Journal of Forestry Research (2026) 37:36
https://doi.org/10.1007/s11676-025-01974-0

ORIGINAL PAPER

In vitro measured leaf-level freezing tolerance predicts photosynthetic impairment during a natural late spring-frost event

Norbert Kunert[1] · **Svenja Gebhard**[1]

Received: 4 June 2025 / Accepted: 13 October 2025

Abstract Climate change has altered the global temperature regimes leading to warmer temperatures occurring earlier in spring in many temperate regions. This has induced an earlier budbreak making trees susceptible to late spring-frost events, however, information on species-specific late spring-frost tolerance is only available from observational studies. Here, we implemented a quantitative study on late spring-frost tolerance determined by in vitro leaf-level measurements of three temperate broad-leaved tree species via the assessment of the maximum quantum yield efficiency of the photosystem II (F_v/F_m). We investigated to what extent in vitro measurements conducted one day before a late spring-frost event can predict the in vivo damage caused by a cold snap. *Fraxinus excelsior* showed the lowest in vitro tested tolerance to late spring-frost, and the leaves lost 50% of F_v/F_m (LT50) at $+0.60 \pm 0.26$ °C. The damage induced by the cold snap the following day (minimum temperature of -3.28 °C) was a fatal decline of F_v/F_m to 5.8% of the maximum. The other two species, namely *Fagus sylvatica* and *Quercus robur,* were characterized by LT50 of -0.17 ± 9.99 °C and -2.29 ± 1.11 °C, respectively. The cold snap induced less damage, F_v/F_m values declined to 46.9% and 53.5% of the maximum in the two species, respectively. The in vitro measurements precisely predicted the damage caused by the late spring-frost event. We suggest that in vitro estimated LT50 values can be used as a comparative leaf trait as it has high predictive power for tree species performance after late spring-frost.

Keywords Cold snap · Spring phenology · Leaf unfolding · Frost damage · Temperate trees

1 Introduction

Climate change causes significant alterations in abiotic growth conditions for plants due to changing temperature regimes in temperate regions. Besides a generally warming climate, with higher mean and maximum temperatures, changes in seasonality have been observed (IPPC 2019). For example, some estimates describe a prolongation of the growing season length of 12.6 days during the period between 1950 and 2011 in Eurasia (Barichivich et al. 2013). However, most studies indicate that springtime warming is much more pronounced than a prolongation of the growing season into fall months (e.g. Menzel and Fabian 1999). Accordingly, in the last decade, a springtime warming rate of 0.060 °C per year has been observed in Europe (Twardosz et al. 2021) and with every 1 °C more an approximately 5–6 days earlier leaf unfolding can be expected (Piao et al. 2019; Wolkovich et al. 2012). Earlier leaf unfolding increases the risk of late spring-frost damage (Augspurger 2011; Bigler and Bugmann 2018) and observations indicate a persistent threat of late spring-frost events despite the warming trend with climate change (Schulze et al. 2020; Düsterhöft et al. 2024). Accordingly, the frequency of late spring-frost events has not declined over the last six decades (Schulze et al. 2020) or even since 1880 (Düsterhöft et al. 2024). Besides a persistent threat of late spring-frost damage, a recent meta-analysis shows that the frequency of late

---

Corresponding editor: Lei Yu.

✉ Norbert Kunert
norbert.kunert@uni-bayreuth.de

[1] Functional and Tropical Plant Ecology, University of Bayreuth, Universitätsstraße 30, 95440 Bayreuth, Germany

spring-frost events has increased by 35% over the last six decades in Europe and Asia (Zohner et al. 2020). Whether the risk frequency of late spring-frosts increases, or the risk remains the same, late spring-frost events occur during a sensitive period of plant development and cause the loss of emerging leaves and reproductive organs (Inouye 2000).

The loss of foliage and reproductive organs has serious consequences on plant performance. The loss of foliage affects the plant's carbon budget in two ways. First, photosynthesizing leaves are lost and cannot assimilate carbon, hence, the start of a net carbon gain is delayed for the subsequent growth period. Second, the lost foliage needs to be replaced by new foliage at the cost of carbon reserves. For example, significant reductions in annual productivity have been found in tree ring studies in response to late spring-frost damage (Dittmar et al. 2006; Sangüesa-Barreda et al. 2019, 2021; Vitasse et al. 2010) probably due to a delayed spring phenology and significant reduction in photosynthesis (Wang et al. 2025). Using recent bomb radiocarbon, D'Andrea et al. (2019) found that temperate trees need to access decade-old carbon reserves to maintain metabolic activity and to re-flush new leaves after a late spring-frost event. Whereas lost leaves can be substituted by new foliage, a late spring-frost event prevents any reproductive success in the same year if the flowers are lost. Therefore, the entire potential reproductive success can be jeopardized by a late spring-frost and greatly limits the establishment of certain sensitive species in late spring-frost prone regions (Augspurger 2011; Dittmar et al. 2006; Príncipe et al. 2017). In general, we know that the damage caused by late spring-frost depends on the species-specific leaf phenology regulating leaf unfolding and flowering, the species-specific resistance to freezing, and on local adaptations of populations to the two mentioned factors (Bigler and Bugmann 2018). The phenological timing of e.g. leaf unfolding comes with a tradeoff between the advantage of assimilating already in early spring and achieving an overall higher carbon gain (Salmela et al. 2013), but a higher risk of late spring-frost damage (Taschler and Neuner 2004; Mura et al. 2025). Unfortunately, physiological damage caused by late spring-frost events is still not well studied (Ummenhofer and Meehl 2017), but a better understanding of species-specific responses would greatly assist in quantifying shifts in plant population and community assemblage under climate change.

Species-specific information on late spring-frost sensitivity mostly comes from observational studies, depending on long-term phenological records, and are based on visual estimates of late spring-frost damage during natural frost events (e.g. Augspurger 2011; Augspurger 2013; Mura et al. 2022). In particular, the dependency on natural events slows down the screening of species for their late spring-frost tolerance and hinders predictions about migrating species and their capacity to establish in future warmer but still late spring-frost prone regions. A variety of in vitro methods exist to quantify frost damage on leaf tissue, such as the assessment of the electrolyte leakage (Mura et al. 2025; van Zuijlen et al. 2024) or differential scanning calorimetry (Švara et al. 2025). However, here we examine an in vitro method that has been implemented successfully to quantify freezing tolerance of plant species, mostly alpine (Taschler and Neuner 2004; Neuner and Pramsohler 2006; Neuner et al. 2013; Bucher et al. 2019) and boreal plants (e.g. Lamontagne et al. 2000), but also on temperate and Mediterranean trees (Perks et al. 2004; Fernández et al. 2008). This quantitative method takes advantage of the temperature sensitivity of the photosystem II (PSII). The PS II is a pigment-protein complex within the thylakoid membranes of the chloroplasts and the most temperature-sensitive component of photosynthesis. Temperature extremes can cause irreversible damage to the PSII and reduce its photochemical efficiency (Cunningham and Read 2006). The photochemical efficiency of the PSII can be measured via chlorophyll fluorescence which expresses the relationship between the variable and maximum chlorophyll fluorescence (F_v/F_m). Criticism of in vitro methods to estimate the thermal dependency of the PSII is the representativeness of those measures for damage occurring during natural events (e.g. for heat resistance: Winter 2024; Winter et al. 2025).

Here, we examine the relationship between in vitro-induced F_v/F_m decline, the in vivo decline of F_v/Fm, and leaf necrosis after a naturally occurring late spring-frost event in three Central European tree species. Our main goal was to show if the in vitro assessed magnitude of frost damage reflects the frost damage observed during a natural cold snap.

2 Material and methods

2.1 Study site and botanical material

The study was carried out on trees on the campus of the University of Bayreuth, Northern Bavaria in Germany (49.9261188° N, 11.5841229° E). Bayreuth is characterized by a transitional climate between oceanic and continental conditions (Zsolnay et al. 2023) with a mean annual precipitation rate of 960 mm and a mean annual temperature of 8.9 °C at an elevation of 340 m a.s.l. (Kunert et al. 2024). Late spring-frost events are common in the area around the 11th of May (Fig. 1). This time of year is folklorically known as the 'Ice Saints' and is believed to bring a brief spell of colder weather including the last frosts during the nighttime in spring in many areas of the Northern Hemisphere.

In this study, we focused on three tree species where long-term phenological information is available (DWD 2025, station identification number: 11454, Fig. 2). Namely, we

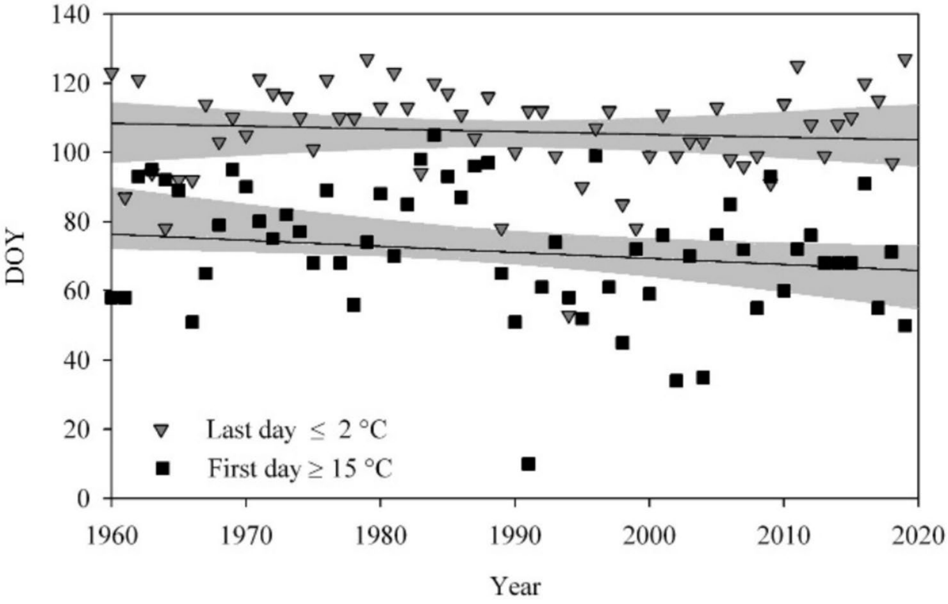

Fig. 1 Changes in spring temperatures indicated by the first warm day of the year (DOY; black squares: maximum daily temperature ≥ 15 °C; $y = -0.285x + 639.58$, $R^2 = 0.07$, $p = 0.003$) and the last cold day (grey triangle: minimum daily temperatures ≤ − 2 °C; $y = -0.013x + 132.19$, $R^2 = 0.007$, $p = 0.370$) since 1960. Grey shaded area indicates the 95% confidence interval of the linear regression

investigated European beech (*Fagus sylvatica*), European Ash (*Fraxinus excelsior*), and pedunculate oak (*Quercus robur*). For those three species, the day of full leaf expansion has been recorded since 1961 (DWD 2021). In mid-April a cold snap was predicted by the weather forecast to happen on the 21st of April 2024 and the following days (Fig. 3). Hence, we decided to sample young leaves to test in vitro their frost tolerance on April 17th, 2024. Therefore, we collected one fully sun-exposed branch from four individuals per tree species from tree individuals growing either in the Ecological-Botanical Garden of the University of Bayreuth or the adjacent University campus. All trees had a diameter of breast height between 25 and 45 cm. The material was bagged and immediately carried to the laboratory and prepared for the in vitro frost treatment. In the laboratory, branches were re-cut to ensure hydration during the entire duration of the sample process. Branches were placed in water-filled buckets and covered with opaque plastic bags until all the leaf material needed was collected.

2.2 In vitro assessment of late spring-frost tolerance

Per individual, we chose eight healthy leaves by measuring the initial maximum photosynthetic efficiency (F_v/F_m, between 0.83 and 0.75, Kunert et al. 2022) with a chlorophyll fluorometer (MINIPAM; Walz, Effeltrich, Germany). The leaves were previously dark-adapted for 30 min. In the following, leaves were randomly assigned to one of eight temperature treatments. Temperature treatments were at − 16, − 8, − 4, − 2, 0, + 2, + 4 and + 10 °C. Leaf discs, 3 cm in diameter, were cut from the leaves. The leaf discs were placed on stiff plastic sheets, fixated with perforated medical tape (Transpore™, 3 M™ GmbH, Austria) on the plastic sheets, and covered with moist Miracloth (Merck, Darmstadt, Germany). We added two drops of "Snomax" solution (Snomax LLC, Englewood, USA) to the Mortcloth tissue. Snomax is based on an extraction from an ice-nucleation-active (INA) bacterium, namely, *Pseudomonas syringae* Van Hall. Snomax acts therefore as an extrinsic nucleator to induce uniform ice nucleation between − 2 °C and − 3 °C (Ralser et al. 2024). This avoids treatment artifacts due to supercooling by inducing a heterogenic ice nucleation (Mittelstädt & Rudolph 1998; Wisniewski et al. 2014) what allows to mimic the earliest possible freezing damage in plants. We used commercial freezers (Liebherr GX 823–21, Biberach an der Riß, Germany) to perform the temperature treatment. The freezers were combined with a heating system to control the temperature in each drawer of the freezers. Each compartment was equipped with a ventilated heater (ventilated car heater 150W, Shenzhen Caiqi Digital Technology, Shenzhen, China). The heaters allow for holding a stable treatment temperature in each compartment. The heaters were controlled with a microcontroller (Arduino Mega, Arduino, Ivrea, Italy) in combination with a thermostat allowing to change the temperature in the compartment. This setup allowed us to cool at naturally occurring cooling rates of 2 °C h^{-1} (Neuner et al. 2013) until the target temperature was reached. The target temperature was kept for 4 h, and leaf discs were thawed at a rate of 2 °C h^{-1}. Leaf discs were incubated under controlled conditions (15 °C, ~ 20 µmol m^{-2} s^{-1} light) for 24 h (Kunert et al. 2022). The recovery of F_v/F_m was measured after a 30-min dark adaptation period. We used a log-logistic curve for the F_v/F_m response as described by Kunert & Hajek (2022):

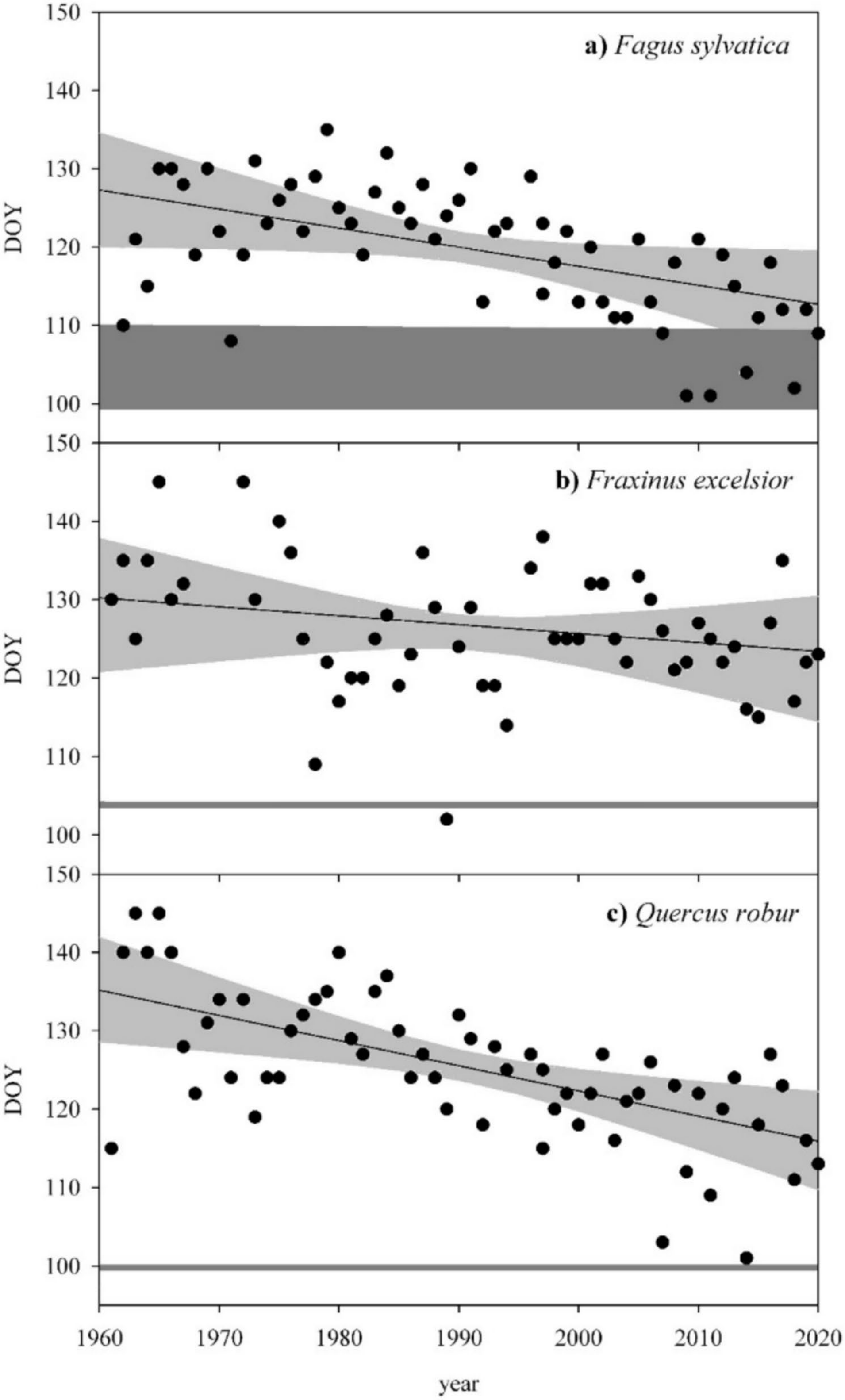

Fig. 2 Timing of leaf unfolding of the three investigated tree species since 1961 (DWD 2025, station 11,454). Long term observations of the spring phenology of (a) *Fagus sylvatica* ($y = -0.243x + 603.0$, $R^2 = 0.26$, $p < 0.001$), (b) *Fraxinus excelsior* ($y = -0.116x + 356.9$, $R^2 = 0.06$, $p = 0.079$), and (c) *Quercus robur* ($y = -0.320x + 762.7$, $R^2 = 0.40$, $p < 0.001$). Dark grey bar indicates the bud break in .2024. Grey shaded area indicates the 95% confidence interval of the linear regression

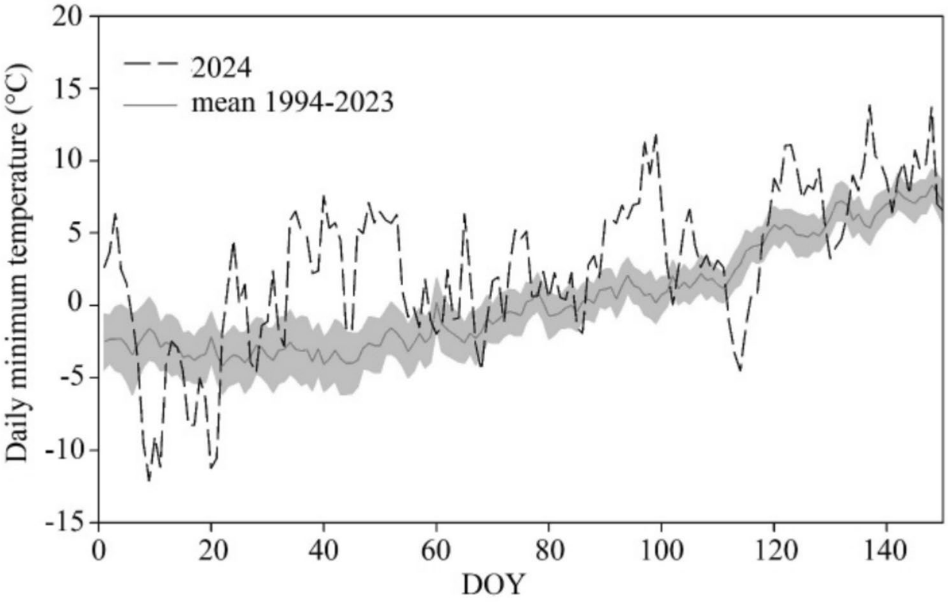

Fig. 3 Course of the daily minimum temperature during springtime measured at the climate station in the Ecological-botanical garden of the University of Bayreuth. The gray line indicates the average minimum temperature over the last 30 years (1994 to 2023, grey shaded area indicates the 95% confidence interval). The black dashed line shows the daily minimum temperatures in 2024

$$F_v/F_m = c + \frac{d - c}{1 + Exp[bLog[T/LT50]]} \tag{1}$$

where T represents the temperature treatment, c is the F_v/F_m of the lower plateau and d is the higher plateau. LT50 represents the turning point of the function and is the lethal temperature at which F_v/F_m reached 50% of the total decrease. Parameter b is the slope of the curve at T = LT50. We further extracted the effective dose at 95% loss of the quantum use efficiency (LT95). Dose–response curves were fitted using the ‘modelFit’ function of the ‘drc’ package using the LL.4 function (Ritz et al. 2015) in R. Akaike’s information criterion was used to decide which model would provide the best fit (European beech and pedunculate oak: W2.3, European ash: W2.4). The analysis was performed in R, version 4.3.1 (R Core Team 2023).

2.3 Assessment of natural frost damage

We assessed the natural frost damage three times after the cold snap. These measurements took place on April 22nd (113), April 25th (116), and May 2nd 2024 (123). For this purpose, ten leaves were collected from each of the tree individuals and stored in a plastic bag with moist tissue to avoid dehydration. In the laboratory, the leaves were dark-adapted for 30 min, and F_v/F_m was measured with the chlorophyll fluorometer. Necrotic leaf damage was visually assessed during the sampling process based on the percentage of damage relative to total leaf area.

2.4 Data and statistical analysis

We harvested long term climate and phenological data from the German Meteorological Service (DWD 2025, station 11,454). We applied linear regressions to assess the long tom development of springtime temperatures and leaf phenologies. To assess species differences in LT50 we fitted a four-parameter log-logistic function to species pairs. We then compared the deviation from the species-specific dose–response curves with an approximate F-test (Ritz et al. 2015) when the null hypothesis was rejected, the ‘compParm’ function was applied to estimate species differences in LT50. The agreement of in vivo and in vitro estimates of were compared with a linear regression. The data analysis was conducted using the R program, version 4.3.1 (R Core Team 2023).

3 Results

3.1 Longterm spring climate and leaf phenology

A springtime warming trend can be observed in Northern Bavaria with the first warm day (minimum temperature 15 °C and warmer) occurring earlier since the 1960ies (Fig. 1, slope of the regression − 0.113, $p = 0.003$). As warmer temperatures occur earlier and earlier in spring, various phenological events, such as bud break and leaf unfolding have been observed to happen leaves earlier in the season (Fig. 2). Despite the warming trend, there is a persistent risk of late spring-frost events around the 11th of May (DOY 132, Fig. 1) when the nighttime temperature drops to − 2 °C and below (slope of the linear regression 0.030, $p = 0.360$).

Besides the climatic warming trend described for the first day with a maximum temperature of 15 °C and warmer, a significant earlier leaf unfolding for two of the three (European beech and pedunculate oak) can be observed in the study area since 1961. During the study year 2024, leaf unfolding happened earlier than in the last years (Fig. 2, red line). The leaf unfolding of European beech was recorded between the 7th and 19th of April (DOY 98 and 110), on the 13th of April in European Ash (DOY 104), and on the 9th of April in pedunculate oak (DOY 100) (for a definition of leaf unfolding see the protocol of the DWD 2021). This early leaf unfolding was probably induced by the warm temperatures during the previous weeks, minimum daily temperatures were several degrees above the long-term mean (Fig. 3).

3.2 In vitro frost tolerance

There was a large variation in the in vitro tested tolerance to late spring-frost among the three species. The least frost-tolerant species was European Ash. Leaves of European ash lost 50% of their photosynthetic quantum efficiency (LT50) above 0 °C at + 0.60 ± 0.26 °C. 95% of F_v/F_m was lost at − 0.40 ± 0.36 °C (Fig. 4). European beech tolerated lower temperatures and had an LT50 of -0.17 ± 9.99 °C. However, the slope of the dose–response curve of European beech was comparably flat indicating a very slow decline in F_v/F_m. LT95 of European beech was reached with − 22.44 ± 31.35 °C at low temperatures. Pedunculate oak was the most tolerant species. LT50 of pedunculate oak was at − 2.29 ± 1.11 °C and LT95 at − 6.81 ± 3.53 °C. However, the z-test suggested that the LT50 values of all three species were no significantly different (all t-values < 1.89 and all p values > 0.084).

3.3 Natural frost damage

The forecasted cold snap occurred on April 21st of 2024 (DOY 112, Fig. 3 and 4). The minimum air temperature in the morning hours on April 21st of 2024, was -1.06 °C. On the 22nd of April, the temperature dropped even lower to -3.28 °C. The cold snap induced a fatal decline in European ash. After exposure to low temperatures during the previous nights, F_v/F_m dropped to 5.9%, relative to the maximum observed F_v/F_m during the in vitro measurements on the 17th of April 2024 (Table 1). The cold snap induced less damage in European beech and pedunculate oak. F_v/F_m declined to 46.9% of the maximum in European beech and to 53.5% in pedunculate oak. After this initial cold snap, a complete die-off of the leaves was observed in European ash, accounting for 100% necrotic leaves (Fig. 4). For the other two species, necrotic leaves made up to approximately 50% of the foliage on the 22nd of April. In the following night the temperature went further down to − 4.51 °C during the morning hours on the 23rd of April and the minimum temperature was at − 1.35 °C on the 24th of April. When we remeasured the chlorophyll fluorescence on the 25th of April, the temperatures were already in the positive range during the night before. However, the F_v/F_m declined further in all species and was at 46.8%, 36.0%, and 1.5% of the maximum in pedunculate oak, European beech, and European Ash, respectively. We estimated more than 50% of the foliage had visible necrosis in pedunculate oak and European beech (Fig. 5). We recorded 100% of dead leaves for European ash. We re-assessed F_v/F_m on the 2nd of May and the surviving leaves and/or non-necrotic parts of the leaves recovered partly their photochemical efficiency. In European beech, F_v/F_m recovered to 85.0% of the previously observed maximum, and in pedunculate oak to 83.1%. All European ash leaves were dead and showed no sign of photochemical activity. In vitro measurements explained 86% of the in vivo observed damage caused by the low temperature during the preceding the nights (Fig. 6, $y = 0.955x - 0.05$, $R^2 = 0.86$, $p = 0.008$).

4 Discussion

4.1 Overview

In vitro measurements decline of photosynthetic quantum use efficiency pictured well the actual frost damage observed in the three different temperate tree species in this study. This gives way to using the applied technique for the quantitative assessment of tree species for their late spring-frost tolerance. We could further show that there are species-specific patterns of late spring-frost tolerance. Species characterized by an average earlier bud break are less prone to damage than species that are adapted to reduce the risk of late spring-frost damage by a late bud break. Late spring-frost tolerant species might benefit most from the prolonged growth period caused by climate change.

4.2 Comparison of in vitro and in vivo damage

The in vitro-induced frost damage reflected the species-specific damage observed after the cold snap. The observed decline of F_v/F_m in European Ash to 5.9% of the maximum after an exposure to − 3.28 °C on the morning of the 22nd of April occurred during the in vitro measurements already at − 0.34 ± 0.35 °C. However, the absolute F_v/F_m (0.045 ± 0.018, Table 1) suggests that leaves were dead after exposure to the low temperatures. European beech and pedunculate oak showed a decline that was equal to the predicted values of an in vitro exposure to − 1.00 ± 10.93 °C

Fig. 4 In vitro induced decline of F_v/F_m measured on a) *Fagus sylvatica*, b) *Fraxinus excelsio*r, and c) *Quercus robur* from samples collected on the 17th, April 2024 before the late-frost event. The solid black line represents the dose response curve of F_v/F_m to the temperature treatment. The grey band indicates the 95% confidence interval of the dose response curve and the small open circles the four replicates per treatment temperature. The LT50 values are indicated as red dots on the dose response curves. The filled symbols in (a), (b) and (c) show the in vivo measurements after the frost event (Full circle: 22nd of April 2024; full triangle: 25th of April 2024; full square: 2nd of May 2024). Values are aligned to d) the minimum daily temperature in the preceding 24 h before the in vivo measurements to show the extent of damage caused by a minimum temperature

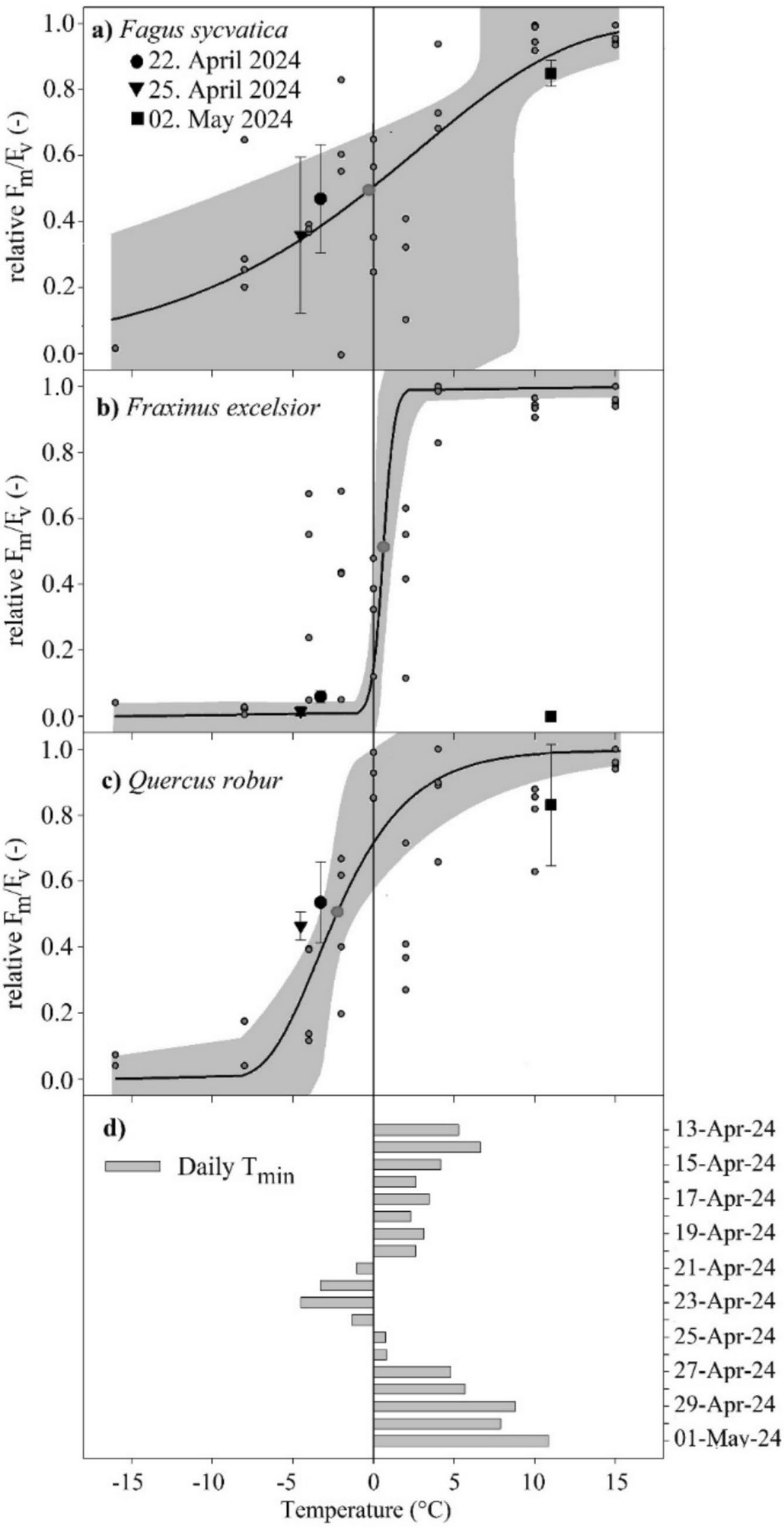

Table 1 Minimum temperatures reached during the cold snap and the following days when in vivo measurement took place. Mean F_v/F_m values and standard derivation for the three tree species, measured in vivo on ten leaves per species (n = 40), on three different dates following the first late frost on April 21st of 2024

Date	Minimum temperature	*Fagus sylvatica*			*Fraxinus excelsior*			*Quercus robur*		
	°C	F_v/F_m	SD	$\%_{max}$	F_v/F_m	SD	$\%_{max}$	F_v/F_m	SD	$\%_{max}$
21/04/2024	− 1.06	–	–	–	–	–	–	–	–	–
22/04/2024	− 3.28	0.382	0.164	46.9	0.045	0.018	5.9	0.382	0.122	53.5
23/04/2024	− 4,51	–	–	–	–	–	–	–	–	–
24/04/2024	− 1,35	–	–	–	–	–	–	–	–	–
25/04/2024	0.74	0.293	0.236	36.0	0.012	0.013	1.5	0.331	0.042	46.4
02/05/2024	11.11	0.692	0.039	85.0	0	0	0	0.593	0.184	83.1

Fig. 5 Photographs of the frost damage observed in vivo on the foliage in (a) *Fagus sylvatica*, (b) *Fraxinus excelsior* and (c) *Quercus robur*. The picture was taken on the 22nd of April 2024

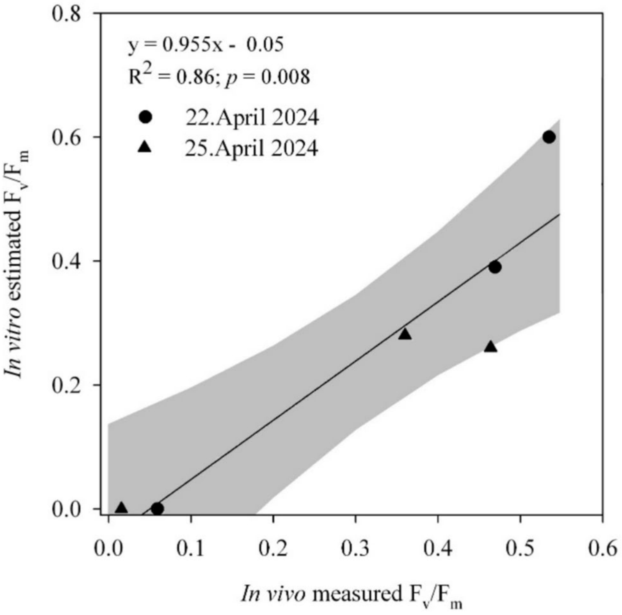

Fig. 6 Linear relationship in vitro estimated decline in F_v/F_m and in vivo measured values after the frost event on the 22nd of April 2024 (circles) and the 25th of April 2024 (triangle). In vitro estimated decline in F_v/F_m was calculated using the minimum air temperatures in the two nights respectively. The grey shaded area indicates the 95% confidence interval

and − 1.97 ± 1.22 °C, respectively. Those estimated temperatures for the observed damage extracted from the dose–response curves are lower than the actual temperatures. We see two possible explanations for this minimal divergence of the in vitro and the in vivo values. First, target temperatures during the in vitro assessment of the late spring-frost tolerance were set to last 4 h, whereas the minimum temperature of − 3.28 °C during the night of the cold snap lasted less than an hour. The duration of the exposure to thermal stress is critical for the manifestation of the magnitude of the functional impairment (Neuner and Buchner 2023). Second, the measurements were conducted on the morning of the 22nd of April only a few hours after the lowest temperature, we assume that the damage associated with the freezing stress had not yet fully developed. The full physiological development of thermal stress to reach a measurable level can take several hours or even days. (e.g. Neuner et al. 2013; Winter et al. 2025). Further, if we take the standard errors of the estimated into account, then the in vitro values and the in vivo values agree well. Three days later the 25th of April, trees had experienced an absolute minimum temperature of − 4.51 °C. The entire foliage of European ash remained dead. We estimated that the 36.0.% and 46.4% remaining of the maximum F_v/F_m in European beech and pedunculate oaks would have been caused by an in vitro treatment at a temperature of − 4.07 ± 14.52 °C and − 2.61 ± 1.06 °C, in the two species respectively. Hence, the further temperature drop caused a further decline in F_v/F_m on the living trees. The in vitro LT50 values also reflected this decline.

Our in vitro estimated LT50 values show that significant damage can be caused by temperatures already around the water freezing point at 0 °C. Sakai and Larcher (1987) reported that leaves of most deciduous woody plants have frost damage after air temperatures well above − 5 °C. In European beech and different oak species fatal late spring-frost damage with almost complete defoliation was described by a minimum temperature of − 4.6 °C leaf loss (Rubio-Cuadrado, et al. 2021), however similar has been observed in beech with temperatures around − 2.3 °C (Dittmar et al. 2006). However, these are observations from the field during extreme events and defoliation was always on a large scale and do not give any information if significant damage can happen earlier. Therefore, in vitro studies are important to predict late spring-frost damage as functional impairment of the photochemistry cannot be visually assessed but can affect plant performance. In a study assessing the late spring-frost tolerance of five temperate tree species, in vitro estimated LT50 ranged between − 8.5 °C and − 4.8 °C during leaf out (Lenz et al. 2013), with European beech the most sensitive tree species. This is far off the range estimated in our study, but our LT50 estimates are much closer to the real-world damage. To our knowledge, the study by Lenz et al. (2013) did not add an extrinsic nucleator and this might have caused artifacts due to supercooling (Mittelstädt and Rudolph 1998; Wisniewski et al. 2014) and might not show the earliest temperature point of causing severe tissue damage.

4.3 Species-specific late spring-frost tolerance

Pedunculate oak was the species characterized by the highest late spring-frost tolerance (LT50: − 2.29 ± 1.11 °C) of the three species investigated. Pedunculate oak was further the species with the steepest slope in the phenological shifts over the last 60 years (Fig. 2). We speculate that the high late spring-frost tolerance allows the species to unfold leaves earlier as it has a reduced risk of suffering from late spring-frost damage. Pedunculate oak might be a real beneficiary of climate change taking advantage of the prolongation of the growth period. However, earlier leaf unfolding might increase the risk of late spring-frost damage (Mura et al. 2022) during future frost events. The species with the next highest late spring-frost tolerance was European beech in our study. European beech is described as very sensitive to late spring-frost (Ellenberg 1978). Our estimates of LT50 were close to the water freezing point, equally like the LT50 values of European ash which is also classified as a very late spring-frost sensitive species. In contrast, Ellenberg (1978) described pedunculate oaks as moderately sensitive, what is basically agreeing with our findings. What is striking are the large standard errors of the LT50 and LT95 values in European beech. This large deviation lies in the non-simultaneous bud break and the phenological stage has great influence on the late spring-frost tolerance (Lenz et al. 2013). In some individuals leaf unfolding occurred on the 7th April, but in some leaves, unfolding was delayed until the 19th of April. This non-simultaneous behavior confirms the high phenological plasticity found in European beech, with high plasticity within local populations or even plasticity on stand level (Vitasse et al. 2010; Capdevielle-Vargas et al. 2015). A wide range of phenological patterns, in particular leaf unfolding, can therefore be considered as a strategy to deal with changing climate conditions or environmental variability (Capdevielle-Vargas et al. 2015; Frank et al. 2017). Oaks usually show similar phenological plasticity as European beech (Vitasse et al. 2010), however, the individuals studied of pedunculate oak showed a low plasticity in the timing of leaf unfolding. In European ash, within population leaf unfolding seems to occur simultaneously and varies more between different provenances (Rosique-Esplugas et al. 2022). Unfortunately, we do not have any information on the type of provenance of our studies individuals, but the tight timing of leaf unfolding leads to the assumption that they were all from the same provenances.

Conclusions

We found considerable variation in the late spring-frost tolerance in the investigated temperature tree species. The good agreement of the in vitro predicted functional impairment and the in vivo observed damage allow us to recommend the in vitro method to test species on larger scale. This will be of great value to test more drought and heat-resistant new tree species or provenances for their sensitivity to late spring-frost and thus to quantify their suitability for forest adaptation purposes in late spring-frost prone areas.

Acknowledgements We would like to thank Gilbert Neuner, Othmar Buchner, and Mattias Stegner for helpful instructions on measuring freezing tolerance in plants. We are thankful to Wolfgang Babel from the micrometeorology group of the University of Bayreuth for providing the climate data from the climate station in the Ecological-botanical Garden. We want to thank Burkhard Stumpf for technical support. Further, we are very thankful to the valuable comments of two anonymous reviewers.

Authors contribution NK designed the study. NK and SG collected the data. NK performed the statistical analysis and wrote the manuscript.

Funding Open Access funding enabled and organized by Projekt DEAL. Open Access funding enabled and organized by Projekt DEAL.

Declarations

Conflict of interest The authors declare no conflict of interest.

References

Augspurger CK (2011) Frost damage and its cascading negative effects on *Aesculusglabra*. Plant Ecol 212(7):1193–1203. https://doi.org/10.1007/s11258-011-9897-z

Augspurger CK (2013) Reconstructing patterns of temperature, phenology, and frost damage over 124 years: spring damage risk is increasing. Ecology 94(1):41–50. https://doi.org/10.1890/12-0200.1

Barichivich J, Briffa KR, Myneni RB, Osborn TJ, Melvin TM, Ciais P, Piao SL, Tucker C (2013) Large-scale variations in the vegetation growing season and annual cycle of atmospheric CO_2 at high northern latitudes from 1950 to 2011. Glob Chang Biol 19(10):3167–3183. https://doi.org/10.1111/gcb.12283

Bigler C, Bugmann H (2018) Climate-induced shifts in leaf unfolding and frost risk of European trees and shrubs. Sci Rep 8(1):9865. https://doi.org/10.1038/s41598-018-27893-1

Bucher SF, Feiler R, Buchner O, Neuner G, Rosbakh S, Leiterer M, Römermann C (2019) Temporal and spatial trade-offs between resistance and performance traits in herbaceous plant species. Environ Exp Bot 157:187–196. https://doi.org/10.1016/j.envexpbot.2018.10.015

Capdevielle-Vargas R, Estrella N, Menzel A (2015) Multiple-year assessment of phenological plasticity within a beech (*Fagus sylvatica* L.) stand in southern Germany. Agric for Meteorol 211:13–22. https://doi.org/10.1016/j.agrformet.2015.03.019

Cunningham SC, Read J (2006) Foliar temperature tolerance of temperate and tropical evergreen rain forest trees of Australia. Tree Physiol 26(11):1435–1443. https://doi.org/10.1093/treephys/26.11.1435

D'Andrea E, Rezaie N, Battistelli A, Gavrichkova O, Kuhlmann I, Matteucci G, Moscatello S, Proietti S, Scartazza A, Trumbore S, Muhr J (2019) Winter's bite: beech trees survive complete defoliation due to spring late-frost damage by mobilizing old C reserves. New Phytol 224(2):625–631. https://doi.org/10.1111/nph.16047

Dittmar C, Fricke W, Elling W (2006) Impact of late frost events on radial growth of common beech (*Fagus sylvatica* L.) in Southern Germany. Eur J Forest Res 125(3):249–259. https://doi.org/10.1007/s10342-005-0098-y

Düsterhöft E, Burgesmeir B, Mayer J, Nitschky N, Schächer L, Kunert N (2024) Spätfröste – Probleme mit neuen Baumarten. AFZ – der Wald 5:20–23

DWD (2021) Anleitung für die phänologischen Beobachter des Deutschen Wetterdienstes, Vorschriften und Betriebsunterlagen Nr. 17, Deutscher Wetterdienst Offenbach 1991, überarbeitet 2021.

DWD (2025) Deutscher Wetterdienst, Climate Data Center. Available from https://opendata.dwd.de/

Ellenberg H (1978) Vegetation Mitteleuropas mit den Alpen in ökologischer Sicht. Verlag Eugen Ulmer, Stuttgart, p 82

Fernández M, Alejano R, Domínguez L, Tapias R (2008) Temperature controls cold hardening more effectively than photoperiod in four Mediterranean broadleaf evergreen species. Tree and Forestry Science and Biotechnology 2(1):43–49

Frank A, Pluess AR, Howe GT, Sperisen C, Heiri C (2017) Quantitative genetic differentiation and phenotypic plasticity of European beech in a heterogeneous landscape: indications for past climate adaptation. Perspect Plant Ecol Evol Syst 26:1–13. https://doi.org/10.1016/j.ppees.2017.02.001

Inouye DW (2000) The ecological and evolutionary significance of frost in the context of climate change. Ecol Lett 3(5):457–463. https://doi.org/10.1046/j.1461-0248.2000.00165.x

IPPC (2019) Summary for Policymakers. In: Climate Change and Land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems [P R Shukla, J Skea, E Calvo Buendia, V Masson-Delmotte, H - O Pörtner, D C Roberts, P Zhai, R Slade, S Connors, R van Diemen, M Ferrat, E Haughey, S Luz, S Neogi, M Pathak, J Petzold, J Portugal Pereira, P Vyas, E Huntley, K Kissick, M Belkacemi, J Malley, (eds.)].

Kunert N, Hajek P (2022) Shade-tolerant temperate broad-leaved trees are more sensitive to thermal stress than light-demanding species during a moderate heatwave. Trees, Forests and People 9:100282. https://doi.org/10.1016/j.tfp.2022.100282

Kunert N, Hajek P, Hietz P, Morris H, Rosner S, Tholen D (2022) Summer temperatures reach the thermal tolerance threshold of photosynthetic decline in temperate conifers. Plant Biol 24(7):1254–1261. https://doi.org/10.1111/plb.13349

Kunert N, Münchinger IK, Hajek P (2024) Turgor loss point explains climate-driven growth reductions in trees in Central Europe. Plant Biol. https://doi.org/10.1111/plb.13687

Lamontagne M, Bigras FJ, Margolis HA (2000) Chlorophyll fluorescence and CO_2 assimilation of black spruce seedlings following frost in different temperature and light conditions. Tree Physiol 20(4):249–255. https://doi.org/10.1093/treephys/20.4.249

Lenz A, Hoch G, Vitasse Y, Körner C (2013) European deciduous trees exhibit similar safety margins against damage by spring freeze events along elevational gradients. New Phytol 200(4):1166–1175. https://doi.org/10.1111/nph.12452

Menzel A, Fabian P (1999) Growing season extended in Europe. Nature 397(6721):659. https://doi.org/10.1038/17709

Mittelstädt H, Rudolph K (1998) Ice nucleation activity of strains from *Pseudomonas syringae* pathovars atrofadens and syringae, mainly isolated from cereals. J Phytopathol 146(11–12):581–586. https://doi.org/10.1111/j.1439-0434.1998.tb04758.x

Mura C, Buttò V, Silvestro R, Deslauriers A, Charrier G, Raymond P, Rossi S (2022) The early bud gets the cold: diverging spring phenology drives exposure to late frost in a *Picea mariana* [(Mill.) BSP] common garden. Physiol Plant 174(6):e13798. https://doi.org/10.1111/ppl.13798

Mura C, Charrier G, Buttò V, Delagrange S, Surget-Groba Y, Raymond P, Rossi S, Deslauriers A (2025) Local conditions have greater influence than provenance on sugar maple (*Acer saccharum* Marsh.) frost hardiness at its northern range limit. Tree Physiol 45(1):tpae167. https://doi.org/10.1093/treephys/tpae167

Neuner G, Buchner O (2023) The dose makes the poison: the longer the heat lasts, the lower the temperature for functional impairment and damage. Environ Exp Bot 212:105395. https://doi.org/10.1016/j.envexpbot.2023.105395

Neuner G, Pramsohler M (2006) Freezing and high temperature thresholds of photosystem 2 compared to ice nucleation, frost and heat damage in evergreen subalpine plants. Physiol Plant 126(2):196–204. https://doi.org/10.1111/j.1399-3054.2006.00605.x

Neuner G, Erler A, Ladinig U, Hacker J, Wagner J (2013) Frost resistance of reproductive tissues during various stages of development in high mountain plants. Physiol Plant 147(1):88–100. https://doi.org/10.1111/j.1399-3054.2012.01616.x

Perks MP, Osborne BA, Mitchell DT (2004) Rapid predictions of cold tolerance in Douglas-fir seedlings using chlorophyll fluorescence after freezing. New for 28(1):49–62. https://doi.org/10.1023/B:NEFO.0000031331.08847.49

Piao SL, Liu Q, Chen AP, Janssens IA, Fu YS, Dai JH, Liu LL, Lian X, Shen MG, Zhu XL (2019) Plant phenology and global climate change: current progresses and challenges. Glob Change Biol 25(6):1922–1940. https://doi.org/10.1111/gcb.14619

Príncipe A, van der Maaten E, van der Maaten-Theunissen M, Struwe T, Wilmking M, Kreyling J (2017) Low resistance but high resilience in growth of a major deciduous forest tree (*Fagus sylvatica* L.) in response to late spring frost in southern Germany. Trees 31(2):743–751. https://doi.org/10.1007/s00468-016-1505-3

R Core Team (2023) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ralser M, Stegner M, Neuner G (2024) When water turns to ice: control of ice volume and freezing dynamics as important aspects of cold acclimation. Environ Exp Bot 227:105957. https://doi.org/10.1016/j.envexpbot.2024.105957

Ritz C, Baty F, Streibig JC, Gerhard D (2015) Dose-response analysis using R. PLoS ONE 10(12):e0146021. https://doi.org/10.1371/journal.pone.0146021

Rosique-Esplugas C, Cottrell JE, Cavers S, Whittet R, Ennos RA (2022) Clinal genetic variation and phenotypic plasticity in leaf phenology, growth and stem form in common ash (*Fraxinus excelsior* L.). Forestry 95(1):83–94. https://doi.org/10.1093/forestry/cpab026

Rubio-Cuadrado Á, Gómez C, Rodríguez-Calcerrada J, Perea R, Gordaliza GG, Camarero JJ, Montes F, Gil L (2021) Differential response of oak and beech to late frost damage: an integrated analysis from organ to forest. Agric for Meteorol 297:108243. https://doi.org/10.1016/j.agrformet.2020.108243

Sakai A, Larcher W (1987) Frost Survival of Plants: Responses and Adaptation to Freezing Stress. Ecological Studies 62, Springer-Verlag, New York.

Salmela MJ, Cavers S, Cottrell JE, Iason GR, Ennos RA (2013) Spring phenology shows genetic variation among and within populations in seedlings of Scots pine (*Pinus sylvestris* L.) in the Scottish Highlands. Plant Ecol Divers 6:523–536. https://doi.org/10.1080/17550874.2013.795627

Sangüesa-Barreda G, Villalba R, Rozas V, Christie DA, Olano JM (2019) Detecting *Nothofagus pumilio* growth reductions induced by past spring frosts at the northern Patagonian *Andes*. Front Plant Sci 10:1413. https://doi.org/10.3389/fpls.2019.01413

Sangüesa-Barreda G, Di Filippo A, Piovesan G, Rozas V, Di Fiore L, García-Hidalgo M, García-Cervigón AI, Muñoz-Garachana D, Baliva M, Olano JM (2021) Warmer springs have increased the frequency and extension of late-frost defoliations in southern European beech forests. Sci Total Environ 775:145860. https://doi.org/10.1016/j.scitotenv.2021.145860

Schulze ED, Hartmann H, Lambert S, Weber U (2020) Folgen von Spätfrösten bei der Buche in montanen Lagen. AFZ der Wald 18:46–48

Švara A, Upchurch D, Goderis B, Colaers M, Deckers T, Keulemans W, Abts W, De Storme N, De Maeyer L (2025) Differential scanning calorimetry: a novel approach to study spring frost tolerance in pome tree floral tissues. Fruit Res. https://doi.org/10.48130/frures-0025-0003

Taschler D, Neuner G (2004) Summer frost resistance and freezing patterns measured *in situ* in leaves of major alpine plant growth forms in relation to their upper distribution boundary. Plant Cell Environ 27(6):737–746. https://doi.org/10.1111/j.1365-3040.2004.01176.x

Twardosz R, Walanus A, Guzik I (2021) Warming in Europe: recent trends in annual and seasonal temperatures. Pure Appl Geophys 178(10):4021–4032. https://doi.org/10.1007/s00024-021-02860-6

Ummenhofer CC, Meehl GA (2017) Extreme weather and climate events with ecological relevance: a review. Philos Trans R Soc Lond B Biol Sci 372(1723):20160135. https://doi.org/10.1098/rstb.2016.0135

van Zuijlen K, Kassel M, Dorrepaal E, Lett S (2024) Frost damage measured by electrolyte leakage in subarctic bryophytes increases with climate warming. J Ecol 112(2):220–232. https://doi.org/10.1111/1365-2745.14236

Vitasse Y, Bresson CC, Kremer A, Michalet R, Delzon S (2010) Quantifying phenological plasticity to temperature in two temperate tree species. Funct Ecol 24(6):1211–1218. https://doi.org/10.1111/j.1365-2435.2010.01748.x

Wang JM, Hua H, Guo J, Huang X, Zhang X, Yang YC, Wang DY, Guo XL, Zhang R, Smith NG, Rossi S, Peñuelas J, Ciais P, Wu CY, Chen L (2025) Late spring frost delays tree spring phenology by reducing photosynthetic productivity. Nat Clim Chang 15(2):201–209. https://doi.org/10.1038/s41558-024-02205-w

Winter K (2024) Are tropical forests approaching critical temperature thresholds? Plant Biol 26(4):495–498. https://doi.org/10.1111/plb.13638

Winter K, Krüger Nuñez CR, Slot M, Virgo A (2025) In thermotolerance tests of tropical tree leaves, the chlorophyll fluorescence parameter F(v)/F(m) measured soon after heat exposure is not a reliable predictor of tissue necrosis. Plant Biol 27(1):146–153. https://doi.org/10.1111/plb.13732

Wisniewski M, Gusta L, Neuner G (2014) Adaptive mechanisms of freeze avoidance in plants: a brief update. Environ Exp Bot 99:133–140. https://doi.org/10.1016/j.envexpbot.2013.11.011

Wolkovich EM, Cook BI, Allen JM, Crimmins TM, Betancourt JL, Travers SE, Pau S, Regetz J, Davies TJ, Kraft NJB, Ault TR, Bolmgren K, Mazer SJ, McCabe GJ, McGill BJ, Parmesan C, Salamin N, Schwartz MD, Cleland EE (2012) Warming experiments underpredict plant phenological responses to climate change. Nature 485(7399):494–497. https://doi.org/10.1038/nature11014

Zohner CM, Mo LD, Renner SS, Svenning JC, Vitasse Y, Benito BM, Ordonez A, Baumgarten F, Bastin JF, Sebald V, Reich PB, Liang JJ, Nabuurs GJ, De-Miguel S, Alberti G, Antón-Fernández C, Balazy R, Brändli UB, Chen HYH, Chisholm C, Cienciala E, Dayanandan S, Fayle TM, Frizzera L, Gianelle D, Jagodzinski AM, Jaroszewicz B, Jucker T, Kepfer-Rojas S, Khan ML, Kim HS, Korjus H, Johannsen VK, Laarmann D, Lang M, Zawila-Niedzwiecki T, Niklaus PA, Paquette A, Pretzsch H, Saikia P, Schall P, Šebeň V, Svoboda M, Tikhonova E, Viana H, Zhang CY, Zhao XH, Crowther TW (2020) Late-spring frost risk between 1959 and 2017 decreased in North America but increased in Europe and Asia. Proc Natl Acad Sci U S A 117(22):12192–12200. https://doi.org/10.1073/pnas.1920816117

Zsolnay N, Walentowitz A, Aas G (2023) Impact of climatic conditions on radial growth of non-native *Cedrus libani* compared to native conifers in Central Europe. PLoS ONE 18(5):e0275317. https://doi.org/10.1371/journal.pone.0275317