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Dealing With Two Stresses: Impact of a Damaging Spring Frost Followed by a Summer Drought on Saplings of Four Temperate Tree Species

Na Luo[1,2,3] | Yann Vitasse[1,4] | Arthur Gessler[1,5] | Manuel G. Walde[1]

[1]Ecosystem Ecology, Forest Dynamics, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland | [2]Key Laboratory for Silviculture and Conservation, Beijing Forestry University, Beijing, China | [3]School of Urban Planning and Design, Shenzhen Graduate School, Peking University, Shenzhen, China | [4]Oeschger Centre for Climate Change Research, University of Bern, Bern, Switzerland | [5]Institute of Terrestrial Ecosystems, ETH Zurich (Swiss Federal Institute of Technology), Zurich, Switzerland

Correspondence: Manuel G. Walde (manuel.walde@wsl.ch)

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ABSTRACT

Global warming increases the likelihood that temperate tree species will face damaging late spring frost (LSF) and severe summer drought during the same growing season. However, the interactive effects of these two stresses are barely explored. We investigated the physiological and growth responses of *Acer campestre*, *Fagus sylvatica*, *Quercus robur* and *Quercus petraea* saplings to artificially induced LSF and drought, focusing on stomatal gas exchange, carbon partitioning, nonstructural carbohydrates (NSCs), phenology and tree growth. LSF depleted NSCs and changed carbon allocation patterns 1 month after the event. Additionally, LSF decreased diameter increment and root growth of *A. campestre* and *F. sylvatica* in the current year. Drought affected gas exchange of all species, decreased NSCs of *A. campestre*, reduced biomass of *Q. robur*, and exacerbated the detrimental LSF effect on *Q. robur's* NSCs. Our findings indicate that saplings prioritized canopy restoration immediately after LSF, and favored reserve replenishment before growth until the end of the growing season. Furthermore, we highlight the risk that LSF and drought in the same year could push tree species beyond their physiological limits and we emphasize the importance of studying multiple stressors' interactions to better understand threshold effects that could profoundly alter forest ecosystems.

1 | Introduction

Plant species in temperate regions colonize their habitats depending on climatic and edaphic factors (Landolt et al. 2010). Trees growing in climates with distinct seasonality (e.g., temperate and boreal regions) have thus evolved to optimize the time at which they start growing in spring (i.e., spring phenology) with regard to potential damage from spring frost while remaining competitive for water, light and nutrient resources (Lenz et al. 2016b). Therefore, the spring phenology of trees growing in seasonal climates with cold winters strongly depends on species- and provenance-specific temperature and photoperiod requirements (Campioli et al. 2024).

In temperate and boreal regions, increasing spring temperatures are expected to advance the vegetation period, thereby increasing the vulnerability to frost damage (Gu et al. 2008; Augspurger 2013; Richardson et al. 2018; Zohner et al. 2020a). The vegetation of central Europe was, for instance, hit by severe and widespread late-spring frosts (LSFs) in 2017 and 2021 after

unprecedented warm temperatures during early spring before the event (Vitasse and Rebetez 2018; Lamichhane 2021; Vautard et al. 2023). LSFs reduce ecosystem productivity and could change forest composition in the longer term (Hufkens et al. 2012; Bascietto et al. 2018), which is why increasing efforts have recently been made to incorporate the impact of LSFs into dynamic vegetation models to simulate future net primary production more accurately (Meyer et al. 2024).

LSF events such as the ones observed in 2017 and 2021 can cause damages ranging from mild leaf injury to complete canopy defoliation (Hufkens et al. 2012), which eventually results in reduced transpiration, reduced carbon assimilation, and depletion of reserves because of the remobilization of stored carbon to rebuild new leaves (D'Andrea et al. 2019; Charrier et al. 2021; Baumgarten et al. 2023). However, sensitivity to LSF depends on species-specific freezing resistance and spring phenological development stage, with newly formed tissues in spring typically being more vulnerable to freezing conditions than mature leaves and shoots (Weiser 1970; Sierra-Almeida et al. 2009; Lenz et al. 2013). Although it is not clear yet how the LSF risk will evolve in the future, the frost-induced damages will likely not decrease due to the spring phenological advances caused by global warming (Vitasse and Rebetez 2018; Zohner et al. 2020b).

In contrast to LSF, the increasing frequency and severity of hot and dry periods during summer (also known as 'hot drought') are among the most evident changes induced by global warming as they cause severe damage to European beech forest stands (Schuldt et al. 2020). Furthermore, the frequency and severity of hot droughts events are projected to further increase in Europe and worldwide in the coming decades (Dai 2012; Trenberth et al. 2013; Spinoni et al. 2017), with the globally occurring heat waves in 2023 potentially being a harbinger of things to come (Perkins-Kirkpatrick et al. 2024). Consequently, the probability that the combination of LSF and summer drought affects plant species in the same year will likely increase solely due to more frequent droughts.

Hot droughts decrease ecosystem productivity and increase the vulnerability of woody species to encounter hydraulic failure (Choat et al. 2012; Choat et al. 2018; Moreno et al. 2024). Damaging LSF may exacerbate the impact of hot droughts on tree vitality, because lower carbohydrate reserves, as typically found after a damaging LSF, might limit the tree's capability to adjust its cells osmotically under drought conditions (Gersony et al. 2020) and reduce the production of defense components against insect pests and pathogens (Huang et al. 2023). Therefore, replenished nonstructural carbohydrate (NSC) pools are crucial to recover from frost damage and resist drought (Luo et al. 2024). The effects of LSF (e.g., Hufkens et al. 2012; Bascietto et al. 2018; D'Andrea et al. 2019; Chamberlain and Wolkovich 2021; Baumgarten et al. 2023) or drought (e.g., Galvez et al. 2013; O'Brien et al. 2014; Huang et al. 2019) on individuals' growth and forest stands' fitness have been well documented separately. However, to our knowledge, no study has yet investigated the combined effects of LSF and summer drought on trees' morphological and physiological attributes, even though the likelihood of these two stresses occurring in the same year is projected to increase.

Temperate tree species mainly adopt three coping strategies for freezing temperatures: escape, avoidance, and tolerance (Körner 2012; Vitasse et al. 2014b). Some species escape frost damage by having more conservative phenological traits (i.e., they are flushing late: escape strategy) (Augspurger 2009), and some developed avoidance and tolerance strategies to prevent the formation of intracellular ice in the meristematic cells (e.g. supercooling and anti-freeze proteins) during budburst (Lenz et al. 2013; Lenz et al. 2016a). Thus, a damaging LSF can cause different degrees of leaf damage due to species-specific freezing resistance and phenological stages (Augspurger 2013; Lenz et al. 2013). Further, recovery of NSC pools and growth after a damaging LSF also varies among temperate deciduous species (Baumgarten et al. 2023). Differences in species-specific drought tolerance are also known for major tree species (Hajek et al. 2022), but species-specific responses to both damaging LSF and drought events combined are largely unknown.

To fill this gap and better understand how the combination of these two stressors affects the performance and vitality of trees, we exposed potted saplings of four temperate deciduous trees (*Acer campestre*, *Fagus sylvatica*, *Quercus robur,* and *Quercus petraea*) to an artificially induced late spring frost (LSF) shortly after leaf emergence (in May) and a summer drought from July to September. In detail, we investigated the physiological and growth responses of the four species to LSF and drought, focusing on stomatal gas exchange, carbon partitioning, nonstructural carbohydrates (NSCs), phenology, and tree growth. We tested the following hypotheses:

1. Frost damages will deplete NSC reserves in storage organs such as coarse roots and stems, leading to prioritization of carbon allocation to above-ground organs to restore the foliage's photosynthetic capacity.
2. Temperate trees will prioritize the recovery of reserves before growth. Therefore, LSF- or drought-induced depletion of NSC pools will reduce tree growth during the year in which the event took place but not necessarily during the subsequent year because of the high turn-over time of NSC pools.
3. The carry-over effect of a damaging LSF will exacerbate the impact of drought in the current and subsequent year (e.g., on reduced growth).

2 | Materials and Methods

2.1 | Study Species

We applied an artificial late-spring frost (LSF) and a drought treatment on four deciduous broadleaf species with contrasting life strategies: *Acer campestre* (L.), an early-flushing, fast-growing, drought-tolerant shrub (rarely tree) species; *Fagus sylvatica* (L.), a late-flushing tree species growing at an intermediate pace susceptible to frost and drought; *Quercus robur* (L.), a late-flushing, drought tolerant tree species growing at an intermediate pace; and *Quercus petraea* (Liebl.) an intermediate-flushing, slow-growing but very drought tolerant tree species. Under natural conditions, *A. campestre* and both species from the *Quercus* genus are rarely hit by frost damage.

However, whether the chance of damaging late-spring frost increases for these species under projected climate warming remains unclear (Vitasse and Rebetez 2018). In natural Central European forests with oceanic climates and well-drained yet moist soils, *F. sylvatica* is most competitive and outcompetes the other three investigated tree species (Ellenberg 2010). This high competitiveness of *F. sylvatica* leads to a displacement of *A. campestre* to locations with sufficient light supply, such as forest edges, a displacement of *Q. robur* to locations too moist for *F. sylvatica* such as soil occasionally affected by waterlogging, and a displacement of Q. *petraea* to highly exposed and dry locations with shallow soils, even though the physiological optimum being pretty similar for all four species. (Ellenberg and Klötzli 1972; Ellenberg 2010). However, the dominance of *F. sylvatica* may decrease in the future as it is less drought-tolerant than other co-occurring species (Leuschner 2020).

In detail, *A. campestre*, *Q. petraea,* and *Q. robur* saplings were raised from local seeds by gardeners of the Swiss Federal Research Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland (47°21′N, 8°27′E, 545 m ASL). *F. sylvatica* saplings were raised from local seeds by Emme Forstbaumschule (Wiler bei Utzensdorf, Switzerland, 47° 9', 7° 33′E, 455 m ASL). As standard practice for tree nurseries in Central Europe, tree seedlings and saplings were exposed to natural environmental conditions and watered when necessary during summer to prevent water stress in 2020 and 2021. In February 2022, 240 2-year-old saplings ($n = 60$ for each species) were transplanted from small containers into 2-L pots (diameter 14 cm, height 17 cm) at the research institute using sandy soil as substrate, which consisted of 20% white peat, 20% expanded scale, 16% pumice stone, 40% quartz sand, and 4% clay. This substrate was chosen to reduce water retention and facilitate drainage. Before moving them outdoors, all potted saplings were stored in a climate chamber at 1°C until March 30 (for 22 days) to homogenize the budburst date between tree species and to delay spring phenological development by a few weeks to minimize the chance of the saplings getting hit by an actual late-spring frost (Figure 1a).

2.2 | Late-Spring Frost and Drought Treatments

We conducted a full-factorial experiment with two treatments, late-spring frost (LSF) and drought, in which 15 out of 60 individuals of each species were randomly assigned to one of four experimental conditions: control, LSF, drought, and LSF +drought. Once leaves of all individuals reached full leaf expansion under environmental conditions in early May, 30 saplings of each species were exposed to an artificial late-spring frost (LSF) event in a climate chamber (Figure 1b). As the saplings were exposed to ambient precipitation and an irrigation system before the initiation of spring phenology, soil water content was close to field capacity for all species before the LSF treatment. The simulated LSF started at about 8.30 pm at a temperature of 10°C. Then, the temperature was gradually decreased by 3.3°C per hour until a target temperature of −5.5°C was reached (see Figure S1). Then, the temperature was kept at −5.5°C for 3 h before gradually increasing to 10°C at the same speed. At around 10.00 am the following day, the LSF-treated individuals were returned to ambient conditions. The temperature threshold of −5.5°C in this experiment was chosen to represent a realistic late-spring frost temperature, which can occur during April for the region and to balance between no harm and severe impairment of tree canopy based on freezing resistance found for the investigated species in previous work (e.g., Till 1956; Vitasse and Lenz 2014a; Baumgarten et al. 2023). During the artificial frost, we added warm water in a bathing tub to protect the roots (Figure S1). The temperatures around the roots and leaves were recorded separately using a waterproof data logger (HOBO MX2203, Onset Computer Corporation, Bourne, MA, USA). The LSF was simulated on May 2 for both *Quercus* species, on May 3 for *A. campestre*, and on May 11 for *F. sylvatica*, considering the species-specific differences in the spring phenological status at the beginning of May. The LSF-induced leaf damage was assessed visually 1 week after the frost treatment using the following six categories: 0%, 1%–20%, 21%–40%, 41%–60%, 61%–80%, and 81%–100% of the leaf area damaged, respectively (Figure S2a). After the LSF treatment, all saplings were exposed to ambient environmental conditions at the research institute for 6–7 weeks (i.e., until the end of June, Figure 1a).

At the end of June, all saplings were transferred into a greenhouse, where half of the saplings previously exposed to LSF and half of the non-LSF controls were exposed to a 2-month drought period during July and August (Figure 1a). Inside the greenhouse, pots were grouped by species and the saplings of each species were further split into four groups based on the LSF and drought treatments to simplify watering. Inside the greenhouse, the groups were randomly distributed and the position of individual pots inside the group was changed regularly. The drought treatment consisted of a 50% water supply reduction, in which the species-specific water demands were calculated based on the combination of the average amount of precipitation of the region and a scaling factor based on the average plant size of a given species. Initially, we calculated the stem volume of each plant as a reference to determine the water requirements for the different species. Subsequently, we adjusted the specified amount of water based on the volumetric water content (VWC) values in the pots during the first week, accounting for evaporation. We manually watered the plants twice a week with a specified amount of water (*F. sylvatica*: 100 mL vs 200 mL; *Q. petraea*: 65 mL vs 130 mL; *Q. robur*: 125 mL vs 250 mL; *A. campestre*: 200 mL vs 400 mL). The soil volumetric water content (VWC) inside the pots was monitored three times a week from July to October to check the efficacy of the drought treatment (Figure S2b), on five randomly selected pots of each species and treatment using a hand-held time domain reflectometry (TDR) probe equipped with a 4.8-inch rod (FieldScout TDR 100 Soil Moisture Meter, Spectrum Technologies Inc., Aurora, IL, USA).

2.3 | Gas Exchange Measurements

On June 11, gas exchange measurements were conducted on five LSF-treated and five control saplings of each species using an infrared gas analyzer system (LI-6800 Portable Photosynthesis System; LI-COR Biosciences, Lincoln, NE, USA). Maximum carbon assimilation (A_{max}), stomatal conductance

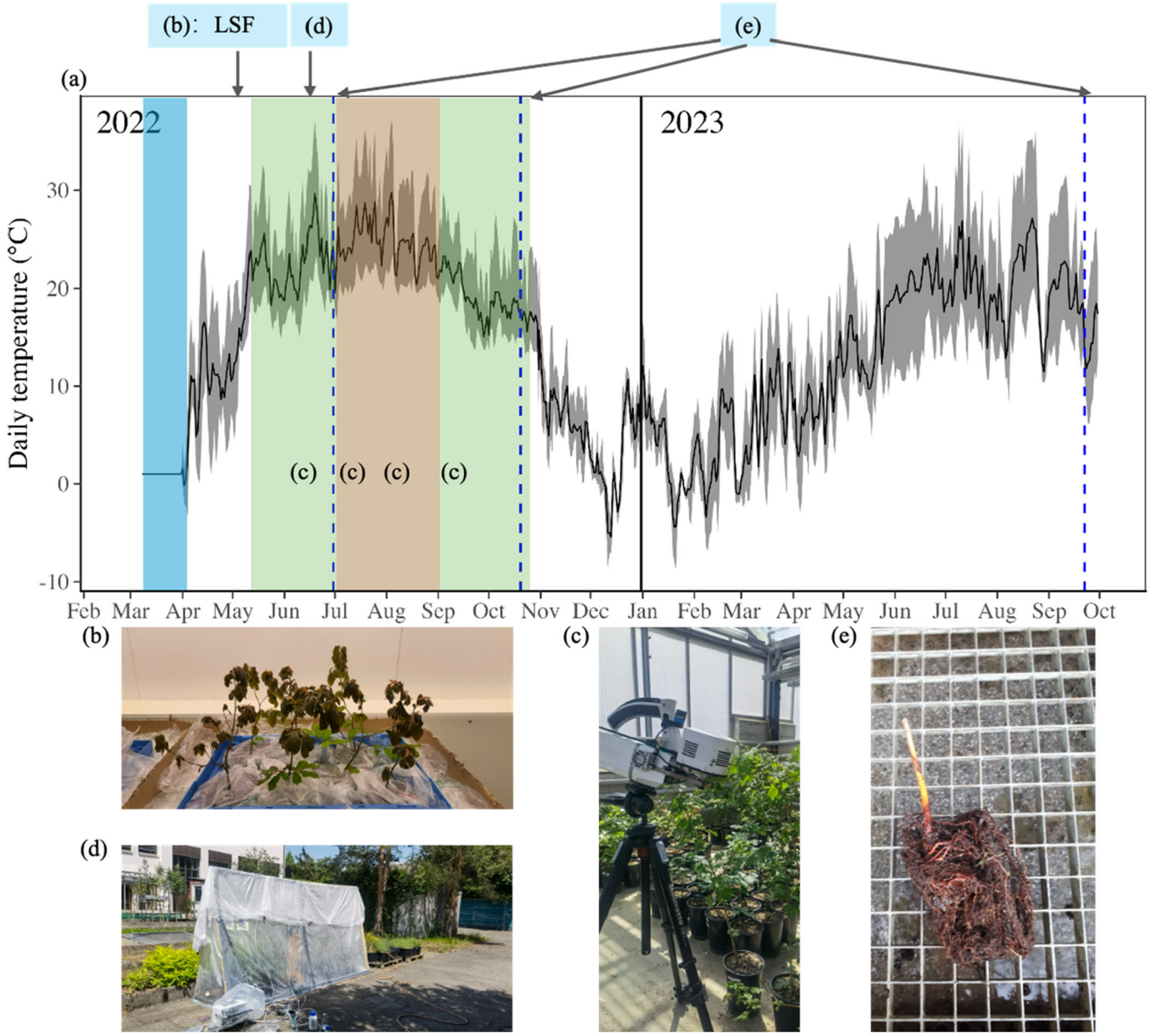

FIGURE 1 | Experimental protocol. Mean (solid line), and daily minimum and maximum air temperature (shaded area) from March 2022 to October 2023 (a). Blue areas indicate the period inside the climate chamber at 1°C, blank areas indicate exposure to ambient temperatures outside, the green area indicates the period inside the greenhouse and is interrupted by the red area that illustrates when the drought treatment was applied. Blue dashed vertical lines correspond to key measurement periods during the experiment, including gas exchange measurements and ^{13}C-labeling conducted around mid-June, and full tree harvests on July 1, 2022, October 20, 2022, and September 22, 2023, respectively. LSF-induced leaf damage in *F. sylvatica* (b). Gas exchange measurements on *A. campestre* (c). $^{13}CO_2$ pulse labeling conducted on saplings of the tree species *F. sylvatica*, *A. campestre*, *Q. petraea*, and *Q. robur* (d). Harvested roots from the experiment (e).

(g_s), and intrinsic water use efficiency (WUE_i, A_{max}/g_s) were extracted and calculated using CO_2 response curves (A-Ci curves). The experiment was conducted under saturating photosynthetic photon flux density of 1500 µmol m^{-2} s^{-1}, air temperature of 25°C inside the cuvette, and vapour pressure deficit (VPD) of 1.4 MPa. The measurements were performed between 9:00 am and 5:00 pm on mature and healthy leaves from the upper most third of the sapling (Figure 1c). Similarly, gas exchange measurements with the same settings were also conducted on July 15, August 20, and September 13 on five control, five LSF-treated, five drought-treated, and five LSF- and drought-treated saplings.

2.4 | Carbon Allocation Patterns Using ^{13}C Pulse Labelling

The effect of frost damage on carbon allocation patterns was tested by ^{13}C-pulse-labelling twenty saplings exposed to LSF and twenty non-LSF controls of each species 1 month after the simulated LSF (i.e., on 15. June). The saplings were placed inside a transparent plastic tent, and the natural CO_2 concentration was allowed to decrease to approximately 300 ppm (Figure 1d). Then, the plants were ^{13}C pulse labelled by adding 5 mL of 0.1 M sulfuric acid at 5-min intervals to a sealed beaker with a mixture of 7.5 g $NaH^{13}CO_3$ (99% purity, Sigma Aldrich)

and 7.5 g $NaHCO_3$ for 3 h and the generated CO_2 was channelled from the beaker into the tent. During the ^{13}C pulse labelling, the $^{12}CO_2$ concentration inside the tent was monitored by a GFS3000 Gas Exchange System (Walz GmbH, Effeltrich, Germany) and kept at around 500 ppm, indicating a total ($^{12}CO_2 + {}^{13}CO_2$) concentration of between 700 and 1000 ppm. A fan ensured uniform air mixing within the tent.

At the end of June 2022, 2 weeks after the ^{13}C labelling experiment, five LSF-treated and five control saplings of each species were harvested (i.e., 40 saplings in total). During the harvest, residual soil was removed by carefully washing the rooting system. Then, the saplings were dissected into coarse roots (CR), fine roots (FR), stems, twigs, and leaves, and the organs were dried separately in an oven at 60°C for at least 72 h (Figure 1e). Then, the dry biomass of each organ was determined, and a representative subset was ground into a fine powder using a ball mill (MM400 ball mill; Retsch GmbH, Haan, Germany) for the subsequent analysis of carbon (C) and nitrogen content (N), stable carbon isotopes ($\delta^{13}C$), and nonstructural carbohydrates (NSCs).

C, N and $\delta^{13}C$ were quantified from 1 ± 0.1 mg of finely ground and homogenized plant organs weighed into tin capsules using an elemental analyzer (Euro EA; Hekatech GmbH, Wegberg, Germany) coupled to an Isotope Ratio Mass Spectrometer (IRMS Delta V Advantage; Thermo Scientific, Bremen, Germany). All samples' carbon isotopic ratios were denoted using the δ notation (‰) relative to the Vienna Pee Dee Belemnite (VPDB) international standard. The $\delta^{13}C$ measurement accuracy was about 0.1‰.

2.5 | Nonstructural Carbohydrates Concentration

Nonstructural carbohydrates (NSCs) were determined following the protocol of Wong (1990) along with adjustments outlined by Hoch et al. (2002), where NSCs are defined as low-molecular-weight sugars (glucose, fructose, and sucrose) and starch. In detail, 10 to 12 mg of finely ground plant powder of each sampled organ (fine root, coarse root, stem, twig, and leaves) from the first and second harvests were weighed and dissolved in 2 mL of distilled water. Afterwards, the samples were steam-boiled in a water bath for 30 min at 400°C. Then, the samples were centrifuged (at 10000 rounds per minute) for 3 min to settle the solid particles at the bottom of the tube. After centrifugation, a 200 μL portion of the sample was transferred into another tube and 100 μL of invertase dissolved in Na-acetate buffer, derived from baker's yeast, was added to hydrolyze sucrose into glucose (Product Code: I4504-250MG, Sigma-Aldrich). Then, the tubes were stored in the dark for at least 1 h before total glucose content (i.e., sugars) was measured. Glucose concentration was measured photometrically at a wavelength of 340 nm using a 96-well microplate photometer (HR 7000 Microplate Photometer; Hamilton Company, Reno, NE, USA). This measurement followed enzymatic conversion to gluconate-6-phosphate through the hexokinase reaction, enabled by using 200 μL reagent solution which consists of 20 mL of hexokinase GHK (Product Code: G3293-20ML, Sigma-Aldrich), 4 μL of isomerase (Product Code: P5381-5KU, Sigma-Aldrich) used to convert fructose concurrently to glucose, and 20 mL distilled water. We incubated 500 μL of the extract (which includes sugars and starch) at 49°C with fungal amyloglucosidase sourced from Aspergillus niger (Product Code: 10115-5G-F, Sigma-Aldrich) for 15 h during the night in a water bath to transform starch into glucose. The next day, overall nonstructural carbohydrate (NSC) content was determined using the same photometric method to quantify the total glucose content (equivalent to NSC). Starch content was determined by subtracting the amount of free sugars from NSC. Pure solutions of starch, glucose, fructose, and sucrose were used as standard references to calibrate the NSC content of the samples. Extraction consistency was achieved by including standard plant powder (Orchard leaves; Leco, St. Joseph, MI, USA). Uniform laboratory settings and unaltered protocols were employed throughout the sample processing phase, effectively circumventing any potential confounds related to inter-method or inter-laboratory comparisons, as reported by (Quentin et al. 2015). NSC contents were determined at the end of June after the artificial LSF (i.e., first harvest) to determine LSF effects on NSC shortly after the summer solstice. In addition, the effects of LSF, drought, and the interaction of LSF and drought on NSCs were determined towards the end of the growing season during mid-October 2022 (i.e., second harvest), after 7 weeks of recovery without water restriction. Total plant NSC pools were calculated by multiplying NSC concentrations by the biomass of individual organs, including coarse roots (CR), fine roots (FR), stem, twigs, and leaves.

2.6 | Assessment of Leaf Phenology

Leaf chlorophyll content was assessed weekly from mid-June until the end of November using a chlorophyll concentration meter (MC-100; Apogee Instruments Inc., Logan, UT, USA). Leaf chlorophyll was monitored for five saplings of each species and treatment. Hence, five mature and healthy leaves from each tree's upper most third were measured for each individual. Leaf senescence was retrieved based on leaf chlorophyll index calculated by the chlorophyll concentration meter (Mariën et al. 2019).

Spring phenology was monitored two times a week for all saplings using a continuous classification scheme based on a five-stage categorial scale, that is, no visible development-stage 0, bud swelling-stage 1, budburst-stage 2, fully emerged but folded, crinkled or pendant leaves-stage 3, and fully unfolded leaves-stage 4). For each monitoring, we quantified the percentage of buds at each stage. Then, linear interpolation was used to calculate the day of the year (DOY) where 50% of buds reached stage 2 (budburst) and where 50% of buds reached stage 4 (leaf-out).

2.7 | Analysis of Tree Growth

Tree height and diameter were measured on regular intervals during 2022 and 2023 using tape measure and callipers (i.e., April 23, May 20, June 10, July 3, July 28, September 12, 2022, and January 24, and July 25, 2023, and September 20, 2023).

In mid-October 2022, after a recovery phase of about 7 weeks without water restriction, total plant NSC pools were determined from NSC concentrations and biomass of the individual organs coarse roots (CR), fine roots (FR), stem, twig, and leaves. In mid-September 2023, residual saplings were harvested, the plants were directly dissected into shoots and roots, and the dry biomass was measured (Figure 1e).

2.8 | Efficacy of the Treatments

The assessment of LSF-induced leaf damage revealed that the foliage of most *Q. robur* and *F. sylvatica* saplings was damaged between 81% and 100%, whereas the LSF-induced foliage damage was less pronounced for *A. campestre* and *Q. petraea* (Figure S2a). Due to the generally relatively low soil water contents at the beginning of July, the difference in soil volumetric water content (VWC) between drought-treated and non-drought-treated saplings was evident from mid-July onwards (Figure S2b). After the LSF treatment, aphids (*Phyllaphis fagi* L.) were detected on the freshly emerging leaves of *F. sylvatica* and powdery mildew (*Erysiphe alphitoides* Griffon & Maubl.) colonized both oak species (Figure S3). We controlled the impact of those pathogens by manually wiping away the aphids and spraying fungicide on mildew-infected leaves at the end of July.

2.9 | Statistical and Data Analysis

All analyses and plots were performed in R 4.1.1 (R Core Team 2023). The effect of LSF on leaf gas exchange (A_{max} and WUE_i) in mid-June, C:N ratios, carbon allocation (i.e., stable carbon isotopes, $\delta^{13}C$) and NSC concentrations were statistically tested using species- and organ-specific *t*-tests and Wilcoxon tests dependent on normality and homoscedasticity of the residuals (*rstatix* package, v0.7.2). Further, we used linear regressions to test the effects of late-spring frost (LSF), drought, and the interaction of both treatments on sapling nonstructural carbohydrate (NSC) pools, diameter increment and sapling biomass. The effect of sampling date, LSF, drought and all possible two-way interactions (all categorical variables) on leaf gas exchange between July and September 2022 was modelled using species-specific linear mixed-effect models from the Gaussian family with identity links and tree identity as random intercepts (*glmmTMB* package, v1.1.10). Leaf chlorophyll content was modelled using species-specific generalized additive models of the Gaussian family (*mgcv* package, v1.9-1) with measurement date (continuous variable), LSF, drought, and all possible two-way interactions as fixed effects, and measurement date as a smoothing parameter. Tukey's multiple comparison tests were used for all linear models as post-hoc tests to assess pairwise differences (*emmeans* package, v1.8.5). All figures were generated using *ggplot2* (v3.4.2).

3 | Results

3.1 | The Direct Effects of Frost on Tree Physiology

On June 11, 30–40 days after exposure to the late-spring frost (LSF) and after full leaf expansion of the untreated saplings, the highest maximum carbon assimilation (A_{max}) and the highest internal water use efficiency (WUE_i) per unit area were found for *Q. petraea* while the lowest values for both parameters were found for *F. sylvatica* reflecting well the distinctively different hydraulic strategy of those species (Figure 2). Overall, the LSF treatment decreased A_{max} and WUE_i of all species, but the A_{max} decrease was only significant for *F. sylvatica* and *Q. petraea* ($p = 0.022$ and $p = 0.004$), while the WUE_i decrease was only significant for *A. campestre* and *F. sylvatica* ($p = 0.043$ and $p = 0.027$).

The stable carbon isotope labelling 35–45 days after the frost treatment significantly enriched $\delta^{13}C$ of all organs of all species (Figure 3A). However, distinct species-specific differences in organ $\delta^{13}C$ between LSF-treated saplings and the control were found after the stable carbon isotope labelling. Significantly higher $\delta^{13}C$ values were measured in the leaves and twigs of LSF-treated *A. campestre*, and the twigs of LSF-treated *F.*

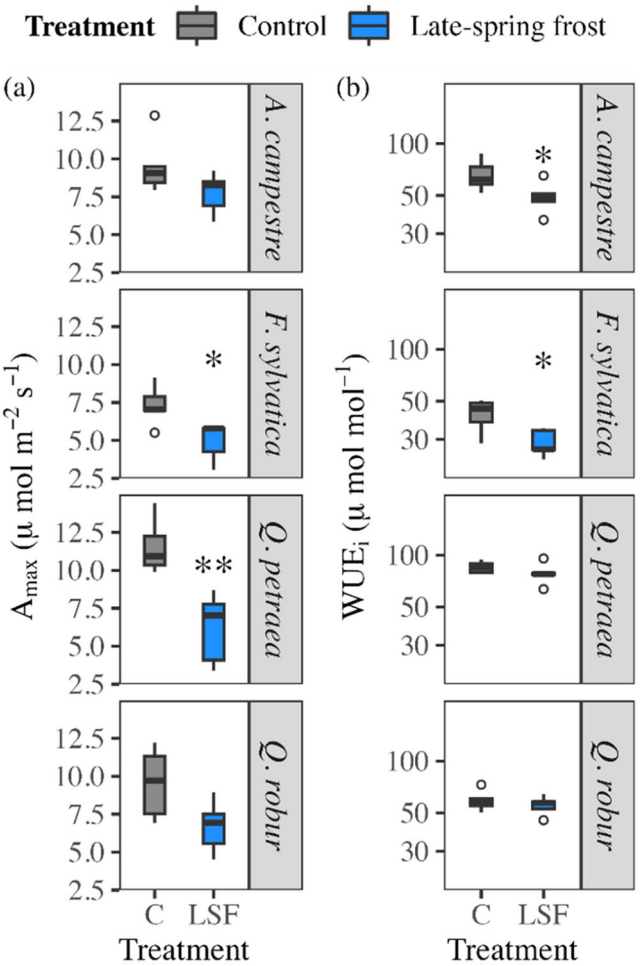

FIGURE 2 | Maximum carbon assimilation (A_{max}, a) and intrinsic water use efficiency of leaves (WUE_i; b) for saplings growing under control (C, $n = 5$) and late-spring frost (LSF, $n = 5$) on June 11. The boxes represent the first quartile, median, and third quartile, while the whiskers indicate 1.5 times the interquartile range in both directions. Species-specific Wilcoxon-tests were applied to detect significant differences between the LSF treatment and the control (labelled with asterisks; ***$p \leq 0.001$; **$0.001 < p \leq 0.01$; *$0.01 < p \leq 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

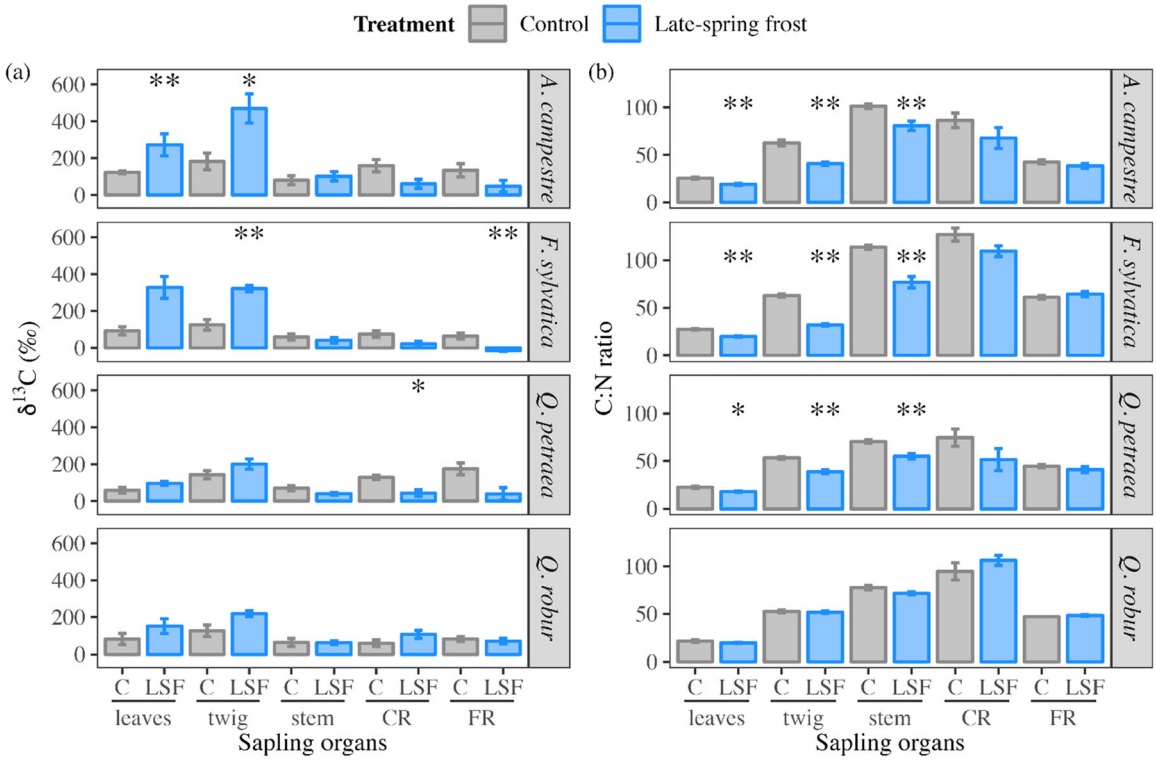

FIGURE 3 | Tissue bulk $\delta^{13}C$ (a) and C:N ratio (b) of leaves, twigs, stem, coarse roots (CR), and fine roots (FR) represented as mean and corresponding standard error for both control saplings (C, $n = 5$) and saplings exposed to damaging late-spring frost (LSF, $n = 5$). Species- and organ-specific Wilcoxon-tests were applied to detect significant differences of the LSF treatment compared to the control (labelled with asterisks; ***$p \leq 0.001$; **$0.001 < p \leq 0.01$; *$0.01 < p \leq 0.05$). Note: depending on species, the natural abundance $\delta^{13}C$ of non-labelled leaves was between −33 ~ −27‰. [Color figure can be viewed at wileyonlinelibrary.com]

sylvatica saplings ($p = 0.008$, $p = 0.016$, and $p = 0.008$), indicating that LSF-treated saplings of these species retained a substantial fraction of the freshly assimilated carbon in those two above-ground organs. In contrast, significantly lower $\delta^{13}C$ values were measured in the FR of LSF-treated *F. sylvatica* and the CR of *Q. petraea* saplings ($p = 0.008$, $p = 0.016$), providing further evidence that tree saplings prioritize above-ground before below ground organs for the allocation of freshly assimilated carbon. Two weeks after the ^{13}C labelling experiment, at the end of June 2022, the highest C:N ratios were found in the stem and CR of all four investigated tree species, while leaves had the lowest C:N ratios regardless of treatment (Figure 3B). The LSF decreased the C:N ratios of leaves, twigs, and stem of all species but *Q. robur* (all $p < 0.050$), while no effect of LSF on the C:N ratios of CR and FR was found for any species (all $p > 0.050$). These findings resulted predominantly from a higher N content of the above-ground organs leaves, twigs and stem in the new leaves produced after the LSF (Figure S4A). Interestingly, lower C content was measured in the CR of LSF-treated *A. campestre* and *F. sylvatica* and in the FR of LSF-treated *Q. petraea* and *Q. robur* saplings (Figure S4B). The simulated LSF affected NSC levels of *F. sylvatica* more strongly than the NSC levels of the other three investigated species (Figure 4). 50 to 60 days after the frost damage, LSF-treated *F. sylvatica* had significantly lower sugar contents in the twigs and FR ($p = 0.008$, $p = 0.016$), and significantly higher sugar contents in the CR ($p = 0.016$; Figure 4A), while all organs of this species were still severely depleted in starch at this point (all $p = 0.008$; Figure 4B). Further, LSF decreased the sugar content of *A. campestre* stem ($p = 0.016$) and the starch content of *A. campestre* leaves and *Q. petraea* CR ($p = 0.018$, $p = 0.032$).

3.2 | LSF and Drought Effects on Gas Exchange, NSCs, Leaf Chlorophyll Content and Growth Until the End of the Growing Season

Both the LSF and the drought treatment affected the leaf gas exchange of the investigated species (Figure 5). However, there were substantial differences between species. The drought treatment decreased A_{max} for *A. campestre* and *Q. petraea* saplings that were not exposed to the LSF treatment (Figure 5A). In contrast, the A_{max} of LSF-treated saplings of these species were unaffected by drought. LSF-treated *F. sylvatica* had consistently higher A_{max} in August and September than the control ($p = 0.041$ and $p = 0.006$), whereas lower A_{max} was found for LSF-treated *Q. petraea* in July and September and for LSF-treated *Q. robur* in July ($p = 0.046$ and $p = 0.002$, and $p = 0.002$, respectively). Saplings of both Quercus species decreased A_{max} with the progression of the vegetation period so that *Q. petraea* had higher A_{max} in July compared to August and September and *Q. robur* had lower A_{max} in September compared to July and August (all $p < 0.001$). All species but *Q. petraea* increased water use efficiency in the drought treatment, with WUE_i being significantly higher for *A. campestre* and *F. sylvatica* in August ($p = 0.007$ and $p = 0.005$) and WUE_i being significantly higher for *Q. robur* in July ($p = 0.002$, Figure 5B). The LSF- treatment decreased WUE_i for *Q. petraea* and *Q. robur* ($p = 0.002$ and $p = 0.004$), indicating that LSF

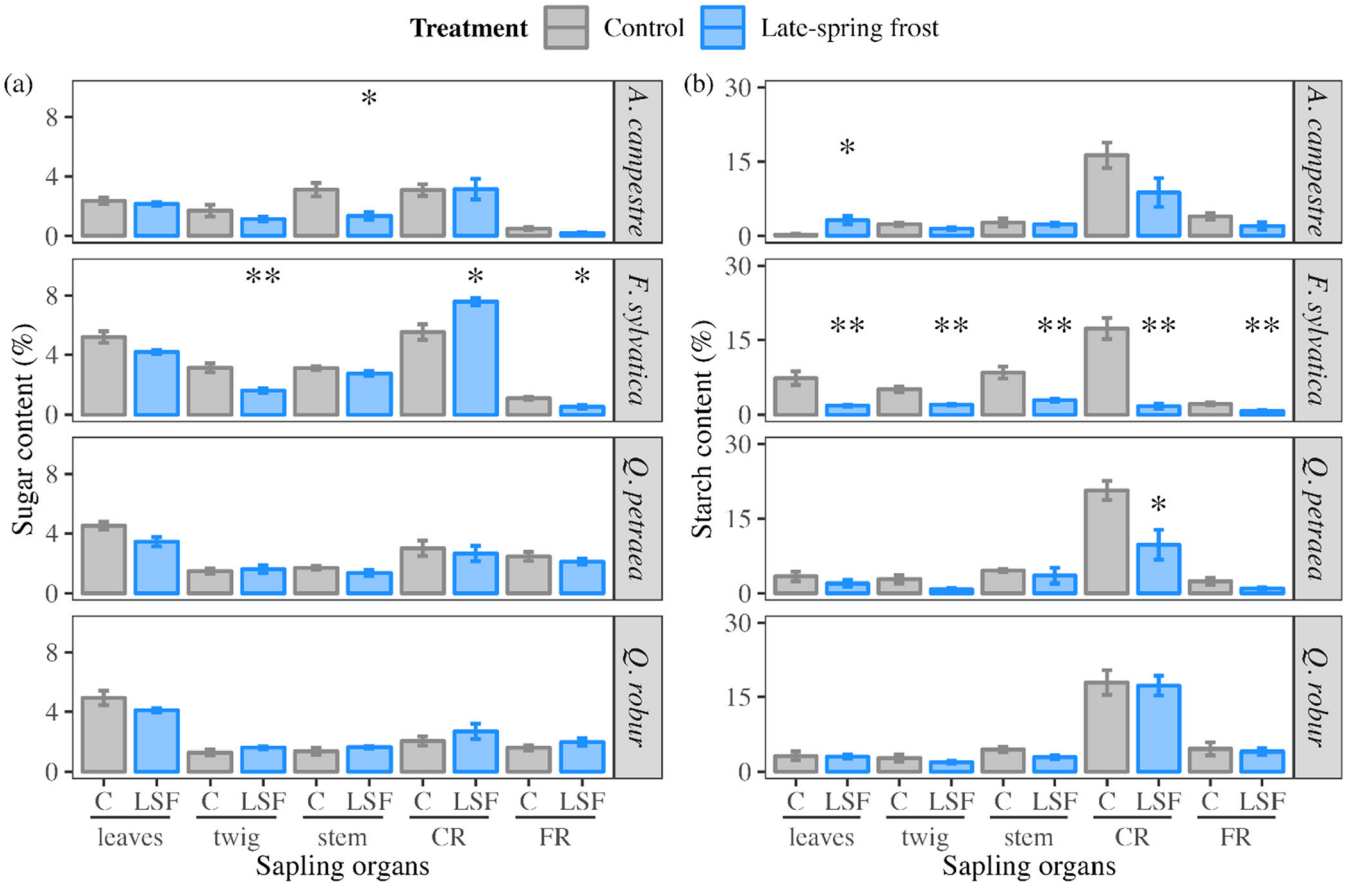

FIGURE 4 | Sugar (a) and starch content (b) of leaves, twigs, stem, coarse roots (CR) and fine roots (FR) represented as mean and corresponding standard error for both control saplings (C, $n = 5$) and saplings exposed to damaging late-spring frost (LSF, $n = 5$). Species- and organ-specific Wilcoxon-tests were applied to detect significant differences of the LSF treatment compared to the control (labelled with asterisks; ***$p \leq 0.001$; **$0.001 < p \leq 0.01$; *$0.01 < p \leq 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

damages on the foliage before drought could reduce the water demand and, thus, drought stress of the sapling. Some species tended to decrease WUE_i with the progression of the vegetation period. Hence, *A. campestre* had higher WUE_i in July compared to August and September ($p < 0.001$ and $p = 0.003$), while *Q. petraea* had lower WUE_i in September than in July and August ($p = 0.002$ and $p = 0.025$).

Species-specific and organ-specific differences in plant NSC pools were observed 7 weeks after the drought treatment (Figure S5). At this time, CR of all species had a higher NSC content than all other organs, but NSCs of CR of *F. sylvatica* and *Q. robur* were also most clearly affected by the treatments. At the plant level, LSF-treated *F. sylvatica* saplings had higher sugar and lower starch contents ($p = 0.001$, $p = 0.046$), while no effect of any treatment on total NSC content was found, suggesting that LSFs could delay the conversion of sugar to starch during autumn (Figure 6a–c). Starch and total NSC content were lower for drought-treated *A. campestre* ($p = 0.017$, $p = 0.021$), but no difference in sugar pools was found for this species. *Q. robur* exposed to both treatments, LSF and drought, had significantly lower starch and total NSC than the control ($p = 0.016$ and $p = 0.010$) and lower starch and total NSC than the saplings exposed to drought only ($p = 0.008$ and $p = 0.006$). In contrast, sugar, starch, and total NSC of *Q. petraea* were not affected by any treatment (Figure 6a–c).

The leaf chlorophyll index (Chl) of the investigated species was affected differently by the LSF- and the drought treatments (Figure 7). LSF increased Chl of *F. sylvatica* and decreased Chl for *A. campestre*, *Q. petraea* and *Q. robur* during summer ($p = 0.020$, $p = 0.005$, $p < 0.001$, and $p = 0.003$, respectively). However, Chl degradation of LSF-treated *A. campestre* and *Q. petraea* progressed slower than the control ($p = 0.005$ and $p < 0.001$). The drought treatment barely affected Chl of any species (Figure S6). Nevertheless, Chl of drought-treated *F. sylvatica* was significantly lower than the control ($p = 0.009$). The LSF treatment decreased the diameter increment of *A. campestre* and *F. sylvatica* saplings after the LSF ($p = 0.024$ and $p = 0.008$). In contrast, the diameter increments of neither of the Quercus species was affected by any treatment (Figure 8).

3.3 | Carry-Over Effects of LSF and Drought on Spring Phenology, Tree Growth and Biomass in the Subsequent Year

Spring phenology timing of the subsequent year, 2023, differed between species, with budburst of *A. campestre* and *Q. petraea* happening about 2 weeks before *F. sylvatica*. However, no effect of any treatment of 2022 on spring phenology 2023 was found (Figure S7). Further, no evidence for a carry-over effect on tree growth was found for any species, as

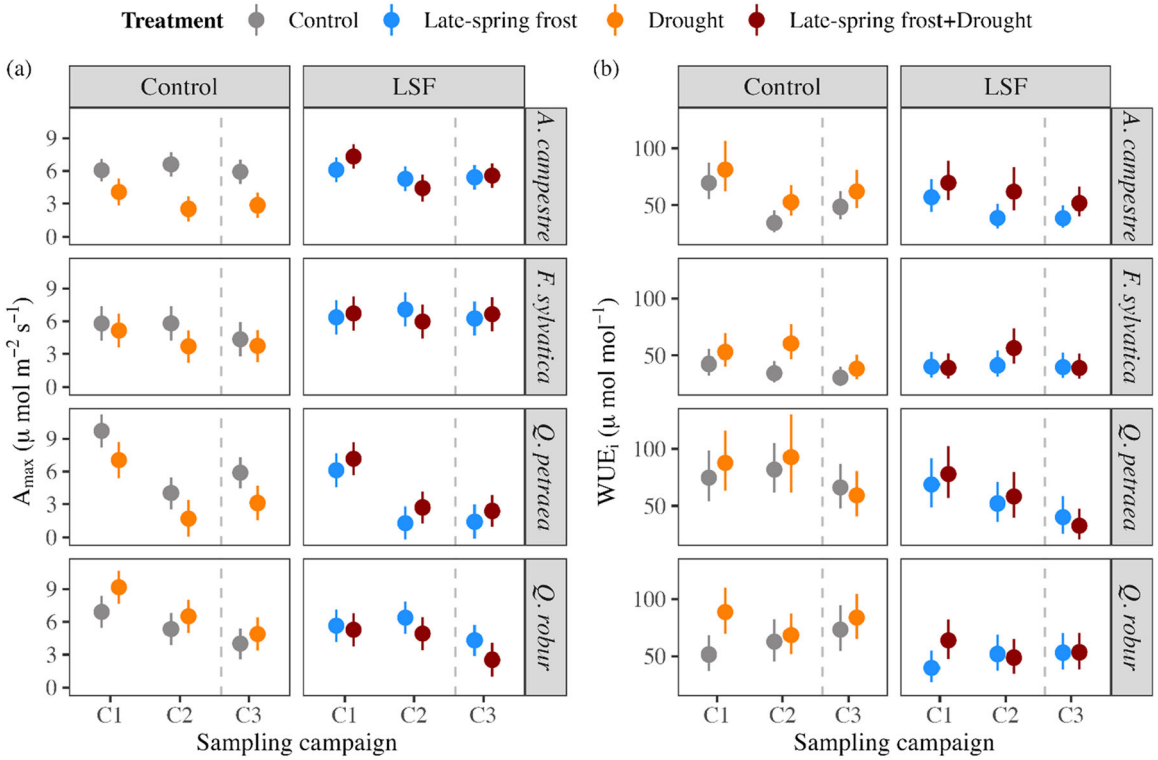

FIGURE 5 | Maximum carbon assimilation (A_{max}, a) and intrinsic water use efficiency of leaves (WUE_i; b) represented as mean and corresponding 0.95 confidence interval prediction for saplings growing under control ($n = 5$), late-spring frost ($n = 5$), drought ($n = 5$) and combined treatments ($n = 5$). Sampling campaigns took place 2 weeks (C1; i.e., July 15) and 7 weeks (C2; i.e., August 20) after initiation of the drought exposure, and 2 weeks after the end of the drought exposure of drought treated saplings (C3; i.e., September 13). The dashed line illustrates the transition from different watering protocols between control and drought treated saplings on the left (C1 and C2) to the same watering protocols on the right (C3). [Color figure can be viewed at wileyonlinelibrary.com]

the diameter increment in 2023 was not affected by any of the 2022 treatments (Figure 8).

In September 2023, the shoot biomass of drought-treated *Q. robur* and root biomass of LSF-treated *A. campestre*, *F. sylvatica,* and *Q. robur* was significantly lower than the control ($p = 0.043$, $p = 0.002$, $p = 0.032$, and $p = 0.043$, respectively; Figure 9a,b). Consequentially, the total biomass of LSF-treated *A. campestre* and *F. sylvatica* and drought-treated *Q. robur* saplings was significantly lower than the control, too ($p = 0.003$, $p = 0.056$, and $p = 0.039$, respectively; Figure 9c). Unexpectedly, LSF-treated *Q. petraea* saplings had lower root and lower total biomass than the control at the end of the vegetation period (both $p = 0.029$), while *Q. petraea* exposed to the combination of the LSF- and the drought-treatment were not any different regarding root and total biomass than the control.

4 | Discussion

Gas exchange measurements in 2022 revealed that our artificially induced late-spring frost (LSF) treatment at the beginning of May decreased maximum photosynthetic capacity (A_{max}) and water use efficiency (WUE_i) for all species at the end of June. However, afterward, the species differed substantially regarding how the LSF-, drought-, and the combination of both treatments affected A_{max} and WUE_i in July, August, and September. Stable isotope labelling provided some evidence that the LSF events may induce a change in carbon allocation patterns, with frost-treated species prioritizing leaves and twigs before roots to store newly assimilated carbon. In addition, the LSF treatment resulted in a tendency for reduced starch pools carbon-to-nitrogen (C:N) ratio of storage organs at the end of June for *A. campestre*, *F. sylvatica* and Q. *petraea*. Interestingly, the LSF treatment decreased starch pools and the C:N ratio of most organs of *F. sylvatica* significantly. In contrast, no significant effect of the LSF treatment on those two parameters was found for *Q. robur*, although the foliage of this species was most severely damaged by the artificial LSF. Towards the end of the vegetation period 2022, total NSC pool of *F. sylvatica* and *Q. petraea* largely recovered, while the drought treatment decreased NSC for *A. campestre* and the combination of the LSF and the drought treatment decreased NSC of *Q. petraea*. LSF decreased the leaf chlorophyll index (Chl) of all species but *F. sylvatica* significantly, whereas the opposite effect was found for LSF-treated *F. sylvatica*. Further, Chl of *F. sylvatica*, but neither of the other species, was significantly affected by drought. The LSF treatment affected diameter increment in the current year (i.e., 2022) for A. *campestre* and *F. sylvatica*, while no effect of any treatment on diameter increment of any species was observed in the subsequent year (i.e., 2023). Nevertheless, the investigation of dry plant biomass at the end of the subsequent year revealed that differences between control, LSF- and drought-treated saplings are retained at least for 1 year with the effect being particularly strong for roots.

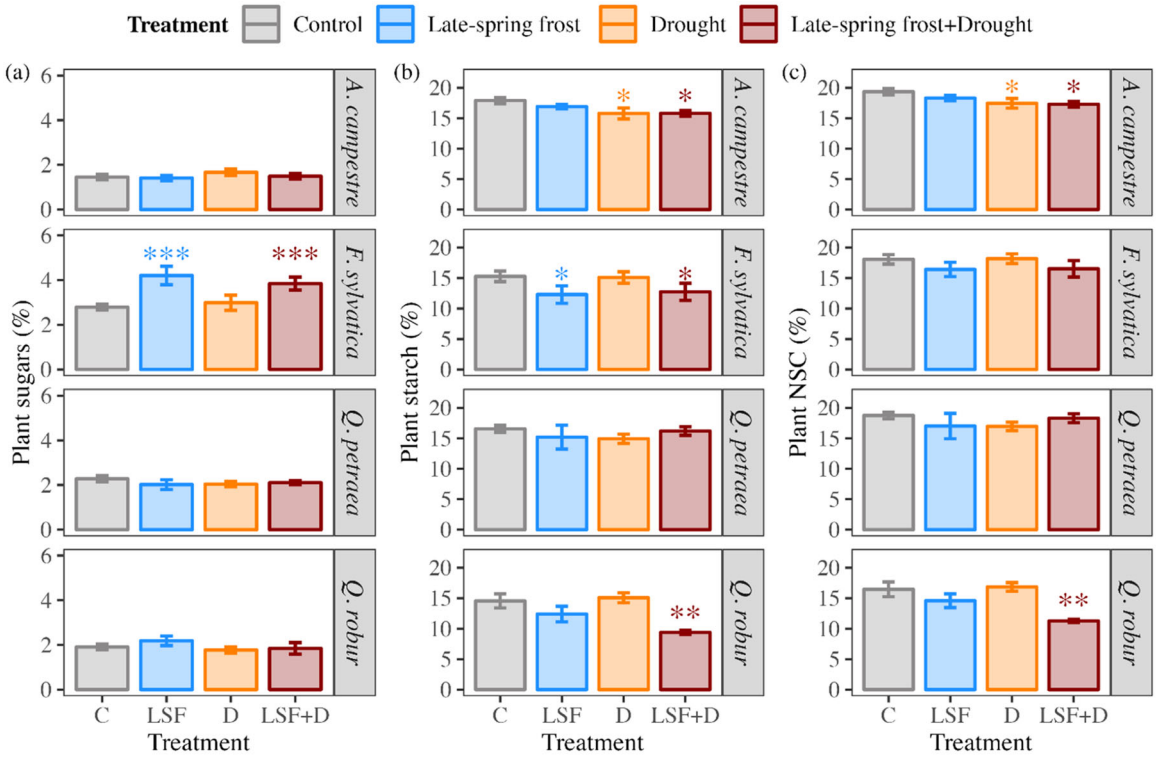

FIGURE 6 | Total plant sugar (a), starch (b), and nonstructural carbohydrate fraction of the biomass (c) represented as mean and corresponding standard error for saplings growing under control (C, $n = 5$), late-spring frost (LSF, $n = 5$), drought (D, $n = 5$) and combined treatments (LSF + D, $n = 5$). Total plant NSC pools were determined from NSC concentrations multiplied by biomass of the individual organs coarse roots (CR), fine roots (FR), stem, twig, and leaves. Species-specific post hoc tests were applied to detect significant differences of the treatment compared to the control (labelled with asterisks; ***$p \leq 0.001$; **$0.001 < p \leq 0.01$; *$0.01 < p \leq 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

4.1 | Saplings Damaged by LSF Prioritize Reconstructing Their Photosynthetic Capabilities and Resource Storage

Stable carbon isotope labelling revealed that LSF-treated saplings preferably retain carbon assimilates in leaves and twigs, which indicates that LSF damage caused source limitation on growth. Trees allocate assimilates such as starch from storage organs to leaves to cope with source limitation (Wiley et al. 2013; Hoch 2015). Further, LSF decreased the carbon-to-nitrogen (C:N) ratio of twigs and stems for *A. campestre*, *F. sylvatica,* and *Q. petraea*, which indicates C depletion of newly formed leaves and an increased N investment of the tree to maximize photosynthesis resulting in significantly higher leaf N content for LSF-treated *A. campestre* and F. *sylvatica* saplings few weeks after the LSF. A significant portion of the nitrogen found in plant leaves is allocated to the photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO), which creates a robust linkage between N investment and the capacity for photosynthesis (Luo et al. 2021). Therefore, N content is often positively related to CO_2 assimilation (Evans 1989). However, our study found lower A_{max} for LSF-treated *F. sylvatica* and *Q. petraea* individuals about 1 month after the frost, in spite of the higher nitrogen content. This discrepancy between leaf N content and A_{max} of LSF-treated saplings might be due to the leaves not being mature yet at the time of the measurements, as they were newly formed about 4 weeks after LSF and, therefore, much younger. In fact, freshly emerged leaves tend to have a slower cyclic electron flow around photosystem I compared to more mature ones (Huang et al. 2017).

The LSF treatment significantly depleted coarse root (CR) starch for *F. sylvatica* and *Q. petraea*, demonstrating well that NSCs are remobilized from storage organs to rebuild damaged organs (D'Andrea et al. 2019). Stored carbon reserves in plants, mainly NSCs in tree species, are remobilized to buffer stress conditions when carbon supply does not meet demand, making them a crucial mechanism for immobile, long-lived organisms (Chapin et al. 1990; Dietze et al. 2014; Peltier et al. 2023). Similar findings were observed in four European broadleaf species, where LSF damage strongly decreased the C/N ratio and NSC content (Baumgarten et al. 2023).

4.2 | NSC Storage Is Prioritized at the Expense of Growth

At the end of the growing season, LSF-induced differences in NSC were most pronounced for coarse roots (CR), which may have caused the reduced root growth during the current (i.e., 2022) and the subsequent year (i.e., 2023). However, the NSC content of all other organs largely recovered from the LSF until the end of the vegetation period 2022, and no significant effect of any treatment on the total NSC pool (sugars+starch) was found for *F. sylvatica* and *Q. petraea* on October 20. However, on the same date, lower total starch and NSC pools were found for drought-treated *A. campestre* and for *Q. robur* treated with

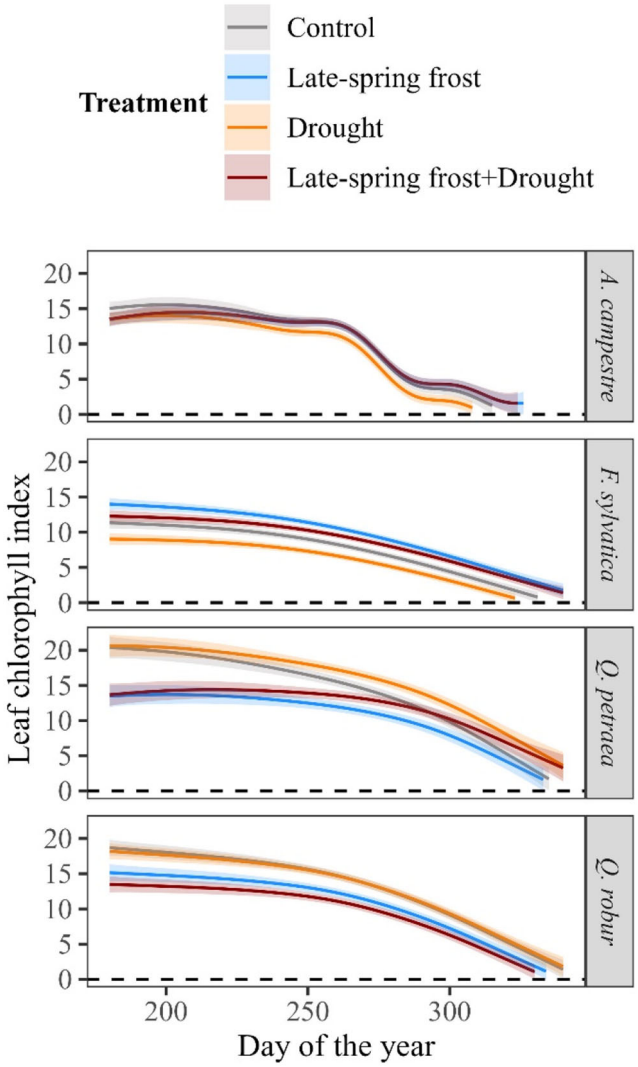

FIGURE 7 | Progression of the leaf chlorophyll index (mean and corresponding 0.95 confidence interval) predicted from species-specific generalized additive models for saplings growing under control (C, $n = 5$), late-spring frost (LSF, $n = 5$), drought (D, $n = 5$) and combined treatments (LSF + D, $n = 5$). [Color figure can be viewed at wileyonlinelibrary.com]

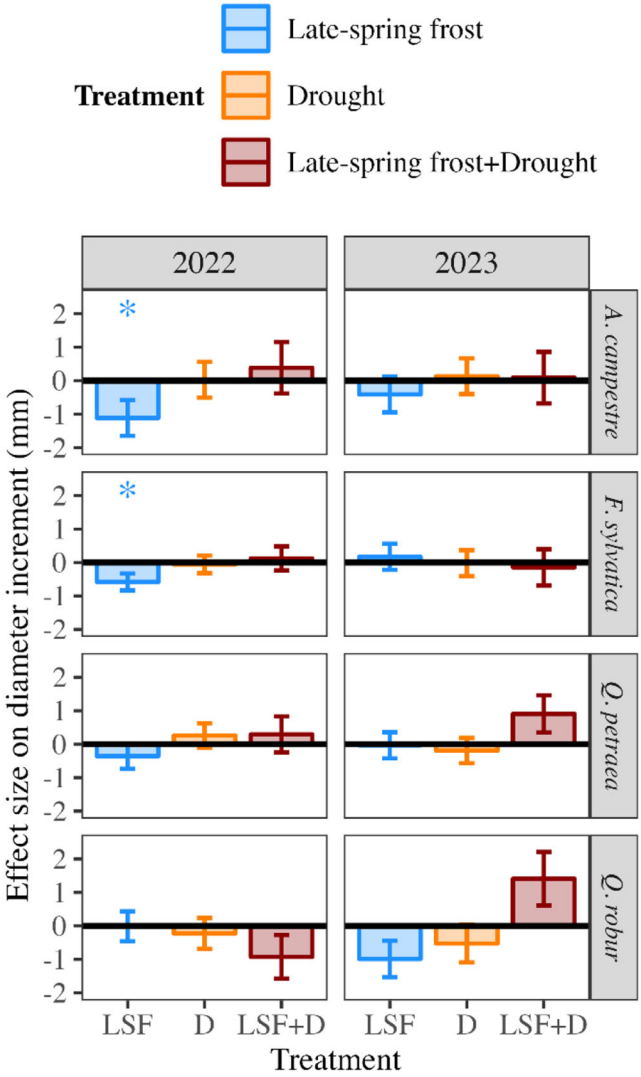

FIGURE 8 | Effect size (i.e., linear model output) of the late-spring frost (LSF, $n = 7$), drought (D, $n = 7$), and combined treatments (LSF + D, $n = 7$) on diameter increment during the vegetation period when the treatments were applied, (2022) and during the subsequent vegetation period with favourable environmental conditions (2023) retrieved from species-specific linear models. Significant effects are labelled with asterisks (***$p \leq 0.001$; **$0.001 < p \leq 0.01$; *$0.01 < p \leq 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

the combination of LSF and drought, which indicates that drought stress, particularly after LSF damage, could prevent tree saplings from replenishing NSC pools. However, other than the total NSC pools that recovered for the most part until the end of the vegetation period after LSF, lower root biomass of LSF-treated saplings was found at the end of the subsequent growing period, indicating a prioritization of reaching an appropriate pool of NSC reserves at the expense of growth for temperate tree saplings. This prioritization of refilling NSC reserves before growth might represent a conservative growth strategy, which, however, pays off in the long run for trees as higher NSC reserves may reduce winter mortality (Galvez et al. 2013) and increase the ability to withstand renewed frost damages (D'Andrea et al. 2019), pathogens (Huang et al. 2019) and drought (O'Brien et al. 2014) in the following years. The results of Weber et al. (2019), who observed much lower biomass increment but similar NSC contents in saplings of ten temperate tree species after 3 years of shading, support this finding.

LSF, and to a lower extent drought, affected leaf chlorophyll (Chl) and thus leaf senescence trajectories. However, there were species-specific differences as LSF increased Chl and delayed leaf senescence of *F. sylvatica*, whereas both LSF-treated *Quercus* species had lower Chl during summer but similar Chl concentrations towards the end of the vegetation period, which indicates that all three species adjust senescence progression after LSF exposure. The consistently higher Chl concentrations and delayed chlorophyll degradation of *F. sylvatica* may be a mechanism to compensate for the carbon losses at the beginning of the growing season (Zohner et al. 2019; Baumgarten et al. 2023). In contrast, the lower Chl of LSF-treated saplings of both *Quercus* species during autumn could be co-driven by a more severe mildew infestation of the second cohort of leaves, as those leaves tend to be more susceptible to mildew infestation as mildew spores concentration in the air

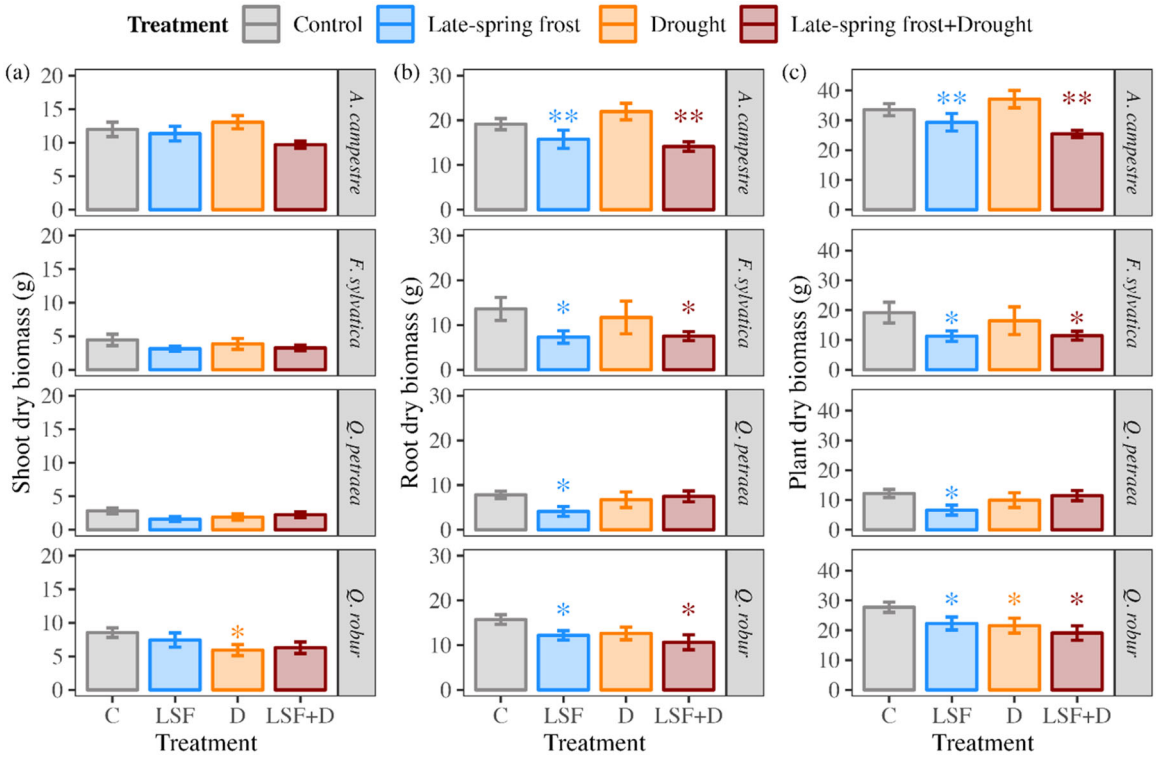

FIGURE 9 | Shoot (a), root (b), and total plant dry biomass (c) represented as mean and corresponding standard error for saplings growing under control (C, $n = 7$), late-spring frost (LSF, $n = 7$), drought (D, $n = 7$) and combined treatments (LSF + D, $n = 7$). Species-specific post hoc tests were applied to detect significant differences of the treatment compared to the control (labelled with asterisks; ***$p \leq 0.001$; **$0.001 < p \leq 0.01$; *:$0.01 < p \leq 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

progressively increases during spring (Desprez-Loustau et al. 2010; Jain et al. 2019), which matches our qualitative visual observations. Indeed, mildew infestation could advance senescence and decrease photosynthesis (Zhang et al. 2022), and may increase the impact of drought stress during summer (Leisner et al. 2023; Aguirre et al. 2024; Choudhary and Senthil-Kumar 2024).

4.3 | Both LSF- and Drought-Stress Impacts Might Still be Visible in the Subsequent Year

We observed significantly lower shoot biomass for drought-treated *Q. robur* towards the end of the subsequent vegetation period, whereas at the same time, significantly lower root biomass for LSF-treated *A. campestre*, *F. sylvatica* and *Q. robur* was found. This outcome illustrates that LSF events create a substantial and prevalent carry-over effect on sapling growth that is potentially stronger than nonlethal droughts, although carry-over effects of the drought-treatment were also found. Previous work showed that LSF affected forest gross primary productivity more severely and longer lasting in a boreal forest than drought (Chen et al. 2023). However, another study showed that LSF had an impact only during the current year, while drought carry-over effects can last up to 4 years, depending on the species (Vitasse et al. 2019). The discrepancy between those two outcomes could result from the timing of the two stresses, where LSF treatment was applied during spring. In contrast, drought stress was applied from mid- to late-summer, representing a period when temperate trees already completed much of the radial growth (Etzold et al. 2022). Thus, the drought treatment may differ depending on tree phenology and result in distinctively different reactions by the saplings, complicating the comparison between the severity of the events.

Root growth reduction was consistent with NSC depletion of storage organs 1 month after the LSF treatment, indicating that a damaging LSF event can indirectly decrease root growth by prioritizing carbon investment into shoot organs since plants tend to shift their resource-use economy in favour of organs that can acquire the most limiting resource (Bloom et al. 1985). Thus, a damaging LSF event may increase carbon investment in shoots to capture light and restore the carbon supply at the expense of root growth. In this sense, juvenile trees may recover leaves and shoots particularly fast from LSF damage as they compete for light for a successful establishment, which, however, leads to a depletion of NSC pools and a marked reduction of root growth. Our study thus shows that the carry-over effect of a carbon investment shift between shoots and roots persists two growing seasons after the event. However, the magnitude of the LSF carry-over effect depended on species and the degree of frost damage. Heavily damaged saplings were more restricted in growth as higher fractions of the NSCs reserves stored in the roots needed to be mobilized to rebuild foliage. Interestingly, the dry biomass of *Q. petraea* saplings was more substantially affected by the LSF-treatment alone than by the combination of both the LSF- and the drought-treatment. This finding suggests that this treatment group may have consisted of a disproportionate number of well-growing *Q. petraea* sapling or that these saplings, although not visible, were less affected by the LSF-treatment or the powdery mildew. Thus,

the magnitude of the carry-over effect after a damaging LSF event may differ between individuals. Further, as our set-up deals with potted saplings growing in nutrient-poor substrate and being exposed to artificial LSF and drought stress treatments, we cannot say which studied species will perform best under natural conditions. Pot experiments may for instance restrict the exploration of deeper soil layers of tree saplings to avoid drought exposure and will make natural stand dynamics such as intra- and inter-specific competition or resource facilitation impossible.

Interestingly, most measurements of LSF-treated *Q. robur* were, unlike the other investigated species, barely different from the control 2 months after the frost damage. However, LSF decreased leaf chlorophyll content, and the combination of both treatments depleted NSC pools of *Q. robur*, which implies that this species may have restored carbon assimilation capacity faster after frost damage than the other species. Presumably, this species recovered relatively fast because of the many spare buds that enable a fast foliage regeneration after defoliation (Baumgarten et al. 2023). Nevertheless, the indirect effects of LSF, such as the mildew infestation, particularly in combination with drought, could have affected *Q. robur* saplings more strongly in the long term than the LSF per se. Thus, future experiments could benefit from quantitative assessments of secondary pathogen attacks and interacting stressors on the defence metabolism of trees.

5 | Conclusion

Artificially induced late-spring frost (LSF) and drought showed that temperate tree saplings prioritize canopy recovery and NSC reserve replenishment at the expense of growth until the end of the growing season. Our results imply that the resilience and vitality of a tree under ongoing climate change will likely depend on its ability to recover from multiple stressors such as LSF, drought, and insect or pathogen infestation. Contrary to our expectations, no clear overall advantage of any species was found in coping with LSF damage and drought at the sapling stage, despite the species-specific differences in susceptibility to both stressors found in the literature. However, the response of natural forests to LSF and drought may differ from our observations on potted saplings. For instance, in natural conditions oak saplings may better avoid drought stress than beech due to their deep rooting system. Therefore, further monitoring and experimental approaches in natural and near-natural forests are needed to validate our results and improve the understanding of how the combination of multiple abiotic and/or biotic stressors will affect Central European forest ecosystems in the future.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.