



University of Zagreb

Faculty of Forestry and Wood Technology

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**THE INFLUENCE OF ATYPICAL
ENVIRONMENTAL FACTORS ON
PHENOLOGY IN DIFFERENT SPECIES
AND GENOTYPES OF FOREST TREES**

DOCTORAL THESIS

Zagreb, 2026



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DOCTORAL DISSERTATION

Mentor:

Saša Bogdan, PhD, Full Professor

Zagreb, 2026



Sveučilište u Zagrebu

Fakultet šumarstva i drvne tehnologije

Marko Bačurin

**UTJECAJ ATIPIČNIH OKOLIŠNIH
ČIMBENIKA NA FENOLOGIJU KOD
RAZLIČITIH VRSTA I GENOTIPOVA
ŠUMSKOG DRVEĆA**

DOKTORSKI RAD

MENTOR:

Prof.dr.sc. Saša Bogdan

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Marko

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The influence of atypical environmental factors on phenology in different species and genotypes of forest trees

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Leaf phenology is a key determinant of the growing season length and ecosystem functioning in temperate forests. In the context of climate change, understanding the factors that influence phenological processes is essential for predicting forest responses to environmental stress. This study investigates the effects of selected atypical environmental factors on leaf phenology in temperate forest tree species.

The main objective was to examine the influence of drought stress, increased phosphorus availability, and rootstock–scion interactions on leaf phenology across different species and genotypes. Experiments were conducted on *Salix caprea* L., *Fagus sylvatica* L., *Quercus petraea* (Matt.) Liebl.), and *Quercus robur* L. Controlled treatments included drought induced at different stages of the growing season, phosphorus enrichment of the substrate, and combined drought–phosphorus treatments. Phenological phases such as budburst, leaf development, and autumn senescence were monitored and compared among treatments, species, and provenances.

The results show that drought stress can induce carry-over effects on phenology in the following growing season, with the direction of phenological shifts depending on the timing of drought. Early-season drought advanced budburst in goat willow, whereas later drought delayed leaf-out. Drought also delayed autumn senescence, particularly when stress occurred later in the growing season. Increased phosphorus availability accelerated autumn leaf senescence in beech and sessile oak, while its influence on spring phenology remained limited. Interactions between drought and phosphorus revealed non-additive effects, where combined treatments often neutralized individual stress responses.

Overall, the results demonstrate that drought, nutrient availability, and rootstock–scion interactions can significantly modify phenological processes in forest trees. These findings

highlight the importance of considering multiple environmental stressors when predicting forest responses to climate change and managing forest reproductive material.

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Keywords: spring leaf phenology, autumn leaf senescence, drought stress, phosphorus availability, phenological plasticity, provenance variation, rootstock–scion interaction, temperate forest trees, climate change.

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Utjecaj atipičnih okolišnih čimbenika na fenologiju kod različitih vrsta i genotipova šumskog drveća

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Fenologija razvoja i odumiranja listova predstavlja jedan od ključnih funkcionalnih pokazatelja u ekosustavima šuma umjerenog pojasa jer određuje trajanje vegetacijskog razdoblja i uvelike utječe na produktivnost, fiziološke procese i prilagodbu šumskih vrsta drveća. Fenološki procesi listopadnih vrsta obuhvaćaju niz razvojnih faza od proljetnog otvaranja pupova i razvoja listova do jesenske senescencije i opadanja lišća. Vrijeme pojave tih fenofaza rezultat je složene interakcije genetskih čimbenika i okolišnih signala. Tradicionalno se smatra da su temperatura i fotoperiod glavni regulatorni čimbenici fenoloških procesa, no sve veći broj istraživanja pokazuje da na fenologiju značajno utječu i tzv. atipični okolišni čimbenici, poput sušnog stresa, dostupnosti hraniva u tlu, patogena i drugih oblika abiotičkog i biotičkog stresa. U kontekstu klimatskih promjena, povećana učestalost sušnih razdoblja te promjene u dostupnosti hraniva predstavljaju važne čimbenike koji mogu uzrokovati promjene u vremenu i dinamici fenoloških procesa kod šumskog drveća.

Glavni cilj ovog istraživanja bio je utvrditi na koji način odabrani atipični okolišni čimbenici utječu na fenološke procese kod različitih vrsta i genotipova šumskog drveća umjerenog klimatskog pojasa. Posebna pozornost posvećena je utjecaju sušnog stresa i vremenu njegove indukcije, povećanoj dostupnosti fosfora u supstratu te interakcijama između sušnog stresa i povećane ishranjenosti fosforom. Istraživanje je također obuhvatilo analizu razlika u fenološkim reakcijama između vrsta i provenijencija te procjenu utjecaja podloge na unutar-klonsku varijabilnost fenologije kod heterovegetativno razmnoženog hrasta lužnjaka. Istraživanja su provedena na vrstama *Salix caprea* L., *Quercus robur* L., *Quercus petraea* (Matt.) Liebl. i *Fagus sylvatica* L.

Eksperimentalni dio rada obuhvatio je nekoliko pokusa provedenih u kontroliranim uvjetima i rasadničkim pokusima. U pokusima koji su se bavili utjecajem vremena indukcije sušnog stresa biljke su bile izložene sušnom stresu u različitim razdobljima tijekom vegetacijske sezone. Sušni stres induciran je u različitim periodima vegetacijskog razdoblja kako bi se utvrdilo ovisi

li fenološki odgovor o vremenu pojave stresa, a istraživanje se provodilo na sadnicama hrasta lužnjaka i vrbe ive. U istraživanjima utjecaja dostupnosti hraniva i suše primijenjen je višefaktorski eksperimentalni dizajn kojim se pratio utjecaj povećane dostupnosti fosfora u supstratu, sušnog stresa te njihova kombiniranog djelovanja. Pokusi su provedeni na mladim biljkama bukve i hrasta kitnjaka podrijetlom iz dviju različitih provenijencija. U navedenom istraživanju praćene su fenološke faze razvoja listova i jesenske senescencije. Eksperimentalni dizajn obuhvaćao je kontrolni tretman, tretman s povećanom dostupnošću fosfora u supstratu, tretman sa sušnim stresom te kombinirani tretman oba čimbenika. Utjecaj podloge na fenologiju plemke istražen je na klonovima hrasta lužnjaka cijepljenima na generativno uzgojene podloge različitog genetskog podrijetla, pri čemu su fenološke faze listanja praćene kroz više vegetacijskih sezona. Fenološka promatranja provedena su pomoću standardiziranih ordinalnih skala, a dobiveni podaci analizirani su odgovarajućim statističkim metodama.

Rezultati istraživanja pokazali su da sušni stres može uzrokovati značajne promjene u fenologiji lista, pri čemu je vrijeme pojave suše tijekom vegetacijske sezone ključan čimbenik koji određuje smjer fenološkog odgovora. Kod vrbe ive (*Salix caprea*) suša u ranijem dijelu vegetacijske sezone uzrokovala je ranije otvaranje pupova u sljedećoj godini, dok je suša u kasnijim fazama vegetacije dovela do odgođenog listanja. Ovi rezultati potvrđuju postojanje tzv. naknadnog učinka stresa, odnosno prijenosnog učinka, pri kojem stres iz prethodne vegetacijske sezone utječe na razvoj biljke u sljedećoj godini. Kod hrasta lužnjaka sušni stres imao je značajan utjecaj na visinski prirast biljaka, pri čemu je kasnija pojava suše tijekom vegetacije uzrokovala izraženije smanjenje rasta. U većini slučajeva suša je također uzrokovala odgodu početka jesenske senescencije listova, što se može interpretirati kao kompenzacijski mehanizam kojim biljke pokušavaju nadoknaditi smanjenu fotosintetsku aktivnost tijekom razdoblja stresa.

Povećana dostupnost fosfora u supstratu pokazala je značajan utjecaj na jesensku fenologiju kod bukve i hrasta kitnjaka. Kod obje vrste zabilježeno je ranije započinjanje i ubrzavanje procesa senescencije listova, dok je utjecaj fosfora na proljetnu fenologiju bio znatno slabiji i ovisio o vrsti i provenijenciji. Zabilježene razlike između provenijencija iste vrste ukazuju na važnu ulogu lokalne prilagodbe i genetske varijabilnosti u fenološkim odgovorima na promjene u dostupnosti hranjiva.

Rezultati pokusa koji su uključivali kombinaciju sušnog stresa i povećane dostupnosti fosfora pokazali su da interakcije između tih čimbenika nisu jednostavno aditivne. U pojedinim slučajevima kombinirani tretmani neutralizirali su fenološke promjene uzrokovane

pojedinačnim stresnim čimbenicima, što ukazuje na složene fiziološke mehanizme prilagodbe biljaka na višestruke okolišne stresove.

Analiza cijepljenih klonova hrasta lužnjaka pokazala je da podloga može značajno utjecati na vrijeme otvaranja pupova kod plemke. Unutar pojedinih klonova zabilježene su razlike u vremenu listanja između rameta cijepljenih na različite podloge, unatoč genetskoj identičnosti plemki. Ovi rezultati ukazuju na važnu ulogu podloge u regulaciji fenoloških procesa te na mogućnost da podloga putem fizioloških i hormonalnih signala utječe na razvoj nadzemnog dijela biljke.

Dobiveni rezultati potvrđuju da fenološki procesi kod šumskog drveća nisu određeni isključivo tipičnim okolišnim signalima, poput temperature i fotoperioda, već i nizom drugih čimbenika koji mogu značajno utjecati na njihovu dinamiku. Istraživanje doprinosi boljem razumijevanju utjecaja atipičnih okolišnih čimbenika na fenologiju šumskog drveća te ima važne implikacije za gospodarenje šumama u uvjetima klimatskih promjena. Stečena saznanja mogu se primijeniti u planiranju proizvodnje šumskog reprodukcijanskog materijala, upravljanju klonskim sjemenskim plantažama te pri odabiru genetski prilagođenih provenijencija i vrsta za buduće okolišne uvjete.

(124 stranica, 5 priloga, 161 literaturnih navoda, jezik izvornika: engleski)

Ključne riječi: proljetna fenologija listanja, jesensko odumiranje listova, sušni stres, dostupnost fosfora, fenološka plastičnost, varijabilnost provenijencija, interakcija podloge i plemke, šumsko drveće umjerenog pojasa, klimatske promjene.

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IZJAVA O AKADEMSKOJ ČESTITOSTI

Izjavljujem da je moj doktorski rad izvorni rezultat mojega rada te da se u izradi istoga nisam koristio drugim izvorima osim onih koji su u njemu navedeni.

U Zagrebu 13. ožujka 2026. godine

Marko Bačurin

STATEMENT OF ACADEMIC INTEGRITY

I hereby declare that my doctoral thesis is the original result of my own work and that I have not used any sources other than those cited in the thesis.

Zagreb, 13.3.2026.

Marko Bačurin

1. INTRODUCTION

Leaf phenology, referring to the timing of leaf development and seasonal progression in deciduous trees, from budburst in spring to senescence and abscission in autumn, constitutes a critical determinant of the growing season length and influences ecological dynamics in temperate forests (Polgar and Primack, 2011). Phenology, through its regulation of the timing and duration of the growing season and reproductive periods, exerts a direct influence on tree fitness and survival (Chuine et al., 2000; Davi et al., 2011; Polgar and Primack, 2011). Given its central role in both ecosystem functioning and individual performance, phenology becomes particularly important in the context of climate change, as forest trees, with their long lifespans, are challenged to keep pace with rising temperatures and increasingly frequent droughts, making phenology one of the most visible indicators of these shifts and a key mechanism of adaptation (Bertin, 2008; Lindner et al., 2010).

Phenological traits are considered to be primarily regulated by climatic factors: spring events are mainly controlled by winter chilling, forcing temperatures (spring thermal accumulation), and photoperiod, whereas autumn events are driven predominantly by temperature and photoperiod (Robson et al., 2013; Basler and Körner, 2014; Way and Montgomery, 2015).

Before woody plants can initiate budburst in the spring, particularly in temperate regions, they must pass through a series of dormancy phases, each reflecting distinct physiological states. As described by Lang et al. (1987) and Arora et al. (2003), dormancy is not a single static condition but a dynamic process that unfolds in three stages: paradormancy, endodormancy, and ecodormancy. In the first stage, *paradormancy*, bud growth is suppressed by signals originating from other parts of the plant. During *endodormancy*, growth is inhibited due to internal factors within the bud itself, regardless of external conditions. Finally, in *ecodormancy*, the buds are physiologically ready to grow, but unfavorable environmental conditions, such as low temperatures, prevent development. Endodormancy is terminated by exposure to chilling temperatures, after which buds enter ecodormancy, a phase during which they become responsive to rising spring temperatures and photoperiod (Pletsers et al., 2015).

This sequence of dormancy phases underscores that budburst is not triggered by a single factor but by the interplay of internal states and environmental cues. Accordingly, in most woody plants, dormancy release and the onset of budburst occur in two steps: chilling or photoperiodic signals unlock the developmental switch, and subsequent warmth determines the speed of bud development (Körner, 2006).

Dormancy induction and release are governed by a trade-off between extending the growing season and avoiding frost damage in both autumn and spring (Bennie et al., 2010). Pioneer species typically rely on a rapid, temperature-driven dormancy release, while late-successional species adopt a more conservative strategy, requiring stronger chilling and exhibiting greater sensitivity to photoperiod (Caffarra and Donnelly, 2011; Basler and Körner, 2012, 2014). In these late-successional species, photoperiod acts as a conservative cue that delays development until day length reaches a genetically determined threshold. This photoperiod tracking protects them from leafing out during short-term warm spells, thereby reducing the risk of frost damage (Zohner and Renner, 2015).

Although phenological traits are under strong genetic control, their expression occurs through interactions with the previously described, typical environmental factors, ultimately leading to the activation of specific phenological processes. These factors, such as temperature and photoperiod, are relatively well understood and are therefore considered the “typical” drivers of phenological processes.

Nevertheless, a range of other biotic and abiotic factors, acting through various stress-related stimuli, can also influence phenological processes. When plants are exposed to such so-called “atypical” factors (Bačurin et al., 2023), they may exhibit shifts in phenological timing, resulting in either an advancement or delay in the onset of particular phenophases, as well as changes in phenological dynamics.

In this context, atypical environmental factors, such as drought (Vander Mijnsbrugge et al., 2016), variations in soil nutrient availability (Fu et al., 2019), elevated ozone concentrations (Skarby et al., 1998), rootstock effects influencing scion phenology (Camisón et al., 2021), and other forms of biotic stress (e.g., pathogenic fungi (Mutz et al., 2021) or insect defoliation induced phenological shifts (Kaitaniemi et al., 1997)), have emerged as critical drivers of phenological variation at both interspecific and intraspecific levels. These factors can significantly influence the timing, duration, and dynamics of phenological events, often leading to deviations from patterns determined by more typical environmental cues such as temperature and photoperiod.

Understanding how atypical factors shape tree phenological patterns is crucial for predicting forest ecosystem responses to various stressors and forms the foundation for effective future forest conservation and management. Atypical environmental factors, such as drought, nutrient imbalances, and rootstock-scion effects, must be considered within the framework of existing

phenological models and management practices. Ensuring the long-term adaptability and resilience of forest genetic resources requires a comprehensive understanding of how these stressors influence phenology across different genotypes. This thesis addresses these challenges by examining the effects of selected atypical factors, specifically the timing of drought, phosphorus enrichment, their interactions, and rootstock-scion interaction, on phenological variation across different species and genotypes of temperate forest trees. The study focuses on *Salix caprea* L., *Fagus sylvatica* L., *Quercus petraea* (Matt.) Liebl.), and *Quercus robur* L., with emphasis on both interspecific and intraspecific responses. This work provides insights for optimizing reproductive material production, establishing clonal seed orchards, and developing adaptive species selection strategies in the face of accelerating environmental change.

1.1. Research Objectives and Hypotheses

The main objectives of the study are as follows:

1. To examine the effects of drought stress, induced at different times of the growing season, on the phenology of a goat willow clone (*Salix caprea* L.) and a pedunculate oak provenance (*Quercus robur* L.), as well as the intragenotypic variation in these effects.
2. To assess the combined and separate effects of drought stress and increased phosphorus content in the substrate on the phenology of common beech (*Fagus sylvatica* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) provenances.
3. To evaluate the levels of interspecific and intraspecific variation in phenological responses among provenances of common beech (*Fagus sylvatica* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) under the influence of drought stress and increased phosphorus availability.
4. To investigate the effect of rootstock on intraclonal phenological variation in pedunculate oak (*Quercus robur* L.).

The following hypotheses have been proposed:

H1: Drought stress causes an “after-effect” (time shift) in the leaf phenology of goat willow (*Salix caprea* L.) and pedunculate oak (*Quercus robur* L.).

H2: Drought stress causes an “after-effect” (time shift) in the leaf phenology of common beech (*Fagus sylvatica* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) provenances

H3: Shifts in leaf phenology depend on the timing of drought stress induction.

H4: The effect of drought stress on height increment depends on the timing of drought stress induction.

H5: Increased phosphorus content and drought stress, both independently and in interaction, affect leaf phenology (time shifts) in common beech (*Fagus sylvatica* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) provenances

H6: Increased phosphorus content mitigates the effects of drought stress, reducing differences between treatments in common beech and sessile oak provenances.

H7: There is interspecific and intraspecific variation in the leaf phenology of sessile oak and common beech in response to increased phosphorus content and drought stress.

H8: Rootstock-scion interactions influence the leaf phenology of pedunculate oak (*Quercus robur* L.) clones

2. LITERATURE REVIEW

2.1. Typical environmental factors influencing phenology in forest tree species

Seasonal phenology in trees reflects adaptive responses to climatic cues, with temperature and photoperiod acting as key regulators of both spring and autumn phenological processes. In spring, flushing phenological processes are influenced by a combination of winter chilling, spring heat accumulation, and photoperiod, while in autumn, leaf senescence is influenced by declining day length and temperature (Robson et al., 2013; Basler and Körner, 2014; Way and Montgomery, 2015).

Importantly, not all species respond uniformly. Some tree species, such as common beech (*Fagus sylvatica* L.), exhibit complex strategies to avoid premature flushing. Heide (1993) showed that both chilling and photoperiod are essential for dormancy release and budburst in beech, allowing it to delay budburst until environmental conditions are stable. In contrast, hornbeam (*Carpinus betulus* L.), studied under similar conditions, did not display a photoperiodic requirement for budburst. Basler and Körner (2014) further demonstrated significant interspecific variability in response to environmental cues. Norway spruce (*Picea abies* (L.) H. Karst.) responded more to temperature, whereas beech was the least temperature-sensitive. Sycamore maple (*Acer pseudoplatanus* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) showed intermediate responses. In terms of photoperiod sensitivity, beech and spruce were highly responsive, oak showed a moderate response, while maple exhibited no detectable photoperiodic sensitivity. Taken together, these findings suggest that the timing of budburst and flushing is largely species-specific, shaped by evolutionary adaptation to specific environmental signals.

When observing autumn leaf senescence, the primary and most common factors triggering the onset of this process are temperature and photoperiod (Rosenthal and Camm, 1997; Way and Montgomery, 2015; Wu et al., 2018). Several authors suggest that, in most tree species, photoperiod acts as the dominant regulator of autumnal phenological processes, as it is considered more stable than temperature (Keskitalo et al., 2005; Basler and Körner, 2012), especially since temperature has become more unpredictable due to climate change. However, the relative importance of these cues can vary between ecosystems. Plants from higher latitudes are generally more sensitive to photoperiod, while those from lower latitudes tend to be more

sensitive to temperature fluctuations (Stinziano and Way, 2014; Way and Montgomery, 2015). This pattern is consistent with provenance trials in common ash (*Fraxinus excelsior* L.), where northern populations exhibited earlier and more consistent timing of autumn senescence, suggesting stronger control by photoperiod, whereas southern populations showed delayed and more variable senescence, indicating a greater sensitivity to temperature cues (Rosique-Esplugas et al., 2022). This is also observed in European aspen (*Populus tremula* L.), where northern provenances tend to initiate autumn senescence at shorter critical photoperiods than southern provenances, suggesting a relatively stronger influence of photoperiod on senescence timing in northern populations (Fracheboud et al., 2009).

In the context of climate change, increased temperature sensitivity has led to an average extension of the growing season (Linderholm, 2006), driven by a significant advancement of spring phenology in most deciduous tree species (Bertin, 2008) and a delayed onset of autumn leaf senescence (Fu et al., 2018). However, these conditions may also increase the risk of frost damage from late spring and early autumn frosts.

2.2. Atypical environmental factors influencing phenology in forest tree species

2.2.1. The impact of drought on phenological processes in forest tree species

Climate change, manifested in rising average temperatures and an increased frequency of drought events, poses a significant and escalating threat to temperate forest ecosystems, particularly in terms of their stability, productivity, and regenerative capacity (Allen et al., 2010; Seneviratne et al., 2012). Forest tree species are particularly vulnerable due to their long lifespans, which limit their capacity to adapt to rapidly changing environmental conditions (Lindner et al., 2010).

In the context of climate change, the effect of drought stress is a frequent topic of many studies, since drought is the main limiting factor that hinders the growth, reproduction, and survival of plants worldwide (Chaves et al., 2003). Drought stress has a profound impact on vegetative growth in forest tree species, suppressing both biomass accumulation and photosynthetic activity (Arend et al., 2013; Bréda et al., 2006; Rubio-Cuadrado et al., 2018), triggering morphological and physiological adjustments such as reduced leaf area, enhanced root growth,

tighter stomatal regulation, and changes in wood anatomy that affect hydraulic efficiency (Hinckley et al., 1980; Thomas and Gausling, 2000; Arend et al., 2013; Balzano et al., 2025). Consequently, water deficit reduces shoot and leaf biomass and the leaf-to-root biomass ratio in sessile oak (Thomas and Gausling, 2000), decreases leaf area and total biomass in beech (Van Hees, 1997), and suppresses seedling height growth in several oak species, including pedunculate, sessile, pubescent, and Turkey oak (Arend et al., 2011; DeliGöz and Bayar, 2018).

Under ongoing climate change, drought has emerged as a key driver influencing not only plant growth but also phenological processes, with phenology recognized as one of the most sensitive and reliable indicators of vegetation response to environmental stress (Chuine et al., 2000; Polgar and Primack, 2011). Although phenological traits are primarily regulated by temperature and photoperiod (Basler and Körner, 2014), a growing body of evidence suggests that so called atypical environmental factors (Bačurin et al., 2023), such as drought, can also modify established phenological patterns.

In this context, long-term observations show that dry conditions are playing an increasingly important role in determining the timing of autumn leaf phenological processes (Wu et al., 2022). Vander Mijnsbrugge et al. (2016) reported that stronger drought stress leads to a more pronounced delay in the onset of autumn leaf senescence in sessile oak seedlings. A similar pattern has been recorded in other forest tree species, for example in beech (Arend et al., 2016; Pflug et al., 2018), as well as in pedunculate oak (Čehulić et al., 2019). Supporting this, Leuzinger et al. (2005) reported that, following the extreme summer drought in Central Europe in 2003, the average leaf senescence date of five deciduous tree species was extended by 22 days. Since the growing season spans the period between budburst and autumn leaf senescence, and drought can disrupt nitrogen uptake and carbon assimilation during this time (McDowell et al., 2008; Joseph et al., 2021), a delayed onset of autumn leaf senescence may serve as a compensatory mechanism to help maintain physiological function following stress conditions. This idea is supported by experimental evidence showing that when carbon assimilation is reduced, as often occurs under drought stress, plants tend to postpone leaf senescence, whereas higher photosynthetic activity is generally associated with an earlier onset of autumn senescence (Zani et al., 2020). By extending the growing season and the duration of active photosynthesis, plants may partially offset the assimilate losses incurred under drought stress (Arend et al., 2016; Pflug et al., 2018), although this compensation is effective only as long as it does not increase the risk of damage from early autumn frosts (Estiarte and Peñuelas, 2015). Postponing leaf senescence increases the risk that early autumn frosts will damage leaves before

nutrient resorption is completed, thereby limiting the nutrient reserves available to support growth in the following season (Fracheboud et al., 2009; Schreiber et al., 2013). In relation to this, a study by Niinemets and Tamm (2005) showed that leaves which fell earlier contained higher nutrient concentrations, while nutrient levels in fallen leaves gradually declined as leaf fall progressed. Although climate change, and drought in that context, are generally understood to delay the onset of autumn leaf senescence and prolong the growing season (Menzel, 2000; Leuzinger et al., 2005; Vitasse et al., 2011; Vander Mijnsbrugge et al., 2020), some studies suggest that this effect may be diminished (Mariën et al., 2021). However, it is known that the drought intensity influences the senescence response: when drought is more severe, the delay in the onset of autumn leaf senescence tends to be greater (Vander Mijnsbrugge et al., 2023). The same study suggests that, beyond drought intensity, the timing of drought stress may also play an important role in triggering the onset of autumn leaf senescence. It indicates that summer drought causes certain shifts in the timing of senescence, while spring drought did not lead to such changes in the study by Mariën et al. (2021), highlighting timing as a potential factor behind the differences in observed outcomes. It is known that the phytohormone abscisic acid (ABA) plays an important role as a trigger of autumnal leaf senescence (Guak and Fuchigami, 2001; Yang et al., 2014; Guo et al., 2021). As a stress-related hormone, ABA regulates plant responses to both abiotic and biotic stresses, as well as various developmental processes (Chaves et al., 2003). Rewatering after drought stress can reduce ABA levels (Wan et al., 2009; Xu et al., 2010), and in this way, drought-related ABA dynamics may interfere with the normal progression of senescence, potentially contributing to a delayed onset (Vander Mijnsbrugge et al., 2023).

In addition to its impact on autumn leaf senescence, drought also influences spring flushing phenology. The spring phenological response to drought reflects the stress experienced during the previous growing season, commonly referred to in the literature as a “carry-over effect” (Yonekura et al., 2004) or “after-effect” (Tahkokorpi et al., 2007; Vander Mijnsbrugge et al., 2020). It reflects the ability of plants to retain a form of physiological “memory” of past stress, arising from epigenetic and biochemical modifications triggered by earlier drought events, which subsequently shape their development by altering both physiological functioning and the timing of spring budburst, potentially enhancing their tolerance to future stress (Bruce et al., 2007). A delay in the onset of bud flush in the subsequent spring (following drought stress during the previous growing season) has been reported in sessile oak seedlings (Vander Mijnsbrugge et al., 2016). The opposite was observed in a study on glossy buckthorn (*Frangula*

alnus Mill.), where budburst showed a significant advancement following drought treatment in the previous growing season (Vander Mijnsbrugge et al., 2020). An advancement of budburst attributed to the carry-over effect has also been documented in oak species (Kuster et al., 2014) as well as in *Fagus crenata* Blume (Yonekura et al., 2004). Interestingly, Čehulić et al. (2019) reported that budburst was delayed in one year and advanced in the next. As previously noted, environmental cues and stresses can induce epigenetic modifications, creating a flexible form of stress “memory” (Bräutigam et al., 2013; Rubio et al., 2023). Stress tolerance is mediated through complex signalling cascades involving signalling molecules that can trigger epigenetic reprogramming (Farooq et al., 2009; Haghpanah et al., 2024). ABA plays an important role in these epigenetic responses, particularly under abiotic stress conditions (Chinnusamy et al., 2008).

2.2.2. *The impact of phosphorus availability on phenological processes in forest tree species*

Nutrient availability is another atypical environmental factor that may influence the timing of phenological processes in forest tree species. While it is well established that nutrients affect various physiological functions in plants, their specific role in regulating phenological events, such as budburst and leaf senescence, remains relatively underexplored. A limited number of studies indicate that nutrient status can lead to either an advancement (Asshoff et al., 2006; Zani et al., 2020) or a delay (Fu et al., 2019; Wang et al., 2022) in the timing of autumnal senescence.

Among essential macronutrients, phosphorus (P) plays a particularly vital role in plant metabolism, productivity, and stress resilience (Zhao et al., 2025; Satheesh and James, 2026). Yet, it is frequently the most limiting nutrient in forest soils (Harrison, 1999), with declining foliar P concentrations reported across European forests (Houdijk et al., 1993). Phosphorus is essential for energy-related processes, enabling plants to capture, store, and utilize solar energy efficiently (Rychter and Rao, 2005). As a crucial element in energy-related processes, phosphorus enables plants to capture, store, and utilize solar energy efficiently. It forms part of key molecules such as ATP and nucleic acids (DNA and RNA), and it is fundamental for the synthesis of enzymes involved in the mobilization and redistribution of carbohydrates and mineral nutrients within the plant system (Poirier and Bucher, 2002; Malhotra et al., 2018). Moreover, as a chemical element incorporated into nucleic acids, phospholipids, and energy-

carrying compounds, phosphorus participates in the regulation of numerous enzymatic and metabolic reactions in plants (Theodorou and Plaxton, 1993). In addition to its functions in growth and metabolism, phosphorus also affects hormone signaling and the expression of senescence-related genes and may influence chromatin-level regulation through SPX domain-mediated signaling (Chiou and Lin, 2011; Wang et al., 2021). Because of these diverse functions, phosphorus availability can influence both the timing and progression of key phenological events, such as spring leaf development and autumn leaf senescence, through metabolic and regulatory pathways. This is supported by findings showing that phosphorus deficiency not only suppresses the development of vegetative and reproductive structures but also significantly delays budburst in apple trees (Taylor and Goubran, 1975). Yang et al. (2016) found that, while the onset of budburst in common beech was unaffected by phosphorus availability, trees from phosphorus-rich sites exhibited faster spring leaf development compared to those from phosphorus-poor habitats, indicating that phosphorus influences the rate and duration of phenological phases rather than their initiation. However, a significant correlation was observed between mean budburst date in *Quercus lobata* Née and soil phosphorus availability, with trees growing in phosphorus-rich soils leafing out earlier (Koenig et al., 2021). Furthermore, Wang et al. (2022) found that the application of nitrogen and phosphorus influences the timing of leaf senescence in two-year-old larch seedlings, showing that supplementing with either nutrient individually or in combination notably delayed the initiation of autumn leaf senescence.

Moreover, phenological responses to nutrients may vary not only between species, but also between provenances of the same species, due to genotypic adaptations to local soil conditions, climate, and rooting strategies (Yang et al., 2016). For instance, while beech and sessile oak often co-occur in European forests, they differ in both root architecture and flushing patterns, with oak typically leafing out earlier than beech. These inter- and intra-specific differences raise important questions about how phosphorus supply may differentially modulate phenological development.

Although current evidence suggests that phosphorus availability can influence the timing and progression of phenological events, research on this topic remains limited. This gap is particularly relevant in the context of evolving forest management practices. As climate-related disturbances become more frequent, artificial regeneration and intensive nursery production of forest reproductive material (FRM) are increasingly widespread across Europe (Forest Europe, 2020). Fertilization is a central component of nursery and seed orchard management, yet its

effects on phenological stability and seedling frost sensitivity remain poorly understood. To address these uncertainties, broader, comparative studies are needed to clarify the role of phosphorus in shaping tree phenology across diverse forest ecosystems.

2.2.3. Interactive effects of drought and elevated phosphorus on phenological processes in forest tree species

It is well known that phosphorus deficiency, through its negative impact on metabolic processes, is often a limiting factor for plant development and physiological processes (Bates and Lynch, 2000; Sardans and Peñuelas, 2004; Aziz et al., 2014). Similarly, drought negatively affects a wide range of metabolic processes, thereby also limiting plant growth and functioning (Thomas and Gausling, 2000; Rubio-Cuadrado et al., 2018). Therefore, while drought and phosphorus deficiency independently constrain plant physiological processes, their interactive effects on plant physiology also need to be considered. One important aspect of this interaction is the negative impact of drought on phosphorus uptake. It is well established that drought restricts phosphorus availability and transport within plants, leading to reduced phosphorus concentrations in the leaves (Sardans and Peñuelas, 2004). The second aspect highlights the beneficial role of phosphorus fertilization in mitigating the adverse effects of drought stress (Gutiérrez-Boem and Thomas, 1998; Jones et al., 2005; Waraich et al., 2011; Tariq et al., 2019). When examining the effects of these factors on phenology, previous studies have shown that both drought (Kuster et al., 2014; Dox et al., 2022) and sufficient phosphorus availability (Taylor and Goubran, 1975; Yang et al., 2016) can influence the timing and progression of phenological processes. However, studies examining the combined effects of drought and phosphorus fertilization on the phenology of woody species are still very limited (Wang et al., 2022; Paquini-Rodríguez et al., 2025).

2.2.4. The impact of rootstock on scion phenology

As previously noted, the demand for high-quality forest reproductive material is steadily increasing. Clonal seed orchards are the most widely used system for producing large quantities of genetically improved seed for operational forest planting (White et al., 2007). They are

practical because selected trees can be easily propagated vegetatively—through rooting, grafting, or tissue culture. In addition, trees propagated vegetatively from mature individuals start flowering earlier than those grown from seed, which means that seed production begins sooner than in generative seed orchards.

Besides securing frequent and abundant seed crops, another crucial objective in the management of clonal seed orchards is to maintain a sufficiently large number of genetically distinct clones for seed production, thereby maximizing the effective population size (Kramer et al., 2008).

For these reasons, clonal seed orchards have been established in Croatia to improve the genetic quality of forest reproductive material and to increase both the frequency and quantity of seed production (Vidaković, 1996). These orchards are formed through vegetative propagation (grafting) of “plus trees,” phenotypically superior individuals selected from natural forest stands.

Grafting is the process of joining two plant parts so that they grow together and share the same vascular system (Pina and Errea, 2005), thereby creating a genetically composite organism that functions as a single plant (Mudge et al., 2009). The scion refers to the vegetative shoot taken from a selected tree, which will develop into the upper part of the grafted plant, while the lower part of the plant is called the rootstock (Mudge et al., 2009). Grafting is the most common form of vegetative propagation used in establishing clonal seed orchards. This method, like any other form of vegetative propagation, ensures that new plants in a clonal seed orchard share the same genotype as the selected trees from which the scions were collected (White et al., 2007).

Budburst is known to be strongly influenced by genetic factors, and its heritability is generally high. Findings from several tree species support this, including studies on pedunculate oak (Scotti-Saintagne et al., 2004), poplar (*Populus* spp.) (Frewen et al., 2000), and birch (*Betula* spp.) (Billington and Pelham, 1991). It is also well known that other key phenological processes are under genetic control and are shaped by environmental factors such as temperature and photoperiod (Robson et al., 2013; Basler and Körner, 2014; Zohner and Renner, 2015). Since grafting combines two genetically distinct components, the rootstock and the scion, it is reasonable to assume that the rootstock may influence shifts in the phenological timing of the scion compared to the original tree (the ortet) from which the scion was taken.

In both forestry and agriculture, rootstocks are often selected to improve graft compatibility, regulate the growth of the scion, and enhance reproductive performance (Jayawickrama et al.,

1991). Studies on fruit tree species have demonstrated that rootstocks can affect vegetative growth, total yield, and a range of phenological and physiological traits expressed by the scion, including leaf and flower development (Wang et al., 1994; Jiménez et al., 2004, 2011; Neilsen et al., 2016; Tworkoski et al., 2016). Furthermore, it has been well documented that certain rootstocks can improve plant tolerance to drought (Tworkoski et al., 2016). Although direct experimental evidence in pedunculate oak (*Quercus robur* L.) remains limited, studies on woody perennials clearly demonstrate that rootstock–scion interactions can modify the timing of key phenological phases, including bud burst and flowering. Differences among rootstocks in hydraulic conductivity and water transport capacity have been shown to influence the onset of shoot growth and bud activation in grafted trees, thereby affecting spring phenology (Atkinson et al., 2003). Beyond hydraulic control, grafting enables long-distance physiological communication between rootstock and scion through vascular tissues, involving hormones and mobile signaling molecules that regulate developmental timing (Goldschmidt, 2014). At the molecular level, experimental evidence confirms that flowering-related signals, including Flowering Locus T (FT) protein and associated mRNAs, can move across graft unions via the phloem, resulting in altered flowering phenology of the scion (Notaguchi and Okamoto, 2015). These findings indicate that phenological traits in grafted trees are not solely determined by scion genotype or environmental conditions, but may also reflect persistent, non-genetic rootstock effects. Consequently, phenological asynchrony observed among genetically identical ramets in clonal seed orchards may partly arise from continuous physiological signaling between rootstock and scion, providing a mechanistic framework for interpreting clone-specific variation in leaf phenology.

2.2.5. Knowledge gaps in understanding the effects of atypical environmental factors on forest tree phenology

Forest tree phenology is strongly regulated by environmental cues (Caffarra and Donnelly, 2011; Vitasse et al., 2011; Basler and Körner, 2012; Vander Mijnsbrugge et al., 2016); however, certain aspects of the effects of atypical environmental factors on forest tree phenology remain poorly understood. Although the influence of drought on phenology is well established, with drought inducing shifts in the onset of phenological processes, the underlying mechanisms remain unclear, as drought can lead to either advances or delays in phenological timing. The causes of differing directions in phenological shifts, particularly in leaf flushing, remain an open

question. Čehulić et al. (2019) reported contrasting shifts in spring phenology across two growing seasons and suggested that differences in the timing of drought exposure may represent a possible explanation for these variable responses. Building on this hypothesis, Bačurin et al. (2025a, 2025b) specifically address the potential role of drought induction timing in shaping phenological responses. Accordingly, the aim of these studies was to examine the effects of drought stress induced at different times during the growing season on the phenology of a goat willow clone (*Salix caprea* L.) and a pedunculate oak provenance (*Quercus robur* L.), as well as to assess intragenotypic variation in these effects.

Following drought, another atypical factor that may influence shifts in phenological processes is phosphorus nutritional status. Most studies to date have focused on the detrimental effects of phosphorus deficiency (Wu et al., 2003; Warren and Adams, 2002; Geilfus et al., 2017), while the consequences of nutrient surplus, including potential physiological stress, are rarely addressed (Pan et al., 2022). The effects of increased phosphorus availability in the substrate, and whether there are interspecific and intraspecific variations in leaf phenological responses, represent knowledge gaps. Thus, the study by Bačurin et al. (2023) aimed to provide insights into these questions dealing with common beech (*Fagus sylvatica* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) provenances.

Furthermore, since phosphorus nutrition is known to help mitigate drought stress and considering that drought often leads to changes in plant phenology, it becomes essential to explore how these two factors may interact. The study by Bačurin et al. (2025c) aims to shed light on this interaction by focusing on interspecific and intraspecific variation in phenological responses among provenances of common beech and sessile oak under the combined influence of drought stress and enhanced phosphorus availability.

Another open question regarding the influence of atypical factors on tree phenology is the effect of rootstock on the scion. Although research on fruit trees and herbaceous crops has clearly shown that rootstock can significantly affect leaf and flowering phenology, this relationship has been far less studied in forest tree species. Therefore, the study by Bačurin et al. (2026) aims to investigate how different rootstocks affect intraclonal phenological variation in pedunculate oak scions.

3. DISCUSSION

3.1. Drought as an atypical environmental factor influencing phenology

3.1.1. Spring leaf phenology

One of the hypotheses of this dissertation was that drought affects leaf phenology not only during the season in which it occurs, but also in subsequent growing seasons, inducing phenological responses commonly referred to as after-effects or carry-over effects (Yonekura et al., 2004; Tahkokorpi et al., 2007; Vander Mijnsbrugge et al., 2016). While the existence of such responses has been documented in several woody species, previous studies have reported contrasting shifts in spring phenology following drought, ranging from delayed to advanced budburst (Čehulić et al., 2019). This variability in phenological responses has raised unresolved questions about the underlying drivers responsible for these divergent outcomes, particularly whether differences in stress timing determine the direction of the phenological shift (Čehulić et al., 2019).

The results of this dissertation indicate that the timing of drought induction during the growing season may represent an important factor contributing to these contrasting responses, helping to explain previously inconsistent findings. In the study conducted on a goat willow clone (Bačurin et al., 2025a) presented in this dissertation, drought imposed at different stages of the growing season resulted in distinct and often opposing phenological shifts in the subsequent year, supporting hypotheses H1 and H3. More specifically, drought stress induced at different periods of the growing season led to significant and contrasting shifts in spring leaf phenology in the following year, confirming the presence of a carry-over effect. Furthermore, early- and late-season drought induction resulted in opposite phenological shifts, suggesting that the response depends on the developmental stage at which the stress occurs, thereby confirming hypothesis H3 in this case.

A clear pattern emerged from the results. Groups exposed to drought from March to early June (groups 1–4) leafed out earlier, on average, than the control group. In contrast, groups exposed to drought from early June to mid-July (groups 5–7) leafed out later than the control. These findings demonstrate that the timing of drought during the growing season determines not only whether a phenological shift occurs, but also its direction. Specifically, early-season drought

resulted in earlier budburst in the following year, whereas drought imposed later in the season caused delayed leaf-out. The observation of opposite responses within the same clonal material strongly supports the conclusion that, in this case, the timing of stress exposure is the key driver of phenological shifts in the subsequent growing season.

Several mechanisms may underlie this phenomenon. Plants are capable of “remembering” stress through epigenetic mechanisms such as DNA methylation, histone modification, and chromatin remodeling, which alter gene expression without changing the DNA sequence (Madlung, 2004; Bruce et al., 2007). In addition, drought stress triggers hormonal responses, particularly involving abscisic acid (ABA), which regulates gene expression and plays a central role in drought signaling (Chaves et al., 2003). These stress-induced physiological and molecular adjustments may persist beyond the season of exposure and ultimately influence budburst timing in the following year, providing a mechanistic explanation for the observed carry-over effects.

At the same time, the results suggest that carbon reserves may also play an important role. The first drought group, which leafed out earliest in 2022, had shown delayed autumn senescence in the previous year. This extended growing season likely allowed a longer period of photosynthesis and carbon assimilation. Consequently, these plants may have accumulated higher concentrations of non-structural carbohydrates (NSCs), which serve as essential energy reserves for budburst and early leaf development. Previous research has shown that low autumn NSC concentrations are associated with delayed spring phenology in several deciduous tree species (Amico Roxas et al., 2021). Accordingly, higher NSC levels could promote earlier budburst, as observed here. Although NSC concentrations were not directly measured in this study, the temporal pattern supports this interpretation and highlights the need for future physiological analyses. Overall, these findings indicate that drought-induced carry-over effects on spring phenology are likely driven by a combination of developmental timing, stress signaling, and resource dynamics.

A comparable experimental design was applied in pedunculate oak (Bačurin et al., 2025b). However, in contrast to goat willow, spring leaf phenology in pedunculate oak was largely unaffected by the drought treatments, indicating that a clear carry-over effect on budburst was not detected in this case. Although numerous studies have reported after-effects in the spring phenology of oak species (Sanz-Pérez and Castro-Díez, 2010; Vander Mijnsbrugge et al., 2016; Čehulić et al., 2019), specific experimental conditions may have contributed to the absence of such effects in the present study. Nutrient limitations caused by substrate leaching or reduced

soil aeration under subirrigation may have influenced carbohydrate accumulation and recovery, thereby affecting phenology, as nutrient limitation is known to reduce photosynthetic capacity (Wilson et al., 2000; Sun et al., 2018). At the same time, such conditions may have induced unintended stress in control plants, thereby reducing differences between treatments. Additional support for this interpretation is provided by net photosynthesis measurements, which showed that drought-stressed plants did not exceed control values during the recovery phase. This contrasts with findings in European beech, where previously drought-stressed individuals maintained higher net photosynthetic activity than controls later in the season (Arend et al., 2016). If we consider the assumption that post-drought photosynthesis may lead to compensatory carbohydrate accumulation, as suggested for goat willow, it is possible that in this case, due to poor nutrient status, such compensatory accumulation did not occur. This interpretation is supported by the lack of enhanced post-drought photosynthesis, which may consequently have limited the expression of stress memory in the subsequent spring phenology. Under these circumstances, the interactive effects of drought and suboptimal nutrient availability likely contributed to the absence of clear carry-over effects on spring phenology in this experiment (Bačurin et al., 2025b). Therefore, although no significant after-effect or shift in budburst or spring leaf phenology was detected, hypotheses H1 and H3, proposing that drought stress induces an after-effect and a temporal shift in leaf phenology, cannot be definitively rejected in the case of pedunculate oak.

3.1.2. Autumn leaf senescence

The two experimental studies on goat willow and pedunculate oak demonstrate that drought induction causes measurable shifts in autumn phenology, delaying the onset of leaf senescence and slowing its progression. The magnitude of these phenological shifts depended on the timing of drought stress during the growing season, thereby supporting hypothesis H3.

In goat willow, early-season drought (induced shortly after leaf emergence) resulted in a modest delay of approximately four days in the onset of senescence compared with the control in the first year. However, in the second year, when drought intensity was higher, all drought-treated groups exhibited delayed autumn senescence, with the most pronounced effect observed in plants exposed to late-season drought. A distinct gradient in the response was evident: the later the drought event occurred within the growing season, the more pronounced the delay in autumn phenology. This pattern can be interpreted as a compensatory response, whereby plants attempt to offset earlier reductions in carbon assimilation caused by water deficit. Following

rehydration, drought-exposed plants may prolong leaf functionality and enhance photosynthetic activity, a post-drought stimulation response that has also been documented in common beech (Arend et al., 2016). This increase in photosynthetic activity may be partially explained by the inverse relationship between photosynthesis and leaf carbohydrate status that has been well documented (Ow et al., 2010; Turnbull et al., 2002). During prolonged drought, reductions in carbon assimilation can result in negative carbon balance, and sustained respiratory demand may lead to significant depletion of leaf starch and whole-plant non-structural carbohydrate reserves (McDowell et al., 2008; Mitchell et al., 2013). Once environmental conditions become favorable again, recovery of photosynthesis may result in elevated assimilation rates as carbon balance is gradually restored. According to Luo et al. (2024), the onset of leaf senescence may occur only after a specific threshold concentration of starch in leaves is reached, with starch potentially acting as a signal triggering senescence initiation. If such a mechanism operates, altered carbohydrate dynamics following drought could influence the timing at which this critical starch threshold is attained, thereby contributing to delayed autumn senescence. Because different treatment groups were exposed to drought at different periods during the growing season, the temporal dynamics of carbon depletion and subsequent starch accumulation likely varied among groups. Consequently, the timing at which the proposed starch threshold was reached may have differed accordingly, resulting in variation in the onset of senescence among treatments.

A comparable pattern was observed in pedunculate oak. Seedlings exposed to drought in late spring and summer exhibited a statistically significant delay and slower progression of autumn senescence. As observed in willow, the magnitude of the delay followed a gradient associated with the timing of stress exposure, with later drought treatments leading to increasingly pronounced delays in autumn senescence.

Importantly, in both experiments, the expression of phenological responses depended on drought intensity. Under comparatively mild drought conditions, such as in the willow experiment in 2021 and in oak in 2022, some treatment groups did not experience sufficient water limitation to induce detectable changes in autumn phenology. In contrast, during the second experimental year in both studies, when drought severity was greater, delayed senescence was consistently observed across all treated groups. Together, these results indicate that both drought intensity and the timing of stress exposure influence the magnitude of phenological reaction.

3.1.3. Influence of drought timing on height increment

Height growth in both goat willow and pedunculate oak was strongly influenced by the timing of drought induction, demonstrating that water deficit does not affect growth uniformly across the growing season. Instead, the developmental stage at which drought occurs determines both the magnitude and direction of the growth response. The results of the two studies indicate that drought timing, rather than drought occurrence per se, represents a critical factor shaping height increment, thereby supporting hypothesis H4.

In pedunculate oak, height growth was significantly reduced when drought occurred later in the growing season, whereas early-season drought had weaker or non-significant effects (Bačurin et al., 2025b). In the same study, seedlings exposed to early-season drought maintained height increments comparable to the control, suggesting that stress shortly after budburst did not constrain final height growth. This capacity for compensation may be related to the episodic growth phenology characteristic of oaks, which allows renewed shoot elongation when favorable conditions are restored. In contrast, drought imposed during later stages of active growth likely overlaps with critical phases of shoot extension and resource allocation, thereby limiting the potential for compensatory growth before seasonal growth cessation. This pattern is consistent with findings by Canham et al. (1999), who reported stronger negative impacts of summer drought in species with episodic growth patterns such as oak. Reductions in height increment under later or more intense drought may also reflect shifts in carbon allocation toward belowground structures under water deficit, aimed at restoring hydraulic functionality and enhancing water and nutrient uptake (Hagedorn et al., 2016), potentially constraining aboveground biomass production and vertical growth (Stober et al., 2000).

In goat willow, growth responses were likewise timing-dependent but exhibited greater interannual variability. During the first experimental year, most drought-treated groups showed reduced height increment, particularly when stress occurred in late spring and early summer. In the second year, when drought intensity was higher, responses differed among treatments: seedlings exposed to drought at the onset of intensive shoot elongation showed the strongest growth reductions, whereas those subjected to later-season drought displayed comparable or even greater height increments relative to the control. Willow species typically complete a substantial proportion of their annual height growth shortly after the onset of vegetative activity (Labrecque et al., 1993), making them particularly sensitive to water deficit during early-season shoot elongation. In line with this, species classified as early-season growers, including

willows, have been shown to exhibit reduced height growth under spring drought conditions (Canham et al., 1999; Van Kampen et al., 2022). In contrast, drought imposed later in the season, after the main growth flush had occurred, did not necessarily constrain final height and in some cases was associated with enhanced growth (Bačurin et al., 2025a, Figure 4). Such responses may reflect compensatory mechanisms operating after rehydration (Arend et al., 2016; Gessler et al., 2020), potentially involving shifts in carbon allocation, utilization of stored non-structural carbohydrates, or prolonged vegetative activity. These patterns indicate that, in goat willow, the effect of drought on height growth is highly dependent on its temporal overlap with the primary growth phase, and that stress occurring outside this critical window may be partially buffered or even followed by compensatory growth.

Although both species were sensitive to the timing of drought, their responses differed. In pedunculate oak, height growth was significantly reduced when drought occurred later in the growing season, potentially reflecting the inhibition of additional shoot flushes characteristic of the species' episodic growth pattern. Early-season drought did not affect height growth, possibly due to compensatory growth and sufficient time for carbon assimilation later in the season, particularly in association with delayed autumn senescence. In contrast, goat willow showed a more variable response. Early-season drought reduced height growth, which is consistent with the species' growth pattern, as a substantial proportion of its annual increment occurs at the beginning of the growing season. Conversely, drought imposed later in the season was sometimes followed by equal or even enhanced height increment, suggesting the presence of compensatory growth mechanisms.

Overall, height growth was influenced by the interaction between drought timing, drought intensity, and species-specific growth phenology. The strongest reductions occurred when drought coincided with intensive shoot elongation, whereas stress outside this critical developmental window allowed partial recovery or, in some cases, enhanced growth. Because these timing-dependent responses were observed under controlled experimental conditions, they are unlikely to be solely the result of environmental heterogeneity. In the context of climate change, where drought regimes are expected to shift not only in intensity and frequency but also in seasonal distribution, these findings (Bačurin et al., 2025a, 2025b) underline the importance of considering the seasonal timing of drought events, as their effects on tree growth vary depending on the developmental stage at which stress occurs.

3.1.4. Species and provenance differences in leaf phenology of sessile oak and beech under drought

In the provenance-based experiment with common beech and sessile oak (Bačurin et al., 2025c), moderate drought did not induce a consistent effect on autumn phenology in either species. Although minor shifts in leaf senescence timing were observed, these changes were not statistically significant. Previous studies have shown that moderate to severe drought can significantly affect autumn phenological responses (Leuzinger et al., 2005; Dox et al., 2022; Vander Mijnsbrugge et al., 2024). However, in the present experiment, the applied drought intensity may have been insufficient to trigger a measurable phenological shift.

The absence of a significant autumn response is consistent with the findings of Bačurin et al. (2025a), where, in the experimental year characterized by lower drought intensity, autumn phenology remained unaffected in several treatment groups of the goat willow clone (see Figure 6 in Bačurin et al., 2025a). A similar pattern was observed in the oak experiment, where the first treatment group was rehydrated immediately after leaf wilting, at a stage when the leaves were still thin and the overall drought effect remained relatively low (Bačurin et al., 2025b; see Figures 2a and 4a). These results suggest that drought-induced shifts in autumn phenology may require a certain threshold of stress intensity before becoming detectable.

Accordingly, autumn leaf senescence remained largely unaffected under drought treatment compared to the control. Under moderate drought conditions, no significant autumn phenological shifts were detected. However, this does not imply that drought has no effect on autumn phenology; rather, it suggests that the applied stress intensity was insufficient to elicit a measurable response. The results indicate that detectable shifts in autumn phenology may require more severe or prolonged water limitation.

However, drought stress significantly influenced spring phenology, and the direction of this response differed between species. This pattern is consistent with previous reports of divergent spring phenological shifts following drought (Vander Mijnsbrugge et al., 2016; Čehulić et al., 2019). In the SB provenance of European beech, drought advanced budburst, whereas in sessile oak from the same provenance it delayed leaf unfolding. These contrasting responses confirm that drought can induce carry-over effects on spring phenology in the subsequent growing season, thereby supporting hypothesis H2. At the same time, the results demonstrate that both the direction and magnitude of these after-effects are species- and provenance-dependent.

In *Fagus sylvatica* (SB provenance), budburst occurred several days earlier under drought compared to control plants (Bačurin et al., 2025c, Figure 3a). This advancement may reflect a drought-escape response, whereby earlier leaf development enables enhanced carbon assimilation before the onset of potential summer water limitation (Campioli et al., 2024). However, advancing budburst may simultaneously increase exposure to late spring frost, indicating a trade-off between maximizing carbon gain and minimizing frost risk.

In contrast, *Quercus petraea* from the same provenance (SB) exhibited a delay in budburst under drought conditions. This delay may reflect a conservative water-use strategy in oaks, associated with stricter stomatal regulation and tighter hydraulic control (Bose et al., 2021). Drought-induced delays in budburst may also involve hormonal regulation, particularly abscisic acid (ABA)-mediated modulation of dormancy release and meristem activation (Van Der Schoot and Rinne, 2011). These opposite responses within the same provenance show that drought does not affect spring phenology in a uniform way, but that the outcome depends on species-specific physiological regulation.

In the KA provenance, no significant drought-induced shifts were detected in either species. This absence of response may indicate reduced phenological plasticity or greater physiological buffering capacity. Previous studies suggest that the KA provenance exhibits enhanced antioxidant activity and increased root biomass allocation (Sever et al., 2023; Vukmirović et al., 2025), traits that may mitigate drought-induced signaling cascades and attenuate downstream effects on phenological timing.

Overall, the results demonstrate that drought alone can induce species- and provenance-specific shifts in spring phenology, whereas autumn phenology remained largely unaffected under the applied moderate stress. The divergence observed between beech and oak within the SB provenance, together with the absence of response in the KA provenance, indicates that drought-induced after-effects in spring leaf phenology are governed by species identity and genetic background rather than occurring uniformly across taxa.

3.2. *The role of increased phosphorus availability in shaping leaf phenology: interspecific and intraspecific responses*

Nutrient availability represents an environmental factor that may modulate phenological processes in forest trees, although its role remains considerably less explored compared with temperature and photoperiod (Asshoff et al., 2006; Fu et al., 2019; Yang et al., 2016). In this dissertation, increased phosphorus availability was investigated for its potential to alter the timing and progression of leaf phenology in common beech and sessile oak. The hypothesis that increased phosphorus availability affects leaf phenology (H5) was tested using juvenile plants originating from two provenances representing contrasting climatic and edaphic conditions. The results reported in Bačurin et al. (2023) demonstrate that elevated phosphorus availability significantly influenced autumn leaf phenology in both species, advancing the onset and accelerating the progression of leaf senescence, while its influence on spring phenology remained generally limited. These findings therefore support the phosphorus component of hypothesis H5 and indicate that phosphorus availability can influence the temporal dynamics of leaf phenological development.

A more detailed examination of the results revealed that both beech provenances responded strongly to phosphorus addition. In the +P treatment, the onset of visible senescence symptoms occurred earlier and the progression of leaf discoloration and mortality was faster than in control plants (Bačurin et al., 2023). Sessile oak exhibited a similar directional response, with earlier autumn senescence under elevated phosphorus availability; however, the magnitude of this response was generally weaker, particularly during the first year of the experiment. These interspecific differences are likely related to differences in phosphorus uptake and internal nutrient allocation. Leaf nutrient analyses showed that beech saplings accumulated higher phosphorus concentrations than oak saplings under the +P treatment, which may partly explain the stronger phenological response observed in beech (Bačurin et al., 2023, Table 3).

Although both oak provenances showed a general tendency toward earlier autumn senescence under elevated phosphorus availability, the response of the Qp_KA provenance was weaker and, in the second experimental year, not statistically significant. This absence of a clear phenological shift is particularly noteworthy given that the Qp_KA provenance exhibited the largest difference in leaf phosphorus concentration between fertilized and control plants. One possible explanation is that both phosphorus limitation and elevated phosphorus availability may promote earlier senescence through different physiological pathways, resulting in a convergence of phenological trajectories between treatments. Under such circumstances, control plants experiencing relatively low phosphorus availability may initiate senescence earlier due to nutrient limitation, while phosphorus-enriched plants may also exhibit earlier

senescence due to accelerated physiological development associated with improved nutrient status. Consequently, the phenological difference between treatments may become less pronounced, leading to statistically non-significant differences despite substantial contrasts in internal phosphorus status.

The advancement of autumn leaf senescence under elevated phosphorus availability is consistent with the physiological role of phosphorus in plant metabolism. Phosphorus is a key component of energy transfer molecules and nucleic acids and is therefore essential for processes such as photosynthesis, carbohydrate metabolism, and nutrient remobilization within the plant. Increased phosphorus availability may consequently enhance plant productivity and carbon assimilation, potentially enabling plants to complete seasonal developmental programs more rapidly. This interpretation is consistent with previous findings showing that increased photosynthetic productivity and assimilate availability can accelerate autumn leaf senescence rather than prolong leaf retention (Zani et al., 2020).

In contrast to its pronounced effect on autumn phenology, the influence of elevated phosphorus availability on spring leaf phenology was weaker and more context-dependent. In the experiments presented in Bačurin et al. (2023, 2025c), budburst timing remained largely unaffected by phosphorus addition in most tested provenances, indicating that phosphorus availability alone generally has a limited influence on the initiation of spring phenology. An exception was observed in sessile oak from the SB provenance, where elevated phosphorus availability (+PW treatment) significantly delayed leaf unfolding compared to control plants. This delay was particularly evident during the early phenophases of leaf development and persisted during subsequent stages of leaf expansion. In contrast, the KA provenance of sessile oak showed no statistically significant response to phosphorus enrichment, and neither provenance of common beech exhibited clear phosphorus-induced shifts in spring phenology.

These results indicate that the effects of phosphorus on spring phenology are species- and provenance-specific. Overall, the absence of consistent responses across species and provenances suggests that phosphorus availability alone does not represent a dominant regulator of budburst timing. In contrast, phosphorus exerted a clearer and more consistent influence on autumn phenology. Elevated phosphorus availability reliably advanced the onset and accelerated the progression of autumn leaf senescence in both species, whereas its influence on budburst timing remained variable and dependent on species identity and provenance origin.

Taken together, these findings support the phosphorus-related component of hypothesis H5, which proposed that increased phosphorus availability can affect leaf phenology in common beech and sessile oak. At the same time, the contrasting responses observed among species and provenances provide additional support for hypothesis H7, indicating the presence of both interspecific and intraspecific variation in phenological responses to increased phosphorus availability. Overall, the results suggest that phosphorus-driven phenological shifts primarily influence the progression and termination of the growing season, whereas their effect on the initiation of spring development appears to be less pronounced.

3.3. *Interactive effects of drought and phosphorus on phenology*

Hypothesis H5 proposed that drought stress and increased phosphorus availability, acting both independently and interactively, influence leaf phenology. This interaction was explicitly tested in Bačurin et al. (2025c), where drought and phosphorus treatments were applied separately and jointly. The results revealed non-additive interactions between these environmental factors, as the combined drought and phosphorus treatment frequently neutralized the phenological shifts observed under single-stressor conditions.

In several species–provenance combinations, phosphorus enrichment modified phenological responses to drought. For example, drought or elevated phosphorus alone significantly altered spring phenology in the SB provenance, whereas the combined treatment resulted in phenological timing similar to the control. Such responses indicate that the phenological outcome of multiple environmental drivers cannot be interpreted as a simple additive combination of individual stressor effects (Tardieu, 2012).

These findings provide only partial support for hypothesis H6, which proposed that increased phosphorus availability mitigates the effects of drought stress. Although phosphorus occasionally reduced the magnitude of drought-induced phenological shifts, this effect was not consistent across species and provenances. In several cases, the combined treatment merely neutralized individual stressor effects without producing a clear buffering response.

The observed variability among species–provenance combinations further highlights the importance of phenological plasticity and local adaptation in shaping plant responses to environmental stress. Differences in phenological strategies among species and populations

often reflect contrasting adaptive responses shaped by climatic history and local environmental conditions (Valladares et al., 2007; Gárate-Escamilla et al., 2019; Müller and Bahn, 2022). Such variation likely contributed to the contrasting responses observed among the studied species and provenances.

Taken together, the results of Bačurin et al. (2023) and Bačurin et al. (2025c) indicate that phosphorus availability plays an active but context-dependent role in shaping leaf phenology. While elevated phosphorus consistently advanced autumn senescence in both species, its influence on spring phenology was weaker and was strongly species- and provenance-specific. Moreover, interactions between phosphorus availability and drought further modulated phenological responses, suggesting that nutrient effects on phenology cannot be interpreted independently of other environmental stressors.

From an applied perspective, these findings have important implications for forest nursery practices and the management of forest reproductive material. Phosphorus fertilization may alter phenological timing and potentially increase the risk of phenological mismatches with local climatic conditions. The observed provenance-specific responses further suggest that fertilization effects on phenology cannot be assumed to be uniform across genotypes, emphasizing the importance of considering genotype \times environment interactions under ongoing climate change.

3.4. *Rootstock influence on intraclonal variability in spring leaf phenology*

The results reported by Bačurin et al. (2026) suggest that rootstock identity may contribute to intraclonal variability in the spring leaf phenology of pedunculate oak. Despite the genetic uniformity of the scion material, differences in the timing of budburst (phenophase 3) were observed among ramets grafted onto genetically diverse seedling rootstocks. Across the study period, a substantial proportion of clones exhibited biologically meaningful within-clone variation in budburst timing, defined here as a difference of more than three days. The highest proportion of clones showing such variation was recorded in 2010 and 2014, when 79.1% and 72.1% of clones, respectively, displayed a range of three days or more between the earliest- and latest-flushing ramet (Bačurin et al., 2026). These findings support hypothesis H8, which proposed that rootstock–scion interactions influence leaf phenology in pedunculate oak clones.

The magnitude of this intra-clonal variability differed markedly among years, suggesting that environmental conditions may influence the expression of phenological differences among ramets. Years characterized by more variable spring temperatures and gradual warming patterns, such as 2010 and 2014, exhibited the highest levels of within-clone phenological divergence. In contrast, years with more synchronized thermal conditions showed greater phenological synchrony among ramets (Bačurin et al., 2026). This pattern is consistent with the well-established genetic control of spring phenology in deciduous trees, where the temperature thresholds required to trigger budburst vary among genotypes (Kramer, 1995; Rousi and Pusenius, 2005; Derory et al., 2006; Körner and Basler, 2010; Vitasse et al., 2010). Consequently, under fluctuating climatic conditions, even small differences in temperature sensitivity may result in noticeable differences in budburst timing.

The observed rootstock effects are consistent with earlier findings from grafted woody plants, where rootstocks have been shown to influence scion phenology through differences in water relations and hormonal signaling. Studies in fruit crops demonstrated that rootstock genotype can alter the timing, synchrony, and rate of budburst and flowering, with important consequences for reproductive success (Durner and Goffreda, 1992; Wang et al., 1994). Clearwater et al. (2007) further showed that differences in the timing of spring root pressure development among rootstocks can affect scion water status at budburst, leading to variation in early shoot development. Similar mechanisms may underlie the intra-clonal phenological variability observed in pedunculate oak, although these processes were not directly assessed in the present study.

An important ecological consequence of rootstock-induced phenological variability was revealed by the late spring frost event in 2012. Analysis of phenophase 4 timing demonstrated that ramets which flushed earlier were more susceptible to frost damage, whereas ramets with delayed development successfully avoided injury. A delay of three days or more in reaching phenophase 4 was associated with avoidance of frost damage during the 2012 frost event. This value was therefore used as a biologically meaningful threshold to define intracolonial variation in budburst timing (Bačurin et al., 2026). This finding supports previous research showing that early-flushing genotypes are more vulnerable to late-spring frost (Utkina and Rubtsov, 2017).

In the context of clonal seed orchard management, these findings have direct practical implications. Phenological synchrony among clones is a key prerequisite for effective cross-pollination and maximizing effective population size (Kramer et al., 2008; Franjić et al., 2011). The results of Bačurin et al. (2026) demonstrate that rootstock choice can influence

phenological timing even within genetically identical clones, potentially increasing phenological asynchrony if not carefully managed. Conversely, the strategic selection of rootstocks with compatible phenological behavior may enhance synchrony while simultaneously improving resilience to late spring frost. Appropriate phenological timing enables trees to minimize the risk of late spring frost damage while maximizing the length of the photosynthetically active growing season, thereby supporting greater biomass accumulation (Lockhart, 1983; Leinonen and Hänninen, 2002; Gömöry and Paule, 2011).

Overall, this study highlights rootstock identity as an additional atypical factor contributing to phenological variability in pedunculate oak. While genetic control, temperature, and photoperiod remain the primary drivers of spring phenology, rootstock–scion interactions may further modulate phenological expression at a biologically meaningful scale. These findings emphasize the importance of considering whole-plant integration and belowground influences when studying phenological dynamics, particularly in managed forest systems that rely on heterovegetative propagation. From a practical perspective, the study also highlights the potential importance of rootstock selection in clonal seed orchard management. Since phenological synchrony among clones is essential for effective cross-pollination and seed production, the phenological characteristics of rootstocks should be considered during propagation. At the same time, rootstock-induced phenological variation may contribute to increased resilience to late spring frost events.

4. CONCLUSIONS

The results of this dissertation demonstrate that leaf phenology in temperate forest tree species is influenced not only by typical environmental drivers such as temperature and photoperiod, but also by a range of atypical environmental factors whose effects depend strongly on their timing, intensity, and interaction with species-specific traits. The findings confirm that phenological responses represent complex outcomes of physiological regulation, environmental conditions, and genetic background.

The experiments investigating drought stress revealed that the timing of drought during the growing season plays a decisive role in determining both the direction and magnitude of phenological responses. In goat willow, drought induced clear carry-over effects on spring phenology, with early-season drought advanced budburst in the following year, while later drought events delayed leaf-out. These findings support hypotheses H1 and H3 and indicate that stress timing can explain previously inconsistent reports of drought-induced phenological shifts. In pedunculate oak, however, drought did not produce a clear carry-over effect on spring phenology, suggesting species-specific differences in drought memory and physiological recovery processes.

Drought also influenced autumn phenology in both species. In general, drought delayed the onset of autumn leaf senescence and slowed its progression, with stronger effects observed when drought occurred later in the growing season and when drought intensity was higher. These results indicate that drought-induced phenological shifts may represent compensatory responses aimed at restoring carbon balance following stress. In addition to phenology, drought timing strongly affected height growth, confirming hypothesis H4. Growth reductions were most pronounced when drought coincided with periods of intensive shoot elongation, whereas drought outside this critical growth window allowed partial recovery or compensatory growth.

The experiments addressing nutrient availability demonstrated that increased phosphorus availability can significantly influence leaf phenology, particularly autumn phenology. Elevated phosphorus consistently advanced the onset and accelerated the progression of autumn leaf senescence in both beech and sessile oak, thereby supporting hypothesis H5. In contrast, phosphorus availability had relatively limited and inconsistent effects on spring phenology, suggesting that nutrient availability primarily affects the termination rather than the initiation of the growing season. At the same time, the magnitude of phenological responses differed

among species and provenances, providing support for hypothesis H7 and highlighting the importance of genetic background in determining phenological plasticity.

The combined drought and phosphorus treatments revealed non-linear interactions between environmental stressors. In several cases, the combined treatment neutralized the individual phenological effects of drought or phosphorus alone, indicating physiological compensation between stressors. These results provide partial support for hypothesis H6 and demonstrate that the phenological outcome of multiple environmental factors cannot be predicted by simple additive effects.

Finally, the study of grafted pedunculate oak clones demonstrated that rootstock identity can influence intraclonal variability in spring leaf phenology, thereby supporting hypothesis H8. Despite the genetic uniformity of scion material, ramets grafted onto genetically diverse rootstocks exhibited measurable differences in budburst timing. These findings highlight rootstock–scion interactions as an additional atypical factor contributing to phenological variability in grafted forest trees.

Overall, this dissertation demonstrates that atypical environmental factors, including drought, nutrient availability, and rootstock–scion interactions, can significantly modify phenological processes in forest tree species. The results emphasize that phenological responses are shaped by complex interactions among environmental stressors, species identity, genetic background, and developmental timing. Understanding these mechanisms is essential for predicting forest ecosystem responses to climate change and for improving forest reproductive material production, nursery management, and the design of clonal seed orchards under increasingly variable environmental conditions.

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6. AUTHOR'S BIOGRAPHY

Marko Bačurin was born on 20 March 1994 in Zagreb. He completed his primary and secondary education in Velika Gorica. He enrolled at the Faculty of Forestry, University of Zagreb, in 2012 and graduated in 2017 with the thesis *Models of identification of casted red deer (Cervus elaphus L.) antlers*. In recognition of his achievements during his studies, he received the Academician Dušan Klepac Award and the Dean's Award. He is also the recipient of the Rector's Award for individual scientific work in the 2016/2017 academic year for the paper entitled *Predicting the Outbreak and Spread of Spruce Bark Beetle (Ips typographus) Infestations after Ice Storm Damage in Gorski Kotar through the Interpretation of WorldView-2 Satellite Imagery*.

He began working at Croatian Forests Ltd. in 2018 as a trainee. In 2020, he was appointed as a teaching assistant in the course Forestry Botany at the Faculty of Forestry and Wood Technology, University of Zagreb, Department of Forest Genetics, Dendrology and Botany. On 21 December 2020, he enrolled in the doctoral programme Forestry and Wood Technology.

He rejoined Croatian Forests Ltd. in 2021, where he worked in the Forest Management Department of the Zagreb Forest Administration. Since 17 January 2022, he has been employed at the Faculty of Forestry and Wood Technology, University of Zagreb, Department of Forest Genetics, Dendrology and Botany, as a teaching and research assistant in the courses Forest Genetics, Forest Tree Breeding, and Conservation of Forest Tree Genetic Diversity, a position he currently holds.

To date, he has co-authored 11 scientific papers and participated in 11 national and international scientific conferences. He is a collaborator on Croatian Science Foundation-funded research projects, including one completed and one ongoing project. He also completed short-term professional training at the Czech University of Life Sciences Prague and at the BFW Training Centre in Traunkirchen, Austrian Research Centre for Forests.

7. SUPPLEMENTS

Supplementary material 1: Bačurin, M., Bogdan, S., Katičić Bogdan, I., Sever, K., 2023. Leaf phenological responses of juvenile beech and oak provenances to elevated phosphorus. *Forests* 14, 834. <https://doi.org/10.3390/f14040834>

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Supplementary material 4: Bačurin, M., Sever, K., Katičić Bogdan, I., Bogdan, S., 2025. Species- and provenance-specific leaf phenological responses to drought and elevated phosphorus in *Fagus sylvatica* and *Quercus petraea*. *Forests* 16, 1402. <https://doi.org/10.3390/f16091402>

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Article

Leaf Phenological Responses of Juvenile Beech and Oak Provenances to Elevated Phosphorus

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Abstract: The few studies dealing with leaf phenological responses to elevated nutrients in forest trees have given ambiguous results, i.e., while some reported delayed leaf-out and autumn leaf senescence, others reported advanced leaf phenology caused by increased nutrition. This study aimed to determine the effects of experimentally increased phosphorus (+P treatment) on the leaf phenologies of two juvenile provenances of common beech and sessile oak. Other objectives were to determine whether there were interspecific differences as well as intraspecific variations. Saplings were excavated in two mixed beech–oak stands and transplanted into four wooden boxes filled with a commercial soil substrate. Phosphorus fertilizer was added to two of the boxes, while the remaining boxes served as controls. Both species responded to +P treatment with advanced autumn leaf senescence in the first year of the experiment. Leaf senescence in common beech began significantly earlier, while in both species, the process was accelerated compared to that in the control. In the second year, the leaf senescence response to +P treatment was even more pronounced in both species. The +P effect on leafing phenology was absent in both common beech provenances and in an oak provenance. However, the other oak provenance showed advanced leafing, indicating the existence of intraspecific differences.

Keywords: *Fagus sylvatica*; *Quercus petraea*; flushing; leaf senescence; nutrition; phosphorus effect; phenology shift



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1. Introduction

The leaf phenology of deciduous trees (i.e., multiphase development of leaves from spring bud burst to autumn senescence and leaf abscission) determines the length of a growth period and is one of the main drivers of ecological processes in temperate-zone ecosystems [1]. It is known that the basic (typical, common) environmental factors that, in interaction with the genome, regulate leaf phenology are temperatures in different parts of the year (chilling, forcing, and autumn temperature) and the photoperiod [2–5]. However, there are additional (atypical, i.e., less-studied) environmental factors that influence phenological processes. Some of these factors are drought stress [6], soil nutrient availability [7–9], pathogen infections [10], increased CO₂ [11], etc. A plant reacts to these atypical environmental factors with shifts in the timing of phenological stages as well as with changes in its dynamics (e.g., acceleration of leafing) [6,12,13]. The results of the few available studies are ambiguous about how and to what extent nutrients affect forest trees' leaf phenology. For example, a delay [14,15] as well as an advance of autumn phenology [16,17] have been reported as effects of the higher availability of nutrients in different tree species.

Spring leaf development is linked with the translocation of carbohydrates and mineral nutrients from storage tissues to apical meristems and/or young leaves in their early stages of development, while autumn leaf senescence is linked with the translocation of mineral nutrients from leaves to storage tissues [18–20]. Both require a supply of energy and appropriate enzymes that regulate translocation within the plant [21]. Phosphorus (P)

is one of the most important macronutrients for plant productivity and response to various stressors, although it is often the least available of all macronutrients in soil. Moreover, P is crucial for the capture, storage, and transmission of solar energy and therefore necessary for the normal functioning of all metabolic and physiological processes. P is part of the energy transport molecule structure (ATP, CTP, GTP, UTP) as well as nucleic acids (DNA and RNA) necessary for the synthesis of enzymes mediating the remobilization of carbohydrates and mineral nutrients [22]. Thus, it may have a significant influence on spring leaf development and/or autumn leaf senescence dynamics.

The influence of elevated P concentrations (as well as the concentrations of other biogenic elements) in soil and/or vegetative organs on shifts in the timing of the spring and autumn leaf phenology of forest trees has rarely been investigated [23]. However, this knowledge could be important for the survival of forest ecosystems in the context of climate change consequences that make the natural regeneration of forest stands difficult [24,25]. Artificial reforestation practices in European forests are increasing, mostly due to various problems caused by climate change (extreme weather events, drought episodes, exotic pest outbreaks, etc.). Today, almost 30% of European forests have been artificially reforested [26]. For this reason, the need for the production of suitable forest reproductive material (FRM) is growing. The FRMs of common beech and sessile oak have significant shares in this process, especially in Central Europe [27]. Therefore, clonal seed orchards (CSOs) of these species are being established in an increasing number of EU countries (<https://ec.europa.eu/forematis/>) (accessed on 15 December 2022). The effective production of quality FRM in CSOs and forest nurseries requires intensive management, i.e., the implementation of the best agrotechnical practices, which includes fertilization. By applying fertilizers that most often contain essential biogenic elements in different proportions (including P), the concentrations of these elements in the soil increases, which ultimately results in an increased concentration of biogenic elements in plant organs [28–30]. This has a positive impact on the survival and later growth of the plants in natural habitats [31–35]. However, the influence of elevated concentrations of biogenic elements on shifts in forest tree spring leafing and/or autumn leaf senescence timing is still quite unknown. Leaf-phenology shifts caused by increased concentrations of biogenic elements in vegetative organs together with the frequent occurrence of late spring and/or early autumn frosts could adversely affect seedling survival [4,5,36]. Most studies address the impacts of P deficiency on forest trees, emphasizing the negative consequences for growth and physiological processes [37]. Due to the results of such research, it is easy to conclude that ample nutrition should provide only benefits to trees. However, too much P in cells and tissues has a harmful effect on plants, which means that it is necessary to maintain a balance of nutrients [38].

Overall, further research is needed to determine the specific effects of elevated P on leaf phenology in different tree species and under different environmental conditions, since there are ambiguous results. Common beech and sessile oak are both deciduous broadleaf tree species, yet they differ significantly in their leaf phenology patterns. It is common for oaks to flush their leaves earlier in the spring than beeches. This is likely since oaks are more tolerant to cold temperatures, which allows them to begin their growing cycle earlier than beeches. Research considering a comparison of leaf phenological responses to various environmental factors between these forest species are lacking, although they often form valuable mixed stands all over Europe [39,40]. Due to differences in their genetics and physiological processes, they may respond differently to elevated P as well. It may be assumed that beech would respond faster than oak due to its shallower rooting system that allows a faster P uptake upon fertilisation. Additionally, factors such as soil type and climate can all influence how different provenances within species respond to elevated phosphorus levels, i.e., within-species variations may be expected. For example, soils in drier habitats may not provide the same amount of phosphorus as soils in wetter habitats. This could lead to different adaptation strategies of provenances and consequently, their various responses to experimentally elevated P-levels.

Consequently, the main objectives of this research were (1) to determine the leaf phenology responses of juvenile common beech and sessile oak to increased phosphorus concentrations available in a growth substrate, (2) to determine whether there were differences in the responses between these two cohabiting yet phenologically different species, and (3) to determine whether there were intraspecific variations, i.e., whether the provenances originating from different habitats differed in the responses.

2. Materials and Methods

2.1. Plant Material

Natural saplings of sessile oak and common beech were sampled at the beginning of March 2021 in two mixed stands dominated by these species. Saplings, 3–4 years old, were dug up from an area of approximately 1 ha, ensuring that they were evenly distributed throughout the stand (to avoid close relatedness and ensure a proper representativeness of the stand) and that their roots were damaged as little as possible. Plants that were of uniform height were selected. In total, 160 plants of each species were excavated in both stands (i.e., 160 saplings \times 2 species \times 2 stands = 640 saplings in total). The saplings were labelled and placed in moist sand (in PVC containers). On the same day, the saplings were transported to the location of the future experimental trial, where they were kept in the shade until transplanting.

The selected stands differed from each other regarding prevailing habitat conditions (Table 1). The first was located within the city of Karlovac (Karlovac stand (KA); 45.4661986 N; 15.5219786 E; 170–185 m a.s.l.). It was a mixed stand of beech and oak, approximately 100 years old, with a north-northeast exposure, on deep soil. We considered this stand to be a representative of wetter and warmer habitats due to the higher average amount of precipitation, exposure, and soil depth as well as the higher average air temperature at this location (Table 1). The second stand was located next to the village of Gornji Slatinik along a road that connects the towns of Našice and Slavonski Brod (Slavonski Brod stand (SB); 45.2733637 N; 17.9727061 E; 230–255 m a.s.l.). This was also a mixed common beech and sessile oak stand, approximately 105 years old, with a south-southeast exposure, on shallow soil. We considered this provenance to be representative of drier colder habitats due to the lower average precipitation, exposure, shallower soil, and lower average air temperature at that location (Table 1).

Table 1. Basic climate data for the sampled stands. Generated for the 1991–2020 period by ClimateEU software (<https://sites.ualberta.ca/~ahamann/data/climateeu.html>; (accessed on 23 June 2022) [41].

Stand/Provenance	Mean Annual Precipitation (mm)	Mean Summer Precipitation (mm)	Mean Annual Temperature (°C)	Mean Coldest Month Temperature (°C)	Mean Warmest Month Temperature (°C)
KA	1099	508	12.3	2.0	22.4
SB	848	414	11.0	0.4	21.1

KA—the Karlovac provenance (stand); SB—the Slavonski Brod provenance.

In the following text, the plants belonging to the sampled stands, i.e., provenances, are labelled as follows:

- (1) Fs_KA—*Fagus sylvatica* provenance from the KA stand
- (2) Fs_SB—*Fagus sylvatica* provenance from the SB stand
- (3) Qp_KA—*Quercus petraea* provenance from the KA stand
- (4) Qp_SB—*Quercus petraea* provenance from the SB stand

2.2. The Experimental Trial

In mid-March 2021, the saplings were transplanted to 4 wooden boxes (W 155 \times L 255 \times D 80 cm (3.2 m³)) located at the nursery of the Faculty of Forestry and Wood Technology in Zagreb, Croatia (45.820642 N; 16.022936 E). Each box was filled with commercial soil

substrate Klasmann-Deilmann TS 3 (pH 6.0; 0.16 g/l P₂O₅). In each box, a total of 100 plants were planted in a random arrangement (spacing: 20 × 18 cm), with 25 plants per beech and oak provenance from the KA stand (Fs_KA and Qp_KA) and 25 plants per beech and oak provenance from the SB stand (Fs_SB and Qp_SB). Then, 1200 g of Triplex mineral fertilizer (45% P₂O₅) was added to the two boxes. Thus, the plants in two boxes (200 individuals in total) were exposed to an increased amount of phosphorus in the growth substrate (treatment +P), while in the remaining two boxes, the plants were exposed to the normal phosphorus content in the substrate (i.e., control—C).

2.3. Chemical Analyses

Soil samples from the original stands were extracted at the beginning of March 2021. The sampling methodology is described by Sever et al. [42]. There were no significant differences between the provenances regarding the chemical features of their soils. The total stocks of N, P, and K at soil depths of 0–15 cm and 16–30 cm were similar, i.e., without significant differences between the provenances.

The sampling of the growth substrate and its chemical analysis were carried out during the establishment of the experimental trial but before adding phosphorus to the +P treatment boxes. The second series of chemical analyses of the substrate was performed in mid-September 2021, i.e., 6 months after the addition of mineral fertilizer. One composite (average) sample was collected from each box. Each composite sample was formed from nine individual samples collected in a diagonal (X) arrangement. The sampling of the substrate was carried out using a pedological probe at a depth of 0–30 cm. The samples were subjected to soluble component extraction by mixing with deionized water in a 1:2 ratio. The material was mixed for 1 h using a rotating mixer. Then, the suspension was filtered through filter paper, and the clear filtrate was subjected to an analytical procedure aiming to determine the pH value, specific conductivity, concentrations of nitrate (NO₃[−]), ammonia (NH₄⁺), total nitrogen (N total), total phosphorus (P₃[−]), potassium (K⁺), calcium (Ca²⁺), magnesium (Mg²⁺), chlorine (Cl[−]), and sodium (Na⁺). The applied methodology for the chemical analyses of the substrate is described in more detail in [43].

The sampling of leaves in the experimental trial was performed twice, in mid-September 2021 and mid-May 2022. From the top of each plant, in all four boxes, three leaves were sampled. Three composite (average) samples were formed for each species provenance. Each sample consisted of a total of 25 randomly selected leaves from 25 plants per species provenance in a box.

The composite leaf samples were subjected to chemical analyses to determine their mineral nutrient contents. The samples were dried at 105 °C to a constant mass, minced, and homogenized. The concentration of N was determined using the Kjeldahl method. Digestion was performed with concentrated nitric acid (HNO₃) and perchloric acid (HClO₄). The concentration of P was determined spectrophotometrically. The concentration of K was determined by a flame photometer. The concentrations of calcium (Ca) and magnesium (Mg) were determined by atomic absorption spectrophotometry. All analyses were carried out according to standardized international protocols [44].

2.4. Leaf Phenology Scoring

Spring leafing and autumn leaf senescence were scored in 2021 and 2022. Leaf phenological phases were scored once or twice a week (during the process) on all plants in the trial using a 1–7 ordinal scale: 1—bud scales completely closed, dormant buds; 2—buds swelling, scales slightly spaced; 3—bud burst, green leaf tips visible; 4—folded leaves visible; 5—leaves unfolding but not yet flattened, small; 6—leaves still relatively small but with flattened blades, blade edges bent downward, withered, lighter green or reddish; 7—leaves appear developed, larger but more tender than fully developed leaves and lighter green or reddish.

Autumn leaf phenological phases were scored (during the process) once a week in 2021 and twice a week in 2022 using a 0–5 ordinal scale: 0—leaves completely green with

no visible discoloration; 1—up to 25% of plant leaves show discoloration; 2—up to 50% of plant leaves show discoloration; 3—more than 50% of plant leaves show discoloration; 4—more than 75% of plant leaves show discoloration; 5—leaves shed. All phenological scorings were done by one experienced person.

The day of the year when a plant reached leaf-out phase 3 was taken as its bud-burst date, and the day of the year when a plant reached autumn leaf phenological phase 3 was taken as its autumn senescence date.

It should be noted that late spring frost affected the plants in the experimental trial from 6 to 8 April 2021. At that time, many oak saplings (as well as few common beech saplings) had already started leafing out. Air temperature dropped to a minimum of $-8\text{ }^{\circ}\text{C}$ on the morning of 6 April and then, for three days, repeatedly rose and fell to negative values. All the leaves on the plants that had started leafing out were destroyed, regardless of which phenophase they were in during that period. However, none of the plants died during this period, and they leafed out again approximately two weeks later.

2.5. Statistical Analyses

Assumptions of residual normality and variance homogeneity were tested by using the Shapiro–Wilk test and Levine’s test [45] with the GLM and UNIVARIATE procedures in SAS Studio software, a free version of SAS OnDemand for Academics by SAS Institute Inc., Cary, NC, USA. Residuals were plotted as a function of fitted values to test for variance homogeneity, and the distribution of residuals was also tested. Since data did not meet the criteria of normal distribution and homogeneity of variances (raw phenological data and mineral nutrition contents of the leaf samples) the nonparametric Kruskal–Wallis and Mann–Whitney U tests were applied. These tests were used to assess the significance ($p < 0.05$) of the +P treatment on the provenance leaf phenology phases on particular days of the year. Additionally, the U test was used to test the significance of leaf nutrition content differences between +P treatment and control plants. The nonparametric tests were performed using RStudio, version 1.2.5001 (©2009–2019 RStudio, Inc., Boston, MA, USA).

3. Results

3.1. Substrate Nutrient Content Differences between the Treatments

The chemical traits of the substrate between the +P treatment and control differed significantly only regarding the concentration of total P. The concentrations of the other nutrients and the pH value in both treatments were similar (Table 2).

Table 2. Mean nutrient contents of the growth substrates in the treatment boxes. The +P treatment had elevated phosphorus content. The control had no added P.

Parameter	+P Treatment		Control	
	Value	Description ¹	Value	Description ¹
pH (H ₂ O)	6.8	Neutral	7.1	Neutral
P ³⁻ (mg/L)	8.4	High	2.7	Moderate
NH ₄ ⁺ (mg/L)	13.2	Optimal	13.8	Optimal
NO ₃ ⁻ (mg/L)	59.5	Optimal	60.0	Optimal
N total (mg/L)	46.9	Medium–normal	49.7	Medium–normal
K ⁺ (mg/L)	63.0	Medium–normal	60.9	Medium–normal
Mg ²⁺ (mg/L)	61.4	Moderate	54.5	Moderate
Ca ²⁺ (mg/L)	204	Low	234	Low
Cl ⁻ (mg/L)	54.8	Medium–normal	52.4	Medium–normal
Na ⁺ (mg/L)	34.79	Moderate	36.6	Moderate
E.C. (mS/cm)	1.185	Medium–normal	1.156	Medium–normal
Salt (%)	0.151	Medium–normal	0.147	Medium–normal

¹ Description according to [46].

3.2. Leaf Mineral Nutrition Variations

According to Mellert and Göttlein [46], the following value ranges in leaf dry matter indicate normal nutrition for common beech and sessile oak: P (1.2–1.9 mg g⁻¹ and 1.4–2.1 mg g⁻¹, respectively), N (18.7–23.2 mg g⁻¹ and 19.8–26.8 mg g⁻¹, respectively), K (6.1–9.7 mg g⁻¹ and 7.2–11.4 mg g⁻¹, respectively), Ca (6.7–14.0 mg g⁻¹ and 5.3–10.2 mg g⁻¹, respectively), and Mg (1.1–2.3 mg g⁻¹ and 1.2–2.4 mg g⁻¹, respectively).

Comparing the measured leaf dry matter nutrient contents from September 2021 (Table 3) with the abovementioned reference values, it is evident that the Fs_SB provenance had a P content in the upper part of the normal range in the +P treatment, while the Fs_KA provenance had a P surplus. The leaf P content of the control plants was within the lower normal limits. It should be emphasized that both common beech provenances had a significantly higher leaf P content in the +P treatment than in the control plants. The sessile oak provenances had a much lower leaf dry matter P content than the common beech provenances in the +P treatment, although it was still in the normal range. The control plants of both oak provenances showed a latent P deficiency. Again, it should be emphasized that both oak provenances in the +P treatment had a significantly higher leaf P contents than the control plants.

Table 3. Mean nutrient contents \pm standard errors (mg g⁻¹) in dried leaf samples of the beech and oak provenances by treatment. Samples were taken in September 2021 and May 2022.

Nutrient	Year	Fs_KA		Fs_SB		Qp_KA		Qp_SB	
		+P	C	+P	C	+P	C	+P	C
P	2021	1.96 * \pm 0.04	1.31 \pm 0.03	1.72 * \pm 0.09	1.24 \pm 0.02	1.36 * \pm 0.08	1.08 \pm 0.04	1.45 * \pm 0.02	1.06 \pm 0.08
	2022	1.82 \pm 0.16	1.64 \pm 0.07	1.95 * \pm 0.10	1.28 \pm 0.08	2.15 * \pm 0.35	1.22 \pm 0.10	1.76 * \pm 0.15	1.31 \pm 0.05
N	2021	21.15 \pm 0.45	23.00 * \pm 0.32	21.75 \pm 0.19	22.50 \pm 0.77	28.67 \pm 0.43	27.30 \pm 0.60	28.08 \pm 0.45	29.00 \pm 0.54
	2022	22.34 \pm 2.26	24.56 \pm 1.16	22.12 \pm 0.98	22.25 \pm 0.85	21.51 \pm 0.84	22.34 \pm 2.26	22.34 \pm 2.26	22.34 \pm 2.26
K	2021	6.38 \pm 0.13	7.19 * \pm 0.30	6.57 \pm 0.15	7.54 \pm 0.13	7.73 \pm 0.09	7.73 \pm 0.17	7.69 \pm 0.10	7.83 \pm 0.11
	2022	6.82 \pm 0.27	8.06 * \pm 0.41	6.57 \pm 0.34	7.31 \pm 0.34	9.67 \pm 0.46	9.13 \pm 0.40	9.59 \pm 0.28	9.15 \pm 0.44
Ca	2021	8.06 \pm 0.27	8.89 \pm 0.43	9.18 \pm 0.10	9.41 \pm 0.08	6.34 \pm 0.27	7.72 * \pm 0.22	6.86 \pm 0.16	6.59 \pm 0.57
	2022	7.47 \pm 0.62	6.71 \pm 0.28	9.18 \pm 0.53	8.68 \pm 0.63	10.41 \pm 0.88	10.91 \pm 0.71	10.32 \pm 0.76	10.98 \pm 1.23
Mg	2021	1.53 \pm 0.12	1.35 \pm 0.06	1.58 * \pm 0.05	1.25 \pm 0.08	1.87 \pm 0.09	2.23 * \pm 0.04	2.00 \pm 0.05	1.97 \pm 0.09
	2022	2.19 \pm 0.10	2.37 \pm 0.09	2.52 \pm 0.11	2.31 \pm 0.09	2.86 \pm 0.14	3.09 \pm 0.21	2.89 \pm 0.13	2.76 \pm 0.18

Fs_KA—*Fagus sylvatica* provenance in the KA stand; Fs_SB—*Fagus sylvatica* provenance in the SB stand; Qp_KA—*Quercus petraea* provenance in the KA stand; Qp_SB—*Quercus petraea* provenance in the SB stand. +P—elevated phosphorus treatment; C—control; an asterisk indicates significantly larger nutrient contents in the treatment (within a provenance). P—phosphorus; N—nitrogen; K—potassium; Ca—calcium; Mg—magnesium.

For the other nutrients, the beech provenances were in the normal range, mostly without significant differences between plants in the +P treatment and control plants. However, within the Fs_KA provenance, the control plants had significantly higher nitrogen and potassium contents than the plants in the +P treatment. On the other hand, the Fs_SB provenance in the +P treatment had a significantly higher magnesium content than the control plants.

The average contents of the other nutrients in the oak provenances were in the normal range (except for nitrogen, of which there was a surplus), without significant differences between the plants in the +P treatment and control plants. However, the control plants of the Qp_KA provenance had significantly higher contents of calcium and magnesium than the plants of that provenance in the +P treatment.

A significant P increase in the oak provenances in the +P treatment was observed from leaf samples taken in May 2022, while the P contents in leaves of common beech provenances remained at approximately the same level as in the previous year (Table 3). The P contents in the leaves from the plants in the +P treatment were higher than those in the control plants in all the provenances, although the difference was not significant in the case of the Fs_KA provenance.

The values for the other nutrients in the beech provenances were in the normal range, mostly without significant differences between the plants in the +P treatment and control.

The exception was the higher mean potassium content in the control plants of the Fs_KA provenance (Table 3). The average contents of the other nutrients were in the normal range for the oak provenances, with no significant differences between the plants in the +P treatment and control. It is interesting that the nitrogen content fell compared to that in the previous year, while the calcium and magnesium values increased above the upper limit of the normal range (Table 3).

3.3. Variations Caused by the Elevated Phosphorus Treatment (+P)

As expected, the effect of increased phosphorus on spring phenology in 2021 was not recorded (Figure 1a). However, a statistically significant difference between the treatments was shown in autumn leaf senescence (Figure 1b). Namely, the plants in the +P treatment showed advanced leaf senescence dates, on average.

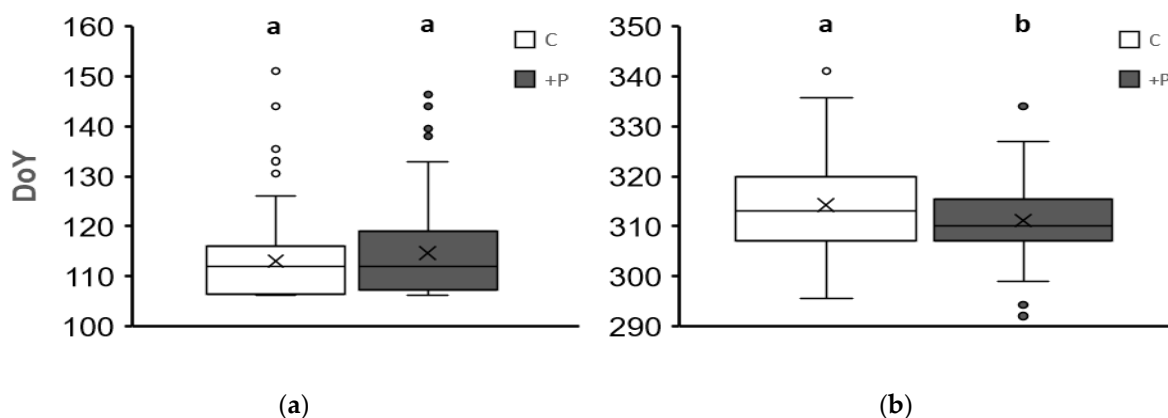


Figure 1. (a) Bud-burst date distribution by treatment in the first experimental year (2021). (b) Leaf senescence date distribution by treatment in the first experimental year. +P treatment—elevated phosphorus; C—control with no phosphorus added. Different letters denote a statistically significant difference between the two treatments (at $p < 0.05$).

With more detailed analyses, it was possible to see that both species responded similarly, i.e., with a significantly advanced autumn leaf phenology of plants in the +P treatment (Figure 2). Thus, the Fs_SB provenance in the +P treatment showed an average of more than 50% dead leaves on day 305; this threshold was reached three days later in the control (Figure 2a). The differences in average leaf senescence dynamics between the +P treatment and control were even more pronounced for the Fs_KA provenance (307 DoY and 311 DoY, respectively) (Figure 2b). For both beech provenances, the visible symptoms of leaf senescence started earlier and proceeded faster in the +P treatment. The dynamics of autumn leaf phenology were significantly different between the treatments (+P vs. control) for the oak provenances as well (Figure 2c,d), although not as intensely.

The effect of elevated phosphorus on leaf flushing phenology in the following year was not recorded for the common beech provenances, i.e., the leafing dynamics of plants in the +P treatment plants were not significantly different from those in the control (Figure 3a,b). However, advanced leaf flushing dynamics in the +P treatment were recorded for the oak provenance Qp_KA (Figure 3d), while there was no significant effect of the +P treatment for the provenance Qp_SB (Figure 3c).

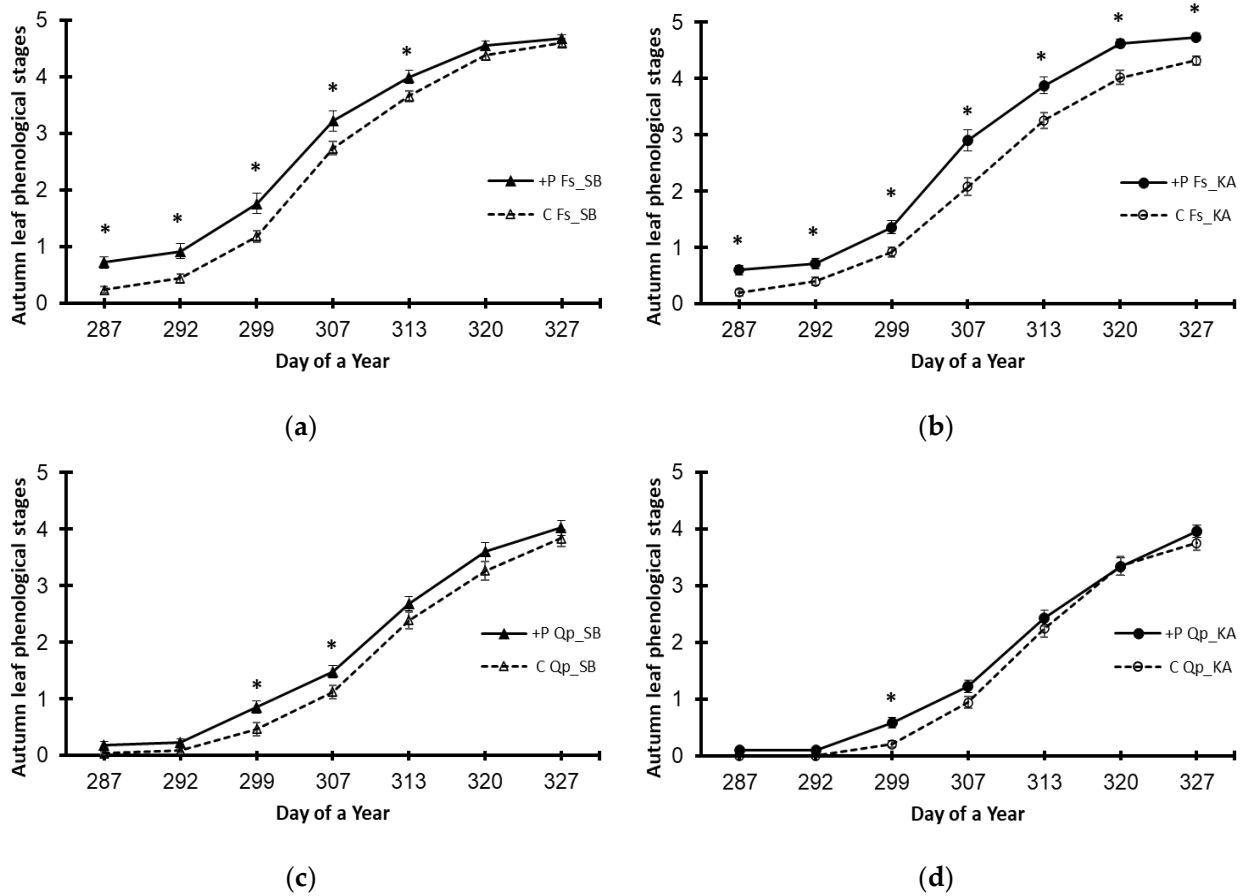


Figure 2. Autumn leaf phenologies of beech (Fs) and oak (Qp) saplings in 2021, by provenance and treatment: (a) beech provenance SB; (b) beech provenance KA; (c) oak provenance SB; (d) oak provenance KA. +P treatment—elevated phosphorus; C—control (no phosphorus added). The markers represent the means \pm S.E. ($n = 50$). A star denotes a statistically significant difference between the treatments on a given day of the year ($p < 0.05$).

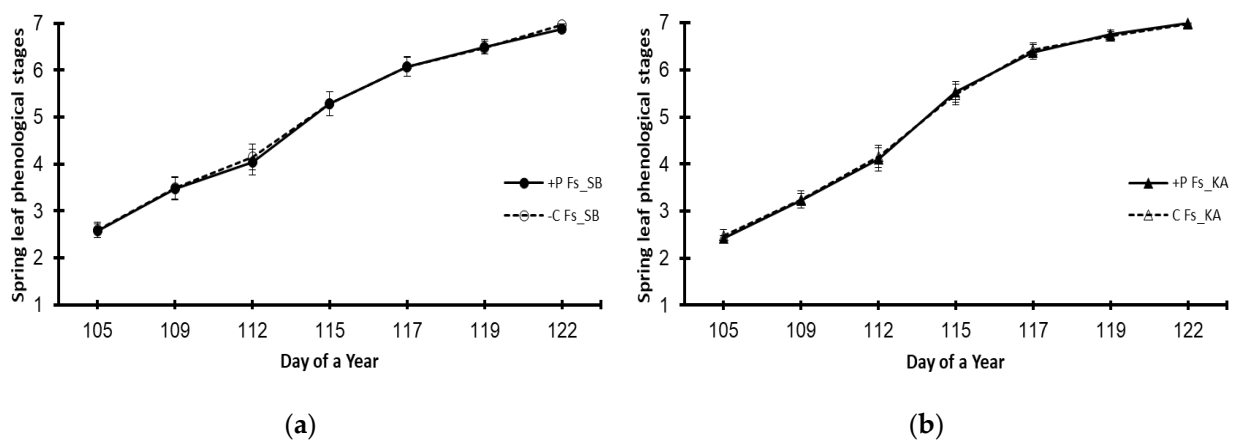


Figure 3. Cont.

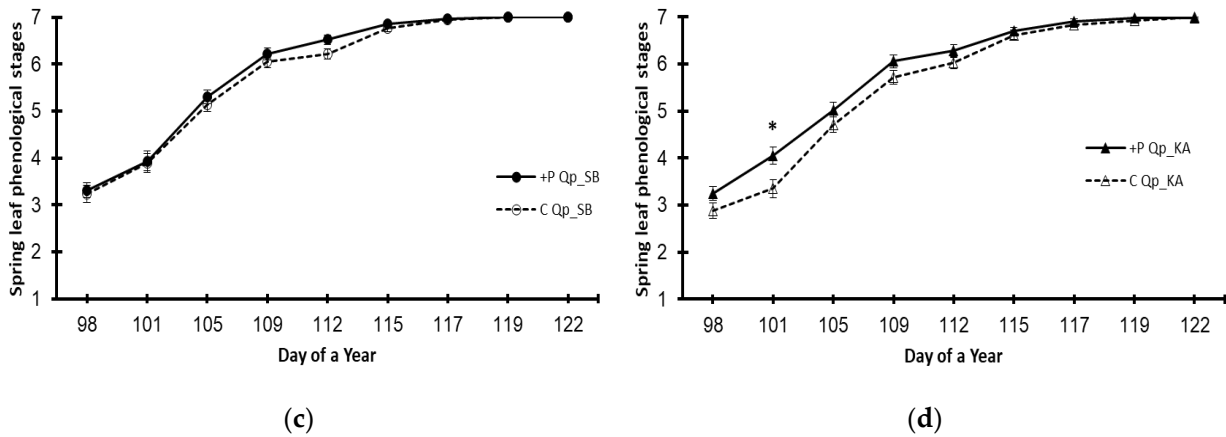


Figure 3. Spring leaf phenology of the beech (Fs) and oak (Qp) saplings in 2022 by provenance and treatment: (a) beech provenance SB; (b) beech provenance KA; (c) oak provenance SB; (d) oak provenance KA. +P treatment—elevated phosphorus; control—no phosphorus was added. The markers represent means \pm S.E. ($n = 50$). A star denotes a statistically significant difference between the treatments on a given day of the year ($p < 0.05$).

The effect of increased phosphorus on autumn leaf phenology was even more pronounced in the fall of 2022 than in the year before (see Figures 2 and 4). Significantly advanced leaf senescence in the +P treatment was recorded for both beech provenances as well as for the oak provenance Qp_SB. Plants of the Qp_KA provenance in the +P treatment also showed advanced leaf senescence compared to the control plants, although the difference was not statistically significant (Figure 4).

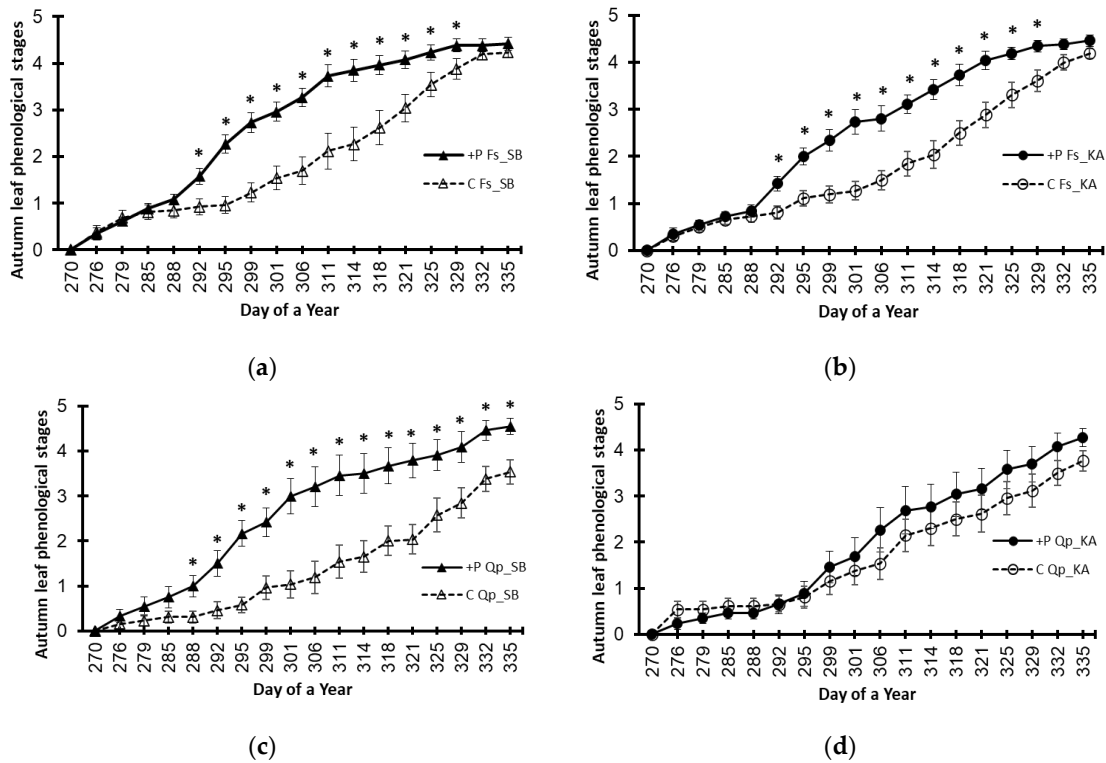


Figure 4. Autumn leaf phenology of beech (Fs) and oak (Qp) saplings in 2022 by provenance and treatment: (a) beech provenance SB; (b) beech provenance KA; (c) oak provenance SB; (d) oak provenance KA. +P treatment—elevated phosphorus; C—control (no phosphorus added). The markers represent the means \pm S.E. ($n = 50$). A star denotes a statistically significant difference between the treatments on a given day of the year ($p < 0.05$).

4. Discussion

Plants growing in phosphorus-enriched substrate showed earlier autumn leaf senescence in the first experimental year (Figure 1b). A closer analysis revealed that both beech provenances reacted to the phosphorus addition with advanced autumn leaf phenology (Figure 2a,b). Both beech provenances started leafing out significantly earlier in the +P treatment, and the process itself was accelerated. The oak provenances also responded with advanced leaf senescence, although the difference was less pronounced than it was for the beech provenances (Figure 2c,d). Autumn leaf phenology between the +P treatment and the control differed even more in the second year in both species (Figure 4).

Autumn leaf phenology is triggered and its dynamics is shaped by the photoperiod [47,48], early autumn temperatures [49,50], available soil moisture [51], and plant nutritional status [15,52]. Phosphorus deficiency induces leaf senescence, i.e., premature leaf discoloration is a typical symptom of P deficiency [53]. However, the influence of various nutrient contents on autumn leaf phenology varies significantly among tree species [15]. Such variations can also be observed in other plant species. For example, phosphorus addition had no effect on soybean (*Glycine max* (L.) Merr.) autumn leaf phenology [54]. On the other hand, a lack of phosphorus in soil delayed the senescence of lower-stem leaves in corn (*Zea mays* L.), although the authors pointed out that this effect was not confirmed for upper-stem leaves [55].

Our study showed advanced leaf senescence in response to increased phosphorus nutrition while, in contrast, delayed autumn leaf phenology due to increased nutrition (a combination of nitrogen, phosphorus, and potassium) was reported by Fu et al. [15] for common beech. The opposing autumn phenology alterations (i.e., advanced or delayed) observed in these two studies can be explained by the fact that the increased contents of several macronutrients (N, P, and K) can have different effects on autumn phenology than the content of only one nutrient (P). Such differences were observed in larch (*Larix gmelinii* var. *principis-rupprechtii* (Mayr) Pilg.) between a combined nutrient increase treatment (N + P) and separate nutrient increase treatments (N or P) [14]. Opposing phenological shifts may also be explained by the different time periods in which fertilizer was applied. Namely, Fu et al. fertilized experimental plants at the end of May, while we added phosphorus immediately after planting (in March). However, since Zavišić and Polle [56] indicated that a significant P uptake from the soil takes place at the end of summer and in autumn, this assumption seems less probable. It is more likely that the interaction effect of nitrogen and phosphorus reported by Fu et al. resulted in an autumn leaf phenological shift that was opposite to that observed in our study.

Advanced leaf senescence as a response to increased sunlight, and increased CO₂ was reported by Zani et al. [16] for common beech, common birch (*Betula pendula* Roth), and Japanese spiraea (*Spiraea japonica* L.f.). The authors concluded that increased productivity, i.e., photosynthetic activity (due to higher sunlight or increased CO₂), resulted in advanced autumn leaf senescence (i.e., the sink limitation hypothesis). Advanced oak autumn leaf phenology due to elevated CO₂ was also reported [17]. Although Zani et al. did not specifically test the increased phosphorus effect on autumn phenology, in our case, it can be assumed that plant productivity may have been increased due to a greater P availability. Therefore, increased P nutrition resulted in the same effect in our research (advanced autumn leaf phenology in the +P treatment). This assumption is supported by the finding that beech photosynthetic activity increased after phosphorus fertilization, which was reported by Zavišić et al. [57]. Advanced autumn leaf phenology caused by elevated P might reduce a plant's susceptibility to early autumn frosts. For example, drought stress induces delayed autumn senescence in forest trees [6]. Since drought episodes during growth period have become more frequent, there is an increasing probability for plants to be exposed to early autumn frosts. Therefore, it seems that improved plant P nutrition can be a part of the solution to this problem, at least considering forest nurseries and/or seed orchards. It would be interesting to see if a +P treatment may reduce the adverse effects of drought. This question will be addressed in the extension of this study.

The observed differences between the species in their response intensities (see Figure 2) can be at least partially explained by significantly lower levels of absorbed phosphorus in oak than in beech in the +P treatment (see Table 3). It is known that these two species differ significantly in terms of rooting depth. Beech roots are shallow, while oak roots are deep due to the intensive growth of their taproots. It was confirmed that oak saplings from studied provenances had significantly longer taproots with fewer fine roots than beech saplings [42]. Due to interspecies differences in root development patterns, it can be assumed that the beech saplings started to absorb nutrients (including P) from shallower parts of the substrate earlier and more intensively. This may be a reason that the oaks, on average, had lower phosphorus contents in their leaves (in September 2021) and therefore showed a less intense response in autumn phenology than the beech provenances.

Statistical nonsignificance of the differences between the Qp_KA in the +P treatment and the control was surprising, though phenology was advanced in the +P treatment (see Figure 4d). We hypothesize that excess and deficient phosphorus could have similar effects on autumn leaf phenology, i.e., earlier senescence occurs in both cases. Based on such an assumption, it seems likely that the Qp_KA provenance could have shown a convergence of the autumn phenology curves due to the occurrence of the largest difference in P content between the +P treatment (2.15 mg g^{-1}) and the control (1.25 mg g^{-1}). Of course, this assumption should be proven experimentally.

As expected, exposure to increased phosphorus contents in the substrate (+P treatment) had no effect on spring leaf phenology in the first year of the study (Figure 1a). The fertilization of the substrate with phosphorus was carried out approximately 15 days before plant leaf-out in the trial. It is evident that the saplings in the +P treatment could not absorb enough phosphorus during that short period to influence their leafing. The probable reason for the inability to absorb the P was insufficient time for the fertilizer to dissolve and become available to the plants. On the other hand, research on uptake dynamics and P allocation in common beech [56] indicates that the P uptake from soil is extremely low in spring (during the leafing phase). The relatively high phosphorus content in leaves is the result of stored phosphorus allocation toward the new photosynthetic apparatuses. This means that plants mainly rely on stored phosphorus reserves during leafing and not on the current source in the soil. The nutrient uptake intensifies after the development of the leaf mass. According to the same study [56], the main P uptake from soil occurs in late summer and fall.

The increased phosphorus effect on spring phenology in 2022 (the second experimental year) was almost completely absent, i.e., neither beech provenances nor an oak provenance showed any differences in flushing phenology between the +P treatment and the control. However, one oak provenance showed advanced spring phenology in the +P treatment (Figure 3d). The advanced flushing of holm oak (*Quercus ilex* L.) seedlings in response to NPK fertilization was reported [58]. Additionally, common beech seedlings originating from a phosphorus-rich habitat showed advanced leafing [23]. The latter report is based on a comparison of two provenances that differed significantly in soil phosphorus content (rich vs. poor P content). Although plants in our study were exposed to significantly different P levels, the soils were not P-rich or P-poor but rather in the upper normal vs. lower normal P content range (or lower normal vs. latent deficiency, in the case of oak—Table 3). In this context, it can be assumed that excess and deficient phosphorus have opposite effects on spring phenology (i.e., a phosphorus surplus induces advanced spring phenology, while a deficiency delays it). However, advanced flushing is shown when comparing groups of plants that strongly differ in phosphorus content, i.e., when comparing a phosphorus-deficit group with a group containing a P surplus. This assumption is supported by the fact that in this study, significantly advanced spring phenology was shown only by one oak provenance (Qp_Ka, see Figure 3), with the largest difference in phosphorus content between the +P treatment and control (Table 3, year 2022). On the other hand, it is possible that plants should be exposed to elevated phosphorus for more than a growing season to show a more intense response in spring leaf phenology. It should be recalled that at the

beginning of leafing, young trees (of common beech) rely on phosphorus reserves stored mainly in coarse roots [56]. In that case, the accelerated flushing of the Qp_KA provenance could have indicated its greater efficiency in storing phosphorus, a phenomenon for which we currently have no direct evidence.

5. Conclusions

In general, the results showed that both species responded similarly to elevated phosphorus content in the substrate, i.e., with advanced autumn leaf phenology, although common beech responded more intensely in the first experimental year. A more pronounced response (i.e., more advanced autumn leaf phenology in the +P treatment than in the control) to the phosphorus treatment was shown in both species in the second experimental year. Advanced leaf senescence as a response to elevated phosphorus may be due to increased plant productivity, which corresponds with the sink-limitation hypothesis.

There was no spring leaf phenology response to elevated phosphorus in common beech and in one sessile oak provenance. However, the other oak provenance responded with advanced leafing indicating within-species variations. Therefore, the effect of elevated phosphorus was more clearly manifested in autumn than in spring leaf phenology, at least in the first growth period after fertilization, especially for common beech.

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Supplementary material 2

The Different Timing of Exposure to Drought Stress Differentially Affects Phenology and Growth in Goat Willow

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ABSTRACT

Periods of drought can substantially alter the phenology of forest tree species, influencing growth dynamics as well as the timing of budburst and leaf senescence. This study investigated whether the plant response varies depending on when during the growing season drought stress occurs.

Female individual of goat willow (*Salix caprea* L.) was propagated by hardwood cuttings, resulting in 151 ramets assigned to eight experimental groups. Seven groups were subjected to drought treatments at different intervals between early March and mid-July 2021, while the eighth served as a well-watered control. The same treatment schedule was repeated in 2022 with increased drought intensity. Each plant remained under drought conditions until visible stress symptoms appeared, and the total mass (container included) decreased to about half of its initial value, after which plants were rehydrated and irrigated regularly for the rest of the season. Control plants were watered continuously.

During autumn 2021, the onset of leaf senescence in the first treatment group was delayed by four days compared with the control. By the following spring (2022), this group flushed about five days earlier than the control, whereas groups 6 and 7 flushed approximately two days later. In autumn 2022, all drought-treated groups showed delayed leaf senescence, most notably those exposed later in the season—by up to 30 days on average compared to the control.

These results indicate that the timing of drought stress strongly modulates leaf phenology in *Salix caprea*, exerting both beneficial and adverse effects on height growth and flower development.

Keywords: *Salix caprea* L.; phenological changes; drought stress; flowering; growth

INTRODUCTION

Drought is a major limiting factor for the growth, reproduction, and survival of plants (Hinckley et al. 1979, Chaves et al. 2003). While forest trees possess some resilience to drought stress, such as through various water-use strategies (Morán-López et al. 2014), the increasingly frequent and intense drought periods in recent years undeniably present a significant threat to forest ecosystems (Arend et al. 2016).

Understanding the impact of drought stress and the possibilities of mitigating its negative consequences on forest trees are of great importance in the context of the increased frequency of drought episodes during the growing season. Morpho-anatomically, plants adapt to drought

stress through a range of defense mechanisms such as by reducing leaf surface area, decreasing cell wall elasticity, and intensifying root growth, thereby reducing aboveground biomass production (Thomas and Gausling 2000, Arend et al. 2013). Physiologically, the adaptation mechanism to drought involves controlling stomatal opening and closure, allowing the plant to regulate transpiration and water loss (Hinckley et al. 1980, Thomas and Gausling 2000). Drought can also trigger adjustments in wood anatomical traits, such as alterations in vessel size and frequency, which affect the balance between hydraulic safety and efficiency (Balzano et al. 2025). In addition to the mentioned morpho-anatomical and physiological mechanisms, phenological processes, such as budburst and autumn leaf senescence, also exhibit responses to drought stress.

Phenological processes are predominantly determined by warm air temperature in spring, cool temperature during spring (i.e., chilling temperatures) and the photoperiod; hence, they can be called typical factors (Robson et al. 2013, Basler and Korner 2014, Kuster et al. 2014, Way and Montgomery 2015). On the other hand, a range of biotic and abiotic factors greatly influence changes in phenological processes through various stress stimuli, and they are called atypical factors (Bačurin et al. 2023). The action of stressful factors affects changes in gene expression, leading to the plant's response to stress stimuli (Ingram and Bartels 1996, Bruce et al. 2007). Epigenetic modifications are regulatory mechanisms that create a kind of “memory” and enable organisms to acclimatize to stressful factors (Bräutigam et al. 2013).

Drought stress has been shown to drive phenological changes in numerous forest tree species, such as reduced growth, shifts in the timing of spring budburst, and earlier or delayed leaf senescence in autumn. (Hinckley et al. 1979, Xie et al. 2015, Čehulić et al. 2019, Wu et al. 2022). Undoubtedly, a delayed (so-called “carry-over”) effect of drought stress on spring leafing has been established in various species of forest trees. The “carry-over” effect manifests through later or earlier budburst in the following spring. Such an effect has been recorded in pedunculate oak and beech (Yonekura et al. 2004, Kuster et al. 2014). Similarly, a negative impact of drought stress has been recorded during the initiation of generative organs in the species *Prunus armeniaca* L. (Bartolini et al. 2020).

The onset of autumn leaf senescence is modified under drought stress conditions; however, some studies report an earlier onset, while others report a delayed one. An earlier onset of leaf senescence, as well as earlier leaf shedding, was observed with extremely low nutrient remobilization caused by drought stress in *Populus tremuloides* Michx. (Killingbeck 2004, Estiarte and Peñuelas 2015). Čehulić et al. (2019) observed interannual phenological changes, noting a delay in budburst during one year and its advancement in the following season. Vander Mijnsbrugge et al. (2016) and Leuzinger et al. (2005) reported a delay in the onset of leaf senescence in *Quercus petraea* (Matt.) Liebl. during autumn. It is also important to note that shifts in leaf phenology, particularly early spring phenology, impact drought. Early leaf-out can reduce soil water reserves, which, in combination with climatic conditions, may result in less water being available during the summer (Denham et al. 2023). In contrast, autumn phenology may be affected by a drought during summer (Lukasová et al. 2020).

However, most studies have investigated the effects of a single drought event on leaf phenology, with stress typically imposed at a specific point during the growing season (Spieß et al. 2012, Kuster et al. 2014, Vander Mijnsbrugge et al. 2016). Only one study has raised the question of variations in the effects of drought stress on leaf phenology, considering different times of stress induction within the growing season (Čehulić et al. 2019).

Goat willow (*Salix caprea* L.), due to its ability for simple autovegetative reproduction, is the model species of this study. By using the same genotype in the study, intergenotypic variations are avoided, i.e., the focus is on

monitoring phenotypic reactions that are predominantly a result of environmental factors.

The main objective of this study was to investigate potential differences in the effects of drought stress when it occurs at different stages of the growing season. In addition, the study aimed to evaluate the influence of such stress on height growth, leaf phenology in spring and autumn, and flower development in the goat willow clone.

MATERIALS AND METHODS

Plant Material and Experimental Design

A mature female goat willow individual was cloned using the hardwood cuttings method in early 2020. A total of 151 two-year-old ramets were cultivated in the greenhouse of the Croatian Forest Research Institute (45.6688° N, 15.6438° E) and divided into eight groups, consisting of seven experimental groups with 19 ramets each, and one control group with 18 ramets. The substrate used for planting the ramets in this experiment was Durpeta Profi 1 peat, characterized by a pH (H₂O) of 5.5–6.5 and nutrient content of N 80–140 mg·L⁻¹, P₂O₅ 100–160 mg·L⁻¹, and K₂O 110–180 mg·L⁻¹. The plants were cultivated in 3-L polyvinyl chloride (PVC) pots.

Drought stress was simulated by withholding water from seven experimental groups, whereas the control group was kept well-watered for the duration of the experiment. In the first year of the experiment (2021), drought stress was induced by termination of irrigation and applied sequentially, with each of the seven groups exposed at different times between early March and mid-July, at intervals of approximately 20 days (Figure 1). Irrigation was discontinued for the first group shortly after leaf emergence (early March) to induce the first drought treatment period and each subsequent group was exposed once the majority of plants in the preceding group exhibited visible symptoms of drought stress and their mass (including container) had declined to about half of the initial value.

In the following year (2022), all the experimental groups were again subjected to drought stress by withholding irrigation. The treatment was again applied sequentially in the same order as in 2021, but it started later due to delayed leaf emergence that spring (early April), with groups exposed at average intervals of 15 days (Figure 1). In both experimental years, drought onset was defined as the day on which irrigation to the plants was suspended, whereas the end of the treatment period was determined by the rehydration of the last plant within each group, since not all plants within a group reached the stress threshold on the same day (Figure 1).

Measurements and Scoring

The mass of drought-exposed plants, including pot and substrate, was measured twice a week with a precision of 1 g to track the progression of water loss and to accurately identify the onset of drought stress symptoms, following the methodology described by Bačurin et al. (2025). Control plants were weighed at the beginning of the treatment period assigned to each group and again at the end of the

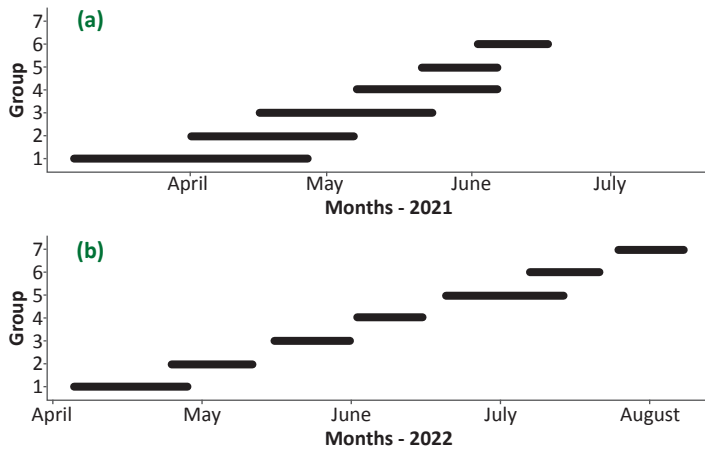


Figure 1. The duration of drought exposure for each group during the 2021 (a) and 2022 (b) growing seasons. Each row represents the treatment period for a group.

treatment. At the beginning of each drought treatment, immediately before irrigation was discontinued, the plants were first irrigated to achieve full saturation and then weighed in this fully saturated state. Because container volume and substrate amount were standardized across all ramets, the initial water content was assumed to be equal.

All plants subjected to drought treatment were regularly monitored for visible signs of water deficit. In the first group of ramets, which experienced drought stress during or immediately after leaf emergence, the earliest observable symptom was the wilting of young, recently developed leaves. In the remaining groups, where leaves were already mature and thicker, the initial response to drought appeared as yellowing, curling, or drying of the foliage. When distinct drought symptoms became evident and the total plant mass (including pot and substrate) had decreased to about half of its initial value, the plants were rehydrated and subsequently maintained under regular watering until the end of the growing season. Control ramets were consistently watered throughout the experimental period.

The intensity of drought stress was calculated as the relative reduction in plant mass within the pots. For each ramet, we determined the ratio between the mass lost up to the point when clear drought stress symptoms were observed (marking the end of the stress period) and its initial mass under fully saturated conditions was obtained, as described by Bačurin et al. (2025).

Plant heights were measured at the beginning and end of the growing season, and spring and autumn leaf phenology were monitored twice a week. Monitoring of spring phenology was conducted for all buds (flower, leaf, and flower-leaf). Leaves developed from flower-leaf buds, with this process being significantly earlier than leafing from purely leaf buds. Additionally, not all ramets possessed flower and/or flower-leaf buds. Therefore, leaf phenology was analyzed only for leaf buds. The number of ramets forming generative organs was also monitored. Leaf phenology was monitored for all plants in the experiment

using an ordinal scale of 1–7: 1 – dormant bud; 2 – bud swelling; 3 – bud burst; buds open, green leaf tips are visible; 4 – folded leaf visible along the shoot; 5 – leaf unfolding, separating from each other; 6 – elongated petiole with multiple rings of leaves; larger leaves, finely serrated with a whitish edge; 7 – significantly larger leaves, serrated edges. Autumn leaf phenological stages were recorded twice per week (scored on a 0–5 ordinal scale), following the method described by Čehulić et al. (2019).

Statistical Analyses

All statistical analyses as well as visualization were carried out in the R environment (version R4.4.1; R Development Core Team 2024.). Data processing was conducted using the tidyverse (v2.0.0) and dplyr (v1.1.4) packages. The assumptions of normality and homogeneity of variance were evaluated using the Shapiro–Wilk and Levene tests, respectively, implemented via the rstatix package (v0.7.2). Height growth data followed a normal distribution; therefore, pairwise t-tests were used to compare each treatment group with the control. Visualization of height growth was generated using ggplot2 (v3.5.1) and ggstatsplot (v0.12.3).

For the proportion of flowering plants, Fisher’s exact test was applied to assess significant differences between groups and the control.

Since phenological scores did not meet the assumption of normality (as shown by the Shapiro–Wilk test), nonparametric analyses—specifically the Kruskal–Wallis and Wilcoxon tests—were employed to detect differences among groups. Statistical significance was set at $p < 0.05$.

RESULTS

Intensity of Drought Stress Based on Ramet Mass Loss

By comparing and statistically analyzing the relative change in total mass (plant + container) measured at the end

of the drought treatment compared to the initial value, we identified a difference in drought stress intensity between 2021 and 2022. In 2022, plants were rehydrated at a lower relative mass than in 2021, indicating exposure to a stronger water deficit before recovery. This difference is consistent with the smaller size and lower transpiration demand of plants in 2021, which caused them to reach higher relative mass values before rehydration. In contrast, the control group maintained a mass ratio close to 1 in both years, confirming that regularly irrigated plants did not undergo stress. Taken together, these findings indicate that drought

stress in 2021 was of lower intensity, whereas in 2022 it can be considered of higher intensity (Figure 2).

The Impact of Drought on Height Growth

By measuring heights at the beginning and end of the growing season in 2021 and calculating the difference between their values, we obtained the growth for each plant within the group. Data processing revealed an average decrease in height growth in all groups after the first year of exposure to drought stress, with the decrease being statistically significant in groups 5 and 6 (Figure 3).

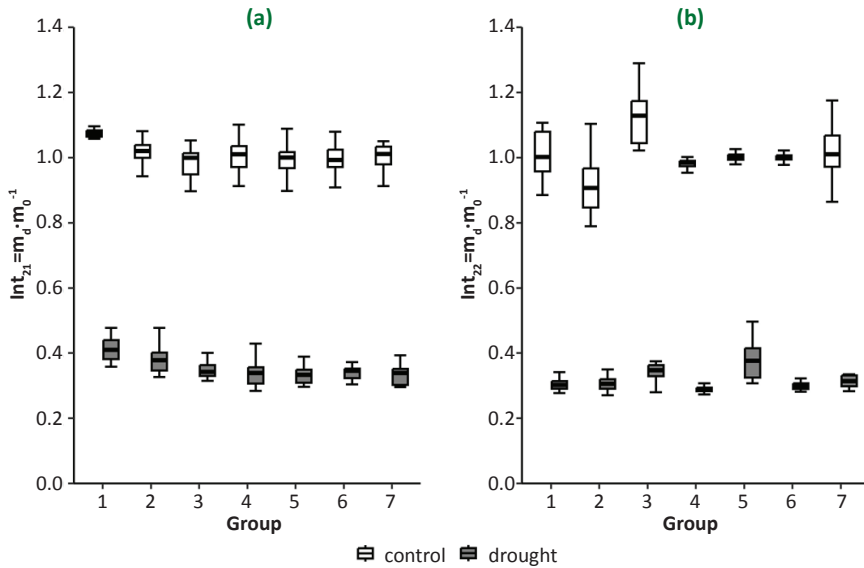


Figure 2. The plant mass ratio at the end of the drought period (upon appearance of visible symptoms, m_d) relative to the initial mass (plant + container, m_0) across groups. **(a)** Int_{21} – mass ratio in 2021; **(b)** Int_{22} – mass ratio in 2022. Control = the control group of regularly irrigated plants, whose mass ratio was recorded repeatedly at the end of each group drought treatment period (groups 1–7); Drought = mass ratio of drought-treated groups. The values are shown as pairs, e.g. 1 = mass ratio of drought group 1 and the control measured at the end of group 1 treatment; 2 = mass ratio of drought group 2 and the control measured at the end of group 2 treatment; and so on until group 7.

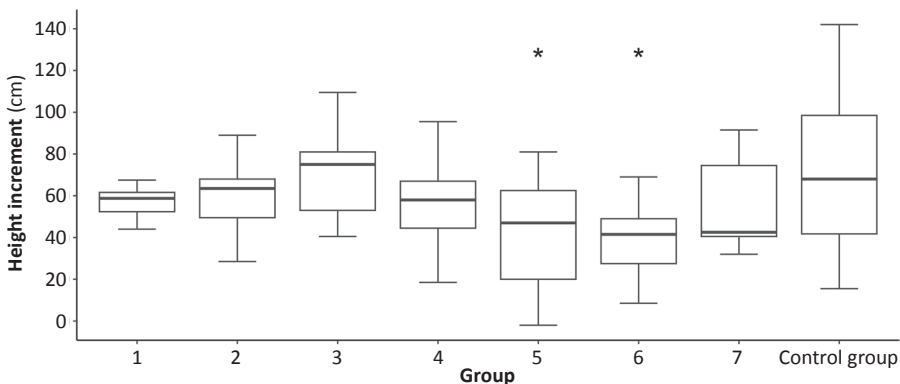


Figure 3. The distribution of height increment for groups 1–7 in 2021, exposed to drought at different times during the growing season. An asterisk highlights a statistically significant difference in annual growth between the groups and the control: *** – highly significant (p-value < 0.001); ** – very significant (p-value < 0.01); * – significant (p-value < 0.05).

By measuring plant height at the beginning and end of the 2022 growing season and by calculating the difference, we determined the height increment for each plant within its respective group. Statistical analysis revealed significant differences between the control group and groups 2, 4, and 5. Group 2 exhibited significantly lower height growth compared to the control, whereas groups 4 and 5 showed significantly greater height growth (Figure 4).

The Impact of Drought Stress on Leaf Phenology
Initial State of Leaf Phenology

The analysis of leaf phenology in the spring of 2021 revealed that there is no statistically significant difference between the other groups and the control group (Figure 5). Such a result is expected because phenological observations took place before the onset of the drought treatment.

Autumn Leaf Phenology after Drought Stress

Analyzing the impact of drought stress in the autumn of 2021, it is evident that the progression of leaf senescence is slower in the first group, exposed to drought from 8 March 2021 to 21 April 2021. A statistically significant delay compared to the control was recorded on the 300th day of 2021 (Figure 6).

Analyzing the impact of drought stress in the autumn of 2022, it is evident that there is slower progress in autumn phenology for all groups compared to the control group. When observing the relationship between groups, it is clear that the groups exposed to drought stress at the beginning of the growing season (1, 2, 3, 4, 5) show faster progress in autumn phenology compared to the groups exposed to drought stress later in the growing season (6, 7).

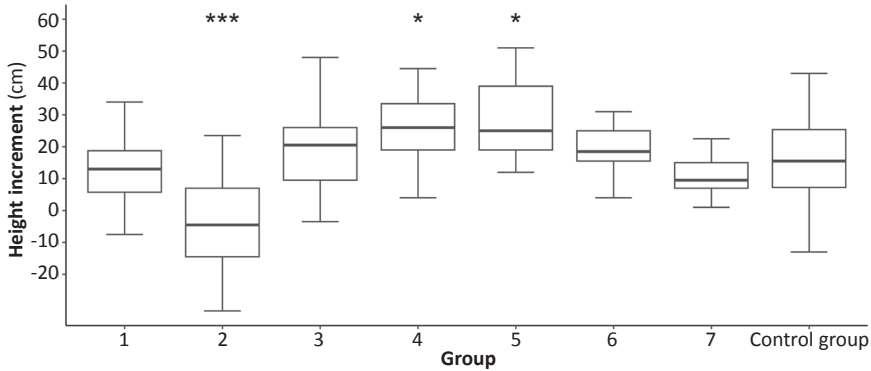


Figure 4. Distribution of height increment in 2022 for groups exposed to drought at different times during the growing season. Asterisks indicate statistically significant differences in growth compared to the control group: *** – highly significant ($p < 0.001$); ** – very significant ($p < 0.01$); * – significant ($p < 0.05$).

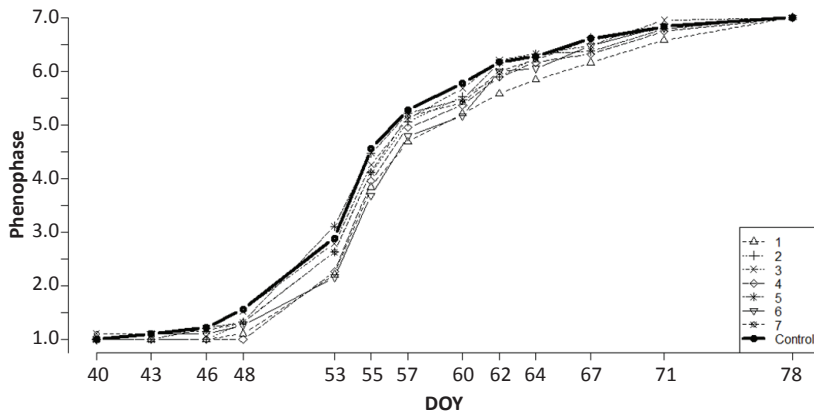


Figure 5. Spring leaf phenology of goat willow ramets in 2021 across experimental groups. The control (bold black line) is compared with drought-treated groups (symbols). The y-axis indicates the phenophase, and the x-axis represents the day of the year (DOY). Points represent mean phenological stage for each DOY.

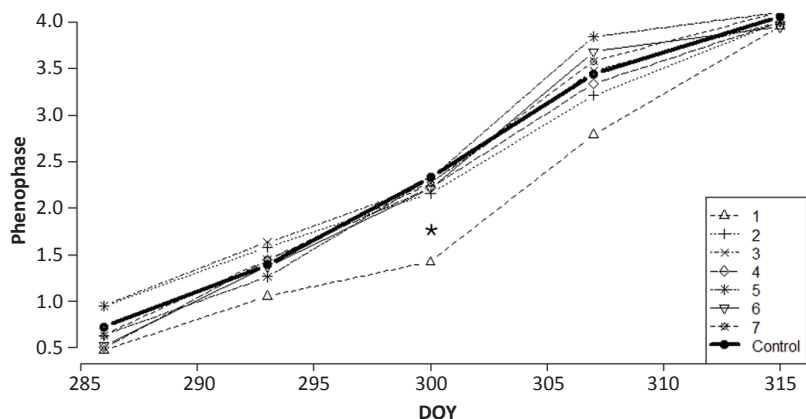


Figure 6. Autumn leaf phenology of goat willow ramets in 2021, across experimental groups. The control (bold black line) is compared with drought-treated groups (symbols). The y-axis indicates the phenophase, and the x-axis represents the day of the year (DOY). Points represent mean phenological stage for DOY. Asterisks marks dates with significant group–control differences ($p < 0.05$).

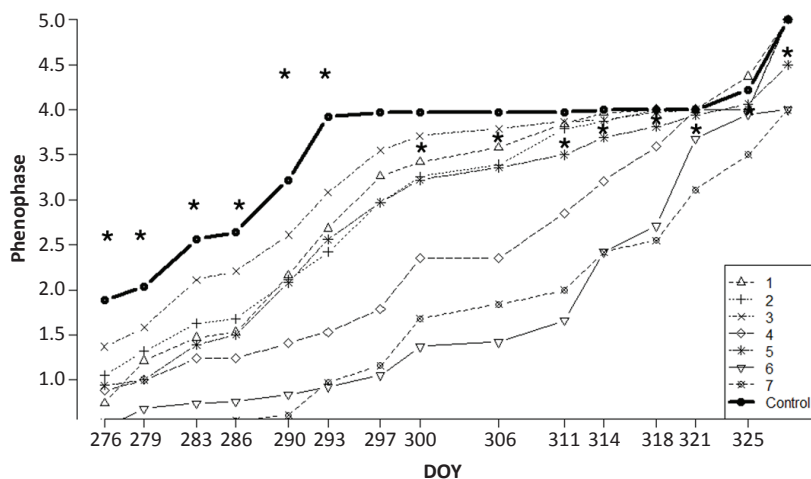


Figure 7. Autumn leaf phenology of goat willow ramets in 2022, across experimental groups. The control (bold black line) is compared with drought-treated groups (symbols). The y-axis indicates the phenophase, and the x-axis represents the day of the year (DOY). Points represent mean phenological stage for DOY. Asterisks marks dates with significant group–control differences ($p < 0.05$).

Spring Leaf Phenology after Drought Stress

An analysis of the impact of drought stress in the spring of 2022 reveals an earlier onset and a faster rate of leaf emergence in the first group. In contrast, delayed leaf emergence and a statistically significant slowdown were observed in groups 6 and 7 (Figure 8).

The Impact of Drought on the Formation of Female Flowers

In 2021, there was no statistically significant difference in the number of flowering ramets between the treatment groups and the control group. In 2022, the results of Fisher’s exact test showed that group 5 (p -value = 0.000547) and group 6 (p -value = 0.006275) had a statistically lower number of ramets that developed flowers compared to the control group.

It was found that in the second year of the study, there was a decrease in the number of plants that developed flowers compared to the previous year (Figure 9). Although the number of flowering ramets also decreased in the control group, the reduction was more pronounced in groups 5, 6, and 7, which had been exposed to drought stress in the previous year (May 21–June 7, June 2–11, and June 30–July 9, respectively). In group 5, only 11% of ramets developed flowers, whereas 74% had developed flowers the year before the drought. In group 6, after the drought stress, 21% of ramets developed flowers, while 58% had developed flowers the year before the drought. In group 7, 42% of ramets developed flowers in 2022, compared to 84% in the previous year.

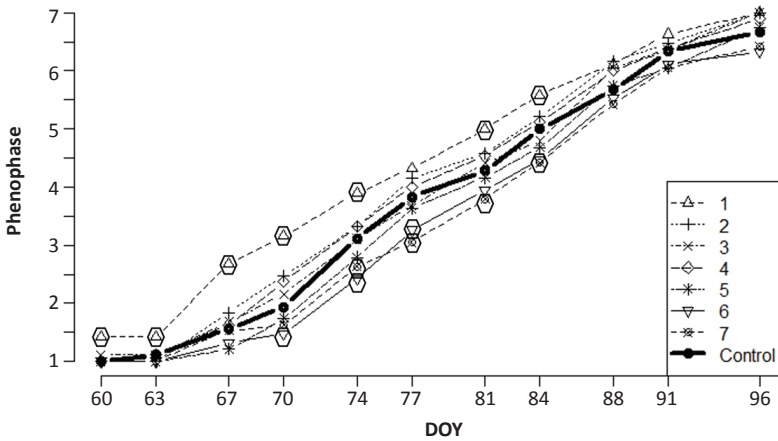


Figure 8. Spring leaf phenology of goat willow ramets in 2022 across experimental groups. The control (bold black line) is compared with drought-treated groups (symbols). The y-axis indicates the phenophase, and the x-axis represents the day of year (DOY). Points represent mean phenological stage for each DOY. Groups marked with a hexagon symbol indicate a statistically significant difference between the group and the control on a specific day of the year ($p < 0.05$).

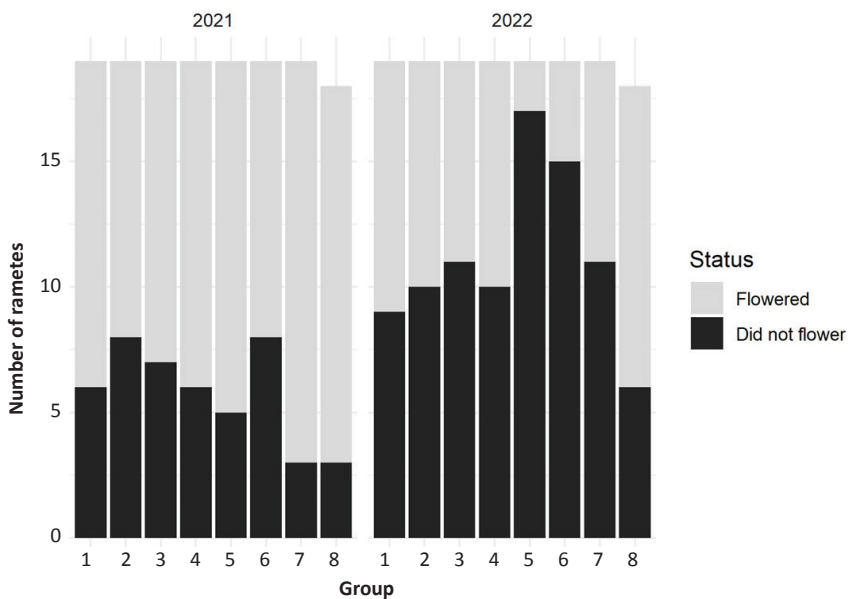


Figure 9. The number of flowering and non-flowering ramets across groups in 2021 and 2022. Bars represent the total number of ramets per group, subdivided by flowering status: black segments indicate ramets that did not flower, while light gray segments indicate ramets that flowered. The figure highlights group- and year-specific variation in flowering incidence.

DISCUSSION

Heights

An average decrease in height growth was observed in all groups after the first year of exposure to drought stress, with a statistically significant decrease in groups 5 and 6 (Figure 3). The minimal growth observed in groups 5 and 6 may be

related to the interaction of drought stress with elevated temperatures during June/July, although this remains a possible explanation rather than a confirmed effect. High temperatures are to be expected during this period of the year, even though they were not directly measured in the greenhouse. Similar interactions between drought and high air temperatures have been reported to negatively affect

growth in *Pinus edulis* Engelm (Adams et al. 2015).

In the following year of the experiment (2022), plants were again exposed to drought stress, but of higher intensity. Significantly, the lowest height growth was recorded in plants from the second group (Figure 4). Budburst date, or the onset of the growing season, is a key determinant of above-ground biomass production in the genus *Salix* (Weiher 2009). Willows complete most of their annual height growth shortly after vegetative activity begins (Labrecque et al. 1993), which makes them particularly sensitive to water deficit at this stage. Indeed, species classified as early-season growers, such as willows, *Betula papyrifera* Marsh., and *Pinus strobus* L., have shown reduced height growth under spring drought conditions (Canham et al. 1999, Van Kampen et al. 2022). Drought reduces carbon assimilation through stomatal closure and lower leaf biomass, while increasing allocation to roots at the expense of above-ground growth (Cowan 1982, Cornelissen et al. 1996, Wikberg and Ögren 2004). Consequently, our finding that drought stress in spring significantly reduced height growth (e.g., group 2 in 2022) is consistent with the general understanding that drought during the period of intensive shoot elongation has the strongest negative impact (Foster et al. 2014).

A significantly higher growth was recorded in groups 4 and 5 compared to the control in 2022. On average, groups 3 and 6 also showed higher growth compared to the control, although these differences were not statistically significant (Figure 4). It can be assumed that the higher height growth is actually a result of the combined effect of drought stress in 2021 and 2022. Namely, this is due to its impact on leaf phenology (prolongation of vegetative activity) and the increase in non-structural carbohydrates (NSC) as a result of compensatory growth. It is important to consider that the plants were subjected to drought stress in 2021, which may have resulted in an increase in NSC. The increase in NSC content in plant tissues is a common physiological response to drought stress, contributing to defense against future drought periods (O'Brien et al. 2014). It has been proven that plants exposed to drought have significantly higher NSC levels than plants not subjected to drought the previous year (Tomasella et al. 2019). Although higher growth was not recorded in 2021 as it was in 2022, it can be assumed that the reason lies in the lower intensity of drought stress that year. Also, although the plants in groups 4 and 5 likely increased assimilation after drought stress, this did not reflect in their current height growth due to the pattern of height growth in willows (i.e., culmination of height growth at the beginning of the growing season). Therefore, it seems convincing that after drought stress in 2021, plants in groups 4 and 5 stored an increased amount of NSC and utilized it during the period of intensive height growth in 2022. In addition to the response to the 2021 drought, plants have the ability of so-called compensatory growth, as a response to recovery in the same year. The concept of compensatory growth involves increasing assimilation above values that were present before the drought stress (Arend et al. 2016, Gessler et al. 2020). Therefore, we can highlight three possible reasons for the higher growth in groups 4 and 5, namely: higher NSC levels as a result of recovery from the 2021 drought; the absence of drought stress during the period of intensive height growth

in 2022; prolonged growing season in 2022 due to drought-induced delay in leaf senescence (Figure 7). More intensive height growth after the second year of exposure to drought stress may be a result of a “carry-over” effect on leaf phenology and growing season length (Figures 7 and 8), as well as a likely increase in NSC. Generally, the growth response to drought stress depends on the interaction between the timing of drought stress induction and the growth phenology specific to each individual tree species (Kozłowski 1964, Kannenberg et al. 2019, Van Kampen et al. 2022).

Autumn Phenology

It is evident that the first group of willow ramets delayed the onset of leaf senescence in 2021 (Figure 6), and the progression of the process was slower compared to the other groups. By delaying the onset of autumn leaf senescence, the plants attempt to compensate for assimilation and growth that were hindered during the period of drought. This delay, following induced drought stress, has been observed in pedunculate oak (Čehulić et al. 2019) and also in beech (Arend et al. 2016). The delay of autumn leaf senescence commonly occurs when the assimilation process during the growing season is disrupted by some stressful factors (Keskitalo et al. 2005, Mariën et al. 2019). The lack of response in the other groups of willow ramets can be at least partially explained by the lower intensity of induced drought stress in the first year of the experiment or by the higher genotype sensitivity to drought stress at the beginning of the growing season.

After the repetition of drought stress in 2022, it is evident that all groups delayed the onset of autumn leaf senescence (Figure 7). Although all groups showed a delayed onset of autumn leaf senescence, groups 6 and 7 had the greatest temporal gap compared to the control, and the process itself occurred slower in the ramets of those groups. Drought stimulates the plant to try to compensate for the previous loss of photosynthetic activity (post-drought stimulation) by delaying autumn leaf senescence and increasing the intensity of photosynthesis, a phenomenon observed in beech (Arend et al. 2016). Groups 6 and 7 were exposed to drought stress in July and August when high air temperatures prevailed. Plants exposed to the interaction of abiotic factors such as drought and high air temperature must balance between preventing additional water loss and protecting against leaf overheating (Zandalinas et al. 2018). Stomata respond to high temperature and drought by closing, leading to a decrease in CO₂ levels in chloroplasts, which reduces the intensity of photosynthesis (Rennenberg et al. 2006). Likewise, negative interaction effects have been recorded through increased production of reactive oxygen species in *Populus yunnanensis* Dode, and it is known that ROS (reactive oxygen species) can cause significant cell damage and inhibit photosynthesis (Baxter et al. 2014).

Groups 4, 5, and 6 demonstrate that manipulating drought stress can even have a positive effect because prolonging growing season indirectly affects greater height growth. However, with the extension of the growing season, the risk of early autumn frosts also increases. From the leaves affected by frost, the plant cannot recycle nutrients, causing a deficiency of nutrients needed for growth at the beginning of the next growing season (Fracheboud et al. 2009).

Spring Phenology

In this study, goat willow ramets exposed to drought at different times during the growing season (in 2021) showed shifts towards earlier or delayed budburst in spring 2022 (Figure 8). Groups exposed to drought stress from March to early June (groups 1, 2, 3, and 4) on average leafed out earlier than the control group. Groups exposed to drought stress from early June to mid-July (groups 5, 6, and 7) leafed out later than the control group. It can be clearly seen that different times of drought stress induction affect shifts in spring phenology in different directions compared to the control group. It is known that drought stress caused delayed budburst in pedunculate oak (*Quercus robur* L.) (Vander Mijnsbrugge et al. 2016), while some authors report earlier budburst in *Quercus ilex* L. and *Quercus coccifera* L. (Sanz-Pérez and Castro-Díez 2010), whereas in pedunculate oak, drought stress caused delayed budburst in one year, followed by early budburst the next year (Čehulić et al. 2019). Čehulić et al. (2019) hypothesize that the different time periods during which plants are exposed to water deficit during the growing season probably cause a “carry-over” effect, resulting in different leaf-out times. Therefore, it can be assumed that drought stress caused an epigenetic (memory) imprint, and the timing of stress induction conditioned different budburst times. Through genetic or biochemical reactions to stressful factors, the plant acquires a so-called stress imprint, which determines the plant’s response to overcoming future stresses (Bruce et al. 2007). It is now known that plants, to cope with acute stress, have developed mechanisms that recognize undesirable conditions and respond to them through DNA methylation, histone modification, or chromatin remodeling without changing the nucleotide sequence (Madlung 2004, Bruce et al. 2007). Additionally, it is important to note the role of plant hormones, such as abscisic acid (ABA), which participate in the expression of specific genes that can be linked to drought stress (Chaves et al. 2003).

When observing the spring leaf phenology of the first group in 2022, it can be noticed that it leafed out earliest compared to all groups (Figure 8). An interesting fact is that the same group experienced delayed onset of autumn senescence the year before (Figure 6). From the above, we can assume that in addition to the epigenetic impression, the phenology of the following growing season may also be influenced by the amount of reserves the plant stores the previous year. Since it extended its growing season in the autumn of 2021, it had a longer period in which assimilation was possible, and as a result of good nutritional status, responded with the earliest budburst. Therefore, we can assume that spring phenology is influenced by the concentration of NSCs the plant accumulates in the previous year’s autumn. Amico Roxas et al. (2021) have demonstrated that low concentrations of NSCs in the autumn affect the delayed onset of spring phenology in several species of deciduous Mediterranean trees. Accordingly, higher levels of NSCs in the autumn could potentially influence earlier budburst, which should definitely be further investigated.

Flowering Analysis

By tracking the number of flowers, it was determined that there was a decrease in the number of flowering ramets in all groups during the second vegetative period, including

the control group that was not exposed to drought (Figure 9). It can be assumed that the general reduction in the number of flowering ramets occurred due to a decrease in starch reserves since the ramets originated from hardwood cuttings. It is known that during rooting, there is a gradual decrease in carbohydrate reserves in the aboveground part of the cutting, as carbohydrates are allocated towards root, leaf, and callus development (Okoro and Grace 1976). Although it is not sufficiently understood how carbohydrate deficiency affects the formation of flower buds and flowering in forest tree species, it has been found that low starch levels negatively affect the differentiation of flower buds in fruit crops. For example, Goldschmidt and Golomb (1982) discovered a positive correlation between starch levels and the number of flower buds in citrus.

Despite the overall decrease in the number of flowering ramets, in groups 5 and 6, which were exposed to drought stress in June/July, there was a significantly more intense decrease in the number of flowering ramets compared to the control (Figure 9). This suggests that flowering is negatively affected by drought stress, especially if induced in late spring or early summer. It is known that drought stress during the month of July slows down the process of flower bud differentiation and prevents subsequent recovery, compromising the regularity of flower differentiation processes in species such as *Prunus armeniaca* L., and negatively impacting fruiting in the following growing season (Bartolini et al. 2020). The initiation of female flower primordia in willows typically occurs after the completion of apical growth (Junttila 1976), i.e., in the early spring period, as is the case with some other tree species, e.g., oaks (Tantray et al. 2017). Therefore, the probable reason for the more intense decrease in the number of flowering ramets in these groups is that drought stress disrupted the initiation process of flower primordia.

CONCLUSIONS

This study demonstrates that the timing of drought stress induction plays a crucial role in shaping the phenological and growth responses of goat willow (*Salix caprea* L.). Drought stress applied at different stages of the growing season resulted in distinct effects on height growth, leaf phenology, and flower formation. Early-season droughts negatively impacted height growth due to overlap with the period of intensive vegetative development, while late-season droughts led to prolonged leaf retention and, in some cases, increased height growth, likely due to physiological compensation and accumulated non-structural carbohydrates (NSCs). Spring and autumn phenological shifts, both advances and delays, indicate the presence of “carry-over” effects, potentially linked to epigenetic memory or changes in resource allocation. Notably, groups exposed to summer droughts showed a marked reduction in flowering, suggesting that drought timing also influences reproductive capacity.

Overall, the findings underline the importance of considering drought timing—not merely its presence—when evaluating the resilience of forest tree species to climate change. The precise mechanisms behind these phenological

and developmental responses, especially the interplay between NSCs, hormonal signals, and epigenetic regulation, warrant further investigation to better inform adaptive forest management strategies under increasing climate variability.

Author Contributions

SB and MB conceived and designed the research, SB and MB developed the methodology and validated the results, MB, SB, and IČ carried out the field measurements, MB performed the formal analysis, MB and SB provided resources, MB and SB handled data curation, MB wrote the original draft, IKB, SB, and IČ reviewed and edited the manuscript, MB prepared visualization, SB provided supervision.

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Conflicts of Interest

The authors declare no conflict of interest.

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Article

The Effects of Drought Timing on Height Growth and Leaf Phenology in Pedunculate Oak (*Quercus robur* L.)

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Abstract: This study examines the effects of drought timing on height growth and seasonal leaf phenology in pedunculate oak (*Quercus robur* L.) seedlings. Drought represents a significant threat to long-lived tree species, impacting growth, phenology, and recovery potential. This research aims to assess whether the timing of drought stress influences height growth and leaf phenology while also investigating possible compensatory mechanisms. The experiment involved five groups of seedlings: four exposed to drought at different periods during the 2022 and 2023 growing seasons, and one regularly irrigated control group. The key monitored parameters included height growth, spring flushing, autumn leaf senescence, and photosynthesis. Preliminary results revealed that late-spring and summer drought had a significant negative impact on height growth and delayed autumn senescence, whereas mid-spring drought allowed for compensatory growth. Spring leaf phenology remained largely unaffected by drought treatments. None of the drought-stressed plants showed increased photosynthesis during the recovery phase compared to the control. These findings highlight the critical role of drought timing in determining growth and phenological outcomes. Relatively late-season droughts were particularly detrimental, limiting recovery and resource allocation, while early-season droughts provided better opportunities for compensation. Further research on drought recovery mechanisms and nutrient interactions is needed to refine forestry management strategies under climate change.



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Keywords: autumn leaf senescence; nutrition; *Quercus robur* L.; spring foliation; stress; subirrigation

1. Introduction

Current climatic changes, including rising temperatures and increased drought frequency, pose significant challenges to ecosystems worldwide [1]. Forest trees are particularly vulnerable due to their long lifespans, which limit their ability to adapt to rapid climate shifts [2]. One of the most observable responses to climate change is phenology, the timing of biological events such as budburst and leaf senescence, which plays a crucial role in vegetation adaptation [3,4]. While phenological traits are primarily driven by temperature and photoperiod [5,6], other environmental stressors, including drought, can significantly alter these processes [7]. As phenology governs the timing and duration of both the growing season and the reproductive phase, it directly influences tree fitness and survival [3,4,8].

Temperature (both chilling and forcing) and photoperiod are the primary drivers of leaf phenology, although sensitivity to these cues varies among and within species [5,6,9,10].

For example, pioneer species such as poplars (*Populus* spp.) and willows (*Salix* spp.) rely mainly on temperature cues and are relatively insensitive to photoperiod, whereas late-successional species like European beech (*Fagus sylvatica* L.) depend more on photoperiod, with temperature acting as a secondary modulator to prevent premature phenological events [11]. Beyond these primary environmental cues, a range of additional stressors—often termed “atypical factors”—can also influence phenological processes [7]. Stressors such as pathogen attacks [12,13], elevated ozone levels [14], nutrient limitations [7,15], and drought [16] have been shown to alter phenology, highlighting the complex interactions between biotic and abiotic factors in shaping plant responses.

Drought profoundly affects both growth and phenology in trees. In sessile oak (*Quercus petraea* (Matt.) Liebl.), drought stress reduces shoot and leaf biomass, lowers the leaf-to-root biomass ratio (due to increased fine root development as an adaptive response to water scarcity), and decreases bud production [17]. Similarly, in pedunculate oak (*Quercus robur* L.), drought caused a 44.5% reduction in seedling height compared to irrigated controls [18]. These effects are consistent across studies, demonstrating significant reductions in growth and biomass under drought conditions [19]. However, the impact of drought on leaf phenology varies considerably. Some studies have reported earlier leaf senescence in pedunculate oak under drought stress [20], whereas others observed delayed autumn senescence in sessile oak [21]. Similarly, Čehulić et al. [22] found that drought induced contrasting spring phenology responses in pedunculate oak, with budburst delayed in one year and advanced the following year, depending on the timing of drought stress. A comparable pattern has been observed in goat willow (*Salix caprea* L.), where drought-induced shifts in spring phenology varied based on the timing of stress exposure [23].

The mechanisms driving these opposing phenological shifts remain poorly understood. One potential explanation is epigenetic modifications, which allow plants to adjust to environmental stressors by altering gene expression without changing DNA sequences. Oaks, for instance, exhibit epigenetic responses to drought and high temperatures, resulting in modified physiological traits such as stomatal regulation, water-use efficiency, and leaf morphology [24]. Another possible mechanism involves metabolic changes during post-drought recovery. Plants recovering from drought often exhibit higher photosynthetic activity than unstressed controls, which may enhance leaf photosynthetic capacity [25,26], activate recovery-related metabolic pathways [27], or improve osmotic adjustments and physiological reorganizations [28]. Increased post-drought photosynthesis has been linked to greater carbohydrate reserve accumulation, which supports regeneration and subsequent growth. For instance, in holm oak (*Quercus ilex* L.), enhanced post-drought photosynthesis was associated with higher carbohydrate reserves [29], a trend that has also been observed in other species [27,30–33].

Despite significant advances in understanding drought responses, most studies focus on stress-induced at a single point in the growing season. It remains unclear whether the timing of drought stress differentially affects leaf phenology and growth, particularly in late-successional species like pedunculate oak compared to pioneer species like goat willow. Additionally, the drivers of opposing phenological shifts under drought stress remain poorly understood.

Based on these knowledge gaps, this study aims to: (1) Determine whether the timing of drought stress exposure variously influences the height growth of pedunculate oak seedlings. (2) Investigate how the timing of drought stress affects leaf phenology responses in pedunculate oak seedlings. (3) Examine whether eventually opposing shifts in spring leaf phenology can be explained by varying levels of photosynthetic activity during post-drought recovery.

We propose the following hypotheses:

H1: *Drought stress induced at different stages of the growing season does not result in uniformly negative effects on height growth.*

H2: *Drought stress followed by rehydration leads to delayed autumn leaf phenology but with varying intensities depending on the timing of stress induction.*

H3: *Drought stress at different times, followed by rehydration, results in contrasting shifts in spring foliation phenology (carry-over effect).*

H4: *The direction of this shift depends on the plants' ability to increase photosynthetic activity during the post-drought period. Increased photosynthetic activity during post-drought recovery boosts non-structural carbohydrate (NSC) reserves, potentially advancing leafing in the following year. Conversely, insufficient photosynthetic recovery leads to lower NSC reserves, causing delayed flushing.*

In the following sections, we examine the extent to which our results support or contradict these hypotheses, providing insights into the physiological mechanisms underlying drought responses in pedunculate oak seedlings.

2. Materials and Methods

2.1. Plant Material and Trail Design

In the fall of 2020, acorns were collected from a natural pedunculate oak stand located southeast of the town of Ivanić-Grad (45.55° N, 16.49° E). The acorns were sown in 1-L polyvinyl chloride (PVC) pots filled with Durpeta peat substrate Profi 1 (pH (H₂O) 5.5–6.5, N: 80–140 mg/L, P₂O₅: 100–160 mg/L, K₂O: 110–180 mg/L) at the “Brestje” nursery (45.84° N, 16.10° E), managed by the Croatian Forests company.

In the spring of 2022, the seedlings were labeled and individually transplanted into 2.5-L PVC pots filled with the same substrate described earlier. The seedlings were divided into five equal groups (24 plants per treatment). Four groups were subjected to drought stress, while the fifth group served as a control and was regularly irrigated.

The plants were irrigated by placing the pots into boxes (dimensions (L × W × H): 60 cm × 40 cm × 18 cm), with each group placed in a separate box. These boxes were half-filled with water, implementing a subirrigation. Subirrigation is an irrigation method that utilizes capillary action to supply plants with water and nutrients from beneath the pots [34,35]. Manual watering was performed twice a week by pouring 1 L of water onto the substrate of each plant, with additional water added to the boxes to maintain their water level at half capacity.

2.2. Drought Treatments

In 2022, four groups were exposed to drought stress, with each group experiencing drought at different times during the growing season. The first group was subjected to water deprivation after leaf emergence (from 19 April to 15 May). The other groups were exposed to water deprivation after most plants in the previous group had already shown signs of stress. The second group experienced drought from 16 May to 29 June, the third group from 7 June to 4 July, and the fourth group from 4 July to 31 July (Figure 1).

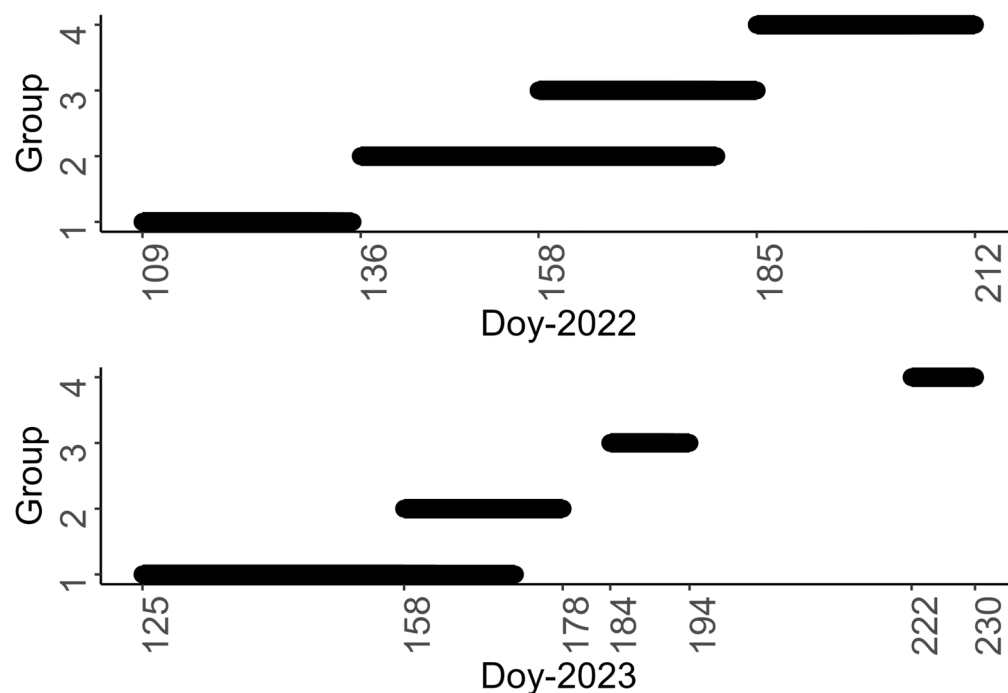


Figure 1. Exposure of groups to water deprivation during the growing seasons. Each row indicates the duration of water deprivation experienced by each group. The upper graph illustrates the length of water deprivation for each group in 2022, while the lower graph presents the corresponding durations for 2023. The start of the treatment was defined as the time when irrigation was stopped, and the end of the treatment was marked as the moment when the last plant in the drought-treated group was rehydrated.

In 2023, the drought periods were as follows: the first group experienced water deprivation from 5 May to 21 June, the second group from 7 June to 27 June, the third group from 3 July to 13 July, and the fourth group from 10 August to 18 August (Figure 1). Differences in the timing of drought exposure between 2022 and 2023 were due to variations in the phenology of leaf emergence in the experimental plants and differences in weather conditions between the two years.

The plants were deprived of water until clear symptoms of drought stress became apparent. At that point, the plants were rehydrated, and regular irrigation was resumed until the end of the growing season. The date on which the last plant was rehydrated was recorded as the treatment's end date.

The control group was irrigated regularly throughout the entire growing season.

2.3. Measurements and Scoring

2.3.1. Mass Weights and Drought Stress Intensity

The mass of plants exposed to drought (including the pot and substrate) was measured twice a week using a scale with a precision of 1 g. The masses of the control plants were also measured on the day the treatment was initiated for each individual group, as well as when the treatment ended. Visual symptoms of drought stress were monitored daily for all plants in the treatment. The first visible symptom of drought stress in the first group (exposed to drought shortly after flushing) was the wilting of newly developed, tender leaves. In the other groups, where the leaves were more developed and thicker, the symptoms included yellowing, curling, and drying of green leaves. When visible symptoms of drought stress appeared, and the mass decreased to approximately 50% of its initial value, individual plants were rehydrated and subsequently regularly irrigated until the end of the growing season. Control plants were regularly irrigated throughout the growing season.

The intensity of drought stress was quantified by calculating the relative weight loss of plants in the pots. This was determined by dividing the weight loss of each plant at the onset of visible drought stress symptoms (marking the end of the stress period) by their initial weight under fully saturated conditions. These values served as input data for subsequent statistical analyses.

2.3.2. Heights and Leaf Phenology

Plant heights were measured with a ruler as the distance from the root collar to the tip of the terminal bud, with an accuracy of 0.5 cm, at the beginning and end of the growing seasons. The two-year height increment was determined by calculating the difference between the measured values at the end of 2023 and the beginning of 2022. These values were used as input data to analyze the impact of repeated drought stress on experimental plants.

Flushing phenology was observed in the spring of 2023 and 2024, while autumn senescence was recorded in the autumns of 2022 and 2023. Flushing phases were scored twice a week during their progression, using a 1–7 ordinal scale described by Čehulić et al. [22]. Autumn leaf phenological phases were scored twice a week on a 0–5 ordinal scale [22]. All phenological scoring was performed by an experienced observer. The phenological phase scores served as input data for subsequent statistical analyses.

2.3.3. Photosynthesis

Instantaneous rates of net photosynthesis (A_{net}) were measured using a portable photosynthesis system (LCpro+, ADC BioScientific Ltd., Hoddesdon, UK) equipped with a broadleaf cuvette. Measurements were conducted on one sun-exposed and healthy leaf per sapling. All measurements were performed between 10:00 and 13:00 (Central European Summer Time), with cuvette conditions maintained at 400 ppm CO_2 , a photon flux density of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ and an air temperature of $25 \pm 2 \text{ }^\circ\text{C}$.

Measurements were conducted on all experimental plants before treatment, on the day most plants in the treated group exhibited visible drought stress symptoms, and twice during the post-drought recovery period for the final treated group.

2.3.4. Statistical Analyses

All statistical analyses of input data, as well as visualizations, were performed using R statistical software (version R 4.4.1; R Development Core Team, 2024). Data processing was performed using the tidyverse (version 2.0.0) and dplyr (version 1.1.4) packages. Normality and homogeneity of variance were tested using the Shapiro–Wilk test and Levene’s test, conducted with the rstatix package (version 0.7.2). Data visualization was performed using the ggplot2 (version 3.5.1) and ggstatsplot (version 0.12.3) packages.

The Shapiro–Wilk test indicated that phenological observation data did not follow a normal distribution. Consequently, the Kruskal–Wallis non-parametric test was conducted to assess statistically significant differences among groups for each Day when phenological observations were done. Post hoc Wilcoxon tests were then performed to evaluate differences between individual groups and the control group. Differences were considered statistically significant when $p < 0.05$.

The Shapiro–Wilk test confirmed that height increment data followed a normal distribution. To assess statistically significant differences between the individual groups and the control group, pairwise comparisons were conducted using t -tests. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Drought Stress Intensity

All groups had significantly lower average mass rates compared to the control group, indicating their significant exposure to drought stress (Figure 2). In the first year of treatment, the first group was rehydrated at a higher average mass rate compared to the other groups, suggesting that this group was exposed to a lower level of stress in comparison to the others (Figure 2a). In the second year of the experiment (Figure 2b), the level of drought stress was similar across all groups. However, Groups 2, 3, and 4 showed a higher average mass rate at the end of the treatment compared to the previous year, indicating that the plants exhibited visible drought symptoms at a lower level of stress.

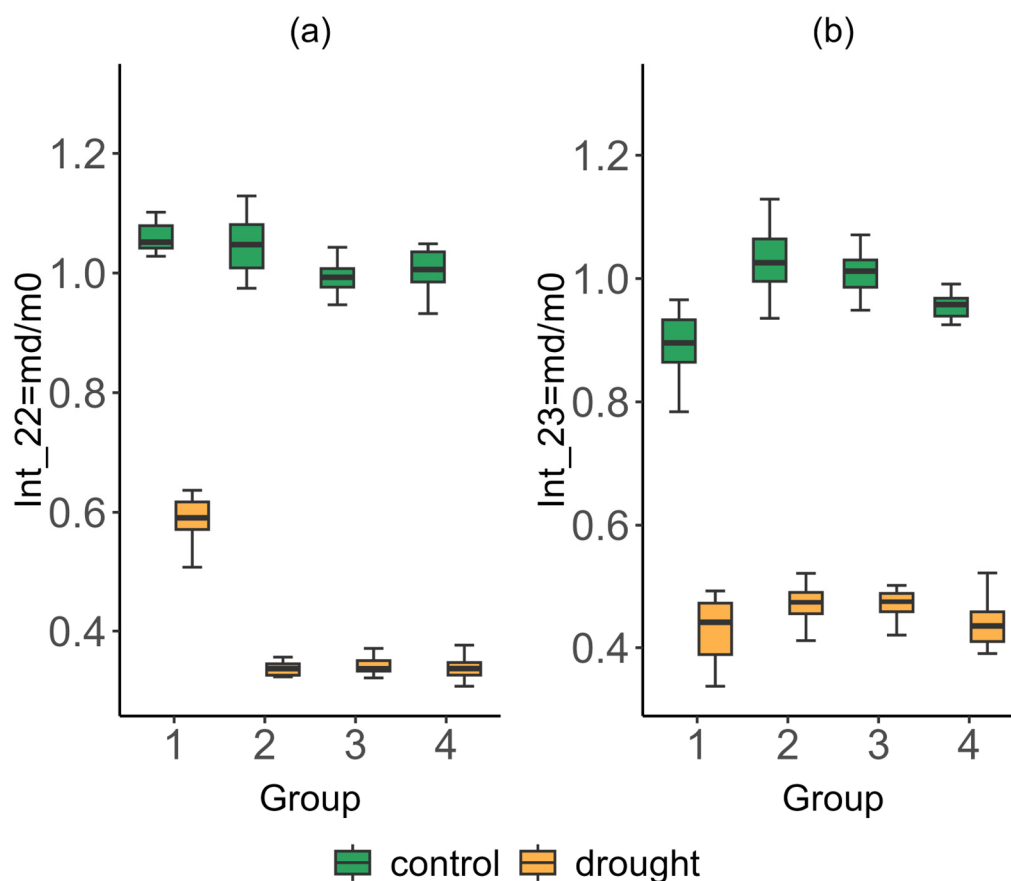


Figure 2. The proportion of mass at the end of drought treatment (upon the appearance of visible symptoms) relative to the initial mass (plant mass with container) by groups in 2022 (a). Int_22—mass ratio in 2022; md—weight at the end of the stress period; m0—initial weight under fully saturated conditions. The proportion of mass at the end of drought treatment (upon the appearance of visible symptoms) relative to the initial mass (plant mass with container) by groups in 2023 (b). Int_23—mass ratio in 2023; md—weight at the end of the stress period; m0—initial weight under fully saturated conditions.

3.2. Height Growth

Plants in drought-treated Groups 2, 3 and 4 that experienced drought stress relatively later in the growing season exhibited significant reductions in height growth increment compared to the control group, while drought-treated plants in Group 1 that experienced drought stress early in the growing season did not differ from control (Figure 3).

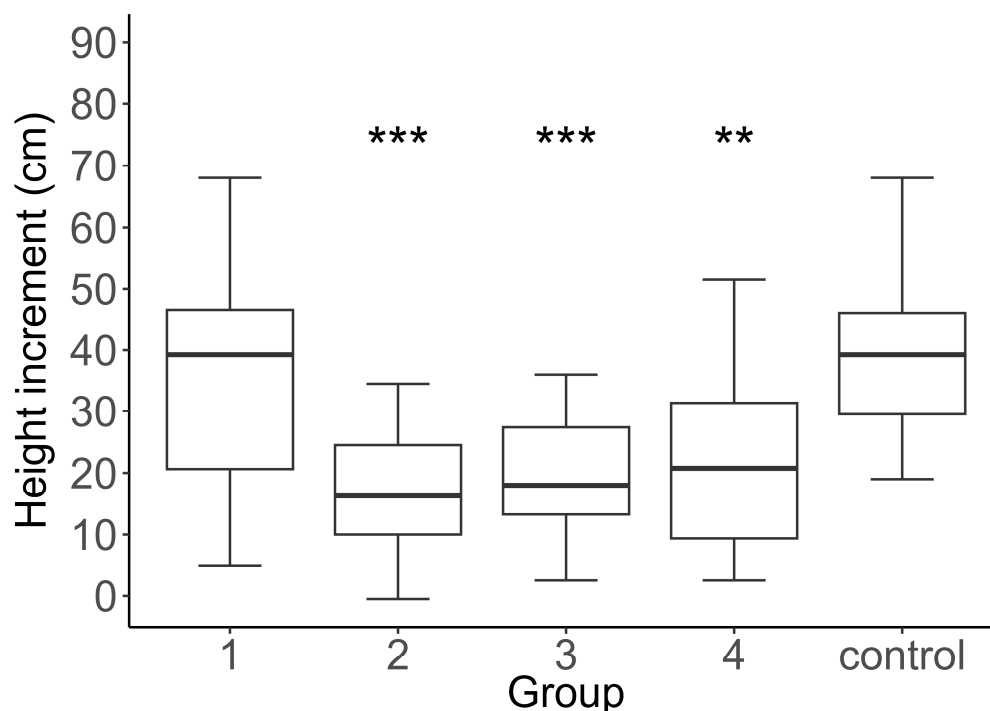


Figure 3. The distribution of the two-year height increment by groups. An asterisk highlights a statistically significant difference in growth between the group and the control: ***: Highly significant (p -value < 0.001) **: Very significant (p -value < 0.01).

3.3. The Impact of Drought Stress on Leaf Phenology

3.3.1. Autumn Leaf Senescence

A delay and slower progression of the autumn leaf senescence process can be clearly seen in all groups except for the first group (Figure 4a). If we consider phenophase 3 (more than 50% of the leaves discolored) as the indicator for the end of the vegetative growth, it can be observed that plants in Groups 2, 3, and 4 (groups exposed to drought stress later) delayed the end of the growth period by an average of more than 25 days. It can also be seen that the curves of leaf senescence dynamics are relatively orderly, following the sequence based on the timing of stress exposure. Group 2 delayed the leaf senescence process the least, on average, while Group 4 showed the highest average intensity of difference compared to the control. However, it should be noted that the differences among Groups 2, 3, and 4 were not statistically significant (Figure 4a).

In 2023, all groups showed significantly delayed leaf senescence compared to the control (Figure 4b). However, this year, the differences between the drought-treated groups and the control were significant only in the initial phenophases (0–2), while as phenophase 3 approached, the statistical significance of the differences disappeared. The difference in the end of vegetative growth between Group 4 and the control (phenophase 3) was only five days on average.

It should be emphasized that in the second year, compared to the previous year, the control group showed the greatest delay in autumn phenology. Namely, while Group 4 (the phenologically latest) entered phenophase 3 an average of 11 days later, the control group entered phenophase 3 an average of 30 days later than in the previous year (Figure 4a,b). Despite this, in the second year, a relatively orderly pattern in the intensity of the delay in autumn phenology could still be observed, ranging from groups exposed to drought earlier (Groups 1 and 2) to those exposed to stress later (Groups 3 and 4), although the differences were not statistically significant (Figure 4b).

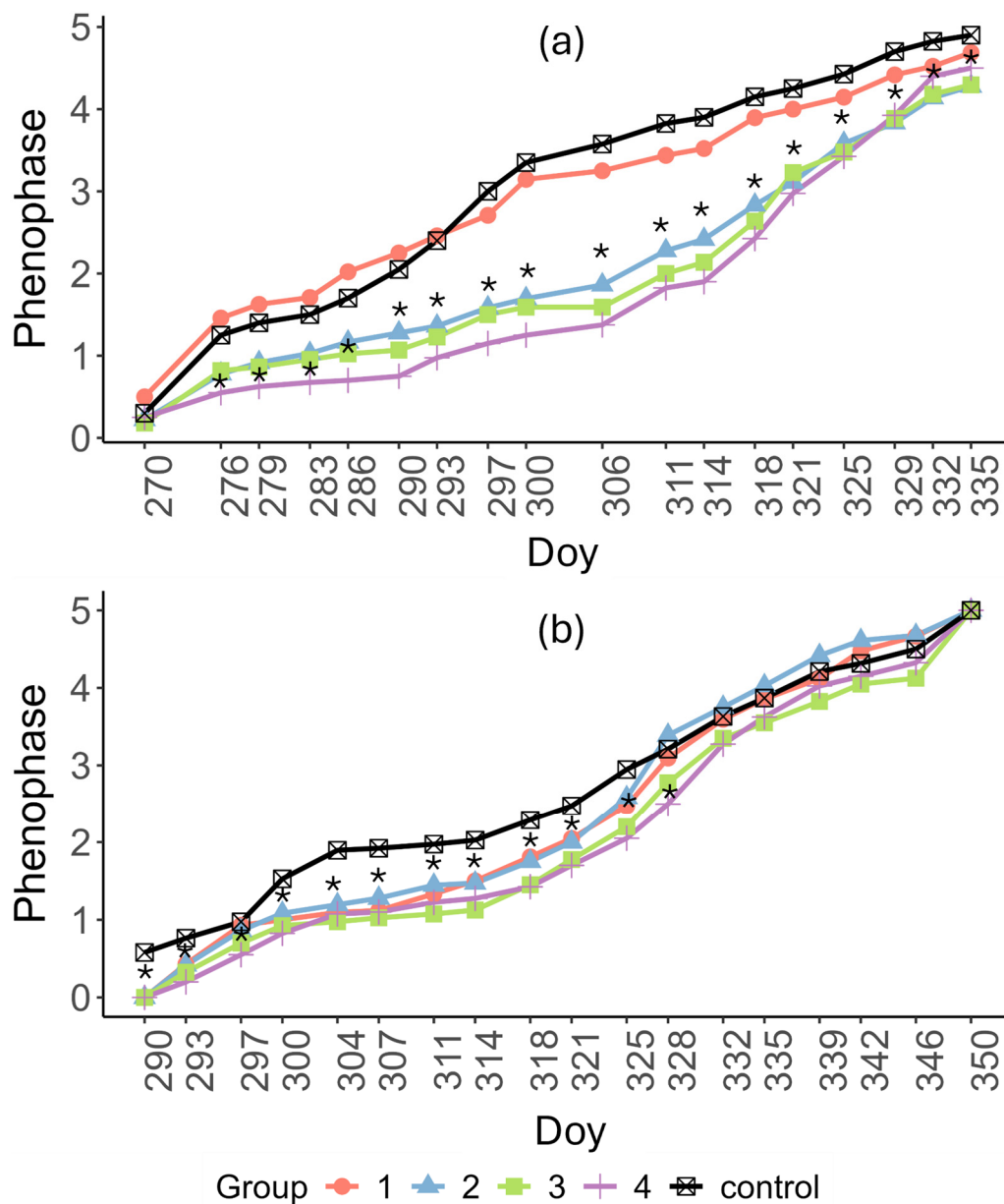


Figure 4. Autumn leaf phenology dynamics of pedunculate oak seedlings in 2022 (a) and 2023 (b) categorized by group. The y-axis indicates the Phenophase and the x-axis represents the day of the year (Doy). Dots correspond to the average phenological phase of a group at each Doy. An asterisk indicates a statistically significant difference between the control group and the groups below the asterisk symbol at Doy ($p < 0.05$).

3.3.2. Spring Leaf Phenology

Spring leaf phenology dynamics over both years showed no significant differences between the treated groups and the control group, indicating the absence of drought stress effect on spring foliation (Figure 5).

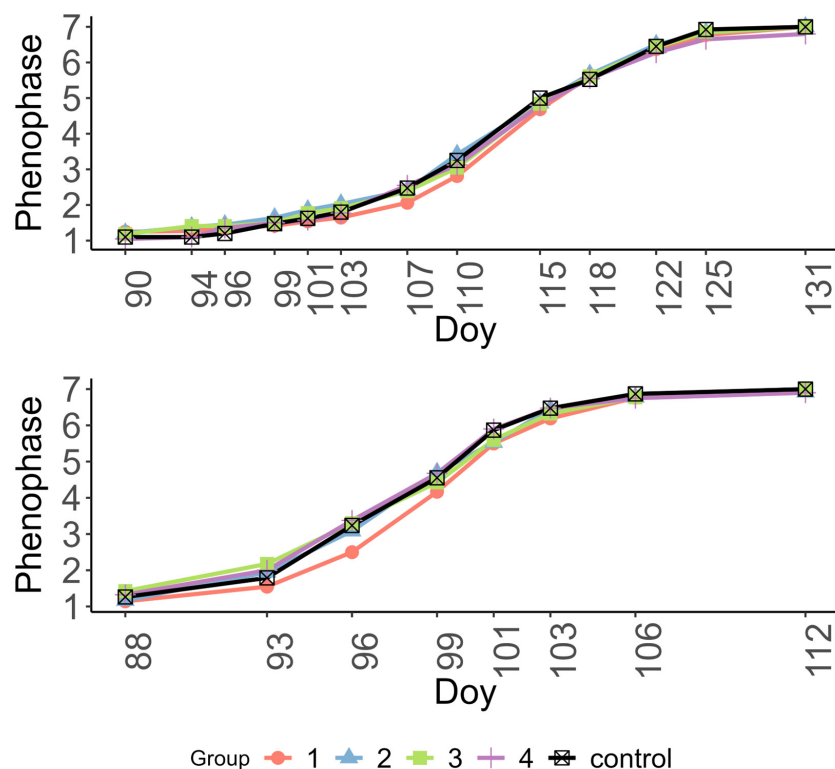


Figure 5. Spring leaf phenology dynamics of pedunculate oak seedlings in 2023 (upper plot) and in 2024 (lower plot), categorized by group.

3.4. Net Photosynthesis

By monitoring changes in seasonal net photosynthesis (A_{net}), it is clear that all groups showed a decline in A_{net} values when the majority of plants within a group displayed symptoms of drought stress. This was followed by A_{net} recovery after rehydration. However, none of the groups attained a higher mean A_{net} value than the control during the recovery phase (Figure 6).

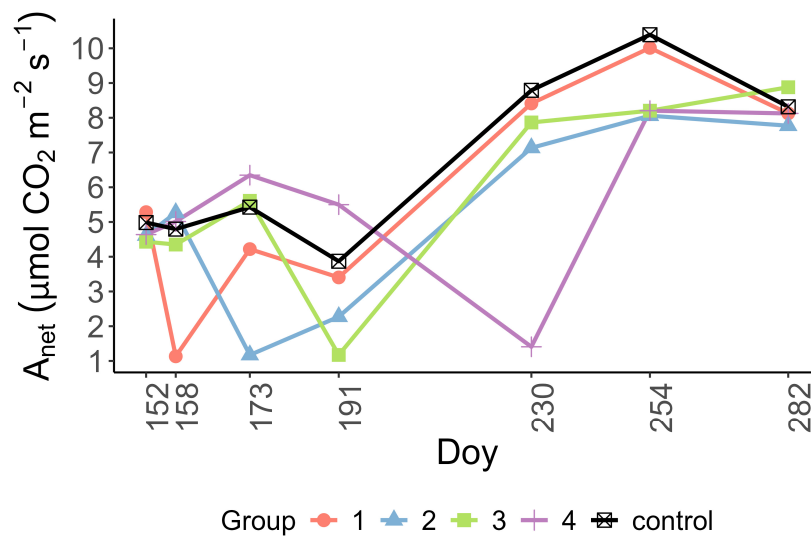


Figure 6. Seasonal change of A_{net} (net photosynthesis) in control group and drought-exposed groups in 2023.

4. Discussion

4.1. Height Increment

Height growth was significantly reduced in Groups 2, 3, and 4, which experienced drought relatively later in the growing season (in late spring and summer, see Figure 3). In contrast, Group 1, exposed to early-season (i.e., mid-spring) drought, showed no significant deviation from the control. These findings confirm that the timing of drought exposure critically influences growth responses.

The ability of Group 1 seedlings to maintain height growth despite early-season drought can be attributed to the species' multiple flushing behavior [36,37]. Similar compensatory growth has been observed in pedunculate oak under short-term drought stress in spring or early summer, as reported by Turcsán et al. [38].

Drought generally has a negative effect on height growth and shoot development, especially if the drought periods are prolonged. However, rewatering enables a strong compensatory effect [20]. Compensatory growth may result from increased assimilation above levels observed prior to drought stress [39], as shown in two studies on European beech [25,26]. In addition to the timing of drought stress induction, the intensity of drought stress is also an important factor. Severe drought stress has been shown to reduce biomass production and non-structural carbohydrate (NSC) concentration in Scots pine (*Pinus sylvestris* L.) [40], whereas moderate drought stress resulted in a greater number of flushes and increased growth compared to well-watered Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings [41]. The stimulating effect of mild drought stress on growth in *Crataegus monogyna* Jacq. was highlighted by Mijnsbrugge et al. [42].

The applied irrigation method may have contributed to suboptimal nutrient availability. Prolonged substrate saturation over both years could have caused nutrient leaching and reduced aeration, potentially affecting growth outcomes. In permeable soils, like the substrate used in this experiment, high moisture levels and water flow during irrigation can leach electrolytes and nutrients from the soil [43]. Excessive water and reduced nutrient availability have been shown to decrease biomass production in Scots pine [40], while reduced root-zone aeration inhibits gas exchange and the absorption of water and minerals in white spruce [44]. Conversely, high nutrient availability positively impacts photosynthesis and stomatal conductivity [45,46]. Height growth increased significantly with soil nutrient availability for Sitka spruce (*Picea sitchensis* (Bong.) Carrière) and Norway spruce (*Picea abies* (L.) H. Karst.) [47]. This experiment likely involved the interaction of drought and poor nutrition, and it is known that this kind of interaction results in reduced height growth [48]. This could explain why the first group did not surpass the control group, although it restored its height growth dynamics compared to the control.

Groups 2, 3, and 4 experienced droughts from late spring to late summer, as well as high temperatures typical of this time of year. It is well known that rising daily air temperatures intensify the negative effects of drought stress, significantly affecting net photosynthesis [49]. Additionally, these groups likely experienced nutrient deficiencies, which further hindered their post-drought recovery. Limited nitrogen availability has been shown to reduce photosynthetic rates and increase carbon allocation to the roots in birch (*Betula pendula* Roth) [50]. Similarly, reduced height and diameter growth were observed under suboptimal nutrient supply in European beech (*Fagus sylvatica* L.), with nitrogen deficiency further influencing resource allocation and root system growth [51].

A known mechanism of response to drought stress involves trees prioritizing carbon allocation to below-ground systems (roots) to restore root functionality, thereby enhancing water and nutrient uptake from deeper soil layers [52]. By prioritizing resources such as carbohydrates and nutrients towards root development rather than stems or leaves, plants reduce height growth and aboveground biomass. While this strategy enables their

physiologically active fine roots to explore a larger volume of soil to meet nitrogen (N) requirements, it comes at the expense of the aboveground biomass and vertical growth [53].

Under such conditions of drought and suboptimal nutrition, our experimental plants likely allocated resources to below-ground sinks, adversely affecting aboveground growth. In addition to nutrient allocation to roots, the interactive stress factors likely reduced assimilation, hindering positive contributions to height growth. On the other hand, the better compensatory growth observed in the first group during the post-drought recovery could be attributed to less severe drought stress in the first year of the experiment (Figure 2a) and more favorable temperature conditions during its recovery phase.

4.2. Autumn Leaf Senescence

In 2022, all drought-treated groups except Group 1 exhibited delayed and prolonged autumn senescence (Figure 4a). This aligns with findings in sessile oak, where intense drought followed by rehydration postponed leaf senescence, likely as a compensatory mechanism for water-deficit-induced losses [21]. Similarly, Čehulić et al. [22] observed that drought conditions induced a delayed onset of autumn leaf senescence in pedunculate oak. Apart from oak species, Arend et al. [25] linked the photosynthetic response to drought with delayed senescence in European beech. Likewise, Bačurin et al. [23] reported delayed senescence in goat willow exposed to drought at various stages of the growing season.

Group 1 exhibited slightly slower senescence than the control, though the difference was not statistically significant, possibly due to lower drought stress intensity (Figure 2a). The leaves of Group 1 were more delicate, and drought treatment was halted upon initial wilting symptoms to prevent mortality. Consequently, leaf damage was less severe than in other groups. Conversely, the other treated groups had tougher leaves at the start of their treatment. Consequently, plants in these groups developed more severe stress symptoms (yellowing and necrosis). The recovery of physiological processes after drought is more effective when tissue damage is minimal [31], which could explain why the first group did not exhibit delayed leaf senescence. This observation is supported by studies on *Cornus sanguinea* L., where plants with less leaf damage from drought stress did not show delayed senescence, unlike those with greater damage [54].

Luo et al. [55] hypothesized that a specific starch concentration threshold in leaves might trigger senescence. It can be assumed that the plants in the first group, due to their less damaged leaf tissue, were able to recover and accumulate sufficient non-structural carbohydrate (NSC) reserves, at least to levels comparable to the control plants. This combination of conditions may explain why the autumn senescence process in the first group did not significantly differ from that of the control plants.

In 2023, all groups exhibited a statistically significant delay in autumn leaf senescence (Figure 4b). In this year, even the first group experienced higher levels of drought stress (Figure 2b), leading to greater leaf mass damage. This supports the earlier assumptions regarding the different behavior of the first group in the previous year. A consistent pattern emerged in the extent of autumn phenology shift depending on the timing of drought stress induction. Plants exposed to drought stress later in the growing season exhibited greater delays in leaf senescence compared to the control. This suggests that the intensity of the senescence response may vary depending on the timing of drought stress induction.

4.3. Spring Leaf Phenology

Contrary to expectations, no shifts in leaf flushing were observed despite evidence that drought stress influences flushing dynamics in oak and other deciduous species. For example, in pedunculate oak, drought stress initially delayed budburst but intriguingly advanced it in the following year [22]. Similarly, Mediterranean oaks such as *Quercus ilex* L.

and *Quercus coccifera* L. showed accelerated budburst under drought conditions [56]. In beech (*Fagus sylvatica* L.), drought-stressed plants also exhibited earlier budbursts in spring following the stress event [57].

Findings by Vander Mijnsbrugge et al. [21] further demonstrated that drought conditions in the previous growing season delayed budburst in sessile oak. Additionally, the timing of drought stress induction caused varying phenological responses in goat willow clones, ranging from advanced to delayed leafing [23].

These findings suggest that spring phenology responses to drought are influenced by stress experienced in the previous growing season, indicating a form of “memory”. This “stress imprint” involves epigenetic and biochemical modifications triggered by previous stress exposure, affecting a plant’s physiological and phenological responses and potentially enhancing resilience to future stressors [58]. These responses are often described as “after-effects” [59,60] or “carry-over effects” [61]. However, the mechanisms driving these effects, whether epigenetic or physiological, remain unresolved. Additionally, why drought stress leads to opposing shifts (advanced or delayed) in spring phenology across studies remains unclear.

The lack of shifts in flushing observed in our study may be due to nutrient deficiencies caused by nutrient leaching and/or poor soil aeration resulting from the irrigation method, as discussed in Section 4.1. Nutrient imbalances can negatively impact photosynthesis and carbohydrate reserve accumulation, disrupting the leafing process. Unintended stress in control plants may have masked differences in spring phenology between groups.

Further evidence for the interaction between suboptimal nutrition and drought in this experiment comes from net photosynthesis measurements (Figure 6). None of the drought-stressed groups achieved higher A_{net} values than the control, contrasting with findings from other studies. For instance, in European beech, net photosynthesis remained high until late summer in previously drought-stressed trees, while it significantly declined in control plants [25].

Thus, the interactive effects of drought and low nutrient availability likely explain the absence of the expected after-effects on spring phenology in our study. Consequently, the hypothesis that the timing of drought stress induction variously influences spring phenology in the subsequent growing season cannot be rejected. On the contrary, future research should investigate this hypothesis (H3, see Section 1) with greater precision.

4.4. Reflection on Hypotheses and Research Implications

Based on the obtained results, we can evaluate the validity of the proposed hypotheses and highlight key insights derived from this research. We expected that the timing of drought stress would have differential effects on height growth and phenology in pedunculate oak, particularly in relation to compensatory growth mechanisms, seasonal leaf dynamics, and post-drought recovery.

Our results support the hypothesis that the timing of drought stress does not uniformly affect height growth. Seedlings exposed to early-season drought (mid-spring) demonstrated compensatory growth after rehydration, ultimately reaching height increments similar to those of the control group. However, seedlings that experienced drought relatively later in the season (late spring and summer) exhibited significantly reduced height growth, indicating that drought at this stage severely limits recovery potential. This suggests that *Q. robur* possesses an ability to mitigate early-season water deficits but is less capable of compensating for late-season drought, likely due to resource depletion and developmental constraints.

Similarly, the hypothesis that drought stress followed by rehydration leads to delayed autumn senescence was confirmed. Seedlings subjected to drought stress later in the season exhibited a pronounced delay in autumn leaf senescence compared to the control group, likely as a compensatory strategy to extend the growing season and optimize carbon assimilation after periods of water deficit. Notably, the intensity of this delay was greater in groups exposed to later drought events, further emphasizing the role of drought timing in shaping leaf phenology.

Contrary to expectations, no significant differences in spring flushing phenology were observed between drought-treated groups and the control, which does not support the hypothesis that the timing of drought stress influences spring phenology in the following year. One possible explanation for this discrepancy is that nutrient availability played a moderating role, potentially limiting the accumulation of non-structural carbohydrates (NSC) necessary for a pronounced carry-over effect.

Similarly, the assumption that enhanced photosynthetic activity during post-drought recovery would lead to shifts in spring leafing was not supported. None of the drought-stressed groups exhibited increased photosynthesis compared to the control after rehydration.

Future research should focus on exploring the long-term impacts of repeated drought events on *Q. robur*, particularly in relation to non-structural carbohydrate dynamics, root-to-shoot allocation strategies, and epigenetic responses. Additionally, investigating the interplay between drought stress and nutrient availability could provide valuable insights into the mechanisms governing tree resilience under climate change scenarios.

5. Conclusions

This study highlights the significance of drought timing in determining growth and phenological responses in pedunculate oak seedlings. Late-spring and summer drought was particularly detrimental to height growth and caused greater delays in autumn leaf phenology, whereas mid-spring drought allowed partial compensation. The absence of anticipated shifts in spring flushing suggests complex interactions between drought timing, nutrient availability, and physiological recovery processes. These findings underscore the need for further research on drought recovery mechanisms to inform adaptive forest management strategies in the face of climate change.

Author Contributions: Conceptualization, S.B. and M.B.; methodology, S.B. and M.B.; validation, S.B. and M.B.; formal analysis, M.B.; investigation, M.B., I.K.B. and S.B.; resources, M.B. and S.B.; data curation, M.B. and S.B.; writing—original draft preparation, M.B.; writing—review and editing, I.K.B., S.B. and K.S.; visualization, M.B.; supervision, S.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Article

Species- and Provenance-Specific Leaf Phenological Responses to Drought and Elevated Phosphorus in *Fagus sylvatica* and *Quercus petraea*

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Abstract

Leaf phenology is a crucial functional trait in temperate forest trees that integrates environmental signals and reflects species' adaptive capacity to stress. This study examined how moderate drought and elevated phosphorus availability, alone and in combination, affect the spring and autumn phenology of juvenile *Fagus sylvatica* and *Quercus petraea* saplings from two climatically distinct Croatian provenances. In a common garden experiment, saplings were subjected to four treatments involving drought and phosphorus addition. Phenological stages were scored using standardized ordinal scales across two growing seasons. Results revealed that phosphorus consistently advanced autumn leaf senescence in both species, independent of drought, while drought effects were species- and provenance-specific. Spring phenology was more sensitive to drought: beech from the drier provenance advanced budburst, suggesting an escape strategy, whereas oak delayed leaf-out under the same conditions. Notably, combined drought and phosphorus treatments often neutralized individual effects, indicating physiological compensation. Provenance-level differences highlighted contrasting strategies—phenotypic plasticity versus stress tolerance—under multi-stressor conditions. These findings underscore the dominant role of phosphorus in regulating phenology and the complex, non-additive nature of drought–nutrient interactions, emphasizing the need for integrative approaches in predicting phenological responses under climate change.

Keywords: abiotic stress; deciduous tree species; nutrient–water interaction; phenological plasticity; spring and autumn phenology



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1. Introduction

In recent decades, global climate change has amplified the intensity and frequency of abiotic stressors such as drought, posing critical challenges to the stability, productivity, and regeneration of temperate forest ecosystems [1]. Altered precipitation regimes and rising temperatures intensify drought events while simultaneously disturbing nutrient stoichiometry in forest soils, particularly the balance between nitrogen and phosphorus [2,3]. Leaf phenology—the seasonal timing of leaf development and senescence—has therefore emerged as a key functional trait of temperate forest trees, integrating environmental cues and mediating tree performance under changing conditions [4].

The timing of phenological events is traditionally governed by photoperiod and temperature [5], yet additional environmental drivers—especially water and nutrient

availability—can substantially modulate developmental transitions [6,7]. Among nutrients, phosphorus (P) is increasingly recognized as a central regulator of phenological timing. Beyond its role in metabolism and growth, P influences hormonal pathways and gene expression, including senescence-associated genes, and can shape chromatin-level control through SPX-domain signaling [8,9]. Empirical work shows that higher P availability can accelerate vegetative development and advance the onset of senescence by enabling earlier completion of developmental phases [6]. However, the extent to which P-driven phenological shifts are modulated by co-occurring drought stress remains poorly resolved. Some studies indicate that P may buffer drought impacts by sustaining metabolic function [10], whereas others report antagonistic or context-dependent outcomes [11].

Drought itself exerts complex—and sometimes opposing—effects on phenology, advancing or delaying developmental phases depending on species identity, stress timing, and severity [12–15]. Despite this, most studies still consider spring and autumn phenology separately, rather than as components of an integrated seasonal cycle [16,17]. This separation hampers our ability to understand transitional dynamics linking leaf flushing and senescence and to predict whole-season responses under multiple stressors. In addition to interspecific variation, local adaptation contributes to divergent phenological strategies among provenances: geographic origin reflects climatic history and ecological filtering, shaping phenotypic plasticity and stress tolerance [18,19]. Yet, provenance-based phenological responses under combined drought and nutrient stress, particularly during juvenile stages, remain underexplored.

To address these gaps, we investigated how elevated phosphorus availability and moderate drought—alone and in combination—affect the spring and autumn leaf phenology of juvenile *Fagus sylvatica* and *Quercus petraea* from two ecologically contrasting Croatian provenances in a common-garden experiment. Based on the background above, we hypothesized that: (i) elevated P generally accelerates phenological development, leading to earlier transitions in both spring and autumn [6,8,9]; (ii) drought modifies leaf phenology in a species- and provenance-dependent manner, reflecting divergent adaptive strategies shaped by climatic origin [12–15,18,19]; and (iii) combined drought–phosphorus effects are non-additive (interactive), potentially buffering or amplifying single-stressor impacts rather than summing linearly [10,11].

We tested these hypotheses using repeated phenological scoring across two growing seasons, enabling an integrated assessment of multi-stressor influences on the full seasonal cycle. Our findings contribute to a better understanding of functional adaptation in temperate tree species and offer insights for climate-resilient forest management.

2. Materials and Methods

2.1. Plant Material and Provenance Sites

This study was conducted on naturally regenerated four-year-old saplings of common beech (*Fagus sylvatica* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) originating from two mature, mixed forest stands located in the continental region of Croatia. The age of the saplings (approximately four years) was determined by counting annual growth rings on a subsample of 25 individuals per each species. The first provenance was located near Karlovac (KA) in the northwest (45.466° N, 15.522° E; 170–185 m a.s.l.), characterized by deeper soils, north–northeast exposure, and higher precipitation. The second provenance was near Slavonski Brod (SB) in the east (45.273° N, 17.973° E; 230–255 m a.s.l.), with shallower soils, south–southeast exposure, and drier conditions.

In March 2021, saplings were collected from both stands beneath 50 mature mother trees (25 per species). A total of 640 saplings were excavated (160 per species per site),

ensuring minimal root damage and even spatial distribution to reduce genetic relatedness. Saplings were temporarily stored in moist sand and shade before being transplanted.

Climatic classification at both sites was humid temperate (Cfwbx'' and Cfwb''x) per Köppen. Mean annual precipitation (1949–2019) was higher at KA (≈ 1112 mm) than SB (≈ 770 mm). Between 2016 and 2020, KA experienced more drought months (17 total, 9 during growing season) than SB (9 total, 4 during growing season). Mean growing season temperature was 18.8 °C, relative humidity 66.7%, and solar radiation 7.6 h/day [20].

2.2. Experimental Design

The experiment was established in mid-March 2021 at the Faculty of Forestry and Wood Technology, University of Zagreb (45.821° N, 16.023° E; 120 m a.s.l.) as a common garden trial. Saplings were planted into four large wooden boxes ($155 \times 275 \times 80$ cm; 3.41 m³ each), filled with commercial Klasmann-Deilmann TS 3 substrate (initially containing 160 mg/L P₂O₅).

Each box contained 100 saplings—25 of each species from each provenance—randomly arranged at 20×18 cm spacing. Two boxes received phosphorus fertilization (1182 g of Triplex, 45% P₂O₅) to raise P₂O₅ levels to 300 mg/L (+P treatment), while two boxes remained unfertilized –P treatment).

During 2021, saplings were exposed to natural conditions with regular watering. In 2022, all boxes were covered with a transparent PVC roof to exclude rainfall, and four treatments were applied:

+PW: elevated phosphorus + regular watering (40 L every 4 days).

Control: no phosphorus + regular watering.

+PD: elevated Phosphorus + drought (minimal watering triggered by visible wilting).

–PD: no phosphorus + drought.

2.3. Leaf Phenology Scoring

Autumn leaf phenological phases were scored twice a week in 2022 using a 0–5 ordinal scale: 0—leaves completely green with no visible discoloration; 1—up to 25% of plant leaves show discoloration; 2—up to 50% of plant leaves show discoloration; 3—more than 50% of plant leaves show discoloration; 4—more than 75% of plant leaves show discoloration; and 5—leaves shed.

Spring leaf phenology was scored twice a week (during the process in 2023) on all plants in the trial using a 1–7 ordinal scale: 1—bud scales completely closed, dormant buds; 2—buds swelling, scales slightly spaced; 3—bud burst, green leaf tips visible; 4—folded leaves visible. 5—leaves unfolding but not yet flattened, small. 6—leaves still relatively small but with flattened blades, blade edges bent downward, withered, lighter green or reddish; and 7—leaves appear developed, larger but more tender than fully developed leaves and lighter green or reddish.

All phenological scorings were performed by one experienced person. In this study, the term phenophase refers to discrete ordinal stages of leaf development or senescence, scored on a predefined scale. The phenological phase scores (i.e., phenophases) served as input data for subsequent statistical analyses.

2.4. Statistical Analyses

All statistical analyses and visualizations were performed in R (version 4.5.1; R Development Core Team, 2025). Data processing relied on the tidyverse (version 2.0.0) and dplyr (version 1.1.4) packages. Assumptions of normality and homogeneity of variance were evaluated using the Shapiro–Wilk and Levene's tests implemented in rstatix

(version 0.7.2). Data visualization was conducted using ggplot2 (version 3.5.1) and ggstatplot (version 0.12.3).

Statistical analyses examined the effects of treatment (+PW, -PD, +PD, control), provenance, and species during two observation seasons (autumn 2022 and spring 2023). To assess multivariate phenological responses, we applied a Permutational Multivariate Analysis of Variance (PERMANOVA) using the *adonis2* function from the *vegan* package (version 2.7.1), with Bray–Curtis dissimilarity and 999 permutations. Analyses were conducted separately for each season (autumn 2022 and spring 2023). The initial model included the main effects (Species, Provenance, Treatment) and all possible two- and three-way interactions. In subsequent models, provenance was nested within species (Species + Species:Provenance + Treatment) to reflect the hierarchical structure of the experimental design.

The same saplings were monitored repeatedly across calendar dates within each season. To account for non-independence, permutations in PERMANOVA were constrained within individuals using the *strata = TreeID* option, ensuring shuffling only among units sharing the same subject identifier. This yields valid permutation *p*-values in a repeated-measures design while preserving full multivariate phenology trajectories.

For univariate, date-wise comparisons of ordinal phenophase scores among treatments, analyses were performed separately for each season, and independently within each species and provenance. Each observation date was analyzed as a cross-section using the Kruskal–Wallis test, followed by Dunn’s post hoc with Benjamini–Hochberg FDR correction. Treating each date as a cross-section avoids violating independence across repeated dates (i.e., no across-date pooling of the same subject within the same test). Differences between treatments were considered statistically significant when $p < 0.05$ on at least two calendar dates.

3. Results

3.1. Autumn Phenology

The PERMANOVA analysis of autumn phenological data revealed that both species and treatment had statistically significant effects on leaf phenology (Table 1). The effect of species was highly significant ($p = 0.001$), explaining 6.3% of the total phenological variation. Likewise, the treatment effect was also highly significant ($p = 0.001$), accounting for 11.7% of the observed variation. This indicates that drought and phosphorus treatments significantly influenced autumn leaf phenological patterns.

Table 1. PERMANOVA Results—Autumn 2022.

Effect	Df	Sum of Squares	R ²	F	<i>p</i> -Value
Species	1	0.5983	0.06291	15.4033	0.001
Provenance	1	0.1058	0.01112	2.7238	0.069
Treatment	3	1.1115	0.11687	9.5392	0.001
Species × Provenance	1	0.0050	0.00053	0.1294	0.921
Species × Treatment	3	0.0766	0.00805	0.6570	0.617
Provenance × Treatment	3	0.1884	0.01981	1.6170	0.160
Species × Provenance × Treatment	3	0.2008	0.02111	1.7232	0.117
Residual	186	7.2242	0.75960		
Total	201	9.5105	1.00000		

Note: Results are based on PERMANOVA analysis (Bray–Curtis distance, 999 permutations). Significance of effect is indicated with red bold *p*-value.

The effect of provenance, although not statistically significant at the conventional 0.05 level ($p = 0.069$), explained an additional 1.1% of the variation and may represent a biologically relevant source of variability.

All interaction terms—including Species \times Provenance, Species \times Treatment, Provenance \times Treatment, and the three-way interaction—were statistically non-significant ($p > 0.1$), suggesting that the effects of treatment and species on autumn phenology were generally additive and consistent across provenances.

The residual variation remained high (75.96%), reflecting expected biological variability and potentially unmeasured environmental factors.

3.1.1. The Treatment Effects on Autumn Phenology in European Beech

Differences in the timing and progression of autumn leaf senescence between drought-exposed and control European beech saplings were not statistically significant (Figure 1). However, provenance SB showed a slight advancement, entering phenophase 3 (>50% discolored leaves) approximately two days earlier under drought conditions compared to the control (Figure 1a). In contrast, leaf senescence dynamics for provenance KA saplings subjected to drought closely matched those of the control (Figure 1b). Thus, it can be concluded that drought had a negligible effect on autumn leaf senescence in European beech saplings, irrespective of provenance.

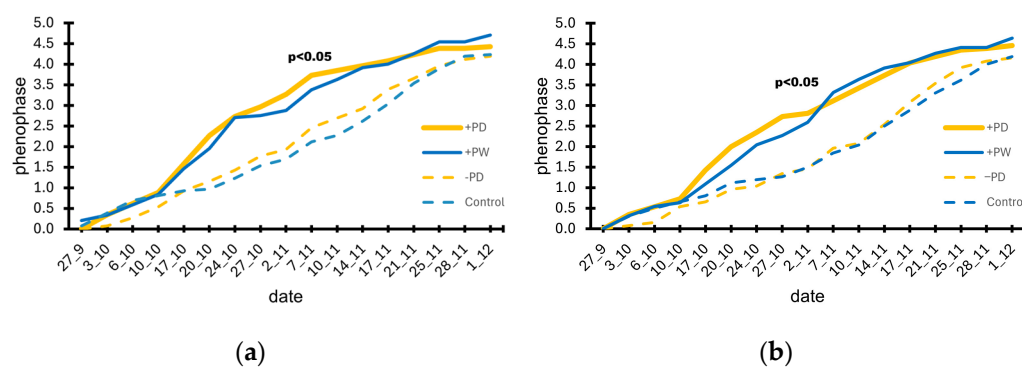


Figure 1. Progression of autumn leaf senescence phenology in European beech saplings exposed to drought (−PD), elevated phosphorus (+PW), combined drought and elevated phosphorus (+PD), and control conditions: (a) provenance SB; (b) provenance KA. Significant differences ($p < 0.05$) indicate that leaf senescence occurred significantly earlier in the +PD and +PW treatments compared to the −PD treatment and control.

Saplings of European beech exposed to combined elevated soil phosphorus and drought conditions (+PD treatment, Figure 1) showed significantly earlier leaf senescence compared to the control in both provenances. The onset of phenophase 3 (>50% discolored leaves) occurred on average 14 and 19 days earlier (provenances KA and SB, respectively). However, the +PD treatment did not significantly differ from the elevated phosphorus alone (+PW treatment). Thus, the interaction effect between drought and elevated phosphorus was not confirmed, indicating that elevated phosphorus predominantly influenced autumn leaf senescence in European beech independently of drought.

3.1.2. The Treatment Effects on Autumn Phenology in Sessile Oak

Differences in the dynamics of autumn leaf senescence between sessile oak saplings exposed to drought and control saplings were also not statistically significant (Figure 2). Similarly to European beech, saplings of provenance SB exhibited slightly earlier leaf senescence under drought conditions, with phenophase 3 occurring on average 2–3 days earlier compared to control saplings (Figure 2a); however, this difference was not significant.

Saplings from provenance KA showed an even less pronounced response to drought (Figure 2b). Overall, drought had a negligible effect on autumn leaf senescence in sessile oak saplings, irrespective of provenance.

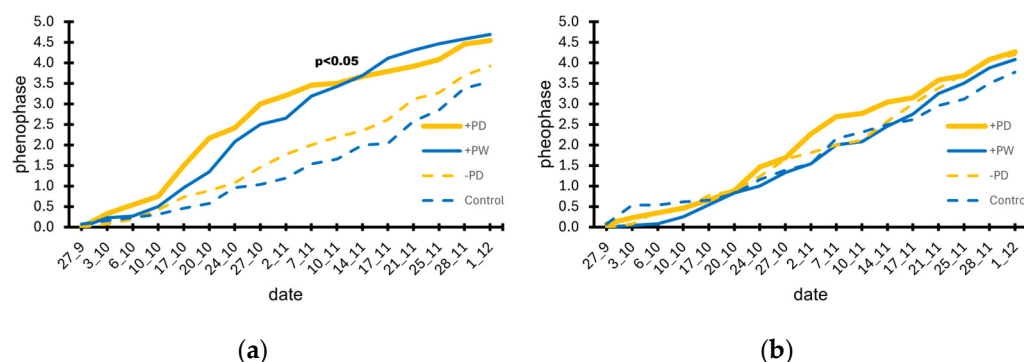


Figure 2. Progression of autumn leaf senescence phenology in sessile oak saplings subjected to drought (−PD), elevated phosphorus (+PW), combined drought and elevated phosphorus (+PD), and control conditions: (a) provenance SB; (b) provenance KA. Significant differences ($p < 0.05$) indicate earlier leaf senescence in the +PD and +PW treatments compared to the −PD treatment and control.

Similarly to European beech, saplings of sessile oak from provenance SB exposed to combined elevated soil phosphorus and drought (+PD treatment, Figure 1a) showed significantly earlier leaf senescence compared to the control. Phenophase 3 occurred on average 29 days earlier in the +PD treatment than in the control (Figure 1a). However, no interaction effect was observed here either; instead, elevated phosphorus predominantly influenced autumn leaf senescence since no significant differences were found between +PD and elevated phosphorus alone (+PW treatment). Conversely, provenance KA showed no significant shifts in autumn leaf senescence timing in either elevated phosphorus (+PW) or combined phosphorus and drought (+PD) treatments. Although phenophase 3 occurred on average 7 days earlier in the +PD treatment, variations in the timing of other phases and within-treatment variability resulted in no statistically significant differences.

3.2. Spring Phenology

The PERMANOVA analysis of spring phenological data showed that species had a dominant and statistically significant effect on leaf phenology ($p = 0.001$), explaining 33.7% of the total multivariate variation (Table 2). This highlights a substantial divergence in spring phenological patterns between *Fagus sylvatica* and *Quercus petraea*.

The effect of treatment was also statistically significant ($p = 0.005$), contributing 3.7% of the phenological variation. This confirms that drought and phosphorus treatments continued to exert measurable influence on spring leaf phenology, albeit to a lesser extent than in autumn.

By contrast, the effects of provenance and all interaction terms (Species \times Provenance, Species \times Treatment, Provenance \times Treatment, and the three-way interaction) were statistically non-significant ($p > 0.1$). The three-way interaction (Species \times Provenance \times Treatment) was marginally non-significant ($p = 0.099$), explaining 1.9% of the variation, and may suggest subtle combined effects worth further investigation.

Overall, a large portion of variation (58.7%) remained unexplained (residual), likely reflecting individual variability and environmental noise.

Table 2. PERMANOVA Results—Spring 2023.

Effect	Df	Sum of Squares	R ²	F	<i>p</i> -Value
Species	1	0.88437	0.33749	110.3181	0.001
Provenance	1	0.00420	0.00160	0.5245	0.489
Treatment	3	0.09735	0.03715	4.0479	0.005
Species × Provenance	1	0.00373	0.00143	0.4658	0.519
Species × Treatment	3	0.01985	0.00757	0.8253	0.527
Provenance × Treatment	3	0.02154	0.00822	0.8956	0.435
Species × Provenance × Treatment	3	0.05020	0.01916	2.0875	0.099
Residual	192	1.53918	0.58738		
Total	207	2.62043	1.00000		

Note: Results are based on PERMANOVA analysis (Bray–Curtis distance, 999 permutations). Significance of effect is indicated with red bold *p*-value.

3.2.1. The Treatment Effects on Spring Phenology in European Beech

The effect of drought on leaf unfolding in European beech saplings was statistically significant for provenance SB (Figure 3a) but not for provenance KA (Figure 3b). In drought-exposed saplings of provenance SB, phenophase 3 (budburst) occurred on average about 5 days earlier compared to control saplings, and subsequent leaf-unfolding phases also started earlier. Saplings of provenance KA subjected to drought exhibited slightly earlier bud swelling (phenophase 2), but differences in subsequent phenophases compared to the control were negligible. Thus, drought significantly accelerated the spring phenology of leaf unfolding only in saplings of provenance SB.

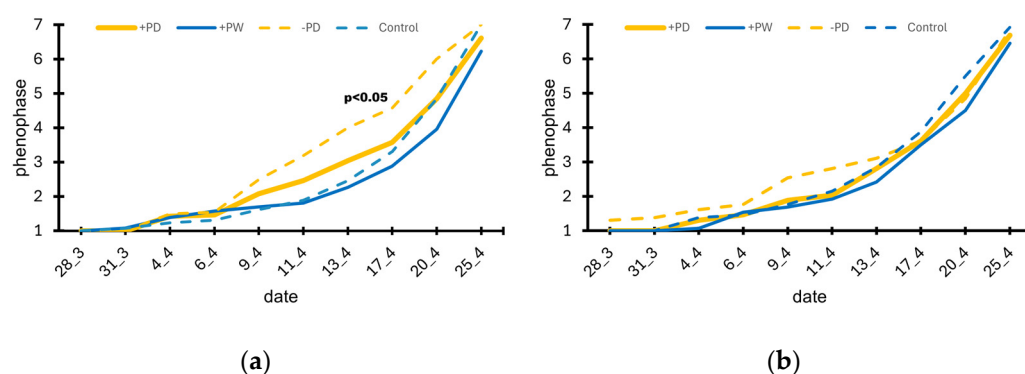


Figure 3. Progression of spring leaf unfolding phenology in European beech saplings subjected to drought (−PD), elevated phosphorus (+PW), combined drought and elevated phosphorus (+PD), and control conditions: (a) provenance SB; (b) provenance KA. Significant differences ($p < 0.05$) indicate earlier leaf unfolding in the drought treatment (−PD) compared to all other treatments.

The combined phosphorus and drought treatment (+PD) significantly differed from both drought alone (−PD) and elevated phosphorus alone (+PW) treatments, but not from the control in the provenance SB (Figure 3a). Therefore, the combined treatment neutralized the individual effects of drought and elevated phosphorus, indicating an interaction effect between drought and elevated phosphorus on leaf unfolding in this provenance. However, such an interaction effect was not observed in provenance KA, nor were there significant individual effects, as none of the treatments significantly differed from the control (Figure 3b).

3.2.2. The Treatment Effects on Spring Phenology in Sessile Oak

In sessile oak, results similar to those observed for European beech were recorded. In provenance SB, the combined phosphorus and drought treatment (+PD) significantly

differed from both drought alone (-PD) and elevated phosphorus alone (+PW) treatments, but not from the control (Figure 4a). This result again suggests an interaction effect between drought and elevated phosphorus on leaf unfolding, whereby the combined treatment neutralized the individual effects of drought and elevated phosphorus. It is important to note that both drought and elevated phosphorus individually significantly delayed leaf unfolding in provenance SB. In provenance KA, there were no significant differences among treatments, indicating the absence of individual or interaction effects. Nevertheless, a potential interaction effect is indicated by the similarity between the +PD treatment and control relative to the -PD treatment (Figure 4b). However, the lack of statistical significance precludes definitive conclusions regarding the interaction effect on leaf unfolding in provenance KA.

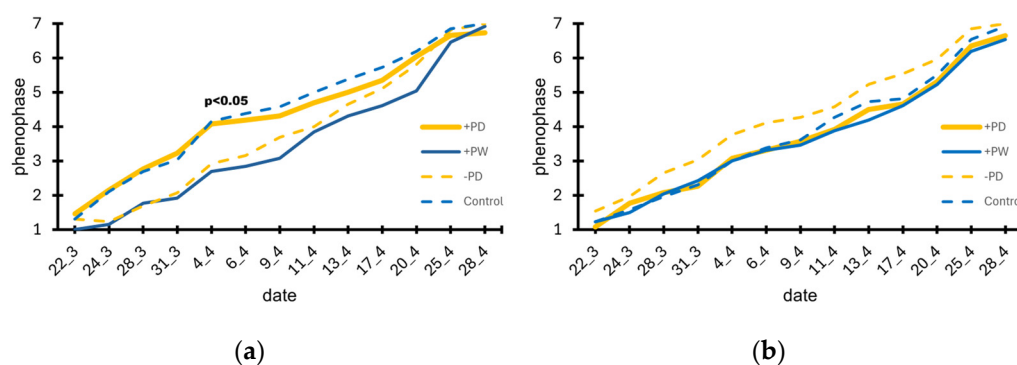


Figure 4. Progression of spring leaf unfolding phenology in sessile oak saplings subjected to drought (−PD), elevated phosphorus (+PW), combined drought and elevated phosphorus (+PD), and control conditions: (a) provenance SB; (b) provenance KA. Significant differences ($p < 0.05$) indicate delayed leaf unfolding in the −PD and +PW treatments compared to the +PD treatment and control.

Contrary to the response observed in European beech, drought significantly delayed leaf unfolding in sessile oak saplings of provenance SB (Figure 4a). On average, phenophase 3 occurred approximately 6 days later in drought-exposed saplings compared to the control, with delays also apparent in subsequent leaf-unfolding phenophases. Although provenance KA saplings exposed to drought exhibited slightly earlier leaf unfolding compared to the control, these differences were not statistically significant (Figure 4b). Notably, control saplings of the two provenances differed significantly from each other (compare control curves in Figure 4a,b), with provenance SB exhibiting earlier leaf unfolding than provenance KA. This provenance difference was not observed in European beech saplings.

4. Discussion

4.1. Autumn Phenology: Negligible Effects of Drought

The PERMANOVA analysis confirmed that both species identity and treatment significantly shaped autumn phenological variation, whereas provenance and interaction effects were weak (Table 1). This statistical outcome supports the interpretation that phosphorus availability, rather than drought or provenance, was the main driver of autumn senescence dynamics. Our experiment revealed negligible drought effects on autumn senescence in both species. Although minor advances were recorded, they were not statistically significant and indicate that the applied drought regime may not have reached thresholds necessary to trigger phenological shifts. Previous studies report highly context-dependent responses: *Fagus sylvatica* sometimes delays senescence after summer drought, especially when rewatering occurs [21], and similar patterns are found in *Quercus petraea* [22]. Other work shows delayed senescence in *Betula pendula* [11] or apple trees [23], while Bačurin et al. [7] observed drought-induced delays in *Q. robur*. Importantly, several studies em-

phasize that only moderate to severe drought during sensitive stages significantly alters senescence [11,21,23]. Thus, our results likely reflect that the moderate stress applied, though ecologically relevant, was insufficient to disrupt hormonal and metabolic cues governing leaf senescence. Although our findings suggest a limited impact of drought on autumn leaf senescence, it is important to recognize that leaf phenology does not fully capture the complexity of tree developmental dynamics. Studies on mature *F. sylvatica* and *Q. petraea* demonstrated that both extremely dry and extremely wet years significantly altered cambial activity, growth rate, and the timing of cambium cessation, even when leaf phenology showed only minor shifts [24]. These results indicate that photoperiod exerts a stronger control over leaf senescence than water availability, while wood formation processes remain more sensitive to climatic extremes. Consequently, leaf and cambium phenology can diverge, especially in oak, underscoring the need for integrative assessments that combine anatomical and foliar observations.

4.2. Dominant Influence of Elevated Phosphorus on Autumn Senescence

In contrast, elevated phosphorus consistently advanced autumn senescence in both species, particularly in SB provenance of *Q. petraea*. This supports growing evidence that phosphorus acts as a developmental signal beyond its metabolic role. Plants sense phosphorus via signaling cascades that regulate senescence-associated genes [8], while elevated P accelerates growth completion and resource reallocation [25]. At the molecular level, SPX-domain pathways and hormone interactions underpin earlier senescence [9]. The similarity of +PW and +PD treatments suggests phosphorus was the dominant driver, overriding drought effects, consistent with studies showing nutrient stoichiometry—especially N:P imbalance—can induce early senescence [26]. Our findings confirm phosphorus as a critical regulator of autumn phenology, with strong capacity to reshape developmental timing.

4.3. Spring Phenology: Contrasting Species Responses to Drought

In spring, PERMANOVA revealed a dominant species effect explaining over 30% of total variation, with additional but smaller contributions of treatments (Table 2). Provenance effects were not significant, highlighting that the contrasting drought responses observed between *F. sylvatica* and *Q. petraea* primarily reflect species-specific strategies rather than geographic origin. Spring phenology showed stronger drought sensitivity and clear interspecific divergence. In *F. sylvatica* (SB provenance), drought advanced budburst by several days, consistent with an “escape” strategy enabling early growth before summer droughts [27]. While potentially adaptive, this increases frost risk and ecological mismatches. In contrast, *Q. petraea* from the same provenance delayed budburst under drought, reflecting a conservative avoidance strategy typical for oaks with deeper rooting and stronger stomatal control [4,28]. Such divergence may stem from species-specific chilling and heat requirements [29] and different hormonal regulation, particularly abscisic acid [30]. Legacy effects from previous seasons may also shape responses [15]. KA provenance in both species showed minimal change, indicating lower phenological plasticity. These contrasting strategies highlight how temperature cues, water status, and hormonal pathways interact to shape spring development [31].

4.4. Interactive Effects of Drought and Phosphorus: Physiological Compensation

The combined drought + phosphorus treatment (+PD) frequently neutralized single-factor effects, resulting in phenological timing close to controls, especially in SB provenances. This suggests compensatory physiological mechanisms where phosphorus mitigates drought stress. Possible processes include improved ATP availability, enhanced root growth, and modulation of ABA signaling [32,33]. Phosphorus is increasingly recognized as a signal regulating development and nutrient balance [8,26], which may explain the ob-

served buffering. Such non-additive interactions are common in multi-stressor contexts [34] and underscore the need for models that integrate nutrient–water–signaling interactions to predict phenological shifts.

4.5. Provenance Differences: Role of Local Adaptation

Clear provenance differences underline the role of local adaptation. SB provenance exhibited greater phenological plasticity: drought advanced budburst in *F. sylvatica* but delayed it in *Q. petraea*. KA provenance, by contrast, remained phenologically stable. Physiological studies support these contrasting strategies: KA saplings display stronger antioxidant defenses [10] and greater biomass allocation to roots [20], consistent with stress tolerance and drought avoidance. Although KA appears wetter on an annual scale, its soils are shallow and drought-prone during growing seasons, favoring conservative phenotypes. SB's higher plasticity aligns with previous reports that southern provenances of beech show stronger responsiveness [18,19]. Together, SB represents a responsive, plastic strategy, while KA reflects conservative stability based on morphology and physiology. These alternative adaptive pathways resonate with ecological trade-offs between plasticity and tolerance [35,36].

5. Conclusions

This study demonstrates that phosphorus availability is a dominant regulator of leaf phenology in *Fagus sylvatica* and *Quercus petraea*, advancing autumn senescence and altering spring development regardless of drought. In contrast, drought effects were weaker and strongly species-specific, with *F. sylvatica* from drier origins showing accelerated budburst, while *Q. petraea* exhibited delayed flushing. Provenances further revealed contrasting adaptive strategies: the Slavonski Brod population expressed higher phenological plasticity, whereas the Karlovac provenance maintained stable phenological timing supported by physiological and morphological stress tolerance traits.

Most importantly, combined drought and phosphorus treatments frequently neutralized single-factor effects, highlighting the non-additive nature of multi-stressor interactions. These findings emphasize that predictions of climate-change impacts on forest phenology cannot rely on single-factor experiments, but must integrate nutrient and water dynamics together with intraspecific variation. From a management perspective, both plastic and conservative provenances provide complementary adaptive value. Their joint use in reforestation may enhance the resilience of European temperate forests under increasing climatic uncertainty.

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Supplementary material 5

Rootstock influence on intra-clonal variability in spring leaf phenology of pedunculate oak (*Quercus robur* L.)

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Abstract

This study aimed to investigate the influence of rootstock on intra-clonal variation in spring leaf phenology of pedunculate oak (*Quercus robur* L.) clones. In the context of clonal seed orchards, phenological synchrony is critical for successful pollination and seed production. While leaf phenology is largely under genetic control, increasing evidence suggests that rootstocks can influence scion phenology.

The experiment was conducted at the Brestje nursery using 43 pedunculate oak clones, each represented by three grafted ramets on genetically diverse seedling rootstocks. Phenological monitoring was carried out from 2010 to 2014, and intra-clonal differences in budburst timing (phenophase 3) were analyzed using bootstrap analysis. Additionally, the impact of a late spring frost in 2012 was assessed by comparing the timing of phenophase 4 between frost-damaged and frost-surviving ramets.

Results showed that, despite genetic uniformity, there was significant intra-clonal variability in budburst timing. In 2010, as many as 79.1% of clones exhibited a budburst range of ≥ 3 days between ramets, which can be considered a biologically meaningful threshold. The highest level of phenological synchrony was recorded in 2013. Notably, ramets that flushed later were less susceptible to frost damage, with the greatest observed difference between damaged and undamaged ramets being 21 days.

These findings highlight the critical role of rootstock in shaping scion phenology, particularly in the context of optimizing seed production and weather adaptability in clonal seed orchards.

Keywords: pedunculate oak, rootstock, leaf phenology, intra-clonal variation, frost resistance, clonal seed orchards

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INTRODUCTION

Pedunculate oak (*Quercus robur* L.) forest stands' regeneration can be achieved naturally through shelterwood cutting. However, in many situations—particularly where natural regeneration proves insufficient—artificial methods such as seed sowing or seedling planting become necessary. To ensure effective forest regeneration in cases requiring artificial methods, it is advisable to rely on high-quality reproductive material that meets genetic and ecological standards.

To enhance the genetic quality of forest reproductive material and increase the frequency and abundance of seed production, clonal seed orchards have been established in Croatia (Vidaković 1996). These orchards are established through heterovegetative propagation (grafting) of “plus trees”—phenotypically superior individuals selected from natural forest stands.

A critical goal in clonal seed orchards' management is to ensure a sufficient number of genotypically distinct clones for seed production, i.e. to maximize effective population size (Kramer et al. 2008). To facilitate successful pollination among these clones, phenological synchrony, the uniformity of developmental timing across clones, is of vital importance (Franjić et al. 2011).

It is well known that budburst is under strong genetic control and exhibits high heritability. This has been confirmed in various tree species, including pedunculate oak (Scoti-Saintagne et al. 2004), poplar (*Populus* spp.) (Frewen et al. 2000), and birch (*Betula* spp.) (Billington and Pelham 1991). Although it is strongly influenced by the genome, the primary environmental triggers for leaf phenological events are temperature and photoperiod. These factors determine the narrow time window during which environmental conditions are optimal for plant development (Robson et al. 2013, Basler and Korner 2014, Zohner and Renner 2015).

The timing and progression of spring leaf phenology, including the phases from budburst to full leaf expansion, significantly influence the length of the growing season and act as major drivers of ecological processes in temperate forests (Polgar and Primack 2011). Trees that set buds too early in the fall or flush too late in the spring may experience a shortened growing season, reducing both competitive ability and growth potential (Frewen et al. 2000).

Optimal phenological timing allows trees to avoid late spring frosts that can damage sensitive tissues and to extend the photosynthetically active period, thereby enhancing biomass production (Lockhart 1983, Leinonen and Hänninen 2002, Gömöry and Paule 2011). While temperature and photoperiod are considered the primary drivers of phenological development, a range of additional abiotic and biotic factors—known in the literature as atypical—can also exert significant influence on phenological traits (Bačurin et al. 2023). These include drought stress (Vander Mijnsbrugge et al. 2016, Bačurin et al. 2025), nutrient availability (Bačurin et al. 2023), and, in cases of severe insect infestation, phenological shifts in spring leaf development (Haukioja et al. 1988, Kaitaniemi et al. 1997).

In addition to the previously mentioned atypical factors, several studies have indicated that grafting can also lead to

rootstock-induced effects on the scion phenology (Young and Houser 1980, Durner and Goffreda 1992, Camisón et al. 2021). In forestry and horticulture, rootstocks are used to improve graft compatibility, regulate scion vigor, and enhance reproductive success (Jayawickrama et al. 1991). Studies in fruit species have shown that rootstocks can affect vegetative growth, fruit yield, as well as the phenological and physiological traits of the scion, including leaf and flower development (Wang et al. 1994; Jiménez et al. 2004, 2011; Tworcoski and Miller 2007; Neilsen et al. 2016). It has also been established that the use of specific rootstocks can improve drought tolerance (Tworcoski et al. 2016).

Although the influence of rootstocks on phenological traits is well known in fruit crops, their effects on scion phenology in forest tree species, such as pedunculate oak, remains insufficiently studied. Given the ecological and silvicultural importance of this species, understanding rootstock-scion interactions is particularly relevant in the context of establishing clonal seed orchards, since grafting is the standard methodology used for their establishment. This study aimed to assess whether different rootstock genotypes influence intra-clonal variability in budburst timing among pedunculate oak clones. Such knowledge is critical to ensuring phenological synchrony among ramets and to maximizing seed production in clonal seed orchards.

MATERIAL AND METHODS

Experimental design and plant material

The data used in this study were collected from an experimental trial established at the Brestje nursery (45.84°N, 16.10°E), managed by Croatian Forests Ltd. The trial was set up using heterovegetatively propagated (grafted) pedunculate oak clones, originating from Kosovac (45°36'09.8"N 17°58'03.3"E), Petkovac (45°08'49.2"N 18°51'36.5"E), and Plešćice I (45°44'52.2"N 16°35'14.7"E) clonal seed orchards. Planting began on 13 March 2008, following a randomized complete block design with three replications. Each of the 150 clones was represented by one ramet per block, resulting in a total of 450 ramets.

Scion material for heterovegetative propagation and trial establishment was collected in the spring of 2007 from the previously mentioned clonal seed orchards and was immediately grafted onto previously prepared rootstocks. These rootstocks had been grown from acorns one year earlier, in the spring of 2006. The acorns were sampled from natural oak populations, and their genetic background is unknown. However, it is assumed that rootstocks are genetically diverse.

Phenological observations of budburst were conducted twice a week until complete leaf development was recorded for all clones. A 1–7 ordinal scale, as described by Franjić et al. (2011), was used to assess phenological phases. All observations were performed by an experienced observer, and the recorded phenological scores were used as input for subsequent statistical analyses.

Data processing and statistical analysis

Although the experimental trial was established in 2008, phenological monitoring data used in this study were an-

alyzed starting from 2010, in order to avoid potential transplant shock effects that may have influenced early plant development. The dataset spans from 2010 to the end of the monitoring period in 2014. It is important to note that data from the year 2012 were excluded from this part of the analysis, which focuses on phenological synchronization, due to the occurrence of a late spring frost that year. This climatic event may have negatively affected plant development and compromised the reliability of the results.

Data were filtered to include only those clones for which all three ramets were consistently represented across all observation years included in the study. In the end, the analysis was conducted on 43 clones, each represented by three ramets.

Data processing and visualization were performed using R statistical software (version 4.4.3; R Development Core Team 2024). Data cleaning, transformation, and preparation were carried out using the packages tidyverse (version 2.0.0), dplyr (version 1.1.4), tidyr (version 1.3.1), zoo (version 1.8-13), rstatix (version 0.7.2), and lubridate (version 1.9.4).

Data visualization was conducted using ggplot2 (version 3.5.1), ggstatsplot (version 0.12.3), ggalt (version 0.4.0), and gridExtra (version 2.3). In addition, summary results were formatted and presented in tabular form using the gt (version 1.0.0) and formattable (version 0.2.1) packages.

For further analysis, the exact date when a plant entered a specific phenological phase was extracted. In cases where the entry into a phenophase was not directly observed, i.e., when the transition occurred between two monitoring dates, interpolation was used to determine the date of phase onset. These interpolated values were subsequently used in statistical analyses.

The Shapiro–Wilk test indicated that the phenological data were not normally distributed. To assess differences in phenological timing among ramets within individual clones, a bootstrap analysis was performed using the boot (version 1.3.31) package. This non-parametric resampling approach provided robust estimates of confidence intervals and test statistics based on 2,000 resamples with replacement from the original dataset. The analysis evaluated whether differences occurred among ramets of the same clone in the date of entry into phenophase 3, corresponding to budburst and marking the onset of vegetative growth—a critical stage influencing both the length of the growing season and a clone’s exposure to environmental stressors such as late spring frost.

Temperature data and biological threshold determination

Daily minimum and mean air temperature data were obtained from the Croatian Meteorological and Hydrological Service and recorded at the Maksimir meteorological station (45°49′19″N 16°02′01″E), the nearest station to the experimental site. On 10 April 2012, a late spring frost occurred, causing significant damage to ramets that had already reached advanced phenological phases (\geq phenophase 4). Using an interpolation model, the exact date each ramet entered phenophase 4 (folded leaf visible) was determined. Ramets that had reached phenophase 4 or higher (≥ 4) on or before 10 April 2012, the date of the frost event, were classified as frost-damaged, while those that entered this phase afterward were classified as frost-surviving. For each clone, the difference in days between the phenophase 4 onset of damaged and surviving ramets was calculated. The analysis revealed that a delay of just three days in reaching phenophase 4 was sufficient to avoid frost injury; this difference was therefore adopted as a biological threshold for frost avoidance. This finding was then used to define a three-day or greater difference in budburst timing (phenophase 3) among ramets within a clone as biologically significant.

RESULTS

Bootstrap analysis of intra-clonal variation in spring leaf phenology

In 2010, the results indicated that 11.2% of clones exhibited statistically significant differences ($p < 0.05$) in phenophase dynamics between their ramets. This proportion increased in 2011 to 17.8%, suggesting greater intra-clonal variability in that year. In 2013, the proportion of clones with significant within-clone differences slightly decreased to 14.4%. However, in 2014, this percentage rose noticeably to 24.8%, representing the highest level of intra-clonal phenological divergence across the analyzed years (Figure 1).

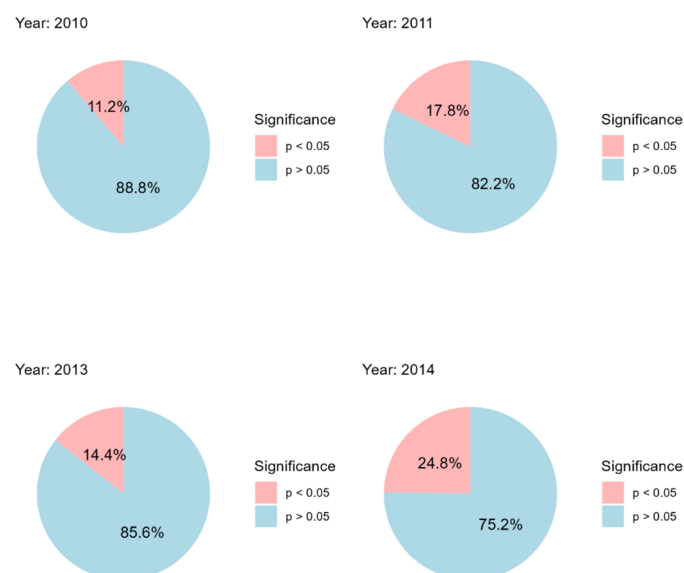


Figure 1 Proportion of clones showing significant phenological differences between their ramets across years (2010, 2011, 2013, and 2014), based on bootstrap analysis in phenophase 3. The red segments ($p < 0.05$) represent clones with statistically significant intra-clonal variation in phenophase progression, while blue segments ($p > 0.05$) indicate clones without significant differences.

Dynamics of spring leaf development in clone BJ 39

The progression of spring phenological phases was tracked in three ramets (a, b, and c) of clone BJ 39 during four years: 2010, 2011, 2013, and 2014. Clone BJ 39 was selected as a representative example to illustrate intra-clonal variation among the clones analyzed in the study. The phenological curves show year-to-year variation in both the onset and rate of development among the ramets (Figure 2).

In 2010, ramet "b" initiated spring leaf phenology slightly earlier than ramets "a" and "c", but all three ramets progressed through phenophases relatively synchronously. Bootstrap analysis for phase 3 in that year confirmed no statistically significant differences between any of the ramet pairs.

In 2011, more pronounced divergence was observed. Ramet

"b" entered budburst earlier than both ramets "a" and "c", and ramet "a" lagged noticeably behind. These differences were statistically confirmed: bootstrap analysis revealed a significant difference between ramets "b" and "c" ($p < 0.001$), while differences between ramets "a" and "b", and "a" and "c", were not significant.

In 2013, all three ramets initiated spring phenology in a relatively synchronized manner; however, ramet "a" exhibited a slower progression of phenophases, indicating a generally slower developmental dynamic compared to the others.

In 2014, ramet "b" initiated spring phenology earlier than both ramets "a" and "c". Bootstrap analysis confirmed a statistically significant difference in the onset timing between ramets "b" and "c", indicating an earlier development in ramet "b".

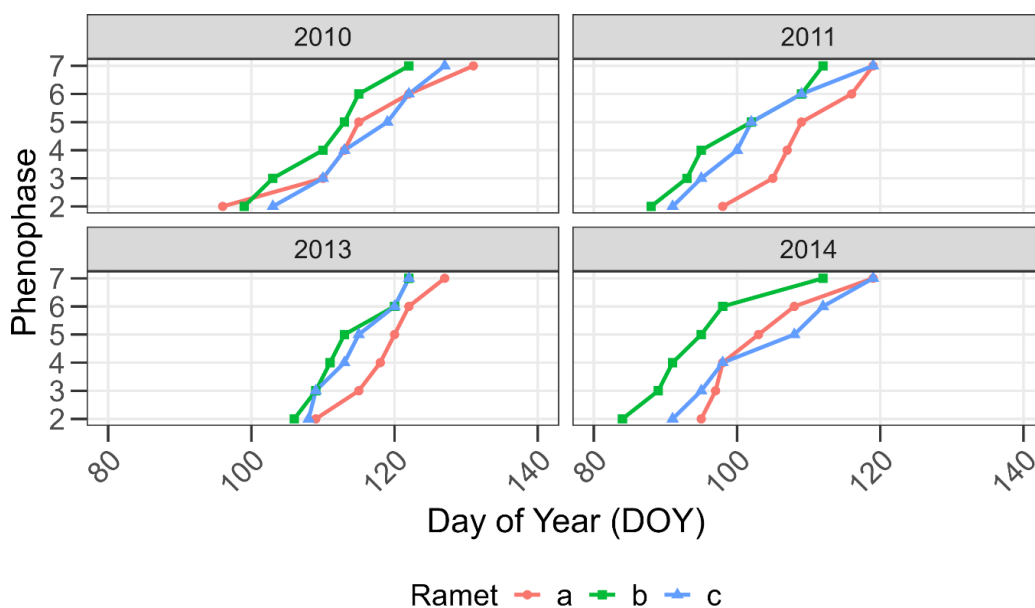


Figure 2 Phenological development of ramets "a", "b", and "c" of clone BJ 39 across four years (2010, 2011, 2013, and 2014). The y-axis represents phenophase values (phases 2–7), while the x-axis shows the day of the year (DOY). Although the general trend of development is similar, the timing and rate of progression vary between ramets and years, indicating intra-clonal variability in phenological dynamics.

Dynamics of spring leaf development in clone NA 02

The progression of spring leaf phenological phases was monitored in three ramets (a, b, and c) of clone NA 02 over four growing seasons: 2010, 2011, 2013, and 2014. Clone NA 02 was selected as a representative example to illustrate intra-clonal variation among the clones analyzed in the study. Visual analysis of phenological curves revealed consistent differences in the onset and progression of development among the ramets (Figure 3).

Particular focus was placed on phenophase 3 (budburst), which marks the visible opening of buds and the initiation of leaf development. In all analyzed years, ramet "a" consistently entered this phase earlier than ramets "b" and "c", while ramet "b" was generally the latest to initiate and complete leaf development.

To assess these differences statistically, bootstrap analysis was performed specifically for phase 3. The results confirmed significant differences between ramets "a" and "b" in 2010 ($p = 0.009$) and 2011 ($p < 0.001$), as well as between ramets "a" and "c" in 2011 ($p = 0.033$). In 2013, although visual differences appeared subtle, statistical analysis revealed that significant differences existed between ramets "a" and "b" and between ramets "a" and "c", while the difference between ramets "b" and "c" was not significant. In 2014, phenological divergence reappeared, with significant differences confirmed between ramets "a" and "b" ($p < 0.001$) and "a" and "c", while again, no significant difference was observed between ramets "b" and "c".

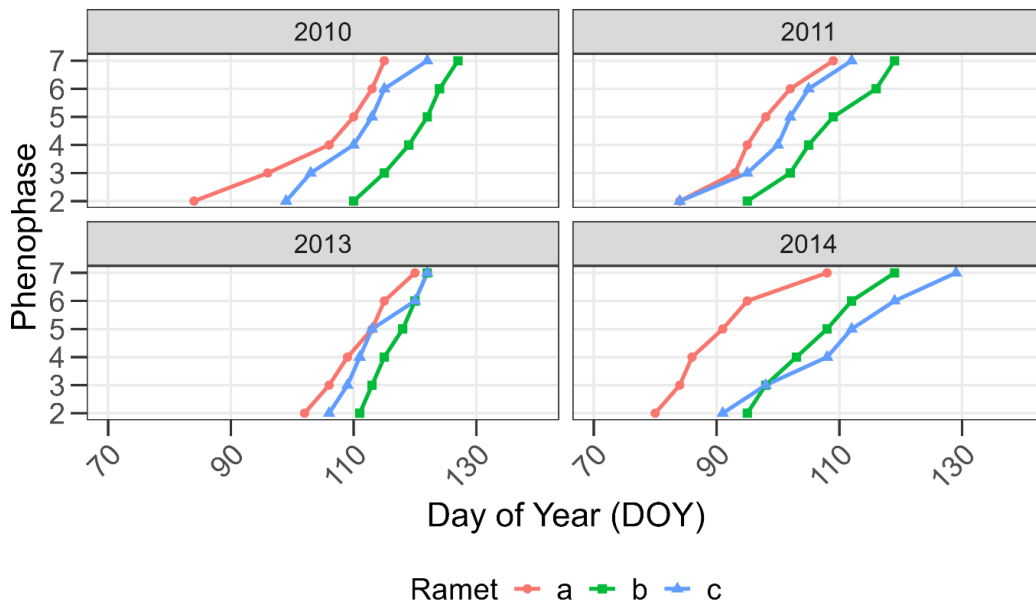


Figure 3 Phenological development of ramets “a”, “b”, and “c” of clone NA 02 across four years (2010, 2011, 2013, and 2014). The y-axis represents phenophase values (phases 2–7), while the x-axis shows the day of the year (DOY). Although the general trend of development is similar, the timing and rate of progression vary between ramets and years, indicating intra-clonal variability in phenological dynamics.

Temperature data and biological threshold determination

The data presented in Figure 4 illustrate the temporal dynamics of five distinct years over a three-month period from late January to late April.

The year 2010 began with temperatures slightly above freezing in early February. This was followed by a brief cooling period and then a gradual warming trend, marked by relatively low variability. The transition to spring conditions was steady and consistent, though less pronounced than in the following years.

The year 2011 started with moderate temperatures but was marked by a highly unstable March, characterized by fre-

quent shifts between warm and cold spells. The warming trend emerged later in the season and became stable only toward the end of April.

The year 2012 featured the coldest start of all the years analyzed, with the lowest temperature recorded in early February (Figures 4 and 5). A noticeable increase in temperature followed; however, the pattern remained highly variable, with repeated alternations between warm and cold periods. Notably, on 10 April 2012, a late spring frost was recorded (Figure 5), which negatively affected ramets that were in phenophase 4 or beyond. Although meteorological station data recorded a frost event on 2 April 2012, no visible impact was observed on the ramets in the experimental trial.

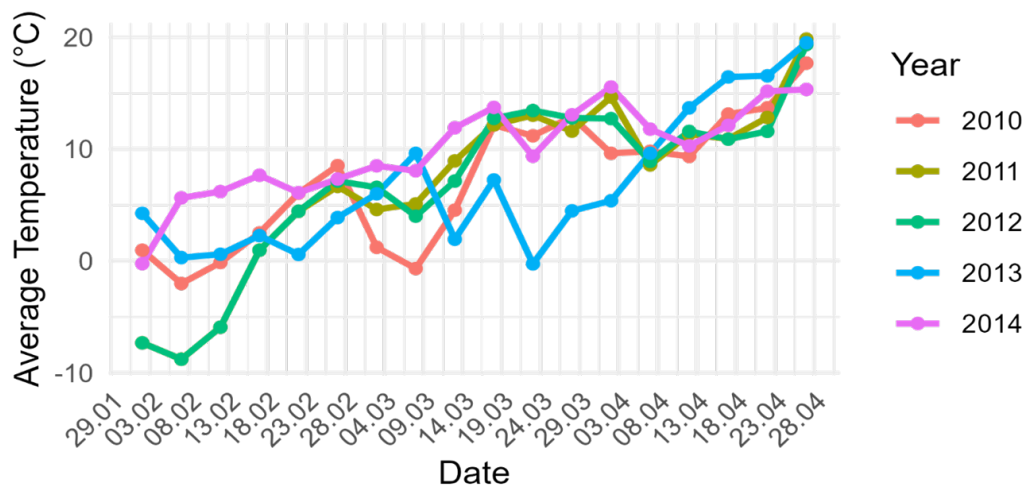


Figure 4 Five-day mean air temperatures (°C) recorded between 29 January and 28 April for five different years (2010–2014). Data were obtained from the Maksimir meteorological station and illustrate interannual variation in early spring temperature.

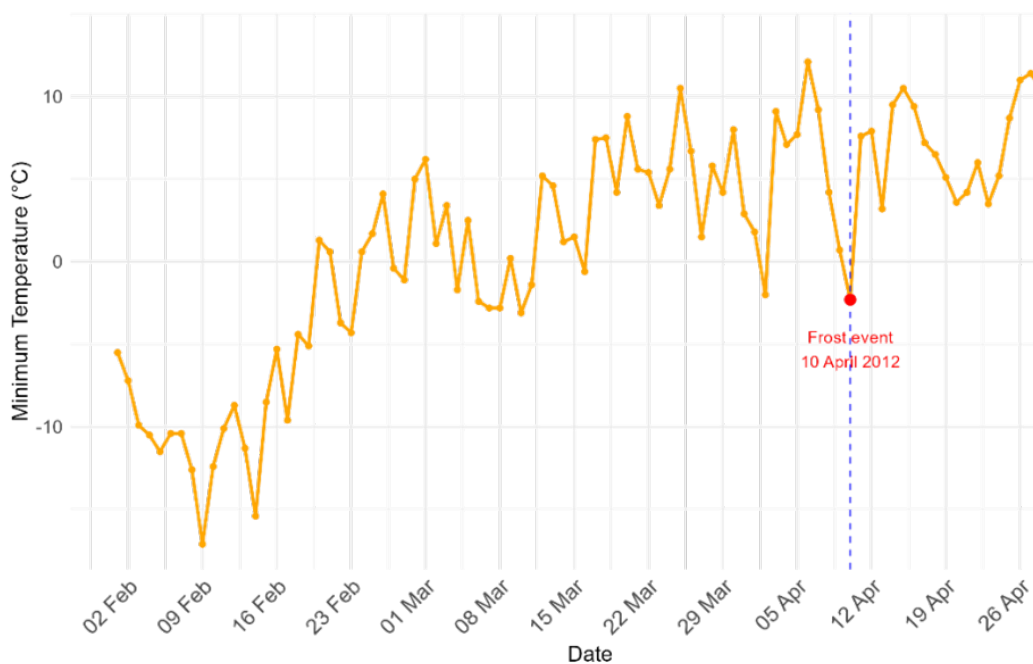


Figure 5 Daily minimum air temperatures (°C) recorded from 1 February to 30 April 2012, based on data from the Maksimir meteorological station. A significant drop in temperature was observed on 10 April 2012, indicating a late spring frost event.

The year 2013 began with temperatures near zero and exhibited considerable variability throughout March, including alternating drops and surges in temperature. Stable and warmer conditions were only recorded from mid- to late April.

The year 2014 showed a stable and gradual warming trend from the beginning of the observation period. However, noticeable cooling periods occurred in mid-March and again from early to mid-April. These cooler phases introduced a degree of instability, precisely during the period when increased budburst activity would typically be expected.

Intra-clonal variability in budburst timing across years

The within-clone variability in the timing of budburst (phenophase 3) was assessed by calculating the range between the earliest and latest ramet transition dates for each clone (Table 1).

In 2013, clones exhibited the highest degree of synchronization, with an average within-clone range of only 3.34 days and a maximum of 7 days. This was further supported by the fact that only 41.9% of clones had a range equal to or greater than three days, indicating limited intra-clonal asynchrony. In contrast, the year 2010 showed the highest variability, with a mean range of 6.31 days and a maximum of 19 days; 79.1% of clones exhibited a range of ≥ 3 days. Similarly, 2014 also showed considerable asynchrony (mean = 5.71 days; max = 16 days), with 72.1% of clones exceeding the 3-day threshold. The year 2011 displayed intermediate variability, with 46.5% of clones falling into this category (Figure 6). These results highlight substantial interannual differences in phenological coherence within clones, likely influenced by varying environmental conditions.

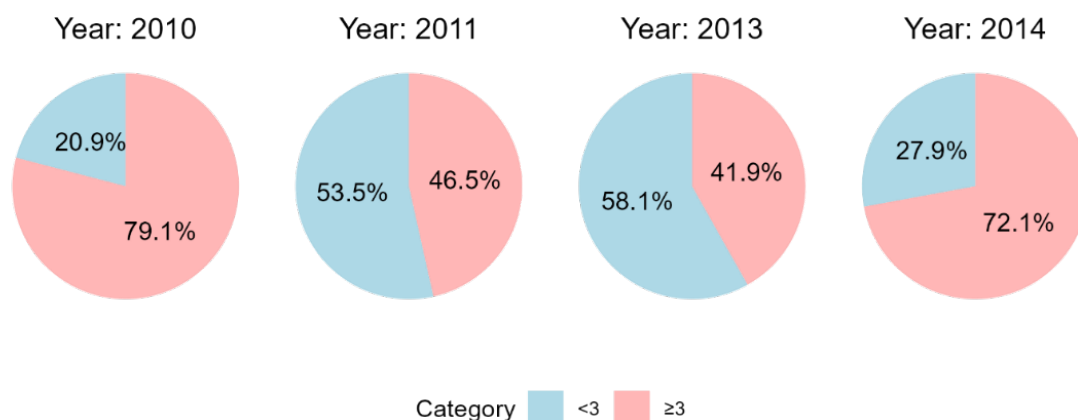


Figure 6 Distribution of clones with fewer than 3 days (<3, blue) and 3 or more days (≥ 3 , pink) of intra-clonal variability in budburst across four years. Each pie chart shows the percentage of clones in each category for the years 2010, 2011, 2013, and 2014. The majority of clones exhibited higher within-clone variability (≥ 3 days) in 2010 and 2014, while in 2011 and 2013 the proportion of clones with lower variability (<3 days) was higher or more balanced.

Table 1 Intra-clonal variability in the timing of budburst (phenophase 3) across four study years (2010, 2011, 2013, and 2014) for each clone. Values represent the range (in days) between the earliest (MIN) and latest ramet (MAX) to reach phase 3 within the same clone, indicating the degree of intra-clonal synchrony. A greater range suggests lower synchrony (i.e., greater asynchrony) among ramets. A difference of three days or more was considered potentially significant.

Clone	2010				2011				2013				2014			
	mean	min	max	range	mean	min	max	range	mean	min	max	range	mean	min	max	range
BJ04	99.00	99	99	0	93.67	93	95	2	108.67	108	109	1	84.67	84	86	2
BJ05	109.33	103	115	12	106.33	105	109	4	115.67	113	117	4	99.67	98	103	5
BJ07	115.67	114	117	3	101.33	100	102	2	113.00	113	113	0	98.00	98	98	0
BJ18	114.00	110	118	8	109.33	105	114	9	116.33	115	117	2	106.00	98	112	14
BJ21	106.33	103	110	7	100.00	98	102	4	111.67	111	113	2	93.67	91	95	4
BJ25	100.33	99	103	4	94.67	93	98	5	108.33	108	109	1	82.00	80	84	4
BJ28	116.67	116	117	1	112.00	112	112	0	118.67	118	120	2	99.67	98	103	5
BJ29	96.67	92	99	7	94.33	93	95	2	108.67	106	111	5	88.00	84	91	7
BJ34	99.33	96	103	7	96.67	93	102	9	109.33	106	111	5	87.00	84	91	7
BJ39	107.67	103	110	7	97.67	93	105	12	111.00	109	115	6	93.67	89	97	8
BJ41	98.00	92	103	11	94.33	93	95	2	107.67	106	109	3	85.67	84	89	5
BJ46	118.00	115	122	7	113.67	113	113	0	123.00	122	125	3	110.67	108	112	4
BJ 50	108.67	106	110	4	93.67	93	95	2	108.67	108	109	1	84.67	82	86	4
NA01	101.67	96	110	14	91.33	88	95	7	107.67	106	111	5	83.67	80	91	11
NA02	104.67	96	115	19	96.67	93	102	9	109.33	106	113	7	93.33	84	98	14
NA04	92.00	92	92	0	88.00	88	88	0	103.67	102	106	4	80.00	80	80	0
NA05	99.33	96	103	7	93.67	91	97	6	107.67	106	109	3	84.33	80	89	9
NA08	97.00	96	99	3	92.67	92	92	0	106.67	106	108	2	84.67	84	86	2
NA09	93.33	92	96	4	92.33	91	95	4	106.00	106	106	0	81.33	80	84	4
NA12	93.67	89	96	7	91.00	91	91	0	105.33	104	106	2	81.00	79	84	5
NA15	98.00	96	99	3	93.00	93	93	0	107.00	106	109	3	83.33	82	84	2
NA16	95.67	92	103	11	93.00	91	95	4	106.67	106	108	2	82.67	80	84	4
NA17	93.33	89	99	10	94.67	93	98	5	108.33	106	113	7	87.67	82	97	15
NA19	98.33	96	103	7	92.33	91	93	2	109.00	109	109	0	84.67	84	86	2
NA20	93.33	92	96	4	91.67	91	93	2	106.00	106	106	0	81.67	77	84	7
NA23	93.00	91	96	5	90.00	88	91	3	108.33	106	113	7	84.67	79	95	16
NA28	99.33	96	103	7	92.33	91	93	2	107.67	106	109	3	84.00	84	84	0
NA29	95.67	92	99	7	93.00	91	95	4	107.67	106	109	3	84.00	84	84	0
NA38	93.33	89	99	10	88.67	88	90	2	104.67	104	106	2	77.00	77	77	0
NA40	99.00	99	99	0	92.33	91	93	2	107.33	106	108	2	84.00	82	86	4
VK04	93.33	92	96	4	90.00	88	91	3	105.33	104	106	2	78.00	77	80	3
VK09	122.00	122	122	0	114.67	112	116	4	121.33	120	122	2	114.00	112	115	3
VK11	115.67	115	118	3	106.33	105	107	2	116.00	115	118	3	103.00	98	108	10
VK16	111.67	110	115	5	110.00	109	112	3	117.33	117	118	1	102.33	102	103	1
VK18	98.00	96	99	3	93.67	93	95	2	108.33	108	109	1	82.67	80	84	4
VK20	104.00	99	110	11	93.00	93	93	0	109.00	109	109	0	85.67	84	89	5
VK29	92.00	92	92	0	91.33	88	95	7	106.00	106	106	0	80.33	79	82	3
VK31	106.00	106	106	0	109.00	109	109	1	116.67	116	117	1	100.00	98	103	5
VK34	113.67	113	115	2	112.00	112	112	0	117.67	117	118	1	102.33	102	103	1
VK38	92.00	92	92	0	86.67	86	88	2	103.67	102	106	4	78.67	77	80	3
VK40	116.33	113	119	6	107.67	102	112	10	115.33	111	118	7	103.00	98	108	10
VK43	93.33	92	96	4	91.00	91	91	0	105.33	104	106	2	77.00	77	77	0
VK56	101.67	92	110	18	96.67	93	102	9	109.67	109	111	2	90.00	86	95	9

Frost avoidance as an outcome of intra-clonal phenological variability

Table 2 presents the timing of phenophase 4 in ramets of oak clones that experienced frost damage in spring 2012, compared to ramets of the same clones that survived without

visible damage. In all cases, frost-damaged ramets reached phenophase 4 earlier than the frost-surviving ramets of the same clone. This finding indicates that early budburst resulted in an earlier onset of phenophase 4, which significantly increased susceptibility to the late spring frost that occurred on 10 April 2012.

Table 2 Comparison of phenophase 4 between frost-damaged and frost-surviving ramets across clones in which at least one ramet experienced frost damage. The table presents the dates on which each ramet reached phenophase 4, along with the difference in days between damaged and surviving ramets within the same clone. Ramets within each clone are labeled as “a”, “b”, and “c”.

Clone	Frost-damaged ramet	Frost-survived ramet	Phenophase 4 (damaged ramet)	Phenophase 4 (survived ramet)	Difference (days)
BJ 25	b	a	2012-04-10	2012-04-13	3.0
BJ 25	c	a	2012-04-10	2012-04-13	3.0
BJ 29	b	a	2012-04-10	2012-04-13	3.0
BJ 29	b	c	2012-04-10	2012-04-13	3.0
BJ 41	b	a	2012-04-06	2012-04-13	6.5
BJ 41	c	a	2012-04-06	2012-04-13	6.5
BJ 50	c	a	2012-04-03	2012-04-17	14.0
BJ 50	c	b	2012-04-03	2012-04-13	10.0
NA 02	a	b	2012-04-06	2012-04-27	20.5
NA 02	c	b	2012-04-10	2012-04-27	17.0
NA 08	a	c	2012-04-06	2012-04-24	17.5
NA 08	b	c	2012-04-10	2012-04-24	14.0
NA 16	b	a	2012-04-06	2012-04-13	6.5
NA 16	c	a	2012-04-10	2012-04-13	3.0
NA 19	b	a	2012-04-10	2012-04-13	3.0
NA 19	c	a	2012-04-10	2012-04-13	3.0
NA 20	a	c	2012-03-30	2012-04-20	21.0
NA 20	b	c	2012-04-10	2012-04-20	10.0
NA 23	b	a	2012-03-30	2012-04-20	21.0
NA 23	c	a	2012-04-10	2012-04-20	10.0

The differences in phenophase timing between damaged and surviving ramets varied considerably across clones, ranging from 3.0 to 21.0 days. Minimal differences (3.0–6.5 days) were observed in clones BJ 25, BJ 29, BJ 41, NA 16, and NA 19, suggesting that although these clones initiated budburst early, the phenological gap between damaged and surviving ramets was relatively small—yet still sufficient to prevent frost damage in the surviving ramets. In contrast, clones BJ 50, NA 02, NA 08, NA 20, and NA 23 exhibited much larger differences, ranging from 10.0 to 21.0 days.

The most extreme case was observed in clone NA 20, where phenophase 4 in damaged ramets occurred on 30 March, while the surviving ramets reached the same phase on 20 April, resulting in a 21-day delay. These findings clearly demonstrate that ramets with earlier budburst were more susceptible to frost damage, and that a delay of three or more days in reaching phenophase 4 was sufficient to prevent such damage.

Figure 7 illustrates the relationship between spring leaf phenology and minimum daily temperature in three ramets of the BJ 25 clone during the spring of 2012. On 10 April 2012, a late spring frost occurred, with minimum temperatures dropping to -2.3°C . At that time, two ramets (“b” and “c”) had already reached phenophase 4 or higher. After the frost event, both exhibited a temporary shift from phase 4 back to phase 2, indicating frost damage to the developing buds and partial loss of newly expanded tissues. Because leaves at advanced developmental stages were destroyed, the trees subsequently initiated renewed leaf growth and were later recorded at lower phenological phases. In contrast, ramet “a” (green line) exhibited slower development, reaching only phase 3 (budburst) at the time of the frost, and showed no signs of damage thereafter. This pattern, shown in Figure 7, illustrates how earlier budburst increased frost exposure risk and led to a phenological setback in the more advanced ramets.

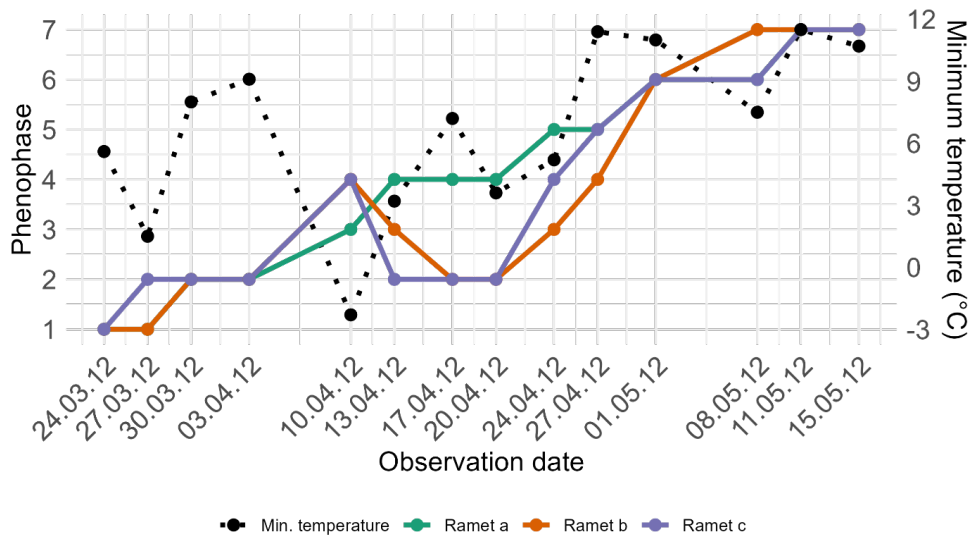


Figure 7 The relationship between spring leaf phenology and minimum daily temperature in three ramets of the BJ 25 clone during the spring of 2012. The graph shows the phenological development stages of three ramets (“a,” “b,” and “c”) of the BJ 25 clone over time, plotted against daily minimum temperature (black line, right y-axis). A late frost event occurred around 10 April, when minimum temperatures dropped below 0°C. Ramets that had already reached or exceeded phenophase 4 by this date (notably ramets “b” and “c”) were damaged by frost, highlighting the impact of early budburst on vulnerability to late spring frosts.

DISCUSSION

Intra-clonal variability in spring leaf phenology

The bootstrap analysis of intra-clonal variability in spring leaf phenology provided an additional statistical perspective on the degree of synchronization among genetically identical ramets across multiple years. While the majority of clones did not exhibit statistically significant differences among ramets, several clones showed clear intra-clonal asynchrony, indicating that temporal variation in budburst can occur even within a single genetic background.

These findings complement the descriptive analysis (Table 1, Figure 6), which captures the range of phenological variation and highlights biologically meaningful differences which may not reach statistical significance. For example, in clone NA 02, graphical analyses of phenological phase dynamics across three ramets over four years consistently revealed a lack of synchronization (Figure 3). The bootstrap test detected statistically significant differences between ramets “a” and “b” in all years except 2013, and further inspection of phenophase 3 onset showed that ramet “a” consistently flushed about seven days earlier than ramet “b”, indicating a difference that is both consistent and biologically relevant.

Taken together, these results show that the bootstrap and descriptive analyses address different but complementary aspects of intra-clonal variability, providing both statistical and biological perspectives on phenological divergence among ramets.

Similarly, if we observe clone BJ 39 (Figure 2), we can see that although all three ramets belong to the same clone, the data clearly demonstrate variability in the timing and dynamics of spring phenophase progression. Throughout all observed years, the ramets of clone BJ 39 exhibited overall consistent

phenological patterns, yet with notable differences among individual ramets. Ramet “b” consistently emerged as the earliest to initiate spring development, regularly reaching budburst and leaf expansion stages ahead of the others. In contrast, ramet “a” showed a clear tendency toward delayed phenological onset, with a slower progression through developmental stages in certain years. This phenological asynchronization among ramets of the same clone may be partly explained by a potential interaction between the rootstock and scion, which could influence the onset of phenological activity.

Such interactions have not been frequently reported in forest tree species; however, there are studies on certain fruit crops that provide relevant insights. For example, Wang et al. (1994) demonstrated that rootstocks significantly influence the spring phenology of ‘Hayward’ kiwifruit, as reflected by notable differences in the timing, synchrony, and magnitude of budburst, with direct implications for subsequent flowering intensity.

Other researchers suggest that shifts in flushing phenology and vegetative growth at the start of the growing season may result from a phenological mismatch between the rootstock and the scion. For instance, the results of the study by Clearwater et al. (2007) clearly indicate that the rootstock can affect the phenology of the scion through differences in the timing of spring root pressure development. High-vigour rootstocks developed positive root pressure before or during scion budburst, enabling uninterrupted shoot growth without signs of water stress. In contrast, low-vigour rootstocks developed root pressure only after shoot growth had already commenced, during which scions exhibited more pronounced water stress and reduced growth. Recent findings by Camisón et al. (2021) show that in chestnut (*Castanea sativa* Mill.), rootstock origin significantly affects scion budburst. Scions grafted onto drought-adapted (xe-

ric) rootstocks flushed earlier than those on humid-origin rootstocks, likely due to differences in hormone signaling and water transport. The study also found that grafting itself can delay budbreak, suggesting that both rootstock genotype and grafting effects can alter phenological timing and contribute to asynchronization among genetically identical ramets. The study demonstrated that in apple trees with upright-narrow (UN) scions, bud break occurred earlier on M.7 and M.9 rootstocks (3.7 days) compared to MM.111 and seedling rootstocks (8.8 days), confirming that rootstock can influence the budbreak phenology of apple scions. Although Tworowski et al. (2016) did not directly monitor leaf-out phenology, their results indicate a connection between rootstock response to drought stress and its influence on scion physiology. They demonstrated that ABA concentrations in scion leaves varied depending on the rootstock, suggesting that distinct patterns of root-derived hormonal signaling can modulate scion responses. This variability in rootstock reaction to environmental conditions highlights the importance of rootstock as a factor in the plant's overall adaptation to stress.

Intra-annual variation in budburst: the roles of temperature sensitivity and rootstock–scion interactions

When examining the range of budburst within the same clone, it becomes evident that this range varies notably from year to year (Table 2). Such variability can be attributed primarily to differences in weather conditions, particularly temperature fluctuations between seasons. A comparison between spring temperature trends (Figure 4) and budburst phenology reveals a strong association between thermal conditions and both the timing and synchrony of budburst across oak clones. Although the exact physiological mechanisms regulating leaf emergence are not fully understood for most plant species, it is well established that temperature plays a key role in leaf development (Linkosalo et al. 2006). This sensitivity to temperature results in considerable interannual variability in the onset of spring phenology (Polgar and Primack 2011).

In 2010, an initially warm early season was followed by a cold spell, with stable temperatures above 10°C occurring only from mid- to late April. That year recorded the highest intra-clonal variability in budburst timing, with a mean range of 6.31 days and a maximum of 19 days. Moreover, 2010 had the largest proportion of clones (79.1%) exhibiting a spread of three or more days between ramets, indicating a pronounced lack of synchrony in budburst among genetically identical individuals. This asynchrony likely resulted from insufficient thermal accumulation early in the season and from differences in the timing at which individual ramets reached their required cumulative temperature thresholds.

Since it is well known that flushing in deciduous forest trees is regulated by genetic mechanisms that define how individuals respond to temperature cues (Derory et al. 2006, Vitasse et al. 2010), several studies have demonstrated that the temperature thresholds required to trigger budburst are genetically controlled (Kramer 1995, Rousi and Pusenius 2005, Körner and Basler 2010, Basler and Körner 2012). The genetic response to rising temperatures may explain the significant variation in budburst timing observed among clones across years (Table 1).

However, the question arises as to why such differences also occur between ramets of the same clone. Given that environmental conditions were uniform across the experimental plot, it is reasonable to assume that this variability arises from interactions between the rootstock and scion. As previously noted, different genotypes can have distinct temperature thresholds (Vitasse et al. 2009, 2010). Since rootstocks were grown from seed and thus genetically different, they may possess varying sensitivities to temperature cues, leading some to trigger budburst in the scion earlier, while others may delay it.

This is particularly plausible in years such as 2010 and 2014, when warm and cold periods alternated over a longer duration, and warming progressed slowly. In such years, it is likely that some rootstocks reached their temperature thresholds earlier, especially those with a faster physiological response to warming. Consequently, greater intra-clonal variability was observed. By contrast, in years when the early season was dominated by low temperatures followed by a rapid warming event, such as in 2011 or 2013, intra-clonal differences in budburst were smaller. The more synchronized thermal signal may have aligned the temperature response thresholds across ramets, minimizing budburst variation.

Interaction between intra-clonal phenological variability and frost susceptibility

Our findings suggest that variability in phenological timing among ramets within the same clone—referred to as intra-clonal phenological asynchronization—may enhance a clone's ability to cope with climatic stressors, particularly late spring frosts. Slower developing ramets are less likely to be affected by low temperatures, thereby increasing the overall frost resilience of the clone. An early flushing strategy can be advantageous by enabling rapid early-season growth and a longer growing period; however, it also increases the risk of frost damage (Vitasse and Rebetz 2018). Foliage loss caused by late spring frost represents a major stress factor for deciduous trees, as it disrupts nutrient allocation, compromises growth and reproduction, and impairs canopy development (Vitasse et al. 2014). Although oaks exhibit a strong ability to regenerate foliage, this compensatory response is energetically costly and shortens the growing season, ultimately diminishing their net annual productivity (Vitasse et al. 2014, Baumgarten et al. 2023). In addition, it is important to highlight that the newly flushed leaves produced during refoliation often coincide with elevated levels of powdery mildew inoculum, and since environmental conditions during this period are generally favorable for fungal development, trees become highly susceptible to severe foliar infections (Marçais et al. 2009).

While ramets in phenophase 3—defined as budburst (widely spaced bud scales and visible green leaf tips)—showed no visible damage, those in phenophase 4 (folded leaf visible) exhibited significant injury, as clearly demonstrated by clone BJ 25 (Figure 7). All ramets that had reached phenophase 4 on the day of the frost event displayed clear symptoms of frost damage, highlighting the increased susceptibility of more advanced phenological phases. This supports previous research indicating that early-flushing genotypes are more vulnerable to spring frost (Utkina and Rubtsov 2017).

In the establishment of clonal seed orchards intended for the production of high-quality forest reproductive material, hetero-vegetative propagation is commonly applied. Therefore, beyond the selection of the scion, it is essential to consider the phenotypic and physiological traits of the rootstock to prevent phenological asynchrony between graft components. This approach aligns with strategies already utilized in agronomy for frost mitigation. A relevant example comes from fruit production, where Durner and Goffreda (1992) demonstrated that the choice of rootstock in peach significantly affects both the timing and rate of flower bud development. Their study showed that certain rootstocks can delay blooming, thereby reducing the risk of frost damage and enhancing yield. Importantly, they emphasized that even a delay of just one to two days in specific bud development stages can markedly reduce frost injury. These findings underscore the biological importance of fine-scale phenological control, the principles that are equally applicable when designing clonal forestry systems where frost sensitivity is a limiting factor.

These findings directly support the biological threshold applied in our research, where a difference of three days in budburst among ramets was considered meaningful. This underlines that even slight shifts in developmental timing—if strategically managed—can play a critical role in minimizing frost damage and ensuring the stability and productivity of clonal seed orchards. In addition, by selecting rootstocks, it may be possible to increase the phenological synchrony of clones in plantations, thereby contributing to an increase in their effective population size and overall productivity.

CONCLUSION

This study confirms that rootstocks can significantly influence intra-clonal variation in the spring phenology of pedunculate oak, despite the genetic uniformity of ramets. Grafting onto different rootstocks resulted in notable differences in budburst timing within clones, with up to 79.1% of clones in 2010 and 72.1% in 2014 exhibiting a budburst range of three or more days between ramets, indicating pronounced phenological asynchrony.

Importantly, even small delays in budburst (≥ 3 days) were associated with successful frost avoidance, suggesting that such intra-clonal asynchrony may enhance the ability of some ramets to escape frost events. Beyond their scientific implications, these findings provide practical guidance for the management of clonal seed orchards and nursery production. When establishing or renewing seed orchards, particular attention should be given to the selection of rootstocks with compatible phenological behavior to minimize asynchrony among ramets and enhance cross-pollination efficiency. In nurseries, monitoring and recording the phenological traits of potential rootstocks could serve as a useful criterion for selecting grafting material. Such an approach could improve both the stability of seed yields and the overall adaptability of orchard trees to variable environmental conditions, thereby supporting long-term, sustainable seed production.

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