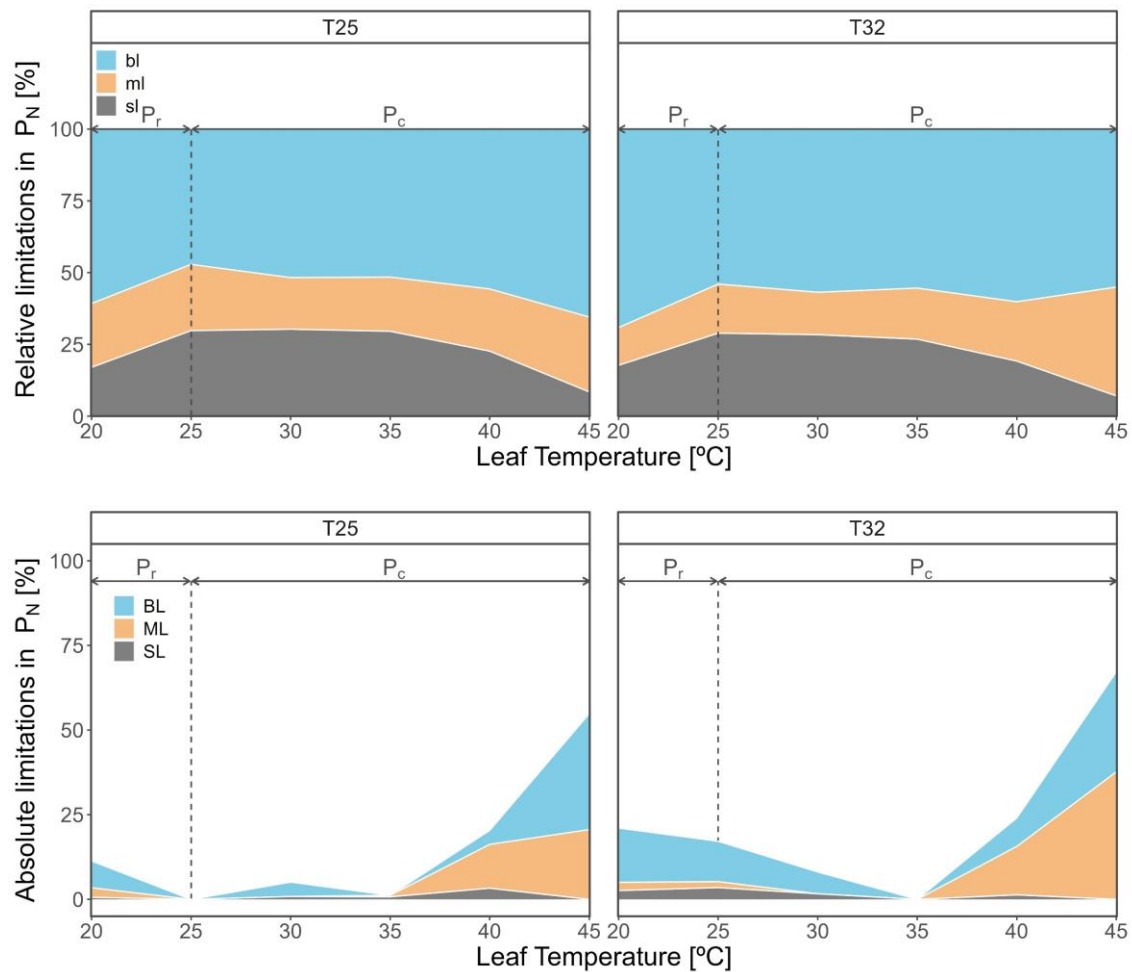


Figure 24. Left T25, right T32. Area graphs that illustrate the absolute and relative limitations to net assimilation (P_N) within a leaf temperature range of 20 to 45°C, according to Grassi & Magnani (2005). The left arrow (P_r) and the right arrow (P_c) signify the main limitation process of the photosynthesis activity, based on equation 2.27 from von Caemmerer (2000). The black vertical dashed line highlights the transition temperature (25°C) from P_r to P_c . The limitations are categorised into three types, each represented by a distinct colour. Absolute limitations are denoted by capital letters, while relative limitations are indicated by lower-case letters: Biochemical limitations (BL or bl) shown in blue; Mesophyll limitations (ML or ml) shown in yellow and stomatal limitations (SL or sl): shown in grey.

Across the entire temperature range, stomatal limitation (SL) to net CO₂ assimilation (P_N) remained negligible in both treatments, never exceeding 3.5% in absolute terms (Fig. 24). SL was particularly low in T25 across all temperatures, and in T32 it was only slightly elevated at 20 °C (2.6%) and 25 °C (3.5%).

At lower temperatures (20–25 °C), biochemical limitation (BL) dominated in both treatments. In T25 seedlings, BL accounted for ~7.9% at 20 °C, while in T32, it reached 16%, more than double the mesophyll conductance limitation (ML), which stayed below 2.5%. At 25 °C, both ML and BL dropped to zero in T25 but remained notable in T32 (ML = 1.8%, BL = 11.9%).

As temperature rose to intermediate levels (30–35 °C), absolute limitations generally declined. In T25, BL decreased to a minimum of 0% at 35 °C, while ML and SL remained below 1%. A similar trend was observed in T32, with BL dropping to 6.4% at 30 °C and reaching zero at 35 °C.

At higher temperatures (40–45 °C), limitations increased sharply. In T25, BL rose from 4.1% at 40 °C to 34.3% at 45 °C, while ML increased from 12.9% to 20.6%. A comparable pattern was seen in T32, with ML reaching 37.7% and BL 29.6% at 45 °C—highlighting a strong rise in both diffusive and biochemical constraints under heat stress.

4.1.4. Discussion

This study reveals that *Fagus sylvatica* seedlings grown under warmer conditions exhibit clear **photosynthetic acclimation**, as assessed through detailed **gas exchange and chlorophyll fluorescence measurements**, despite maintaining a conserved overall temperature-response pattern. By isolating the effects of growth temperature under controlled VPD conditions, we show that reductions in carbon assimilation arise primarily from biochemical downregulation through nitrogen allocation, rather than diffusive constraints. These photosynthetic limitations are accompanied by a compensatory strategy of elevated stomatal conductance and transpiration, supporting evaporative cooling and CO₂ influx at the cost of greater water loss. At the same time, seedlings grown at higher temperatures invest more heavily in photoprotective mechanisms—particularly non-photochemical quenching—indicating a shift toward energy dissipation to safeguard electron transport. Despite these divergent acclimation responses, all plants followed a common trajectory of thermal sensitivity across traits, transitioning

similarly between photosynthetic limitations (from P_r to P_c) and converging on shared biochemical and diffusive constraints under heat stress.

4.1.4.1. Photosynthetic Acclimation to Warming in *Fagus sylvatica*

Across the full temperature range tested (20–45 °C), *Fagus sylvatica* seedlings grown at elevated temperature (32 °C) consistently exhibited ~30% lower net CO₂ assimilation (P_N) than those grown at 25 °C, except around 35 °C, where differences were minimal (Fig. 19). This reduction closely mirrored a ~30% decline in both V_c , J_f , V_{cmax} and J_{max} , suggesting that warm-grown seedlings experienced primarily biochemical limitations to photosynthesis. In contrast, mesophyll conductance (g_m) and stomatal conductance (g_s) remained largely unchanged between treatments (Fig. 20), ruling out a significant role of diffusive constraints. Notably, despite differences in absolute trait values, the temperature-response curves of these traits displayed similar shapes across both treatments, with no detectable shift in their theoretical temperature optima (T_{opt}).

This convergence in thermal response profiles indicates that core photosynthetic functions scaled proportionally across growth regimes—not only from a biochemical perspective, but also in terms of diffusive capacity. The absence of any upward shift in the thermal optimum (T_{opt}), despite prolonged exposure to elevated temperatures, further underscores this conservative acclimation pattern. While numerous studies report that T_{opt} tends to align with growth conditions (Berry & Björkman, 1980; Kumarathunge et al., 2019; Yamori et al., 2014), our results support a growing body of evidence showing stable T_{opt} coupled with decreased photosynthetic capacity under warming (K. R. Carter & M. A. Cavaleri, 2018; Crous et al., 2025; Dusenge et al., 2021).

Still, plants do not necessarily require a shifted T_{opt} to acclimate effectively, as thermal acclimation often maintains or even improves carbon gain under higher growth temperatures through mechanisms such as heat-stable Rubisco activase (Crafts-Brandner & Salvucci, 2000; Crafts-Brandner et al., 1997; Law & Crafts-Brandner, 1999), adjustments to thylakoid membrane composition (Murakami et al., 2000; Samtani et al., 2022), or enhanced mesophyll conductance (Evans & Von Caemmerer, 2013; von Caemmerer & Evans, 2015). This suggests that *Fagus sylvatica* does not rely on T_{opt}

adjustment for acclimation but instead maintains proportional scaling among photosynthetic processes across temperatures.

Mechanistically, this downregulation appears to reflect reduced nitrogen investment in photosynthetic proteins. Leaf nitrogen content was significantly lower in T32 seedlings, likely limiting Rubisco abundance and electron transport capacity—key constraints on V_{cmax} and J_{max} (Dusenge et al., 2020; Jin et al., 2011). Notably, both carboxylation and photorespiration processes acclimated in parallel, with V_c and R_{PR} showing similarly reduced capacities and thermal optima in T32 plants, suggesting a coordinated regulation of Rubisco function under warming, potentially mediated by Rubisco activase (Sage et al., 2008; M. E. Salvucci & S. J. Crafts-Brandner, 2004). The similar shape of the photosynthetic temperature response curves across treatments further supports this interpretation: photosynthetic traits scaled coherently with temperature, but absolute performance was constrained by nutrient allocation rather than enzyme kinetics or structural adjustments. In this context, thermal acclimation in *Fagus sylvatica* appears to function more through baseline resource economy than through active optimization of temperature responses.

Although photosynthetic performance declined under warming, carbon balance is not solely determined by CO_2 assimilation. Respiratory costs—including both mitochondrial respiration and photorespiration—also impose a substantial metabolic burden. Interestingly, despite their lower P_N , T32 seedlings exhibited reduced rates of mitochondrial respiration (R_i) and inferred photorespiration (R_{PR}) compared to T25 when measured at the same temperatures. This suggests that warmer-grown plants downregulated baseline metabolic fluxes as part of a resource-saving strategy, potentially contributing to the observed lower nitrogen allocation to photosynthetic proteins.

However, this reduction in respiratory loss does not imply improved carbon economy. Our results show that T32 seedlings invested disproportionately in non-photochemical quenching (NPQ), maintaining levels comparable to T25 despite lower P_N . This pattern indicates a compensatory shift toward photoprotective heat dissipation under warming—reflecting a physiological trade-off between photosynthetic efficiency and stress avoidance (Zahra et al., 2023).

Taken together, these responses point to a constrained form of thermal acclimation: *Fagus sylvatica* seedlings prioritize photoprotection and metabolic conservation over maximizing carbon gain. The maintenance of high NPQ likely entails increased investment in heat shock proteins and protective pigment systems, further diverting resources from carbon assimilation. In turn, this strategy may buffer plants under chronic moderate heat but narrows their safety margin under acute stress, especially given this species' known sensitivity to high VPD even in the absence of soil drought (Lendzion & Leuschner, 2008).

4.1.4.2. Uncoupling of Electron Transport and Carbon Assimilation Under Warming Conditions

Despite substantial differences in CO₂ assimilation between treatments, the effective quantum yield of PSII (Φ_{PSII}) and the fraction of open PSII reaction centers (qL) followed similar temperature-response curves in both T25 and T32-grown *Fagus sylvatica* seedlings (Fig. 23). In other words, PSII photochemical efficiency and the openness of PSII centers remained largely insensitive to growth temperature. This stability implies constrained acclimation of core light-dependent processes—likely a mechanism to preserve redox homeostasis and minimize proton leakage across the thylakoid membrane, thereby protecting the coupling efficiency of ATP synthesis (Yamori et al., 2014). Such regulation, which maintains a stable Φ_{PSII} across thermal gradients, suggests a robust control of the proton motive force and redox poise in the photosynthetic electron transport chain (PETC). However, at higher temperatures than the optimum, Φ_{PSII} and qL tends to deeply decrease, pointing potential inhibition to PSII as a consequence of heat stress (Nievola et al., 2017).

In contrast, the electron transport rate derived from chlorophyll fluorescence (J_f) showed clear sensitivity to growth temperature: T25 seedlings maintained higher J_f across the entire thermal range, whereas T32 plants failed to enhance J_f capacity under warming, probably by lower nitrogen investment in PETC machinery (Duarte et al., 2021). This decoupling from CO₂ fixation resulted in a steep rise in the J_f/P_N ratio—particularly in T32 plants—which surpassed the canonical stoichiometric threshold of ~8 electrons per CO₂ assimilated (Perera-Castro & Flexas, 2023). Elevated J_f/P_N values indicate excess excitation pressure and suggest that, under heat, photochemistry remains active while downstream biochemical sinks become

saturated (Li & Ma, 2021). This imbalance likely necessitates enhanced photoprotective energy dissipation via alternative sinks or antioxidant responses (Nievola et al., 2017; Yamori et al., 2014). Altogether, these findings align with a conservative acclimation strategy in *Fagus sylvatica*, in which PSII integrity is maintained at the cost of reduced carbon gain and increased oxidative risk.

4.1.4.3. Trade-off between stomatal conductance, thermal regulation and water loss

Across the full temperature gradient, transpiration rates in well-watered *Fagus sylvatica* seedlings increased exponentially with rising leaf temperatures. This intensification of water loss enabled effective evaporative cooling, as evidenced by a narrowing leaf–air temperature difference (ΔT) at higher temperatures (Fig. 25). Notably, stomatal conductance (g_s or g_{sw}) remained largely stable across all temperatures and growth treatments, suggesting that stomata remained open even under high vapor pressure deficit (VPD). This behavior promoted both CO₂ uptake and latent heat dissipation, highlighting a physiological strategy that prioritizes carbon assimilation and thermal regulation over strict water conservation (Cowan & Farquhar, 1977; Damour et al., 2010; Fredeen & Sage, 1999; Gates, 1968).

Such a strategy, while effective under non-limiting water conditions, introduces a significant trade-off: the benefit of thermal protection through enhanced transpiration comes at the cost of elevated water expenditure (Drake et al., 2018). This trade-off is particularly important under high VPD, where maintaining open stomata can exacerbate water loss, even as it supports gas exchange. Our results support this interpretation, as high transpiration rates coincided with effective evaporative cooling and sustained carbon uptake (Fig. 22). However, given that mesophyll conductance (g_m) constrains photosynthetic rate (P_N), the interaction between stomatal behavior, g_m , and photosynthetic performance becomes a critical axis of thermal acclimation (Fig. 23). In this context, *Fagus sylvatica* seedlings appear to prioritize thermal homeostasis even at the risk of accelerated water depletion.

In addition to physiological adjustments, seedlings grown under warmer conditions exhibited clear morphological acclimation. These plants developed smaller individual leaves but increased total leaf number—an architectural

strategy that enhances heat dissipation while minimizing the proportion of total leaf area lost in case of hydraulic failure (Rubio et al., 2025). This shift likely reflects an adaptive response aimed at distributing hydraulic risk and maximizing thermal stability under chronic warmth.

Finally, the observed for E and ΔT reinforce the complexity of these acclimation patterns. These traits response distinctly from photosynthetic but similarly to photoprotective traits, suggesting a coordinated but modular response to heat. As temperature rose, ΔT narrowed, transpiration increased, and non-photochemical quenching (NPQ) intensified (Fig. 22 and 23). This co-activation of evaporative cooling and photoprotective mechanisms underscores the integrated nature of the thermal tolerance strategy in *Fagus sylvatica*. Taken together, these findings highlight a finely tuned response in which water loss, leaf architecture, and biochemical safeguards interact to sustain function under thermal stress—albeit at considerable resource cost (Drake et al., 2018; Gates, 1968; Mondal et al., 2023).

4.1.4.4. Thermal Sensitivity in *Fagus sylvatica*: Convergent Photosynthetic Responses Across Contrasting Growth Temperatures

Despite clear acclimation differences in absolute photosynthetic capacity, *Fagus sylvatica* seedlings displayed remarkably consistent temperature-response patterns across both growth environments. Linear mixed-effects (LME) models revealed that while growth temperature significantly influenced trait magnitudes, allowing for treatment-specific slopes did not improve model fit. This indicates that seedlings grown at 25 °C and 32 °C followed parallel thermal trajectories—pointing to a shared physiological response shaped more by temperature per se than by growth history.

Importantly, we observed no indication that warm-grown plants shifted their resource allocation toward one photosynthetic module at the expense of another. Instead, all major components—Rubisco capacity, electron transport, mesophyll conductance—declined in unison, suggesting a systemic response rather than a targeted adjustment (Fig. 24). While the 32 °C seedlings maintained lower photosynthetic efficiency overall, they shared the same limiting steps and inflection points with the 25 °C group, emphasizing the common biochemical and structural vulnerabilities that define the species' thermal ceiling.

Moreover, all individuals exhibited a similar transition from RuBP regeneration–limited assimilation (P_r) to Rubisco-limited assimilation (P_c) above 25 °C, reinforcing the idea that core photosynthetic processes—biochemical and diffusive alike—respond in a coordinated manner to rising temperatures, regardless of prior thermal exposure. This convergence was further supported by stable trait ratios, similar T_{opt} , and overlapping curve shapes for V_{cmax} , J_{max} , and g_m across treatments (Table 2), suggesting that photosynthetic limitations emerge along conserved thresholds.

Above 40 °C, however, thermal sensitivity sharply increased. Rubisco activase, known to be more heat-labile than Rubisco itself, likely contributed to observed deactivations in V_{cmax} , R_{PR} , and J_{max} , consistent with declines in carbon assimilation and the onset of photosystem inactivation (Gounaris et al., 1986; Haldimann & Feller, 2004; Sharkey & Zhang, 2010)³. At the same time, g_m declined substantially, imposing a parallel constraint on CO₂ diffusion. This convergence of biochemical and diffusive limitations at high temperatures underscores the necessity of incorporating both into predictive models of photosynthesis under climate stress—particularly since g_m is often overlooked despite its clear role in curtailing P_N under extreme conditions (Knauer et al., 2020).

Beyond biochemical downregulation, thermal stress also amplified energetic trade-offs. As temperatures rose, both cohorts increased non-photochemical quenching (NPQ), indicating raising of photoprotective mechanisms that diverts excess energy from photosystems. However, in T32 seedlings, this investment occurred without a commensurate increase in carbon gain, leading to a decoupling of protection and productivity. At 45 °C, T32 seedlings exhibited particularly severe declines in P_N , elevated R , but also high NPQ—hallmarks of rising oxidative stress and an over-reliance on dissipation pathways (Drake et al., 2018; Mondal et al., 2023; Zahra et al., 2023).

These results suggest a tipping point in thermal resilience: although photoprotective mechanisms remain active, their energy cost may outweigh their benefit when respiration and photorespiration accelerate. This trade-off—between thermal safety and photosynthetic efficiency—highlights a structural limitation in *Fagus sylvatica*'s acclimation strategy. The species does not appear to fully rewire its carbon metabolism under prolonged warming but instead modulates existing pathways within a conservative physiological envelope.

Taken together, our findings demonstrate that the temperature sensitivity of photosynthesis in *Fagus sylvatica* is both consistent and inherently limited. While seedlings exhibit clear biochemical acclimation and increased investment in photoprotective mechanisms, these responses converge toward a common functional ceiling beyond 40 °C—where both carbon assimilation and thermal regulation begin to fail. At this threshold, plants grown under more favorable thermal conditions retain a broader safety margin, likely due to higher nitrogen allocation and superior carbon balance, which together buffer the effects of acute heat stress. This convergence highlights a dual vulnerability to resource depletion and metabolic instability under extreme temperatures, underscoring the need for further investigation into the transient dynamics of thermal stress in temperate forest species as climate extremes intensify.

4.1.4.5. Fitting parameters from an Arrhenius temperature model as a proxy of acclimation

As detailed above, growth temperature markedly influences photosynthetic traits such as CO₂ assimilation (P_N), dark respiration (R_D), maximum carboxylation rate (V_{cmax}), and maximum rate of electron transport (J_{max}), as shown by our temperature-response curve fittings.

However, we found no significant correlation among the empirical Arrhenius parameters—other than a pronounced linear relationship between E_d and ΔS . The primary objective of applying these equations to P_N , V_{cmax} , J_{max} is to derive robust parameters for modelling photosynthetic processes at scales ranging from individual plants to ecosystems.

Yet, these models stem from bacterial growth equations (Sharpe & DeMichele, 1977) originally based on studies of isolated proteins (Eyring & Stearn, 1939). Caution is therefore warranted when extending them to more complex organisms (Moore et al., 2021): although they are widely used (Bernacchi et al., 2001; Bunce, 2000; Crous et al., 2022; Dreyer et al., 2001; Kumarathunge et al., 2019), Lowry and Ratkowsky (1983) observed that these formulations lack full thermodynamic considerations, particularly for irreversible enzyme reactions.

For example, when fitting a given dataset to these models, P_{Tref} determines the “height” of the curve, while ΔS and E_d , together with E_a , govern both the peak and curvature—thus defining the T_{opt} value. However, as previously

noted, no single parameter dictates the function's shape across the tested temperature range (Fig. Supplementary, partial derivatives), particularly given the strong correlation between E_d and ΔS . Analysis of these partial derivatives shows that curvature and shape result from a balance among the empirical parameters (P_{Tref} , E_a , E_d , ΔS). Consequently, interpreting them requires caution: the interplay between E_d and ΔS can be as crucial as their individual magnitudes, and relying on a single parameter alone fails to capture the full complexity of each modelled trait.

4.1.5. Conclusions

This study demonstrates that *Fagus sylvatica* seedlings exhibit clear photosynthetic acclimation to elevated growth temperatures, primarily through biochemical adjustments constrained by reduced nitrogen allocation. Despite this downregulation in net CO₂ assimilation, Rubisco capacity, and electron transport, temperature-response curves for gas exchange and mesophyll conductance remained stable, indicating a conserved thermal sensitivity independent of growth regime.

Warm-grown seedlings also adopted a water-intensive strategy to maintain leaf thermal balance, with consistently high stomatal conductance and transpiration rates enabling evaporative cooling under high VPD. Morphological shifts—smaller but more numerous leaves—further suggest adaptation aimed at distributing hydraulic risk and enhancing heat dissipation.

At the photoprotective level, increased investment in non-photochemical quenching (NPQ) highlights a physiological trade-off between photosynthetic efficiency and thermal safety. However, this investment did not offset the decline in carbon gain at high temperatures, particularly beyond 40 °C, where both biochemical and diffusive limitations converged.

Overall, our findings reveal a constrained acclimation strategy: *Fagus sylvatica* adjusts within a narrow physiological envelope, prioritizing survival mechanisms over metabolic optimization. Despite acclimatory shifts in resource allocation and stress mitigation, all seedlings exhibited a remarkably consistent response trajectory, underscoring a common thermal ceiling for this species. As climate extremes intensify, this dual vulnerability—to carbon imbalance and water loss—may critically limit the

resilience of *Fagus sylvatica* in warming environments, particularly in Mediterranean populations already near the species' thermal threshold.

4.1.6. Supplementary

Temperature response models of P_N , V_{cmax} , J_{max} , g_m , and R_l normalized to unity

Additionally, a normalized temperature response formulation was applied for comparative analyses across parameters (P_N , V_{cmax} , J_{max} , g_m , and R_l), where all values were scaled relative to their maximum (Bernacchi et al., 2002; Harley & Tenhunen, 1991; Nobel, 2009a, 2009b):

$$P(T) = \frac{e^{(c - \frac{E_a}{RT})}}{\left(1 + e^{\left(\frac{\Delta S T - E_d}{RT}\right)}\right)} \quad (20)$$

and, when deactivation was not observed:

$$P(T) = e^{(c - \frac{E_a}{RT})} \quad (21)$$

In these formulations, where E_a (kJ mol⁻¹) is the activation energy, R (0.0083143 kJ K⁻¹ mol⁻¹) is the ideal gas constant, E_d (kJ mol⁻¹) is the deactivation energy, ΔS (kJ K⁻¹ mol⁻¹) is an entropy term, and T (K) is leaf temperature, and c it is a scaling constant.

T_{opt} in this normalized framework was computed analogously to Eq. 18:

$$T_{\text{opt}} = \frac{-E_d}{RLn\left(\frac{-c + E_a}{-c + E_a - E_d}\right) - \Delta S} \quad (22)$$

Table 3. Acronyms, definitions, variables and units used

Symbol	Definition	Values (units)
P_N	Net assimilation rate	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
C_c	Chloroplastic CO_2 concentration	($\mu\text{mol CO}_2 \text{ mol}^{-1}$)
C_i	Intercellular CO_2 concentration	($\mu\text{mol CO}_2 \text{ mol}^{-1}$)
E	Leaf transpiration	($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)
R_{PR}	Rate of photorespiratory CO_2 release	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
F_v/F_m	Maximum quantum efficiency of PSII	(dimensionless)
F_v'/F_m'	Operating quantum efficiency of PSII	(dimensionless)
g_m	Mesophyll conductance to CO_2	($\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
g_s	Stomatal conductance to CO_2	($\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
g_{sw}	Stomatal conductance to water vapour	($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)
J_f	Electron transport rate corrected according to Valentini et al (1995)	($\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}$)
J_{high}	Electron transport rate derived from the curvature method (J_{max} in Farquhar notation)	($\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}$)
J_{max}	Maximum rate of electron transport	($\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}$)
NPQ	Non-photochemical quenching	(dimensionless)
Φ_{PSII}	Effective quantum yield of photosystem II	(dimensionless)

q_L	Fraction of open PSII centres	(dimensionless)
R_D	Respiration rate in darkness	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
R_l	Mitochondrial respiration rate in the light	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
V_c	Rubisco carboxylation rate	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
V_{cmax}	Maximum Rubisco carboxylation rate	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
V_o	Rubisco oxygenation rate	($\mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$)
WUE	Water use efficiency	($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$)
WUE_i	Intrinsic water use efficiency	($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$)
I^*	Chloroplastyc CO_2 compensation point	($\mu\text{mol CO}_2 \text{ mol}^{-1}$)

Table 4. Summary table of mean values ($\pm$ standard error) of the parameters describing temperature responses of leaf photosynthetic capacity (fitted to eq. XX) in seedlings of *Fagus sylvatica* growing at two different temperatures for net assimilation rate (P_N), maximal rate of carboxylation (V_{cmax}), maximal rate of electron flow (J_{max}), mesophyll conductance (g_m) and respiration in darkness (R_D) and the same parameters but normalized to their average values at 25°C. The fitting parameters are the following: scaling constant (c), activation energy (Ea), deactivation energy (Ed), entropy factor (ΔS), explained variance (EVA), optimal temperature (T_{opt}), modeled value of each parameter at the optimal temperature (P_{opt}), and the ratio of the T_{opt} as T32 divided by T25.

Parameter	Tgrowth	c	Ea	Ed	S	EVA	Topt	Popt	T32/T25
	(° C)	-	(kJ mol ⁻¹)	(kJ mol ⁻¹)	(kJ mol ⁻¹ K ⁻¹)	-	(° C)	(Parameter units)	%
P_N	25	5.8 $\pm$ 2	8 $\pm$ 5	230 $\pm$ 39	1 $\pm$ 0.1	0.65	27.5 $\pm$ 1.7	12.5 $\pm$ 0.9	18
	32	8.5 $\pm$ 0.8	15 $\pm$ 2	296 $\pm$ 42	1 $\pm$ 0.1	0.71	32 $\pm$ 1.6	10.2 $\pm$ 0.7	
V_{cmax}	25	25.9 $\pm$ 1.1	53 $\pm$ 3	212 $\pm$ 17	1 $\pm$ 0.1	0.68	33.7 $\pm$ 0.5	140.3 $\pm$ 10.2	12
	32	32.9 $\pm$ 1.8	72 $\pm$ 4	249 $\pm$ 21	1 $\pm$ 0.1	0.95	34.6 $\pm$ 0.7	108.9 $\pm$ 3.2	
J_{max}	25	23.3 $\pm$ 0.6	46 $\pm$ 1	312 $\pm$ 36	1 $\pm$ 0.1	0.77	34.2 $\pm$ 1.3	187.7 $\pm$ 6.2	34
	32	26.8 $\pm$ 2.7	56 $\pm$ 7	243 $\pm$ 28	1 $\pm$ 0.1	0.85	32.6 $\pm$ 1.2	124.1 $\pm$ 6.2	
g_m	25	30.11 $\pm$ 6.2	79 $\pm$ 15	233 $\pm$ 21	1 $\pm$ 0.1	0.87	30 $\pm$ 0.8	0.226 $\pm$ 0.012	12
	32	18.3 $\pm$ 3.3	50 $\pm$ 8	271 $\pm$ 10	1 $\pm$ 0.1	0.94	30.9 $\pm$ 0.5	0.199 $\pm$ 0.007	
R_D	25	18.5 $\pm$ 0.6	46.4 $\pm$ 1.5		0.09	0.98		0.81	
	32	17.7 $\pm$ 0.9	45.2 $\pm$ 2.4		0.11	0.96			
P_N (25)	25	3.2 $\pm$ 2	8 $\pm$ 5	230 $\pm$ 39	1 $\pm$ 0.1	0.65	30 $\pm$ 1.7	1 $\pm$ 0.1	
	32	6.3 $\pm$ 0.8	15 $\pm$ 2	296 $\pm$ 42	1 $\pm$ 0.1	0.71	32.7 $\pm$ 1.6	1.2 $\pm$ 0.1	
V_{cmax} (25)	25	21.5 $\pm$ 1.1	53 $\pm$ 3	212 $\pm$ 17	1 $\pm$ 0.1	0.68	34.3 $\pm$ 0.4	1.7 $\pm$ 0.1	
	32	28.9 $\pm$ 1.8	72 $\pm$ 4	249 $\pm$ 21	1 $\pm$ 0.1	0.95	34.9 $\pm$ 0.7	2 $\pm$ 0.1	
J_{max} (25)	25	18.4 $\pm$ 0.6	46 $\pm$ 1	312 $\pm$ 36	1 $\pm$ 0.1	0.77	34.7 $\pm$ 1.2	1.5 $\pm$ 0.1	
	32	22.4 $\pm$ 2.7	56 $\pm$ 7	243 $\pm$ 28	1 $\pm$ 0.1	0.85	33.1 $\pm$ 1.2	1.6 $\pm$ 0.1	
g_m (25)	25	31.93 $\pm$ 6.2	79 $\pm$ 15	233 $\pm$ 21	1 $\pm$ 0.1	0.87	29.8 $\pm$ 0.8	1.394 $\pm$ 0.073	
	32	20.2 $\pm$ 3.3	50 $\pm$ 8	271 $\pm$ 10	1 $\pm$ 0.1	0.94	30.7 $\pm$ 0.5	1.32 $\pm$ 0.047	
R_D (25)	25	18.7 $\pm$ 0.6	46 $\pm$ 1			0.11	0.98		
	32	18.25 $\pm$ 0.9	45 $\pm$ 2			0.18	0.96		

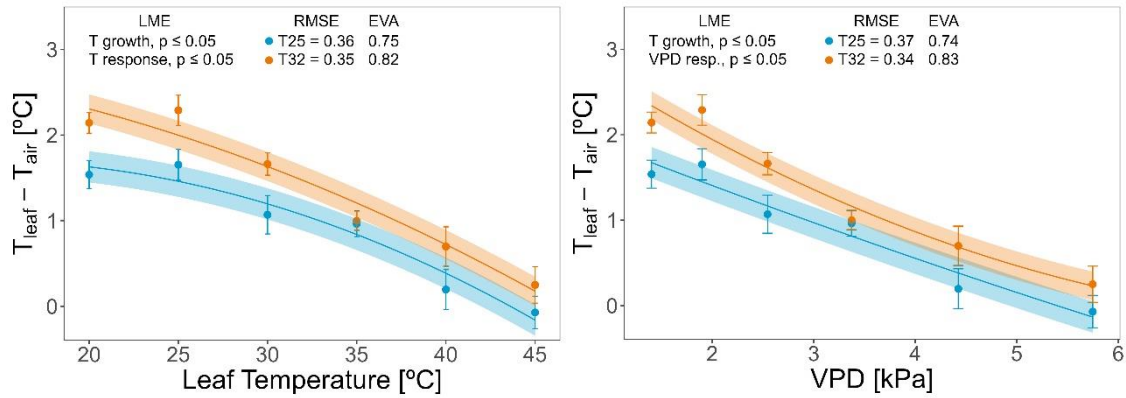


Figure 25. Leaf-to-air temperature difference ($T_{\text{leaf}} - T_{\text{air}}$) as a function of (A) leaf temperature and (B) vapor pressure deficit (VPD) in *Fagus sylvatica* seedlings grown at 25 $^{\circ}\text{C}$ (T25, blue) and 32 $^{\circ}\text{C}$ (T32, orange). Points represent mean values $\pm$ standard error; solid lines represent model fits, and shaded ribbons indicate 95% confidence intervals. Linear mixed-effects models (LME) revealed significant effects of growth temperature and response variable (leaf temperature or VPD). Model performance metrics are shown in each panel: root mean squared error (RMSE) and explained variance (EVA).

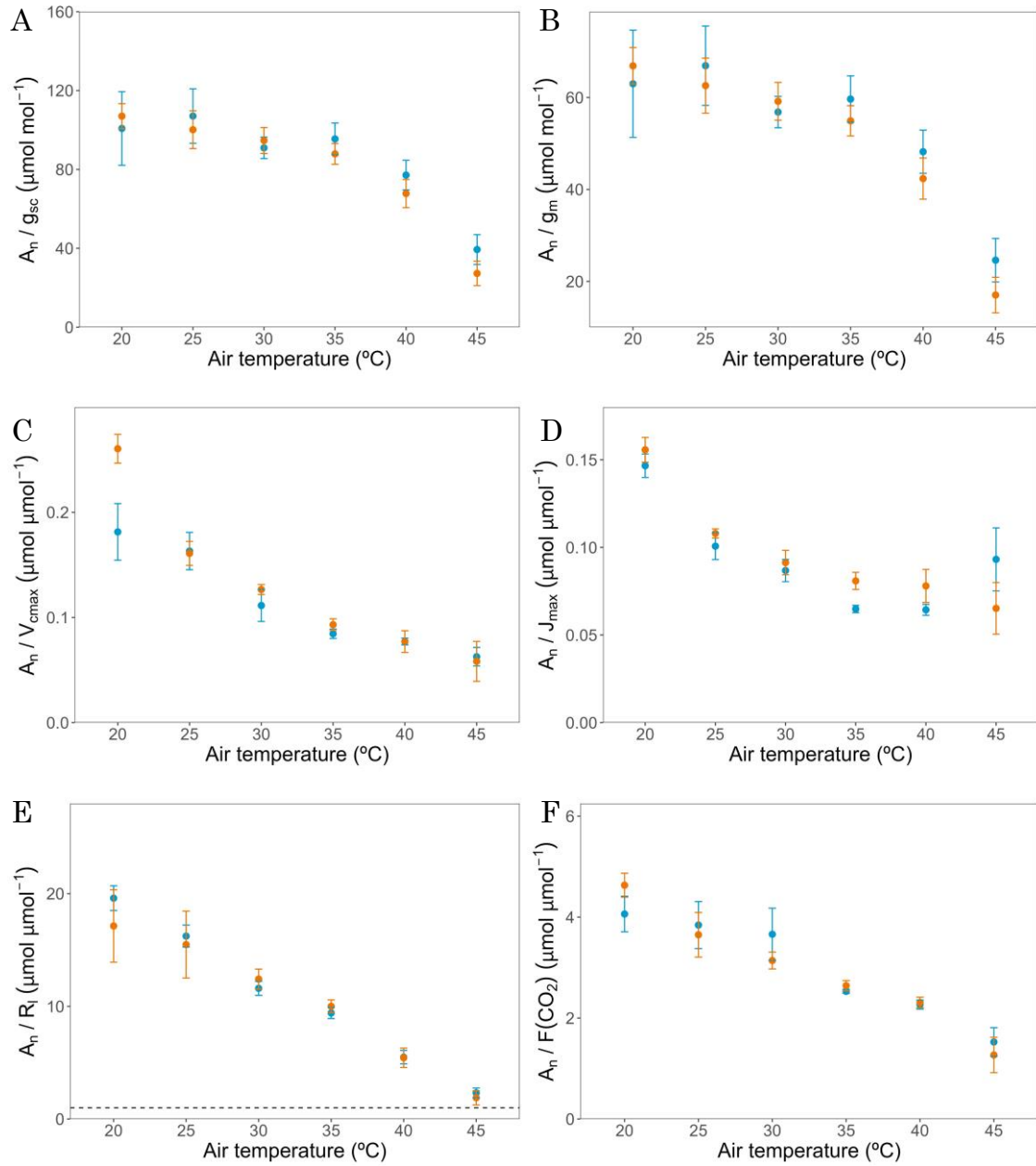


Figure 26. Temperature responses of photosynthetic coordination ratios in *Fagus sylvatica* seedlings grown at two contrasting growth temperatures. Plants were cultivated at 25 °C (T25, blue) and 32 °C (T32, orange), and physiological measurements were conducted across six target leaf temperatures (20–45 °C). Each panel shows the ratio of net assimilation rate (A_n) to a different limiting or supporting factor: A) stomatal conductance to CO_2 (g_{sc}); B) mesophyll conductance (g_m); C) maximum carboxylation rate of Rubisco (V_{cmax}); D) Maximum rate of electron transport (J_{max}); E) Mitochondrial respiration rate in the light (R_l); F) rate of photorespiratory CO_2 release ($F(\text{CO}_2)$). Values represent means $\pm$ standard error ($n = 4$ plants per treatment).

Partial derivates from Ec 11 to each fitting parameter

dP(Tref)/dP

$$P * \exp\left(0.0004 * Ea * \left(1 - \frac{298}{x}\right)\right) * \frac{1 + \exp(-0.0004 * Ed + 0.12 * S)}{1 + \exp\left(0.12 * \frac{S * x - Ed}{x}\right)}$$

dP(Tref)/dEa

$$dEa := P * \left(0.0004 - \frac{0.12}{x}\right) * \exp\left(0.0004 * Ea * \left(1 - \frac{298}{x}\right)\right) * \frac{1 + \exp(-0.0004 * Ed + 0.12 * S)}{1 + \exp\left(0.12 * \frac{S * x - Ed}{x}\right)}$$

dP(Tref)/dΔS

$$\begin{aligned} dS := & -0.12 * P * \exp\left(0.0004 * Ea * \left(1 - \frac{298}{x}\right)\right) * (1 + \exp(-0.0004 * Ed + 0.12 * S)) * \frac{\exp\left(0.12 * \frac{S * x - Ed}{x}\right)}{\left(1 + \exp\left(0.12 * \frac{S * x - Ed}{x}\right)\right)^2} + 0.12 * P \\ & * \exp\left(0.0004 * Ea * \left(1 - \frac{298}{x}\right)\right) * \frac{\exp(-0.0004 * Ed + 0.12 * S)}{1 + \exp\left(0.12 * \frac{S * x - Ed}{x}\right)} \end{aligned}$$

dP(Tref)/dEd

$$\begin{aligned}
 dEd := & -0.0004 * P * \exp\left(0.0004 * Ea * \left(1 - \frac{298}{x}\right)\right) * \frac{\exp(-0.0004 * Ed + 0.12 * S)}{1 + \exp\left(0.12 * \frac{S * x - Ed}{x}\right)} + 0.12 * P * \exp\left(0.0004 * Ea * \left(1 - \frac{298}{x}\right)\right) \\
 & * (1 + \exp(-0.0004 * Ed + 0.12 * S)) * \frac{\exp\left(0.12 * \frac{S * x - Ed}{x}\right)}{\left(1 + \exp\left(0.12 * \frac{S * x - Ed}{x}\right)\right)^2 * x}
 \end{aligned}$$

Impact on the partial derivates from the equation 11 in the range between 280K and 320K (3°C to 47°C) to:

P_N

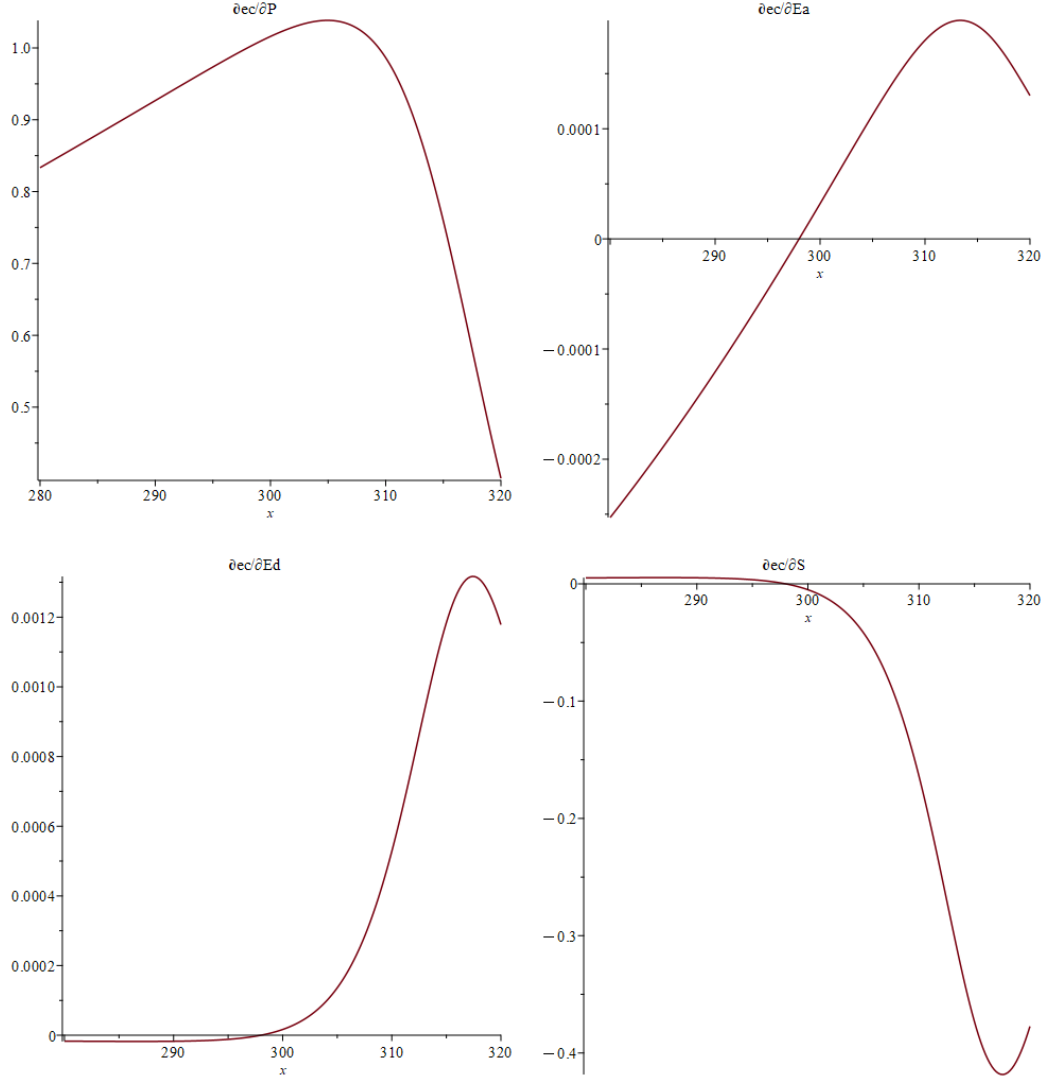


*Figure 27. Sensitivity of modelled net CO₂ assimilation (A_n) to individual Arrhenius parameters across a physiological temperature range (280–320 K). Each panel shows the partial derivative of the fitted exponential temperature response function with respect to one of the four fitting parameters, evaluated using the same parameter set fitted to A_n . The derivatives represent the influence of changes in each parameter on the temperature-dependent response of A_n . Shown are: **(Top left)** $\partial ec / \partial P$: sensitivity to the scaling factor (P), which modifies the overall amplitude of the response curve. **(Top right)** $\partial ec / \partial E_a$: sensitivity to the activation energy (E_a), which governs the initial temperature dependence and rise of A_n ; **(Bottom left)** $\partial ec / \partial E_d$: sensitivity to the deactivation energy (E_d), which controls the decline in A_n at supraoptimal temperatures; **(Bottom right)** $\partial ec / \partial S$: sensitivity to the entropy term (S), which shapes the peak position and symmetry of the curve.*

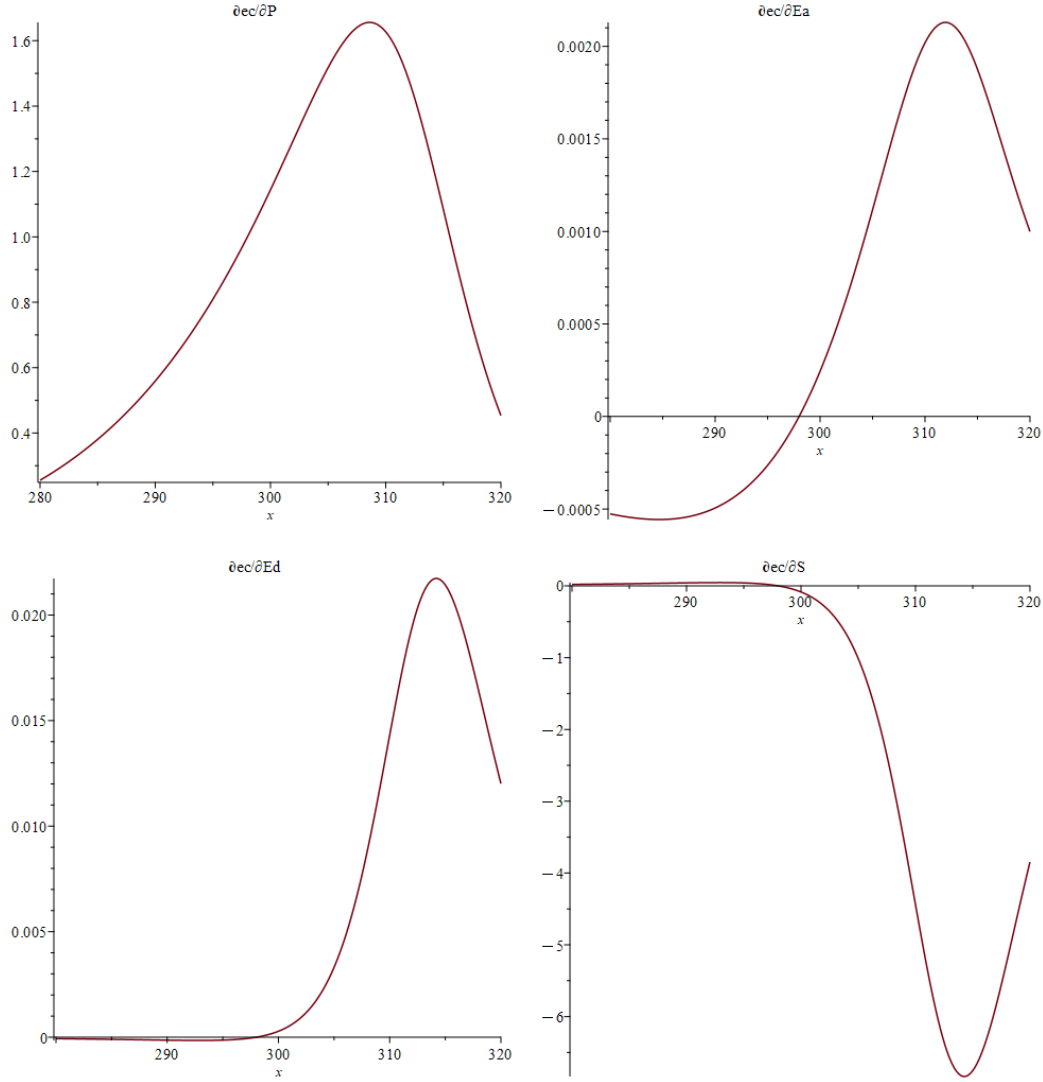
$J_{\max}$ 

Figure 28. Sensitivity of modelled maximum rate of electron transport ($J_{\max}$) to individual Arrhenius parameters across a physiological temperature range (280–320 K). Each panel shows the partial derivative of the fitted exponential temperature response function with respect to one of the four fitting parameters, evaluated using the same parameter set fitted to $J_{\max}$. The derivatives represent the influence of changes in each parameter on the temperature-dependent response of $J_{\max}$. Shown are: **(Top left)** $\partial ec / \partial P$: sensitivity to the scaling factor (P), which modifies the overall amplitude of the response curve. **(Top right)** $\partial ec / \partial E_a$: sensitivity to the activation energy (E_a), which governs the initial temperature dependence and rise of $J_{\max}$; **(Bottom left)** $\partial ec / \partial E_d$: sensitivity to the deactivation energy (E_d), which controls the decline in $J_{\max}$ at supraoptimal temperatures; **(Bottom right)** $\partial ec / \partial S$: sensitivity to the entropy term (S), which shapes the peak position and symmetry of the curve.

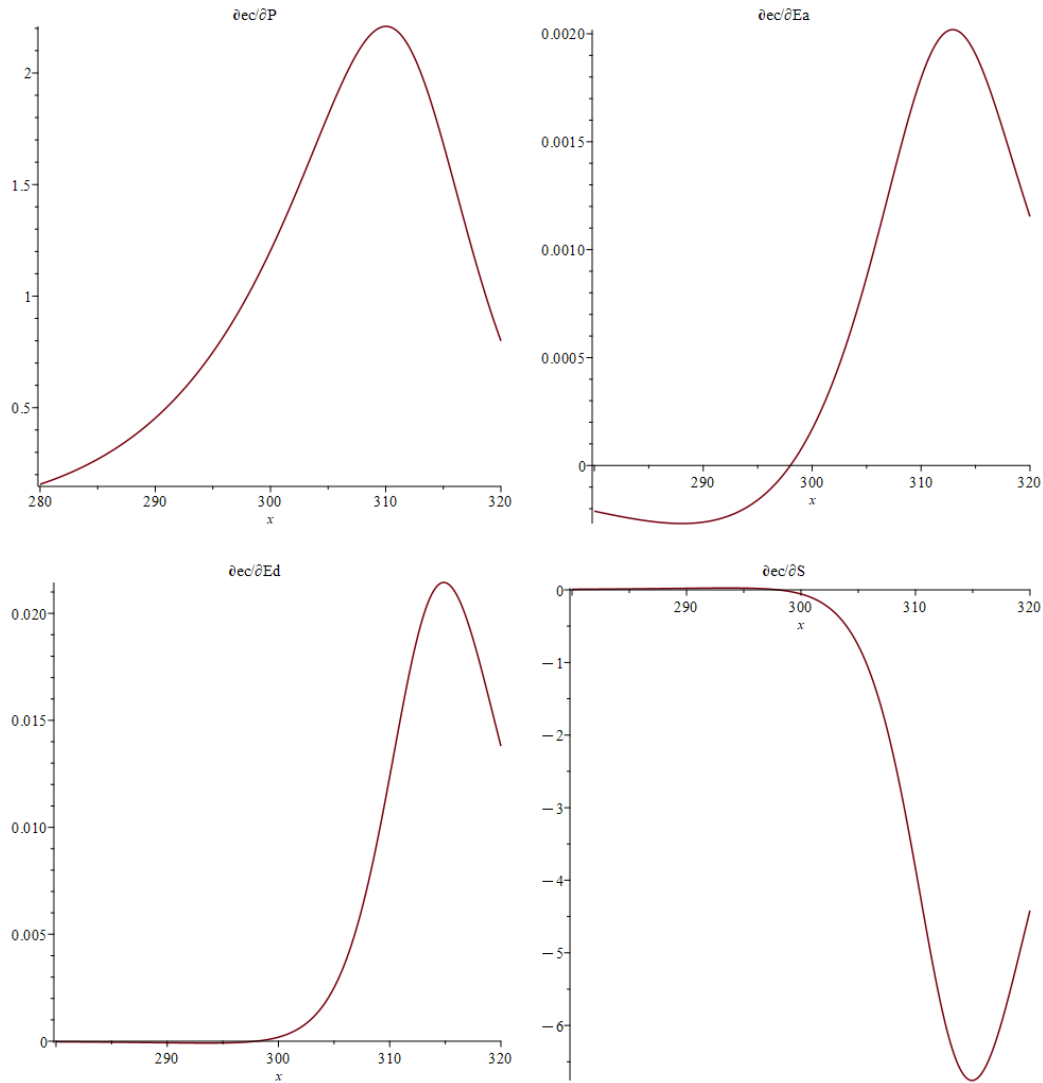
V_{cmax} 

*Figure 29. Sensitivity of modelled maximum rate of carboxylation of Rubisco (V_{cmax}) to individual Arrhenius parameters across a physiological temperature range (280–320 K). Each panel shows the partial derivative of the fitted exponential temperature response function with respect to one of the four fitting parameters, evaluated using the same parameter set fitted to V_{cmax} . The derivatives represent the influence of changes in each parameter on the temperature-dependent response of V_{cmax} . Shown are: **(Top left)** $\partial ec / \partial P$: sensitivity to the scaling factor (P), which modifies the overall amplitude of the response curve. **(Top right)** $\partial ec / \partial Ea$: sensitivity to the activation energy (Ea), which governs the initial temperature dependence and rise of V_{cmax} ; **(Bottom left)** $\partial ec / \partial Ed$: sensitivity to the deactivation energy (Ed), which controls the decline in V_{cmax} at supraoptimal temperatures; **(Bottom right)** $\partial ec / \partial S$: sensitivity to the entropy term (S), which shapes the peak position and symmetry of the curve.*

4.2. Study 2: Temperature Govern Gas Exchange and Stomatal Dynamics in *Fagus sylvatica* L. Seedlings: Insights into Multiple Stress Responses under Constant VPD



4.2.1. Introduction

Global climate change is driving a progressive increase in mean air temperature together with greater frequency of heatwaves and summer droughts in temperate regions (IPCC, 2023; Dai, 2013). These changes intensify evaporative demand and create scenarios of concurrent stresses that have no historical analogue (Schönbeck et al., 2022).. European beech (*Fagus sylvatica*) a mesic and late-successional species, is particularly vulnerable to warming because of its narrow climatic niche and limited drought tolerance (Cavin & Jump, 2017)), (Leuzinger et al., 2005). Although adult trees can persist for decades under suboptimal conditions, seedling establishment and survival represent the most critical bottleneck for population renewal (Fotelli et al., 2009; Rozas et al., 2019). Reports of recruitment failure at the southern range edge highlight that acclimation mechanisms during the juvenile stage are decisive for the future distribution of this species. (Muffler et al., 2021).

Plant responses to warming have been studied extensively, with a general focus on shifts in photosynthetic capacity, respiratory costs, and thermal optima (Way & Oren, 2010; Yamori et al., 2014). However, in natural and experimental systems, temperature is often confounded with variation in vapor pressure deficit (VPD), since both parameters typically covary in the atmosphere (Mills et al., 2024). Because VPD directly drives stomatal closure, experiments without explicit VPD control cannot separate whether observed reductions in assimilation result from biochemical downregulation or from stomatal limitations linked to atmospheric dryness (Buckley et al., 2017). This distinction is critical: growth temperature per se may elicit acclimation in photosynthetic biochemistry, whereas high VPD constrains stomatal opening regardless of thermal acclimation (Way et al., 2013). By maintaining constant VPD, the direct role of temperature on gas exchange can be clarified. Temperature plays a central regulatory role in plant physiology, particularly in shaping how photosynthesis and respiration interact to determine carbon balance under stress. Net CO₂ assimilation (A_n) typically follows a unimodal temperature response: A_n increases with rising temperature up to a thermal optimum (T_{opt}), beyond which it declines steeply. This downturn is driven by a suite of interlinked limitations. The core processes that constrain photosynthesis under warming—Calvin cycle activity, energy supply from the electron transport chain, and CO₂ diffusion within the leaf—are often studied through model-derived parameters. Specifically, the maximum carboxylation rate of Rubisco (V_{cmax}) is used as a proxy for the enzymatic capacity of the Calvin cycle to fix CO₂, while the maximum rate of electron transport (J_{max}) reflects both the activity of the photosynthetic electron transport chain and its role in regenerating RuBP, the CO₂ acceptor. Mesophyll conductance (g_m), meanwhile, captures the diffusional pathway from intercellular airspaces to chloroplasts and is closely tied to membrane permeability and aquaporin function. At supraoptimal temperatures, V_{cmax} and J_{max} often decline due to reduced Rubisco activation and destabilization of the thylakoid membrane system (Bukhov et al., 1999; Crafts-Brandner & Salvucci, 2000; Falcioni et al., 2024). In parallel, g_m may decrease as membrane permeability and aquaporin function are disrupted, further limiting CO₂ supply to the chloroplast (Evans, 2021; Flexas et al., 2012). At the same time, the rates of respiration (R_d) and photorespiratory CO₂ release (R_{PR}) continue to rise with temperature, leading to increased metabolic costs (Dusenge et al., 2019). The resulting imbalance—where assimilation plateaus or declines while

respiratory demand accelerates—can push the seedling carbon balance into deficit, especially under co-occurring drought or shade (Rubio et al., 2025).

These warming-induced constraints are tightly intertwined with stomatal behaviour. Stomata represent a central control point of plant carbon–water relations, as they simultaneously regulate CO₂ uptake for photosynthesis and transpirational cooling of the leaf (Hetherington & Woodward, 2003; Buckley, 2019). Under warming, some species maintain high g_s to enable leaf cooling, often at the cost of reduced intrinsic water-use efficiency (Urban et al., 2017a), while others restrict g_s to conserve water, limiting carbon assimilation (Day, 2000). Because stomatal behaviour integrates multiple signals—including leaf temperature, soil water availability, light intensity, and hormonal regulation—it is especially sensitive to the convergence of different stressors (Brodribb et al., 2020). For instance, warming may increase stomatal conductance under well-watered conditions, but accelerate closure when combined with soil water deficit. Similarly, high light can amplify transpiration and increase risk of desiccation, whereas shade may buffer thermal stress but restrict carbon gain (Niinemets, 2010). (Feller, 2016; Slot et al., 2019).

When evaporative cooling is limited by stomatal closure, plants increasingly depend on alternative photoprotective strategies, such as non-photochemical quenching (NPQ), to dissipate excess excitation energy and prevent thermal damage to the photosynthetic apparatus (Baker, 2008; Falcioni et al., 2024). Although effective in preventing acute stress, these mechanisms are metabolically costly, diverting resources away from carbon gain and further exacerbating the strain on seedling carbon balance under warming and multistress conditions (Fahad et al., 2017; Feller, 2016).

Although the underlying mechanisms remain debated, stomatal responses are shaped by both passive hydraulic signals (e.g., declining turgor or osmotic potential) and active hormonal regulation, primarily via abscisic acid (ABA) (Damour et al., 2010). Moreover, drought and low light can further constrain stomatal function by reducing hydraulic capacity or altering ABA biosynthesis and sensitivity, sharpening the physiological bottleneck imposed by warming (Buckley, 2019; Hernández et al., 2020; Martins et al., 2016; Torres-Ruiz et al., 2015).

Crucially, warming, drought, and low light do not act independently. Evidence from multi-stress experiments indicates that plant responses are

rarely additive. Instead, combined stresses often trigger emergent syndromes that cannot be predicted from single-factor studies (Mittler, 2006; Zandalinas et al., 2021; Brosché et al., 2021; Zandalinas & Mittler, 2022).. For beech seedlings, this implies that the simultaneous occurrence of heat, drought, and shade may constrain plasticity by forcing trade-offs among carbon gain, hydraulic safety, and photoprotection.. Shade-acclimated leaves, for instance, typically exhibit reduced LMA, lower osmotic plasticity, and limited root biomass, all of which heighten vulnerability when soil moisture declines or leaf temperatures rise (Ismael Aranda et al., 2001; Aranda et al., 2015; Aranda et al., 2005). Similarly, when stomatal closure under drought limits transpirational cooling, leaf temperatures may exceed optimal thresholds even under moderate ambient warming, compounding biochemical impairments to photosynthesis and increasing the demand for photoprotective mechanisms (Falcioni et al., 2024; García-Plazaola & Becerril, 2000; Ware et al., 2015). These adaptive trade-offs illustrate how warming acts as a central driver of physiological failure under compound stress.

Despite the recognized importance of multi-stress scenarios, little is known about how temperature governs stomatal dynamics when atmospheric demand is held constant. This gap hinders our ability to predict regeneration success under climate change, particularly in rear-edge populations where water limitation and heat extremes co-occur (Jump et al., 2009; Galiano et al., 2012). A mechanistic understanding of stomatal regulation under combined warming, drought, and light limitation is thus urgently needed. (Deluigi et al., 2025; Didion-Gency et al., 2024; H. L. Duan et al., 2013; Fotelli et al., 2005; O. Ghannoum, N. G. Phillips, J. P. Conroy, et al., 2010; Küppers & Schneider, 1993).

In this study, we investigate how *F. sylvatica* seedlings integrate growth temperature, soil water availability, and light intensity under constant VPD conditions. By combining factorial treatments, we aimed to disentangle the direct effects of growth temperature from those of evaporative demand. Specifically, we hypothesize that: (1) An at elevated growth temperatures and under well-watered conditions would be sustained by compensating for increased respiratory demand through enhanced g_s and upregulated V_{cmax} and J_{max} . (2) warming would accelerate stomatal closure under drought through stronger ABA signalling and impaired osmotic adjustment; (3) shade

would promote an acquisitive strategy with greater leaf production and lower LMA, maintaining assimilation at the cost of reduced water-use efficiency; and (4) the convergence of warming, drought, and shade would narrow the scope for plastic acclimation, shifting photosynthetic limitations from biochemical to predominantly stomatal.

4.2.2. Materials and methods

4.2.2.1. Plant material, growth conditions and treatments

Beech seedlings were established from nuts collected in the Natural Reserve of ‘Hayedo de Montejo’ in 2021 (458,249; 4,551,615 UTM ETRS89). In February 2022, after three months at 4 °C in darkness and moist conditions, one seed per pot germinated within a few days of difference in 9 L pots, filled with a mixture of peat and sand (75–25% v:v). Each pot was fertilized with a slow-release fertilizer (Nutricote®, 16N:4.4P:8.3K, 2.5 g L⁻¹ of substrate). Pots were randomly arranged in two different walk-in growth chambers set at two different temperatures but with the same air *VPD*. One chamber was set with day/night temperatures and relative humidity of 25/15 ± 0.5 °C and relative humidity of 40/68 ± 1% (*VPD* of 1.91/0.86 ± 0.06 kPa) (named T25), close to the temperature optimum for photosynthesis for beech (Šigut et al., 2015), and the other to 32.5/22.5 ± 0.5 °C and 61/80 ± 1% (*VPD* of 1.91/0.86 ± 0.06 kPa) (named T32), both with a 12-h photoperiod. A +7.5 °C increase in mean temperature is expected from June to August for Western and Central Europe and the Mediterranean region according to the scenario SSP5-8.5 for 34 models of AR6 CMIP6 (interactive-atlas.ipcc.ch) (IPCC, 2023). In each chamber, we established two treatments of light intensity: high PPFD (ca. 600 μmol photons m⁻² s⁻¹ at the canopy height) and low PPFD (ca. 200 μmol photons m⁻² s⁻¹ at the canopy height). Low PPFD (e.g., shaded conditions) was provided by gray polyethylene shade nets mounted on frames and was measured as the average of six different measurements using a quantum meter (MQ 200; Apogee Instruments Inc., Logan, UT, USA) in a horizontal position on leaves. Plants developed under well-watered conditions for two months when seedlings with similar height and number of leaves were assigned to two contrasted watering treatments. Well-watered plants were watered daily to full capacity. Water-stressed plants were kept without watering until stomatal conductance declined to one third of that in well-watered plants (ranging from ca. 10 days in high PPFD to ca. 24 days in low

PPFD) and then plants were watered every three days with an initial amount of 150 mL of water for plants under high PPFD and 65 mL of water for plants under low PPFD (about 1/3 of daily evapotranspiration in both cases). Once a week, plants were weighed and the volumetric soil water content (*SWC*) was measured by time domain reflectometry (TDR, Trase System I, Soil Moisture Equipment, Logan, UT, USA) and watering amounts were adjusted. At this time the final multifactorial design (two factors: water and light) was set for each growing temperature: (1) high PPFD (ca. 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the canopy height) and well-watered conditions (HW); (2) high PPFD and moderate water stress (HS); (3) low PPFD (ca. 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the canopy height) and well-watered conditions (LW); (4) low PPFD and moderate water stress (LS). Each week, plants were randomly repositioned within their treatment group. Every three weeks, plants were rotated between chambers to minimize chamber effects, while maintaining their assigned watering treatment.).

4.2.2.2. Minimum leaf conductance ($g_{\min}$), leaf mass per area (LMA) and nitrogen content

Two days before starting gas exchange (GE) and α chlorophyll fluorescence (CF) measurements, on June 12th, we collected healthy, fully expanded leaves from six individuals per growth chamber which will be the plants also used for gas exchange measurements . Each leaf was immediately stored in a plastic zip bag with moist tissue paper and kept in a portable cooler at 4 °C until taken to the laboratory. In the lab, leaves were stored at 4 °C in darkness with the petiole submerged in water overnight until the next day for measurements. We estimated $g_{\min}$ through bench dehydration of the rehydrated leaves with petioles sealed with a high-strength adhesive (Loctite, Prism 401) (Araus et al., 1991). Briefly, the leaf area was measured at the beginning of dehydration, and leaves were placed on top of a perforated rack at constant air temperature and relative humidity (ca. 25 °C and 46%, respectively) without direct light. They were repeatedly weighed with an analytical balance to determine the steady-state rate of water lost by unit of leaf area once stomata were closed, i.e., epidermal transpiration ($E_{\min}$, $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$). To achieve the epidermal minimum conductance by one side of the leaf ($g_{\min}$, $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$), we followed the next approximation ($g_{\min} = E_{\min} P / (2 \text{ VPD})$), where P is the atmospheric pressure (94 kPa) and VPD is the air vapor pressure deficit (1.65 kPa), and the boundary layer

conductance was assumed negligible. Leaf mass area (LMA) was determined for these same leaves, that were oven-dried at 70°C until 48h and then weighed to estimate LMA as the ratio of dry mass to leaf area. The same leaves were then ground to a fine powder using a ball mixer mill (Retsch Mixer MM301, Leeds, UK). A subsample of the homogenized powder was weighed into tin foil capsules and sent to the Davies facilities, where nitrogen concentration was quantified by combusting the material with an elemental analyzer interfaced to an isotope ratio mass spectrometer (PDZ Europa 20-20; Sercon Ltd., Cheshire, UK), yielding calibrated nitrogen concentrations. Finally, these nitrogen values were expressed as area basis using the LMA relationship.

4.2.2.3. Gas exchange and chlorophyll fluorescence

Gas exchange (GE) and chlorophyll *a* fluorescence (CF) measurements were conducted from 14 June to 20 July 20, 2022, on fully expanded, and unshaded leaves from six plants per growth chamber and treatment. All measurements were performed using a portable infrared gas analyzer (LI-6400XT, LI-COR, Lincoln, NE, USA) equipped with a fluorescence leaf chamber (LI-6400-40). To ensure full thermal equilibration, the IRGA was placed inside each chamber at least one hour before measurements began. Leaf cuvette conditions were matched to those of the growth chambers: 25 °C and 40% relative humidity (RH) for T25 plants, and 32 °C and 61% RH for T32 plants.

Measurements were carried out under controlled, near steady-state conditions. Leaf temperature was regulated by adjusting the IRGA block temperature to the desired target. The reference CO₂ concentration was maintained at 400 µmol mol⁻¹ with a flow rate of 300 µmol s⁻¹. Actinic irradiance was set to 1,200 µmol photons m⁻² s⁻¹ for high-light and 800 µmol photons m⁻² s⁻¹ for low-light treatments, including 10% blue light in both.

Simultaneous chlorophyll fluorescence measurements were recorded using the multiphase flash (MPF) method (Loriaux et al., 2013) to determine maximum fluorescence in the light-adapted state (F_m'). Steady-state fluorescence (F_s) and minimal fluorescence in light (F_o') were also measured, with F_o' obtained using a brief far-red pulse at the end of light induction (Baker, 2008). These measurements were used to calculate the effective quantum yield of PSII (F_v'/F_m'). Under dark conditions, maximum fluorescence (F_m) was elicited with a saturating pulse (0.8 s, 8,000 µmol

photons $\text{m}^{-2} \text{s}^{-1}$), and minimum fluorescence (F_0) was recorded following a far-red pulse at the end of the dark induction period, allowing calculation of F_v/F_m .

From these readings, additional fluorescence-derived parameters such as the fraction of open PSII centres (q_L), non-photochemical quenching (NPQ), and effective PSII quantum yield (Φ_{PSII}) were derived according to Kramer et al. (2004). All measurements were taken from the same position of the same leaf to ensure consistency.

After introducing the common settings for measurements, we detail this comprehensive measurement protocol, that was systematically applied to the same leaf and included the following: i) initial gas exchange and fluorescence measurements; ii) a low O_2 A_n -PPFD response curve; iii) dark respiration measurement; iv) an A_n - C_i response curve.

First, GE readings were allowed to stabilize before the initial light-adapted measurements were recorded. A light-response curve was then conducted under a 1% O_2 atmosphere by switching from ambient air to a premixed gas containing 1% O_2 balanced with N_2 . The LI-6400XT software was reconfigured accordingly while maintaining the same flow rate. PPFD was stepwise reduced from 1,200 to 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in the following sequence for high light: 1,200, 1,000, 800, 500, 300, 200, 150, 100, 50, and 20. For low light treatments, the sequence was 800, 600, 500, 300, 200, 150, 100, 50, and 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. At each level, GE and CF parameters were recorded after stabilization (typically within 120–240 s).

Upon completing this curve, the auxiliary gas supply was removed and the system reset to ambient O_2 . To quantify dark respiration (R_d), all light sources were turned off and the plant was kept in darkness for 20 minutes. GE and CF were recorded immediately after this dark period. Mitochondrial respiration in the light (R_l) was calculated as $0.5 \times R_d$ (Busch, 2013).

Subsequently, a A_n - C_i response curve was performed under ambient O_2 (21%) at 1,200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Once photosynthesis stabilized (approximately 20–30 min), the reference CO_2 concentration was adjusted stepwise through 300, 220, 150, 100, 70, 350, 600, 900, 1,000, and 1,200 $\mu\text{mol mol}^{-1}$, with data collected after stabilization at each step (120–240 s).

To avoid measurement artifacts associated with prolonged exposure to the IRGA, a second set of A_n -PPFD curves was conducted on the same

experimental plants one day later. These measurements were performed using a second LI-6400XT/LI-6400-40 system, cross-calibrated under in situ conditions against the primary instrument. The same stepwise light-response sequences were used (from 1,200 or 800 down to 20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, depending on light treatment), and measurements were acquired after reaching steady-state.

All GE data—including curve-based readings—were corrected for potential CO_2 leakage as described in Flexas et al. (2007), and for epidermal conductance following Araus et al. (1991). Light-saturated intrinsic water-use efficiency (WUEi) was calculated as A_n/g_{sw} , and total water-use efficiency (WUE) as A_n/E .

4.2.2.4. Calculation of mesophyll conductance (g_m)

Mesophyll conductance (g_m) was estimated using the **variable J method**, combining gas exchange and chlorophyll fluorescence measurements as described by (Harley, Loreto, et al., 1992). To calibrate electron transport under non-photorespiratory conditions (1% O_2), we used light-response curves (A_n -PPFD) to establish a relationship between the theoretical electron transport rate ($J_a = 4(A_n + R_l)$)—assuming four electrons per CO_2 molecule fixed in the absence of photorespiration—and the electron transport rate derived from chlorophyll fluorescence (J_f). **J_f was calculated as $J_f = (\alpha \times \beta) \times \text{PPFD} \times \Phi_{\text{PSII}}$** , where Φ_{PSII} is the effective quantum yield of PSII, α is leaf absorptance, and β is the fraction of absorbed photons partitioned to PSII (Cano et al., 2014). The product $\alpha\beta$ was determined according to (Valentini et al., 1995).

Mesophyll conductance (g_m , $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) was then calculated as:

$$g_m = \frac{A_n}{C_i - \frac{I^* [J_f + 8(A_n + R_l)]}{J_f - 4(A_n + R_l)}} \quad (23)$$

where A_n is the net CO_2 assimilation rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), C_i is the intercellular CO_2 concentration ($\mu\text{mol mol}^{-1}$), I^* is the CO_2 compensation point in the absence of mitochondrial respiration ($\mu\text{mol mol}^{-1}$) according to (Bernacchi et al., 2001), and R_l is mitochondrial respiration in the light ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$).

The chloroplastic CO_2 concentration (C_c , $\mu\text{mol mol}^{-1}$) was defined as:

$$C_c = C_i - \frac{A_n}{g_m} \quad (24)$$

Finally, when feasible, we incorporated our own estimations of R_l and g_m into subsequent calculations.

4.2.2.5. Calculation of maximum rate of carboxylation of Rubisco (V_{cmax}), apparent light saturated rate of electron transport (J_{high}), and light saturated maximum rate of electron transport (J_{max})

A_n – C_i response curves were fitted using the Farquhar model of photosynthesis (Farquhar et al., 1980) incorporating established modifications (Harley, Thomas, et al., 1992; Sharkey, 1985; Wullschlegel, 1993). Net assimilation (A_n , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) was modeled as:

$$A_n = V_c - 0.5V_o - R_l = \min\{P_c, P_r, P_p\} \quad (25)$$

A_n is also limited by one of three processes: Rubisco activity (P_c), RuBP regeneration via electron transport (P_r), or triose phosphate utilization (P_p), defined as:

$$P_c = \frac{(C - \Gamma^*)V_{cmax}}{C + K_c\left(1 + \frac{O}{K_o}\right)} - R_l \quad (26)$$

$$P_r = \frac{(C - \Gamma^*)J_{max}}{4C - 8\Gamma^*} - R_l \quad (27)$$

$$P_p = 3TPU - R_l \quad (28)$$

In these expressions, V_c and V_o are rates of carboxylation and oxygenation of Rubisco ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); R_l is the rate of CO_2 evolution in light resulting from processes other than photorespiration ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); $\min\{\}$ denotes the “the minimum of”; P_c , P_r and P_p are the net CO_2 assimilation rate limited by Rubisco, RuBP and TPU, respectively ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); TPU is the rate of phosphate release in triose phosphate utilization (starch and sucrose production); C and O are the partial pressures of CO_2 and O_2 at RubisCO (Pa); K_c and K_o are Michaelis-Menten constants for carboxylation and oxygenation respectively; C_i is the partial pressures of CO_2 in the intercellular air space (Pa); J_{max} is the maximum rate of electron transport ($\mu\text{mol e}^- \text{ m}^{-2} \text{ s}^{-1}$) that is dependent upon incident light irradiance (I); the factor 4 in Eqn 5 indicates that for the regeneration of RuBP in the Calvin cycle, four electrons are necessary for produce enough ATP and NADPH (Farquhar et al., 1980).

The parameter J_{high} —the apparent saturation point of electron transport—was initially derived from A_n – C_i curve fitting. However, as noted by Buckley

and Díaz-Espejo (2015), J_{high} underestimates the true asymptotic capacity for electron transport. Therefore, to obtain J_{max} we applied the non-rectangular hyperbola model from von Caemmerer (2000), using the following equations:

$$I_2 = I \cdot \frac{\text{abs}(1-f_1)}{2} \quad (29)$$

$$J_{\text{high}} = \frac{I_2 + J_{\text{max}} - \sqrt{(I_2 + J_{\text{max}})^2 - 4\theta I_2 J_{\text{max}}}}{2\theta} \quad (30)$$

Where I_2 it is the useful light absorbed by PSII, I it is the incident irradiance, abs it is the absorptance of leaves (0.85), f_1 corrects for spectral quality of light (0.15), θ is an empirical curvature factor and 0.7 is a good average value and J_{high} it is the apparent saturated rate of electron transport derived from A_n - C_i curve (von Caemmerer, 2000).

Solving for J_{max} yields:

$$J_{\text{max}} = \frac{J_{\text{high}}(J_{\text{high}}\theta - I_2)}{J_{\text{high}} - I_2} \quad (31)$$

This correction ensures that J_{max} reflects the theoretical maximum capacity for electron transport under saturating light, rather than the empirical saturation point estimated from A_n - C_i fitting.

Estimates of V_{cmax} and J_{max} at 25 °C ($V_{\text{cmax}25}$ and $J_{\text{max}25}$) were derived by standardizing temperature response curves previously calibrated for these *Fagus sylvatica* seedlings under well-watered, high-light conditions at each growth temperature (unpublished data).

Finally, the rate of photorespiratory CO_2 release (R_{PR}) can be derived as half the oxygenation rate:

$$R_{\text{PR}} = 0.5V_o = \frac{A_n - R_l}{\frac{C_c}{\Gamma^*} - 1} \quad (32)$$

The factor 0.5 is added based on the assumption that one molecule of CO_2 is released for every two oxygenation reactions.

4.2.2.6. Analysis of gas diffusional and biochemical limitations of photosynthesis

The limitations to A_n imposed by the stomatal and mesophyll conductance, and biochemical processes, that could be attributed to V_{cmax} or J_{max} (eq. 6) were examined using the following equations as described by (Grassi & Magnani, 2005):

$$\frac{dA_n}{A_n} = L_s + L_m + L_b = l_s \frac{dg_s}{g_s} + l_m \frac{dg_m}{g_m} + l_b \frac{dV_{cmax}}{V_{cmax}} \text{ (or } l_b \frac{dJ_{max}}{J_{max}} \text{)} \quad (33)$$

$$l_s = \frac{g_{tot}/g_s \cdot \delta P_c \text{ (or } \delta P_j)/\delta C_c}{g_{tot} + \delta P_c \text{ (or } \delta P_j)/\delta C_c} \quad (34)$$

$$l_m = \frac{g_{tot}/g_m \cdot \delta P_c \text{ (or } \delta P_j)/\delta C_c}{g_{tot} + \delta P_c \text{ (or } \delta P_j)/\delta C_c} \quad (35)$$

$$l_b \text{ (by } V_{cmax} \text{ or } J_{max}) = \frac{g_{tot}}{g_{tot} + \delta P_c \text{ (or } \delta P_j)/\delta C_c} \quad (36)$$

Where g_s is stomatal conductance to CO_2 (g_s); g_{tot} is total conductance to CO_2 between the leaf surface and carboxylation sites ($1/g_{tot} = 1/g_s + 1/g_m$); L_s , L_m and L_b are the contributions of stomatal conductance, of mesophyll conductance and of maximum carboxylation rate (or maximum rate of electron transport) to dA_n/A_n , respectively; and l_s , l_m and l_b are the corresponding relative limitations ($0 < l_i < 1$, $i=s, m, b$). Additionally, $\partial P_c/\partial C_c$ and $\partial P_j/\partial C_c$, the partial derivatives of Equations 4 and 5 to C_c , were calculated using the following equations

$$\delta P_c/\delta C_c = \frac{V_{cmax} (\Gamma^* + K_c (1 + \frac{O}{K_c}))}{((C_c + K_c (1 + \frac{O}{K_c}))^2)} \quad (37)$$

$$\delta P_r/\delta C_c = \frac{3 \cdot J_{max} \cdot \Gamma^*}{4(C_c + \Gamma^*)^2} \quad (38)$$

4.2.2.7. Statistical analyses

After checking the normality of the data, three-way analyses of variance (ANOVA) were performed in R v. 4.3.2 (R Development Core Team 2024) using 'Tgrowth', 'light' and 'water' as fixed factors. In all cases, the data met the assumptions of normality and homoscedasticity. , Significance was set at $P \leq 0.05$ and model assumptions were checked. Statistically significant differences between groups were assessed using Tukey's HSD post-hoc test in R v. 4.3.2 using the "agricolae" package. Principal Component Analysis (PCA) was performed in R version 4.3.2, utilizing the "stats" package for matrix calculations and the "factoextra" package for visualization support. The PCA was based on the correlation matrix, and the eigenvectors generated were analysed to identify variables exhibiting a strong association with specific principal components (PCs). Pearson correlation coefficients were estimated using the "rcorr" function in R. Results are presented as means $\pm$ standard error (SE).

4.2.3. Results

4.2.3.1. Plant growth and morphological traits

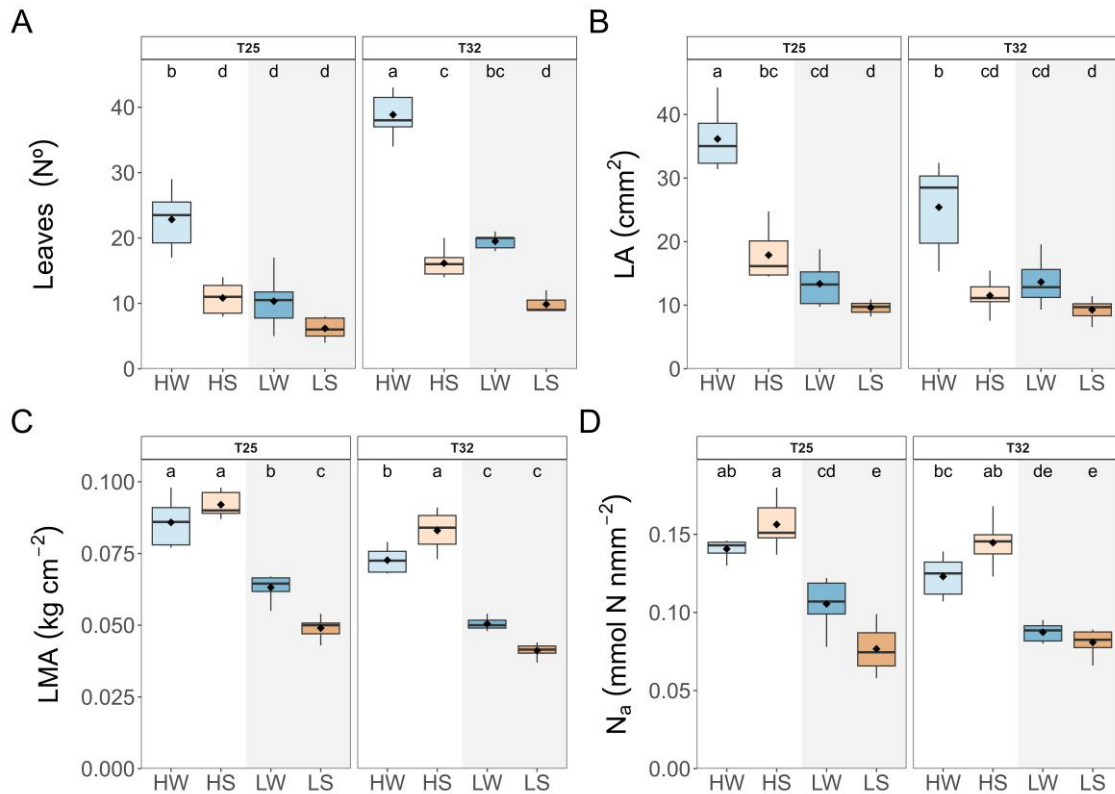


Figure 30. Plasticity of leaf morphology acclimation in *Fagus sylvatica* seedlings grown under a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L). Shown are: A) number of fully developed leaves (Leaves); B) Average leaf area (LA); C) leaf mass area (LMA) and D) nitrogen content by leaf area (N_a). Boxplots display the interquartile range, with horizontal lines representing medians and solid black dots denoting means. Different lowercase letters indicate significant differences among treatments ($p < 0.05$).

At the morphological level, warming exerts a significant effect on most evaluated traits (table 5). Higher growth temperature promotes an increase in the number of leaves, but a decrease in individual leaf area (Fig. 32A-B). Notably, leaf mass per area (LMA) was consistently lower in seedlings grown at elevated temperature (T32), particularly under well-watered conditions, reflecting thinner, lighter leaves (Fig. 32C). This trend was more pronounced under high light conditions. In contrast, seedlings grown under shade exhibited the lowest LMA values across treatments. Nitrogen content per unit leaf area (N_a) closely mirrored the pattern observed for LMA, with reduced

Na values at higher temperatures and lower light intensities (Fig. 32D), highlighting a consistent decrease in structural investment under these conditions. Overall, these morphological adjustments indicate an integrated acclimation response that modulates leaf construction strategies to balance trade-offs among resource capture, structural costs, and tolerance to environmental stress.

4.2.3.2. Leaf water relations and ABA

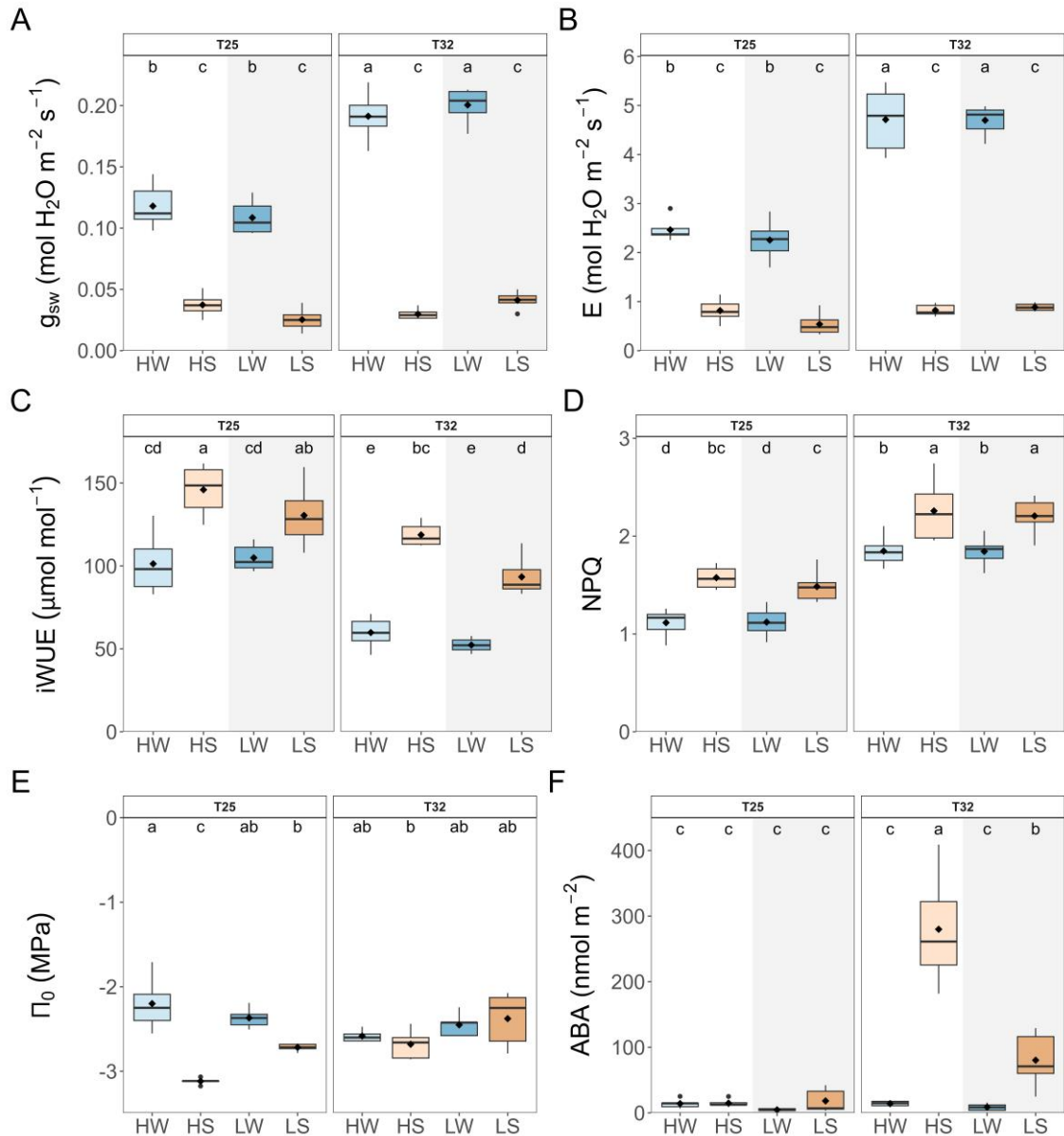


Figure 31. Leaf gas exchange and α chlorophyll fluorescence parameters in *Fagus sylvatica* seedlings grown under a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L). Shown are: A) stomatal conductance to water vapour (g_{sw}); B) transpiration (E); C) osmotic potential at turgor loss point (Π_0); D)

leaf abscisic acid concentration (ABA); E) intrinsic water use efficiency (iWUE); and F) non-photochemical quenching (NPQ). Boxplots display the interquartile range, with horizontal lines representing medians and solid black dots denoting means. Different lowercase letters indicate significant differences among treatments ($p < 0.05$).

Growth temperature exerted a strong influence on water-related gas exchange traits. Under well-watered conditions, both stomatal conductance to water vapor (g_{sw}) and transpiration (E) increased at 32 °C compared to 25 °C. For instance, g_{sw} rose from 0.12 ± 0.01 to 0.19 ± 0.01 mol H₂O m⁻² s⁻¹ and E from 2.46 ± 0.09 to 4.71 ± 0.27 mol H₂O m⁻² s⁻¹ in HW plants (T25HW vs. T32HW; Fig. 33A–B). Although A_n remained similar across temperatures (Fig. 34A), this increase in water loss reduced intrinsic water use efficiency (iWUE), which declined from 101.3 ± 7.4 to 59.8 ± 3.8 μ mol mol⁻¹ (T25HW vs. T32HW; Figure 6C). Warming also enhanced non-photochemical quenching (NPQ), rising from 1.12 ± 0.06 at T25 to 1.85 ± 0.06 at T32 (Fig. 33D), indicating greater photoprotective energy dissipation. Comparable responses were observed in shaded, well-watered plants, with increased g_{sw} and E , reduced iWUE, and increased NPQ. In contrast, warming had little effect on osmotic potential at the turgor loss point (π_0) or leaf abscisic acid (ABA) concentration under these conditions (Fig. 33E–F).

Drought reduced both g_{sw} and E within each growth temperature, with stronger declines at 32 °C (e.g., g_{sw} : 0.191 ± 0.010 to 0.030 ± 0.004 ; E : 4.71 ± 0.27 to 0.82 ± 0.05 mol H₂O m⁻² s⁻¹; T32HW vs. T32HS). These reductions increased iWUE at both temperatures, but warming consistently lowered iWUE under drought. For example, iWUE decreased from 146.0 ± 6.1 at 25 °C to 118.7 ± 2.9 μ mol mol⁻¹ at 32 °C in HS plants, and from 130.5 ± 7.5 to 93.4 ± 4.7 μ mol mol⁻¹ in LS plants. NPQ also increased with drought, and this response was further amplified by warming (from 1.58 ± 0.05 at 25 °C to 2.26 ± 0.13 at 32 °C in HS), suggesting additive effects of water and heat stress on photoprotection.

Interestingly, drought exerted contrasting effects on π_0 and ABA depending on growth temperature. At 25 °C, drought decreased π_0 , particularly under high light, while ABA levels remained unchanged. At 32 °C, however, π_0 remained stable whereas ABA concentrations increased markedly, indicating that stomatal regulation mechanisms shift with growth temperature.

Under well-watered conditions, light availability had only minor effects. Low-light seedlings maintained g_{sw} values comparable to their high-light

counterparts (e.g., 0.110 ± 0.010 vs. 0.121 ± 0.011 mol H₂O m⁻² s⁻¹ for T25LW vs. T25HW), resulting in similar iWUE (104.9 ± 3.3 vs. 101.3 ± 7.4 μmol mol⁻¹). A similar pattern occurred at 32 °C. NPQ was also unaffected by light availability, suggesting that photoprotective energy dissipation remained stable across light treatments when water was not limiting.

4.2.3.3. Net assimilation and stomatal conductance

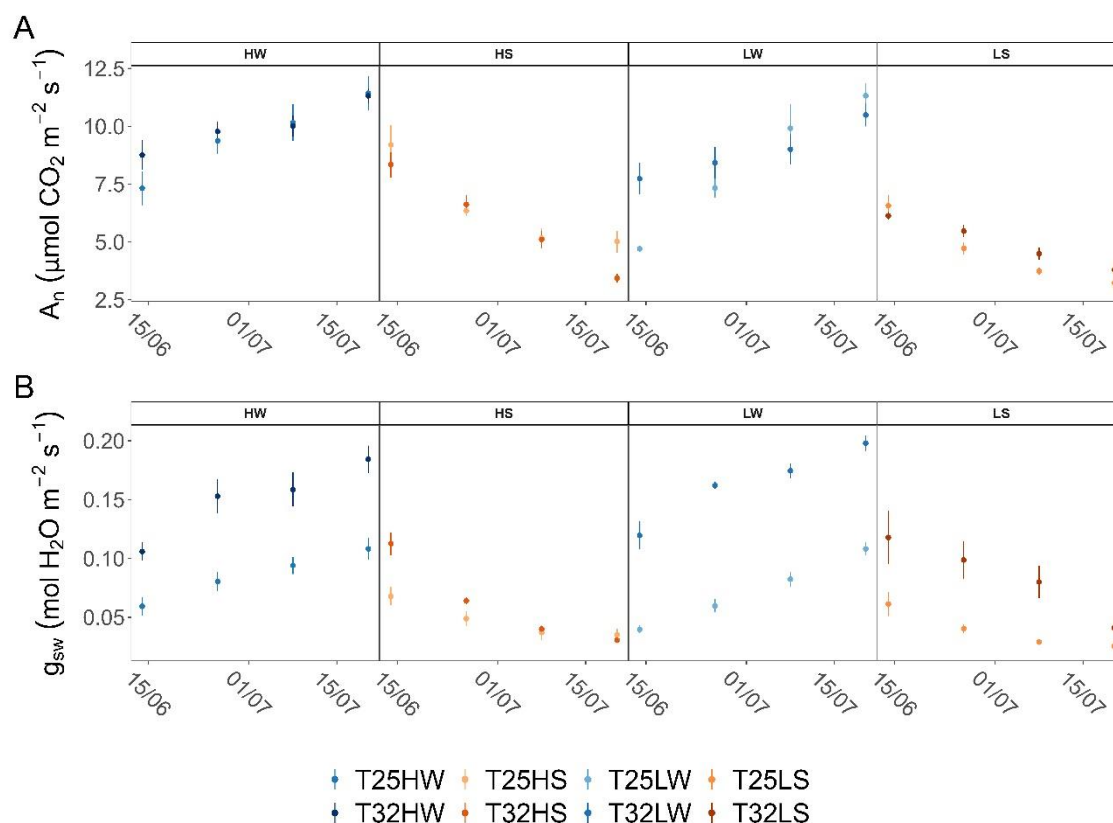


Figure 32. Temporal dynamics of gas exchange in *Fagus sylvatica* seedlings grown under a factorial combination of growth temperature (25 °C [T25] vs. 32 °C [T32]), light intensity (high [H] vs. low [L]), and soil water availability (well-watered [W] vs. water-stressed [S]). A) Net assimilation rate (A_n); B) Stomatal conductance to water vapor (g_{sw}). Gas exchange was measured every 14 days from the onset of drought treatments. Each panel corresponds to one of the eight treatment combinations. Circular markers indicate mean values $\pm$ standard error. Blue tones denote well-watered treatments (HW, LW), and orange tones indicate water-stressed treatments (HS, LS); lighter colors correspond to T25, and darker colors to T32.

Across both thermal regimes, well-watered seedlings (HW, LW) gradually increased their net assimilation rates (A_n) throughout the experimental period, with an average increase of approximately 15%. Differences between T25 and T32 plants remained minor ($\leq 5\%$), indicating broadly similar photosynthetic performance under well-watered conditions. In contrast, water-stressed seedlings (HS, LS) showed a consistent decline in A_n ,

reflecting the cumulative impact of drought (Fig. 32A). For example, compared to their initial values, A_n in HS plants declined by over 45% under T25 (from 9.20 ± 0.80 to 5.01 ± 0.55 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and more than 59% under T32 (from 8.34 ± 0.45 to 3.44 ± 0.43 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) by the final measurement date.

Temporal patterns in stomatal conductance to water vapor (g_{sw}) closely paralleled those of A_n . Well-watered seedlings maintained or increased g_{sw} , while water-stressed plants showed progressive declines over time. Notably, g_{sw} was consistently higher in T32 plants than in T25, particularly under well-watered conditions (Fig. 32B). For instance, T25-HW plants maintained g_{sw} values approximately 42% lower than T32-HW counterparts across the experimental range (averaging 0.087 ± 0.006 vs. 0.150 ± 0.004 $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), suggesting enhanced stomatal responsiveness to warming when water was not limiting.

4.2.3.4. Temperature modulation of gas exchange under drought and light stress

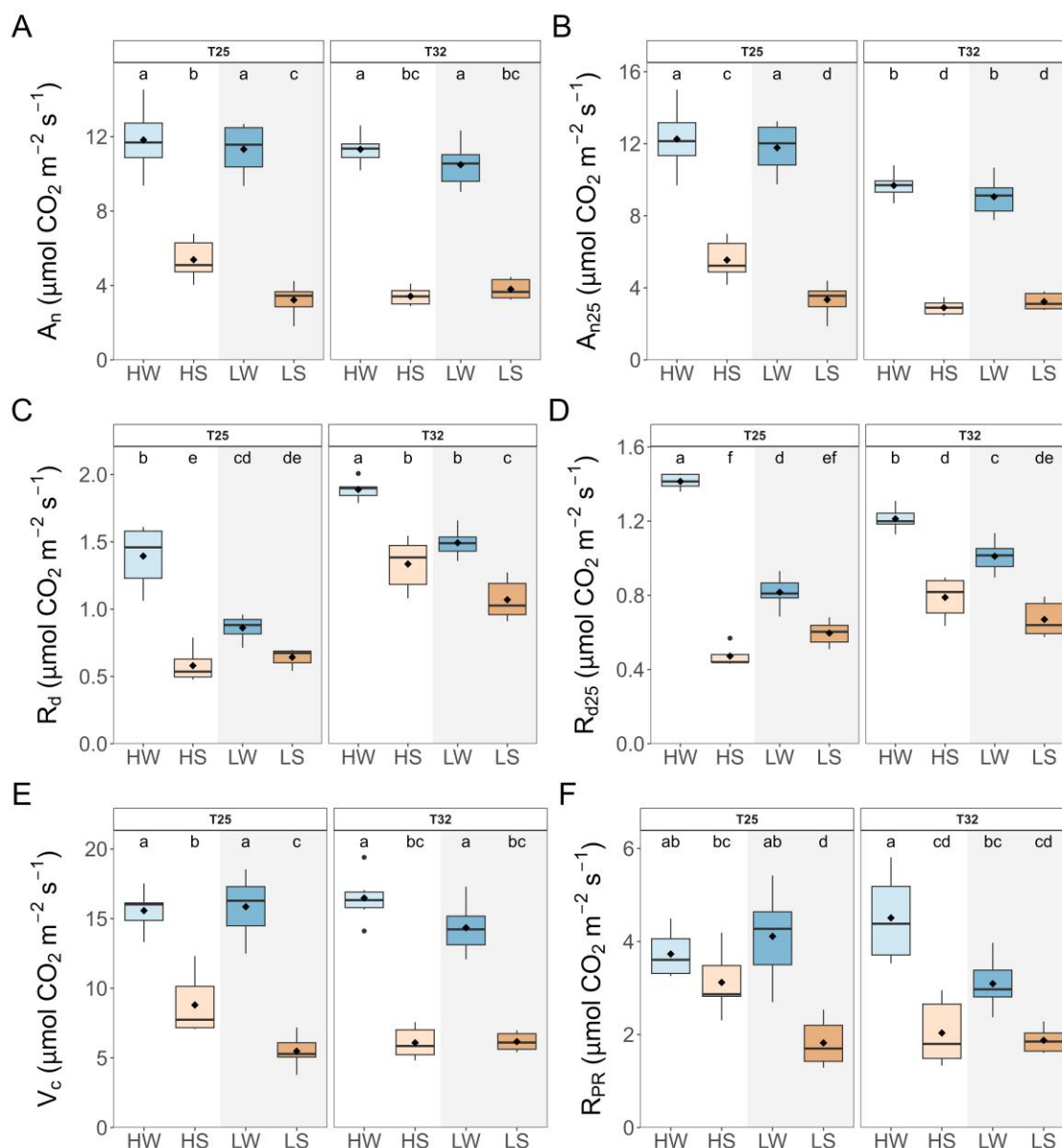


Figure 33. Leaf gas exchange parameters in *Fagus sylvatica* seedlings grown under a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L). Shown are: A) net assimilation rate (A_n); B) net assimilation rate scaled to 25°C (A_{n25}); C) respiration in darkness (R_d); D) respiration in darkness scaled to 25°C (R_{d25}); E) Rubisco carboxylation rate (V_c); and D) rate of photorespiratory CO_2 release (R_{PR}). Boxplots display the interquartile range, with horizontal lines representing medians and solid black dots denoting means. Different lowercase letters indicate significant differences among treatments ($p < 0.05$).

Growth temperature did not significantly affect carbon assimilation parameters such as net assimilation rate (A_n) or Rubisco carboxylation rate (V_c). A_n remained similar between HW plants at 25 °C and 32 °C (11.8 ± 0.7 vs. $11.3 \pm 0.3 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), as did V_c (15.6 ± 0.7 vs. $16.5 \pm 0.7 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). In contrast, temperature significantly influenced CO_2 -releasing processes: respiration in darkness (R_d) increased from 0.7 ± 0.05 to $0.94 \pm 0.02 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, and photorespiratory CO_2 release (R_{PR}) slightly increased from 3.7 ± 0.2 to 4.5 ± 0.4 (T25HW vs. T32HW). When scaled to a common temperature (25 °C), warming generally reduced photosynthetic performance across treatments, but also downregulated R_d , suggesting potential acclimation of respiratory metabolism (Fig. 33).

At 25 °C, drought markedly impaired photosynthetic performance. A_n declined from 11.8 ± 0.7 to $5.4 \pm 0.5 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ (T25HW vs. T25HS), while R_d and R_{PR} were slightly reduced (0.70 ± 0.05 to 0.29 ± 0.02 , and 3.7 ± 0.2 to $3.1 \pm 0.3 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, respectively), indicating an overall decline in carbon gain. Under elevated temperature (32 °C), these drought effects were further aggravated: A_n dropped to 3.4 ± 0.2 , and R_d more than quadrupled relative to T25HS (1.33 ± 0.08 vs. $0.29 \pm 0.02 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). Although R_{PR} also increased with warming ($2.0 \pm 0.3 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in T32HS), the rise was comparatively moderate (Fig. 33A&C). This imbalance — a sharp rise in respiratory costs alongside only a modest increase in photorespiration — underscores an exacerbated loss of carbon efficiency under combined drought and heat stress, and to a lesser extent, even under well-watered conditions.

Interestingly, under saturating light, low-light well-watered seedlings (LW) performed similarly to high-light well-watered plants in both A_n (11.3 ± 0.6 vs. 11.8 ± 0.7) and V_c (15.8 ± 0.9 vs. 15.6 ± 0.7 at 25 °C), yet exhibited significantly lower R_d (0.43 ± 0.02 vs. 0.7 ± 0.05) with comparable R_{PR} (4.1 ± 0.4 vs. $3.7 \pm 0.2 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). This pattern suggests equivalent Rubisco activity but reduced mitochondrial respiration, pointing to enhanced photosynthetic efficiency under lower light loads in non-stressed conditions.

4.2.3.5. Diffusive and biochemical photosynthetic adjustments to warming, drought and shade

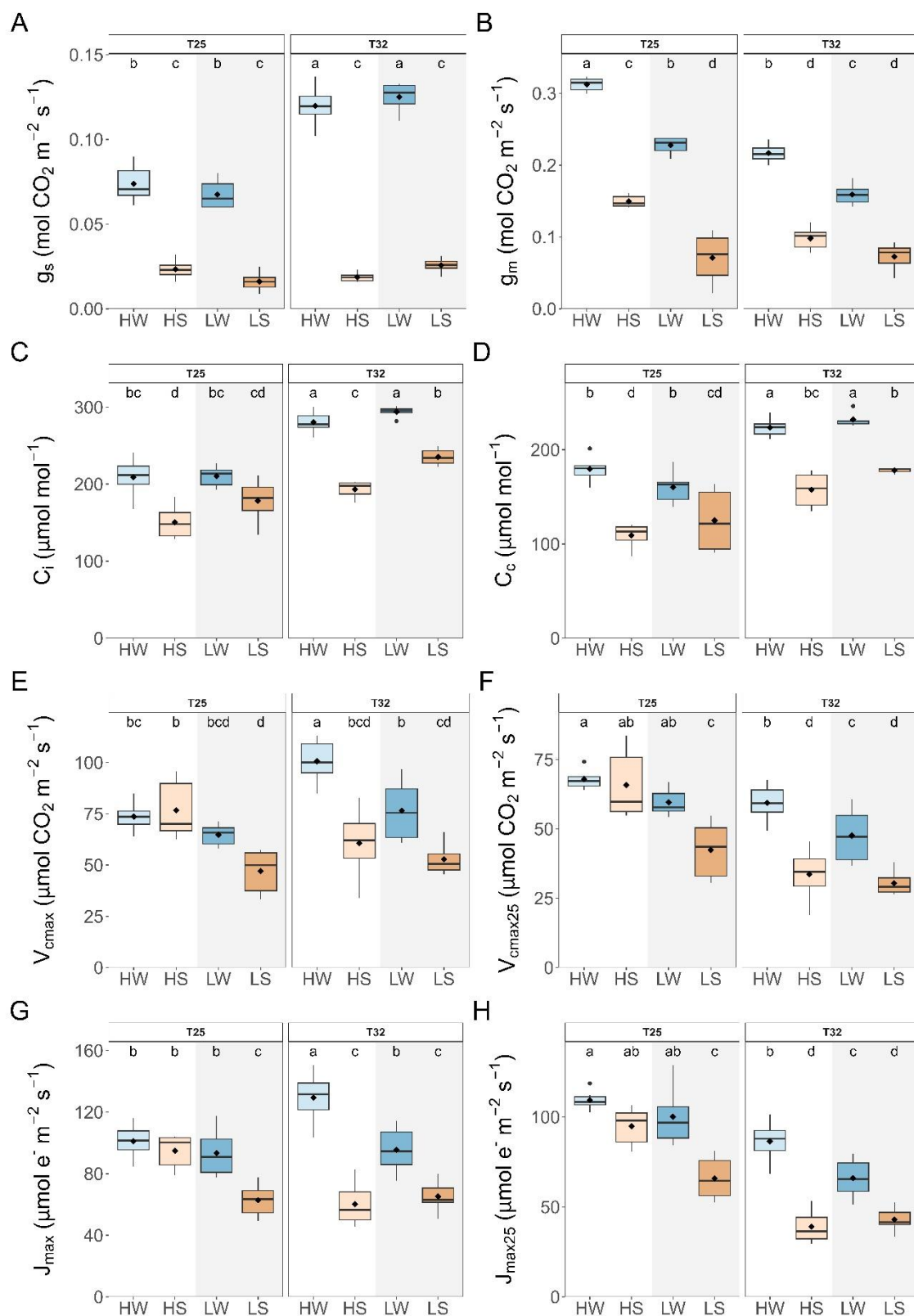


Figure 34. Leaf gas exchange parameters in *Fagus sylvatica* seedlings grown under a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs.

low (L). Shown are: A) stomatal conductance (g_s); B) mesophyll conductance (g_m); C) intercellular CO_2 concentration (C_i); D) chloroplastic CO_2 concentration (C_c); E) maximum rate of carboxylation of Rubisco (V_{cmax}); F) maximum rate of carboxylation of Rubisco at 25 °C ($V_{\text{cmax}25}$); G) maximum rate of electron transport (J_{max}); maximum rate of electron transport at 25 °C ($J_{\text{max}25}$). Boxplots display the interquartile range, with horizontal lines representing medians and solid black dots denoting means. Different lowercase letters indicate significant differences among treatments ($p < 0.05$).

Growth temperature exerted contrasting effects on diffusive photosynthetic traits. Stomatal conductance (g_s) significantly increased at 32 °C compared to 25 °C under well-watered conditions, rising from 73.8 ± 4.6 to 119.8 ± 4.9 $\text{mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in high-light plants (T25HW vs. T32HW) and from 67.5 ± 3.6 to 125.2 ± 3.5 in low-light plants (T25LW vs. T32LW). In contrast, mesophyll conductance (g_m) declined from 312.3 ± 4.0 to 216.7 ± 5.3 in HW (T25 vs T32) and from 227.5 ± 4.8 to 159.2 ± 6.0 $\text{mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in LW (T25 vs T32) plants (Fig. 34A–B). These opposing responses were accompanied by increased intercellular (C_i) and chloroplastic CO_2 concentrations (C_c) at higher temperatures, for example from 209.2 ± 10.4 to 280.3 ± 5.8 $\mu\text{mol mol}^{-1}$ (C_i , T25HW vs T32HW) and 179.5 ± 5.6 to 223.7 ± 4.1 $\mu\text{mol mol}^{-1}$ (C_c , T25HW vs T32HW) (Fig. 34C–D), suggesting that higher g_s under warming partially offset reduced internal diffusion.

Growth temperature also affected biochemical traits: V_{cmax} increased from 73.6 ± 2.9 to 100.6 ± 4.4 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, and J_{max} from 101.2 ± 4.5 to 129.4 ± 6.8 in HW seedlings. In shade seedlings, increases were more modest, with V_{cmax} rising from 64.7 ± 2.1 to 76.5 ± 6.1 and J_{max} from 93.4 ± 6.4 to 95.6 ± 6.2 (T25LW vs. T32LW; Fig. 34E–G).

Within each temperature, drought reduced both g_s and g_m , with sharper declines at 32 °C. (Fig. 34A–B). These reductions were accompanied by declines in C_i (280.3 ± 5.8 to 193.3 ± 4.5) and C_c (223.7 ± 4.1 to 157.4 ± 7.7 $\mu\text{mol mol}^{-1}$), as well as in V_{cmax} and J_{max} (100.6 ± 4.4 to 60.7 ± 7.0 ; 129.4 ± 6.8 to 60.2 ± 5.8 , respectively). Warming further accentuated these reductions under drought.

At both temperatures, low-light well-watered seedlings (LW) maintained g_s values similar to their high-light plants (e.g., 67.5 ± 3.6 vs. 73.8 ± 4.6 $\text{mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) but exhibited lower g_m (227.5 ± 4.8 vs. 312.3 ± 4.0), indicating a light-driven constraint on internal diffusion. C_i and C_c remained stable, and reductions in V_{cmax} and J_{max} under shade were moderate, with only minor impacts on A_n .

Finally, temperature-normalized biochemical capacity at 25°C ($V_{\text{max}25}$, $J_{\text{max}25}$) declined consistently with warming across all treatments. For example, in HW plants, $V_{\text{max}25}$ decreased from 67.9 ± 1.5 to 59.3 ± 2.8 , and $J_{\text{max}25}$ from 109.4 ± 2.2 to 86.5 ± 4.7 , with similar patterns for the rest of treatments (Fig. 34F–H). These patterns indicate a general reduction in photosynthetic efficiency under higher growth temperatures, pointing to an overarching carbon limitation in response to warming.

Table 5. F-statistics from a full-factorial mixed effect ANOVA using growing temperature (Tgrowth), watering (Water) and light intensity (Light) as fixed effects with n = 6 replicates as random effect. Significant outcomes are indicated in bold with p -values < 0.05 indicated as *, p < 0.01 as ** and p < 0.001 as *** in the table. The factor or combination of growing factors with highest F score for each variable were marked in gray. Same abbreviations for variables than in Table 1.

Variable	Tgrowth	Light	Water	Tgrowth × Light	Tgrowth × Water	Light × Water	Tgrowth × Light × Water
A_n	4.57*	5.96*	520.46***	3	0.0019	0.13	5.03*
LMA	23.38***	540.68***	10.63*	3.41	0.43	19.37***	4.96*
g_{sw}	138.29***	0.0046	1077.09***	8.09*	113.02***	0.0005	0.1
g_s	138.78***	0.02	1078.73***	7.87*	115.15***	0.0082	0.07
C_i	153.17***	16.87**	131.87***	1.71	7.19*	7.13*	0.0072
E	177.16***	1.33	852.39***	1.99	131***	0.0004	0.15
WUE	99.11***	0.08	52.59***	0.0014	0.23	0.3	1.35
iWUE	121.19***	9.65*	139.94***	2.14	4.29*	6.59*	0.0077
R_d	204.56***	49.1***	154.85***	1.4	0.12	20.23***	8.32*
R_l	204.45***	48.97***	154.79***	1.39	0.12	20.22***	8.31*
J_f	2.57	6.41*	164.55***	0.01	0.43	0.37	7.77*
R_{PR}	2.58	9.91*	69.41***	0.68	1	0.29	13.75**
g_m	109.34***	145.79***	659.69***	15.5**	31.59***	3.53	1.58
NPQ	185.6***	0.45	57.85***	0.02	0.06	0.47	0.06
V_{cmax}	4.38*	26.88***	33.07***	0.24	13.03**	0.12	7.34*
J_{max}	0.01	19.85***	78.3***	0.51	16.37**	0.88	16.71**
C_c	105.22***	1.35	117.26***	2.4	0.56	5.31*	1.16
V_c	0.95	5.25*	338.61***	0.37	0.41	0.4	8.85*
N_a	4.51*	126.81***	0.02	0.54	1.37	12.48*	0.85
LA	14.39**	89***	74.64***	12.26*	0.89	26.41***	1.16
Leaves	143.77***	205.93***	261.18***	5.33*	22.43***	36.47***	5.9*

4.2.3.6. Limitations in net assimilation (A_n) in response to growth temperature and multi stress

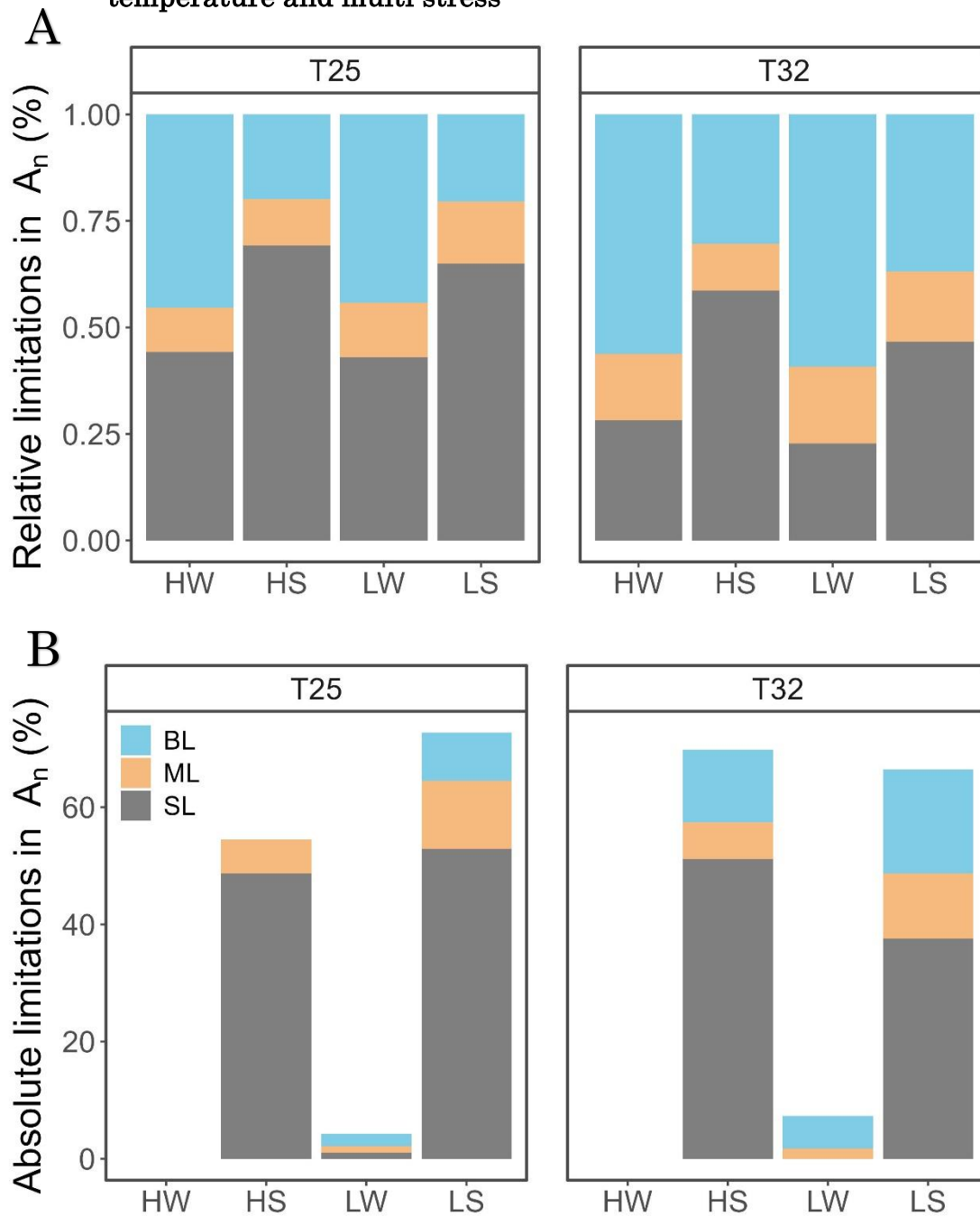


Figure 35. Relative (A) and absolute (B) contributions of stomatal (SL, dark gray), mesophyll (ML, orange), and biochemical (BL, light blue) limitations to net photosynthesis (A_n) in *Fagus sylvatica* seedlings grown under a factorial combination of growth temperature (25 °C [T25] vs. 32 °C [T32]), water availability (well-watered [W] vs. water-stressed [S]), and light intensity (high [H] vs. low [L]). Top panels display the proportional (stacked) contributions of each limitation type (SL + ML + BL = 100). Bottom panels show the absolute limitation values (%), highlighting total reductions in photosynthetic capacity under each treatment.

Across all treatments, photosynthesis was primarily Rubisco-limited (A_c). At 32 °C, biochemical limitations (l_b) increased under well-watered conditions (e.g., T32LW = 0.59), while stomatal limitations (l_s) dominated under drought (e.g., T32HS = 0.59; T25HS = 0.69). At 25 °C under well-watered conditions (HW, LW), limitations were more evenly partitioned (e.g., T25HW: l_s = 0.44, l_b = 0.45), whereas at 32 °C well-watered seedlings became more biochemically constrained (T32HW: l_b = 0.56).

Absolute limitation analysis provided a clearer perspective on treatment effects on A_n (Fig. 35B). Under well-watered conditions, and saturating light (HW, LW), constraints were negligible across both temperatures. In contrast, drought substantially increased total limitations. At 25 °C, T25-HS showed a 54.5% reduction in A_n , driven mainly by stomatal limitation (SL = 48.7%) with a smaller mesophyll contribution (ML = 5.8%). T25-LS plants experienced the strongest total limitation (72.8%), combining high SL (52.9%), moderate ML (11.6%), and some biochemical limitation (BL = 8.2%). Warming exacerbated these effects: T32-HS seedlings showed a comparable stomatal limitation (SL = 51.2%) but with a larger biochemical component (BL = 12.4%). In T32-LS seedlings, stomatal limitation was lower (SL = 37.6%) but biochemical limitation increased markedly (BL = 17.8%). These patterns indicate that warming under drought shifts part of the limitation burden from diffusional to biochemical processes, further constraining carbon assimilation.

Overall, growth temperature, water availability, and light intensity jointly shaped the performance of beech seedlings, with warming consistently amplifying the effects of drought. Morphological adjustments under higher temperature favored thinner, less costly leaves, particularly under shade, but were accompanied by reductions in structural investment (LMA , N_a). At the physiological level, warming increased stomatal opening and transpiration, enhancing water loss and photoprotective energy dissipation, yet reducing intrinsic water-use efficiency. Under drought, photosynthetic capacity declined sharply, with warming further aggravating carbon losses through elevated respiratory costs. Diffusive traits revealed opposing adjustments, with g_s increasing but g_m decreasing at high temperature, partly offset by higher C_i and C_c . Biochemical capacity (V_{cmax} , J_{max}) initially rose under warming, but temperature normalization (V_{cmax25} , J_{max25}) exposed an overall downregulation of efficiency. Finally, limitation analysis revealed a clear shift in the burden of photosynthetic constraints: stomatal control

dominated under drought at moderate temperature, while warming increasingly transferred this limitation towards biochemical processes. Together, these patterns highlight that warming not only intensifies drought stress but also alters the relative importance of diffusional and biochemical controls, ultimately constraining carbon assimilation across environmental scenarios.

4.2.4. Discussion

Our experimental design, which controlled for vapor pressure deficit (VPD), allowed us to isolate the effect of growth temperature on beech seedlings and revealed a pattern of partial acclimation of photosynthesis to warming, consistent with our first hypothesis (H1). Net CO₂ assimilation rates remained relatively stable across temperatures under well-watered conditions, despite enhanced respiratory costs and reduced intrinsic water-use efficiency (iWUE). This balance was achieved through compensatory adjustments in diffusive traits: stomatal conductance (g_s) increased while mesophyll conductance (g_m) declined, leading to higher intercellular and chloroplastic CO₂ concentrations (C_i , C_c). Nevertheless, when photosynthetic traits were standardized to 25 °C, both $V_{\text{cmax}25}$ and $J_{\text{max}25}$ declined (Fig. 33 & 31), supporting H1 by indicating a downregulation of biochemical capacity and incomplete acclimation of photosynthesis under warming (Way & Yamori, 2014; Slot & Winter, 2017; Kumarathunge et al., 2019).

Our findings also support H2, as respiration responded more strongly to temperature than assimilation, potentially driving an imbalance in carbon gain under heat stress (Atkin & Tjoelker, 2003; Heskell et al., 2016; Dusenge et al., 2019). Enhanced g_s under warming (H3) has been reported as a mechanism to increase evaporative cooling and delay thermal damage (Drake et al., 2018), but this comes at the expense of lower iWUE (Fig. 31A-C). In contrast, the reduction in g_m we observed agrees with reports that mesophyll conductance is particularly sensitive to both temperature and drought, acting as a key diffusional bottleneck (Flexas & Medrano, 2002; Tosens et al., 2016). Finally, shade buffered the negative impacts of warming (H4), consistent with earlier findings that low-light conditions promote thinner leaves with lower structural costs and reduced metabolic demand (Niinemets, 2010; Valladares et al., 2007). Altogether, these responses illustrate that warming does not lead to complete photosynthetic failure under well-watered conditions but

instead shifts the balance between diffusional and biochemical processes, reducing the efficiency of carbon assimilation under combined stress scenarios. Thermal Acclimation of Photosynthesis under Warming

Rising growth temperatures triggered marked adjustments in the photosynthetic machinery of *Fagus sylvatica* seedlings, indicative of partial thermal acclimation. While seedlings grown at 32 °C displayed net assimilation rates (A_n) comparable to those at 25 °C, this apparent similarity masked a constrained carbon balance driven by elevated respiration (R_d), consistent with the classical thermal response (Fig. 33A & C) of plants (Wang et al., 2020). As temperature rises beyond the photosynthetic optimum, enzyme efficiency typically declines while respiratory costs continue to increase, eventually tipping the carbon balance toward net loss and limiting growth (Rubio et al., 2025). Indeed, although warming may transiently stimulate metabolic processes, prolonged exposure often leads to suppressed photosynthesis and enhanced respiration, as we observed in T32 seedlings.

However, plants have evolved acclimation strategies to mitigate these effects. Thermal acclimation involves the adjustment of photosynthetic parameters to match the prevailing temperature environment. In warm-grown leaves, this frequently entails a downregulation of base biochemical capacities—specifically reduced V_{cmax} and J_{max} when standardized to 25 °C (i.e., $V_{\text{cmax}25}$ and $J_{\text{max}25}$)—coupled with an upward shift in the temperature optimum for photosynthesis (Kumarathunge et al., 2019; Scafaro et al., 2017; Yamori et al., 2014). In our experiment, seedlings grown at 32 °C likely reconfigured their photosynthetic apparatus in this way: although absolute rates of V_c and A_n under growth conditions remained similar between treatments, parameter estimates recalculated at 25 °C using temperature response models of study 1 revealed lower A_{n25} , $V_{\text{cmax}25}$, and $J_{\text{max}25}$ in T32 plants (Fig. 33). This suggests that the observed assimilation was sustained through increased enzyme activation at higher temperature, rather than through enhanced biochemical capacity.

Respiratory traits mirrored this trend. Although absolute R_d was higher in warm-grown seedlings, normalization to 25 °C (R_{d25}) showed a clear downregulation (Fig. 33C-D). This pattern is consistent with a typical acclimatory response aimed at minimizing respiratory carbon loss under sustained heat exposure (Way & Yamori, 2014). Such adjustments represent a functional compromise: by downscaling photosynthetic and respiratory

capacities at lower temperatures, plants conserve nitrogen and energy while maintaining assimilation capacity at higher thermal regimes (Wang et al., 2020).

This trade-off has been quantified in global syntheses, which report reductions of approximately 4–5% in $V_{\text{cmax}25}$ and $R_{\text{d}25}$ for every 1 °C increase in growth temperature (Scafaro et al., 2017; Wang et al., 2020). Our results align well with this pattern, reinforcing the interpretation of optimal acclimation. Still, these physiological adjustments were insufficient to fully compensate for thermal stress. Seedlings in the 32 °C treatment exhibited reduced growth in diameter and total leaf area, likely reflecting a persistently limited carbon budget (Rubio et al., 2025). These outcomes highlight the limits of acclimation: while warm-grown plants may operate more efficiently under elevated temperature, this comes at the cost of reduced flexibility and efficiency under cooler reference conditions. At the same time, certain species exhibit a higher degree of acclimation to warming (Dusenge et al., 2020; Way & Yamori, 2014; Yamori et al., 2014), suggesting that *Fagus sylvatica* seedlings possess only a moderate capacity for thermal adjustment.

Ultimately, this reflects the core trade-off underlying photosynthetic acclimation: maintaining performance at elevated temperatures necessitates the tempering of potential capacity at lower ones, a strategy that helps avoid unsustainable respiratory expenditure when operating near thermal limits (Atkin & Tjoelker, 2003). These findings set the physiological foundation for understanding how photosynthetic acclimation interacts with stomatal regulation and water use, both of which are intricately modulated under warming conditions.

4.2.4.1. Compound Heat and Drought Stress: Photoprotection and Carbon Balance

When drought stress coincided with high temperature in our experiment, European beech seedlings experienced synergistically greater strain than under either stress alone. Photosynthetic inhibition was disproportionately strong under this compound stress, but the seedlings activated defensive adjustments to cope. A striking response was in photoprotective energy dissipation: under simultaneous heat and water deficit, we infer that excess absorbed light energy (which could not be used for assimilation due to stomatal closure and heat-inactivated enzymes) was shunted into non-

photochemical quenching (NPQ). NPQ is a photoprotection mechanism in which excess excitation energy is harmlessly dissipated as heat in the chloroplast antennae (Ruban, 2016). Indeed, studies on combined drought–heat stress show that NPQ capacity is sharply upregulated when both stresses coincide. In drought-and-heat treated eggplant, for example, PSII efficiency and quantum yield plummet while NPQ surges to dissipate the surplus light energy (Hannachi et al., 2022) a clear indicator that photoprotective thermal dissipation is safeguarding the photosystems.

We faced similar results in our beech seedlings, since water-stressed individuals showed lower effective PSII yield and elevated NPQ relative to single-stress plants (Fig. 32D & 36B). High NPQ under heat–drought reflects the seedlings’ reliance on thermal energy dissipation to prevent photodamage when normal photochemistry is impaired. This response, however, carries a carbon cost: energy diverted to NPQ is not used for CO₂ fixation, compounding the decline in A_n .

In parallel, combined stress affected respiratory metabolism. Heat alone tends to increase R_d , and drought can also raise respiration through heightened ion pumping, osmoprotection, and repair processes, especially when tissues experience oxidative stress (Sharkey & Zhang, 2010). Our warm-drought seedlings likely sustained a high R_d even as A_n fell to minimal levels (Fig. 33A&C). Consequently, the both ratio A_n/R_d and J_f/A_n collapsed under compound stress (Fig. 36E-F) – a clear sign of carbon balance impairment (Perera-Castro & Flexas, 2023). In practical terms, this means that a much larger fraction of the carbon fixed in photosynthesis was immediately respired (or that very little carbon was fixed at all) under the worst stress conditions. Such a situation can lead to carbon deficits where maintenance respiration exceeds carbon gain during parts of the day, risking growth stagnation or carbon starvation. The literature confirms this trend: plants under severe heat and drought commonly show drastic drops in net assimilation accompanied by sustained respiratory release, driving down the A_n/R_d ratio and sometimes even leading to tissue carbon loss (Deluigi et al., 2025; H. Duan et al., 2013; Feller, 2016; Hannachi et al., 2022).

Our observations align with these findings – growth was most suppressed in the combined stress treatment, indicating that any carbon gained through occasional photosynthesis was largely consumed by respiration and protective mechanisms. Importantly, the interplay of intense light with heat–drought

stress was somewhat moderated by our low-light (shade) treatment. Under shade, excess light energy was intrinsically limited, which may have constrained how much NPQ was needed or how severe photoinhibition became in those seedlings. Nonetheless, even in shade, combined drought and heat likely pushed seedlings into a highly conservative mode (low A_n , some NPQ activation, and basal metabolism). This synergistic stress effect emphasizes that photoprotection and metabolic regulation are in full force when water shortages and high temperatures co-occur. It bridges to the question of how light availability, especially low light, interacts with these stress responses – as shade can both relieve excess light damage and impose its own limitations, influencing the acclimation outcome.

4.2.4.2. Water-Use Efficiency, Evaporative Dynamics and stomatal regulation under Warming and Drought

Stomatal regulation emerged as a key factor modulating water-use efficiency (WUE) in warmed beech seedlings. Since we fixed the VPD for both growth temperatures, we isolated their net effect in stomatal conductance. We also observed a dual response in stomatal conductance (g_{sw}) depending on water availability (Fig. 31A-C).

Under well-watered conditions, warming tended to increase g_{sw} , enabling greater transpirational cooling at the leaf surface (Gates, 1968). This was observed as an upward shift in g_{sw} and transpiration (E) in seedlings at 32 °C without drought stress (relative to cooler controls), suggesting that the plants opened stomata to dissipate heat via evaporation, but also by biochemical mechanisms, since these plants showed also higher NPQ (Fig. 31A-D). Such behavior has been documented in other species, where high temperatures trigger stomatal opening, as a means of evaporative cooling values (Falcioni et al., 2024; Gauthey et al., 2024; Mills et al., 2024). Notably, this can lead to a decoupling between A_n and g_{sw} at extreme temperatures: photosynthesis plateaus or drops while stomatal conductance remains high, resulting in excess water loss (Diao et al., 2024; Marchin et al., 2023).

Our results are consistent with this pattern – warmed, well-watered beech seedlings experienced a reduction in intrinsic water-use efficiency (iWUE). The cost of this response is lower carbon gain per unit water lost (iWUE decline), and a heightened risk of dehydration, as more water is expended for cooling. However, the benefit is cooler leaf tissues and avoidance of heat

damage, which can preserve photosynthetic apparatus in the short term (Gauthey et al., 2024).

In contrast, under drought conditions warming had the opposite effect on stomata. When soil moisture was limiting, our 32 °C seedlings markedly reduced g_{sw} relative to well-watered counterparts. This closure is an adaptive response to conserve water under high evaporative demand. By curtailing stomatal aperture, drought-stressed seedlings at elevated temperature limited transpirational water loss (reducing E) and thus improved their water use efficiency (WUE) compared to non-drought plants. Indeed, we observed higher WUE and $\delta^{13}C$ (a longer-term proxy for WUE) in water-limited treatments, indicating a shift toward water conservation. However, despite the drought-induced improvements in both iWUE and WUE, warming counteracted these gains, leading to an overall decline in water use efficiency under elevated temperature.

Under drought, warming induced contrasting mechanisms of stomatal regulation. At 25 °C, water-stressed seedlings exhibited a lower osmotic potential at the turgor loss point (Π_0) compared to well-watered controls, with no significant differences in leaf abscisic acid (ABA) concentration. In contrast, at 32 °C, osmotic adjustment capacity appeared impaired, while ABA concentrations increased markedly, particularly under high light (Fig. 31 E-F).

The trade-off here is that tighter stomatal closure under heat drought protects hydration at the expense of CO₂ supply, causing a sharper drop in A_n . In our study, warmed seedlings facing water deficit had the lowest net photosynthesis rates among treatments (Fig. 33A), reflecting this carbon cost of water saving. However, at growth terms, should be noted that light availability it is the most limiting factor (Rubio et al., 2025).

Taken together, these responses highlight that warming alone can push stomata to either extreme: spend water to shed heat, or save water and endure heat, depending on moisture context (Duan et al., 2015; Rubio et al., 2025). The overall effect on seedling performance was a drop in WUE under warm-wet conditions (due to excess transpiration) but an increase in WUE under warm-dry conditions (due to restricted stomata), with attendant impacts on growth. This dynamic sets up complex outcomes when compound stresses occur – for instance, simultaneous heat and drought impose both limitations, leading changes in terms of photoprotection and metabolic strain.

4.2.4.3. Shade and Warming: Plasticity in Photosynthesis and Leaf Traits

Light availability fundamentally altered the way *Fagus sylvatica* seedlings responded to warming. Under low irradiance conditions, seedlings experienced substantial carbon source limitation, as photosynthesis was primarily constrained by photon capture rather than enzymatic capacities. This scenario resulted in markedly reduced early-season photosynthetic performance compared to seedlings under high irradiance, driven largely by slower phenological development—specifically delayed leaf maturity, a known limitation in young, expanding leaves (Ellsworth & Reich, 1992; Mishra et al., 2012). Consequently, shade-grown (LW) seedlings initially exhibited significantly lower net assimilation rates (A_n), stomatal conductance (g_s), and mesophyll conductance (g_m) than HW ones (Fig. 32A-B & 34B).

Nevertheless, shade-acclimated leaves typically optimize their limited light environment by enhancing quantum yield efficiency, reducing compensation points, and exhibiting lower structural investments (higher SLA, lower LMA) (Mathur et al., 2018). Consistent with this strategy, LW seedlings in our experiment maintained high quantum yields (Φ_{PSII}), lower light compensation points (LCP), and superior intrinsic light-use efficiency (Φ_{CO_2}) as shown by chlorophyll fluorescence and gas exchange analyses (Fig. 36A-D). However, such optimized efficiency did not mitigate the severe carbon acquisition constraints, as reflected by fewer, smaller leaves and considerably reduced total leaf area relative to high irradiance (HW) counterparts.

Importantly, this physiological gap between HW and LW seedlings was dynamic rather than fixed. Over the growing season, LW seedlings underwent a gradual but substantial acclimation process. Temporal gas exchange measurements revealed that initial significant disparities in A_n and g_s diminished over time. By late summer, LW seedlings reached comparable photosynthetic capacities to HW plants when measured under saturating irradiance, supporting earlier reports of shade-induced photosynthetic acclimation in beech (Cascio et al., 2010; Robakowski & Antczak, 2008).

Elevated temperature (32 °C) under low irradiance conditions further accelerated this physiological convergence. Warm-grown LW seedlings exhibited increased dark respiration (~25%), indicative of enhanced metabolic activity (Hikosaka et al., 2016; Way & Yamori, 2014). While higher respiration incurred a carbon cost, it likely facilitated faster enzymatic

activation and tissue development, mitigating the phenological delay typical in shaded conditions (Hikosaka et al., 2016). As a result, seedlings grown in warm shade produced more leaves and developed a greater total leaf area composed of thinner, lower-LMA leaves (Fig. 30A-C), characteristic of an opportunistic, resource-acquisitive growth strategy (Rubio et al., 2025).

Enhanced leaf morphological plasticity under warming also implied improved thermal regulation and photoprotection. The thinner leaves facilitated better heat dissipation, and higher non-photochemical quenching (NPQ) improve this process, which our chlorophyll fluorescence measurements confirmed was notably higher under combined warming and shading. Such physiological responses suggest an up-regulated baseline capacity for thermal energy dissipation, preparing seedlings for sporadic increases in irradiance or temperature fluctuations, despite generally lower overall light intensities.

Thus, warmth combined with shade triggered a nuanced plastic response: it mitigated the negative impacts of shade-induced carbon limitation by stimulating rapid leaf proliferation, enhancing leaf-level thermal regulation, and maintaining photosynthetic function under resource constraints. This intricate balance underlines the species' inherent plasticity, highlighting that moderate warming can beneficially modulate shade stress through accelerated leaf phenology and morphological adjustments. Consequently, the interaction of thermal and irradiance stresses in *Fagus sylvatica* illustrates a complex yet adaptive response, where environmental context crucially determines the trajectory of acclimation.

4.2.4.4. Morphological and Nitrogen-Use Plasticity across Thermal and Light Gradients

Our study reveals that *Fagus sylvatica* seedlings adjust a suite of morphological traits (leaf size, leaf thickness/density and number) and leaf nitrogen distribution in response to light and temperature, underlining the integrative nature of acclimation. Leaf morphology exhibited clear plasticity: in high light, cool conditions, beech seedlings developed bigger and thick leaves (less average area on a single leaf, but higher total leaf area and LMA, whereas in shade, cool conditions, they produced smaller and thinner leaves (lower total leaf area and low LMA; Fig. 30A-C) characteristic of shade acclimation (Poorter et al., 2009; Poorter, 2009). These structural differences are a primary driver of differing photosynthetic capacities – as reported in

natural beech canopies, variations in LMA due to light availability largely explain differences in net assimilation normalized at 25°C (A_{n25}) (sun leaves with high LMA have higher A_{n25} than shade leaves - Figure 30C and 33B) (Bachofen et al., 2020). In our controlled setting, we saw the same pattern at 25 °C: high-light seedlings had higher LMA, higher A_n , J_{max25} and V_{cmax25} per unit area (Fig. 33), while low-light seedlings had low LMA and higher chlorophyll-per-N ratios (Fig. 30), optimizing light capture over raw capacity.

Warming imposed additional morphological shifts. Elevated temperature led to the production of smaller leaves and a general reduction in LMA across both light regimes (Rubio et al., 2025). However, under high-light conditions, warm-grown leaves were able to sustain their similar LMA than cool-grown leaves by decreasing their average leaf area, which helps reduce the boundary layer and facilitate heat dissipation (Leigh et al., 2017; Marchin et al., 2022).

For instance, our 32 °C seedlings, we observed a differential approach. Under high light conditions, our seedlings displayed a conservative strategy by lowering individual leaf area and lower total leaf area than 25 °C seedlings, even though they compensated by increasing leaf number (Fig. 30A-B). However, under low light conditions they displayed a different approach. These seedlings were able to sustain this leaf dimensions and increasing their number for displaying a higher total leaf area by reducing their LMA (Fig. 30C). This suggests a strategy shift under low light, where T32 plants adopt a more acquisitive phenotype (e.g., a higher number of cheaper leaves with lower *LMA*) to offset reduced carbon gain (Poorter et al., 2009; Poorter, 2009). This reduced LMA in warm conditions also aligns with field studies showing that foliage developing in hotter, drier microclimates tends to be constructed with less dry mass per area, which can help enhance convective cooling (a thinner leaf sheds heat faster) (Gates, 1968). However, a trade-off is implicit: a lower LMA means less structural investment and often less nitrogen per area (Figure 12), which can diminish photosynthetic capacity unless counterbalanced by higher foliar nitrogen concentration (Bachofen et al., 2020; Duarte et al., 2021).

4.2.5. Conclusion

Our findings demonstrate that *Fagus sylvatica* seedlings exhibit substantial physiological and morphological plasticity—particularly at the leaf level—in response to warming, drought, and low light, three environmental stressors expected to intensify under climate change. Thermal acclimation was evident: elevated temperatures modulated photosynthetic performance and respiration, enabling partial buffering of heat stress (Atkin & Tjoelker, 2003; Way & Yamori, 2014; Yamori et al., 2014). However, this acclimation incurred costs. Under well-watered warming, seedlings maintained photosynthesis but experienced carbon constraints due to elevated respiration and investment in photoprotection (e.g., NPQ), along with increased water use (Atkin & Tjoelker, 2003; Falcioni et al., 2024; Rubio et al., 2025), as it is reflected in their photosynthetic traits normalized at 25°C (Figures XX and XX).

Stomatal behavior varied with water availability: while warming under ample moisture promoted evaporative cooling at the cost of reduced intrinsic water-use efficiency (iWUE), drought combined with heat triggered stomatal closure and enhanced NPQ, exacerbating carbon limitations (Hannachi et al., 2022). Additionally, warming under drought also altered the dominant mode of stomatal regulation, shifting from osmotic-driven (π_0 -sensitive) closure at cooler temperatures to ABA-mediated control under high-temperature conditions. Morphologically, warming led to notable changes: in high light, seedlings produced a higher number of smaller leaves with similar LMA, reducing total leaf area and thus overall productivity. In contrast, under shade, seedlings maintained total leaf area by increasing leaf number and reducing LMA, thereby compensating for heat stress—likely aided by lower irradiance and reduced thermal load.

These results suggest that *Fagus sylvatica* seedlings may tolerate moderate warming, especially in moist, shaded understories. However, regeneration is likely to be constrained under compound stress scenarios, such as concurrent heatwaves and drought. The species' resilience will depend on the frequency of such extremes and the buffering effects of microclimatic conditions. Management practices promoting understory moisture and light heterogeneity could bolster regeneration under future climates.

4.2.6. Supplementary

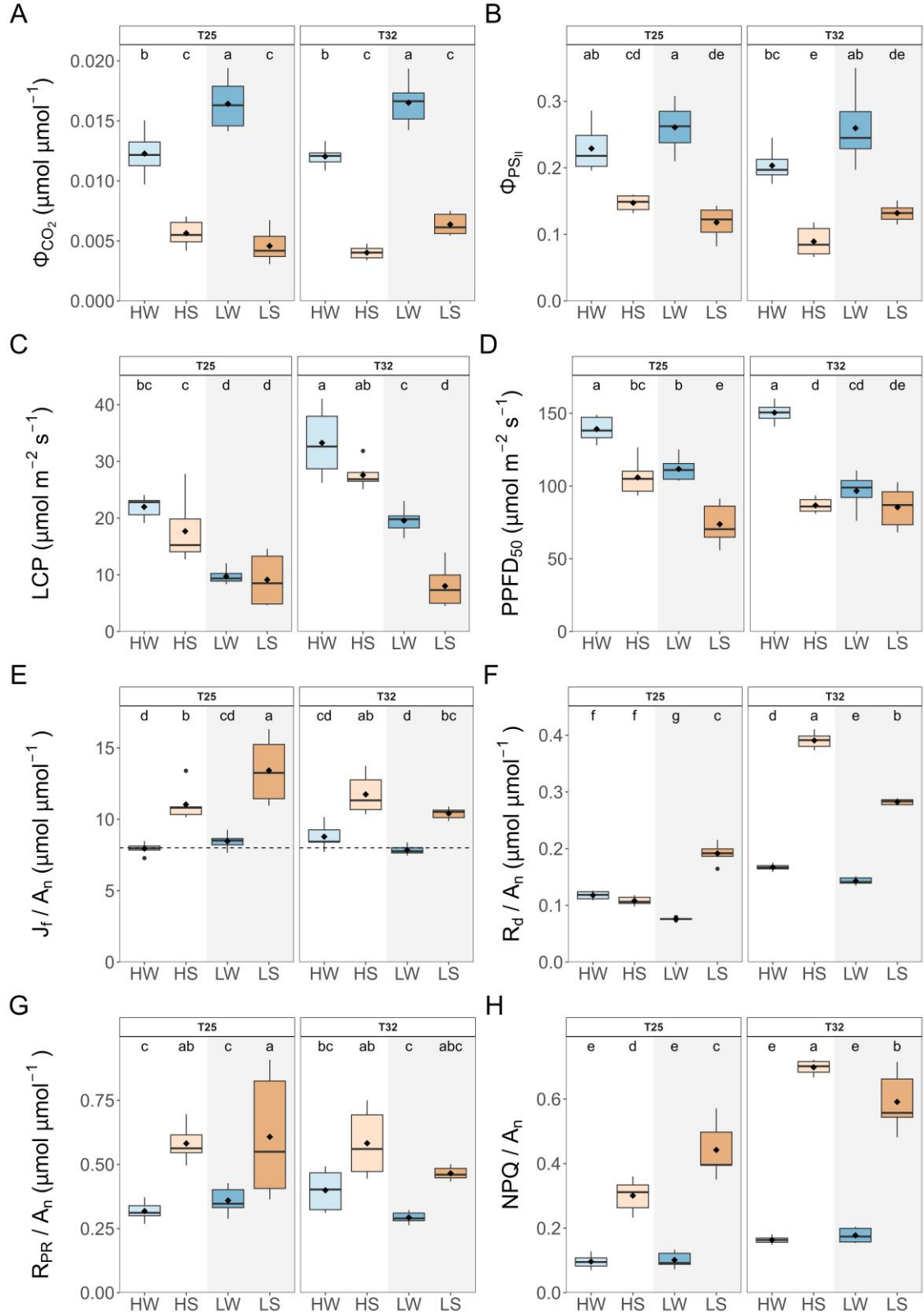


Figure 36. Leaf gas exchange parameters in *Fagus sylvatica* seedlings grown under a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L). Shown are: (A) quantum yield of CO_2 assimilation (Φ_{CO_2}); (B) effective

quantum yield of PSII (Φ_{PSII}); (C) light compensation point (LCP); (D) photosynthetic photon flux density at which the leaf reaches 50% of its light-saturated net photosynthetic rate ($PPFD_{50}$); (E) ratio of electron transport (Valentini et al., 1995) to net assimilation (J/A_n); (F) ratio of dark respiration to net assimilation (R_d/A_n); (G) ratio of photorespiratory CO_2 release to net assimilation (R_{PR}/A_n); and (H) non-photochemical quenching relative to net assimilation (NPQ/A_n). Boxplots display the interquartile range, with horizontal lines representing medians and solid black dots denoting means. Different lowercase letters indicate significant differences among treatments ($p < 0.05$).

Table 6. F-statistics from a full-factorial mixed effect ANOVA using growing temperature (Tgrowth), watering (Water) and light intensity (Light) as fixed effects with n = 6 replicates as random effect. Significant outcomes are indicated in bold with p -values < 0.05 indicated as *, p < 0.01 as ** and p < 0.001 as *** in the table. The factor or combination of growing factors with highest F score for each variable were marked in gray. For abbreviations check the Abbreviations and Acronyms section.

Variable	Tgrowth	Light	Water	Tgrowth:Light	Tgrowth:Water	Light:Water	Tgrowth:Light:Water
A _n	4.572*	5.96*	520.461***	2.997	0.002	0.127	5.03*
A _{n25}	40.83***	5.594*	485.566***	3.612	4.092*	0.368	4.521*
R _d	204.555***	49.1***	154.851***	1.397	0.121	20.235***	8.315**
R _{d25}	20.261***	69.213***	382.595***	2.579	12.402**	67.019***	45.498***
V _c	0.95	5.249*	338.608***	0.37	0.413	0.404	8.85**
R _{PR}	2.58	9.912**	69.412***	0.675	0.996	0.289	13.751***
g _s	138.778***	0.016	1078.73***	7.867**	115.151***	0.008	0.074
g _m	109.343***	145.788***	659.686***	15.505***	31.59***	3.53	1.583
C _i	153.172***	16.872***	131.873***	1.708	7.194*	7.133*	0.007
C _c	105.219***	1.348	117.256***	2.404	0.56	5.308*	1.161
V _{cmax}	4.376*	26.88***	33.066***	0.236	13.033***	0.118	7.34**
V _{cmax25}	51.607***	22.21***	43.001***	3.529	5.855*	0.411	5.709*
J _{max}	0.013	19.854***	78.302***	0.505	16.367***	0.877	16.714***
J _{max25}	120.748***	18.666***	91.039***	3.112	3.024	0.138	12.394**
Leaves	100.86 ***	145.62 ***	188.08 ***	7.40 **	23.78 ***	34.68 ***	2.88
LA	9.40 **	300.24 ***	276.75 ***	39.46 ***	45.09 ***	150.84 ***	60.06 ***
LMA	14.39 ***	89.00 ***	74.64 ***	12.26 **	0.89	26.41 ***	1.16
N _a	8.394**	205.359***	0.018	1.051	3.589	23.605***	1.193
g _{sw}	138.293***	0.005	1077.092***	8.088**	113.023***	0.001	0.1
E	177.158***	1.326	852.392***	1.986	130.999***	0	0.147
iWUE	121.186***	9.65**	139.945***	2.137	4.286*	6.59*	0.008
NPQ	185.6***	0.448	57.848***	0.021	0.057	0.471	0.056
Π ₀	5.02 *	1.43	2.46	0.11	11.08 **	6.28 *	1.67

ABA	54.13***	22***	61***	19.53***	51.54***	16.24***	21.18***
Φ_{CO_2}	0.001	37.419***	537.359***	5.005*	0.029	20.13***	3.636
Φ_{PSII}	3.94	8.089**	170.636***	7.312*	0.231	4.397*	1.772
LCP	43.578***	142.26***	23.878***	7.55**	7.401**	0.226	4.432*
PPFD ₅₀	0.819	83.587***	135.753***	0.131	0.086	14.527***	20.835***
J_p/A_n	2.761	0.142	107.274***	14.648***	3.535	1.32	2.892
R_d/A_n	2038.213***	88.956***	1747.666***	246.745***	560.824***	10.467**	353.703***
R_{PR}/A_n	1.041	1.625	46.112***	4.906*	1.459	0.043	0.001
NPQ/ A_n	151.877***	0.091	640.515***	16.929***	48.307***	0.023	19.112***

4.3. Study 3: Elevated Growth Temperature Modifies Drought and Shade Responses of *Fagus sylvatica* Seedlings by Altering Growth, Gas Exchange, Water Relations, and Xylem Function

The findings presented in this chapter have contributed to the development of the following publication:

Plants 2025, 14(10), 1525; <https://doi.org/10.3390/plants14101525>



4.3.1. Introduction

The significant increase in temperature due to ongoing climate change, along with more frequent and severe droughts (e.g., scenario SSP5-8.5) (IPCC, 2023), poses challenges for forest species with long reproductive cycles, slow migration capacity, and largely unknown limits of acclimation. Plant acclimation to warmer growing temperatures remains an underexplored process and becomes increasingly complex when combined with other environmental stressors. Many plant species experience a triple interaction of stressors: sustained warming, episodic water deficit, and regeneration under the low-light conditions typical of forest understories (Pineda-García et al., 2013). To gain insights into how future warming will affect regeneration niches of key late-successional species, and how tree populations may respond to rapid environmental changes, it is essential to examine the acclimation capacity and the phenotypic plasticity, i.e., the capacity of an organism to modify its physiological, morphological, or developmental traits in response to environmental changes, compared to its baseline behavior under control conditions, of essential functional traits (Ramírez-Valiente, 2025 #873; Valladares et al., 2014). Shifts in traits such as gas exchange, water relations, and biomass allocation can influence ecological strategies like drought and shade tolerance, ultimately affecting the species' ability to compete and regenerate, thereby altering forest dynamics (Reich, 2014; Valladares & Niinemets, 2008).

The temperature dependence of most biological processes, such as metabolic and growth, follows an asymmetric Arrhenius equation, typically peaking at the optimal temperature (Arroyo et al., 2022). For instance, warming can promote tree growth and increase leaf area at the expense of root investment, as long as water and nutrients are not limiting. However, growth tends to decline when temperatures exceed the optimum (Kumarathunge et al., 2020; Way & Oren, 2010), mainly due to a reduction in leaf area and net photosynthesis (A_n) (Dai et al., 2021; Kumarathunge et al., 2020; Yamori et al., 2014). Meanwhile, respiration rates (R_d) continue to rise beyond the optimum for A_n , leading to further declines in growth (Atkin & Tjoelker, 2003). Reduced carbon availability at higher temperatures is also reflected in lower leaf mass per area (LMA) (Poorter, 2009), a trait associated with plant ecological strategies, including drought and shade tolerance (Westoby et al., 2002). Additionally, leaves that develop under elevated temperatures tend to

be smaller, which helps reduce the boundary layer and facilitate heat dissipation (Leigh et al., 2017; Marchin et al., 2022). Both A_n and R_d acclimate to changes in growth temperature typically by the downregulation of R_d (Atkin & Tjoelker, 2003; Crous et al., 2022) and the upregulation of the temperature optimum for A_n (Crous et al., 2022; O. Ghannoum, N. G. Phillips, M. A. Sears, et al., 2010; Way & Oren, 2010), which allows plants to maximize carbon gain with warming (Kumarathunge et al., 2019). However, a decoupling between A_n and stomatal conductance (g_{sw}) has been reported at high temperatures in many species, particularly those adapted to warm climates. In such cases, A_n decreases while g_{sw} increases due to the so-called evaporative cooling (Diao et al., 2024; Drake et al., 2018; Urban et al., 2017b), with the concomitant reduction in intrinsic water use efficiency ($iWUE$) and increased risk of dehydration (Schönbeck et al., 2022).

The extent to which stomata acclimate to warming depends on a complex balance among their key roles. Stomata regulate not only photosynthesis and carbon uptake but also leaf water loss through transpiration (E), leaf temperature via evaporative cooling (Drake et al., 2018; Urban et al., 2017b), responses to oxidative stress (Suzuki & Mittler, 2006), and leaf water balance (Hetherington & Woodward, 2003). Maintaining water potential within specific safety limits is crucial to prevent hydraulic failure (Choat et al., 2018) and leaf dehydration (Mantova et al., 2023). Transpiration is driven by the vapor pressure difference between the leaf interior and the atmosphere (VPD_L) multiplied by the total conductance for water vapor. Therefore, for a given stomatal aperture, more water is lost to the atmosphere as air temperature increases, due to higher VPD_L (Diao et al., 2024; Grossiord et al., 2017). To sustain higher water flow at higher evaporative demand, leaf water potential at the turgor loss point (π_0) often decreases in seedlings grown at warmer temperatures (Way et al., 2013), an adjustment frequently observed in species adapted to warm and dry habitats (Bartlett et al., 2012). However, it remains unclear whether these changes are a direct response to warming alone or to its interaction with other stressors, such as soil or atmospheric moisture (Aranda et al., 2021).

Acclimation of the hydraulic architecture to warming in forest species remains understudied even though changes are expected due to increased evaporative demand (Way et al., 2013; Way & Oren, 2010). For example, well-watered *Populus tremuloides* seedlings grown at warmer temperatures than

ambient showed higher growth, and greater allocation to roots, which increased hydraulic conductance and a higher sapwood area to leaf area (Huber value, H_V) (Way et al., 2013). Conversely, in a xeric site, both *Juniperus monosperma* and *Pinus edulis* showed a lack of acclimation of H_V or xylem anatomy after four years of atmospheric warming and only the juniper reduced leaf-specific hydraulic conductivity (K_L), associated with a decrease in canopy conductance under warming (Grossiord et al., 2017). Well-watered plants exposed to warming can develop larger and/or more frequent xylem conduits (Maherali & DeLucia, 2000; Way et al., 2013), potentially enhancing conductivity, without necessarily affecting embolism resistance. By contrast, in *Eucalyptus camaldulensis* and *E. grandis*, seedlings grown above optimal growth temperatures developed smaller vessels, lower specific conductivity (K_s), and higher wood density (ρ) (Thomas et al., 2004, 2007). Besides changes in xylem anatomy, increased sap temperature reduces viscosity while maintaining constant sap density, thereby enhancing whole-plant hydraulic conductance independently of xylem structural traits (Thomas et al., 2004).

Although warming increases the water deficit experienced by plants, the water deficit itself also amplifies the negative effects of supraoptimal growth temperatures (Osonubi & Davies, 1980; Rodríguez-Calcerrada et al., 2010). Water deficit reduces the temperature optima for both photosynthesis, as a consequence of reduced diffusional CO_2 conductance (Cano et al., 2014; Cano et al., 2013), and tree growth due to decreased photosynthesis rates, reduced leaf area, and increased *LMA* (Kumarathunge et al., 2020). However, active osmotic adjustment in warmed trees can enhance drought tolerance (Bartlett et al., 2012). Water deficit-induced stomatal closure increases leaf temperature and the risk of tissue damage, even when thermal acclimation occurs (Al-Salman et al., 2023; Marchin et al., 2022). When stomata are partially or fully closed due to soil water deficit, residual water loss through the leaf cuticle becomes crucial for plant survival (López et al., 2021). This leaf minimum conductance ($g_{\min}$) typically decreases under drought; however, short-term high temperatures can increase $g_{\min}$ (Duursma et al., 2019) whereas prolonged exposure to elevated temperatures under sustained higher VPD tends to reduce it (Schönbeck, 2022 #1227}.

Another major environmental stressor limiting the growth and regeneration capacity of many temperate tree species is light availability (Bachofen et al.,

2020). Under shaded conditions, plants allocate more resources to stem elongation and increased leaf surface area to capture light (Bachofen et al., 2020), leading to trade-offs that can compromise acclimation to water stress (Aranda et al., 2002; Aranda et al., 2008). For example, plants grown under shade often exhibit a lower capacity of osmotic adjustment (Ismael Aranda et al., 2001; Tschaplinski et al., 1998), lower *LMA* (Poorter et al., 2009), and lower biomass allocation to roots (Rodríguez-Calcerrada et al., 2008), which collectively impair drought and heat tolerance. Conversely, large forest gaps expose seedlings to high light intensity and warmer temperatures, promoting higher metabolic activity and growth rates but also exposing them to greater water stress and thermal damage (Marchin et al., 2022; Weston & Bauerle, 2007). Understanding how elevated temperatures, water stress, and light conditions interact is critical for predicting challenges to forest regeneration under climate change since the combined response to multiple stressors is not simply the additive sum of individual stress responses (Mittler, 2006; Zandalinas & Mittler, 2022).

Most studies on temperature acclimation have not controlled for air humidity, resulting in elevated VPD that can confound the interpretation of warming effects, given the high sensitivity of transpiration to VPD (Way et al., 2013). Furthermore, transpiration responses are known to acclimate to the prevailing VPD during growth (Seversike et al., 2013), making comparisons across studies conducted at different VPD levels potentially controversial (Koehler et al., 2023). By contrast, studies that directly manipulate temperature while maintaining constant VPD are rare (Mills et al., 2024). Disentangling the direct effects of warming from soil and atmospheric drought thus requires further investigation, as shifts in resource allocation, along with morphological and physiological acclimation to warming, can increase vulnerability to subsequent drought events (H. Duan et al., 2013; Way & Oren, 2010).

In this study, we exposed one-year-old seedlings of *Fagus sylvatica* L. to a factorial combination of optimal (25 °C; T25) and supraoptimal (+7.5 °C; T32) temperatures, soil water availability (well-watered vs. water-deficit), and light intensity (high vs. low), while maintaining a constant VPD. Our goal was to disentangle the direct effects of elevated temperature from atmospheric drought and to investigate how warming modulates the physiological, morphological, and anatomical responses of beech seedlings to

simultaneous water and light stress (see Table A1 for measured traits and abbreviations). By addressing these questions, our study provides novel insights into the multifactorial stress responses of a key late-successional species and advances our understanding of its regeneration potential under projected climate change scenarios (Zandalinas & Mittler, 2022; Zandalinas et al., 2024). We hypothesized that: (1) Warming will reduce plant growth, primarily through a reduction in leaf area rather than proportional declines in photosynthesis, exacerbating the negative effects of concurrent water deficits and shade. (2) Stomatal conductance will increase under warming when water is not limiting to promote evaporative cooling, but it will decrease under water deficits. (3) Xylem anatomical traits related to hydraulic conductivity will adjust to match changes in leaf transpiration, maintaining water balance. (4) Phenotypic plasticity to water and light availability will be reduced due to warming.

4.3.2. Materials and Methods

4.3.2.1. Plant Material, Growth Conditions, and Treatments

Beech seedlings were established from nuts collected in the Natural Reserve of ‘Hayedo de Montejo’ in 2021 (458,249; 4,551,615 UTM ETRS89). In February 2022, after three months at 4 °C in darkness and moist conditions, one seed per pot germinated within a few days of difference in 9 L pots, filled with a mixture of peat and sand (75–25% v:v). Each pot was fertilized with a release fertilizer (Nutricote®, 16N:4.4P:8.3K, 2.5 gr L⁻¹ of substrate). Pots were randomly arranged in two different walk-in growth chambers set at two different temperatures but with the same air *VPD*. One chamber was set with day/night temperatures and relative humidity of 25/15 ± 0.5 °C and relative humidity of 40/68 ± 1% (*VPD* of 1.91/0.86 ± 0.06 kPa) (named T25), close to the temperature optimum for photosynthesis for beech (Šigut et al., 2015), and the other to 32.5/22.5 ± 0.5 °C and 61/80 ± 1% (*VPD* of 1.91/0.86 ± 0.06 kPa) (named T32), both with a 12 h of photoperiod. A +7.5 °C increase in mean temperature is expected from June to August for Western and Central Europe and the Mediterranean region according to the scenario SSP5-8.5 for 34 models of AR6 CMIP6 (interactive-atlas.ipcc.ch) (IPCC, 2023). In each chamber, we established two treatments of light intensity: high PPFD (ca. 600 μmol photons m⁻² s⁻¹ at the canopy height) and low PPFD (ca. 200 μmol photons m⁻² s⁻¹ at the canopy height). Low PPFD (e.g., shaded conditions)

was provided by gray polyethylene shade nets mounted on frames and was measured as the average of six different measurements using a quantum meter (MQ 200; Apogee Instruments Inc., Logan, UT, USA) in a horizontal position on leaves. Plants developed under well-watered conditions for two months when seedlings with similar height and number of leaves were assigned to two contrasted watering treatments. Well-watered plants were watered daily to full capacity. Water-stressed plants were kept without watering until stomatal conductance reached 1/3 of well-watered plants (ranging from ca. 10 days in high PPFD to ca. 24 days in low PPFD) and then plants were watered every three days with an initial amount of 150 mL of water for plants under high PPFD and 65 mL of water for plants under low PPFD (about 1/3 of daily evapotranspiration in both cases). Once a week, plants were weighed and the volumetric soil water content (*SWC*) was measured by time domain reflectometry (TDR, Trase System I, Soil Moisture Equipment, Logan, UT, USA) and watering amounts were adjusted. At this time the final multifactorial design (two factors: water and light) was set for each growing temperature: (1) high PPFD (ca. 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the canopy height) and well-watered conditions (HW); (2) high PPFD and moderate water stress (HS); (3) low PPFD (ca. 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the canopy height) and well-watered conditions (LW); (4) low PPFD and moderate water stress (LS). Each week, plants were randomly repositioned within their treatment group. Every three weeks, plants were rotated between chambers while maintaining their assigned watering treatment (i.e., identical conditions but a different growth chamber).

4.3.2.2. Growth and Biomass Allocation

Growth and biomass were measured in six randomly selected plants of each treatment at the beginning of the experiment. These plants were not used for any other measurement until harvest. Height, stem diameter, and the number of fully developed leaves were measured six times throughout the experiment (every 15–20 days) (see Figure A1). After 90 days under their assigned treatments, the same plants were harvested, and biomass was divided into roots, stems, and leaves. Roots were separated from the soil by gently washing under clean tap water. Samples were oven-dried at 60 °C for 7 days and weighed. We calculated root (*RM*), stem (*SM*), and leaf mass (*LM*) fractions as the ratio of organ dry mass to total plant dry mass. One representative fully mature leaf from the top canopy of each plant was

scanned to obtain the leaf area using ImageJ 1.53t (NIH, Bethesda, MD, USA) (Schneider et al., 2012) and then oven-dried at 70 °C for 48 h for constant dry mass (Mettler Toledo AB 204, Columbus, OH, USA) to determine the leaf mass per area (LM) as the ratio of leaf dry mass to projected area. The total leaf area (TLA) of each plant was estimated from LM and LM . The mean leaf area for each plant (LA) was calculated as TLA divided by the number of leaves.

4.3.2.3. Gas Exchange and Water Use Efficiency

One fully mature leaf from the upper plant canopy was selected to measure the net photosynthetic rate (A_n , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance (g_{sw} , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), and transpiration rate (E , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$). These parameters were measured twice during the experiment in five plants per treatment using a portable IRGA system (LI-6400XT) with the transparent chamber (6400-08 clear chamber) to record the leaf gas exchange under growth conditions. Measurements were conducted under growth conditions of air temperature (block temperature was set at 25 °C for T25, and 32 °C for T32) and relative humidity to achieve a VPD close to 1.9 kPa. The reference CO_2 concentration was set at 400 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ and a flow rate of 300 $\mu\text{mol m}^{-2} \text{ s}^{-1}$. After recording gas exchange under growing conditions, the light source was switched off inside the chambers, and after 20 min of acclimation in the dark, dark respiration (R_d) was measured. All gas exchange variables were corrected with the minimum conductance ($g_{\min}$) (see below) according to (Warren et al., 2011). The leaf water use efficiency (WUE) was calculated as (A_n/E) and the intrinsic water use efficiency ($iWUE$) was determined using (A_n/g_{sw}).

4.3.2.4. Stomatal Traits and Carbon Isotope Composition ($\delta^{13}\text{C}$)

The same leaves used for gas exchange measurements were collected for carbon isotope composition ($\delta^{13}\text{C}$) and then for stomatal traits. Two leaf disks taken from each side of the leaf blade were dried for 3 days at 65 °C and analyzed at the Stable Isotope Facility of UC Davis (CA, USA). Isotope composition was measured with an elemental analyzer (PDZ Europa ANCA-GSL, Sercon Ltd., Cheshire, UK) interfaced to an isotope ratio mass spectrometer (PDZ Europa 20-20; Sercon Ltd., Cheshire, UK) with internal standards reaching $\delta^{13}\text{C}_{\text{VPDB}}$ standard deviation $<0.07\text{‰}$. Leaf trichomes were removed using duct tape and three negative impressions were taken of the

middle portion of the leaf avoiding main veins and using nail varnish. The impressions were attached to a microscope slide using transparent tape and imaged under a light microscope (Leica DM2500 LED) using the software Leica Application Suite X 3.7.2.22383 (Leica Microsystems GmbH, Wetzlar, Germany). Photomicrographs were analyzed using ImageJ (Schneider et al., 2012). Stomatal density (S_D) was calculated as the number of stomata per unit leaf area ($\text{no}^\circ \text{mm}^{-2}$) on three leaf areas of 1.15 mm^2 each using $\times 10$ magnification. Within each leaf area, 10 stomata were randomly selected to measure in μm the guard cell length (S_L), stomatal pore length (Pl_s), stomatal complex width (W_s) and guard cell width (GW_s) using $\times 40$ magnification. The potential conductance index (PCI) was computed as $PCI = (GW_s)^2 \text{ SD } 10^{-4}$ (Holland & Richardson, 2009), as a surrogate of potential maximum stomatal conductance.

4.3.2.5. Minimum Leaf Conductance or Residual Conductance ($g_{\min}$)

At the same time as gas exchange measurements, one healthy, fully expanded leaf from the same plants was immediately stored in a plastic zip bag with moist tissue paper and kept in a portable cooler at 4°C until it was taken to the laboratory. In the lab, leaves were stored at 4°C in darkness with the petiole submerged in water overnight until the next day for measurements. We estimated $g_{\min}$ through bench dehydration of the rehydrated leaves with petioles sealed with a high-strength adhesive (Loctite, Prism 401) (Araus et al., 1991). Briefly, the leaf area was measured at the beginning of dehydration, and leaves were placed on top of a perforated rack at constant air temperature and relative humidity (ca. 25°C and 46%, respectively) without direct light. They were repeatedly weighed with an analytical balance to determine the steady-state rate of water lost by unit of leaf area once stomata were closed, i.e., epidermal transpiration ($E_{\min}$, $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$). To achieve the epidermal minimum conductance by one side of the leaf ($g_{\min}$, $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), we followed the next approximation ($g_{\min} = E_{\min} P / (2 \text{ VPD})$), where P is the atmospheric pressure (94 kPa) and VPD is the air vapor pressure deficit (1.65 kPa), and the boundary layer conductance was assumed negligible.

4.3.2.6. Pressure-Volume Curves and Related Traits

Leaf water relations were measured using pressure-volume (P-V) curves of leaves sampled at the end of the night period on five seedlings per treatment.

Leaves were rehydrated for six hours in the dark and at 4 °C with only the petiole immersed in distilled water. After that, leaves were scanned to obtain the initial leaf area and were allowed to slowly dehydrate at constant room temperature using the free transpiration method (Robichaux, 1984; TYREE & HAMMEL, 1972). Leaf water potential (Ψ_{leaf}) was measured with a pressure chamber (model 1000; PMS Instrument Co., Albany, NY, USA) and leaf mass with a precision balance at intervals, starting with $\Psi_{\text{leaf}} > -0.05$ MPa and until ca. -3.5 MPa for HW and LW treatments and -6 MPa for HS and LS treatments. We checked all curves for oversaturation during the first steps of dehydration and when present was corrected (Dreyer et al., 1990; Kubiske & Abrams, 1990). Leaf dry mass was determined, as described above. The osmotic potential at full turgor (π_{100}), the turgor loss point (π_0), leaf capacitance before the turgor loss point ($C_{\pi_{100}}$) and after (C_{π_0}), the maximum modulus of elasticity (ϵ_{leaf}), and the relative water content at the turgor loss point (RWC_{π_0}) were derived from the P-V curves according to (Brodribb & Holbrook, 2004; Robichaux, 1984).

4.3.2.7. Predawn and Midday Water Potentials

The same leaves used for gas exchange measurements were used to determine midday leaf water potentials (Ψ_{leaf}) after cutting the petiole and sealed inside a plastic bag that had been exhaled and stored in ice boxes. They were then transported to the lab and measured with a pressure chamber (PMS instrument; model 1505D) within 1–2 h of excision (Al-Salman et al., 2024). One adjacent leaf was previously covered and allowed to equilibrate for at least 3 h before midday with cling wrap and aluminum foil to prevent transpiration, and to determine the midday branch water potential (Ψ_{stem}) (i.e., non-transpiring leaf). Leaf predawn water potential (i.e., end of the night period) (Ψ_{pd}) was measured the night before on the same plants. The safety margin (SM_{leaf}) was calculated as the absolute difference between the turgor loss point (π_0) and the transpiring leaf water potential at midday (Ψ_{leaf}).

4.3.2.8. Hydraulic Conductivity and Huber Value

Loss of hydraulic conductivity and maximum hydraulic conductivity were measured in nine seedlings per treatment at the end of the experiment, following (López et al., 2019). Plant transpiration was stopped 3 h before harvesting by switching off the lights and enclosing plants in black bags. Then, pots were immersed in water and the stem was cut at the base. The

entire above-ground portion was kept under water, tearing leaves away from shoots, and the plant distal end was cut to ensure xylem relaxation for 30 min (Aranda et al., 2015). The stem was then sequentially cut back underwater, and a 5 cm long segment above the cotyledons was connected to a XYL'EM apparatus (Bronkhorst, Montigny les Cormeilles, France) to measure hydraulic conductivity at low pressure (≤ 2 kPa) before (K_{init}) and after flushing the sample with degassed, filtered 2 mmol KCl solution at high pressure (0.2–0.3 MPa) for 20 min (K_{max}). Loss of hydraulic conductivity (PLC) was determined as:

$$PLC = 100(1 - K_{\text{init}}/K_{\text{max}}), \quad (39)$$

Maximum specific hydraulic conductivity (K_s) was calculated by dividing K_{max} by the sapwood area and leaf-specific conductivity (K_L) by dividing K_{max} by the total supported leaf area. The Huber value (H_V) was determined as the ratio of K_L and K_s (i.e., sapwood area relative to leaf area). Finally, the hydraulic conductance of the whole plant (k_{plant}) was estimated as in (Robson et al., 2012):

$$k_{\text{plant}} = \frac{E}{(\Psi_{\text{pd}} - \Psi_{\text{leaf}})} \quad (40)$$

where E was the leaf transpiration rate and $(\Psi_{\text{pd}} - \Psi_{\text{leaf}})$ was assumed to be the water potential gradient from the soil to leaf (MPa), assuming full equilibration of plant and the soil at the end of the night period (i.e., $\Psi_{\text{soil}} \approx \Psi_{\text{pd}}$).

4.3.2.9. Stem Xylem Anatomy

Five stem segments per treatment, those used for hydraulic conductivity measurements, were selected for anatomical measurements. One centimeter in length was used to determine the wood density (ρ) following (Aranda et al., 2015). Fifteen μm -thick cross and tangential sections were cut using a sliding microtome (Leica SM 2400), stained with 0.1% safranin, and mounted for subsequent image analysis. Digital images of the thin sections were taken using a Moticam A1 camera (Motic, Hong Kong, China) attached to an Olympus BX50 light microscope. Images were analyzed using ImageJ (Schneider et al., 2012). We measured three sectors per sample from the cross-section to quantify the average vessel lumen area (V_A), vessel density (V_D), the equivalent circle diameter (D), and the hydraulic diameter (D_h) following (Scholz et al., 2013):

$$D = \sqrt{\frac{4V_A}{\pi}} \quad (41)$$

$$D_h = \left(\frac{\sum D^4}{N} \right)^{\frac{1}{4}} \quad (42)$$

where V_A is the lumen conduit area, N it is the number of vessel elements. To determine the parenchyma surface area, a further three sectors per sample were taken from the tangential section.

4.3.2.10. Trait Variation and Plasticity

We calculated the effect of each treatment individually by expressing the variation in each measured trait (see Table A1 for definitions) as a normalized value ranging from -1 to 1 . Negative values indicate a decrease in the trait as a consequence of the treatment, while positive values indicate an increase. When a trait changes—either increasing or decreasing—compared to a reference value, the following formulas were used to quantify plasticity:

If the treatment mean of a variable ($\mu_{\text{treatment}}$) < reference mean ($\mu_{\text{reference}}$), the plasticity is negative:

$$NP = \frac{(\mu_{\text{treatment}} - \mu_{\text{reference}})}{\mu_{\text{reference}}} \quad (43)$$

If the mean of treatment ($\mu_{\text{treatment}}$) > the mean of reference ($\mu_{\text{reference}}$), the plasticity is positive:

$$NP = \frac{(\mu_{\text{treatment}} - \mu_{\text{reference}})}{\mu_{\text{treatment}}} \quad (44)$$

Note that we can obtain the individual trait response ratio as:

$$\frac{\mu_{\text{treatment}}}{\mu_{\text{reference}}} = NP + 1 \quad (45)$$

Or:

$$\frac{\mu_{\text{treatment}}}{\mu_{\text{reference}}} = \frac{1}{1 - PP} \quad (46)$$

where NP and PP are the values of the negative or positive plasticity for the trait, respectively.

The Phenotypic Plasticity Index (PPi) was calculated to assess the overall plasticity of *Fagus sylvatica* seedlings to different environmental conditions, evaluating the effects of growth temperature on light and water plasticity changes, as described by (Valladares et al., 2006). This form of expressing plasticity is relevant when several traits are analyzed together, so we can visualize which traits are more plastic than others. We calculated the PPi for each trait as the difference between the maximum and minimum mean values ($\max \mu - \min \mu$) divided by the maximum mean value:

$$PPi = \frac{\max \mu - \min \mu}{\max \mu} \quad (47)$$

4.3.2.11. Statistical Analyses

After checking the normality of the data, three-way analyses of variance (ANOVA) were performed in R v. 4.3.2 (R Development Core Team 2024) using 'Tgrowth', 'light', and 'water' as fixed factors. In all cases, the data met the assumptions of normality and homoscedasticity; a significance level of $p \leq 0.05$ was used, and model assumptions were checked. Statistically significant differences between groups were assessed using the HSD Tukey post hoc test in R v. 4.3.2 using the "agricolae" package. Principal Component Analysis (PCA) was performed in R version 4.3.2, utilizing the "stats" package for matrix calculations and the "factoextra" package for visualization support. The PCA was based on the correlation matrix, and the eigenvectors generated

were analyzed to identify variables exhibiting a strong association with specific principal components (PCs). Pearson correlation coefficients were estimated using the “rcorr” function in R. Results are presented as means $\pm$ standard error (SE).

4.3.3. Results

4.3.3.1. Changes in Morphology and Biomass Allocation

Elevated growth temperature (T32) significantly reduced overall plant growth and altered biomass allocation in *Fagus sylvatica* seedlings compared to optimal conditions (T25). After 90 days, seedlings grown at T25 developed thicker stems and larger leaves and accumulated more total biomass (Figure 37; Table A2). In contrast, those grown at T32 produced a greater number of smaller leaves, leading to lower total leaf area (*TLA*) despite an increase in leaf number (Figure 37). This pattern was consistent across all water and light treatments, indicating a direct effect of supraoptimal temperature on growth morphology. Temperature also reshaped biomass partitioning. Seedlings at T32 invested proportionally more in leaves and less in shoots than those at T25. The shift toward greater leaf allocation may reflect a compensatory response to reduced leaf size, but it did not offset the reduction in carbon gain. Growth at T32 was tightly coupled with lower net photosynthesis (A_n), as confirmed by strong correlations between stem diameter and A_n across treatments (Figure A6). Both water deficit and low light reduced growth, biomass, and the number of fully developed leaves, although the cumulative effect of light intensity exerted a stronger effect than water deficit (Figure 37). Water deficit enhanced root biomass allocation in both temperature regimes and increased water use efficiency, while shade decreased overall plant biomass (Figure 37). However, under all stress combinations, seedlings at T32 showed lower growth performance and reduced plasticity in allocation patterns compared to T25 (Table A2; Figure A2). These results demonstrate that warming directly constrains growth through reduced carbon assimilation and altered morphology, irrespective of resource availability.

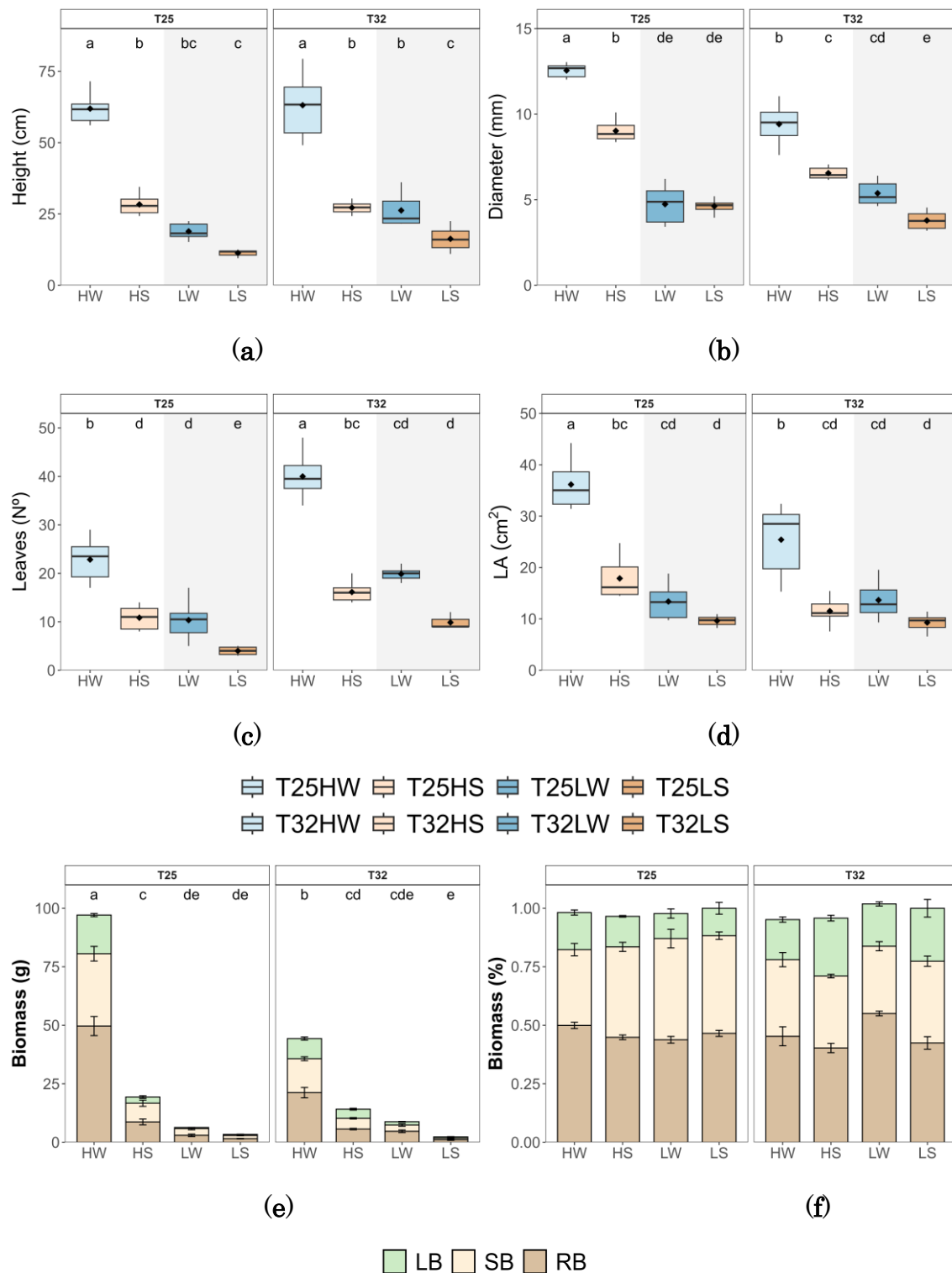


Figure 37. Boxplots of growth and biomass allocation of beech seedlings grown in a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L). (a) height (h); (b) diameter (d); (c) a number of developed leaves (leaves); (d) leaf area (LA); (e) total biomass of leaves (LB), shoots (SB), and roots (RB) and (f) relative proportion of biomass (%) of leaves (LB), shoots (SB), and roots (RB). The straight horizontal black line within each boxplot indicates the median, while the mean value is represented as a solid black point. Different letters indicate significant differences among treatments.

4.3.3.2. Leaf Gas Exchange, Water Use Efficiency, and Stomatal Traits

Leaf gas exchange was significantly affected by temperature, with T32 reducing photosynthetic performance and water-use efficiency compared to T25. Seedlings at T32 exhibited significantly lower A_n and higher mitochondrial respiration (R_d), which together reduced the net carbon balance across all treatments (Figure 38; Table 8). Notably, this decline in A_n occurred even though VPD was held constant across temperature regimes, highlighting a direct temperature effect independent of evaporative demand. Interestingly, stomatal conductance (g_{sw}) increased under T32, but only in well-watered conditions, suggesting that stomata remained more open to enhance evaporative cooling at higher leaf temperatures. This increase in g_{sw} , however, was not matched by higher A_n , leading to significantly lower intrinsic water use efficiency ($iWUE$) at T32. The more negative $\delta^{13}C$ values observed under T32 further confirmed the decreased water-use efficiency (Figure 38e,f). Under water stress, g_{sw} decreased across both temperature regimes, but the reduction in gas exchange was more pronounced at T32, indicating a compounding effect of warming and drought (Table 8). Temperature also modulated residual water loss. Minimum leaf conductance (g_{min}) was significantly higher at T32, particularly in well-watered plants, suggesting greater cuticular or incomplete stomatal closure under warm conditions. Stomatal traits were not directly affected by warming although warming had significant interaction with the other two growing factors. Stomatal density (S_b) increased with light intensity only at T25, but stomatal length (L_s) was shorter under water deficit only at T32.

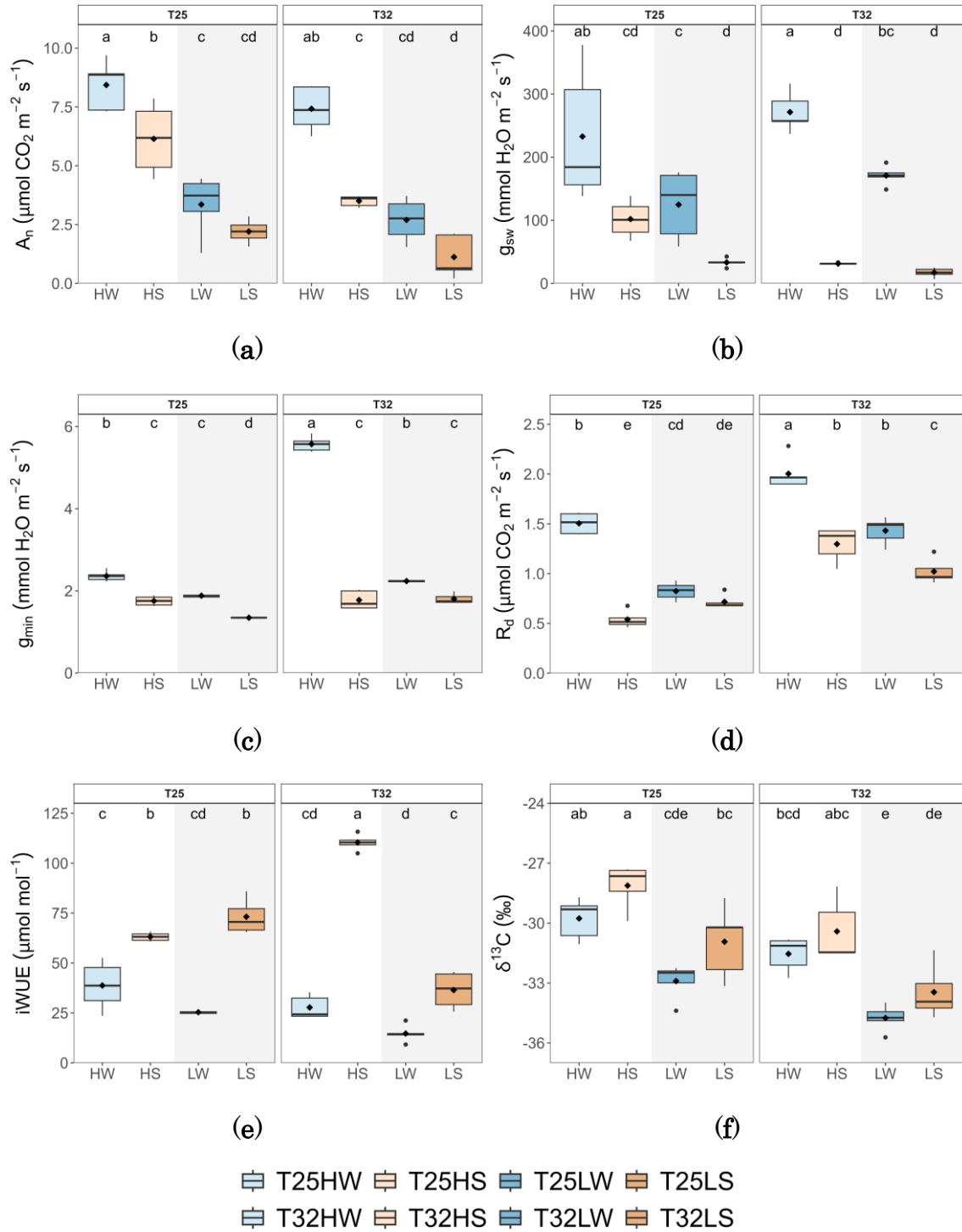


Figure 38. Boxplots of leaf gas exchange and isotopic carbon composition of beech seedlings grown in a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L): (a) net assimilation (A_n); (b) stomatal conductance to water vapor (g_{sw}); (c) minimal conductance (g_{min}); (d) mitochondrial respiration (R_d); (e) intrinsic water use efficiency (iWUE); (f) carbon isotope composition ($\delta^{13}\text{C}$). The straight horizontal black line within each boxplot indicates the median, while the mean value is represented as a solid black point. Different letters indicate significant differences among treatments.

4.3.3.3. Leaf Water Relations

Elevated temperature (T32) negatively affected leaf water status, primarily by lowering midday leaf water potential (Ψ_{leaf}), stem water potential (Ψ_{stem}), and predawn water potential (Ψ_{pd}), regardless of water or light treatment (Figure 39; Table 8). These reductions were most pronounced under water deficit, but even well-watered plants at T32 exhibited more negative water potentials compared to those at T25, pointing to a direct thermal effect on plant water balance. The capacity for osmotic adjustment was also compromised at T32. At T25, seedlings under water stress showed clear osmotic adjustments, with more negative osmotic potential at full turgor (Π_{100}) and turgor loss point (Π_0), especially under high light (Figure 39). These responses were accompanied by higher leaf capacitance ($C_{\Pi 100}$) and lower leaf tissue elasticity (ε_{leaf}), allowing for greater water retention and stress buffering. However, at T32, seedlings failed to significantly adjust Π_0 or Π_{100} under stress, particularly under high light, suggesting a loss of drought acclimation capacity. Notably, warming also reduced the leaf safety margin (SM_{leaf})—the difference between midday Ψ_{leaf} and Π_0 —across all treatments. This decrease was the most severe under combined water deficit and shade, where SM_{leaf} values approached critical thresholds, increasing the risk of turgor loss and hydraulic failure.

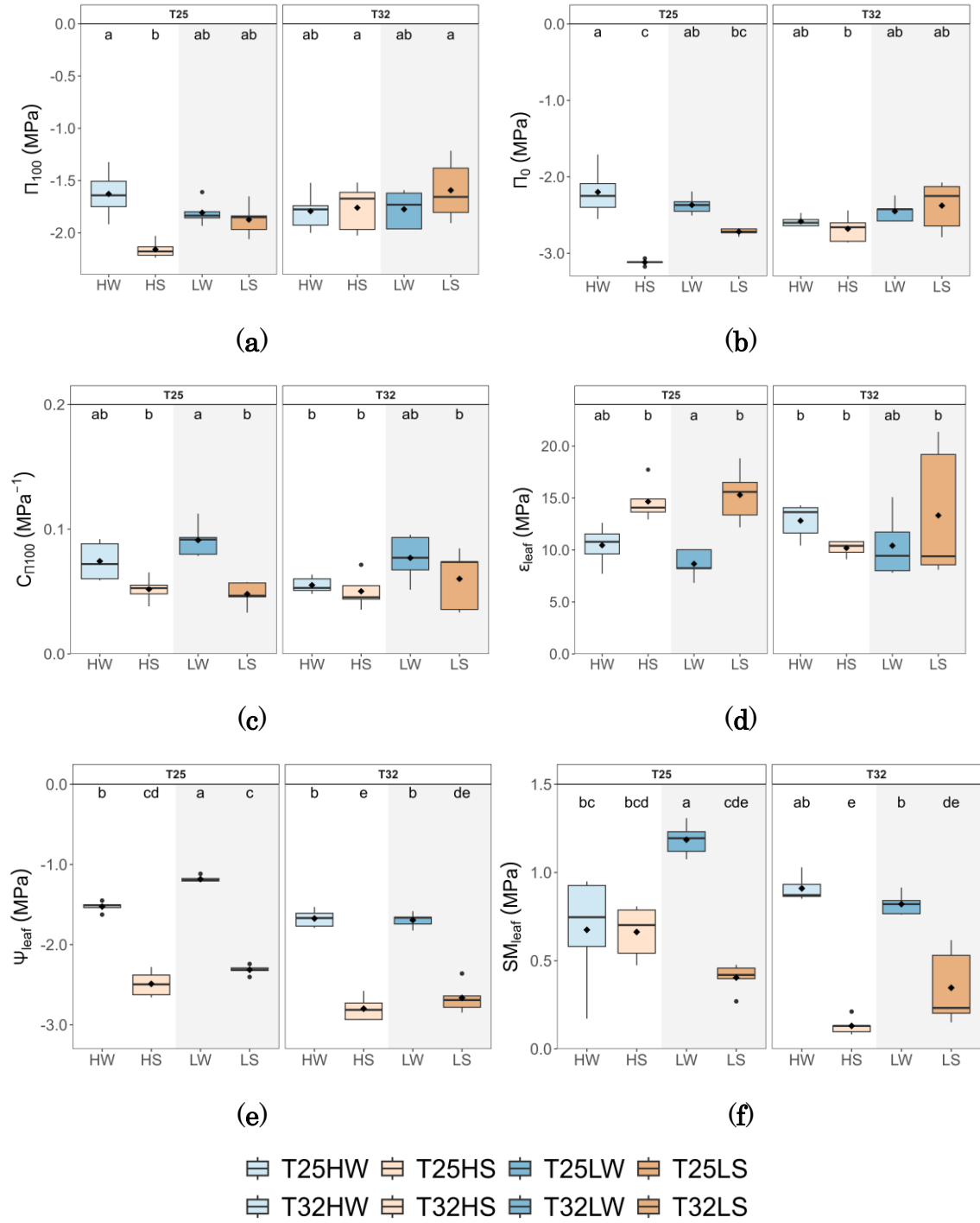


Figure 39. Boxplots of leaf water relations of beech seedlings grown in a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L): (a) osmotic potential at full turgor (Π_{100}); (b) osmotic potential at turgor loss point (Π_0); (c) leaf capacitance before the turgor loss point ($C_{\Pi 100}$); (d) maximum modulus of elasticity (ϵ_{leaf}); (e) leaf midday water potential (Ψ_{leaf}); and (f) safety margin (SM_{leaf}). The straight horizontal black line within each boxplot indicates the median, while the mean value is represented as a solid black point. Different letters indicate significant differences among treatments.

4.3.3.4. Hydraulic Traits and Stem Anatomy

Elevated temperature (T32) significantly affected several key hydraulic traits, particularly in interaction with light and water availability (Table 8). While stem-specific hydraulic conductivity (K_s) was primarily influenced by light intensity, leaf-specific conductivity (K_L) and the Huber value (H_v) showed consistent reductions under warming, especially under water stress or shade (Figure 40, Table 8). Notably, plant hydraulic conductance (k_{plant}) increased slightly only under high light and well-watered conditions at T32, suggesting a limited compensatory response to warming when resources were not limiting. Contrary to expectations, warming alone did not significantly alter K_s , but it reduced K_L and H_v in most treatments (Figure 40), indicating a decoupling between stem-level and whole-plant hydraulic efficiency. This effect was particularly evident in shaded or drought plants, where warming exacerbated the reduction in water transport efficiency. The mean loss of xylem conductivity (PLC) was lower than 10% in well-watered plants and lower than 16% in water-stressed plants pointing to moderate drought during the whole experiment.

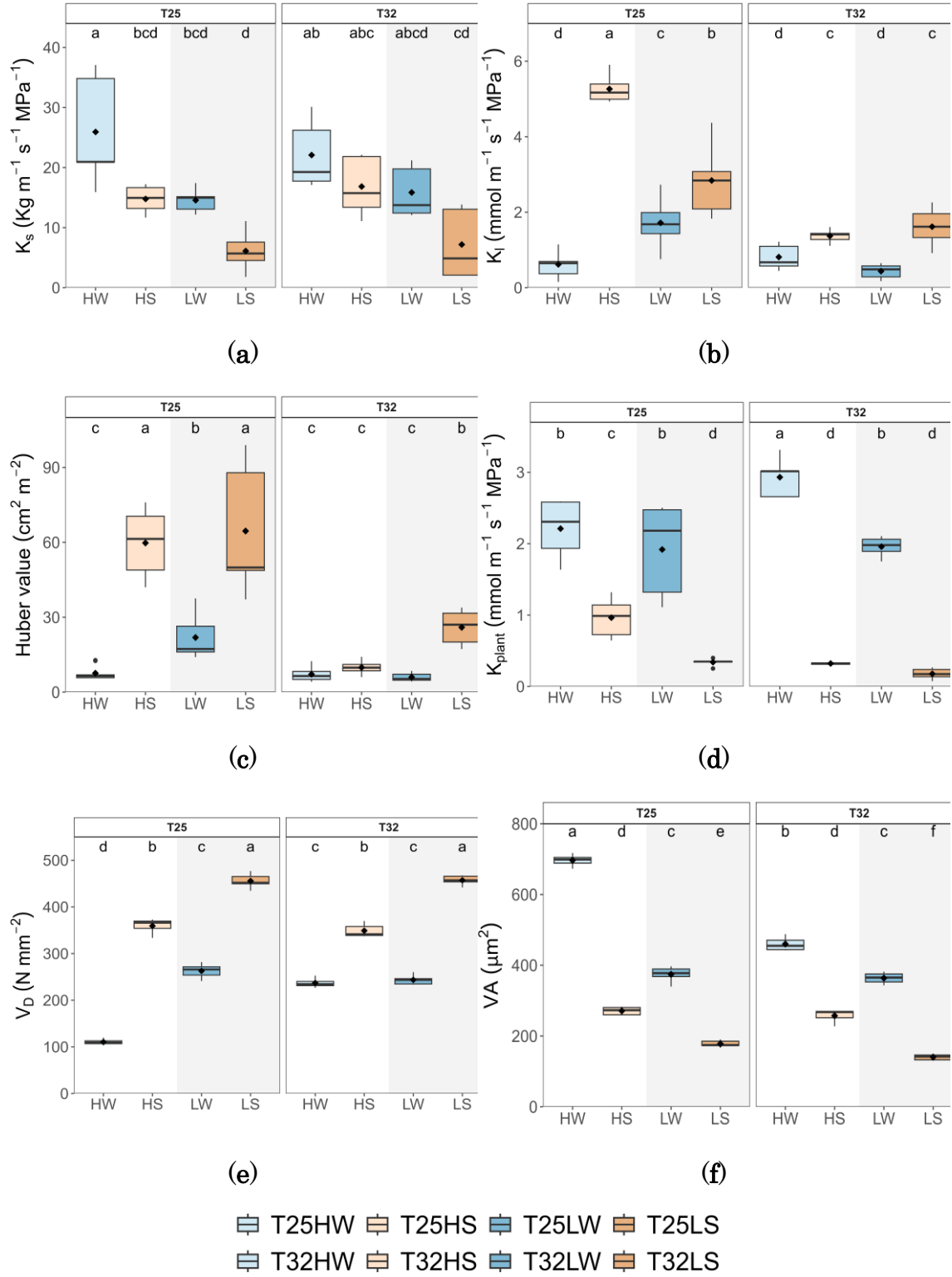


Figure 40. Boxplots of hydraulic traits and stem anatomy of beech seedlings grown in a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L): (a) stem specific hydraulic conductivity (K_s); (b) leaf-specific hydraulic conductivity (K_{leaf}); (c) Huber value (H_v); (d) plant hydraulic conductance (k_{plant}); (e) vessel density (V_D); and (f) average vessel area (V_A). The straight horizontal black line within each boxplot indicates the median, while the mean value is represented

as a solid black point. Different letters indicate significant differences among treatments.

Stem xylem anatomy was remarkably affected by water stress, which induced an increase in vessel density (V_D) but lowered vessel area (V_A) and hydraulic diameter (D_h). High light favored wider vessels (higher V_A) and lower vessel density (V_D), which were positively correlated with total leaf area (TLA) across treatments (Figure 51). Importantly, warming constrained this anatomical plasticity by limiting the increase in V_A typically observed under high light. No significant differences between treatments were observed in the xylem area occupied by radial parenchyma.

4.3.3.5. Plasticity in Response to Temperature, Light, and Water Availability

Warming (T32) consistently reduced the plasticity of many traits (quantified by the phenotypic plasticity index, PPi), especially those related to hydraulics, growth, and leaf water relations, indicating a dampened capacity to adjust to environmental variation under elevated temperature (Figure 41). Under well-watered conditions, PPi in response to light was higher at T25 for most traits related to growth, gas exchange, and biomass allocation. Under water stress, T32 seedlings exhibited reduced plasticity in leaf water relations and hydraulics, especially under high light, suggesting that warming constrains the range of physiological responses available to cope with drought (Figure 41). Under shade, warming enhanced the plasticity of some traits, particularly those related to gas exchange. This suggests a strategy shift under low light, where T32 plants adopt a more acquisitive phenotype (e.g., a higher number of cheaper leaves with lower LMA) to offset reduced carbon gain. However, this came at the expense of hydraulic safety, as shown by reductions in K_L , H_v , and SM_{leaf} , emphasizing the trade-offs induced by warming. The overall PPi for all treatments and variables is presented in Figure 50.

The degree of plasticity differed among trait categories. Growth-related traits and biomass partitioning were among the most plastic across environments. (Figure 42). In contrast, wood density (ρ) showed little plasticity, although it was significant for many treatments, including an increase due to water deficit or warming (Figure 42). Stomatal morphology showed limited plasticity, and S_D was more plastic than L_s . Among the most temperature-sensitive traits were g_{min} , C_{Pi0} , H_v , V_D , K_L , k_{plant} , number of leaves, and root biomass (Figure 42).

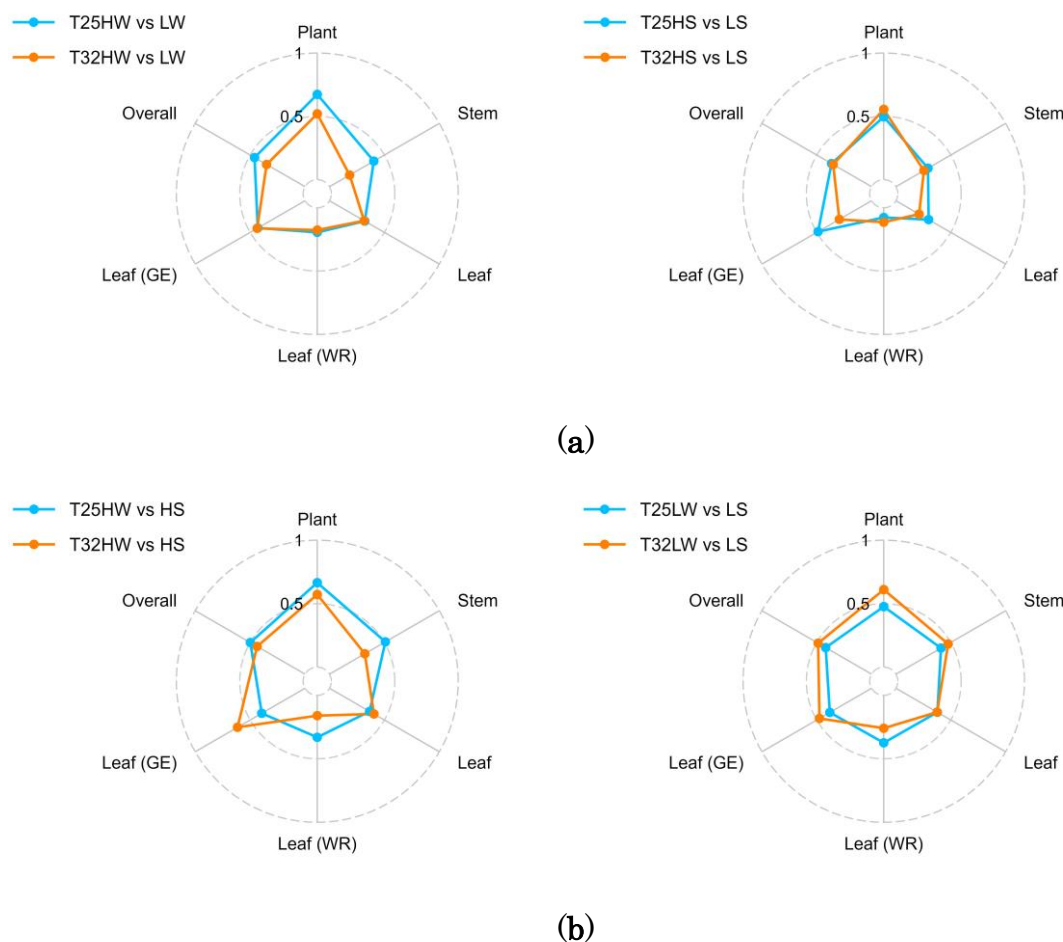


Figure 41. Radar plots of temperature influence over the Phenotypic Plasticity Index (PPI) in response to different stressors: (a) light intensity and (b) water availability. Traits were grouped into different organizational levels according to Table A1 into Plant, Stem, Leaf (including both Water relations + Gas exchange), Leaf (WR) (leaf water relations only), Leaf (GE) (leaf gas exchange only), and overall. Treatments are as follows: growth temperatures (T25, T32); HW (high light intensity and well-watered), HS (high light intensity and water stress), LW (shade and well-watered), LS (shade and water stress).

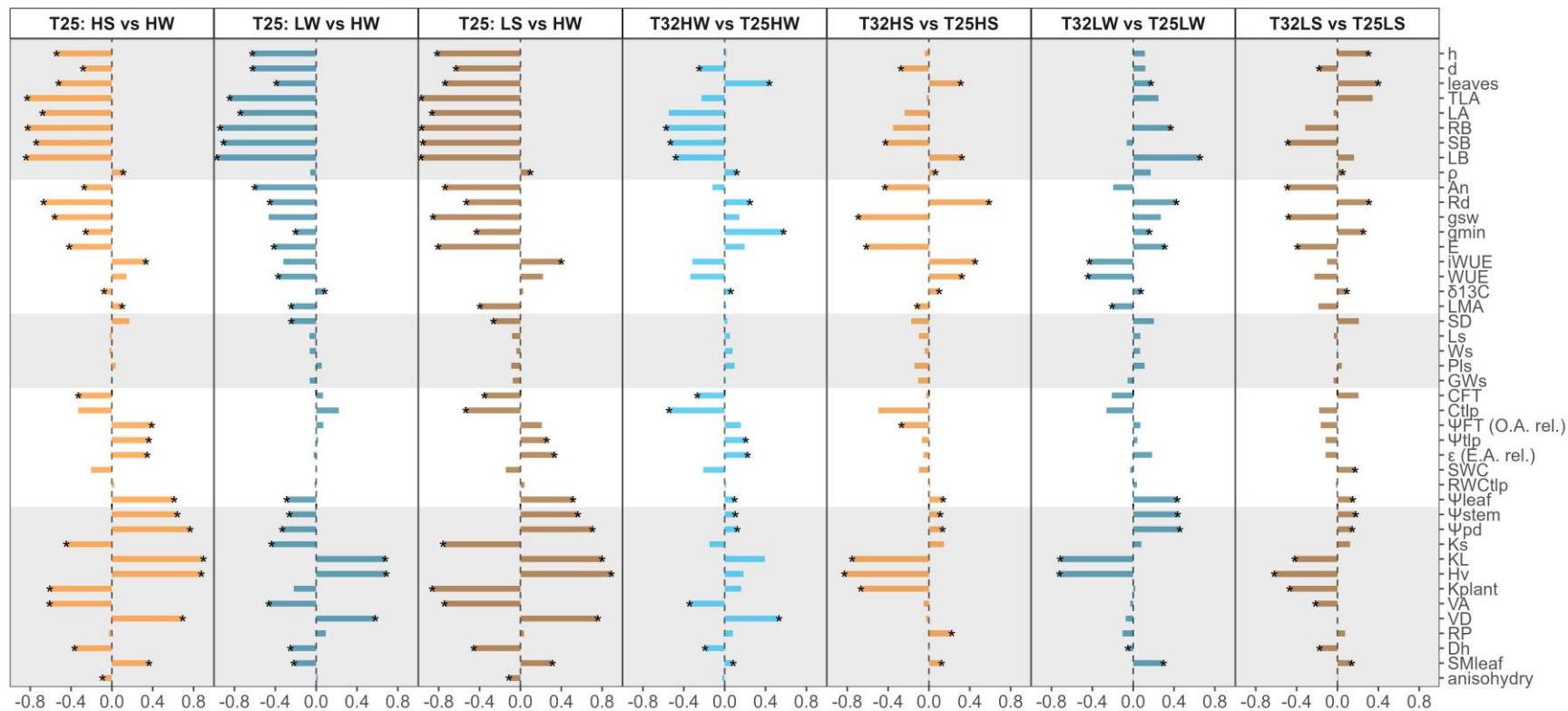


Figure 42. Plasticity score of each trait to each growing factor. From left to right, the first three columns analyze the effect of water deficit at high light (HS vs. HW), the effect of light intensity under well-watered conditions (LW vs. HW), and the combined effect of water deficit and shade (LS vs. HW) at T25. The next columns reflect the effect of growing temperature under well-watered conditions and high light intensity (T32HW vs. T25HW), the effect of growing temperature under water deficit conditions and high light intensity (T32HS vs. T25HS), the effect of growing temperature under well-watered conditions and shade (T32LW vs. T25LW), and finally, the effect of growing temperature under water deficit and shade (T32LS vs. T25LS). A negative plasticity score (NP, ranging from -1 to 0 , see Materials and Methods for equation) was used when the first treatment (the one on the left side of the comparison) had lower values than the reference treatment (the one on the right side) and is referred to mean values of the reference treatment. By contrast, the positive plasticity score

(PP ranging from 0 to 1) uses the difference between the treatment and the reference mean but is divided by the treatment mean. Asterisks indicate significant differences at $p < 0.05$ by t -test between each growing condition. Note that water potentials were considered positive here, hence positive plasticity (PP) means lower water potential and also lower SM_{leaf} .

4.3.3.6. Correlations Among Traits and Principal Components Analysis (PCA)

Warming substantially altered the coordination among functional traits in *Fagus sylvatica* seedlings. Some traits showed strong positive correlations at both temperatures: plant growth traits (height and diameter) with A_n , K_s , and V_A (Figure 43). At T25 we found significant positive correlations between plant height and ρ and trade-offs between height and H_v , ρ and H_v , and ρ and $iWUE$. In contrast, at T32, many of these correlations weakened or changed direction, suggesting a restructuring of trait integration in response to warming (Figure 43). For example, at T32, plant diameter was significantly correlated with LMA and k_{plant} . At T25, high $iWUE$ negatively correlated with g_{sw} , ρ , leaf C_{H100} , k_{plant} , leaf water potentials, and V_D , and positively correlated with $\delta^{13}C$, ϵ_{leaf} , RWC_{H0} , and H_v . However, at T32, $iWUE$ became decoupled from most hydraulic traits and only retained weak negative correlations with g_{sw} and leaf water potentials. This indicates that warming disrupted the typical trade-offs and synergies that underlie water-use strategies. Similarly, stomatal length (L_s) showed significant correlations with multiple traits at T32, including number of leaves, g_{sw} , g_{min} , and SM_{leaf} , relationships that were absent or weaker at T25 (Figure 43). This suggests that under warming, stomatal traits may gain importance as integrative regulators of leaf-level water dynamics and thermal regulation.

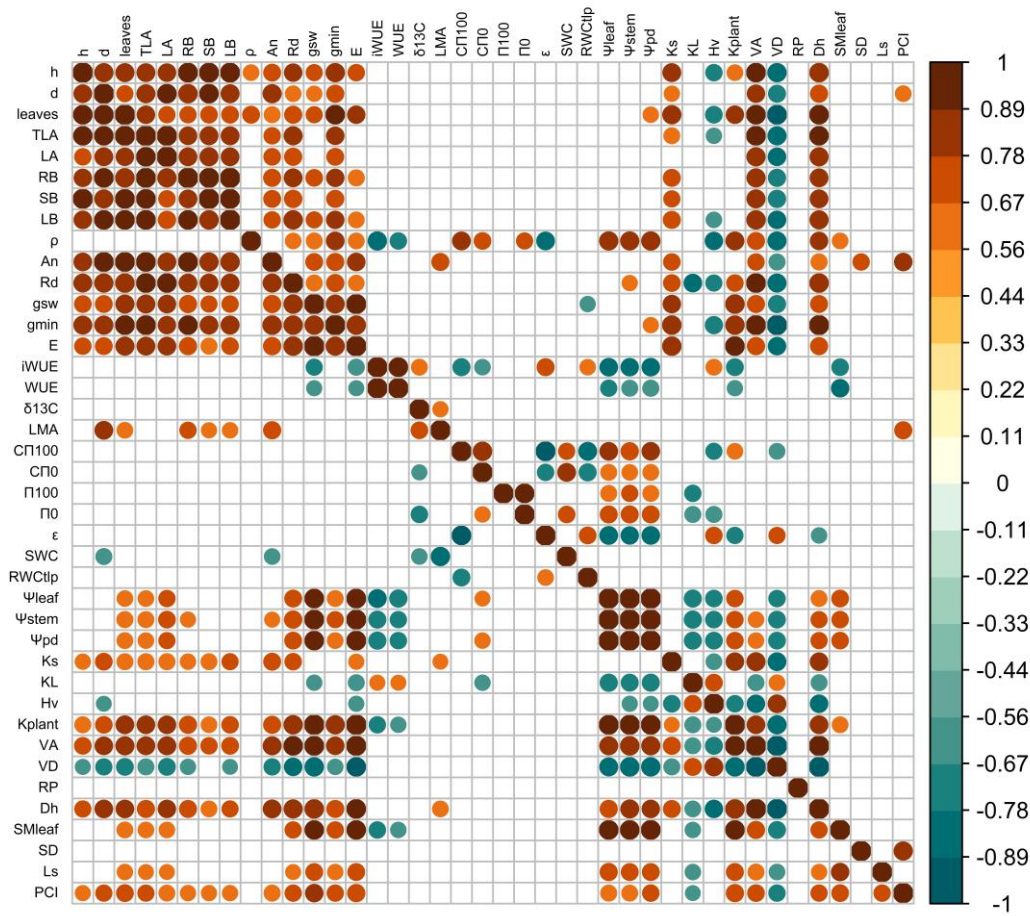


Figure 43. Pearson's correlation matrix for traits in plants growing at T25 (upper triangle) and at T32 (lower triangle). Significant correlations are color-coded, with positive correlations in warm colors and negative correlations in cool colors. See Table A1 for abbreviations.

Principal Component Analysis (PCA) further clarified how temperature modulated the main axes of phenotypic variation (Figure 44). The first component (PC1, 45% of variance) was associated with water availability, separating well-watered and water-stressed plants. Traits with high positive loadings on PC1 included k_{plant} , V_A , g_{sw} , g_{min} , Ψ_{leaf} , and TLA , reflecting efficient water transport and growth. Negative scores were associated with traits favored under drought, such as $iWUE$, V_D , K_L , H_v , and ε_{max} . The second component (PC2, 19% of variance) reflected light intensity effects. Traits with positive loadings include LMA , $\delta^{13}C$, A_n , and S_D , while negative loadings include π_0 and $C_{\pi 100}$. Importantly, warming shifted the position of treatments in PCA space, particularly under water stress. For example, T32 HS plants were further displaced from the HW quadrant, driven by reduced k_{plant} , Ψ_{leaf} , and A_n (Figure 44).

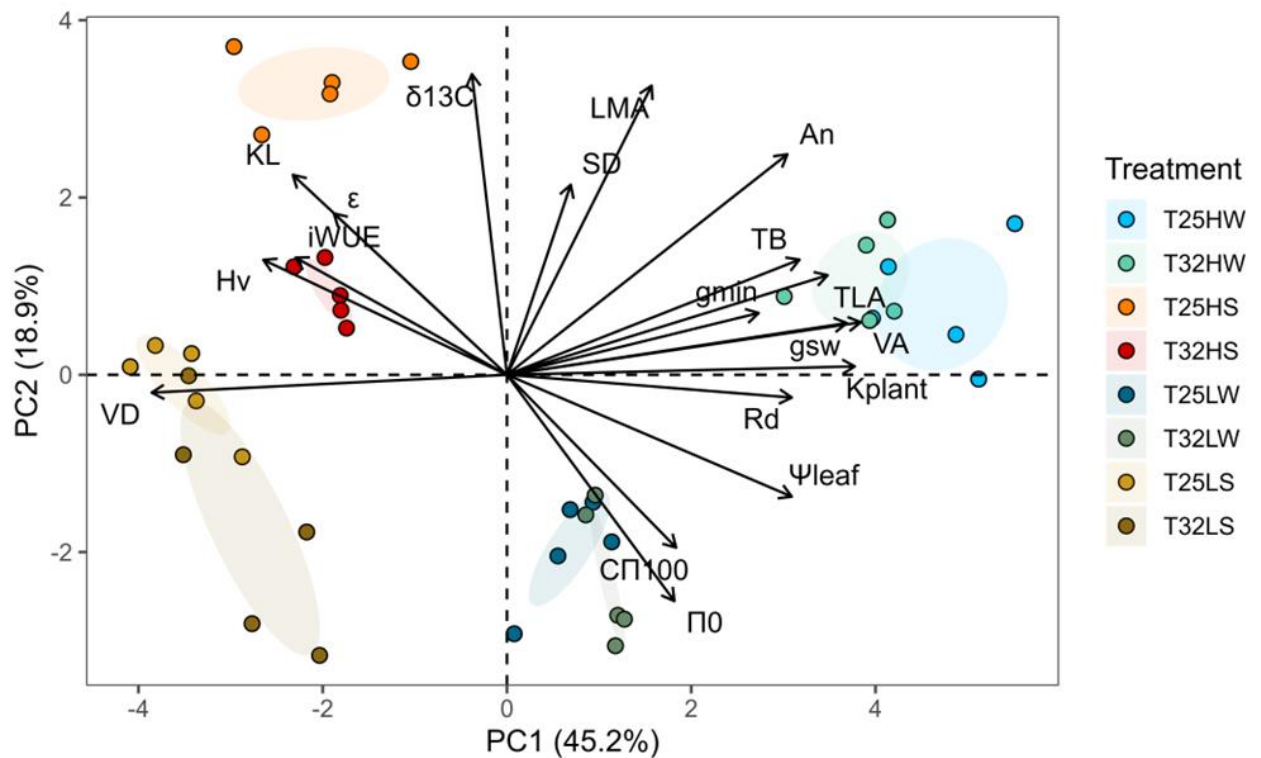


Figure 44. Principal Component Analysis (PCA) for the evaluated traits. Arrows represent the eigenvectors of each variable for the first two principal components (PC1 and PC2). The direction and length of the arrows indicate the contribution and importance of each variable to the principal components. Each plant is represented by a different color depending on the treatment. The oval areas represent the centroids of each treatment $\pm 75\%$ of the variation within the treatment.

4.3.4. Discussion

Temperature is one of the primary drivers of plant function and development. However, compared to other environmental stressors such as drought or shade, the direct effects of elevated temperature on tree physiology and performance remain relatively understudied. The present study is among the first to isolate the direct effect of growth temperature on key morphological, physiological, and anatomical traits in forest tree seedlings while controlling for vapor pressure deficit (VPD). Furthermore, we address a gap of knowledge by examining how warming interacts with concurrent stressors such as water deficit and shade in tree species, within the framework of “multifactorial stress combination”, a concept recently explored mainly in model plant species (Zandalinas et al., 2024).

Our findings show that warming exerted a direct and predominantly negative effect on several functional traits related to plant growth and physiology

(Figure 42), reduced phenotypic plasticity, especially in response to drought, and impaired certain acclimation responses observed at temperatures close to the optimum (T25) that were absent at supraoptimal temperatures (T32). Nevertheless, *Fagus sylvatica* seedlings showed some adaptive strategies that may facilitate acclimation to warming. In the following sections, we discussed the extent of plant acclimation to warming and how warming may have affected the shade and drought tolerance of this late-successional species.

4.3.4.1. Impact of Warming on Plant Morphology, Physiology, and Anatomy at High and Low Irradiance

Warming consistently reduced plant biomass and stem diameter (Figure 37), whereas height was less affected or even enhanced under shade, consistent with previous studies of trees growing near their warmer range limit (Reich et al., 2015; Wertin et al., 2011). Wood density (ρ) increased at T32, likely reflecting an investment in mechanical support and xylem safety (Van Gelder et al., 2006). Anatomically, warming increased V_D and decreased V_A at high irradiance (Figure 40), supporting the formation of denser wood with narrower conduits. These anatomical changes can hamper hydraulic conductivity and efficiency of water transport but limit the spread of xylem embolism. These anatomical changes suggest a trade-off between hydraulic efficiency and safety, consistent with previous studies in warming-exposed hardwoods (Hacke et al., 2001; Sperry, 2003). Importantly, K_s was not significantly altered by warming alone (Figure 40), indicating that the physical properties of sap (e.g., reduced viscosity) may partially buffer xylem conductivity under higher temperatures (Cochard et al., 2000), as observed in trembling aspen (Way et al., 2013). Our results highlight the acclimation capacity of beech seedling xylem to warming, thereby supporting our third hypothesis. This contrasts with the limited xylem adjustment of gymnosperms exposed to prolonged experimental warming (Grossiord et al., 2017).

At high irradiance, warming (T32 HW) decreased total leaf area (TLA), leaf mass per area (LMA), and leaf size (LA), despite an increased number of leaves (Figure 37). These morphological adjustments likely facilitate thermal regulation by reducing the boundary layer thickness and promoting evaporative cooling (Al-Salman et al., 2023; Gates, 1968). The same pattern was observed in warm-adapted *Populus* trees, which down-regulated leaf

temperature by producing smaller and thinner leaves with higher stomatal density (Blasini et al., 2022). Hence, warming promoted a conservative production of transpiring tissue under high irradiance, which correlated with a concomitant decrease in V_A , indicating a close correlation between plant transpiration rate and water supply (Mencuccini et al., 2019). The reduction in the net assimilation rate (A_n) by 15% under warming, coupled with a 25% increase in respiration (R_d), confirms the cost of elevated temperature on carbon balance and supports our first hypothesis (Figures 2 and A2). The temperature optimum for A_n in beech was estimated at 24.5 °C (Holišová et al., 2013); hence the slight reduction of A_n at 32 °C could be attributed to thermal acclimation, possibly by raising the A_n optimum temperature at T32 (Kumarathunge et al., 2019; Way & Oren, 2010). In any case, the depletion of A_n might be associated with metabolic constraints, particularly the downregulation of leaf photochemistry, which frequently occurs under elevated temperatures (Ameye et al., 2012), rather than diffusional limitations to CO_2 . Increased stomatal conductance (g_{sw}) and transpiration (E) at T32, even under constant VPD, demonstrate a shift toward evaporative cooling mechanisms to mitigate leaf overheating (Mills et al., 2024). This rise in g_{sw} and E correlated also with higher k_{plant} , suggesting strong coordination between water supply and demand at the plant level. Higher temperatures also promoted faster water use and higher leaf minimum conductance (g_{min}), which eventually led to faster depletion of soil water for warmed plants (Duan et al., 2015). Thus, reduction in TLA at T32 HW was important to avoid lowering Ψ_{leaf} , beyond the leaf safety margin (SM_{leaf}) since π_0 did not change with temperature. Additionally, the reduced LMA observed in warmed plants limited their leaf capacitance before or after turgor loss (Figure 42), partially aligning with previous findings (Way et al., 2013).

Growing under shaded conditions, when water was not limited (LW), mitigated the negative effects of warming described above and stimulated plant growth and biomass. Indeed, T32 LW seedlings produced greater biomass, higher wood density, more leaves, and showed increased allocation to leaf tissue with lower LMA (Figures 1 and A3), suggesting an acquisitive strategy adapted to low light and thermal stress, aimed to compensate for the reduced A_n but higher R_d . These plants prioritized maximizing TLA and transpiration, including g_{min} , to maintain CO_2 diffusion, increase C_i , and reduce leaf temperature (Figures 2 and 4), in a way to minimize photorespiration losses (Van Gelder et al., 2006), at the cost of reduced

hydraulic safety. Indeed, K_L , H_V , and SM_{leaf} were significantly lower, indicating increased vulnerability to water stress. Regarding changes in g_{min} , at moderately high temperatures ($<40^\circ\text{C}$), the stomatal component of g_{min} is of greater importance than at higher temperatures and VPD, where the cuticle component governs g_{min} (Garen & Michaletz, 2025). Accordingly, we found strong correlations between g_{min} and stomatal traits, especially PCI , which suggested a lack of complete closure of the stomata as the main cause explaining the rise in g_{min} at T32. On the other hand, T25 LW followed a more conservative strategy, with lower TLA and gas exchange, but higher Ψ_{leaf} and tougher leaves (high $LDMC$ and LMA). This fast-growing strategy of T32 LW could be risky if water is limited, as it features a higher leaf area but reduced leaf capacitance and Ψ_{leaf} , which lowers SM_{leaf} (Figure 3). This makes beech-shaded phenotypes more vulnerable to water stress under warming (Cochard et al., 1999).

4.3.4.2. Impact of Warming and Water Deficit at High and Low Light Intensity

The interaction between elevated temperature and water deficit imposed significant constraints on the hydraulic functioning and carbon economy of *Fagus sylvatica* seedlings. Seedlings grown under T32 with limited water availability (HS and LS treatments) showed pronounced reductions in A_n , LA , TLA , LMA , and g_{sw} , while simultaneously exhibiting increased respiration and reduced osmotic adjustment capacity (Figure 42).

As water availability diminishes, elevated growth temperatures lead to a steeper reduction in A_n , primarily influenced by increased stomatal closure to avoid hydraulic dysfunction but at the cost of decreased carbon uptake, increased hydraulic resistance, and growth (Cano et al., 2014) (Figures 2 and 4). Reduced A_n in water-stress T32 plants may also arise from lowered synthesis of protective enzymes, antioxidants, and heat dissipation proteins, essential for mitigating the higher thermal and oxidative stress produced by limited leaf cooling capacity (Watson-Lazowski et al., 2024). Furthermore, water-stressed plants showed lower K_s but a more redundant xylem, consistent with a strategy aimed at damage mitigation rather than efficiency (Sperry, 2003). The start of embolism formation in *Fagus sylvatica* was estimated at Ψ_{stem} below -1.8 MPa (Aranda et al., 2015; Hacke & Sauter, 1995), although beech seedlings from the same population and acclimated to water deficit reached this point at much lower levels (-2.4 MPa), suggesting

a very narrow xylem safety margin to preserve xylem functioning under drought (Aranda et al., 2015). Lower Ψ_{stem} and Ψ_{leaf} in T32 water-stressed plants may be the consequence not only of higher biomass allocation to leaves and decreased water viscosity (Aranda et al., 2015) but also of a lack of leaf osmotic and elastic adjustments. The limited osmotic adjustment, together with the lower Ψ_{leaf} , decreased SM_{leaf} to almost 0.2 MPa. A lack of thermal acclimation was also observed for leaf capacitance and ϵ_{max} , decreasing further the plant's ability to tolerate fluctuations in Ψ_{leaf} and maintain turgor pressure (Figure 3) (Aranda et al., 2015). Interestingly, shade intensified the negative effects of combined warming and water stress. T32 LS plants exhibited the lowest values of K_L , H_V , k_{plant} , and SM_{leaf} , indicating an impaired ability to supply water to leaves and protect against desiccation. Although shaded seedlings generally displayed an acquisitive strategy under warming when water was available, this response became maladaptive under concurrent drought, leading to decreased performance and potential mortality risk (Rodríguez-Calcerrada et al., 2009).

In contrast, T25 plants under drought showed more effective acclimation strategies. They were able to double A_n of T32 HS, achieve more than 0.8 MPa of active osmotic adjustment, lowering $\Psi_{\pi 0}$ below 3 MPa (Figures 2 and 3; Table A3), and increasing ϵ_{max} . T25 HS also showed higher leaf shedding, which increased H_V and the supply of water to the remaining leaves (higher K_L), lowering vessel area and producing more redundant xylem (Figure 3; (Aranda et al., 1996)), overall improving the whole-plant water balance (Munné-Bosch & Alegre, 2004) and sustaining higher leaf gas exchange rates (Figure 38) (Fanourakis et al., 2013; Schönbeck et al., 2022). Both temperature treatments decreased g_{min} in response to water deficit, which may offer significant advantages for the conservation of xylem function, particularly during warmer and drier (Fanourakis et al., 2013; Schönbeck et al., 2022).

Collectively, these findings reveal that warming exacerbates the negative effects of drought and that while moderate drought or warming alone can be tolerated through structural or physiological adjustment, their combination imposes compounded stress that exceeds the plastic limits of key traits. The results underscore the importance of trait coordination in stress resilience and suggest that *Fagus sylvatica* seedlings may face severe regeneration challenges under future climate scenarios characterized by higher

temperatures, reduced soil moisture, and light limitation beneath forest canopies.

4.3.4.3. Phenotypic Plasticity and Regeneration Niche of Beech Seedlings Under Multi-Stress Factors

A central finding of this study is that elevated temperature reduced the phenotypic plasticity of key functional traits in *Fagus sylvatica*, particularly under stressful combinations of drought and shade. These results are in agreement with our fourth hypothesis related to the cost associated with maintaining plasticity in resource-limited environments (Van Buskirk & Steiner, 2009). The calculated plasticity index (PPI) showed clear reductions at T32 for traits involved in hydraulics, leaf water relations, and growth, indicating a narrowing of the seedlings' capacity to respond to environmental heterogeneity (Figures 5 and 6). These reductions were especially pronounced under drought, suggesting that warming compromises acclimation to water limitation. Phenotypic plasticity allows plants to modify their phenotype to match their current environment. However, there are limits and costs associated with plasticity. We show that plasticity was trait- and context-dependent probably due to the complex interaction among traits. Traits related to whole-plant biomass showed greater plasticity than those measured at the organ or tissue levels (Figure 41). While leaf gas exchange retained moderate plasticity under warming, hydraulic and anatomical traits were less flexible. Wood density (ρ), xylem anatomy (V_A , V_D), and stomatal morphology exhibited minimal plastic responses, implying structural constraints (Figures 6 and A1) and indicating higher costs (Schlichting & Pigliucci, 1998). Interestingly, warming in the absence of water deficit enhanced plasticity at low light intensity (Figure 42), enabling a compensatory increase in leaf number and area and revealing the strong adaptation of this shade-tolerant species to grow in the humid forest understory (Fotelli et al., 2005; Robson et al., 2009). However, this strategy was not viable under drought, revealing a trade-off between plasticity for carbon acquisition and vulnerability to hydraulic failure. Extensive phenotypic plasticity in key functional traits is often considered favorable for the persistence of populations under rapid climate change (Valladares et al., 2014). Nevertheless, our results emphasize that plasticity itself is temperature-sensitive and that warming may push plants beyond the threshold where plastic responses remain beneficial. The reorganization of

trait correlations under warming (as revealed by PCA and correlation analysis) further suggests that trait integration shifts under stress, potentially limiting coordinated responses.

From an ecological perspective, these findings raise concerns about the capacity of *Fagus sylvatica* to regenerate in future forests, particularly near its southern distribution limit.

4.3.5. Conclusions

Our findings underscore the significant impact of elevated temperature on plant growth, biomass partitioning, and physiology of European beech seedlings. Specifically, we observed that warming decreased overall plant plasticity, reduced leaf size, allocated higher biomass to leaves, reduced leaf shedding, and increased metabolic and water demands, suggesting that global warming could constrain the adaptive capacity of beech regeneration, especially near the drier margins of its distribution. Warming reduced biomass due to a lower total leaf area and net photosynthesis but increased respiratory costs, diminished the potential for beech seedlings to perform the osmotic adjustment, and decreased the leaf safety margin, which may compromise the future survival of the species under dry conditions. Nevertheless, beech growth under shade might increase due to more and less costly leaves (lower *LMA*), which enhance transpiration rate and help cool down the leaf if warming occurs with deep soil and wet atmospheric conditions. This highlights the importance of understanding species-specific responses to multifactorial stress factors, which is essential for projecting the long-term persistence and ecological success of forest species under future warming scenarios.

4.3.6. Supplementary

Table 7. List of studied traits, with abbreviations and units and the classification to calculate the Phenotypic Plasticity index (PPi).

Symbol	Level for PPi	Definition	Values Units
h	Plant	Plant height	(cm)
d	Plant	Plant diameter	(mm)
leaves	Plant	Number of fully developed leaves	(n°)
TLA	Plant	Total plant leaf area	(cm ²)
LA	Leaf GE relations	Average leaf area	(cm ²)
RB	Plant	Root biomass	(g)
SB	Plant	Shoot biomass	(g)
LB	Plant	Leaf biomass	(g)
ρ	Plant	Wood density	(g cm ⁻³)
A_n	Leaf GE relations	Leaf net assimilation rate	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
R_d	Leaf GE relations	Leaf respiration	($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
g_{sw}	Leaf GE relations	Leaf stomatal conductance to water vapor	($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)
g_{min}	Leaf GE relations	Leaf minimum conductance to water vapor	($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)
E	Leaf GE relations	Leaf transpiration	($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)
iWUE	Not used for PPi	Intrinsic water use efficiency	($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$)
WUE	Not used for PPi	Water use efficiency	($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$)
$\delta^{13}\text{C}$	Not used for PPi	Carbon isotope composition	(‰)
LMA	Leaf GE relations	Leaf mass per area	(g m ⁻²)
S_D	Stomatal anatomy	Stomatal density	(n° stomata per mm ²)
L_s	Stomatal anatomy	Stomatal length	(μm)
PCI	Stomatal anatomy	Potential conductance index	($\mu\text{m}^2 \text{ mm}^{-2} 10^{-4}$)
W_s	Stomatal anatomy	Stomatal complex width	(μm)
Pl_s	Stomatal anatomy	Stomatal pore length	(μm)
GW_s	Stomatal anatomy	Guard cell width	(μm)
C_{1100}	Leaf water relations	Leaf capacitance at full turgor	(MPa ⁻¹)
C_{110}	Leaf water relations	Leaf capacitance at turgor loss point	(MPa ⁻¹)
Π_{100}	Leaf water relations	Leaf osmotic potential at full turgor	(MPa)
Π_0	Leaf water relations	Leaf turgor loss point	(MPa)
ε_{leaf}	Leaf water relations	Leaf maximum Young's modulus of elasticity	(MPa)
RWC_{110}	Leaf water relations	Relative water content at the turgor loss point	(%)

SWC	Leaf water relations	Leaf-saturated water content	(g g ⁻¹)
Ψ_{leaf}	Leaf water relations	Leaf midday water potential	(MPa)
Ψ_{stem}	Leaf water relations	Stem midday water potential	(MPa)
Ψ_{pd}	Not used for PPI	Predawn water potential	(MPa)
SM_{leaf}	Not used for PPI	Leaf safety margin	(MPa)
K_S	Stem	Hydraulic specific conductivity	(kg m ⁻¹ s ⁻¹ Mpa ⁻¹)
K_L	Stem	Leaf hydraulic conductivity	(mmol m ⁻¹ s ⁻¹ Mpa ⁻¹)
H_v	Plant	Huber value	(m ² m ⁻²)
k_{plant}	Plant	Plant hydraulic conductance	(mmol m ⁻² s ⁻¹ Mpa ⁻¹)
V_A	Stem	Average vessel lumen area	(μm^2)
V_D	Stem	Vessel density	(n ^o vessels per mm ²)
RP	Stem	Xylem area occupied by radial parenchyma	(%)
D_h	Not used for PPI	Hydraulic diameter	(μm)

Table 8. F-statistics from a full-factorial mixed effect ANOVA using growing temperature (Tgrowth), watering (Water) and light intensity (Light) as fixed effects with $n = 5$ replicates as random effect. Significant outcomes are indicated in bold with p -values < 0.05 indicated as *, $p < 0.01$ as ** and $p < 0.001$ as *** in the table. The factor or combination of growing factors with highest F score for each variable were marked in gray. For abbreviations check the Abbreviations and Acronyms section.

Variables	Tgrowth	Light	Water	Tgrowth × Light	Tgrowth × Water	Light × Water	Tgrowth × Light × Water
h	6.70 *	297.50 ***	193.51 ***	3.16	0.76	67.01 ***	<0.01
d	42.33 ***	520.49 ***	98.20 ***	42.15 ***	1.05	29.80 ***	6.72 *
leaves	100.86 ***	145.62 ***	188.08 ***	7.40 **	23.78 ***	34.68 ***	2.88
TLA	9.40 **	300.24 ***	276.75 ***	39.46 ***	45.09 ***	150.84 ***	60.06 ***
LA	14.39 ***	89.00 ***	74.64 ***	12.26 **	0.89	26.41 ***	1.16
RB (abs)	41.51 ***	240.44 ***	149.30 ***	45.23 ***	24.45 ***	109.47 ***	35.29 ***
SB (abs)	19.35 ***	145.18 ***	74.65 ***	15.00 ***	5.46 *	55.17 ***	6.45 *
LB (abs)	8.48 **	267.33 ***	122.04 ***	15.91 ***	18.65 ***	90.60 ***	27.50 ***
RB (rel)	0.30	0.71	9.25 **	4.69 *	5.96 *	0.02	5.29 *
SB (rel)	15.35 ***	4.59 *	1.84	3.93	0.03	<0.01	4.80 *
LB (rel)	25.41 ***	1.53	2.84	1.08	4.37 *	<0.01	1.17
A _n	18.99 ***	169.38 ***	52.01 ***	2.37	2.77	7.88 **	0.95
R _d	195.44 ***	66.12 ***	229.84 ***	3.53	0.19	67.24 ***	10.88 **
g _{sw}	0.01	26.59 ***	119.13 ***	1.21	9.21 **	4.94 *	0.70
g _{min}	652.60 ***	700.90 ***	1149.90 ***	232.70 ***	379.90 ***	470.70 ***	432.10 ***
E	0.15	50.60 ***	195.30 ***	2.74	26.13 ***	2.79	2.03
iWUE	1.02	108.38 ***	390.07 ***	77.93 ***	17.56 ***	18.80 ***	84.06 ***
WUE	7.26 *	59.41 ***	97.25 ***	38.82 ***	2.68	6.81 *	44.05 ***
LMA	264.22 ***	505.64 ***	166.07 ***	22.42 ***	111.74 ***	7.13 **	118.75 ***
δ ¹³ C	31.65 ***	50.34 ***	11.99 **	0.08	0.37	0.13	0.32
S _D	0.59	10.49 **	0.29	4.55 *	0.77	0.62	0.82
L _S	<0.01	2.98	11.35 **	0.67	6.55 *	0.33	0.22
PCI	1.00	27.23 ***	6.87 *	7.18 *	11.39 **	0.03	2.03
C _{Π0}	1.49	5.69 *	21.60 ***	1.00	5.47 *	3.01	0.22
C _{Π100}	33.79 ***	22.46 ***	86.54 ***	0.49	14.93 ***	22.86 ***	0.33
Π ₀	5.02 *	1.43	2.46	0.11	11.08 **	6.28 *	1.67
Π ₁₀₀	0.27	4.25 *	16.44 ***	1.40	16.27 ***	4.50 *	0.88
ε _{leaf}	0.28	0.04	9.40 **	0.11	7.08 *	4.19 *	0.66
Ψ _{leaf}	69.36 ***	29.73 ***	737.58 ***	6.85 *	0.01	<0.01	4.305 *
Ψ _{stem}	59.97 ***	15.49 ***	710.44 ***	12.86 **	0.07	0.35	0.75

Ψ_{pd}	46.04 ***	11.67 **	852.71 ***	1.87	0.03	3.55	4.87 *
SM _{leaf}	13.28 ***	3.69	107.36 ***	0.41	5.42 *	5.53 *	29.57 ***
K _s	0.14	32.26 ***	28.46 ***	0.38	0.81	0.01	0.93
K _l	90.41 ***	0.40	221.90 ***	0.85	74.61 ***	27.33 ***	54.54 ***
H _v	76.66 ***	15.01 ***	99.57 ***	1.45	55.79 ***	1.05	6.37 *
k _{plant}	0.39	23.98 ***	256.35 ***	0.01	7.94 **	2.54	3.85
V _D	48.24 ***	447.68 ***	2158.38 ***	55.35 ***	42.96 ***	5.88 *	87.85 ***
V _A	261.49 ***	893.15 ***	2680.29 ***	90.51 ***	90.14 ***	111.22 ***	148.31 ***
RP	2.17	0.05	0.93	3.67	3.00	0.06	<0.01
D _h	245.32 ***	1165.82 ***	3405.51 ***	11.11 **	52.28 ***	14.03 ***	190.31 ***

Table 9. Phenotypic plasticity index: (max mean–min mean)/max mean temperature response to light for each group of variables. Bold numbers represent significant differences between light treatments.

Group of Variables	Measured Variables	Well-Watered		Water Deficit	
		T25	T32	T25	T32
		Response to Light (HW vs. LW)	Response to Light (HW vs. LW)	Response to Light (HS vs. LS)	Response to Light (HS vs. LS)
Plant level	10	0.67	0.52	0.50	0.56
Stem	6	0.40	0.18	0.29	0.26
Leaf (PV + GE)	13	0.32	0.32	0.30	0.21
Leaf hydric relations	6	0.19	0.18	0.08	0.11
Leaf GE relations	7	0.43	0.44	0.49	0.29
Overall	29	0.46	0.35	0.37	0.35

Table 10. Phenotypic plasticity index: (max mean–min mean)/max mean temperature response to water for each group of variables. Bold numbers represent significant differences between watering treatments.

Group of Variables	Measured Variables	High Light		Low Light	
		T25	T32	T25	T32
		Response to Water (HW vs. HS)	Response to Water (HW vs. HS)	Response to Water (LW vs. LS)	Response to Water (LW vs. LS)
Full plant	10	0.67	0.57	Full plant	10
Stem	7	0.51	0.32	Stem	7
Leaf (PV + GE)	12	0.37	0.41	Leaf (PV + GE)	12
Leaf water relations	6	0.33	0.16	Leaf water relations	6
Leaf GE relations	6	0.39	0.61	Leaf GE relations	6
Overall	29	0.50	0.44	Overall	29

Table 11. Summary table of all the measured parameters, expressed as mean $\pm$ standard error. Sample size is referred as n. Variables are described in Table A1.

Growth Temperature		T25				T32			
Treatment		HW	HS	LW	LS	HW	HS	LW	LS
Sample Size (n)		6	6	6	6	6	6	6	6
h	(cm)	61.9 $\pm$ 2.3	28.4 $\pm$ 1.6	18.9 $\pm$ 1.2	11.3 $\pm$ 0.5	63.1 $\pm$ 3.9	27.2 $\pm$ 0.8	26.2 $\pm$ 2.3	16.3 $\pm$ 1.6
d	(mm)	12.6 $\pm$ 0.2	9.0 $\pm$ 0.3	4.7 $\pm$ 0.5	4.6 $\pm$ 0.2	9.4 $\pm$ 0.4	6.6 $\pm$ 0.1	5.4 $\pm$ 0.3	3.8 $\pm$ 0.2
leaves	(n°)	23 $\pm$ 2	11 $\pm$ 1	10 $\pm$ 2	3 $\pm$ 0	41 $\pm$ 3	16 $\pm$ 1	20 $\pm$ 1	10 $\pm$ 0
TLA	(cm ²)	1881.76 $\pm$ 113.43	214.56 $\pm$ 8.03	165.04 $\pm$ 54.89	31.34 $\pm$ 8.24	986.67 $\pm$ 68.17	328.22 $\pm$ 31.02	273.95 $\pm$ 26.37	86.74 $\pm$ 22.89
LA	(cm ²)	36.16 $\pm$ 2.02	17.88 $\pm$ 1.71	13.37 $\pm$ 1.48	9.62 $\pm$ 0.42	25.41 $\pm$ 2.98	11.51 $\pm$ 1.1	13.65 $\pm$ 1.37	9.28 $\pm$ 0.71
RB	(g)	30.9 $\pm$ 3.1	8 $\pm$ 1.3	2.9 $\pm$ 0.5	1.4 $\pm$ 0.1	14.5 $\pm$ 0.8	4.6 $\pm$ 0.3	2.7 $\pm$ 0.6	0.7 $\pm$ 0.1
SB	(g)	16.3 $\pm$ 0.8	2.6 $\pm$ 0.6	0.5 $\pm$ 0	0.4 $\pm$ 0.1	8.6 $\pm$ 0.7	3.9 $\pm$ 0.4	1.4 $\pm$ 0.2	0.5 $\pm$ 0.1
LB	(g)	49.7 $\pm$ 4.1	8.7 $\pm$ 1.2	3 $\pm$ 0.5	1.5 $\pm$ 0.1	21.2 $\pm$ 2.2	5.6 $\pm$ 0.3	4.7 $\pm$ 0.6	1 $\pm$ 0.2
TB	(g)	95.8 $\pm$ 6.5	19.3 $\pm$ 2.5	6.6 $\pm$ 1.1	4.1 $\pm$ 0.8	46.9 $\pm$ 2.6	14.8 $\pm$ 1	9 $\pm$ 1.3	2.5 $\pm$ 0.5
RB	(%)	32.4 $\pm$ 2.6	38.6 $\pm$ 1.9	43.2 $\pm$ 4	41.8 $\pm$ 1.6	32.7 $\pm$ 3	30.8 $\pm$ 0.8	28.7 $\pm$ 2	34.9 $\pm$ 2.2
SB	(%)	15.9 $\pm$ 1.1	13.1 $\pm$ 0.3	10.7 $\pm$ 2	11.7 $\pm$ 2.5	17.1 $\pm$ 1.1	24.7 $\pm$ 1.2	18.1 $\pm$ 0.9	22.6 $\pm$ 3.8
LB	(%)	50 $\pm$ 1.3	44.9 $\pm$ 1	43.8 $\pm$ 1.4	46.5 $\pm$ 1.3	45.3 $\pm$ 4.1	40.3 $\pm$ 2	55 $\pm$ 1	42.4 $\pm$ 2.7
K _s	(kg m ⁻¹ s ⁻¹ Mpa ⁻¹)	25.94 $\pm$ 4.21	14.78 $\pm$ 0.92	14.56 $\pm$ 0.91	6.11 $\pm$ 1.31	22.09 $\pm$ 2.58	16.85 $\pm$ 2.23	15.86 $\pm$ 1.93	7.19 $\pm$ 2.61
K _L	(mmol m ⁻¹ s ⁻¹ Mpa ⁻¹)	0.62 $\pm$ 0.11	5.26 $\pm$ 0.15	1.72 $\pm$ 0.32	2.84 $\pm$ 0.45	0.81 $\pm$ 0.12	1.37 $\pm$ 0.07	0.44 $\pm$ 0.08	1.62 $\pm$ 0.23
K _{plant}	(mmol m ⁻² s ⁻¹ Mpa ⁻¹)	2.46 $\pm$ 0.31	0.96 $\pm$ 0.13	1.92 $\pm$ 0.29	0.34 $\pm$ 0.02	2.93 $\pm$ 0.12	0.32 $\pm$ 0.01	1.96 $\pm$ 0.06	0.18 $\pm$ 0.03

H _v	(cm ² m ⁻²)	7.6 ± 1	59.8 ± 6.4	21.9 ± 3.8	64.5 ± 12.1	7.2 ± 0.8	9.9 ± 0.9	6 ± 0.7	26 ± 3.2
LMA	(g m ⁻²)	82.9 ± 1.4	90.2 ± 2.1	62.2 ± 1.4	49.6 ± 1.5	80.1 ± 1.9	43.9 ± 1.1	49.5 ± 0.7	40.5 ± 0.7
A _n	(μmol m ⁻² s ⁻¹)	8.4 ± 0.5	6.1 ± 0.7	3.4 ± 0.6	2.2 ± 0.2	7.4 ± 0.4	3.5 ± 0.1	2.7 ± 0.4	1.1 ± 0.4
R _l	(μmol m ⁻² s ⁻¹)	0.75 ± 0.02	0.25 ± 0.01	0.41 ± 0.02	0.35 ± 0.02	1.00 ± 0.04	0.61 ± 0.04	0.72 ± 0.03	0.51 ± 0.03
g _{sw}	(mmol m ⁻² s ⁻¹)	232.7 ± 46.7	101.9 ± 13	124.8 ± 24	33.2 ± 3	271.4 ± 14	31.3 ± 0.5	171 ± 6.8	17.2 ± 3.2
g _{min}	(mmol m ⁻² s ⁻¹)	2.36 ± 0.06	1.75 ± 0.05	1.88 ± 0.02	1.35 ± 0.01	5.57 ± 0.08	1.78 ± 0.1	2.24 ± 0.01	1.81 ± 0.05
E	(mol m ⁻² s ⁻¹)	3.2 ± 0.4	1.8 ± 0.2	1.9 ± 0.3	0.6 ± 0	3.9 ± 0.1	0.7 ± 0	2.7 ± 0.1	0.4 ± 0.1
iWUE	(μmol mol ⁻¹)	38.8 ± 5.3	63.1 ± 0.9	25.4 ± 0.4	73.2 ± 4.7	27.8 ± 2.6	110.4 ± 1.7	14.7 ± 1.9	36.5 ± 5
WUE	(μmol mol ⁻¹)	2.7 ± 0.2	3.3 ± 0.1	1.8 ± 0	4 ± 0.3	1.9 ± 0.1	5.3 ± 0.4	1.1 ± 0.2	1.5 ± 0.2
δ ¹³ C	(‰)	-29.8 ± 0.5	-27.6 ± 0.7	-32.3 ± 0.7	-30.9 ± 0.8	-31.5 ± 0.4	-30.4 ± 0.7	-34.8 ± 0.3	-33.5 ± 0.6

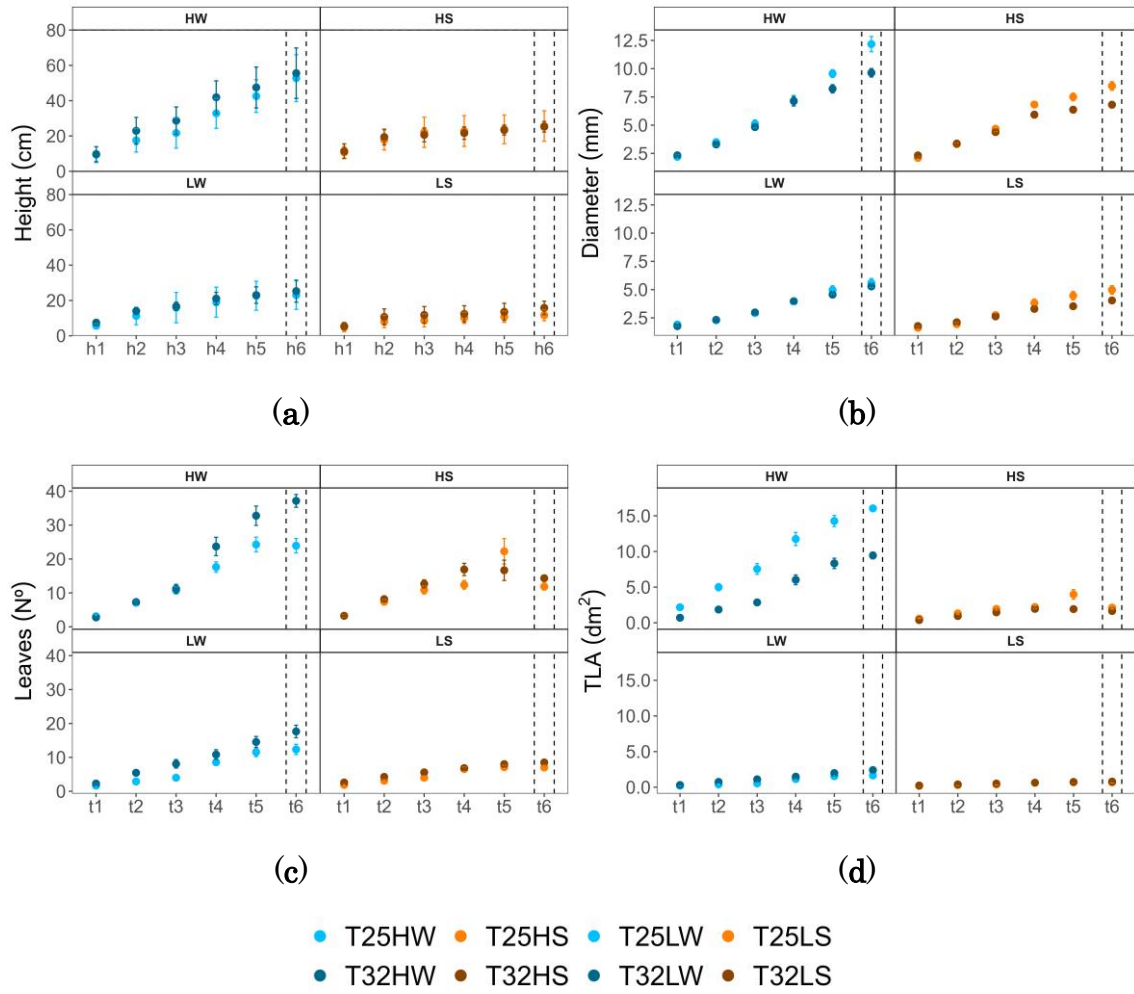
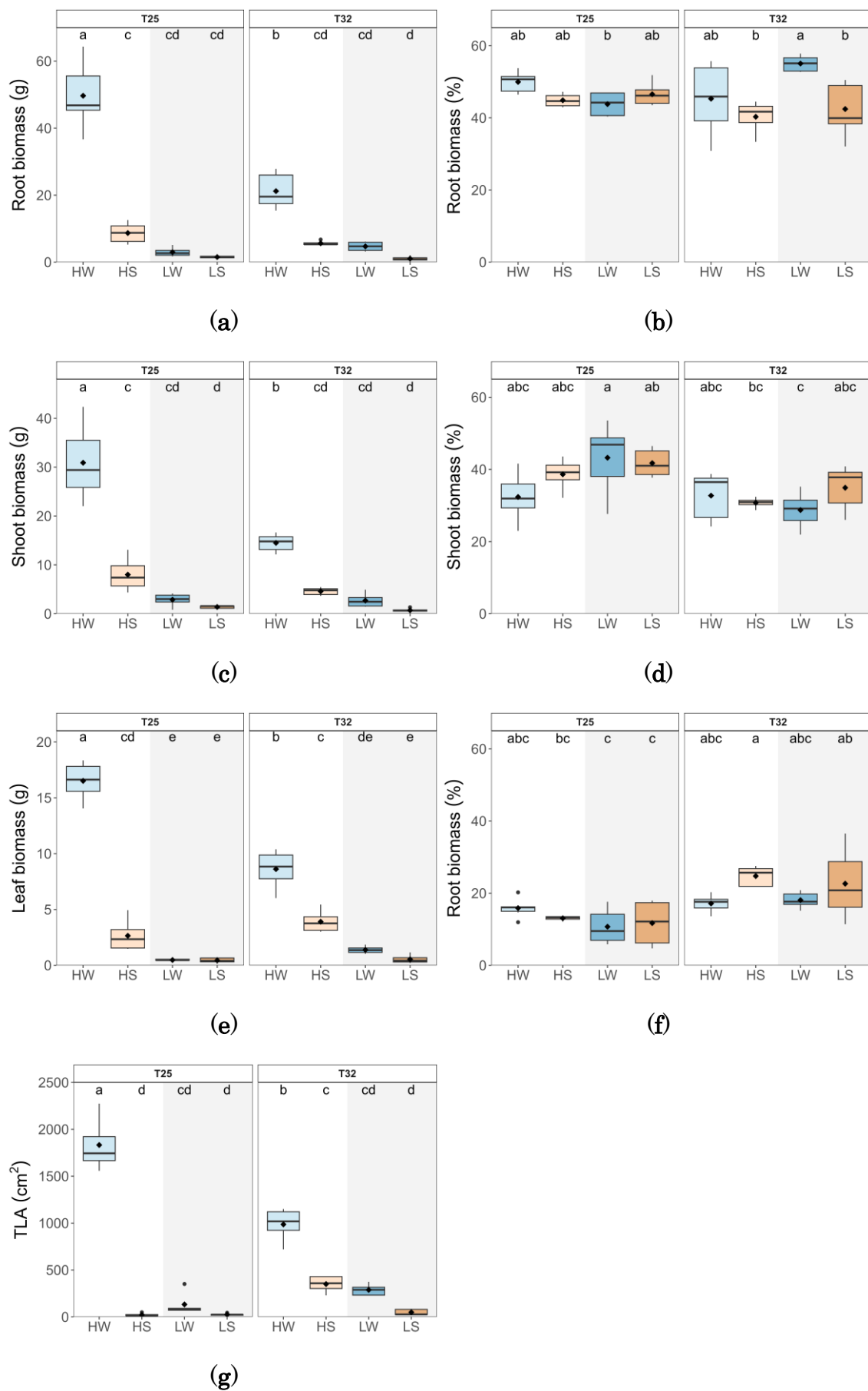


Figure 45. Temporal dynamics of plant morphological traits: (a) height (h, cm), (b) diameter (d, mm), (c) leaves number (Leaves, no.), and (d) Total leaf area (TLA, cm²) measured over six different assessments (h1–h6, d1–d6, L1–L6, and LA1–LA6, respectively) to different treatments: HW (high light well-watered), HS (high light water-stressed), LW (low light well-watered), LS (low light water-stressed) and growth temperatures (25 °C, T25, and 32 °C, T32). Data points represent means $\pm$ standard error (SE). Dashed vertical lines indicate the final measurement point, corresponding to the moment of sampling and measurement for the results discussed in this study.



 T25HW
  T25HS
  T25LW
  T25LS
 T32HW
  T32HS
  T32LW
  T32LS

Figure 46. Boxplots of biomass traits and of beech seedlings grown in a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L): (a) absolute root biomass (RB, g); (b) relative root biomass to total biomass (RB, %); (c) absolute shoot biomass (SB, g); (d) relative shoot biomass to total biomass (SB, %); (e) absolute leaves biomass (LB, g); (f) relative leaves biomass to total biomass (LB, %) and (g) total leaf area (TLA, cm²). The straight horizontal black line within each boxplot indicates the median, while the mean value is represented as a solid black point. Different letters indicate significant differences among treatments.

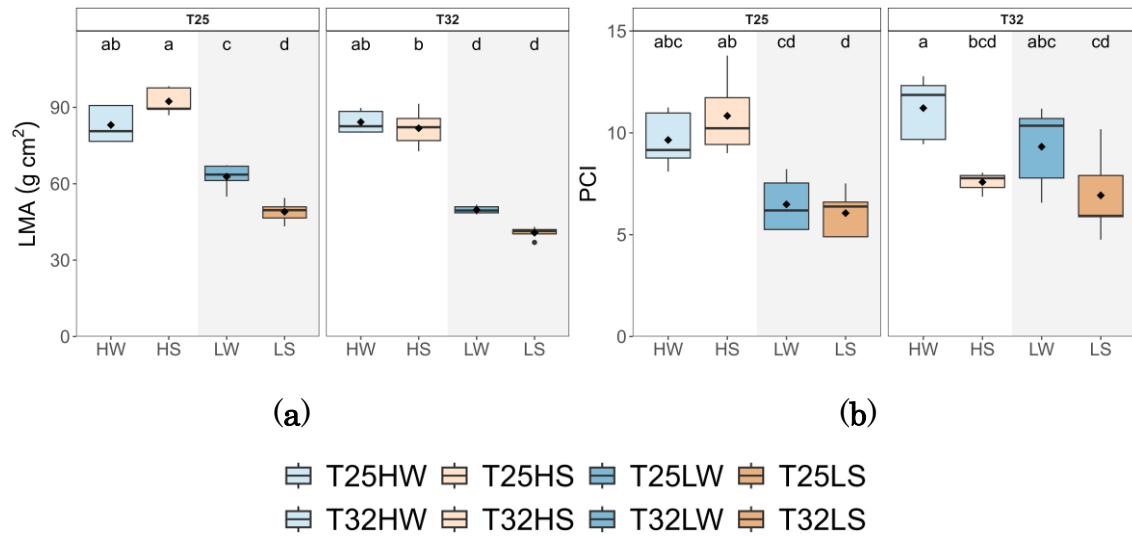


Figure 47. Boxplots of leaf morphological traits of beech seedlings grown in a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L): (a) leaf mass area (LMA, kg m⁻²); (b) potential conductance index (PCI). The straight horizontal black line within each boxplot indicates the median, while the mean value is represented as a solid black point. Different letters indicate significant differences among treatments.

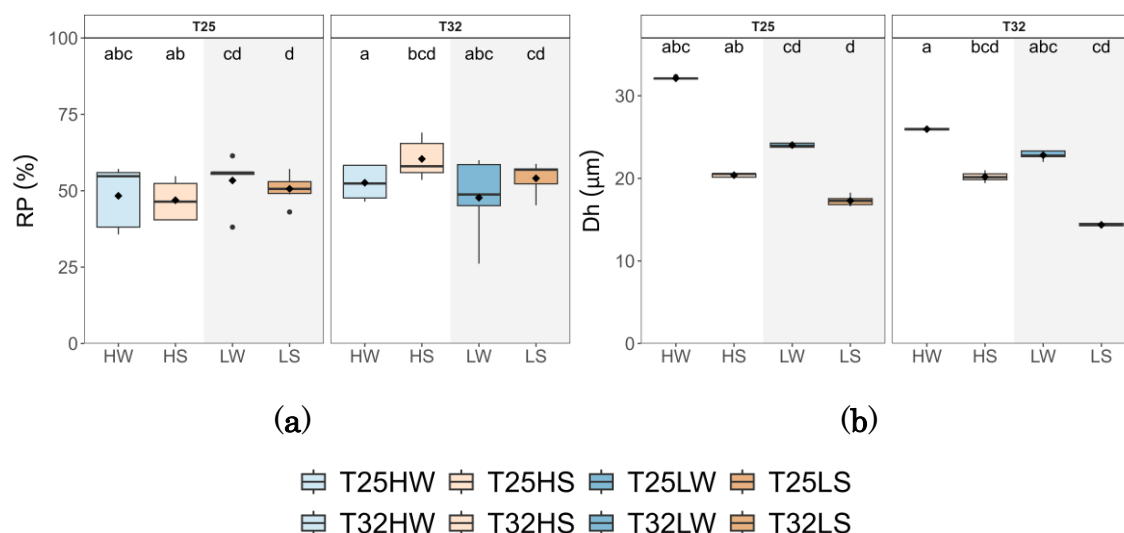


Figure 48. Boxplots of xylem morphological traits of beech seedlings grown in a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L): (a) Xylem area occupied by radial parenchyma (Radial parenchyma, %); (b) hydraulic diameter (Dh, μm). The straight horizontal black line within each boxplot indicates the median, while the mean value is represented as a solid black point. Different letters indicate significant differences among treatments.



Figure 49. Relative response, or plasticity score, of each trait to the effect of each growing factor according to the Phenotypic Plasticity Index (PPI) as described by

(Valladares & Sánchez-Gómez, 2006). Gray and white shading highlights the different *Fagus sylvatica* organization levels.

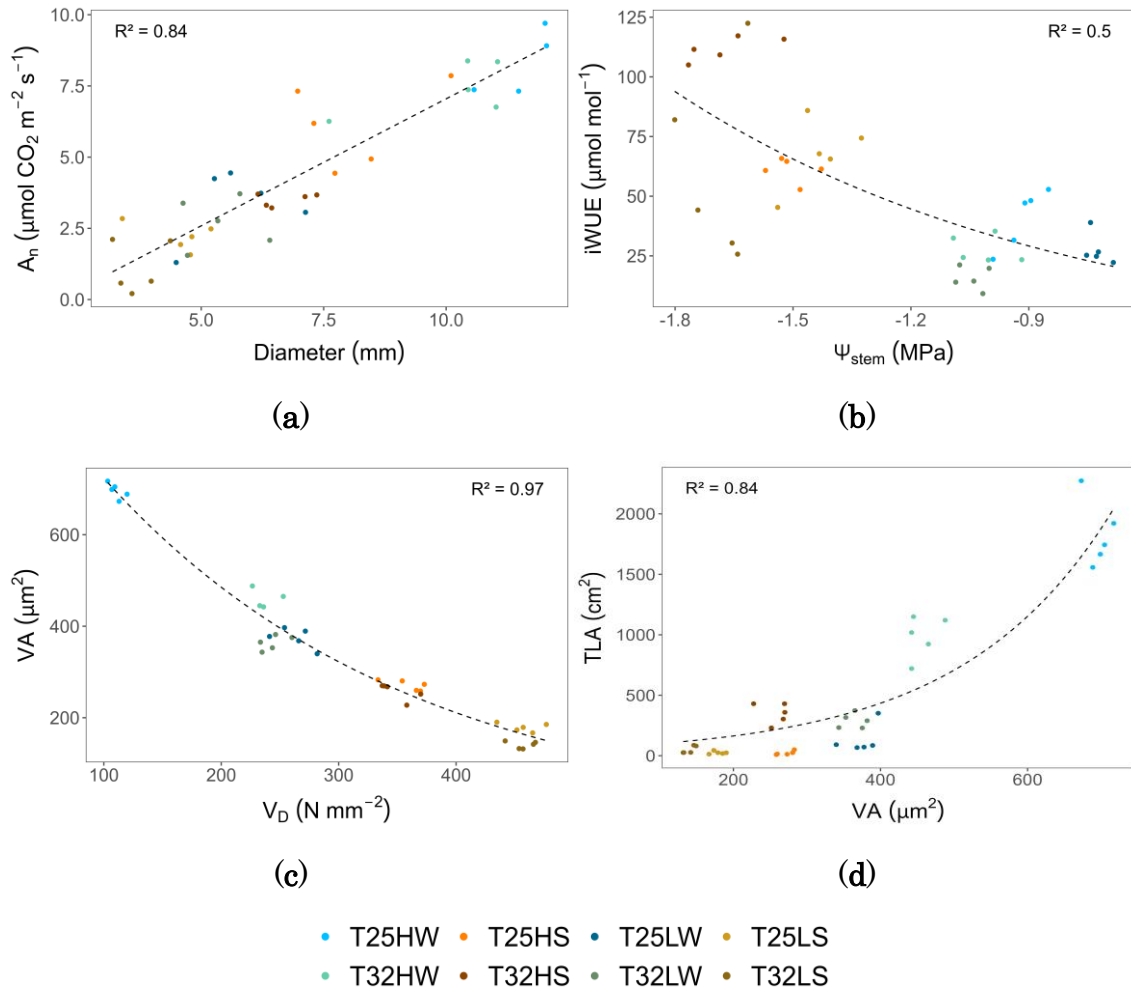


Figure 50. Relationships between functional traits: (a) straight line regression between net photosynthesis (A_n) and stem diameter (d); (b) Quadratic (second-order polynomial) regression between intrinsic water use efficiency ($iWUE$) and stem water potential at midday (Ψ_{stem}); (c) Negative quadratic relationship between vessel density (V_D) and mean vessel cross-sectional area (V_A) and (d) Positive quadratic relationship between vessel area (V_A) and total leaf area (TLA). Colored symbols represent different treatments: HW (high light well-watered), HS (high light water-stressed), LW (low light well-watered), LS (low light water-stressed) and growth temperatures (25 °C, T25, and 32 °C, T32). Dashed lines indicate the fitted regressions; coefficients of determination (R^2) are reported in the upper corner of each panel.

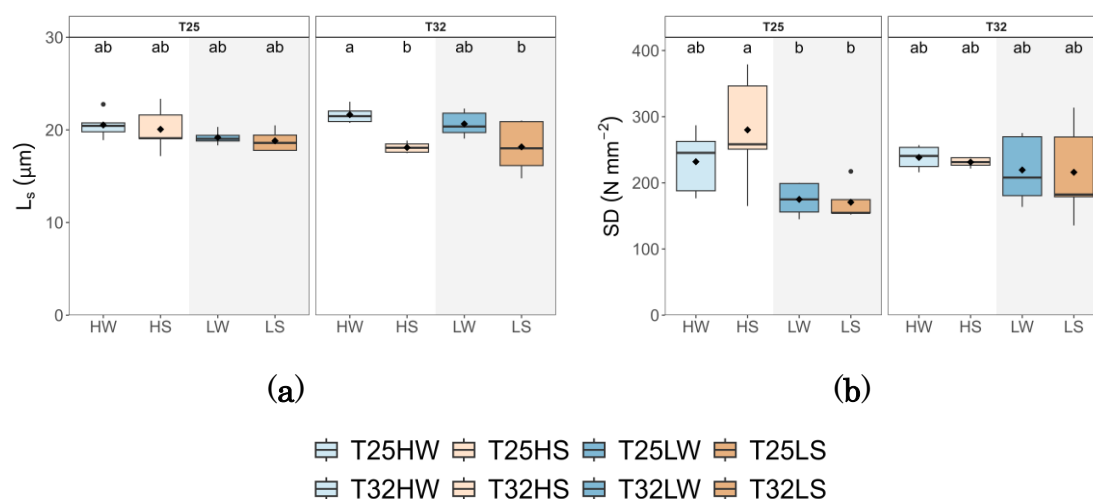


Figure 51. Boxplots of stomatal traits of beech seedlings grown in a factorial combination of temperature: 25 °C (T25) vs. 32 °C (T32), soil water availability: well-watered (W) vs. water-stressed (S), and light intensity: high (H) vs. low (L): (a) stomatal length (L_s , μm) and (b) stomatal density (SD, number of stomatas per mm^{-2}). The straight horizontal black line within each boxplot indicates the median, while the mean value is represented as a solid black point. Different letters indicate significant differences among treatments.

5. Discussion

The persistence of long-lived tree species under rapid climate change will largely depend on their capacity for phenotypic plasticity. At the southern range margin, European beech (*Fagus sylvatica*) faces unprecedented exposure to warming, recurrent droughts, and intense irradiance, conditions that threaten seedling establishment and long-term population renewal. Because evolutionary adaptation is too slow to match the pace of current environmental change, short-term acclimation mechanisms represent the primary buffer against demographic collapse. Previous research has shown that beech exhibits substantial but incomplete plasticity, particularly in traits related to photosynthesis and hydraulics, yet uncertainties remain about how multiple stressors interact to constrain regeneration.

This thesis contributes to closing this gap by experimentally disentangling the single and combined effects of warming, drought, and shade on the physiology and morphology of beech seedlings. Factorial experiments under controlled conditions allowed us to integrate responses across photosynthesis, water relations, and structural traits. Together, these findings provide an evidence-based assessment of how well beech seedlings can acclimate to future high-end climate scenarios. The following sections synthesize the results of the three experimental chapters in relation to the central hypotheses of this dissertation. Specifically, we discuss (i) the extent and limits of photosynthetic acclimation to warming, (ii) the consequences of combined warming and drought for carbon and water balance, (iii) the interactive role of light availability, (iv) hydraulic and anatomical adjustments, and (v) the ecological implications of plasticity constraints for beech regeneration at the rear edge of its distribution. This thesis addresses that gap by experimentally disentangling the roles of warming, drought, and light in shaping photosynthetic function, water balance, and structural adjustments in beech seedlings. The general discussion integrates the results of the three experimental chapters to evaluate how these acclimation processes operate under single and combined stressors, and whether they provide sufficient plasticity to buffer future climate extremes. In the following sections, we examine (i) the extent and limits of photosynthetic acclimation to warming, (ii) the combined effects of warming and drought on carbon–water balance, (iii) the interactive role of light availability, (iv) hydraulic and

anatomical adjustments, and (v) the broader ecological implications and constraints of plasticity for beech regeneration at its southern range margin. We found that under well-watered conditions, elevated temperature significantly reduced net photosynthesis due to the downregulation of V_{cmax} and J_{max} , despite there was a trend toward increased stomatal and mesophyll conductances (hypothesis **H1**). Notably, the optimal temperature for photosynthesis (T_{opt}) largely remained unchanged. This indicates only partial thermal acclimation, without a shift in the temperature optimum (hypothesis **H1**).

When warming was combined with drought, seedlings exhibited pronounced limitations on carbon assimilation and experienced intensified respiratory losses, leading to a strong decline in water-use efficiency (hypotheses **H1** and **H2**). Stomatal conductance declined sharply under elevated temperature and drought. At the same time, intrinsic water-use efficiency (iWUE) increased under combined drought, shade, and warming, confirming the opposing trends predicted by **H2**. These patterns highlight that warming exacerbated drought effects on gas exchange and carbon balance (hypothesis **H2**).

Under shaded conditions, seedlings maintained moderate photosynthetic activity at saturating light. However, shade combined with warming and drought strongly constrained physiological and anatomical plasticity (hypotheses **H3** and **H4**). Leaf area, biomass, and growth were most suppressed when all three stresses were combined, supporting **H5**. Seedlings exposed to combined heat, drought, and shade showed denser wood, narrower xylem conduits, and reduced K_{leaf} and K_{s} . These changes are consistent with a shift toward hydraulic safety and align with **H4**.

Taken together, these results confirm that warming alone reduces photosynthetic performance through biochemical downregulation despite enhanced stomatal conductance to CO_2 (**H1**), and that its combination with drought produces stronger, non-additive impacts on gas exchange and water balance (**H2**). Light availability further modulates these responses, with shade strongly constraining acclimation (**H3**), while morphological and anatomical plasticity is progressively limited under resource scarcity (**H4**). Moreover, seedlings faced unavoidable trade-offs, prioritizing carbon gain, light harvesting, or hydraulic safety but not optimizing all simultaneously (**H5**). Ultimately, the combination of warming, drought, and shade imposes the strongest constraint on seedling function and acclimation potential,

demonstrating the non-additive nature of multifactorial stress responses (H6), and highlighting the necessity of incorporating these interactions into predictive models and management strategies for European beech under a climate change scenario.

5.1. Photosynthetic Adjustments to Warming: Limits and Compensations

Numerous studies have demonstrated the capacity of temperate trees to photosynthetically acclimate to elevated temperatures (Dusenge et al., 2019; Slot & Winter, 2017; Yamori et al., 2014). However, whether this acclimation is maintained under complex and interacting stress conditions remains unclear. Our results demonstrate that *Fagus sylvatica* seedlings exhibit clear photosynthetic acclimation to elevated growth temperature, yet with significant trade-offs. As predicted (H1), seedlings exposed to chronic warming of 32 °C reduced photosynthetic capacity by ~20–30%, while the thermal optimum of assimilation (T_{opt}) remained largely stable (T_{opt}) compared to control seedlings (25 °C) (Study 1; Fig. 19A; Table 1). This apparent paradox—unchanged T_{opt} with decreased A_{opt} —was linked primarily to a downregulation of the biochemical components of photosynthesis, including the maximum rate of Rubisco carboxylation (V_{cmax}) and the maximum electron transport rate (J_{max}), rather than stomatal limitations (Study 1; Fig. 20 & 24). These findings align with previous studies reporting biochemical downregulation as a central acclimation mechanism in temperate species (Way & Yamori, 2014; Yamori & von Caemmerer, 2009). Study 2 provided independent support: although A_n appeared similar at growth temperatures, recalculation to a common reference (25 °C) revealed equivalent reductions in photosynthetic capacity (Fig. 33A-B).

This pattern suggests strategic nitrogen reallocation between RuBP regeneration and carboxylation capacity (Scafaro et al., 2017). While we did not quantify nitrogen partitioning, reduced V_{cmax} combined with unchanged chloroplastic CO_2 concentration (C_c) suggest enzyme reallocation or deactivation at supra-optimal temperatures (Study 2; Fig. 34D; Table 5). Parallel adjustments in respiration further supported acclimation: leaf dark respiration (R_d), measured at a common temperature, was lower in warm-grown seedlings (Study 2; Fig. 33C-D). Such downregulation is consistent with thermal acclimation theory, where plants reduce baseline maintenance

costs at higher growth temperature (Way & Yamori, 2014). Although this partially offset carbon losses, reduced photosynthetic input still led to a net carbon deficit (Studies 1 and 2).

Gas-exchange dynamics further illustrate this trade-off. Stomatal conductance (g_s) tended to increase, counterbalancing the observed decrease in mesophyll conductance (g_m). This led to higher CO₂ partial pressures in both the intercellular air spaces (C_i) and the chloroplasts (C_c), indicating that CO₂ diffusion capacity was largely maintained to compensate for biochemical downregulation (Study 2; Fig. 34A-D). Such partial acclimation—maintaining CO₂ diffusion but downscaling biochemical capacity—has also been observed in other tree species (Kelsey R. Carter & Molly A. Cavaleri, 2018; Dusenge et al., 2021), reinforcing the idea of a narrow thermal niche in European beech. Collectively, these findings confirm H1: warming reduces photosynthetic performance because enhanced CO₂ diffusion only partially offsets the respiratory costs and biochemical downregulation of photosynthesis.

Notably, the high stomatal conductance across the leaf temperature range (from 20°C to 45°C), enabling sustained transpiration for evaporative cooling (Studies 1–3). Under well-watered conditions, warmed plants kept stomata open (or more open) than control seedlings, even at high vapor pressure deficit, leading to ~50% lower intrinsic water-use efficiency (iWUE) due to “excess” water loss (Study 2; Fig. 31A&C). This behavior aligns with an evaporative cooling strategy: by spending more water, leaves avoided overheating at the cost of water efficiency (Chaves et al., 2016). Such responses have been documented in other species, where high temperature triggers stomatal opening to allow transpirational cooling (Mills et al., 2024; Sadok et al., 2021). Correspondingly, warm-grown seedlings developed morphological traits that facilitate heat dissipation. They produced smaller leaves but more leaves, resulting in a reduced average leaf size and lower total leaf area compared to controls, especially under high light conditions (Studies 2 and 3). This shift in crown architecture (many small leaves instead of a few large ones) diminishes boundary layer thickness around leaves and distributes thermal and hydraulic risk across more foliage (Leigh et al., 2017). Such morphology is considered adaptive under warming (Deluigi et al., 2025; Poorter et al., 2009): small leaves can cool faster, and if drought strikes, losing a few leaves out of many incurs a smaller penalty to the plant’s total carbon

gain. Indeed, our warmed seedlings allocated proportionally more biomass to leaves (higher leaf mass fraction) while reducing leaf mass per area (thinner leaves) – an acquisitive strategy to maximize light capture and transpiration cooling per unit leaf mass (Study 3; Fig. 37C-F).

Beyond morphology, photoprotection was also reinforced. Non-photochemical quenching (NPQ) under warming (Study 2; Fig. 31D) increased significantly, especially when combined with drought or high irradiance (Studies 1 and 2). This suggests a shift toward photoprotective dissipation of excess energy under conditions of impaired carbon assimilation. Such mechanisms may buffer the photosystems from oxidative damage, albeit at the cost of carbon gain (Hannachi et al., 2022; Ruban, 2016).

This enhanced photoprotective investment is a double-edged sword: it prevents acute heat/light damage to photosystem II (stabilizing the electron transport function), but it also diverts energy and resources away from growth (Zha et al., 2017). Our data support this trade-off, as NPQ in warm-grown seedlings remained as high as in controls despite lower photosynthesis and biomass, reflecting a prioritization of survival over productivity (studies 2 and 3). Such responses are consistent with known heat acclimation patterns where plants upregulate heat dissipation to avoid photoinhibition at the expense of carbon gain (Falcioni et al., 2024; Hannachi et al., 2022; Ruban, 2016).

Together, our results reveal that *Fagus sylvatica* seedlings exhibit clear signs of photosynthetic acclimation to moderate warming, mainly through biochemical downregulation and photoprotection. However, this capacity is contingent on resource availability: under low light or combined drought stress, acclimation is substantially weakened, indicating that carbon and energy constraints under these conditions limit the expression of thermal plasticity. These findings reinforce the need to integrate multiple environmental variables in climate impact models, as single-factor experiments may overestimate the capacity of trees to cope with warming. In particular, the interactive roles of light and water availability appear critical in determining whether beech seedlings can maintain functional photosynthetic performance under future high-temperature scenarios.

5.2. Carbon and Water Economy Under Compound Warming and Drought

Warming and water deficit interact **synergistically** to depress carbon gain and strain water balance in *Fagus sylvatica* seedlings beyond the effects of either factor alone. Under drought, net CO₂ assimilation (A_n) declined by more than half relative to well-watered plants; when drought co-occurred with elevated growth temperature (32 °C), A_n fell to ~30% of the value observed in ambient temperature droughted seedlings, indicating a strong amplification of drought impacts by warming (Study 2; Fig. 33). Concomitantly, dark respiration increased sharply—up to four-fold relative to ambient-drought plants—driving a collapse of the A_n/R_d ratio and pointing to periods of negative diurnal carbon balance (Studies 2–3), the little carbon gained during brief stomatal opening was largely consumed by respiration, and in many warm-drought seedlings probably insufficient to meet baseline metabolic needs. Such a state (extended periods of carbon deficit) risks tipping seedlings into carbon starvation, especially if the stress prolongs (López et al., 2022; McDowell, 2011). In line with these observations, biomass accumulation was nearly halted in the warm-drought treatment (T32-HS); seedlings showed stunted growth or even signs of carbon reserve depletion, whereas those under drought alone (T25-HS) maintained at least some growth by reallocating resources (e.g. shedding leaves to conserve water; Study 3; Fig. 37). Similar interactive effects of drought and warming on carbon balance have been documented in other temperate trees (Adams et al., 2009; Birami et al., 2020; Teskey et al., 2015).

Because VPD was controlled across growth temperatures, the observed shifts in gas exchange primarily reflect temperature-driven regulation rather than atmospheric dryness. Under well-watered conditions, warming increased stomatal conductance and transpiration, consistent with an evaporative-cooling strategy that lowered intrinsic water-use efficiency (iWUE) (Study 2–3). Under drought, stomata closed, but in warm-grown plants this did not translate into higher iWUE than in cool-grown droughted plants (Study 2; Fig. 31A–C). This pattern can be explained by (i) elevated $g_{\min}$ (residual epidermal/cuticular conductance) that sustains water loss due to a greater tendency to leak water via cuticle or incompletely closed stomata (Garen & Michaletz, 2025; Yang et al., 2012) (Studies 2 and 3); and (ii) high respiratory costs that reduce the carbon return per unit water lost (Hüve et al., 2012).

The net effect is comparatively lower iWUE in warm-drought relative to ambient-drought, despite similar stomatal closure—an outcome consistent with the distinct syndrome of *hot drought* (Adams et al., 2009; Choat et al., 2018).

Photochemically, warm-drought seedlings showed a marked rise in non-photochemical quenching (NPQ) alongside reduced PSII efficiency (Study 2; Fig. 31D & 36B), indicating enhanced dissipation of excess excitation energy when CO₂ supply and biochemistry constrain electron use. This photoprotective shift likely averts oxidative damage but diverts energy and metabolites from assimilation, reinforcing carbon limitation (Allakhverdiev et al., 2008; Flexas & Medrano, 2002; Murchie & Lawson, 2013; Way & Yamori, 2014). This mirrors findings in other species that combined drought and heat cause a surge in thermal energy dissipation to protect chloroplasts when CO₂ assimilation is limited (Hannachi et al., 2022). While necessary to avoid photodamage, this response carries a substantial carbon cost: energy sent to NPQ or antioxidant production is energy not invested in fixing CO₂, compounding the decline in A_n (Study 2; Fig. 36F-H). Thus, under compound stress our beech seedlings were forced into a defensive mode – closing stomata to save water and ramping up photoprotection – but as a result, their carbon gains were minimal and largely consumed by respiration and protective metabolism (Study 2; Fig. 36F-H) (Feller, 2016).

Collectively, these findings confirm H2, showing that warming amplifies drought constraints on leaf gas exchange through non-additive effects: on the water side, warming reduces iWUE under well-watered conditions by increasing g_{sw} and residual conductance, while under drought it aggravates carbon imbalance by elevating respiratory costs (R_d) and enhancing photoprotective energy dissipation (NPQ), so that seedlings sustain similar assimilation but with substantially higher metabolic costs. In practical terms, moderate warming already pushes seedlings close to the limits of their carbon–water budget, and superimposed drought rapidly exceeds those limits—even when photoprotection and hydraulic acclimation are engaged. These results highlight the potential for carbon starvation and hydraulic failure to interact in shaping seedling mortality during future hot drought episodes, echoing broader patterns observed in tree die-off events (Adams et al., 2009; Anderegg et al., 2020; McDowell et al., 2008).

5.3. Shade × Temperature Interaction and Implications for Beech Regeneration

Light availability is a key determinant of tree seedling performance, shaping both carbon acquisition and hydraulic safety (Mathur et al., 2018; Valladares & Niinemets, 2008; Zivcak et al., 2014). Shading acts as a primary environmental filter, constraining photosynthetic capacity while driving morphological adjustments that favor light capture. However, it remains uncertain how shade modulates the capacity of temperate tree seedlings to acclimate to warming. In our experiment, shade strongly altered the acclimation responses of *Fagus sylvatica* seedlings to elevated temperature, generating contrasting outcomes for carbon gain and hydraulic safety. Specifically, seedlings growing under deep shade showed substantial carbon limitations, expressed through reduced net assimilation (A_n) and stomatal conductance (g_s). Nevertheless, shaded seedlings compensated in part by maximizing light-use efficiency, sustaining higher quantum efficiencies and lower light requirements under low irradiance (Study 2; Fig. 36A–D). They also developed a typical shade-tolerant phenotype (Studies 2–3; Fig. 30A–C; Fig. 37A–B): fewer and smaller leaves with low leaf mass per area (LMA).

Despite this initial limitation, shade-grown seedlings acclimate over time, ultimately achieving similar CO₂ assimilation rates under saturating light as their high-light counterparts, but with lower respiration, indicating higher carbon-use efficiency (Study 2; Fig. 33A–D). This capacity to adjust confirms the well-established shade tolerance of beech (Cascio et al., 2010; Robakowski & Antczak, 2008).

When shade is coupled with warming, seedlings adopt an acquisitive phenotype. Leaf area expands, LMA decreases, and biomass allocation shifts toward shoots, enhancing resource acquisition under well-watered conditions (Rubio et al., 2025). These traits support high assimilation rates and biomass production that can exceed those of low-light seedlings at moderate temperatures (Studies 2–3; Fig. 30 & Fig. 33A–B; Fig. 37C–F & 38A–D). Although warming narrows the margin for positive carbon balance under deep shade (Studies 2–3; Fig. 33A–D & Fig. 38A–D), seedlings still optimize their acclimation by reinforcing an acquisitive strategy that sustains high performance at operative light levels. This strategy parallels known shade-acclimation responses in forest understorey species (Poorter et al., 2009) and

highlights beech's capacity to exploit warm, shaded microhabitats—albeit with trade-offs.

However, under high irradiance, acclimation to warming was only partial. Seedlings maintained comparable A_n under saturating light, but this required elevated metabolic expenditure and was accompanied by reduced total leaf area, thereby limiting whole-plant carbon gain. Morphological plasticity was expressed conservatively—denser, fewer leaves with high LMA and reinforced xylem architecture—prioritizing hydraulic safety over growth (Aranda et al., 2005; Lens et al., 2011; Martínez-Vilalta et al., 2014; Rubio et al., 2025). Consistent with this syndrome, conservative traits constrain carbon gain but improve resistance to drought in high-light environments (Choat et al., 2018). This pattern partially supports H3: shade constrained acclimation as expected, whereas high irradiance did not enhance metabolic plasticity; instead, it promoted only partial acclimation and a conservative, stress-resilient configuration.

These contrasting syndromes reflect a central ecological trade-off mapped onto the leaf economic and hydraulic spectra (Grossiord et al., 2020; Martínez-Vilalta et al., 2014): acquisitive traits in shaded seedlings enhance carbon economy when water is ample, whereas conservative traits in high-light seedlings prioritize hydraulic safety at the cost of growth. In warm shade, rapid development—fast height growth and early leaf expansion (Reich et al., 2015; Wertin et al., 2011) —is accompanied by high specific leaf area and shallow rooting; together with poor osmotic adjustment (Π_0) and limited elastic adjustment (ϵ_{leaf}), these characteristics narrow hydraulic safety margins by impairing turgor maintenance, reducing stem-specific and whole-plant conductance (K_s , K_{plant}), and delaying hormonal regulation (ABA accumulation), leaving seedlings vulnerable to desiccation (Studies 2-3; Fig. 31F; Fig. 38; (Buckley & Diaz-Espejo, 2015; Valladares & Pearcy, 2002).

This interplay between light environment and stress resilience has critical implications for *Fagus sylvatica* regeneration under climate change. Beech typically establishes in shaded microsites—under closed canopies or on north-facing slopes—where light is limited but soil moisture tends to be higher (Rubio-Cuadrado et al., 2024). These environments have been considered climatic refugia (Bolte et al., 2016; Grubb et al., 1996), and our findings partly support this view. However, under hot drought, the shade-induced acquisitive

phenotype collapses, and seedlings experience steep functional declines and high mortality.

Neither shaded nor fully sun-exposed microsites guarantee persistence. The combination of high temperature, drought, and shade produced the greatest functional declines and highest mortality in our experiments—pointing that triple-stress environments are most lethal (Studies 2–3)—and field observations indicate higher drought mortality for shaded seedlings than for those in partial light (Valladares & Pearcy, 2002).

This convergence of stressors may already be driving regeneration failures at beech's dry distribution margins. Even short-duration heatwaves, in the absence of severe soil drought, have led to seedling death in understories. These patterns suggest that the future regeneration niche of *Fagus sylvatica* will be tightly constrained by interactions between microsite light and climatic extremes. While shaded microsites may allow establishment under moderate warming, they risk becoming demographic traps under compound stress. Conversely, more open microsites may filter for conservative, drought-resilient phenotypes—albeit at a cost to growth. Predicting future forest dynamics will therefore require detailed attention to microsite heterogeneity and local climate–light interactions (Grossiord et al., 2020; Valladares et al., 2014; Way & Pearcy, 2012).

5.4. Hydraulic Safety Margins and Anatomical Acclimation

Hydraulic acclimation is a central axis of tree responses to climate stress, as anatomical and physiological adjustments determine the trade-off between carbon gain and hydraulic safety (Grossiord et al., 2017; Hacke et al., 2001; Sperry, 2003). Our results showed that *Fagus sylvatica* seedlings displayed marked plasticity in hydraulic traits under warming, drought, and shade, generating contrasting outcomes for carbon gain and hydraulic safety.

Under elevated temperature alone, safety margins were moderately reduced but remained within functional limits, indicating that warming by itself does not critically compromise xylem integrity. Instead, anatomical adjustments such as reductions in vessel size and increases in vessel density point to a conservative acclimation strategy that prioritizes safety over efficiency (Study 3). This supports H4, suggesting that warming in the absence of water

limitation promotes a conservative phenotype aimed at maintaining hydraulic stability, even at the expense of potential growth..

By contrast, drought alone elicited stronger compensatory responses in both water relations and tissue properties.. Osmotic adjustment (>0.8 MPa) lowered π_0 , and leaf turgor loss point shifted toward more negative values (Rubio et al., 2025). Leaf elasticity increased, consistent also with enhanced drought tolerance (REF). Additionally, seedlings reduced leaf area and biomass, thereby lowering transpirational demand and increasing the Huber value, thus supplying more water to the remaining leaves, higher K_L . Whereas “drought-type” xylem with smaller vessels and redundancy buffered transport (Study 3; Fig. 40). All these adjustments correspond to classical drought-resistance mechanisms described in woody plants (Tyree & Zimmermann, 2002; Bartlett et al., 2012), and they illustrate the capacity of beech seedlings to buffer water deficits through a combination of morphological and physiological strategies. Although these changes improve resistance to desiccation, they come at a clear cost in carbon gain and growth, underscoring the central trade-off between drought resistance and productivity..

The most critical scenario occurred under combined warming and drought, where safety margins collapsed to near zero. In this context seedlings failed to adjust osmotically and tissue elasticity was impaired,—and relied instead on ABA-mediated stomatal closure (Schönbeck et al., 2022; Warren et al., 2011). Without osmotic buffering, turgor was lost at higher water potentials, while midday Ψ_{leaf} dropped below -2.0 MPa, close to embolism thresholds for beech (Cochard et al., 1999). Here, the anatomical acclimations that buffered warming proved insufficient, and the efficiency–safety trade-off tipped decisively toward failure. Growth was curtailed well before photosynthesis fully declined, indicating a reallocation of resources toward hydraulic maintenance. Hydraulic conductivity declined markedly, reflecting a situation where neither anatomical adjustments nor stomatal regulation could prevent vulnerability to cavitation. This outcome highlights that acclimation mechanisms effective under single stressors become overwhelmed when heat and drought co-occur, reinforcing the notion of non-additive interactions (Anderegg et al., 2016; Adams et al., 2017).

Shade further intensified this imbalance by amplifying evaporative demand while restricting water supply. Larger leaf area and reduced root allocation

increased the mismatch between transpiration and uptake, leaving warm–shade–drought seedlings with the lowest K_L , K_{plant} , and safety margins, with some individuals irreversibly wilting. In this context, traits beneficial for carbon gain under well-watered conditions became maladaptive liabilities, exposing the limits of plasticity when multiple stresses converged (Ramírez-Valiente et al., 2021; Zandalinas et al., 2024). These findings confirm H5: seedlings cannot simultaneously optimize carbon gain, light capture, and hydraulic safety—particularly under warm–drought and shaded conditions.

Crucially, these effects were synergistic rather than additive. The joint action of warming, drought, and shade compressed hydraulic safety margins beyond those observed under any single or dual stress. This reduced both water transport and redundancy, heightening desiccation risk, and leading to mortality in the most vulnerable individuals. Such outcomes provide clear experimental support for H6, demonstrating that the combined stresses generate unique syndromes of decline not predictable from single-factor experiments.

These mechanistic limits translate into strong constraints on regeneration. Warming alone can be accommodated through xylem acclimation, but the addition of drought erodes safety margins, while shade exacerbates the imbalance, transforming potential refugia into demographic traps. In shaded, warm, and dry microsites, acquisitive traits such as large leaf deployment and shallow rooting accelerate soil water depletion and overextend the hydraulic system.. By contrast, seedlings in more open microsites tend to develop conservative traits that limit carbon gain but improve water security.

With extreme events projected to become more frequent and severe (IPCC, 2023), these trade-offs imply a narrowing regeneration window for *Fagus sylvatica* at its southern range margin (Roibu et al., 2022). Although beech seedlings display notable plasticity in xylem and physiology that supports acclimation to moderate warming, this flexibility is rapidly exhausted under compound stress. Ultimately seedling fate is governed by the trade-off between carbon acquisition and hydraulic safety, with shade further intensifying vulnerability. Future regeneration will depend less on warming alone than on the combined effects of heat, drought, and light environment, underscoring the need to integrate microsite-dependent vulnerability into predictive models of beech forest recruitment.

5.5. Plasticity Constraints and Ecological Consequences

Our findings highlight that phenotypic plasticity in *Fagus sylvatica* seedlings is strongly constrained under warming, particularly when combined with drought and shade. Although beech seedlings expressed a broad capacity for acclimation in single-stress scenarios, the overall plasticity index (PPi) declined markedly with the addition of heat, and even more so under compound stress. This erosion of plasticity indicates that warming narrows the range of viable trait adjustments, leaving seedlings less capable of buffering environmental fluctuations. These patterns confirm H4 by showing that the ability to flexibly deploy acquisitive or conservative strategies depends critically on the stress context, and that under warming such flexibility is progressively curtailed.

Plasticity differed strongly among traits. Morphological and biomass-related traits displayed the highest levels of variation, allowing seedlings to adjust allocation patterns across environments. By contrast, anatomical and hydraulic traits were comparatively less plastic, reflecting stronger developmental or biophysical constraints. While this rigidity confers stability to core functions, it also limits the scope for rapid adjustment under extreme events. The imbalance between flexible morphological traits and conservative hydraulic traits reinforces the idea that plasticity is a double-edged sword: while it permits adjustments in growth and allocation, it cannot fully protect the hydraulic system when multiple stressors converge (Van Buskirk & Steiner, 2009; Nicotra et al., 2010).

Warming tended to impose uniform shifts—such as higher ρ across all treatments—rather than enabling context-specific variability. This reflects developmental constraints: xylem anatomy is largely fixed early in the season and cannot be restructured in response to later drought (Gricar et al., 2015; Sultan, 2000). Even traits expected to be flexible, such as stomatal conductance and photosynthetic capacity, showed only moderate plasticity under warming (Rubio et al., 2025). While cool-grown seedlings varied widely in stomatal conductance between wet and dry treatments, warm-grown plants showed a reduced range, suggesting that high temperature exposure locks them into narrower regimes, potentially due to metabolic stress or reduced resource availability (Meier & Leuschner, 2008; Rodríguez-Calcerrada et al., 2010; Saltré et al., 2015).

Moreover, certain plastic responses that are advantageous under benign conditions became maladaptive in stressful environments. For instance, large leaf areas and shallow rooting promoted carbon gain under ample moisture but turned into liabilities under warm–shade–drought conditions, where excessive transpiration depleted water reserves and reduced safety margins (Studies 2 and 3). This context-dependent maladaptation underscores that plasticity does not guarantee adaptive value across all environments (Ramírez-Valiente et al., 2021), but instead may increase vulnerability when trait shifts amplify resource mismatches. Such outcomes resonate with our findings in hydraulic traits (section 5.4), showing that seedlings cannot optimize carbon gain, light capture, and hydraulic safety simultaneously, particularly under compound stress.

Finally, the integration of traits revealed that under multiple stressors, previously coordinated responses became decoupled, leading to disintegration of trait networks. (PCA; Study 3). For example, increased leaf area was not matched by proportional root investment under warm shade, creating imbalances. Similarly, stomatal density no longer scaled with photosynthetic capacity. Such decoupling suggests that warming reconfigures the coordination of trait networks (Schneider, 2022; Valladares & Pearcy, 2002), undermining the holistic acclimation strategy of seedlings. This loss of coordination compromises the capacity of seedlings to maintain functional balance between photosynthesis, water use, and structural safety. Together, these results confirm that plasticity in beech seedlings is both trait- and context-dependent, and that its limits are rapidly reached under combined warming, drought, and shade. Ecologically, such constraints imply that regeneration success at the southern distribution edge will depend not only on the absolute magnitude of stress but also on the availability of microsites that mitigate extremes. In this sense, the observed limits of plasticity strongly restrict the capacity of rear-edge beech populations to cope with future climate extremes, reinforcing the urgency of integrating trait-based vulnerability into regeneration and distribution models. Such scenarios are not theoretical: southern European forests already combine summer drought, frequent heat, and shaded understories, and recruitment failure of beech is documented there (del Río et al., 2018). These field patterns align mechanistically with our finding that plasticity collapses under the same stress combinations (Zandalinas et al., 2024).

5.6. Synthesis and Outlook: Tolerance Limits and Regeneration Risk

The integration of the three experimental studies confirms H6: the interaction of warming, drought, and shade exerts non-additive effects that cannot be predicted from single-factor responses (CITE). Across scales of organization—from photosynthetic metabolism to hydraulic safety and whole-plant allocation—our results reveal that beech seedlings display substantial plasticity under moderate warming or single stresses, yet this flexibility rapidly collapses under compound extremes (CITE). Thus, the capacity of *Fagus sylvatica* to cope with climate change at its southern distribution limit is not determined by one isolated mechanism but by the convergence of multiple stress pathways that jointly define tolerance limits (CITE).

An important theoretical context for these findings is the concept of **multiple resource limitation**. Classical physiological theory (*Liebig's Law of the Minimum*) posits that growth is constrained by the most limiting resource (CITE); however, in natural environments, plants are often co-limited by several resources simultaneously (CITE). Under such conditions, acclimation reflects a continuous adjustment to balance carbon, water, and nutrient constraints rather than to overcome a single limitation (CITE). The acclimation responses observed in our beech seedlings exemplify this principle. Under combined heat and drought—especially when accompanied by moderate shade—carbon uptake became the overriding limitation even when light was available, essentially expressing Liebig's principle under multifactorial stress (CITE). Yet the coordinated adjustments in water use and nitrogen allocation indicate that seedlings were not passively constrained but actively negotiating trade-offs among co-limiting processes to maintain functional balance (CITE). The downregulation of V_{cmax} and J_{max} under warming can thus be interpreted as an optimized reallocation of nitrogen from the photosynthetic machinery to maintain water-use efficiency (CITE), aligning with trait-based theories that plants balance acquisitive traits (maximizing resource uptake) and conservative traits (minimizing resource loss) according to environmental pressures (CITE). This framing highlights that beech acclimation is not governed by a single dominant factor but by an integrated carbon–water–nutrient economy operating under multiple simultaneous constraints (CITE).

A central outcome of this work is the progressive erosion of carbon and water balance as stress factors accumulate (CITE). Warming alone reduced photosynthesis and respiration while sustaining growth when water was not limiting, consistent with projections of physiological resilience to mild warming (Geßler et al., 2007). Drought elicited classical acclimation mechanisms such as osmotic adjustment, elastic tissues, and leaf shedding (CITE). However, under hot-drought conditions these strategies proved insufficient, as safety margins shrank to near zero and hydraulic conductivity declined irreversibly (CITE). Shade further amplified this imbalance by promoting acquisitive traits (large leaf area, shallow rooting) that became maladaptive under water scarcity (CITE). The combined result is a narrowing window of functional viability, in which seedlings are unable to reconcile carbon acquisition, water security, and light capture (CITE). These tolerance thresholds define the physiological bottleneck for regeneration at the rear edge (CITE).

Ecologically, these mechanistic limits translate into strong demographic consequences (CITE). Microsites that might act as refugia under warming or drought alone can turn into demographic traps under compound stress, as acquisitive strategies accelerate soil-water depletion and overextend the hydraulic system (CITE). By contrast, seedlings in more open microsites tend to develop conservative phenotypes that secure water status but at the cost of carbon gain and growth, potentially limiting recruitment success (CITE). The frequency and severity of compound extremes projected for coming decades (IPCC, 2023) will therefore determine whether regeneration windows close entirely in certain habitats (CITE). As regeneration failure is often the first signal of range contraction (Dullinger et al., 2004; Jump et al., 2009), our results suggest that the southern edge of beech distribution is particularly vulnerable to climate change, not only through adult decline but also through impaired recruitment (CITE).

From a management perspective, these findings emphasize the need to integrate microsite-dependent vulnerability into models and silvicultural practices (CITE). Maintaining canopy heterogeneity and promoting light–water balance at the stand scale could enhance regeneration opportunities by mitigating extremes of radiation and evaporative demand (CITE). In addition, conservation strategies should consider the use of drought-tolerant provenances and the identification of natural refugia where stress

combinations are less severe (CITE). Ultimately, the tolerance limits identified here indicate that while beech seedlings retain capacity to acclimate to moderate warming, their persistence under future climates will depend critically on the frequency of compound events and the availability of safe microsites (CITE). Failure to incorporate these multifactorial constraints into regeneration and distribution models risks overestimating the resilience of rear-edge populations in the decades to come (CITE).

5.7. Limitations of the study

As with any controlled experiment, this thesis has several limitations that need to be acknowledged when interpreting the results and applying these findings to natural forest systems. The first concerns the experimental design. Growth chambers provided a high level of environmental control, but at the cost of simplifying natural variability. Vapor pressure deficit (VPD) was kept relatively constant, diel temperature fluctuations were absent, and soils were homogeneous. These conditions differ substantially from those in the field, where seedlings are exposed to highly dynamic VPD, abrupt heat waves, heterogeneous soil profiles, and fluctuating water availability. (Alejandro Gabriel et al., 2025). Notably, in natural settings, elevated temperatures are often accompanied by high vapor pressure deficits (VPD), which intensify atmospheric water demand. Because we held VPD constant, our warm-grown seedlings likely experienced milder water loss than they would under field conditions, where high VPD exacerbates stomatal closure and carbon limitation (Grossiord et al., 2020). Additionally, drought in nature occurs gradually and across spatially variable soils, allowing for potential deep-water access or uneven stress exposure (Werner et al., 2021). In contrast, our pot-based system imposed a relatively uniform and accelerated drought due to limited rooting volume, potentially amplifying drought effects compared to natural soils. While this design allowed us to disentangle the independent and interactive effects of warming, drought, and shade, it may have underestimated the intensity and unpredictability of stress in natural forests.

A second limitation relates to the biological material used. The study examined a single provenance of *Fagus sylvatica*; given the species' broad distribution and known genetic variability in drought tolerance, responses may differ across populations (Robson et al., 2012). Although this approach

minimized genetic and environmental noise, it does not capture the intraspecific variation that exists across populations. Provenances from drier or more continental origins may exhibit different hydraulic thresholds, acclimation strategies, or degrees of plasticity, which could alter the patterns observed here

Third, the temporal scale of the experiment was relatively short. Seedlings were exposed to stress during a single growth cycle, which is relevant for the regeneration bottleneck but insufficient to assess carry-over effects, long-term acclimation, or cumulative damage over multiple years. (Julia et al., 2013). Traits such as xylem anatomy, non-structural carbohydrate reserves, or leaf morphological adjustments may require more than one season to fully express. Therefore, while the results highlight key mechanisms operating in the short term, they cannot be directly extrapolated to multi-year responses or to the persistence of older cohorts.

Finally, several biotic and ecological interactions were not included. Seedlings were grown without mycorrhizal symbionts, herbivores, or competitors, all of which can significantly influence stress tolerance (Pugnaire et al., 2019; Van Der Heijden et al., 2008). Mycorrhizas, in particular, are known to enhance nutrient uptake and modulate drought resistance, while neighborhood competition for light and water strongly shapes seedling establishment in the field. Their absence implies that the stress responses documented here represent a simplified version of the natural regeneration process.

Taken together, these limitations caution against direct extrapolation of our results to mature trees or heterogeneous forest environments. Nevertheless, the controlled design provided a unique opportunity to isolate causal mechanisms and quantify tolerance limits under factorial stress combinations, an approach rarely possible in field studies. Thus, while the absolute values of plasticity, hydraulic safety margins, or carbon balance reported here may differ in natural conditions, the patterns and trade-offs identified remain highly informative. They provide mechanistic insights into how warming, drought, and shade interact to constrain regeneration at the rear edge of the distribution, offering a valuable foundation for predictive models and for future experiments that incorporate broader genetic diversity, longer timescales, and more complex biotic interactions.

5.8. Future directions

Building on this work, several avenues for future research emerge. A first priority is to conduct longer-term and field-based experiments to validate whether beech seedlings exhibit the same acclimation patterns and physiological limits under natural climate variability as in controlled environments. For example, field manipulations using infra-red lamps or open-top chambers, combined with experimental droughts, could track seedling survival, growth, and trait expression over multiple years. Such studies would help uncover carry-over effects, delayed mortality, and potential acclimation of traits such as rooting depth, carbohydrate storage, or mycorrhizal associations.

A second research direction involves exploring responses to extreme climatic events, particularly heatwaves superimposed on drought. Our findings indicate that seedlings tolerate moderately elevated temperatures under benign conditions, but even short episodes of 40 °C may trigger hydraulic collapse if water is limiting. Identifying physiological thresholds — such as maximum tolerable leaf temperature or critical water potential for embolism — will be key for improving mechanistic models of mortality risk under compound extremes.

Third, it is essential to investigate intraspecific genetic variation in acclimation capacity. Beech populations span a wide climatic gradient, from mesic central Europe to drought-prone southern margins, and likely harbor adaptive differences. Comparative experiments across provenances would clarify whether certain populations maintain broader plasticity or stronger drought recovery under warming, thereby informing strategies of assisted migration, seed transfer, or selective breeding aimed at reinforcing resilience.

A fourth line of research concerns **community-level interactions**. Beech typically regenerates in mixed stands, where facilitative or competitive dynamics with other species strongly modulate stress outcomes. Multifactorial experiments combining beech with more drought-tolerant or shade-tolerant neighbors would reveal whether such interactions buffer or exacerbate climate impacts. Integrating these dynamics into regeneration studies will be crucial for predicting future forest composition.

Finally, future efforts should focus on **model integration**. Mechanistic parameters derived from this thesis — such as reductions in photosynthesis

at +7 °C, impaired osmotic adjustment under warm–drought, or mortality rates linked to narrow hydraulic safety margins — can be incorporated into gap models, dynamic vegetation models, and Earth system simulations. Embedding trait-based vulnerability thresholds into these frameworks will allow projections of regeneration bottlenecks and range shifts under high-emission scenarios (e.g., SSP5-8.5). This integration will enhance our capacity to identify regions where beech may persist, where management interventions are required, and where local extinction risks are highest.

Furthermore, since beeches often coexist with other tree species, community-level experiments exposing mixed seedlings to warming and drought could

6. Conclusions

This thesis demonstrates that *Fagus sylvatica* seedlings possess limited yet context-dependent acclimation capacity to high-end climate change scenarios. By experimentally disentangling the effects of warming, drought, and shade, we provide a mechanistic understanding of how these factors interact to constrain regeneration at the species' southern distribution limit.

1. Warming alone downregulated photosynthetic capacity, reducing net CO₂ assimilation without shifting the thermal optimum. Seedlings responded with a water-intensive strategy — high stomatal conductance and transpiration, coupled with the production of many small leaves — that buffered overheating through evaporative cooling. While partially effective under ample water supply, this strategy strongly reduced intrinsic water-use efficiency, highlighting a trade-off between carbon gain and thermal safety.
2. Warming × drought produced synergistic effects rather than additive ones.. Elevated respiration, failure of osmotic adjustment, narrowing of hydraulic safety margins drove strong declines in carbon assimilation and water-use efficiency, pushing seedlings towards carbon starvation and hydraulic failure. These results confirm that compound stress combinations can exceed the tolerance limits inferred from single-stressor experiments.
3. Warming × shade interactions were context dependent. Shade buffered thermal load under benign moisture conditions and facilitate an acquisitive strategy. However, in warm drought microsites, these same traits became maladaptive, exacerbating water imbalance and causing mortality in the most vulnerable seedlings. This demonstrates how microsite conditions can transform shade from a refuge into a demographic trap.
4. Across treatments, seedlings displayed plasticity in photosynthetic, morphological, and hydraulic traits, but this plasticity was progressively constrained under stress convergence. Warming reduced plastic responses in osmotic adjustment, leaf shedding, and stomatal regulation. While beech seedlings adjusted traits to optimize either carbon gain, hydraulic safety, or light capture under isolated stressors, they could not

optimize all simultaneously under combined warming, drought, and shade. This confirms that regeneration success depends not only on physiological flexibility but also on the costs and limits of plasticity.

5. Taken together, these findings highlight that the fate of beech regeneration under climate change will be determined less by warming alone than by its interactions with drought and light environment. Seedling survival ultimately hinges on trade-offs between carbon acquisition and water security, with stress convergence pushing individuals beyond their physiological limits.

From an ecological perspective, our results underscore the vulnerability of rear-edge populations. While moderate warming can be tolerated, recurrent compound stress events — heatwaves coinciding with drought in shaded understories — are likely to narrow the regeneration window and contract the species' range. Incorporating microsite-dependent vulnerability and trait-based thresholds into predictive models will therefore be essential for anticipating regeneration bottlenecks and informing adaptive management.

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