

**CZECH UNIVERSITY OF LIFE SCIENCES
PRAGUE**



Faculty of Forestry and Wood Sciences
Department of Forest Protection and Entomology

**Interaction between Spruce and *Ips typographus*: The
Role of Spruce Physiology**

A dissertation thesis submitted for the degree of Doctor of Philosophy
in

Global Changes Forestry

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CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Forestry and Wood Sciences

Ph.D. THESIS ASSIGNMENT

Vivek Vikram Singh

Global Change Forestry

Thesis title

Interaction between Spruce and Ips typographus: the role of Spruce physiology.

Objectives of thesis

The main objective of this thesis is to investigate how drought stress affects the physiological and molecular responses of Norway spruce (*Picea abies* [L.] Karst.) and how these responses relate to its vulnerability to bark beetle (*Ips typographus*) attack. In Eurasian boreal forests, *I. typographus* plays a crucial ecological role by decomposing dead wood and recycling essential nutrients. However, when populations exceed epidemic levels, they can cause extensive tree mortality. One of the leading hypotheses explaining such outbreaks is that prolonged or recurrent drought weakens a tree's defenses, making it more susceptible to colonization. The structural and microclimatic characteristics of forest stands also play a critical role in their susceptibility to drought and bark beetle disturbance. In this context, sap flow will be used as a proxy for tree-level water use, allowing assessment of how stand density and neighboring tree competition influence transpiration dynamics under drought.

Furthermore, at the molecular scale, drought triggers a cascade of physiological adjustments, including increased abscisic acid (ABA) accumulation, stomatal closure, reduced CO₂ assimilation, and the progressive depletion of non-structural carbohydrate (NSC) reserves, which are required for both metabolic maintenance and the synthesis of defensive compounds. Consequently, drought not only compromises the carbon balance but also limits the production of protective molecules such as LEA proteins, dehydrins, and heat shock proteins, which are vital for cellular stabilization during water deficit. Although these processes are recognized as key components of drought tolerance in model species, the molecular mechanisms that regulate them in Norway spruce remain insufficiently understood. Thus, this work aims to integrate ecophysiological measurements with gene expression analyses to advance our understanding of drought response in Norway spruce.

Methodology

This thesis will combine field-based and controlled experiments to study drought-related processes in Norway spruce at multiple scales. Sap-flow measurements in mature spruce stands will provide insights into tree water relations and stand-level responses. Controlled greenhouse experiments will facilitate a mechanistic understanding of drought-induced transcriptional changes by utilizing RNA sequencing (RNA-Seq) on needles, phloem, and roots. Differentially expressed genes and key pathways will be identified and validated using quantitative RT-qPCR. By linking whole-tree water relations with tissue-specific molecular responses, this research aims to provide a more integrated understanding of how drought stress affects Norway spruce resilience and influences its susceptibility to bark beetle attack under a changing climate.

The proposed extent of the thesis

70 SP

Keywords

Climate change, drought, *Picea abies*, sap flow and transpiration, *Ips typographus*, differential gene expression, RT-qPCR, tree defence

Recommended information sources

- Biedermann, P.H., Müller, J., Grégoire, J.C., Gruppe, A., Hagge, J., Hammerbacher, A., Hofstetter, R.W., Kandasamy, D., Kolarik, M., Kostovcik, M. and Krokene, P., 2019. Bark beetle population
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Declaration

I hereby declare that I have independently prepared this PhD thesis entitled “Interaction between Spruce and *Ips typographus*: The Role of Spruce Physiology”, using the referenced literature and drawing upon consultations and guidance provided by my advisors. I consent to the publication of this dissertation in accordance with Act No. 111/1998 Coll., on Higher Education Institutions, as amended, irrespective of the outcome of its defense.

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दुर्गम वनों और ऊँचे पर्वतों को जीतते हुए
जब तुम अंतिम ऊँचाई को भी जीत लोगे-
जब तुम्हें लगेगा कि कोई अंतर नहीं बचा अब
तुममें और उन पत्थरों की कठोरता में
जिन्हें तुमने जीता है-
जब तुम अपने मस्तक पर बर्फ का पहला तूफ़ान झेलोगे
और काँपोगे नहीं-
तब तुम पाओगे कि कोई फ़र्क नहीं
सब कुछ जीत लेने में
और अंत तक हिम्मत न हारने में।

- कुँवर नारायण

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List of original publications

Publications included in the thesis

This thesis is based on the work contained in the following papers and referred to using Roman numbers in text:

- I. **Singh, V.V.**, Naseer, A., Mogilicherla, K., Trubin, A., Zabihi, K., Roy, A., Jakuš, R. & Erbilgin, N. (2024a). Understanding bark beetle outbreaks: exploring the impact of changing temperature regimes, droughts, forest structure, and prospects for future forest pest management. *Reviews in Environmental Science and Bio/Technology*, 23, 257–290. <https://doi.org/10.1007/s11157-024-09692-5>.
- II. **Singh, V.V.**, Zabihi, K., Trubin, A., Cudlín, P., Korolyova, N., Jakuš, R. & Blaženec, M. (2025a). Effect of diurnal solar radiation regime and tree density on sap flow of Norway spruce (*Picea abies* [L.] Karst.) in fragmented stand. *Central European Forestry Journal*, 71. <https://doi.org/10.2478/forj-2024-0021>.
- III. **Singh, V.V.**, Naseer, A., Sellamuthu, G., Mogilicherla, K., Gebauer, R., Roy, A. & Jakuš, R. (2024b). Robust reference gene selection in Norway spruce: essential for real-time qualitative PCR across diverse tissue, stress and developmental conditions. *Frontiers in Forests and Global Change*, 7, 1458554. <https://doi.org/10.3389/ffgc.2024.1458554>.
- IV. **Singh, V.V.**, Naseer, A., Mogilicherla, K., Jamnická, G., Blaženec, M., Jakuš, R., Gebauer, R. & Krokene, P. (2025b). Tissue-specific drought transcriptome atlas of Norway spruce reveals coordinated yet distinct adaptive strategies. Original manuscript

Publications not included in the thesis

- V. Karpov, A., Pirtskhalava-Karpova, N., **Singh, V.V.**, Trubin, A., Korolyova, N., Modlinger, R. & Jakuš, R. (2025). Response of mature Norway spruce to experimental thermal and drought stress in relation to *Ips typographus* attack: Crown temperatures and sap flow. *Forest Ecology and Management*, 600, 123290. <https://doi.org/10.1016/j.foreco.2025.123290>.
- VI. Jakuš, R., Blaženec, M., Pivovar, M., Marešová, J., **Singh, V.V.**, Karpov, A. & Korolyova, N. (2025). Differentiating Acute and Chronic Stress in Norway Spruce using Hyperspectral and Thermal Remote Sensing in Experimental Plots. *The International Archives of the Photogrammetry, Remote Sensing and Spatial Information Sciences*, 48, 133. <https://doi.org/10.5194/isprs-archives-XLVIII-2-W11-2025-133-2025>.
- VII. Singh, U., Nasreen, S., Tripathi, G., Mehrishi, P., Pradhan, R.K., Bestakova, P., **Singh, V.V.**, Gouda, K.C., Sharma, L.K., Jalem, K., Maca, P. et al. (2025). Disaggregating IMERG satellite precipitation over Czech Republic: an innovative approach using hybrid Extreme Gradient Boosting based on Fuzzy

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- XV. Zabihi, K., Surový, P., Trubin, A., **Singh, V.V.** & Jakuš, R. (2021). A review of major factors influencing the accuracy of mapping green-attack stage of bark beetle infestations using satellite imagery: Prospects to avoid data redundancy. *Remote Sensing Applications: Society and Environment*, 24, 100638. <https://doi.org/10.1016/j.rsase.2021.100638>.

List of Abbreviations

ABA - Abscisic acid.

cDNA - Complementary DNA.

DEGs - Differentially Expressed Genes.

FDR - False Discovery Rate.

GST - Glutathione S-transferase.

HSPs - Heat Shock Proteins.

IPM - Integrated Pest Management.

IPP - Isopentenyl pyrophosphate.

LEA proteins - Late Embryogenesis Abundant proteins.

PCA - Principal Component Analysis.

RIN - RNA Integrity Number.

RT-qPCR - Reverse Transcription Quantitative Polymerase Chain Reaction.

SWP, Ψ_w - Soil Water Potential.

TFs - Transcription Factors.

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Abstract

Norway spruce [*Picea abies* (L.) Karst.] is among the most ecologically and economically significant coniferous species in Europe. Naturally adapted to cool, moist environments, its distribution range spans from central and eastern Norway across Fennoscandia, the Baltic states, Belarus, Russia, and into Central and Southeastern Europe. As a dominant component of boreal forests and a primary host of the Eurasian spruce bark beetle (*Ips typographus*), the species is highly vulnerable to climatic changes. Drought and rising temperature are critical stressors that predispose Norway spruce to bark beetle attack; however, the physiological and molecular mechanisms underlying this vulnerability are not fully understood. The presented thesis contributes to a better understanding of spruce responses to abiotic stress by combining field-based observations with molecular analyses. Firstly, the ecology and infestation dynamics of *I. typographus* are reviewed to identify key factors that impair spruce defenses and promote the development of outbreaks. Current management approaches are critically assessed, and molecular-based strategies for future bark beetle control are proposed. Secondly, the influence of stand density on spruce physiology is examined through sap flow measurements, providing insights into tree transpiration, forest stand structure, and their relationship with susceptibility to infestation. At the functional genomics level, methodological limitations are addressed by identifying reliable internal control genes across tissues, developmental stages, and environmental conditions, thereby improving the accuracy of gene expression studies in spruce. Finally, genome-wide transcriptome analysis was performed to identify genes associated with drought stress, followed by functional enrichment analyses, Transcription factor identification, pathway mapping, and RT-qPCR validation of key genes, providing a robust drought transcriptome atlas. Collectively, this research aims to advance the understanding of spruce responses to drought and temperature stress at both ecological and molecular scales. The generated housekeeping genes and the comprehensive transcriptome catalogue together provide a robust foundation for future research, including breeding and conservation initiatives aimed at climate-smart forest management. These resources will support efforts to enhance the resilience of *P. abies* to increasingly severe environmental stressors.

Keywords: Climate change, drought, *Picea abies*, sap flow and transpiration, *Ips typographus*, differential gene expression, RT-qPCR, housekeeping gene, tree defence.

1. Introduction

Global climate change has intensified the frequency and severity of extreme events such as droughts, windstorms, and heatwaves, fundamentally reshaping forest ecosystems worldwide. In Eurasian boreal and temperate forests, these disturbances have caused large-scale tree mortality, reduced productivity, shifts in species composition, and weakened ecosystem resilience (Price et al., 2013; Hlásny et al., 2021; Altman et al., 2024; Singh et al., 2024a). Among these drivers, drought represents a particularly critical stressor because it directly impairs hydraulic function and reduces the synthesis and mobilization of defensive compounds that are essential for resistance against herbivores and pathogens (Netherer et al., 2015; Erbilgin et al., 2021).

Norway spruce [*Picea abies* (L.) Karst.] is one of the most important conifer species in European boreal and montane forests, valued for its rapid growth and high-quality timber. Its natural range extends across much of northern and central Europe, where it is adapted to cool and moist environments (Danielsen et al., 2021). However, extensive planting outside its ecological optimum, driven by economic demand, has increased its exposure to heat stress, drought, and pest pressure (Caudullo et al., 2016; Hlásny et al., 2019). This maladaptation is now evident. In recent decades, Norway spruce has experienced unprecedented levels of mortality, with bark beetles emerging as the most damaging biotic agent. Climate warming accelerates beetle population dynamics, enabling earlier swarming, extended breeding seasons, and faster brood development (Hlásny et al., 2019; Hinze and John, 2020). Between 1970 and 2010, recurrent droughts and heatwaves caused annual timber losses of 2–14 million m³ in central Europe, culminating in catastrophic losses of 118 million m³ in 2019 (Ebner, 2020). Despite more than a century of research on bark beetles, knowledge gaps remain regarding how host physiology and molecular stress responses shape outbreak dynamics.

Outbreaks of bark beetles are not solely determined by climate. They are strongly mediated by stand structure, tree vitality, and disturbances. Unstable stands weakened by fungal infection or excessive slenderness are highly vulnerable to windthrow, which in turn initiates large-scale bark beetle infestations and subsequent tree mortality (Mezei et al., 2014b; Hroššo et al., 2020). Windthrow also modifies forest microclimates by opening the canopy, allowing more solar radiation to reach the forest floor, and creating conditions

favorable for beetle colonization (Emmel et al., 2013; Vanderhoof et al., 2014). Forest management further shapes these dynamics. Salvage logging, commonly applied to recover economic value from damaged timber, can inadvertently increase the vulnerability of residual trees. By exposing stand edges to direct solar radiation, salvage operations intensify heat stress and reduce defense capacity, thereby increasing bark beetle susceptibility (Kautz et al., 2013; Marešová et al., 2020). Stand density also plays a critical role: dense forests increase competition for water, nutrients, and light, lowering individual tree resilience, while sparse or fragmented stands increase canopy gaps, elevate heat loads, and drive higher evapotranspiration (Zabihi et al., 2021, 2023; Singh et al., 2025). Together, these interacting ecological factors strongly influence Norway spruce survival during beetle outbreaks (Korolyova et al., 2022).

While stand-level processes shape exposure and disturbance regimes, the ultimate survival of trees depends on their ability to withstand water deficit at the physiological and molecular levels. Drought occurs when soil water content drops below thresholds that increase hydraulic resistance at the root–soil interface. In response, trees deploy a coordinated suite of physiological and molecular adjustments that reconfigure signaling networks, ion transport, and transcriptional programs to maintain cellular integrity (Kizis et al., 2001; Osakabe et al., 2014; Haswell and Verslues, 2015; Choat et al., 2018). Recent advances in high-resolution sequencing now enable genome-wide transcriptome profiling, even in non-model species such as conifers, uncovering the regulatory networks mobilized under stress. These analyses reveal the central role of phytohormone-mediated signaling. Abscisic acid (ABA) rises rapidly in roots and shoots under water deficit, is translocated to guard cells, and triggers stomatal closure while activating widespread transcriptional reprogramming to support drought acclimation (Osakabe et al., 2014; Polle et al., 2019; Haas et al., 2021; Ribeyre et al., 2025). The canonical ABA cascade involves *PYR/PYL/RCAR* receptors, *PP2C* phosphatases, and *SnRK2* kinases, which activate ABF/AREB transcription factors binding to ABA-responsive promoters.

In parallel, ABA-independent pathways also contribute to drought resilience. The DREB/CBF subfamily of AP2/ERF transcription factors, acting on DRE/CRT motifs, regulates genes encoding osmoprotectants, antioxidant enzymes, and late embryogenesis abundant (LEA) proteins (Yamaguchi-Shinozaki and Shinozaki, 2006; Yoshida et al., 2014; Yao et al., 2021; Mader et al., 2023). Other regulators, including MYB, NAC, and HD-ZIP transcription factors, coordinate osmotic adjustment, redox balance, and ion homeostasis,

integrating drought responses into a cohesive molecular framework (Haas et al., 2021; Yao et al., 2021). Collectively, these mechanisms determine whether drought stress results in acclimation or irreversible damage, ultimately influencing susceptibility to secondary pests such as bark beetles.

Understanding spruce vulnerability to bark beetles, therefore, requires integration across ecological, physiological, and molecular scales. Disturbances, such as windthrow and salvage logging, alter forest structure and microclimates, thereby shaping exposure risk. Stand density influences resource competition and resilience, while drought stress regulates the physiological and molecular capacity of trees to withstand attack. This thesis investigates how drought and stand structure influence spruce physiology across ecological and molecular scales, thereby shaping vulnerability to *I. typographus*. Bridging these perspectives is essential to clarify the multi-level mechanisms of vulnerability and resilience under climate change and to provide a foundation for adaptive forest management and breeding efforts.

2. Aims and Objectives

The overall aim of this thesis is to provide a multi-scale understanding of how drought and stand structure influence spruce physiology across ecological and molecular scales in the context of bark beetle outbreaks. By integrating ecophysiological analyses of stand-level transpiration dynamics with molecular investigations of drought stress physiology, this work seeks to clarify the mechanisms underlying tree vulnerability and resilience.

Article I examines the ecology of bark beetles, outbreak dynamics, and management approaches. The specific objective was:

- To identify ecological, structural, and climatic drivers shaping *Ips typographus* outbreaks, and to evaluate the potential of cutting-edge molecular-based approaches for bark beetle management.

Article II focused on the influence of stand structure on susceptibility to bark beetle infestation, with a particular emphasis on how stand density affects vulnerability. The specific objective was:

- To quantify how stand density and solar exposure modify spruce water use (sap flow) and its implications for spruce susceptibility.

Articles III and **IV** explore the molecular mechanisms underpinning Norway spruce drought response. The specific objectives were:

- To resolve molecular mechanisms of spruce drought response through (a) reference gene selection and (b) a tissue-wide drought transcriptome atlas to identify key genes, pathways, and regulatory networks underlying drought stress responses across multiple tissues.

3. Research background

3.1 Climate Change and Forest Ecosystems

Forests cover nearly 30% of Earth's land surface and represent major global carbon reservoirs, regulating climate, supporting biodiversity, and providing essential ecosystem services ranging from timber production to recreation and cultural values (Keenan, 2015; Gullino et al., 2022; Baciu et al., 2021; Hernández-Blanco et al., 2022). Despite their ecological and societal importance, forest ecosystems are increasingly threatened by climate change and anthropogenic pressures. Rising temperatures, altered precipitation regimes, and human-driven disturbances such as land-use change, drought, storms, wildfires, and pest and pathogen outbreaks are undermining forest health, productivity, and resilience (Allen et al., 2010; Choat et al., 2018; Teshome et al., 2020; Hlásny et al., 2021; Balla et al., 2021; Stanturf et al., 2024).

Climate impacts on forests are multifaceted. While warming may enhance growth in some regions, it often leads to declines in growth in others, particularly where heat and water limitations intersect. Longer growing seasons can increase evapotranspiration and intensify drought risk, with consequences for species distribution and community composition (Keenan, 2015). Fragmented forests are especially vulnerable, as reduced connectivity limits their capacity to adapt compared to intact systems (Vose et al., 2018). Forest types with narrow climatic tolerance, such as boreal and Mediterranean ecosystems, are expected to undergo profound structural and functional shifts, whereas the future of tropical forests remains uncertain due to complex interactions between warming, rainfall variability, and deforestation (Keenan, 2015).

Beyond these general patterns, climate change has amplified disturbance regimes that exert long-lasting effects on forest dynamics. Among these, biotic stressors such as insect and pathogen outbreaks are projected to intensify, reshaping forest composition and ecosystem services at regional and global scales (Price et al., 2013; Trumbore et al., 2015; Seidl et al., 2017; Stanturf et al., 2024). In particular, bark beetles have emerged as one of the most destructive disturbance agents across the Northern Hemisphere. Their outbreaks interact strongly with climate extremes, especially drought, leading to unprecedented levels of tree mortality (Bentz et al., 2010; Sommerfeld et al., 2018). Central European forests provide striking examples of these dynamics. Large-scale bark beetle infestations, particularly in Norway spruce (*P. abies*) monocultures, have become the dominant disturbance in recent

decades (Seidl et al., 2016; Hlásny et al., 2019, 2021). The susceptibility of Norway spruce is influenced by both ecological and physiological factors. As a species adapted to cool, moist conditions, spruce is inherently vulnerable to drought stress, which reduces hydraulic conductivity and limits the synthesis and mobilization of defensive compounds. Extensive planting beyond its ecological optimum, often in even-aged monocultures, has further amplified vulnerability by creating dense, uniform stands that favor the spread of pests and hinder adaptive resilience (Hlásny et al., 2019; Singh et al., 2024a).

Climate warming compounds these risks by directly enhancing bark beetle population dynamics. Warmer conditions accelerate brood development, increase voltinism, and extend swarming periods, enabling more generations per year and expanding beetle ranges into previously unsuitable regions (Schlyter et al., 2006; Ghimire et al., 2015; Marini et al., 2017). At the same time, prolonged drought and irregular frost cycles weaken tree defenses and reduce vitality, leaving stands more susceptible to colonization. Disturbance interactions amplify these vulnerabilities: drought-induced mortality and wind damage create breeding substrates for beetles, facilitating explosive population growth that quickly overwhelms forest resilience (Netherer et al., 2022).

The convergence of climate extremes, forest management legacies, and bark beetle biology has therefore reshaped disturbance regimes in European conifer forests. The episodic outbreaks have increasingly become recurrent and widespread phenomena, driving shifts in forest structure and function that are likely to intensify under future climate scenarios. Norway spruce, given its ecological traits, economic significance, and current patterns of decline, stands at the center of this challenge. Understanding how climate change alters spruce vulnerability, through interactions between drought, physiology, and bark beetle dynamics, is thus critical for developing adaptive management and conservation strategies in the decades ahead.

3.2 Main drivers of forest disturbance in the Bark beetle context

3.2.1 Wind

In recent years, the frequency of extreme wind events has increased, resulting in the uprooting of trees (Peterson, 2000; Wohlgemuth et al., 2022; Lehmannski et al., 2024). Bark beetle outbreaks are often preceded by windthrow events since windfalls provide abundant development substrate for large numbers of pest beetles (Hanewinkel et al., 2008; Gochnour et al., 2022). The vulnerability of forest ecosystems to wind damage is influenced by several

factors, including the traits of individual trees, the overall stand structure, and specific site conditions (Peterson, 2000; Mitchell, 2013; Mezei et al., 2014; Seidl et al., 2014). As spruce stands mature, their vulnerability to wind disturbance increases, and structurally unstable individuals are typically the first to be lost. Early wind events typically cause the fall of isolated trees without initiating large-scale collapse; however, successive storms or bark beetle infestations can subsequently lead to additional, progressive damage within the stand (Schütz et al., 2006; Mezei et al., 2014). Once the strongest and most stable trees, known as "skeleton trees," begin to break down, gaps are created in the forest, followed by a total dissolution of stands when wind events continue. These larger openings create forest edges, which have been found to attract bark beetles and often serve as starting points for infestations (Marešová et al., 2020). Small canopy openings act as interspace gaps (Zabihi et al., 2023, 2025). However, the presence of larger gaps or forest edges tends to increase the likelihood of destabilization, whether caused by wind alone (Schütz et al., 2006) or through a combination of wind and bark beetle damage (Jakuš, 1998; Mezei et al., 2014).

Prolonged drought, when combined with windthrow or other abiotic disturbances, greatly intensifies bark beetle outbreaks by allowing populations to exceed the threshold required to attack healthy trees. Windthrown trees provide ideal breeding material for *I. typographus*, leading to rapid increases in beetle numbers. As populations grow, the insects can overwhelm the natural defenses of even vigorous trees, resulting in extensive mortality and widespread forest decline (Christiansen and Bakke, 1988; Hlásny et al., 2021). These combined stressors, including drought, wind, and bark beetle activity, create a reinforcing cycle that accelerates forest degradation.

3.2.2 Stand structure and composition

Forest structure, particularly stand density, age class, and spatial arrangement, plays a critical role in shaping bark beetle outbreak dynamics (Fettig et al., 2007; Sproull et al., 2015). Along an elevation gradient, stand density can substantially shape individual tree traits, including transpiration. Increased density elevates crown closure, which in turn influences bark surface temperature, DBH, and related attributes such as tree height and crown area by intensifying competition for limited water and nutrient resources (Zabihi et al., 2023; Figure 1). In particular, dense single-age plantations established for commercial production tend to exhibit reduced resilience, as sustained resource competition progressively weakens the trees. This vulnerability is compounded by reduced carbon-based defenses, such as resin production, which is essential

for resisting bark beetle infestations (Zhang et al., 2015; Young et al., 2017; Netherer et al., 2019). Another factor, windthrow, as discussed in the previous section, fragments forest stands by creating canopy gaps and exposed edges, which attract bark beetles. These conditions, characterized by increased solar radiation, wind exposure, and lower humidity at forest edges, alter the microclimate and amplify beetle reproduction and infestation (Hanson and Lorimer, 2007; Marešová et al., 2020).

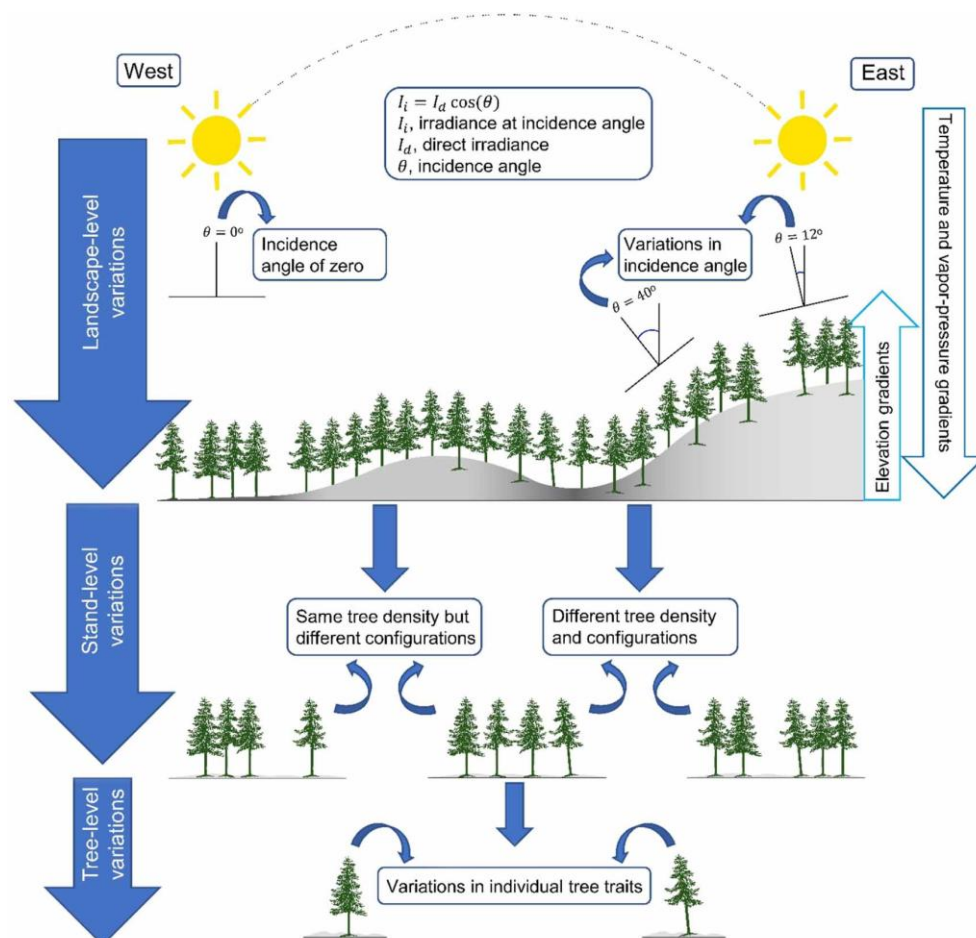


Figure 1 | A schematic representation of influential factors on sap flow variability at nested scales from the landscape- to stand- and tree-level. The size and direction of the blue arrows represent the magnitude and direction of factors acting or interacting at different scales. At the landscape level, when elevation increases, the decrease in air temperature and vapor pressure influences sap flow. Higher elevations are generally associated with steeper landscapes, which cause variations in the incidence angle. When the incidence angle decreases (either due to landscape terrains, as shown here, or sun positions at different times of the day, season, or year), the intensity of solar irradiance increases. At the stand level, the density and spatial configurations of trees determine the allocation of resources (water, nutrients, and solar irradiance) among trees, as well as the heat loads on trees (influencing bark temperature) and interspace forest grounds, which collectively influence sap flow. At the tree level, variations in DBH and above- and below-ground tree structures are other potential driving factors of sap flow variabilities (Zabihi et al., 2023).

Monocultures were introduced in Europe in the 18th and 19th centuries to address timber shortages by prioritizing high-yield species (Griess and Knoke, 2011). Norway spruce, for instance, has become widely cultivated for its rapid growth and favorable timber qualities (Spiecker, 2000; Caudullo et al., 2016). However, frequent droughts and heatwaves now threaten these monocultures, with significant increases in spruce mortality rates observed in Europe from 1970 to 2019. These drought events, along with structural changes in forest composition, have contributed to an estimated 50% increase in bark beetle outbreaks (Seidl et al., 2011). In the Czech Republic, Norway spruce constitutes roughly 50% of forest species composition, predominantly in monoculture stands with vulnerable age classes (Čermák et al., 2021). In addition to extensive planting outside its native range, increased growing stocks and altered age structures have heightened the susceptibility of spruce forests to *I. typographus* infestations (Seidl et al., 2011; Hlásny et al., 2021).

Intensive management practices, such as sanitation felling, can paradoxically increase forest vulnerability by creating new edges, which expose trees to additional environmental stress and bark beetle attacks (Hanson and Lorimer, 2007; Özçelik et al., 2022). In contrast, strategies such as thinning reduce density and competition, promoting resilience by enhancing individual tree vigor and resistance to beetle infestations (Fettig et al., 2007; Hood et al., 2016). A more adaptive approach that tailors forest management to incorporate topographic and structural variations may, therefore, improve forest resistance (Zabihi et al., 2023, 2025; Thomas et al., 2024).

To counter the vulnerability of monoculture forests, increasing tree diversity and varying age classes has been widely tested and recommended for enhancing forest resilience to both biotic and abiotic stresses (Lohbeck et al., 2016; Singh et al., 2025). There is growing evidence that forests composed of multiple species contribute positively to environmental health, economic outcomes, and community values. Carefully selecting species with complementary structural and functional traits in mixed plantations supports greater ecosystem stability and productivity (Hartley, 2002; Forrester et al., 2005; Pawson et al., 2013; Carnol et al., 2014; Alem et al., 2015; Drössler et al., 2015).

3.2.3 Thermal stress

Thermal stress in plants refers to the physiological and metabolic disruptions that occur when temperatures exceed optimal growth thresholds. When high temperatures coincide with limited water availability, conifers experience reduced photosynthetic capacity, constrained

carbohydrate allocation, and impaired growth and reproduction (Bita and Gerats, 2013). These changes have direct consequences for bark beetle susceptibility. Norway spruce relies on sustained production and pressure of oleoresin to physically expel and chemically deter attacking beetles. Heat and drought together reduce resin duct formation, lower resin pressure, and limit terpenoid biosynthesis, leading to a weakened primary defense. Under these conditions, trees are less capable of resisting mass attack events initiated by pheromone-coordinated groups of bark beetles. Recent findings show that acute drought can rapidly depress photosystem efficiency, increase needle temperature, and reduce terpene and monoterpene production in spruce, which directly correlates with increased successful colonization by *I. typographus* and *I. duplicatus* (Basile et al., 2024).

Thermal stress also interacts strongly with bark beetle population dynamics. Warmer summers and milder winters increase overwintering survival, accelerate larval development, and often permit additional beetle generations per year. This results in faster population build-up and a higher likelihood of outbreak conditions (Hlásny et al., 2019; Gullino et al., 2022). Heat-stressed trees emit stress volatiles and display reduced canopy vigor, signals that beetles use during host selection. Trees growing at forest edges or in recently thinned stands are particularly vulnerable because they experience higher solar radiation, trunk heating, and vapor-pressure deficits compared to trees in closed canopies (Emmel et al., 2013; Vanderhoof et al., 2014; Hroššo et al., 2020; Stříbrská et al., 2023). However, bark beetle attacks are not restricted to forest edges. Hyperspectral imaging studies have demonstrated that drought and acute thermal stress produce detectable spectral shifts in needles and crowns across entire stands, including interior trees that do not experience direct edge exposure (Pivovar et al., 2025). Such stress-induced physiological signatures occur before visible canopy decline, indicating that bark beetles may locate weakened hosts deep within stands by responding to subtle stress volatiles rather than relying solely on microclimate differences near edges.

At the stand scale, abrupt changes in microclimate such as increased radiation, elevated temperatures, reduced humidity, and stronger wind exposure influence both species composition and tree water relations (Kautz et al., 2013). These conditions reduce transpiration control and further deplete internal water reserves (Herbst et al., 2007; Marešová et al., 2020; Özçelik et al., 2022). As water stress intensifies, the tree's ability to maintain resin flow declines, and beetles gain a substantial advantage. The combination of rapid physiological decline (Basile et al., 2024) and early stress detectability from above-ground spectral signals (Pivovar et al., 2025) suggests that bark beetle outbreaks can initiate and expand within stand

interiors long before edge trees exhibit visible decline. Thus, thermal stress, weakened resin-based defenses, and climate-driven increases in bark beetle reproduction interact to create conditions that favor frequent and severe infestation events.

3.2.4 Drought Stress

Intense and recurring drought events compromise the defense mechanisms of host trees, making them more susceptible to bark beetle infestations (Berryman, 1972; Christiansen et al., 1987; Erbilgin et al., 2021). Drought stress occurs when soil water levels drop below a critical threshold, resulting in reduced soil water content and increased hydraulic resistance at the root-soil interface. This can cause a breakdown in water cohesion and xylem vessel embolism, ultimately leading to the disruption of irreversible water transport and premature mortality in roots and twigs (Cruziat et al., 2002; Cochard et al., 2009). Research suggests that while moderate drought may limit tree growth, it enhances carbon allocation to defense mechanisms; however, severe drought restricts the production of defense metabolites (Desprez-Loustau et al., 2006; Ferrenberg et al., 2015).

Conifer species differ greatly in their ability to manage drought, but species lacking the ability to cope with different water regimes are more likely to be susceptible to bark beetle attack, e.g., Ponderosa pine and Norway spruce. Isohydric conifer species, such as Norway spruce, delay the decline in xylem water potentials by reducing transpiration through stomatal closure (Rothe et al., 2002; Schume et al., 2004). Although stomatal regulation helps maintain water potential in the xylem, it also reduces sap flow and gas exchange between leaves and the atmosphere, limiting photosynthesis while respiratory carbon consumption continues (Bréda et al., 2006; Rennenberg et al., 2006). As a result, repeated bark beetle attacks deplete stored carbon reserves (i.e., non-structural carbohydrates), and trees struggle to replenish them due to impaired photosynthesis, leading to a reduction in the production of defense metabolites (Erbilgin et al., 2021). Additionally, while stomatal regulation helps prevent a lethal drop in xylem water potential, it can also shrink tracheid diameters due to increased water tension. This shrinkage, combined with a decrease in turgor pressure inside the epithelial cells of resin ducts, reduces the physical pressure on oleoresin, thereby lowering resin exudation rates upon wounding. This compromises the tree's ability to defend against additional drought episodes, frost, and pest attacks, potentially leading to tree mortality (Figure 2; Cruziat et al., 2002; Bréda et al., 2006; Cochard et al., 2009; Rissanen et al., 2016).

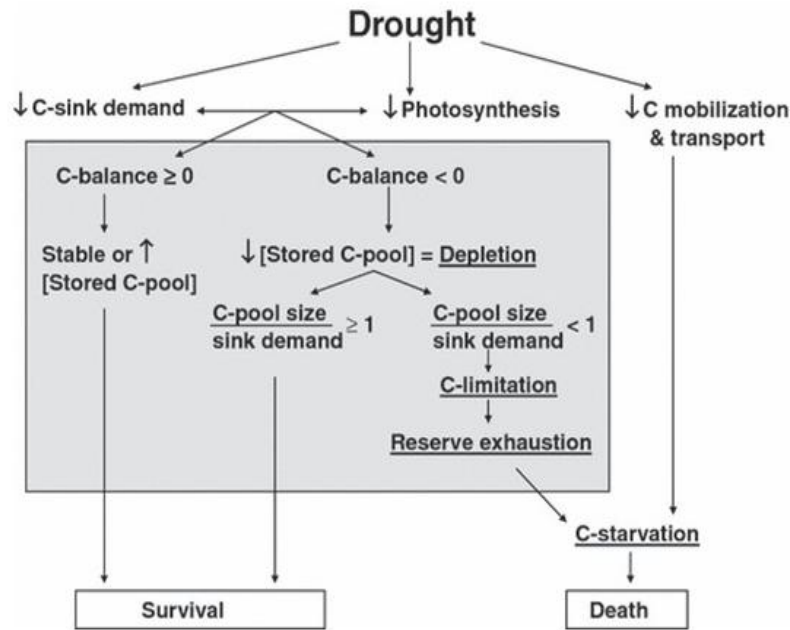


Figure 2 | Effects of drought on carbon assimilation, demand, and transport, and possible consequences for plant survival. The grey area depicts the possible effects of drought on whole-plant C balance and on the concentration of stored C pools, assuming no constraints on C mobilization and transport (Sala et al., 2010).

3.3 Bark beetles

Bark beetles (Coleoptera: Curculionidae: Scolytinae) represent one of the most species-rich and economically impactful groups of forest insects, with a particularly strong influence on coniferous ecosystems. Globally, more than 6,000 species have been described, many of which primarily colonize dead, dying, or physiologically compromised trees and thus play an important role in forest nutrient turnover. In contrast, a subset of bark beetle species is capable of coordinated mass attacks on living, healthy hosts, leading to large-scale forest damage and extensive tree mortality, especially across forests of the Northern Hemisphere (Raffa et al., 2015; Valeria et al., 2016; Hlásny et al., 2021; Singh et al., 2024a). These insects belong to the weevil family and display a wide range of mating and reproductive strategies. In certain taxa, such as *Dendroctonus*, reproduction is typically monogamous, with females acting as the primary colonizers and pairing with a single male. In contrast, species within the genus *Ips* exhibit a polygamous system, in which males initiate host attacks and subsequently mate with multiple females (Bleiker et al., 2013; Biedermann et al., 2019; Singh et al., 2024a). Bark beetles identify suitable host trees primarily through kairomones, which are host-derived chemical cues that guide beetles to stressed or susceptible trees. After locating an appropriate

host, they often mount coordinated mass attacks, penetrating the stem and establishing their galleries within the intercortical tissues, where reproduction and larval development take place. Although bark beetles play an important ecological role by facilitating decomposition and nutrient redistribution through the colonization of dead or dying trees, large-scale attacks on living hosts can severely alter forest structure and function. Such outbreaks may lead to extensive tree mortality and ecosystem disruption, rendering bark beetles among the most damaging insect pests in forested landscapes (Vega and Hofstetter, 2014). This chapter focuses on the ecological characteristics and attack strategies of the Eurasian spruce bark beetle, *Ips typographus*.

3.3.1 Eurasian spruce bark beetle, *I. typographus*

The Eurasian spruce bark beetle, *I. typographus*, is a small insect (4.5-5 mm) that exhibits sexual dimorphism. They are typically univoltine but may shift to polyvoltine due to the influence of high temperature, resulting in accelerated growth (Hlásny et al., 2011). During the endemic phase, these beetles primarily infest mature trees that have been weakened by biotic or abiotic stress factors, such as high temperatures, drought, disease, or herbivory. The beetles maintain associations with microbial symbionts, including bacteria and ophiostomoid fungi, which significantly contribute to overcoming tree defenses (Lieutier et al., 2009; Zhao et al., 2019). After overwintering, the new generation of beetles emerges from the forest litter or Norway spruce trunks and disperses, traveling distances ranging from 100 meters to several kilometers in search of suitable host trees (Biedermann et al., 2019). Such host selection strategies are based on the plant volatiles in the forest, which may serve as attractants or repellents (Raffa et al., 2015). The interaction between host trees and bark beetles is multifaceted, influenced by various factors operating at multiple spatial and temporal scales (Jakuš et al., 2011; Kautz et al., 2011; Netherer et al., 2024). Additionally, *I. typographus* exhibits density-dependent host colonization behavior. In the endemic phase, beetle populations target windthrown, dead, or weakened trees, whereas during the epidemic phase, they can colonize healthy trees (Økland and Berryman, 2004; Økland and Bjørnstad, 2006).

In the Czech Republic, Norway spruce forests experienced severe mortality in 2019, with an estimated loss of 118 million m³ due to droughts, heatwaves, and bark beetle outbreaks (Figure 3; Knížek et al., 2020; Ebner, 2020). Among the factors previously discussed, the structure of Czech forests, which is highly susceptible to such disturbances, has contributed to these large-scale outbreaks (Figure 4; Hlásny et al., 2021b). Conventional management

methods, including pheromone-based mass trapping, salvage logging, insecticides, and anti-attractants, have shown limited effectiveness in controlling bark beetles during peak outbreaks. As a result, the focus has shifted toward advanced functional genomics approaches such as RNA interference (discussed in detail in Chapter 5.1).

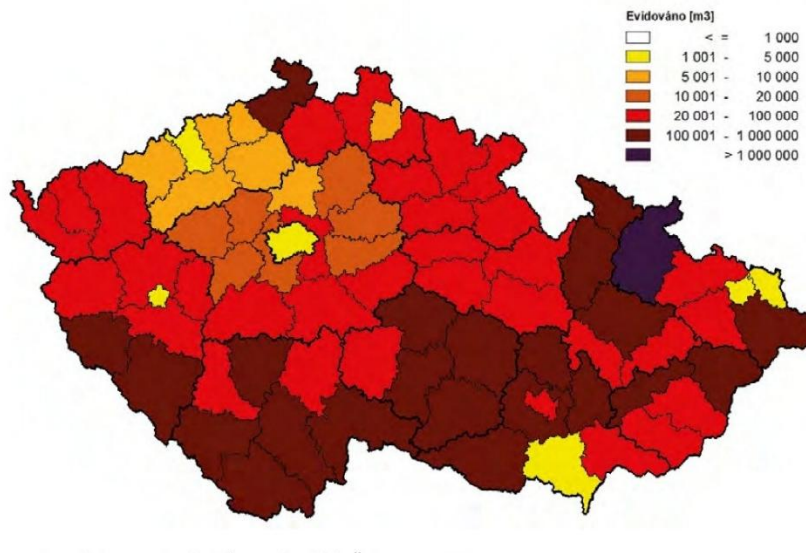


Figure 3 | Reported volume of spruce bark-infested wood resulting in losses across the Czech Republic. (Knížek et al., 2020).

3.3.2 Attack dynamics

At the stand level, once pioneer male beetles successfully breach the host tree's defenses, they release aggregation pheromones—2-methyl-3-buten-2-ol (MB) and (-)-*cis*-verbenol (cV)—which attract additional beetles to the site (Keeling et al., 2021; Figure 5B.a). During the construction of the nuptial chamber, male beetles emit large amounts of MB, while cV production varies depending on the concentration and enantiomeric composition of α -pinene in the Norway spruce phloem (Birgersson et al., 1984; Figure 5B.b). The pheromones released by the infested trees attract both male and female beetles to the area, and the newly arriving beetles further emit these aggregation pheromones to coordinate a mass attack on the host tree (Blomquist et al., 2010). Once inside the host, adult beetles construct mating chambers, mate, and create oviposition galleries where they lay eggs. The eggs hatch into larvae that feed and develop beneath the bark before emerging from the parent trees. Depending on factors such as the host's phloem thickness, the population of *I. typographus* can increase by up to 15-fold from one generation to the next (Hlásny et al., 2019).

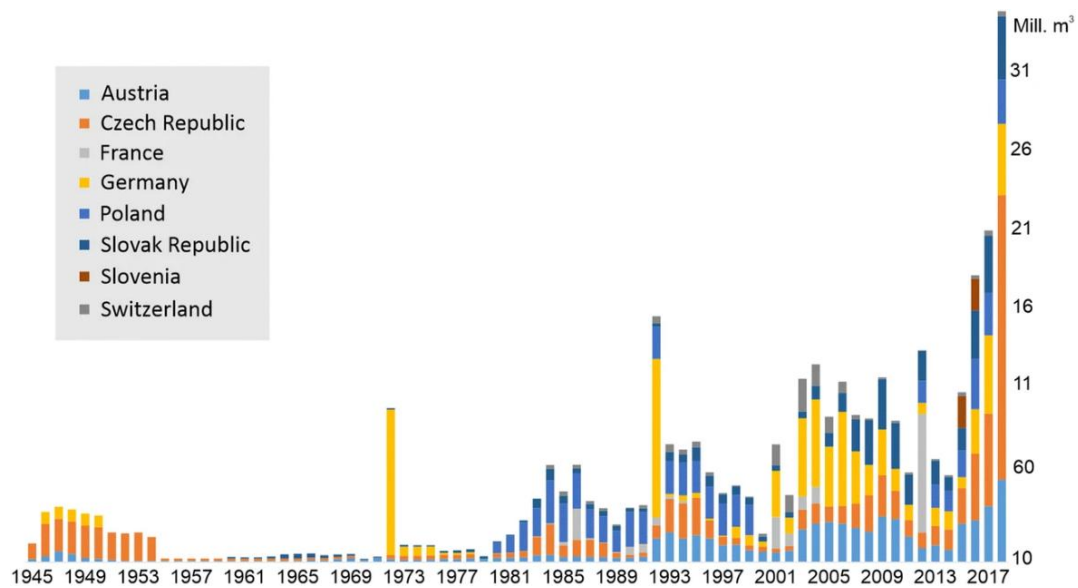


Figure 4 | Volume of Norway spruce killed by *Ips typographus* and other bark beetles in European countries since 1945 (Hlásny et al., 2021b).

In the mass attack phase, the first attacked tree is completely taken over by several male and female beetles. Once the host tree is fully occupied, the production of anti-attractant compounds, such as Verbenone, starts either by the beetle's symbiotic gut microbiome or the tree microbiome as a stop signal for further invasion to avoid overcrowding (Xu et al., 2015), alongside the declining production of aggregation compounds. This leads to an increased attraction towards the second tree (nearby fresh host), where a small proportion of beetles land to initiate the new mass attack phase (Figure 5Bc and Bd). This whole mechanism marks the completion of the first “switching” as very few beetles are attracted towards the first tree due to less concentration of MB + cV and increasing amounts of inhibitory compounds Ipsdienol (Id) and Ipsenol (Ie). It is crucial to note that semiochemicals Id and Ie may act as an attractant in smaller amounts but have an inhibitory effect in higher concentrations, and during the late phase of host colonization, they act as “shut-off” signal regulating attack density (Schlyter and Anderbrant, 1989; Figure 5B.e). The second attacked tree becomes the locus of attack, while a

few beetles deflect towards an adjacent tree. This way, the beetles increase in number, and with an increased population, they can attack healthy trees.

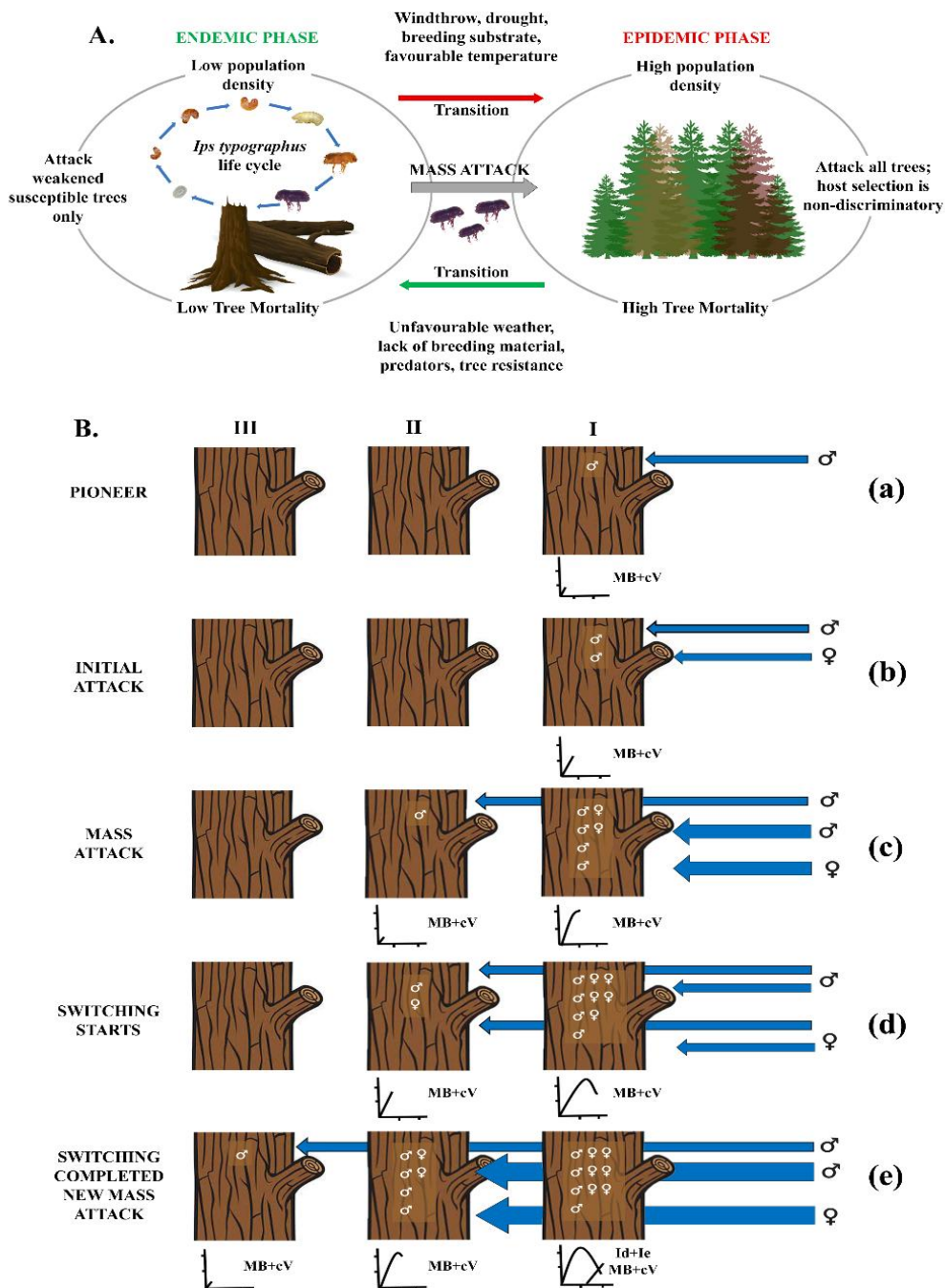


Figure 5 | Conceptual overview of *Ips typographus* population dynamics. (A) During the endemic stage, beetles typically utilize weakened or recently disturbed material such as wind-felled trees, logs, and stumps for feeding and reproduction. Under conducive conditions, populations may shift to an epidemic state. This transition is driven by multiple interacting factors, including favorable climatic conditions, increased availability of suitable breeding substrates, drought stress, and wind disturbance. In the epidemic phase, population densities become high enough to enable coordinated mass attacks on physiologically intact trees, often leading to extensive tree mortality. Subsequently, population levels may decline and revert to the endemic phase due to adverse weather, depletion of breeding material,

increased pressure from natural enemies, and enhanced resistance in surviving host trees (modified after Kautz et al., 2014). **(B) Host colonization and pheromone-mediated attack dynamics.** (a) Host entry is typically initiated by pioneer individuals—males in *Ips* species and females in *Dendroctonus*. In *I. typographus*, pioneering males emit aggregation pheromones, primarily 2-methyl-3-buten-2-ol (MB) and (–)-cis-verbenol (cV), to recruit conspecific males and females. (b) If the initial beetles successfully overcome host defenses, the arrival of additional individuals results in intensified pheromone production, promoting mass attack. This collective assault aims to suppress host defenses sufficiently to allow successful reproduction. (c) While the primary host tree (I) is undergoing mass colonization, a fraction of males disperses to adjacent, previously unattacked trees (II), initiating new colonization events. (d) As emission of attractive pheromone components declines at the original host, beetle attraction gradually shifts away from tree (I) and toward the neighboring tree (II), marking the onset of an attraction switch. (e) Once this switching process is complete, aggregation pheromone output remains low, whereas inhibitory pheromones (ipsdienol and ipsenol) are produced in greater quantities. At this stage, beetle attraction is focused on tree (II), where a new mass attack develops, and a subset of males again disperses to another uncolonized tree (III), perpetuating the cycle. The accompanying graphs illustrate logarithmic pheromone release patterns based on Birgersson et al. (1984). Symbols along the x-axis correspond to attack phases 1, 3, and 6, representing males boring into the bark (1), completion of nuptial chamber construction (3), and pairing with females (6), respectively. (Singh et al., 2024a).

3.4 Norway spruce and its defense mechanisms

Norway spruce employs complex defense mechanisms to protect against bark beetles, utilizing both morphological and chemical strategies. Morphological defenses include features such as necrotic lesions, thickened bark, and stone cells, which act as physical barriers. Chemical defenses, on the other hand, involve the production of toxic substances, including terpenoid oleoresins stored in resin ducts and phenolic compounds within parenchyma cells (Biedermann et al., 2019; Hlásny et al., 2019).

The chemical defenses of conifers primarily depend on terpenes and phenolic compounds, with stilbenes and flavonoids playing a key role in deterring bark beetles and their associated fungi. Phenolic compounds are synthesized through the shikimic acid pathway, using phenylalanine as a precursor. Terpenes, on the other hand, are produced via two distinct pathways: the mevalonate pathway in the endoplasmic reticulum/cytosol and the 1-deoxyxylulose-5-phosphate pathway in plastids. Both pathways produce isopentenyl pyrophosphate (IPP), a five-carbon building block. Through the action of prenyltransferases, IPP is converted into the central intermediates geranyl pyrophosphate, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate, which are then transformed by terpene synthases into monoterpenes, sesquiterpenes, and diterpenes (Celedon and Bohlmann, 2019; Hammerbacher et al., 2019).

Conifer defenses may be either constitutive (inherent) or activated in response to environmental stress. Induced defenses, both anatomical and chemical, involve the formation

of traumatic resin ducts, increased terpene levels, alterations in phenolic composition, and the proliferation of polyphenolic parenchyma cells after stimuli such as beetle attack, fungal infection, methyl jasmonate (MeJA) application, or physical damage (Martin et al., 2002; Zhao et al., 2011). Fungal inoculation and MeJA treatment have been shown to trigger terpene production similarly to bark beetle attacks (Martin et al., 2002; Zhao et al., 2011; Schiebe et al., 2012). For example, Zhao et al. (2011) reported that mono-, sesqui-, and diterpene levels in certain conifers rose to 240-fold within 35 days of inoculation with the pathogenic blue-stain fungus *Ceratocystis polonica*, an associate of *I. typographus*. Trees exhibiting these induced defenses experienced only a five percent attack rate from beetles compared to untreated trees.

3.4.1 Molecular Mechanisms Underpinning Drought Stress Response

Drought stress triggers a complex array of physiological, biochemical, and molecular responses in plants, which are essential for survival in water-limited environments (Farooq et al., 2009; Mahmood et al., 2019). Transcriptome analyses reveal various functional gene groups involved in these responses, reflecting a sophisticated interplay of signaling pathways, gene expression, and metabolic adjustments that enable adaptation to drought conditions. For instance, many genes associated with plant metabolism, particularly carbohydrate metabolism, are upregulated under drought stress, with the accumulation of sugars linked to desiccation tolerance; these compounds help protect plant structures from mechanical and metabolic damage during dehydration (Oliver et al., 2010).

A central molecular response to drought involves the regulation of gene expression through transcription factors (TFs) such as dehydration-responsive element-binding proteins (DREBs) and NACs, which coordinate the activation of stress-responsive genes (Takahashi et al., 2018; Soma et al., 2021; Zhao et al., 2023). Drought-responsive gene promoters feature a DRE/CRT motif that binds ABA-independent DREB/CBF TFs, serving as a coupling element for ABA-dependent gene expression through ABRE elements. Notably, DREB1A/CBF3, DREB2A, and DREB2C proteins interact with AREB/ABF TFs, indicating a crosstalk between ABA-dependent and independent signaling pathways. Under osmotic stress, AREB/ABF TFs and SnRK2 kinases activate DREB2A transcription, suggesting complex interactions at both transcript and protein levels (Joshi et al., 2016). Another crucial response group includes genes associated with defense and cellular rescues, such as those encoding late embryogenesis abundant (LEA) proteins, heat shock proteins (HSPs), and dehydrins. HSPs function as molecular chaperones, facilitating proper protein folding and protecting against unfolding and

denaturation, playing roles in responses to various abiotic stressors (Wang et al., 2004). LEA proteins facilitate detoxification and may also serve as chaperones, with a well-established role in desiccation tolerance (Battaglia et al., 2008). Dehydrins, a large family of LEA proteins, are specifically linked to responses to water deficiency and are thought to protect cellular metabolism under stress, as documented in various forest tree species (Bae et al., 2009; Vornam et al., 2011).

Transport processes are equally critical for the mobilization and accumulation of solutes and hormones. For instance, sugar transporters help adjust osmotic pressure, while ABC transporters respond to diverse biotic and abiotic stresses (Wanke and Kolukisaoglu, 2010). Aquaporins, as channel proteins in cellular membranes, play a crucial role in maintaining water balance under drought conditions. A significant portion of drought stress-related proteins participates in two primary regulatory pathways: the ABA-dependent and ABA-independent pathways, which are interconnected (Hirayama and Shinozaki, 2010). In the ABA-dependent pathway, transcription factors such as bZIPs, NACs, MYBs, and MYCs play key roles, while the ABA-independent pathway prominently features NAC and DREB transcription factors (Olsen et al., 2005).

The advent of next-generation sequencing (NGS) has revolutionized the field of genome analysis, providing deeper insights into the complex genomes of plants. In the case of Norway spruce, a significant breakthrough was achieved when its genome was sequenced, marking a milestone in conifer genomics (Nystedt et al., 2013). This genome sequence was the first draft among conifers, and it has been instrumental in advancing studies on gene expression and molecular regulation under stress conditions. However, despite extensive research, the functions of many drought-responsive genes in Norway spruce remain unknown due to a lack of homology with known sequences. The massive size and complexity of the Norway spruce genome, nearly 20 gigabases, pose substantial challenges similar to those faced by other conifer genomes. Due to its extremely large size and complexity, significant research efforts have been directed toward transcriptomic analysis.

Despite the importance of accurate gene expression analysis in forestry, breeding, and the study of seed plant traits, suitable internal control genes have not been identified for Norway spruce to date. Reference genes, also known as internal controls, are genes that are stably expressed and required for basic cellular functions. Their stability is critical for reliable real-time quantitative polymerase chain reaction (RT-qPCR), a widely used method valued for its sensitivity, specificity, reproducibility, and speed. Because the accuracy of RT-qPCR depends

on the stability of reference genes, which often encode proteins involved in cell cycle regulation, DNA replication, or metabolism, an ideal reference gene should maintain uniform expression across tissues, developmental stages, genotypes, and experimental conditions (Han et al., 2012; Lü et al., 2018). Identifying such genes is therefore critical for improving gene expression profiling, providing deeper insights into biological processes and regulatory networks, and ultimately enhancing our understanding of factors that support spruce resistance, resilience, and survival (Wise et al., 2007). We aimed to address this gap by identifying suitable reference genes in Norway spruce across various stress conditions (drought, heat, pathogen infection) in seedlings, tissues (needle, phloem, root), and developmental stages (seedlings, mature trees) in Chapter 5.3.

Overall, this thesis aims to investigate the impact of stress factors on Norway spruce through a range of approaches, from field studies to molecular-based analyses, in the context of bark beetle infestations. These studies contribute to a deeper understanding of stress factors at both the landscape and molecular levels, which may be crucial in influencing the species' susceptibility to bark beetles.

4. Materials & Methods

This subchapter provides a brief description of the materials and methods used in the studies. The studies II, III, and IV refer to data papers corresponding to Chapters 5.2, 5.3, and 5.4, respectively.

4.1 Field Experiments

The field study described in Chapter 5.2 was conducted in research plots of the Czech University of Life Sciences, managed by the School Forest Enterprise (ŠLP) in Kostelec nad Černými lesy (49.9940° N, 14.8592° E) in the eastern district of the Central Bohemian region of the Czech Republic. The area is dominated by coniferous stands with fir (*Abies* spp.) and beech (*Fagus* spp.), underlain by granodiorite bedrock with Luvisol soils. The climate is characterized by warm, dry summers, a vegetation period of 150–160 days, a mean annual temperature of 7.0–7.5°C, and a mean annual precipitation of 600 mm.

The experiment was established in 2018 as part of the EXTEMIT-K project across six mature Norway spruce (*P. abies*) stands aged 90–100 years. Each plot was divided into four subplots (A–D) with 8–10 trees per subplot. Trees had a mean DBH of 40.5 ± 5.7 cm. For the present study, five plots (excluding plot 2) were included (Figure 6A). Plots 3 and 4 were separated by ~430 m, whereas plots 1, 5, and 6 were located 2–9 km away.

4.1.1 Experimental Setup

Ecophysiological Measurements

Sap flow was measured using EMS81 sensors (EMS Brno, Czech Republic), installed in 2018 at a height of 2 m on mature trees selected for comparable height and DBH (Figure 6B). The sensors operate on the trunk heat balance (THB) principle, consisting of four electrodes, three of which are electrically heated and positioned above a reference electrode located 10 cm below. Climatic data were obtained from an automated meteorological station (Minikin QTHi, EMS Brno, Czech Republic) installed in an open area between experimental plots 3 and 4 (<200 m distance). Precipitation was measured using a Minikin ERi device equipped with a Pronamic Pro Rain gauge (EMS Brno). The meteorological station continuously recorded relative air humidity (%), air temperature (°C), and radiation levels, with photosynthetically active radiation (PAR) used to derive global radiation (W m^{-2}) for the study period. Soil water potential

(SWP, Ψ_w) was monitored with hexagonal sensors (Teros 21, Meter Group, München, Germany) installed at a depth of 20 cm, with five sensors deployed per subplot.

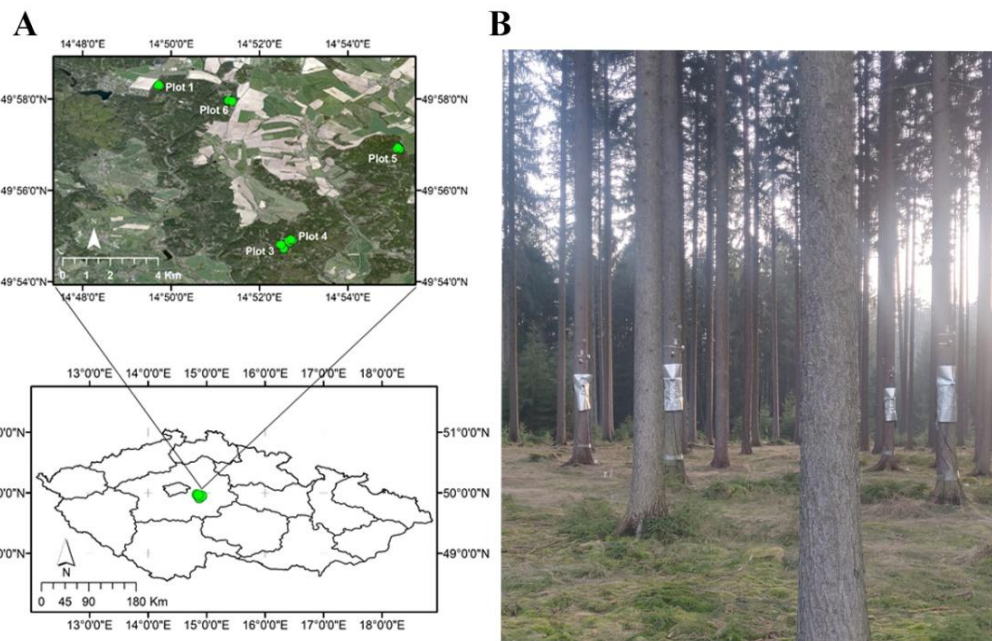


Figure 6 | (A) Study area showing Norway spruce tree positions (green circles) within five out of six established study plots (17 subplots) under the EXTEMIT-K project, located at the School Forest Enterprise (ŠLP) of the Czech University of Life Science near Kostelec nad Černými Lesy, Czech Republic. (B) Picture of the experimental setup from one of the subplots with trees equipped with sap flow sensors. (Singh et al., 2025a)

Tree density and solar irradiation data

Stand density and solar irradiation were quantified using UAV imagery at a 20 cm resolution, processed in ArcGIS Desktop (version 10.8.1; ESRI, 2020). For each monitored tree, a 10 m buffer was delineated and divided into four sectors (north, south, east, west). Trees within each sector were counted, and crowns overlapping boundaries were weighed according to the proportion of crown area in each sector. Solar azimuth angle, sun trajectory, and day length were derived from SunCalc.org (SunCalc, 2017; Figure 7).

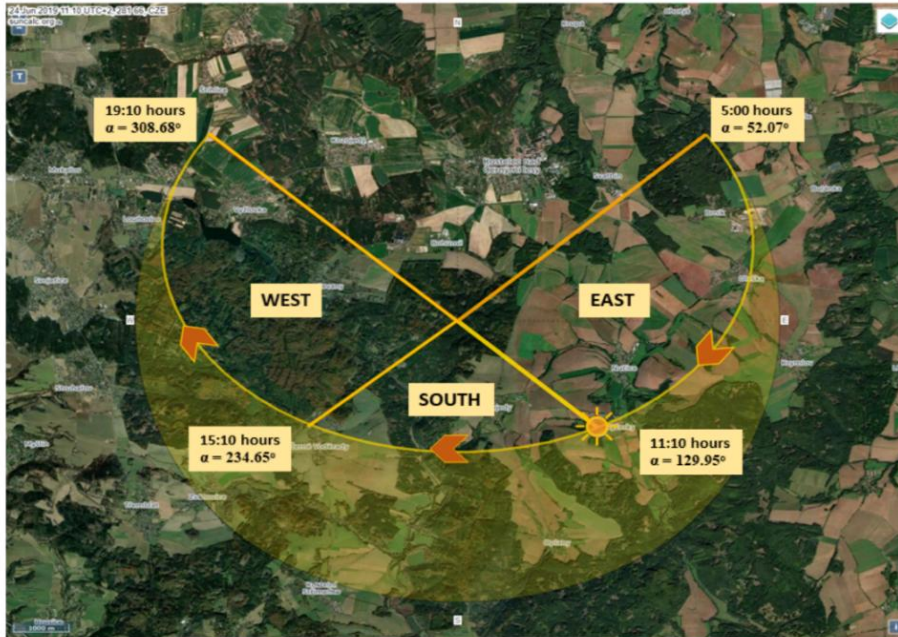


Figure 7 | Sun trajectory across sectors: East (05:00–11:10), South (11:10–15:10), and West (15:10–19:10). The yellow arrow indicates the observed sun path, while α denotes the azimuth angle between sector boundaries. Dawn and dusk occurred at 05:00 and 21:10, respectively. (Singh et al., 2025a)

4.2 Greenhouse experiments

For Chapters 5.3 and 5.4, three-year-old containerized Norway spruce seedlings, approximately 40 cm high, were used. Seedlings were procured from a local nursery and planted in a moist peat/perlite mixture in May 2022. The seedlings originated from seed sources collected in the Bohemian-Moravian Highlands, Czech Republic, a region situated at 500–600 m above sea level and receiving approximately 600–750 mm of annual precipitation. The seedlings were transferred to the greenhouse at the Czech University of Life Sciences, Prague. Before any treatment, the plants were irrigated regularly with tap water to acclimate them to the greenhouse environment (Figure 8).

4.2.1 Greenhouse Measurements and Sampling

The seedlings were acclimated to greenhouse conditions at the Czech University of Life Sciences, Prague, under a temperature of $23 \pm 2^\circ\text{C}$ and a 16-hour light/8-hour dark photoperiod. Before treatments, seedlings were irrigated regularly with tap water. They were then randomly assigned to either the control or drought treatment groups. Control plants received regular irrigation, while drought-stressed plants underwent a six-week period of water supply withdrawal.



Figure 8 | Experimental setup in the greenhouse. The left panel shows Norway spruce seedlings at the beginning of the acclimatization phase. The right panel shows seedlings after three weeks of drought treatment, with the drought-stressed seedling on the left and the well-watered control seedling on the right.

For Study III (Chapter 5.3), the plants were subjected to the following treatments: **Control treatment:** This group was irrigated regularly and was not subjected to any abiotic or biotic treatment. **Water deficit treatment:** One group was subjected to drought stress by gradually reducing the water supply until the mean plant water potential decreased to below -2.1 MPa. The degree of drought stress was quantified by measuring the predawn shoot water potential using a Scholander pressure chamber (PMS Instruments, Corvallis, Oregon, USA). **Heat stress:** Seedlings were placed in a climate chamber (FytoScope FS-SI 3400, PSI, Drásov, Czech Republic) at 35°C for ten days with a constant relative humidity of 80% and regular irrigation. **Biotic stress:** The seedlings were inoculated with the Ophiostomatoid fungus *Ophiostoma flexuosum* and incubated for one month in the controlled climate chamber mentioned above. Throughout the treatment period, the plants were carefully maintained under optimal humidity, light, and nutrient conditions. At the end of treatments, current-year needles, bark phloem, and fine roots were collected, snap-frozen in liquid nitrogen, and stored at -80°C.

For the identification of housekeeping genes under developmental conditions, tissue samples (needle, phloem, and root) were collected from mature trees in August 2022 from the field experimental plots of the School Forest Enterprise (ŠLP) in Kostelec nad Černými lesy. Needles were collected by shooting branches with a shotgun (Burnett et al., 2021). Phloem was

sampled using a 5 mm cork borer at a trunk height of ~2 meters, while lateral roots ~1 m from the trunk were sampled at 15 cm depth following Kalyniukova et al. (2024). All collected tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C for future analysis.

For the study in Chapter 5.4, a total of 48 seedlings were randomly allocated to control and drought treatment groups, with each group further divided into four biological replicates containing six seedlings. The control group was irrigated regularly, whereas the drought group experienced a gradual reduction in watering over a six-week period to induce a severe water deficit. Drought progression was assessed weekly by measuring midday shoot water potential on current-year lateral shoots using a Scholander pressure chamber (PMS Instruments, Corvallis, USA) in both control and treated seedlings. The treatment continued until water potential in the stressed group declined below -2.1 MPa, a level indicative of severe drought and associated with increased risk of xylem embolism in Norway spruce (Rosner et al., 2019). Temperature and photoperiod were maintained uniformly across treatments throughout the experiment. Following sampling, tissues were immediately snap-frozen in liquid nitrogen and preserved at -80°C for subsequent analyses.

4.3 Molecular Protocols

4.3.1 Samples used for RNA isolation

Study III

In Study III (Chapter 5.3), a total of 16 plants were used per treatment (drought, control, heat, fungi infection). The plants were grouped into sets of four, which were pooled to create one biological replicate. As a result, each treatment consisted of four biological replicates, with each replicate representing pooled samples of four plants. RNA isolation and cDNA library preparation were performed according to the protocols outlined in the subsequent section.

Study IV

Chapter 5.4 study involved a total of 24 plants for each treatment (drought and control) in the greenhouse experiment. For each treatment and tissue type, six plants were pooled to generate four biological replicates. The detailed methods and protocols are described below.

4.3.2 Protocols Used

RNA isolation

Total RNA from the needle, phloem, and root tissues was extracted using a modified cetyltrimethylammonium bromide (CTAB) (Singh et al., 2024c). Briefly, 100 mg of homogenized tissue was mixed with CTAB extraction buffer, followed by the addition of Chloroform:isoamyl alcohol (24:1) and centrifugation. The aqueous phase was then collected, and 0.5 volume of 5M lithium chloride was added, with the mixture incubated at -20°C for 1 hour. After centrifuging at 12,000 rpm for 10 minutes at 4°C , the supernatant was discarded, and the RNA pellet was washed twice with 70% ethanol. The dried pellet was dissolved in 50 μL of RNase-free water. To eliminate any genomic DNA, the RNA was treated with the TURBO DNase Kit (Invitrogen, USA). RNA quality and purity were assessed using a 1.2% agarose gel and a NanoDrop spectrophotometer (Thermo Scientific, USA), with absorbance ratios checked at 260/280 and 260/230 nm. Complementary DNA (cDNA) was synthesized from 1 μg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) following the manufacturer's instructions and stored at -20°C until further use.

Primer design and RT-qPCR

Primers for RT-qPCR assays in Study III & IV were designed using the PrimerQuest™ Tool (Integrated DNA Technologies, USA). For study III, fifteen candidates were selected from previously published literature and our in-house transcriptome dataset (Table 1). The RT-qPCR reaction mixture consisted of 5.0 μL of SYBR® Green PCR Master Mix (Applied Biosystems, USA), 1.0 μL of cDNA from fivefold dilutions, 0.5 μL forward and reverse primers each of 10 μM concentration, and 3.0 μL of RNase-free water (Invitrogen, USA) to make up a total volume of 10.0 μL . Amplification was conducted with initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The reactions were performed using an Applied Biosystems StepOne Real-Time PCR System (Applied Biosystems, USA). For Study III, melting curve analysis was performed to ensure gene-specific amplification and verify primer specificity, with a gradual increase in temperature from 60°C to 95°C , and *PaDHN5* from the dehydrin family was used for validation. The expression levels of the target genes were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001). For Chapter 5.4, thirteen genes were validated using RT-qPCR (Table 2), and *18S rRNA* was used as a reference gene for expression normalization after Singh et al. (2024b).

Table 1. Primer information of the reference genes and target gene used in study III.

Gene symbol	Gene name	Primer sequence		Amplicon length (bp)	PCR efficiency	Regression coefficient
<i>RPL26</i>	ribosomal protein L26	F:	CATCTAGTGTGCGGCGTATT	114	96.72	0.999
		R:	CTCGTACCACCTGAACTTCATC			
<i>PP2A2</i>	Serine/threonine-protein phosphatase PP2A-2	F:	GGCCTATGTGTGATCTACTGTG	113	96.99	0.997
		R:	GTGATTGAATTGGGCTGCTATG			
<i>RPL7Ae</i>	Ribosomal protein L7Ae	F:	CCACTGTTGGCTGAGGATAAG	119	100.47	0.996
		R:	CCCTCATTGGTTGTACAGAA			
<i>UBCP</i>	Ubiquitin-like domain-containing CTD phosphatase	F:	CCACAGAATGGGCTTGTGATA	126	96.96	0.999
		R:	GTGGCTCAGATCATCCAGTTC			
<i>SKIP22</i>	F-box protein	F:	TCCCCACAGAGCTCAAAGT	134	91.19	0.995
		R:	CTCAGCCGCATACTTCTTCTT			
<i>HSP90</i>	Heat shock protein	F:	GGCGATCAAGATGAAGCAAAG	138	111.92	0.991
		R:	AAGCACACATGGCGAAGA			
<i>CCZ1</i>	Vacuolar fusion protein	F:	CAAACAGCAATAGCAGTGAAGG	133	93.88	0.999
		R:	ACAGAGAGCTGGCTAGTAAGA			
<i>SDH5</i>	Succinate dehydrogenase	F:	GCTCTACGGGCTGCATATAAA	119	106.87	0.879
		R:	ACCAACAGCGTCACTAACC			
<i>RID2</i>	18S rRNA	F:	GAGGAGCACGAGCTGTATTG	104	93.72	0.987
		R:	CACAACCAGACCACCTGAAA			
<i>JMJ16</i>	Putative lysine-specific demethylase	F:	AGCAGATGTGGAGACTAGGA	122	93.88	0.993
		R:	GGAGCCGTGCAATGTTATTTAG			
<i>COG7</i>	Conserved oligomeric Golgi complex	F:	CCTCGGCTGAAGAAGACAAT	135	103.63	0.997
		R:	TTGTCCCAGCACCCAATAC			
<i>RPS10</i>	ribosomal protein S10	F:	CCTCGATTTGGTGACAGAGATG	110	96.72	0.999
		R:	GCCCTAAATTGAGGCTGGTATT			
<i>SP1</i>	ubiquitin-protein ligase SP1	F:	GGCACTACTCTGACTGTTGT	117	114.86	0.999
		R:	GGCAGATCCTAGCCGTTTC			
<i>TULP6</i>	Tubby-like F-box protein	F:	CCTGAGCCTAATCCATCAGTTT	104	94.79	0.999
		R:	AGTGGGTAGCGATAATCCATTG			
<i>ARP9</i>	Actin-related protein 9	F:	CCTGGAAAGGTGGTGCTATT	120	93.65	0.999
		R:	ATCTCTGTACTTTCGACCACTC			
Target gene						
<i>PaDhn5</i>	Dehydrin-like protein	F:	ATCAATGTGCGGGTGAAG	122	120.48	0.951
		R:	ACTCCCACTGATCTGAA			

Table 2. List of genes and their primer information used in RT-qPCR validation in study IV.

Transcript ID	Gene symbol	Gene name	Primer sequence (5'-3')	Amplicon length (bp)	T _m (°C)
MA_75192g0010	<i>NAC68</i>	NAC domain-containing protein 68	F: TTCGATCCGTGGCAACTA R: CCAGCAGTAACAGGTTTGTC	161	61 61
MA_9957372g0010	<i>IP3</i>	ABA/WDS-induced protein	F: GCATTGCACGCAAAGAAG R: GTGATGCTCGTGGAAAGATG	114	60 60
MA_125113g0010	<i>GPX4</i>	Probable phospholipid hydroperoxide glutathione peroxidase	F: GGATCTCAGCATCTACAAGG R: GGTTCTGTCCACCAAAT	168	59 59
MA_104187g0010	<i>TPS6</i>	alpha-trehalose-phosphate synthase	F: ACAGGGTAGCTCCAAAGT R: GCATCAGCAACAGCTTCTA	153	60 60
MA_10380599g0010	<i>PTI</i>	PINTA (-)-alpha-pinene synthase	F: CACTTGGGCTTCTCCATC R: GGACGGATCCCATCTCTTA	166	60 60
MA_10212729g0010	<i>TPSD2</i>	Pinene synthase	F: TAGAGCAACCGAGGAAGAT R: CTGCTGATGTCGAAAGTAGG	138	60 60
MA_5806734g0010	<i>GSTUN</i>	Glutathione S-transferase U23	F: CTCCAAGAGCGAACTGTTAC R: TATGCATTGGAGGGCAGA	156	60 61
MA_4505582g0010	<i>DHN5</i>	Dehydrin-like protein	F: ATCAATGTGCGGGTGAAG R: TTCAGATCAGTGTGGGAGT	122	60 60
MA_11183g0010	<i>BCH2</i>	Beta-carotene hydroxylase 2	F: AAGAGCCAGGAAGAAGGT R: CCGAACATCTCTGTGAAAGG	150	60 60
MA_104214g0010	<i>DHN2</i>	Dehydrin	F: ACCATCTGCAGAGACTGT R: CGGAGGATTCGTGGATAAA	121	60 60
MA_79644g0020	<i>PXG</i>	Peroxygenase	F: ATCCGGACAGACTCACATA R: TCCTCTGATGCTCTCCTTT	161	60 60
MA_18439g0010	<i>GDL62</i>	GDSL esterase/lipase At4g10955	F: GGCCTTCAGAGGAACAAATG R: GGACACATACTTCTGGACGA	139	61 61
MA_222634g0010	<i>LEA88</i>	Late embryogenesis abundant protein	F: CACTGAATCTGCCTCTTCTG R: TGGTGTCTTGACTTCCT	131	60 60

4.4 Spectrophotometric measurements

For study IV, free proline content was quantified using a ninhydrin-based colorimetric assay following the method of Bates et al. (1973) with minor modifications. For each treatment (control and drought), current-year needles from six individual seedlings were pooled to constitute one biological replicate, with four biological replicates per treatment. Approximately 0.5 g fresh mass (FM) of needle tissue was homogenized in 10 mL of 3% sulfosalicylic acid. The homogenate was filtered, and 2 mL of the filtrate was combined with 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent. The mixture was incubated in a water bath at 100°C for 1

h. After cooling, 4 mL of toluene was added and vortexed for 20 s. The absorbance of the toluene phase was recorded at 520 nm using a cuvette spectrophotometer (GBC Scientific Equipment, Victoria, Australia), with toluene serving as the reference.

Photosynthetic pigments were determined spectrophotometrically according to Lichtenthaler (1987). For each biological replicate, approximately 1 g of needle tissue was homogenized and extracted with 80% acetone. Absorbance was measured at 470, 646, and 663 nm using a spectrophotometer (Cintra, GBC Scientific Equipment, Victoria, Australia). Concentrations of chlorophyll a (chl a), chlorophyll b (chl b), total chlorophyll (chl a+b), and total carotenoids (Car x+c) were calculated, and values were expressed on a dry mass basis (mg g⁻¹).

4.5 RNA Sequencing, Library Preparation, and Analysis

Total RNA samples with RNA integrity number (RIN) ≥ 7.5 and 260/280 ratio ≥ 2.0 were selected for sequencing on the Illumina NovaSeq 6000 platform (NovoGene, China), generating approximately 50 million paired-end reads (PE150) per sample. mRNA was enriched using poly-T oligo-attached magnetic beads, and cDNA libraries were prepared with random hexamer primers. Raw sequencing data (FASTQ format) were processed using NovoGene's in-house scripts to remove adapter sequences, poly-N, and low-quality reads, resulting in high-quality clean reads. Quality metrics, including Q20, Q30, and GC content, were calculated. All downstream analyses were performed using clean data. Clean reads were aligned to the Norway spruce reference genome (*P. abies* v1.0; Nystedt et al., 2013) with Hisat2 v2.0.5 (Mortazavi et al., 2008). Differential gene expression analysis was conducted using the DESeq2 R package (ver. 3.22.5) (Robinson et al., 2010; Love et al., 2014). P-values were adjusted using the Benjamini-Hochberg approach to control the false discovery rate (FDR). Genes with adjusted p-values ≤ 0.05 and absolute log₂ fold change ± 1 were considered significantly differentially expressed.

Functional Annotation, Hierarchical clustering, Transcription Factor identification, and MapMan analysis of Transcripts

Functional annotation of DEGs was performed to characterize their biological roles and provide insight into molecular responses to drought stress. Gene Ontology (GO) enrichment analysis was conducted to assign DEGs to three principal domains: biological processes (BP), cellular components (CC), and molecular functions (MF). Significantly enriched GO terms were

identified using a corrected p-value threshold of < 0.05 . Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was carried out to identify metabolic and signaling pathways associated with DEGs. Both GO and KEGG analyses were performed using the `enrichGO` and `enrichKEGG` functions in the `clusterProfiler` R package (ver. 4.8.3; Yu et al., 2012) referencing the GO (<http://www.geneontology.org/>) for ontological data and metabolic pathways from the KEGG database (<http://www.genome.jp/kegg/>) for metabolic pathway annotations (Kanehisa et al., 2020). Statistical enrichment of DEGs in KEGG pathways was assessed within `clusterProfiler` using a corrected p-value < 0.05 as a threshold, ensuring robust identification of enriched categories. Hierarchical clustering analysis (HCA) of DEGs was conducted using log₂ fold change data, centroid linkage, and Euclidean distance in Cluster 3.0 (Eisen et al., 1998), generating dendrograms to visualize expression patterns and clustering dynamics. Transcription factors (TFs) were identified by extracting coding sequences (CDS) from DEGs and translating them into protein sequences with ESTScan 3.0, which facilitated the identification of open reading frames. The resulting protein sequences were queried against PlantTFDB 4.0 (Jin et al., 2016) using an E-value cutoff of $\leq 10^{-5}$ to classify the TF families.

For functional categorization and pathway visualization, MapMan v3.6.0RC1 (Thimm et al., 2004) was employed, utilizing a custom mapping file specifically designed for *P. abies*. MapMan organizes gene expression data into a hierarchical tree structure of functional categories, termed “Bin,” which are predefined based on biological processes and pathways, facilitating a comprehensive overview of metabolic and regulatory shifts. A custom mapping file was generated using the Mercator4 online annotation platform, which assigned functional Bin terms to *P. abies* sequences from a reference FASTA file, ensuring accurate gene-to-function associations for this non-model species. DEGs uniquely regulated in each tissue, as well as those common across all tissues, were identified based on a false discovery rate (FDR) < 0.05 and an absolute log₂ fold change of ± 1 . Annotated DEGs were then mapped onto functional Bins and visualized in MapMan to assess metabolic and regulatory pathways, supporting evaluation of tissue-specific and systemic regulation. All pathway diagrams were systematically examined to identify regulatory patterns and adaptive responses across tissues. Statistical enrichment of pathways in MapMan was determined by the Wilcoxon rank-sum test, comparing the distribution of expression values for genes within each Bin versus those outside; Bins with $p < 0.05$ were considered functionally enriched.

4.6 Statistical Analyses

Study II

Total sap flow in each sector (in kg) was first calculated using equation (I) to examine how sap flow responds to diurnal solar radiation. Sap flow data were then normalized with equations (II) and (III) to obtain relative sap flow values in percentages.

$$\text{Total SF sector } n = \sum \text{SF } n(\text{day1} + \dots + \text{day9}) \quad (\text{I})$$

$$\text{SF sum normalized } n = \frac{\text{SF sum } n}{\text{SF sum max} * 100} \quad (\text{II})$$

$$\text{Normalized coefficient} = \frac{\text{SF sum normalized } n}{\text{SF sum}} \quad (\text{III})$$

Generalized Linear Models (GLMs) with θ as the natural parameter and ϕ as the dispersion parameter were used to relate tree groups to sap sums per sector, using the "glm" function from the "stats" package (R Core Team, 2021). Additionally, Pearson's correlation test ("cor.test" function) was performed at a 0.05 significance level to analyze the relationship between tree density sets and sap flow (R Core Team, 2021).

Study III

Five algorithms were employed to evaluate the expression stability of 15 candidate reference genes in Norway spruce: geNorm, NormFinder, BestKeeper, ΔCt , and RefFinder. RefFinder, a web-based platform, was used to provide a comprehensive ranking by integrating the results of the other four algorithms. The geNorm algorithm calculates the expression stability value (M) and performs pairwise variation (V) analysis, where lower M values indicate greater stability (Vandesompele et al., 2002). NormFinder ranks candidate genes according to their stability across sample groups, considering both intra- and inter-group variation (Andersen et al., 2004). BestKeeper evaluates stability based on standard deviation and correlation coefficient of quantification cycle (Cq) values (Pfaffl et al., 2004). The ΔCt method assesses relative expression stability by comparing Cq differences between gene pairs within each sample (Silver et al., 2006).

For all analyses, mean Cq values of each candidate gene were used as input. RefFinder then calculated the geometric mean of individual rankings to provide an overall comprehensive ranking of reference gene stability. To determine the optimal number of reference genes

required for accurate RT-qPCR normalization, pairwise variation (V_n/V_{n+1}) was estimated using geNorm, where the V value reflects the effect of adding an additional reference gene to the normalization factor. To validate the selected reference genes, the relative expression levels of the dehydrin gene *PaDhn5* were analyzed using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) with both the most and least stable reference genes. Data normality was assessed using the Kolmogorov-Smirnov test. Target gene expression levels were then analyzed using single-factor ANOVA in XLSTAT Cloud (ver. 1.0), with a significance threshold of $\alpha=0.05$ to identify differences between the control and treatment groups.

Study IV

The spectrophotometric and RT-qPCR data for the treatment and control groups were analyzed for normality using the Shapiro–Wilk test, followed by an assessment of variance homogeneity between the control and treatment groups with Levene’s test. Subsequently, an independent t-test was conducted, applying either the student’s t-test for equal variances (if Levene’s $p > 0.05$) or Welch’s t-test for unequal variances (if Levene’s $p < 0.05$). The p-values indicating significant differences between the control and treatment groups at a 95% confidence interval were calculated using RStudio (version 4.2.3). Differential gene expression analysis was conducted using the DESeq2 R package (ver. 3.22.5) (Robinson et al., 2010; Love et al., 2024). P-values were adjusted using the Benjamini-Hochberg approach to control the false discovery rate (FDR) (Benjamini and Hochberg, 1995). Genes with adjusted p-values ≤ 0.05 and absolute \log_2 fold change ± 1 were considered significantly differentially expressed.

5. Results

This dissertation comprises four chapters, including three published articles and one manuscript. The first chapter (5.1) is an overview article that examines bark beetle population dynamics, addressing the primary drivers and assessing various management strategies, with recommendations for future molecular-based tools to control these pests. The second chapter (5.2) investigates the impact of forest stand fragmentation on Norway spruce physiology through ecophysiological measurements in the context of bark beetle susceptibility. The third chapter (5.3) addresses a critical gap in the literature by identifying suitable internal control genes (reference genes) for normalizing gene expression in Norway spruce across various experimental conditions. The final chapter (5.4) analyzes gene expression in Norway spruce seedlings under drought stress, highlighting key pathways involved in drought tolerance.

5.1 Understanding bark beetle outbreaks: exploring the impact of changing temperature regimes, droughts, forest structure, and prospects for future forest pest management.

Published as: **Singh, V. V.**, Naseer, A., Mogilicherla, K., Trubin, A., Zabihi, K., Roy, A., Jakuš, R. & Erbilgin, N. (2024a). Understanding bark beetle outbreaks: exploring the impact of changing temperature regimes, droughts, forest structure, and prospects for future forest pest management. *Reviews in Environmental Science and Bio/Technology*, 23, 257–290. <https://doi.org/10.1007/s11157-024-09692-5>.

Contributions: conceptualization, reviewing the literature, visualization, writing - original draft, writing- reviewing and editing.

This study delivers a comprehensive evaluation of bark beetle ecology by analyzing population processes at both stand and landscape scales and by identifying the principal factors driving outbreak formation under ongoing climate change. It explores how alterations in forest structure influence beetle colonization and dispersal, how drought and water limitation compromise host tree vitality, and how temperature regimes regulate bark beetle development and activity. Particular emphasis is placed on the interaction between spruce trees and bark beetles, alongside a critical appraisal of existing forest management practices and their effectiveness in mitigating damage. The findings highlight the growing need for innovative, mechanism-based solutions to complement traditional forest protection strategies.

Conventional management approaches are shown to have limited efficacy, especially as climate change continues to intensify the frequency and severity of bark beetle outbreaks. Accordingly, this work advocates for the integration of emerging molecular technologies with established control measures. Approaches such as RNA interference (RNAi) and CRISPR/Cas9 offer the potential for highly specific, targeted pest management, yet their application to bark beetles, particularly *Ips typographus*, remains largely unexplored. While substantial research is still required to assess feasibility, effectiveness, and ecological safety, incorporating such molecular tools could significantly enhance adaptive forest management in the Anthropocene.



Understanding bark beetle outbreaks: exploring the impact of changing temperature regimes, droughts, forest structure, and prospects for future forest pest management

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Abstract Climate change has increased the susceptibility of forest ecosystems, resulting in escalated forest decline globally. As one of the largest forest biomasses in the Northern Hemisphere, the Eurasian boreal forests are subjected to frequent drought, windthrow, and high-temperature disturbances. Over the last century, bark beetle outbreaks have emerged as a major biotic threat to these forests, resulting in extensive tree mortality. Despite implementing various management strategies to mitigate the bark beetle populations and reduce tree mortality, none have been effective. Moreover, altered disturbance regimes due to changing climate have facilitated the success of bark beetle attacks with shorter and multivoltine life cycles,

consequently inciting more frequent bark beetle-caused tree mortality. This review explores bark beetle population dynamics in the context of climate change, forest stand dynamics, and various forest management strategies. Additionally, it examines recent advancements like remote sensing and canine detection of infested trees and focuses on cutting-edge molecular approaches including RNAi-nanoparticle complexes, RNAi-symbiotic microbes, sterile insect technique, and CRISPR/Cas9-based methods. These diverse novel strategies have the potential to effectively address the challenges associated with managing bark beetles and improving forest health in response to the changing climate.

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1 Introduction

Over the last several decades, a shift in climatic conditions has increased the frequency of severe windstorms and drought events, favouring the emergence and proliferation of insect outbreaks. Bark beetles (Coleoptera: Curculionidae: Scolytinae) are widespread globally, and several species are associated with extensive mortality of major tree species in the Northern Hemisphere (Vega and Hofstetter 2015; Hlásny et al. 2021). For instance, the Eurasian spruce bark beetle, *Ips typographus* (L.) plays a vital role in forest succession and nutrient recycling by decomposing dead and dying trees (Edmonds and Eglitis 1989; Hofstetter et al. 2015; Raffa et al. 2015). However, once their population density reaches the epidemic threshold, they have the potential to cause widespread forest mortality (Berryman et al. 1989; Hlásny et al. 2021). These outbreaks have gone beyond economic implications by disrupting forest succession and nutrient cycling and turning forests from carbon sink to source (Seidl et al. 2014; Aldea et al. 2023).

As the primary host for *I. typographus* and one of the major tree species in the Eurasian boreal forests, Norway spruce (*Picea abies*) is highly vulnerable to rising temperatures. For instance, unprecedented Norway spruce mortality occurred from 1970 to 2010 due to frequent drought events and heatwaves, which resulted in an average annual loss of up to 14 million m³ per annum, especially 118 million m³ in 2019 alone (Ebner 2020). Furthermore, its primary pest, *I. typographus* has benefited from warming temperatures, which resulted in accelerated and extended brood development and early swarming (Hlásny et al. 2011; Netherer et al. 2014; Hinze and John 2020).

Management of bark beetles is driven by two primary objectives: prevention and containment (Wermelinger 2004). Prevention strategies aim to keep the beetle populations at low densities by implementing measures such as removing infested

trees, employing traps, and clearing fallen trees. Containment involves salvage and sanitation logging in outbreak areas or nearby areas to halt or slow the spread of outbreaks. However, the current management strategies are still inadequate, and their effectiveness has been frequently questioned (Dobor et al. 2020). Consequently, exploring innovative strategies for bark beetle management is imperative, leveraging techniques emerging from functional genomics. Methods such as RNA interference (RNAi) and CRISPR/Cas9 are rapidly advancing as pest control tools, with their effectiveness proven in controlling agricultural pests, such as *Spodoptera* and the Colorado potato beetle (Gui et al. 2020; Mezzetti et al. 2020; Vatanparast and Park 2022). Recent reports demonstrate the presence of core machinery genes that can be utilized for gene silencing for *Ips* management using molecular tools (Powell et al. 2021; Joga et al. 2021). However, previous reports have not highlighted or addressed the applicability of these techniques. This review emphasizes the future usability and efficiency of such techniques in *Ips* bark beetle management. Unlike many conventional pest management approaches, these molecular methods are tailored to target specific species. Nevertheless, they require further exploration and functional validation for *I. typographus* management.

Despite a century of extensive research on bark beetles, there are several gaps in our understanding of the main drivers of bark beetle outbreak dynamics and the strategies for their management. Moreover, the recent advancements in the bark beetle management techniques are poorly elaborated. Here, we aim to provide a brief overview of the ecology and population dynamics of *I. typographus* and discuss the major drivers of outbreaks in the context of climate change. This overview focuses on *I. typographus*, but we also discuss other eruptive bark beetle species. We preliminarily examine how changes in a forest structure affect the initial host colonization and spread, how droughts and water stress affect host tree physiology and vigour, and how temperature regimes affect bark beetle activity. We analysed the present management approaches and their efficacy in protecting the forests and suggested cutting-edge molecular-based approaches to face new challenges that could be used to guide future forest management practices in the Anthropocene.

2 Eurasian spruce bark beetle ecology

Ips typographus is a relatively small insect (4.5–5 mm) and exhibits sexual dimorphism. They are univoltine (*producing one generation per year*) but may shift to polyvoltine (*more than two generations per year*) due to the influence of warm temperatures, resulting in accelerated development (Hlásny et al. 2011). During the endemic phase, the beetles infest mature trees that are stressed and weakened due to various biotic or abiotic factors (e.g., high temperature, drought, disease, herbivory). The beetles are associated with a variety of microbial symbionts, including bacteria and ophiostomoid fungi, that play a significant role in exhausting tree defenses (Lieutier et al. 2009; Zhao et al. 2019b; Chakraborty et al. 2023). After overwintering, a new generation of beetles emerge from the litter or Norway spruce trunks and disperses between 100 m to tens of kilometers while searching for a suitable host tree (Biedermann et al. 2019; Fig. 1A). Host tree-bark beetle interaction is generally considered multifaceted, and various factors across different spatial and temporal scales shape their interactions (Jakuš et al. 2011; Kautz et al. 2011; Netherer et al. 2024). Furthermore, *Ips typographus* shows density-dependent host colonization behaviour. Beetle populations in the endemic phase rely on the availability of windthrown, dead, or weakened trees; in contrast, in the epidemic phase, they colonize healthy trees (Økland and Berryman 2004; Økland and Bjørnstad 2006). We will review factors in the context of the population dynamics of *I. typographus*.

2.1 Stand-level dynamics

At the stand level, once the pioneer male beetles overcome the host defenses, they produce aggregation pheromones, 2-methyl-3-buten-2-ol (MB) and (-)-*cis*-verbenol (cV) (Keeling et al. 2021; Ramakrishnan et al. 2022; Fig. 1B.a). Male beetles produce high amounts of MB when initiating the nuptial chamber, while the production of cV changes depending on the amount and enantiomeric composition of α -pinene in Norway spruce phloem (Birgersson et al. 1984; Fig. 1B.b). Emission of these aggregation pheromones from trees under attack brings additional male and female beetles to the foci trees. Arriving male and female conspecifics release more of the

same pheromones to coordinate mass attacks on the host (Blomquist et al. 2010). Upon entering the host, adult beetles create mating chambers, mate, and excavate oviposition galleries where eggs are deposited. Eggs hatch into larvae, feed, complete their development under bark, and exit from the parental trees. Depending on the host characteristics, such as phloem thickness, *I. typographus* can increase its population size by up to 15-fold from the preceding generation (Hlásny et al. 2019).

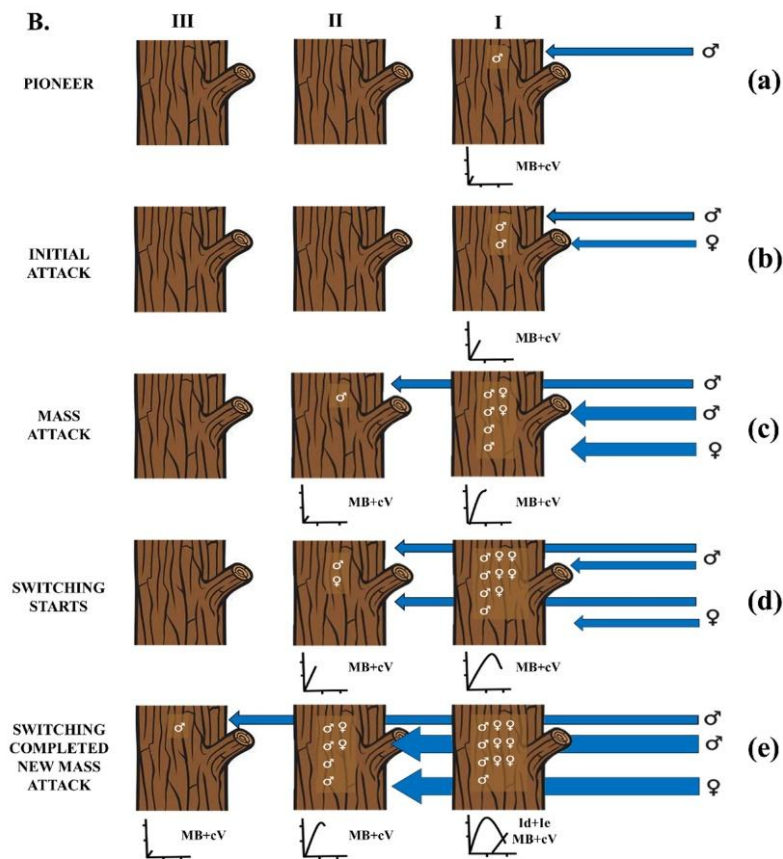
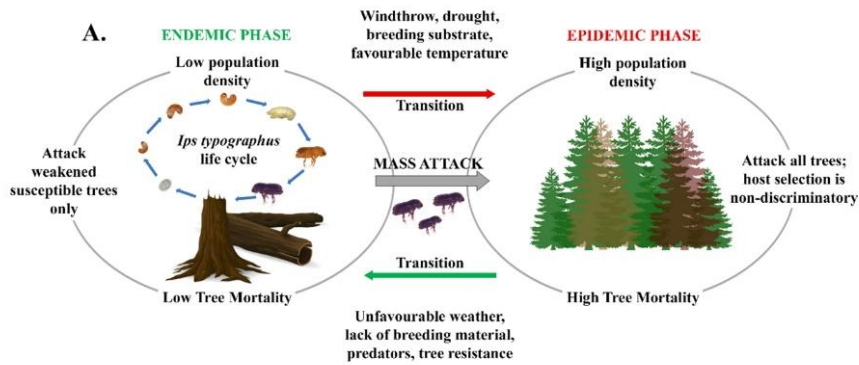
Once the foci host trees are fully occupied, the production of anti-attractant compounds such as verbenone starts to avoid overcrowding (Xu et al. 2015) while the production of aggregation pheromones declines. This increases attraction towards nearby uninfested trees, initiating a new wave of mass attacks (Fig. 1B.c, 1B.d). This event marks the completion of “switching” of bark beetle attacks from heavily colonized trees to neighbouring uncolonized trees (Schlyter et al. 1987; Fig. 1B.e). This process may result in several mass-attacked trees at the landscape level.

2.2 Landscape-level dynamics

The spatial distribution of mass-attacked trees, the host availability, and environmental factors can influence the initiation and spread of bark beetle infestations. However, the effects of these factors vary between endemic and epidemic phases of *I. typographus* (Mezei et al. 2014a; Potterf et al. 2019).

2.2.1 Endemic phase

When bark beetle population densities are low, they are primarily associated with stressed or defensively compromised trees (Raffa et al. 2008; Boone et al. 2011; Biedermann et al. 2019). Factors such as tree resistance, stand structure, abundance of natural enemies and competitors, and weather can potentially limit the beetle’s population size at the endemic phase, whereas natural disturbances and climatic events can disrupt this equilibrium by reducing tree resistance and hence increasing the beetle population (Raffa et al. 2008; Ruel et al. 2023). The capacity of bark beetle populations to cross the epidemic threshold and move from the endemic to the outbreak phase depends critically on the availability of suitable breeding materials, such



as susceptible host trees, stumps, and windblown trees (Hroššo et al. 2020). Weather events like extensive wind damage or intense droughts can provide ample breeding grounds for population build-up. As the population has built up and depleted the available substrate, they begin to attack healthier trees (Biedermann et al. 2019).

2.2.2 Epidemic (outbreak) phase

Once the beetle population reaches the epidemic phase, the discriminatory behaviour in host selection becomes less adaptive because there is a greater possibility of recruiting sufficient numbers of conspecifics to overcome the defenses of large healthy trees, which tend to have greater resources

Fig. 1 Scheme of *Ips typographus* population dynamics. (A.) In the endemic phase, beetles feed and reproduce in windthrown trees, trunks, and stumps and later move to the epidemic phase under favourable conditions. There are several factors, such as favourable weather, availability of breeding substrate, droughts, and windthrows, that influence the transition of the beetle populations to the epidemic phase. In the epidemic phase, the beetle population is high enough to mass attack healthy trees and cause widespread mortality. Later, certain factors such as unfavourable weather, exhaustion of the breeding substrate, abundance of predators, and increased tree resistance of surviving trees can cause high beetle mortality and limit the population to the endemic phase (Adapted from Kautz et al. 2014). (B.) a. The initial host colonization stage is usually initiated by pioneer beetles, males in *Ips* and females in *Dendroctonus*. In *Ips typographus*, aggregation pheromones, 2-methyl-3-buten-2-ol (MB) and (-)-*cis*-verbenol (cV) are released to attract additional male and female beetles. b. If the pioneering beetle survives host tree defenses, with the arrival of additional beetles, they produce larger amounts of aggregation pheromones which usually ends the mass colonization of host trees. The goal of mass colonization is to exhaust tree defense to secure reproduction. c. During the mass colonization stage, while the initial tree (I) is mass colonized, a small portion of male beetles land on a neighbouring uncolonized tree (II) and starts a new colonization sequence. d. As the production of attractive pheromone components diminishes, the attraction towards the initial tree declines, while simultaneously, attraction towards the neighbouring tree increases, starting a switch in attraction. e. When the initial "switching" process is completed, only small amounts of aggregation pheromones are produced, while larger amounts of inhibitory compounds (ipsdienol & ipsenol) are continuously produced. The attraction is now shifted to the neighbouring tree (II), where a new mass attack starts, with a small proportion of males once more being deflected and ending up on the new uncolonized neighbouring tree (III). The small graphs represent log pheromone release from Birgersson et al. (1984). The marks on the x-axis represent the attack phases 1, 3 and 6, where (1) males are bored in bark, (3) have completed nuptial chamber formation, and (6) are joined with females. (Adapted from Schlyter et al. 1987)

(i.e., thicker phloem) to support substantial beetle populations, resulting in higher number of broods (Raffa et al. 2008). The factors governing the bark beetle outbreaks also change as the infestation progresses (Walter and Platt 2013).

In the early stages of the outbreak, beetles still infest susceptible trees with weakened defenses (Raffa et al. 2008), with most beetles targeting windthrown trees. As a result, the beetle infestation is still relatively small at this stage, and tree mortality is primarily attributed to windthrow events (Potterf et al. 2019). However, the infestation of standing trees intensifies up to three years after a major windthrow event and enters a patch-driven outbreak phase,

signified by the establishment of many infestation spots, benefiting from a large pool of available healthy host trees (Økland et al. 2016; Potterf et al. 2019). Still, the rate of infestation spread is minimal, and the distance between new and old infestation sites is maximum (Jakuš et al. 2002). These spots typically emerge close to windthrow areas within previously unaffected stands, appearing as small, rounded patches (Potterf et al. 2019).

As the epidemic spreads across the landscape, beetle pressure becomes the most crucial determinant of tree mortality because the population size of bark beetles is large enough to mass attack healthy trees and overcome their defenses (Walter and Platt 2013; Mezei et al. 2014b). The outbreak emerges within one to three years following a major disturbance event (Økland and Berryman 2004). Although healthy trees can withstand a certain number of attacks, once a certain threshold is surpassed, such a tree cannot repel the attackers (Krokene 2015). As a result of the coalescence of nearby infestation spots, the distance between freshly and previously infested locations is smaller during the epidemic phase than the endemic phase (Kautz et al. 2011; Potterf et al. 2019). As each successive generation of bark beetles exhausts resources needed by subsequent generations (Raffa et al. 2008), higher population pressure further facilitates the initiation and spread of bark beetle infestations at higher elevations (Jakuš et al. 2002) and other neighbouring less affected areas.

2.2.3 Epidemic to the endemic phase transition

In the later phase of the outbreak, as the significant portion of available host trees has already been depleted, bark beetles target marginal host trees in less favourable habitats. This phase is characterized by relatively lower densities of beetle populations than the earlier phases of the outbreak, and the beetle numbers are too low to kill healthy trees. Surviving trees are likely to fend off attacking beetles due to their anatomical and chemical defenses (Nelson et al. 2007; Raffa et al. 2008; Erbilgin et al. 2017; Zhao and Erbilgin 2019; Zhao et al. 2019a). In the post-epidemic phase, while the number of new bark beetle infestation sites decreases, the expansion of existing infestation spots reaches its maximum, with the beetles more likely to target less suitable resources

neighbouring old spots due to limited available resources.

The mechanisms behind the decline in bark beetle populations in the later stage of an outbreak are not well understood. However, when the host tree supply is depleted, the beetle population decreases significantly (Walter and Platt 2013). Additionally, adverse weather conditions, pathogens, and natural enemies can significantly increase beetle mortality, ultimately limiting their population to the endemic phase (Boone et al. 2011; Wegensteiner et al. 2015).

3 Effects of forest stand structure on population dynamics of *I. typographus*

The structure of the forest stand (e.g., stand density and age classes) strongly influences bark beetle outbreak (Fettig et al. 2007; Sproull et al. 2015). Windthrow alters stand structure as a primary form of disturbance by creating openings in the forest canopy (Marini et al. 2017). Promoting forest diversity (discussed in Sect. 6.2) and thinning are the highly recommended practices to reduce overall vulnerability and have been effective against *Dendroctonus ponderosae*, *D. frontalis*, and *D. brevicomis* (Egan et al. 2010; Fettig and McKelvey 2010; Zhang et al. 2013; Hood et al. 2016).

Within outbreak stands, foresters employ intensive measures like sanitation felling and salvage logging to reduce damage, including cutting and removing infested or dead trees (Stadelmann et al. 2013). However, intensive management activities such as sanitation can further amplify the destabilization of the stand. For example, intense sanitary interventions in outbreak-impacted stands could cause the fragmentation of forest stands by creating canopy openings and numerous margins or edges (Hanson and Lorimer 2007; Zabihi et al. 2021; Özçelik et al. 2022; Singh et al. 2023). Notably, fragmented forests exhibit increased susceptibility to wind damage and render freshly formed forest edges to bark beetle infestation (Kautz et al. 2013; Marešová et al. 2020). This susceptibility is likely due to sudden changes in microclimatic conditions, such as increased solar radiation and temperature, reduced humidity, increased vapour pressure demands, and greater wind exposure (Hanson and Lorimer 2007; Herbst et al. 2007; Marešová et al. 2020; Özçelik et al. 2022). The

resulting changes may further facilitate increased bark beetle generations and temporal scale of infestation and increase the damage risk.

Likewise, highly dense plantations, often driven by commercial goals, exhibit poor growth and resilience to stress. Increased tree density can increase intraspecific competition among individual trees and constrain resource availability (Zabihi et al. 2023; Thomas et al. 2024). Research indicates that increased competition within a forest stand can exacerbate the impacts of drought and increase the likelihood of mortality, particularly if water is the limiting resource (Zhang et al. 2015; Young et al. 2017; Korolyova et al. 2022). Consequently, constraints on resource availability can influence many plant functions, such as resin exudation, photosynthesis, and subsequent biosynthesis of carbon-dependent defense metabolites (Bazzaz et al. 1987; Netherer et al. 2014; Erbilgin et al. 2021), and predispose them to bark beetle attacks (Netherer et al. 2019).

Practices like thinning can enhance forest resilience by providing growing space to individual trees (Fettig et al. 2007; Hood et al. 2016; Knapp et al. 2021) but its impact on the dynamics of *I. typographus* remains unknown. Forest structures vary across elevation, slope, and aspect gradients, with elevation playing a crucial role in tree mortality during all phases of *I. typographus* infestation (Mezei et al. 2014a; Sproull et al. 2015). For example, trees tend to be more densely packed at lower elevations within a limited altitude range (Mazón et al. 2020; Zabihi et al. 2023). This difference in tree density due to elevation also affects tree characteristics like bark temperature and diameter (Fig. 2; Zabihi et al. 2023) which are important predictors of host susceptibility to bark beetles. Since topography and inter-tree spacing significantly modulate ecosystem functions, forest management strategies should be tailored considering these variables to foster improved overall health and resilience of secondary spruce forests (Zabihi et al. 2023; Thomas et al. 2024).

4 Impact of drought stress on population dynamics of *I. typographus*

The intense and recurring drought events compromise the host tree defense mechanisms, rendering them more susceptible to infestation by bark beetles

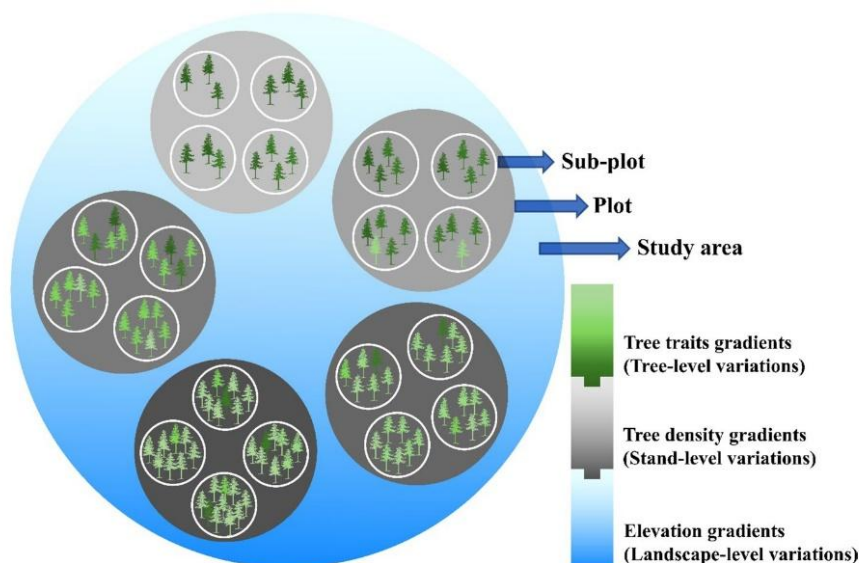


Fig. 2 A representation showing how variations in landscape, stand, and tree characteristics interact at different scales of elevation, tree density, and tree traits. The five circles in the diagram represent tree density gradients, with darker grey indicating higher density and lighter grey indicating lower density. The blue circle represents changes in elevation,

with darker blue indicating higher altitude and lighter blue indicating lower altitude. The individual tree traits, such as bark temperature and diameter at breast height, shown in green color gradients, were found to be positively and negatively related to tree density, respectively. (Adapted from Zabihi et al. 2023)

(Berryman 1972; Christiansen et al. 1987; Erbilgin et al. 2021). Research indicates that severe drought events limit the production of defense metabolites, unlike moderate drought, which is hypothesized to limit tree growth but enhance carbon allocation to defenses (Desprez-Loustau et al. 2006; Ferrenberg et al. 2015). Drought stress occurs when the available water in the soil falls below a specific threshold, leading to decreased soil water content and increased hydraulic resistance at the root-soil interface. Water transfer through xylem tissues may become irreversibly disrupted due to water cohesion breakdown and vessel embolism, increasing the risk of premature mortality of roots and twigs (Cruziat et al. 2002; Cochard et al. 2009).

Isohydric conifer species avoid or delay the decline in xylem water potentials by restricting transpiration through stomatal closure (Rothe et al. 2002; Schume et al. 2004). Although stomatal regulation helps in maintaining water potential in the xylem, reduction in sap flow and leaf-atmosphere gas exchange declines the photosynthetic product assimilation regardless of continued respiratory consumption of

carbon (Bréda et al. 2006; Rennenberg et al. 2006). Eventually, stored carbon reserves (i.e., non-structural carbohydrates) are depleted by repeated bark beetle attacks, and due to no or low replenishment as trees cannot sustain the production of defense metabolites (Erbilgin et al. 2021). At the same time, while tighter stomatal regulation can help avoid a decline in lethal xylem water potentials, the tension in the water conduits may cause shrinkage in tracheid diameters. This, together with a decrease in turgor pressure inside the epithelial cells lining the resin ducts, reduces the physical pressure on the oleoresin and subsequently reduces the exudation rate upon wounding of the bark, which may compromise the tree's defense against frost, another drought episode and pest attacks, ultimately leading to tree mortality (Cruziat et al. 2002; Bréda et al. 2006; Cochard et al. 2009; Rissanen et al. 2016).

Despite the well-known negative impacts of drought events on tree physiology and inherent defensive mechanisms, there is no conclusive evidence connecting drought-induced physiological stress and the tree's attractiveness to bark beetles

(Netherer et al. 2014). The [Rosalia Roof experiment](#) on drought manipulation addresses the issue of resource distribution in Norway spruce, focusing mainly on secondary metabolism (Netherer et al. 2014, 2019; Matthews et al. 2018). The project aimed to determine the impact of drought stress on both inherent and triggered defense mechanisms of trees against bark beetle attacks, including factors like resin flow and hypersensitive reactions to blue stain fungus inoculation. Furthermore, a recent field study has shown that when bark beetles infest drought-stressed trees, it not only decreases the local availability of carbohydrates crucial for essential tree functions but also hinders the tree's capacity to replenish carbohydrate reserves (Erbilgin et al. 2021). Nevertheless, further research is needed to determine whether there is a difference in the attractiveness of stressed and control trees and validate the hypothesis that susceptible host trees are more attractive to pioneer beetles (Wermelinger 2004; Netherer et al. 2014).

5 Changing temperature regimes and their impact on population dynamics of *I. typographus*

Bark beetle outbreaks have been correlated with shifts in temperature and precipitation regimes as host tree vigor is affected by warmer spring and summer temperatures combined with increased water stress (Powell and Logan 2005; Berg et al. 2006). Bark beetle population growth and survival depend on thermal conditions as it can shorten development time and increase the number of generations per year (Bentz et al. 2010; Marini et al. 2012). Moreover, winter mortality is another critical component in bark beetle population dynamics (Hinze and John 2020). While the cold temperature adaptations and cold hardening mechanisms of many bark beetle species remain relatively unexplored, some *Ips* and *Dendroctonus* beetles stand out for their ability to accumulate cryoprotectant compounds (e.g., glycerol) during the colder periods of autumn, resulting in the decreased mortality (Lombardero et al. 2000; Bentz et al. 2010; Košťál et al. 2011). Most bark beetle species have symbiotic relationships with microorganisms like blue-stain fungi and bacteria that increase their tolerance to cold temperatures and

provide nutrients to the larvae (Bleiker and Six 2007; Ayres et al. 2000; Guevara-Rozo et al. 2020).

The ambient temperature also influences the flight activities of bark beetles. The optimal flying activity occurs from 22 to 26 °C, while *I. typographus* do not swarm below 16.5 °C (Hinze and John 2020). Swarming of beetles in search of suitable breeding material is affected by two factors: the emergence from overwintering, which can be anticipated by thermal sum, and the mass flight of beetles, which occurs at a temperature above 20 °C (Annala 1969; Wermelinger 2004). A recent study showed that most *I. typographus* were caught on the hottest day (maximum temperature of 33.4 °C) of the observation period (mean air temperature 19.2 °C), suggesting that its ability to find hosts and mass flight is not compromised by increased thermal conditions (Hinze and John 2020). Furthermore, the average flight distance of *I. typographus* increases significantly on days with moderate temperatures than cold temperatures (Wermelinger 2004; Hinze and John 2020). Due to the rapid genetic adaptation of insects to seasonal changes in temperature regimes, range expansion of bark beetles beyond their habitat has also been observed where species move into new niches facilitated by increasing temperature (Balanyá et al. 2006; Battisti et al. 2006; Bradshaw and Holzapfel 2006; Nealis and Peter 2008; Erbilgin et al. 2014; Erbilgin 2019). Bark beetle dispersal-related ecological and environmental factors are reviewed in detail by Jones et al. (2019).

Regional-scale flight activity periods of *I. typographus* have been established by analysing climate data, focusing on temperature parameters. For instance, in southern Sweden, the onset of spring flight among *I. typographus* occurred when the accumulated thermal sum, averaging above 5 °C, persisted for roughly 47 ± 24 days (Öhrn et al. 2014). This period aligns with the findings from Denmark (Harding and Ravn 1983) and southern Finland (Annala 1969; Öhrn et al. 2014), where a similar flight duration of 45 days was observed. The flight period in Denmark lasted from early May to mid-August, whereas, in central Europe, it occurred between April and September (Faccoli and Stergulc 2004; Baier et al. 2007; Öhrn et al. 2014). In the region of southern Sweden, the flight activity period of *I. typographus* was observed to take place from mid-April to mid-August, which can be attributed

to the effects of climate change resulting in warmer spring and summer temperatures compared to three decades ago (Öhrn 2012; Öhrn et al. 2014). In central European forests, sister broods of *I. typographus* with a minimum of two subsequent generations have been documented during the summer (June to August). Consequently, these temperature conditions have led to an increased frequency and extension of the flight activity periods of bark beetles (Netherer et al. 2019).

6 The current and potential future management practices

Bark beetle management strategies involve a range of approaches and typically start with removing windthrown and trees recently infested by *Ips typographus*. Anti-attractants or trap trees are commonly used to control beetle populations, whereas methods like remote sensing and canine detection are employed for early detection and mitigating attack damage. Furthermore, modern molecular tools are being investigated for controlling forest pests. In this section, we will discuss the traditionally adopted measures (salvage and sanitation harvesting, pheromone or trap-tree, short rotation forestry), progressive-practical methods (remote sensing, detection dogs) and explore promising cutting-edge molecular methods (Sterile insect technique (SIT), clustered regulatory interspaced short palindromic repeats/cas9 (CRISPR/Cas9) and RNA interference (RNAi)).

6.1 Reduction of rotation periods

Norway spruce forests often have rotation periods exceeding 100 years in many regions of Europe, resulting in extensive forest areas that are particularly prone to wind and bark beetle disturbances. For example, in Slovakia, between 1998 and 2009, less than a quarter of Norway spruce could reach 100 years of age (Hlásny et al. 2017). Because of these vulnerabilities, reducing the duration of rotation can be an effective strategy for forests to adapt to the growing bark beetle pressures (Zimová et al. 2020). Given that the optimal rotation period varies depending on the forest management system, site productivity, and species mixture (Hlásny et al. 2017), forest managers should consider the length of the

current rotation period based on regional conditions. However, reducing the rotation period may also result in a decline in biodiversity and the amount of carbon stored in forests.

6.2 Optimizing forest composition to enhance resilience against bark beetles

Due to rapid population and economic growth, forests are under increasing pressure to meet demands for wood and associated products (Liu et al. 2018). Consequently, extensive areas across the globe are being cleared and transformed into plantation forests to cater to this demand (West 2014). Plantation forests vary in their intended purposes, and different species are chosen accordingly. Monocultures emerged in Europe during the eighteenth and nineteenth centuries as a response to timber scarcity, aiming to achieve high quality products (Griess and Knoke 2011). For instance, Norway spruce trees were extensively harvested in most parts of Europe due to their rapid growth rate and favourable timber characteristics (Spiecker 2000; Caudullo et al. 2016). However, frequent drought events are threatening the harvesting plantations. For instance, the significant increase in spruce mortality in Europe from 1970 to 2019 was partly attributed to the frequent drought events and heatwaves, with alterations in the structure and composition of forests accounting for approximately half of the documented increase in bark beetle outbreaks (Seidl et al. 2011). Furthermore, ecosystem services such as carbon sequestration, water regulation, and habitat provision are compromised in monocultures (Barrette et al. 2023) because a higher number of species with diverse functional traits can collectively contribute to a wide range of ecological functions (Lefcheck et al. 2015). Apart from the deliberate cultivation of Norway spruce beyond its native range, the augmentation of growing stocks and alterations in age-class distributions have significantly enhanced the susceptibility of spruce forests to *Ips typographus* attacks (Seidl et al. 2011; Hlásny et al. 2021).

In response to diebacks of monoculture forests, increasing tree diversity and age classes has been tested and recommended to increase forest resilience against biotic and abiotic disturbances (Lohbeck et al. 2016; Singh et al. 2023). In fact, there is a large number of empirical evidence suggesting

that planting multiple species can provide several environmental, economic, and social benefits, and careful selection of species in mixed plantations can facilitate the promotion of complementary structural and functional traits (Hartley 2002; Forrester et al. 2005; Pawson et al. 2013; Carnol et al. 2014; Alem et al. 2015; Drössler et al. 2015). Thus, adopting mixed plantations as a strategy is more favourable than monocultures (Zhang et al. 2022). For instance, Dedrick et al. (2007) evaluated risks for several forest types and found that monoculture Norway spruce forests were more susceptible to biotic and abiotic disturbances than mixed-species forests. Including trees with varying age classes in mixed forests offers the potential for improved canopy coverage, leading to reduced solar radiation reaching the forest floor, which may minimize ground-level evaporation (Singh et al. 2023).

The argument that fostering tree species diversity lowers the risk of bark beetle infestation (Klapwijk et al. 2016) can be attributed to two main factors: Firstly, as discussed, mixed forests provide greater resilience against windthrow and storm damage; both of which are crucial drivers of the initiation of bark beetle outbreaks. In addition, an abundance of diverse tree species fosters various natural enemies of bark beetles including predators, pathogens, and parasitoids (Jactel and Brockerhoff 2007; Klapwijk and Björkman 2018; Stemmelen et al. 2022) suppressing the bark beetle population. Secondly, bark beetles rely on several cues for host selection, including olfactory, gustatory, and visual (Campbell and Borden 2006), and these cues help beetles to discriminate, for instance, defensively compromised trees (Rodriguez and Redman 2008; Schiebe et al. 2019). However, such cues may be masked by diverse volatile emissions from non-host trees in mixed forests during the host selection (Schiebe et al. 2011). Furthermore, tree diversity benefits the host tree species preferred by bark beetles; as such trees benefit from being hidden among non-host trees in the stand (Berthelot et al. 2021). However, tree species richness only works to suppress bark beetle outbreaks and not to avoid them, and even when the proportion of host plants is below 40% in the stand (de Groot et al. 2023). These facts demonstrate the importance of species richness in the context of bark beetle infestation. Yet, more evidence from field studies involving choice bioassays is needed to determine

the effects of non-host volatiles on beetle infestation dynamics.

6.3 Early detection of infested trees, monitoring, and mass trapping

6.3.1 Detection dogs

Successfully implementing a management strategy that relies on rapid detection of bark beetle infestations and removing recently infested trees is necessary for forest protection. However, human detection generally requires close inspection (≤ 1 m) of trees and is therefore time-consuming, costly, and not always possible (Svensson 2007). Thus, aerial detection of infested trees generally occurs 2–3 months after an infestation, when tree crown colour fades, and bark falls off. At this point, the majority of bark beetles have already left infested trees and might target other uninfested trees. Using trained detection dogs has proven an effective alternative to locating infested trees (Johansson et al. 2019). The main advantages of using trained detection dogs in finding infested trees are their incredible sense of smell and capacity to explore large areas quickly (Hepper and Wells 2015; Mosconi et al. 2017). Vošvrđová et al. (2023) reported that trained sniffer dogs can locate infested trees up to 150 m away. Thus, such dogs can extend the time window for finding and removing infested trees, potentially avoiding the growth of larger infestations. As a limitation, detection dogs cannot be used for larger and remote areas.

6.3.2 Pheromone traps

Various trapping techniques have been widely used in bark beetle management in addition to sanitation felling and removal of windthrown trees. The rationale for using pheromone traps is to monitor beetle populations or reduce beetle numbers below the outbreak density by mass-trapping. The approach uses pheromone trap barriers, which aim to reduce the bark beetle population to a level where trees can successfully defend themselves against attacks (Jakuš 1998, 2001). Trapping techniques include using trap trees or log traps baited with species-specific pheromones or host compounds that may attract beetles. Once attracted, beetles can be killed

with pesticides or by removing the infested trees or logs. Limited scientific evidence supports the efficacy of using trap trees to effectively reduce beetle populations or the number of attacked trees (Klutsch et al. 2017). However, there is a shortage of research on the efficacy of trapping techniques in reducing the risks of outbreaks and rates of damage, particularly for large-scale applications. While pheromone traps typically capture only 3–10% of beetle populations at a relatively high cost (Wermelinger 2004), using pheromone trap barriers as part of the integrated forest protection system makes it possible to reduce tree mortality significantly (Jakuš 1998, 2001). Due to relatively high labour costs, using pheromone traps has been discontinued in Scandinavia and most parts of Germany and France. The use of pheromone traps for mass trapping of bark beetles is further constrained by the “spillover effect,” where commercial pheromones attract more beetles than the traps could handle, causing attacks in trees adjacent to the traps (Niemeyer 1997; Jakuš et al. 2022). Nevertheless, the primary purpose of such traps should continue to monitor the bark beetle population rather than population reduction and outbreak suppression. Further improvement in using pheromone trap barriers is possible with the new mixtures of attractants (Blaženec et al. 2021; Jirošová et al. 2022).

6.3.3 Anti-attractants

Anti-attractants are semiochemicals used to disrupt the host-finding behavior of pests. By emitting repelling signals to pests or masking the attractive signals emitted by potential hosts, anti-attractants can protect trees from being located and colonized by these pests. Among the range of active anti-attractant compounds identified for *I. typographus* (Schiebe et al. 2011), the first notable one is verbenone, generated from the host compound α -pinene or converted from *I. typographus*'s main pheromone component *cis*-verbenol (Birgersson and Leufvén 1988). The second group comprises non-host volatiles (NHVs): *trans*-conophthorin, an important synergistic compound found in the bark of broad-leaf trees (Zhang and Schlyter 2003); green leaf volatiles (GLV; 1-hexanol; (*Z*)-3-hexen-1-ol; 1-2-hexen-1-ol), detected in non-host birch (*Betula* spp.) and aspen (*Populus tremula*) (Zhang et al. 1999); and C8

alcohols (3-octanol; 1-octen-3-ol), emitted from the bark of the mentioned species. Attempts to protect logs or fallen trees with anti-attractants have not been successful in stopping bark beetle attacks, including *I. typographus* (Jakuš and Blaženec 2003).

Currently, the most promising methods for defending standing spruce trees against *I. typographus* attacks involve dispensers with a blend of verbenone and NHV compounds (Schiebe et al. 2019). A recent study reported a strong “switching effect,” of using anti-attractants, which involves pushing away beetles from areas with anti-attractants into areas without non-attractants (Jakuš et al. 2022). Furthermore, installing anti-attractant dispensers at two different heights on trees showed no difference, suggesting that using anti-attractants is ineffective in areas affected by severe drought and extremely high bark beetle populations (Jakuš et al. 2022). Unusually, (+)-*trans*-4-thujanol repelled female *I. typographus*, demonstrating its efficacy to be on par with known anti-attractants such as 1,8-cineole and verbenone, making it an innovative anti-attractant for forest protection (Jirošová et al. 2022). A recent meta-analysis by Afzal et al. (2024) on the push–pull strategy, which involves using attractive and repellent semiochemicals, reports that this strategy can reduce *Ips* and *Dendroctonus* populations by 66% and 54%, respectively, compared to the control.

6.3.4 Remote sensing (RS) of forests

Gathering precise and current spatial information on the presence and dynamics of the bark beetle infestation is still challenging in large areas with limited access (Stereńczak et al. 2019). Recently, several research teams in Europe and North America have focused on studying the spatio-temporal analyses of bark beetle population dynamics (Simard et al. 2012; Kärverno et al. 2014; Meddens and Hicke 2014; Senf et al. 2015; Havašová et al. 2017; Mezei et al. 2017; Marvasti-Zadeh et al. 2024). Forest managers will likely need to face widespread and frequent infestations of bark beetles in the near future, necessitating the need to develop efficient tools that can assess the current spread and dynamics of insect outbreaks in a given area rapidly and precisely. Precise and up-to-date spatial information on bark beetle outbreaks (e.g., locations of dead trees) is essential when planning protective and sanitary

actions (e.g., pheromone or tree trapping) to control or limit the intensity of an outbreak in a given area (Fassnacht et al. 2014; Fettig and Hilszczański 2015). The ability to identify and map tree mortality caused by bark beetle outbreaks depends mainly on the forest structure. For example, detecting and monitoring forest stands that consist solely of coniferous host species is relatively straightforward. New dead trees in such stands mostly appear in large, easily detectable groups as bark beetles primarily attack trees adjacent to those already killed (Lausch et al. 2011; Seidel et al. 2016). Detection of individual-infested trees can change in complex forest structures consisting of mosaics of various stands with mixed tree species where the typical host tree species occur in smaller scattered groups or individual trees. Identifying individual infested trees in such a situation is a challenging task, and as financial resources typically limit fieldwork, RS methods are an effective alternative and supplement to field surveys. Multispectral aerial and satellite imagery, in combination with artificial intelligence and machine learning, has been successfully used for mapping insect outbreaks and other forest disturbances (Roberts et al. 2003; White et al. 2007; Long and Lawrence 2016; Senf et al. 2017; Marvasti-Zadeh et al. 2024). For instance, the significant spectral differences between the healthy and stressed trees (i.e., drought) were found on imageries of the Enhanced Vegetation Index (EVI) and Visible Atmospherically Resistant Index (VARI) at the beginning of the growing season before the new attacks. The results highlight the potential of using SVIs derived from high-resolution multispectral imagery to detect pest infestations early and manage forest ecosystems (Trubin et al. 2023, 2024).

We could rely on various RS data and sources to estimate tree health characteristics. Remote sensing generally uses satellite- or aircraft-based (e.g., Unmanned Aerial Vehicle) sensor technologies. In forestry, especially in the case of examination of the spectral properties of trees, we can use active (such as LIDAR) and passive sensors (such as Multi- and Hyperspectral sensors) (Niemann et al. 2015). The most recent methods in using, processing, and analysing RS in the context of tree predisposition determination combine sensor data and represent multi-temporal GIS analysis based on hyperspectral and airborne laser scanning data (Abdullah et al.

2019; Stereńczak et al. 2019). Hyperspectral and airborne laser scanning data are widely used to identify prominent forest disturbances in the bark beetle outbreak sites and dead trees in the red and grey attack stages. The change in foliage colour in trees affected by infestation becomes noticeable after approximately 6 to 8 months from the onset of the infestation. This transformation in the crown of infested trees from green to yellow to red is due to the gradual loss of moisture and the deterioration of pigments within the foliage. These distinct stages in the colour change are referred to as the “red-attack” and “grey-attack” phases, respectively (Safranyik and Carroll 2007). Analysis of red and grey attack data allows us to understand the dynamics of the bark beetle outbreak, and the accumulation of additional data, such as climatic (temperature, wind speed, precipitation), will allow us to understand the most critical drivers of the outbreak.

The possibility of identifying all attacked trees is limited by the output data available for analysis from visible dominant trees in the first forest layer. Another associated challenge is incorrectly identifying dead trees as living due to reflection from the lower layer (Stereńczak et al. 2019). Similarly, early detection of trees attacked by bark beetles depends on the availability of ground truth data (Nardi et al. 2023; Trubin 2024; Kautz et al. 2024). However, acquiring ground truth data is often labour-intensive (Zabihi et al. 2021) and only limited to areas accessible by the field crew. Additionally, early attacked trees are challenging to identify using remote sensing when there are stressed trees around and due to changes in the foliar colour of spruce trees at the beginning of the season. Another limitation relates to the limited computational power for analysing large-scale data.

6.4 Biological control agents

6.4.1 *Natural enemies*

Biological control involves suppressing a pest by introducing a naturally occurring antagonist to achieve sustainable and eco-friendly control over insect pests (Kenis et al. 2017). Natural enemies such as predators, parasites and parasitoids impact bark beetles' population dynamics and ecology. The variety of antagonists that target bark beetles is extensive and includes the order Hymenoptera,

beetles (Coleoptera), flies (Diptera), true bugs (Heteroptera), snake flies (Raphidioptera), and mites (Acari) (Table 1).

Bark beetle predators are highly diverse, and can consume all life stages of bark beetles, from eggs to adults, and significantly influence their population dynamics. Although numerous predator species are associated with bark beetle galleries, only a few actively prey on eggs, larvae, pupae, and/or adults. Most predators targeting bark beetles belong to Coleoptera (beetles), including Cleridae, Trogossitidae, and Rhizophagidae (Hopping 1947; Mills 1985; Wermelinger 2002). The most notable coleopteran predators of bark beetles are the checkered beetles (Cleridae). This small family includes several early-season predatory species, such as *Thanasimus dubius* (F.) from North America and *Thanasimus formicarius* (L.) from Europe (Stephen and Dahlsten 1976; Herard and Mercadier 1996; Lawson et al. 1997). Clerid beetles arrive at the infested trees shortly after bark beetles and consume the beetles directly on the bark surface. *Thanasimus formicarius* is among the most studied predators of *I. typographus* (Kenis et al. 2007). Its females lay approximately 100 eggs, and adults can consume up to three beetles per day, while larvae can consume approximately 50 bark beetle larvae throughout their larval development stages (Mills 1985; Dippel et al. 1997). While clerid beetles are typically considered generalist predators, trogossitids are believed to have a more specialized approach (Kohnle and Vite 1984; Lawson and Morgan 1992). For instance, *Temnochila virescens* primarily responds to attractants produced by *Ips* species, and their larvae feed on bark beetle larvae and pupae within the tree's phloem, whereas adult *Temnochila* species consume the adults of various other bark beetles (Billings and Cameron 1984).

Furthermore, parasitoids can be an important part of the population dynamics of bark beetles. The parasitoids prefer a specific developmental stage of the host. Most parasitoids associated with Scolytids belong to the Hymenoptera order, including Braconidae and Pteromalidae. They can attack various developmental stages of bark beetles. Each parasitoid larva typically consumes one beetle larva or pupa. The most effective parasitoid of *I. typographus* is *Coeliodes bostrichorum*, and it appears to be exclusively associated with bark beetle

species breeding in Norway spruce (Feicht 2006; Kenis et al. 2007).

Various organisms, including wasps, ants, birds, shrews, mites, and pathogens like viruses and microsporidia are potential antagonists of *I. typographus*, but their impact on population dynamics requires further understanding (Kenis et al. 2007). Muratoğlu et al. (2011) identified five pathogenic bacterial genera associated with *I. typographus* mortality, with *Serratia liquefaciens* showing notable effectiveness, causing 53.3% mortality. Commercially produced entomopathogenic microbes, including fungal species like *Beauveria bassiana*, *Metarhizium anisopliae*, *Hirsutella guignardii*, *Isaria farinosa*, and *Lecanicillium lecanii* have been utilized as microbial control agents against *Ips* and *Dendroctonus* (Kreutz et al. 2004; Popa et al. 2012; Lacey et al. 2015; Mann and Davis 2021; Rosana et al. 2021; Fernandez et al. 2023). Entomopathogens serve as effective pest controllers, efficiently managing insect populations while also being environmentally friendly towards non-target organisms (Hajek and Bauer 2009). However, despite their potential, challenges such as susceptibility to ultraviolet light, low moisture, temperature fluctuations, plant secondary metabolites, and competition with other microorganisms limit the widespread field application (Mann and Davis 2021).

While biological control strategies offer innovative and eco-friendly methods for bark beetle management, only a few biological control strategies against bark beetles have been implemented so far. The bark beetles are often attacked by polyphagous enemies, which typically have minimal impact on regulating the beetle populations (Kenis et al. 2007). In most cases, the lack of specificity of many natural enemies of bark beetles would make them unsuitable as biological controls, except the successful use of the specific predatory beetle *Rhizophagus grandis* to effectively control the great spruce bark beetle in areas like the Caucasus and Western Europe (Grégoire et al. 1992; Averbeke and Grégoire 1995). Apart from that, biological control programs also struggle to prove their financial benefits. Politically, this impedes government investment in biological control research and development and reduces academic interest in further exploration. At the management level, lack of engagement leads to skepticism about the effectiveness and financial advantages compared to pesticides (Barratt et al. 2018). Understanding

Table 1 Overview of Bark Beetle Management Approaches: A summary outlining key strategies employed for bark beetle management, highlighting traditionally used, progressive-practical, and cutting-edge molecular methods to mitigate infestations and promote forest health

Approach type	Method	Targeted trees/bark beetles	Recommendations/comments	References
Resilience	Rotation period	Coniferous sp.	Shortening of the rotation period	De Groot et al. (2019), Zimová et al. (2020)
Sanitation	Mixed plantations		Mixing diverse species	Fares et al. (2015), De Groot et al. (2019), De Groot et al. (2023)
	Salvage		Harvesting of windthrown timber	Göthlin et al. (2000), Stadelmann et al. (2013)
Monitoring/population control	Sanitation felling		Removal of infested trees	Stadelmann et al. (2013), Seidel et al. (2016)
	Pheromone traps	<i>Ips</i> and <i>Dendroctonus</i> sp.	Use of pheromones to lure beetles	Jakuš (1998), Jakuš and Blaženeč (2003), Faccoli and Stergulec (2004), Blaženeč et al. (2021), Duduman et al. (2022), Jakuš et al. (2022)
	Tree traps		Use of standard tree traps with large diameters	Raty et al. (1995), Faccoli and Stergulec (2004), Lubojacký and Holuša (2014), Holuša et al. (2017)
Early Detection	Anti attractants		Spruce forest protection with chemicals to achieve the anti-attractive effect	Zhang and Schlyter (2003), Schiebe et al. (2019), Jakuš et al. (2022), Jirošová et al. (2022), Jakuš et al. (2024)
	Remote sensing	Coniferous sp.	Satellite-mediated detection of susceptible host tree/attack detection	Candotti et al. (2022), Dalponte et al. (2022), Safonova et al. (2022), Gao et al. (2023), Huo et al. (2023), Jamali et al. (2023), Trubin et al. (2023, 2024)
	Detection dogs	<i>Ips typographus</i>	Faster detection of bark beetle-infested trees	Johansson et al. (2019), Vošvrđová et al. (2023)

Table 1 (continued)

Approach type	Method	Targeted trees/bark beetles	Recommendations/comments	References
Biological Control Agents	Coleopteran predators A. Cleridae 1. <i>Thanasimus</i> sp. 2. <i>Enoclerus</i> sp. B. Histeridae 3. <i>Plegaderus</i> sp. 4. <i>Platysoma</i> sp. C. Monotomidae 5. <i>Rhizophagus</i> sp. D. Staphylinidae 6. <i>Phloeonomus</i> sp. 7. <i>Phacophallus</i> sp. 8. <i>Quedius</i> sp. E. Tenebrionidae 9. <i>Corticus</i> sp. F. Trogossitidae 10. <i>Tenebroides</i> sp. 11. <i>Tennochila</i> sp. G. Zopheridae 12. <i>Lasconotus</i> sp. 13. <i>Aulonium</i> sp.	<i>Ips</i> and <i>Dendroctonus</i> sp.	Natural enemies of <i>Ips</i> and <i>Dendroctonus</i> sp.	Otvos (1965), Billings and Cameron (1984), Kohnle and Vite (1984), Grégoire et al. (1985, 1992), Mills (1985), Lawson and Morgan (1992), Lawson et al. (1996, 1997), Dippel et al. (1997), Reeve (1997), Aukema et al. (2000a, 2000b), Wermelinger (2002), Erbilgin and Raffa (2001), Dahlsten et al. (2003), Aukema and Raffa (2005), Boone et al. (2008), Wermelinger et al. (2012, 2013), Martin et al. (2013)
	Non-Coleopteran Predators A. Diptera B. Heteroptera C. Acari			Dahlsten (1970), Moser and Roton (1971), Nicolai (1995), Ulrich (2004), Feicht (2004), Hedgren and Schroeder (2004), Hulcr et al. (2005), Moser (1975), Moser et al. (1978, 1989, 2005), Hofstetter et al. (2009), Sarikaya and Avci (2009), Wermelinger et al. (2012), Sousa et al. (2023)
	Parasitoids A. Hymenoptera <i>Braconidae</i> sp. <i>Pteromalidae</i> sp. <i>Coeliodes</i> sp. B. Microbial parasitoids <i>Bacillus</i> sp. <i>Acinetobacter</i> sp. <i>Proteus</i> sp. <i>Serratia</i> sp. <i>Beauveria</i> sp. <i>Metarhizium</i> sp. <i>Hirsutiella</i> sp. <i>Isaria</i> sp.			Kreutz et al. (2004), Feicht (2006), Kenis et al. (2007), Wegensteiner (2007), Muratoglu et al. (2011), Popa et al. (2012)

Table 1 (continued)

Approach type	Method	Targeted trees/bark beetles	Recommendations/comments	References
Molecular techniques	Sterile insect technique (SIT)	<i>I. typographus</i>	Induce sterility in insects via irradiation	Turčáni and Vakula (2007), Čičková et al. (2018)
	RNA interference (RNAi)	Coleoptera (<i>Dendroctonus ponderosae</i> , <i>D. frontalis</i> , <i>Agrilus planipennis</i> , <i>Anoplophora glabripennis</i>)	mRNA degradation-mediated gene silencing	Rodrigues et al. (2017a, 2017b, 2018), Kyre and Rieske (2022), Kyre et al. (2019, 2020), Dhandapani et al. (2020a, 2020b)
	Clustered regulatory interspaced short palindromic repeats (CRISPR/cas9)	*Coleoptera	Gene editing tool	*Successfully tested and proven effective on agricultural beetles like Colorado potato beetles but not yet tested on forest beetles such as <i>Ips</i> and <i>Dendroctonus</i> species

ecological and environmental factors (i.e., bark beetle attack dynamics, predator population and their co-existences, the impact of changing climates on prey and predators, and the effect of other pesticide-based control measures on natural enemies) determining the efficiency of biological control methods against bark beetles need to be thoroughly investigated for successful incorporation of natural enemies in forest pest management programs.

6.4.2 Sterile insect technique (SIT)

The sterile insect technique involves dispersing a large number of sterile male insects into the population in a given area, with the expectation that these males mate with normal females, resulting in non-viable offsprings and lower the population densities of pests in the generation that follows (Lance and McInnis 2005; Diallo et al. 2019; Tam et al. 2023). Both sterile sexes can be released when it is impossible to distinguish between the sexes, making the procedure more successful while helping to keep the pest population below the epidemic threshold (Hendrichs et al. 2021; Ikegawa et al. 2021). The SIT relies on the irradiation of insects by gamma radiation from isotopic elements (cobalt-60 or cesium-137) as well as high-energy electrons and X-rays. To effectively sterilize the insect's reproductive cells while still keeping them alive enough to compete for mating, the radiation dose must be tightly managed because radiation can harm the sex cells of insects by causing chromosome fragmentation (dominant lethal mutation, translocations, and/or chromosomal aberrations), which results in the production of unbalanced gametes. Hence, mitosis is inhibited, and fertilized egg/embryo development is impaired (Bakri et al. 2021; Klassen and Vreysen 2021; Robinson 2021). The lethality of the dose–effect on two lepidopteran pests (*Ostrinia nubilalis* and *Phyllocnistis citrella*) indicates that higher doses of gamma radiation reduce the life span of these insects in both sexes. However, the larval emergence of the F1 was skewed towards males. The effectiveness of this technique is studied and confirmed in agricultural insect pests like sweet potato weevil (*Cylas formicarius*), pepper weevil (*Anthonomus eugenii*), and *I. typographus* (Turčáni and Vakula 2007; Čičková et al. 2018; Ikegawa et al. 2022; Basso et al. 2023). While SIT proves to be an

economical, sustainable, and efficacious approach, its potential for extensive implementation in large-scale field scenarios has not been examined and continues to pose a constraint.

6.5 Genetic approaches

6.5.1 Utilizing genetic information of host-tree

The degree and frequency of drought events have recently increased for forests worldwide due to rapid climate change, and adaptation of trees to the new disturbance regimes can be locally scaled at the gene level and includes substantial selection pressure on tolerant phenotypes (Zacharias et al. 2022). Norway spruce exhibits higher lignin synthesis and increased expression of defense-related genes in response to shade, potentially enhancing its resilience against pest attacks (Ranade et al. 2019, 2022). Tree responses to heat stress are influenced by signaling factors (protein kinases and transcription factors), heat shock proteins, heat stress factors, and catalase enzymes. In contrast, ascorbate peroxidase and histidine kinases remove reactive oxygen species during heat stress, while dehydration-responsive element-binding proteins shield trees from osmotic stress.

According to spatial and differential gene expression examination of conifers, stomatal closure and cuticular wax on the surface of needles can reduce water loss. A study on needles of maritime pine (*Pinus pinaster*) and Norway spruce reported that in non-irrigated saplings, the cuticular wax and the genes involved in its synthesis were overexpressed as compared to the irrigated ones (Blödner et al. 2007; Le Provost et al. 2013). With the advancement of molecular techniques and high throughput sequencing methods, the use of molecular tools to target genes for pest management is highly popularized (Singh et al. 2024). Various genes have been employed in agricultural pest management since the *cry* gene was identified in the 1980s, which was among the initial unique genes utilized for GM crops to combat insect pests. These advancements, however, are still in their early stages and are constrained by factors like tree size, density, and application at the forest stand level. Hence, additional technologies were diverted to insect-level genetic modifications, which led to the development of various new techniques such as sterile insect technique, gene silencing via RNAi and

CRISPR, and other pre- and post-translation level modifications. Identifying genetic markers of tree resistance can be applied to forest protection, tree health diagnosis, and breeding bark beetle-resistant trees (Korecký et al. 2023).

6.5.2 RNA interference (RNAi)

In insects, RNAi is a conserved cellular process that turns off gene function by interfering with mRNA breakdown and protein production (Fire et al. 1998; Zhu and Palli 2020; Mogilicherla and Roy 2023a). This process involves silencing specific genes by using short RNA molecules to target and degrade messenger RNA (mRNA), preventing the production of proteins encoded by those genes. The silencing or “turning off” of vital genes for the survival of insects ultimately results in mortality (Zhu and Palli 2020).

Three distinct RNAi mechanisms have been identified: small interfering RNA (siRNA), microRNA (miRNA), and piwiRNA (piRNA). The siRNA pathway has received the most attention in insect pest management (Zhu and Palli 2020). In a nutshell, the siRNA machinery is activated by the successful delivery of double-stranded RNA (dsRNA) into the cell, then Dicer-2 (ribonuclease type III) enzyme converts the dsRNA into siRNAs (~21–24 bp), which are subsequently integrated into the RNA-induced silencing complex. Eventually, Argonaute2 cleaves and removes the sense strand of the siRNA. The remaining antisense strand then directs the RNA-induced silencing complex to sequence-specific targeting of complementary mRNA strand, which leads to degradation of the mRNA strand and post-transcriptional gene silencing (Zhu and Palli 2020). This sequence-specific mechanism of RNAi can be harnessed to effectively target vital genes in insects, including bark beetles, offering a means of pest management (Zhu and Palli 2020; Joga et al. 2016, 2021; Mogilicherla and Roy 2023b).

Researchers have developed RNAi-biopesticides that effectively silenced the target genes and caused decent mortality in beetles such as the Colorado potato beetle (*Leptinotarsa decemlineata*), emerald ash borer (*Agrilus planipennis*), Asian long-horned beetle (*Anoplophora glabripennis*), mountain pine beetle (*Dendroctonus ponderosae* Hopkins) and southern pine beetle (*Dendroctonus frontalis*) (Table 1) (Yoon et al. 2016, 2018; Rodrigues et al.

2017a, 2017b; Máximo et al. 2020; Dhandapani et al. 2020a, 2020b; Kyre et al. 2019, 2020; Kyre and Rieske 2022). The putative variability of RNAi among genetically variable beetles in geographically distinct populations was also recently documented (Kyre et al. 2024). Recent research has led to the development of the RNAi-based biopesticide Ledprona against *L. decemlineata*, which prevents the expression of enzymes, promotes protein breakdown, and ultimately results in mortality (Pallis et al. 2023). RNAi provides excellent promise for minimal environmental impact, attributed to its precise targeting and the transient nature of its active molecules. However, additional research is needed before widespread use of such commercial products in forestry. To ensure its safety, a combination of bioinformatics and ecologically sound bioassays with selected focal insect species will help a thorough understanding of potential off-target effects and impacts on non-target organisms (Christiaens et al. 2022; Mezzetti et al. 2022). These products must be used only to maintain the bark beetle populations under endemic conditions and preserve the beneficial role of bark beetles as decomposers in forest ecosystems.

Bark beetles are more susceptible to RNAi, but the effectiveness depends on the target gene selection, dsRNA stability, and expression of the RNAi core machinery genes (Mogilicherla et al. *unpublished data*). Effectiveness, off-target and non-target effects, and a lack of reliable dsRNA delivery mechanisms are the main obstacles to the widespread use of RNAi for bark beetle pest management. In contrast, feeding techniques combined with advanced development technologies (symbiont-mediated and nanoparticle-enabled) are critical for improved dsRNA transport, stability, endosomal escape, and dsRNA processing (Joga et al. 2021; Mogilicherla and Roy 2023b). Recently, Mogilicherla and Roy (2023b) comprehensively reviewed chitosan-dsRNA nanopesticides and their applications in managing bark beetles. The bacteria and fungi that live symbiotically with bark beetles have been successfully isolated and identified and can be considered putative candidates for symbiont-mediated RNAi (SMT) for tropical application to control bark beetles (Chakraborty et al. 2020a, 2020b, 2023; Gupta et al. 2023; Mogilicherla and Roy 2023b). These studies may pave the way for developing and

using RNAi-biopesticides as a secure, efficient, and innovative method to safeguard forest trees. Fortunately, current research conducted globally and recent work on the genome, transcriptome, and proteome of bark beetles and their symbiotic microbes will significantly enhance the information on these insects and facilitate the development of species-specific RNAi-based biopesticides in the future (Powell et al. 2021; Ashraf et al. 2023; Naseer et al. 2023; Sellamuthu et al. 2023). Nonetheless, only a limited number of RNAi-based insecticides have obtained licensing and are on the verge of becoming accessible in the market (Li et al. 2023; Pallis et al. 2023).

6.5.3 CRISPR/Cas9

Numerous studies have made considerable use of the ground-breaking genome editing tool known as clustered regulatory interspaced short palindromic repeats/cas9 (CRISPR/Cas9) (Sun et al. 2017; Singh et al. 2022; Yan et al. 2023). CRISPR/Cas9 is a gene editing technology used to alter the DNA of organisms. In the CRISPR/Cas system, CRISPR comprises DNA sequences found in prokaryotic organisms like bacteria and archaea, while Cas9 is an enzyme that uses CRISPR sequences as a guide to locate and open specific DNA strands. The Cas9 enzyme attaches to the target DNA and cleaves it, deactivating the targeted gene. This process, known as gene “knock-out,” is a reliable method for identifying genes of interest or gaining deeper insights into genome complexities (Doudna and Charpentier 2014; Jiang and Doudna 2017).

CRISPR is a more intuitive and user-friendly technology because it needs only one guide RNA (gRNA) for target identification and Cas9 nuclease for implementation (Richter et al. 2013; Upadhyay 2021). The relevant derivative has a single chimeric guide RNA (sgRNA) that recognizes and binds to the intended target sequence, as well as a CRISPR-associated (Cas) nuclease that cleaves DNA at specific locations by producing site-specific double-strand breaks (Horvath and Barrangou 2010; Wiedenheft et al. 2012). The plasmid DNA, RNA, or ribonucleoprotein complex (RNPC) are some of the different delivery mechanisms that can be used to introduce the CRISPR/Cas components (sgRNA and Cas9 protein) into the target organism

(Ogaugwu et al. 2013). CRISPR/Cas9 has an advantage over RNAi due to its ability to bring about enduring and inheritable genomic alterations. In contrast, RNAi only produces immediate effects unless a steady supply of dsRNA is kept available. Nevertheless, CRISPR technology has the potential to revolutionize pest control, but both the advantages and disadvantages must be carefully considered (Perkin et al. 2016). The CRISPR/Cas9 technology has been used against a wide variety of insect species; however, in the case of coleopteran insects, it has only been applied to the red flour beetle (*Tribolium castaneum*) and Colorado potato beetle (*Leptinotarsa decemlineata*) (Gilles et al. 2015; Gui et al. 2020; Singh et al. 2022).

6.5.4 Ethical and legal implications of RNAi and gene editing technologies in forest pest management

Development of regulatory protocols for modern biotechniques such as RNAi and CRISPR is essential before intended deployment. However, the regulatory protocols can vary across jurisdictions, predominantly emphasizing the process or the end product due to associated risks and the need to effectively manage toxicity levels to non-target organisms (Ahmad et al. 2021; Távora et al. 2022). The regulatory framework for genetically modified (GMO) products in the European Union and New Zealand is process-based and necessitates significant time and financial expenditure. In contrast, the end-product-based regulatory structure used by the US, Canada, China, and selected European countries is comparatively efficient in terms of time and cost. Before commercialization, the RNAi and gene-edited products must be checked for potential adverse effects on non-target organisms, including microorganisms in the habitat (i.e., forest soil). Therefore, discussing several regulatory regimes regarding RNAi and CRISPR systems in numerous jurisdictions worldwide is necessary.

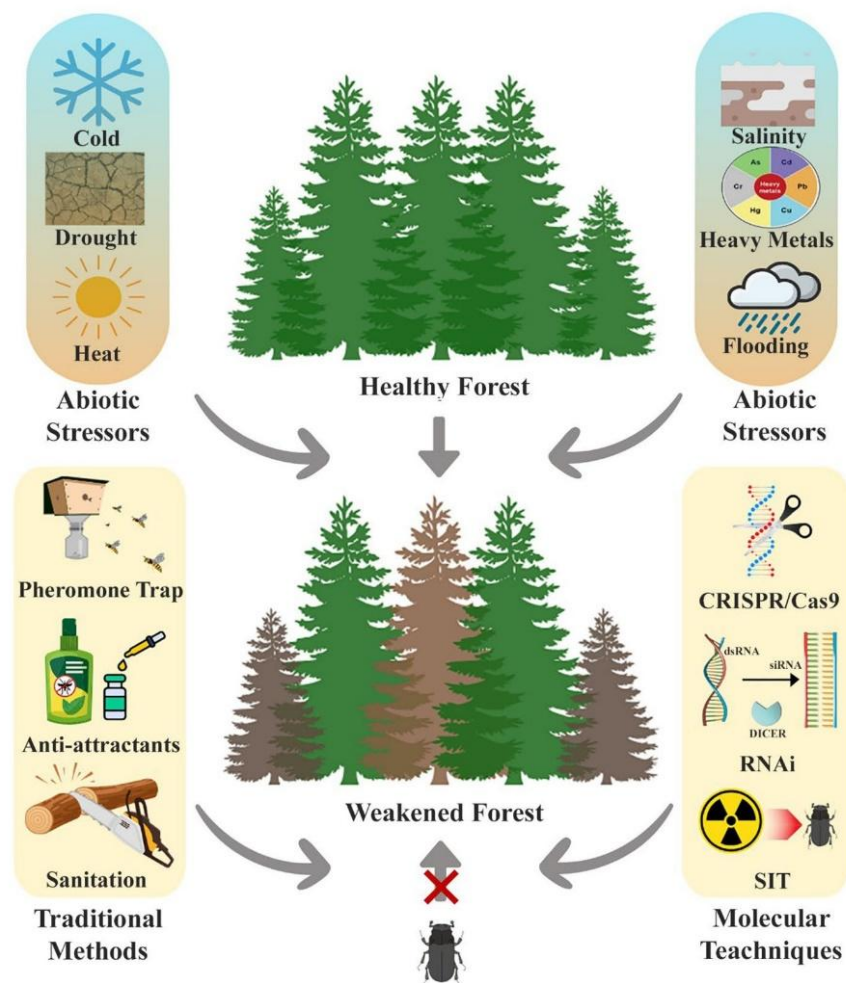
Only a limited number of RNAi and CRISPR-based products have been approved for global commercial release, likely due to persistent regulatory obstacles and unsettling consumer perception and acceptance (Mat Jalaluddin et al. 2019). Besides, several important issues must be addressed before society accepts biotechnological products (Taning et al.

2021). Regular communication among researchers, foresters, and other pertinent participants (i.e., state forest enterprises and forest owners) is essential for reporting biotechnological breakthroughs. Furthermore, the contemporary acceptance of CRISPR and RNAi-based bioproducts, such as biotic stress-resistant plant varieties and biopesticides, depends on transparent communication referring to technical intricacies like gene editing and silencing mechanisms alongside a thorough exploration of potential risks and benefits. Scientists from relevant sectors are pivotal in facilitating open dialogue with forest organizations and sustaining educational efforts to foster informed decision-making (Rank and Koch 2021; Taning et al. 2021). Nevertheless, it is crucial to address ethical and moral considerations at the outset of developing CRISPR/Cas and RNAi-based technological solutions (Frewer et al. 2013; Gupta et al. 2015; Beghin and Gustafson 2021).

Interestingly, RNAi technique does not always require a transgenic expression of target dsRNA; the use of topically applied RNAi-based biopesticides or spray-induced gene silencing (SIGs) for the management of tree diseases may see a rise in public acceptance (Shew et al. 2017). Regulatory frameworks for topical RNAi-based products in agriculture or forestry are still nascent worldwide. Despite this, extensive research has been conducted into plant protection using topically applied RNAi-based bioproducts, driven by their promising benefits. Topical RNAi-based products offer clear advantages over most used crop protection methods using chemical pesticides. However, the environmental fate and stability of the naked dsRNA or dsRNA conjugates used in SIGs and their impact on beneficial microorganisms in the habitat must be assessed from case to case.

Although there is still much debate about the regulations surrounding CRISPR-edited organisms, a few nations have established regulatory frameworks designed to assess these products (reviewed by Devos et al. 2022). Some genetic engineering methods do not entail the introduction of exogenous DNA sequences. Such methodologies may not produce a final product classified as genetically modified (Podevin et al. 2013; Entine et al. 2021). In many countries, legislation governing GMOs or traditional chemical and biological pesticides does not cover these genetic engineering methods. Nevertheless, an

Fig. 3 The Scheme illustrates the currently used traditional methods and prospective molecular techniques that can help manage bark beetles in weakened forest ecosystems. The upper panel shows the major abiotic factors that weaken the forest ecosystem and increase its susceptibility to bark beetle infestations. The traditional methods are helpful in monitoring and controlling bark beetle populations to some extent, but novel cutting-edge technologies can play a crucial role in eliminating pest outbreaks



approach based on scientific parameters to develop and validate biotechnological products is fundamental to formulating the most appropriate risk assessment protocols (Mezzetti et al. 2020).

7 Conclusions and future perspectives

A considerable proportion of global plantation forests consisting of monocultures, preferred for timber production owing to their uniformity and ease of management, are under threat. However, as extreme weather conditions like droughts and rising temperatures favour forest pest outbreaks, there is a pressing need for eco-friendly strategies to reduce the vulnerability of forests to climatic change. To enhance

overall forest resilience, it is proposed that cultivating plantations with diverse species and promoting complementary traits could be a viable solution. However, despite being a longstanding priority on the political agenda in central Europe, realizing such goals has been slow. A recent report from the European Commission aims to promote a transition toward a sustainable and resilient silviculture practice by 2030 (European Commission 2021).

Understanding the mechanism of bark beetle infestation and outbreak dynamics at different spatial scales has serious implications for protecting and managing the Norway spruce ecosystem. Despite the ecological and economic impacts of bark beetle outbreaks, the current control measures are ineffective in stopping the spread of bark beetle outbreaks.

Existing methods like pheromone and tree traps, anti-attractants, remote sensing, and canine detection of infested trees against bark beetles will continue to aid in managing bark beetles amidst climate change. However, additional approaches such as nanoparticle-coated RNAi complexes and symbiont-mediated RNAi, SIT, and CRISPR/Cas9-based approaches are needed and may potentially replace some of the current management approaches in combating bark beetles in forest management (Fig. 3). These new approaches have been very successful in controlling agricultural pests. They are relatively less explored for forest pest management, especially for bark beetles, for various reasons, including the availability of sequenced genomes, difficulties in rearing on an artificial diet, and obtaining viable eggs from beetles for injection. Regardless, extensive product development and testing their effectiveness in field studies are required before commercial manufacturing and widespread field application. In addition, the success of the potential application of the recent molecular techniques is contingent upon understanding their mechanisms, assessing potential risks, and navigating the global regulations governing similar genetic materials. It can be anticipated that molecular approaches will continue evolving in the research domain aimed at combating forest pests, and thus, the environmental safety of such approaches should be carefully monitored for safe deployment.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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5.2 Effect of diurnal solar radiation regime and tree density on sap flow of Norway spruce (*Picea abies* [L.] Karst.) in fragmented stand.

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Contributions: Investigation, Validation, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

This study examined the impact of tree density and diurnal solar radiation on sap flow dynamics in Norway spruce growing within fragmented stands. Sap flow responses were assessed across three directional sectors aligned with the sun's position: morning (05:00–11:10; East), midday (11:10–15:10; South), and evening (15:10–21:10; West). Tree density was calculated within a 10 m radius buffer around each sap flow measured tree using high spatial resolution aerial imagery acquired by an Unmanned Aerial Vehicle. Sap flow was measured at 10-minute intervals for 25 selected trees during the nine hottest summer days in 2019.

Results revealed an inverse relationship between tree density and sap flow rate. Trees in high-density stands exhibited reduced sap flow compared with those in lower-density stands, with the strongest effects occurring during midday. Maximum sap flow rates were observed in the southern sector at midday, coinciding with peak solar radiation and evaporative demand. These patterns demonstrate that stand density regulates water use and transpiration, indicating that lower-density forests may display greater resilience to extreme climatic conditions, while simultaneously facing higher susceptibility to pest outbreaks due to increased solar exposure and subsequent temperature stress. Overall, the findings underscore the central role of stand density in mediating forest physiological functioning and highlight its relevance for developing silvicultural strategies that enhance resilience under climate change. Further works based on these results are presented in Zabihi et al. (2023, 2025).

Effect of diurnal solar radiation regime and tree density on sap flow of Norway spruce (*Picea abies* [L.] Karst.) in fragmented stand

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Abstract

The continuous threat of ongoing climate change and related weather anomalies challenge forest ecosystems. The phytosociological structure of forests plays a crucial role in determining their resilience to various abiotic and biotic stressors. Furthermore, stand density, which partly regulates the allocation of resources within individual trees, is a vital aspect of understanding forest functioning. This study was conducted in Norway spruce (*Picea abies* [L.] Karst.) forests in the Czech Republic, where we investigated the influence of tree density on sap flow rates within three pre-defined directions corresponding to sun position during the morning (5:00–11:10 hours; East), noon (11:10–15:10 hours; South), and evening (15:10–21:10 hours; West) intervals. Tree density was calculated within a 10 m radius buffer around each sap flow measured tree using high spatial resolution aerial imagery acquired by Unmanned Aerial Vehicle. Sap flow was measured at 10-minute intervals for 25 selected trees during the nine hottest summer days in 2019. Our data reveals an inverse correlation between sap flow and tree density, underscoring the substantial impact of neighboring tree density on transpiration. This relationship was most pronounced during midday, followed by the evening and morning hours, suggesting higher heat loads on the trees and forest grounds during the midday. Our findings emphasize the critical role of stand density in modulating forest functioning and underscore the importance of maintaining specific canopy densities as part of effective silvicultural practices in the face of ongoing climate change.

Key words: solar radiation; sun positioning; tree density; bark beetle outbreaks; transpiration; competition effect

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1. Introduction

In recent decades, there has been a notable change in global climatic conditions, leading to an increased occurrence of severe windstorms and droughts. This shift has contributed to widespread forest dieback (Singh et al. 2024a). Within Eurasian boreal forests, the spruce bark beetle, *Ips typographus* L. (Coleoptera: Curculionidae: Scolytinae), plays a crucial role in forest succession and nutrient recycling by decomposing dead trees (Hlásny et al. 2021). However, when their population density surpasses an epidemic threshold, these beetles can cause extensive forest mortality (Hlásny et al. 2019; Powell et al. 2021). Between 1970 and 2010, the

Eurasian boreal forests experienced an unprecedented level of Norway spruce (*Picea abies* [L.] Karst.) mortality. This was primarily due to the increased frequency of drought events and heatwaves during this period. The impact of the Norway spruce mortality was significant, leading to an annual average loss ranging from 2 to 14 million m³ in the Czech Republic alone. In 2019, the impact was particularly severe, with a loss of 118 million m³ of Norway spruce in the region (Ebner 2020; Singh et al. 2024b). The impact extended beyond economic losses, as it disrupted the natural processes of forest regeneration and nutrient recycling, ultimately transforming these forests from carbon sinks to carbon sources (Seidl et al. 2014).

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Typically, bark beetle outbreaks are preceded by windthrow events since windfalls provide an ideal development substrate for large numbers of pest beetles (Hanewinkel et al. 2008). To reduce the damage caused by bark beetles, foresters use the traditional outbreak management strategy of sanitation felling and salvage logging, which includes cutting down and removing infested or dead trees (Stadelmann et al. 2013). Broad applications of such management strategies against bark beetles leave the forest stands fragmented in the outbreak area (Özçelik et al. 2022). These patched forest stands create many forest margins that act as fresh forest edges (Zabihi et al. 2021). Fragmented open-canopy forests with lower tree density are exposed to higher solar radiation loads than closed-canopy forests (Emmel et al. 2013; Vanderhoof et al. 2014). The resulting sudden changes in microclimatic conditions, such as increased temperatures and solar radiation, reduced humidity, increased vapor-pressure demands, and wind exposure affect the physiological responses of trees, including sap flow (Herbst et al. 2007; Marešová et al. 2020; Özçelik et al. 2022).

The phytosociological structure of the forest, determined by the number and size of individual trees, regulates the amount of light, water, and soil nutrients available to each tree (Kholdaenko et al. 2022, 2023). In addition, tree growth and its ability to respond to environmental abiotic and biotic drivers are also affected by the density of trees in a given area and the competition for resources (Bello et al. 2019; Steckel et al. 2020). For instance, an increase in the number of trees per unit area leads to greater competition for resources, such as water, nutrients, and sunlight, affecting transpiration and photosynthetic capacity (Zavdilová et al. 2023). In contrast, when tree density is lower and there is more space between the trees, increased solar radiation exposure and heat loads on tree canopies and forest grounds increase evapotranspiration (Niccoli et al. 2020; Zabihi et al. 2023). In this context, several studies have shown that reduced forest density increases the productivity of the remaining trees by increasing average resource availability while reducing the vulnerability of forests to wildfires and insect infestations (Latham & Tappeiner 2002; Fettig et al. 2014). Conversely, others have shown that thinning can increase drought vulnerability due to increased evaporative demands (Aussenac 2000; Lagergren et al. 2008; Brooks & Mitchell 2011), as well as increased competition for soil water (Nilsen et al. 2001). In forest ecosystems, the tree population density serves as an indirect measure of the intensity of competition, impacting the growth and mortality rates in forests globally (Bottero et al. 2017). While some of the effects of tree density have been explored on water stress (Eastham et al. 1990; D'Amato et al. 2013), resistance towards pathogens (Zausen et al. 2005; Zhang et al. 2013), and scarcity of nutrients (Latham & Tappeiner

2002; Perevalova 2019), how the sap flow rate is influenced by neighbouring tree density in a stand remains unexplored. Sap flow, as an integral part of calculating water balance, photosynthesis, and other quantities of interest in process models can explain and predict the functioning of forest ecosystems.

We hypothesize that the increased neighboring tree density may intensify competition for available water resources and result in lower sap flow rates. Additionally, we hypothesize that the sap flow rates vary over the course of a day due to temporal variations in solar radiation. In this study, we aim to understand the interplay between tree density in a forest stand and sap flow rates, which could be important in shaping forest management practices toward establishing a sustainable ecosystem, particularly in the context of prevailing extreme climatic events. The goal was to assess diurnal sap flow patterns as a function of temporal variation in incoming solar radiation loads on target trees and the effect of neighboring tree density in each sector (East, West, and South) on overall sap flow rates. We aim to answer the following research questions: (i) how does the sap flow react to incoming solar radiation over the course of a day? (ii) how does the number of neighbouring trees in close proximity affect sap flow rates? and (iii) how these findings relate to forest susceptibility to bark beetle outbreaks?

2. Material and methods

2.1. Study area

This study was carried out in a Norway spruce-dominated area with an altitude of 430 m a.s.l., located near Kostelec nad Černými Lesy (49.9940° N, 14.8592° E) in the eastern district of the Central Bohemian region of the Czech Republic (Fig. 1A). The vegetation in this area primarily consisted of coniferous forests, with a mixture of fir (*Abies* sp.) and beech (*Fagus* sp.). However, at the time of the study, spruce (*Picea* sp., 50%) and pine (*Pinus* sp., 16%) were the dominant tree species (Trubin et al. 2023). The prevailing mineral bedrock across the study area was granodiorite covered with Luvisol (IUSS Working Group WRB 2006). The climate in this region is characterized by dry and warm summers, with a growing season of 150–160 days and an average annual temperature and precipitation of 7–7.5 °C and 600 mm, respectively (Tolasz et al. 2007; Trubin et al. 2024).

The study site had six plots of mature (90–100-year-old) Norway spruce stands, established in 2018 under the EXTEMIT-K project, located in a mixed oak-beech vegetation zone. The project's objective was to conduct field experiments and provide practical solutions to mitigate the increasing bark beetle populations and protect

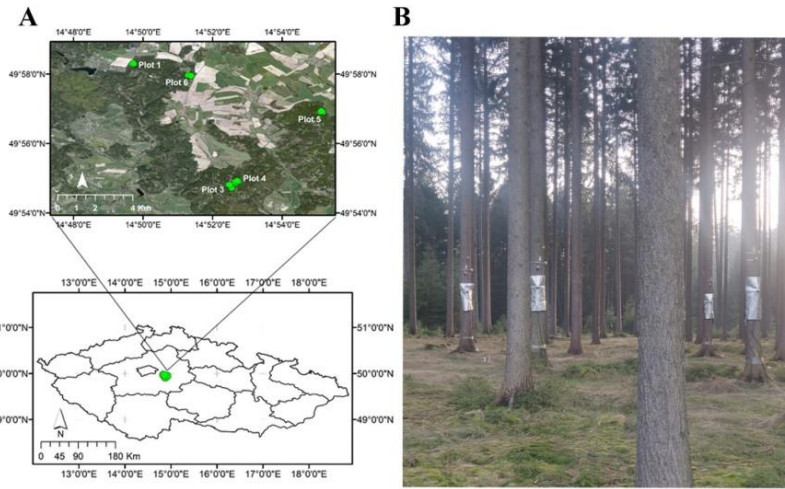


Fig. 1. (A) Study area showing Norway spruce tree positions (green circles) within five out of six established study plots (17 subplots) under EXTEMIT-K project, located at the School Forest Enterprise (ŠLP) of the Czech University of Life Science near Kostelec nad Černými Lesy, Czech Republic. (B) Picture of the experimental setup from one of the subplots with trees equipped with sap flow sensors.

spruce forests (Trubin et al. 2023, 2024). Each plot had four subplots (A, B, C, and D) with a stand of 8–10 Norway spruce trees, having an average diameter at breast height (DBH) of 40.49 cm (SD = 5.70 cm). Plots 3 and 4 were situated close to each other, within a distance of 430 m, while plots 1, 5, and 6 were relatively far, with distances ranging from 2 to 9 km. Plot 2 was excluded from the analysis due to the inability to collect sap flow data. The forest stands were managed using classical silvicultural practices, primarily thinning.

2.2. Climatic data and meteorological measurements

Climatic data were collected by an automated meteorological station (Minikin QTHi, EMS Brno, Czech Republic) located in an open space between experimental plots 3 and 4 (distance < 200 m, Table 1). Precipitation was recorded with a Minikin ERI equipped with a Pronamic Pro Rain gauge (EMS Brno). The meteorological station recorded relative air humidity (%), air temperature

Table 1. Average, maximum, and minimum values of selected variables measured and calculated at the study site during the target days of the study period. ‘All Study days’ represent the nine days used in the study. E, S, and W represent the sectors defined by the time intervals of the sun transition. As Ψ_w and ET_0 could not be calculated for hourly intervals, and thus the rows are left empty.

Variables	Type of value	All Study days	East 5:00–11:10	South 11:10–5:10	West 15:10–21:10
Air temperature (°C)	avg	26.45	22.26	31.01	27.83
	min	11.85	11.85	25.19	20.05
	max	35.56	32.05	35.37	35.56
Relative air humidity (%)	avg	48.44	63.71	34.28	41.75
	min	17.78	30.14	17.98	17.78
	max	94.32	94.32	56.83	79.29
Global radiation ($W; m^{-2} day^{-1}$)	avg	6,303.68	2,403.05	2,943.5	957.13
	min	5,996.47	2,242.81	2,834.01	888.50
	max	6,654.42	2,539.82	3,072.25	1,058.79
Vapour Pressure Deficit (Pa)	avg	2,051.63	1,148.65	3,038.95	2,346.56
	min	96.09	96.09	1,671.41	557.52
	max	4,802.86	3,192.85	4,579.32	4,802.86
Soil Water Potential (Ψ_w ; kPa)	avg	-102.52	—	—	—
	min	-1,377.40	—	—	—
	max	-9.60	—	—	—
Potential Evapotranspiration (ET_0 ; mm)	avg	4.73	—	—	—
	min	4.35	—	—	—
	max	5.34	—	—	—

(°C), and radiation levels. The photosynthetically active radiation (PAR) was used to calculate global radiation ($W \cdot m^{-2}$) for the study period. Soil water potential (SWP, Ψ_w) was monitored using hexagonal sensors at a depth of 20 cm (Teros 21, Meter group, München, Germany), using five sensors at each subplot. The vapor pressure deficit (VPD) was calculated for each sector and all study days following the Monteith equation (Goudriaan & Monteith 1990). To calculate potential evapotranspiration (ET_0), we used the “PE_Oudin” function from the ‘airGR’ package in R, which uses Oudin’s formula (adjusted PE model). This model has high accuracy and requires fewer environmental variables than other ET_0 estimation models (Oudin et al. 2005; Singh et al. 2023). Days with high solar radiation during June and July of 2019, i.e. the four hottest days in June (24, 25, 26, and 30) and the five hottest days in July (6, 23, 24, 25, and 26) were selected. The average temperature was 26.5 °C across the study days, while the maximum air temperature of 35.5 °C was recorded on the 30th of June 2019. The average precipitation was 631 mm in 2019 and the maximum precipitation across the study period was 5.4 mm on 21st July 2019. The average Ψ_w was high throughout the study period, and the lowest Ψ_w was observed at one subplot (−1,377 kPa; Table 1). However, Ψ_w values did not drop below the permanent wilting point of −1,500 kPa, indicating no significant drought during the study period (Brodrribb et al. 2014; Özçelik et al. 2022).

2.3. Sap flow, tree, and crown characteristics measurements

Sap flow sensors (EMS81, EMS Brno, CZ) were installed at the height of 2 m in 2018 on mature trees, considering their similarities in height and DBH (Fig. 1B). The sensors were based on the trunk heat balance (THB) method consisting of four electrodes where the three electrodes placed on top are electrically heated, and the fourth one is set 10 cm below, serving as a reference. The temperature difference between heated and reference electrodes was adjusted to 1K. The method for calculating the heat balance of the heated space is determined by the following equation (Čermák et al. 2004):

$$Q = \frac{P}{C_w} \times d \times \Delta T - \frac{Z}{C_w} \quad [1]$$

where Q is the sap flow rate ($kg \cdot s^{-1} \cdot cm^{-1}$), P is the power of heat input (W), ΔT is the temperature difference at the measuring points (K), C_w is the specific heat of water ($J \cdot kg^{-1} \cdot K^{-1}$), d is the circumferential distance (cm) covered by the electrodes, and Z/C_w is the heat loss determined in the absence of sap flow. The THB method provides sap flow in kilograms. Therefore, to calculate the total water consumption of the tree, Q for the measured area was multiplied by the xylem circumference at installation height (Čermák et al. 2004).

Additionally, macroscopic crown characteristics, such as crown length and total crown defoliation, were recorded during field surveys (Polák et al. 2006; Cudlín et al. 2017). The total crown defoliation, an important proxy of the tree’s physiological condition, was visually estimated as the percentage of missing foliage in the total crown volume as described by Cudlín (2015). The length of the juvenile, productive parts, and defoliation pattern of the tree crown, which determines photosynthetic rates, were used to recalculate the unit of sap flow. All meteorological, SWP, and sap flow data collected from the field were stored in 10-minute intervals in a single data logger (GreyBox N2N 3P, EMS Brno, CZ) connected to a cloud system that delivers the data online.

2.4. Tree buffer and neighboring tree density estimation

The solar azimuth angle (α), sun trajectory, and sunlight phases (time of sunrise, sunset, dusk) for the study days were determined using SunCalc.org (SunCalc 2017, Fig. 2). The daylight was observed from 5:00 to 21:10 hours ($\alpha = 52^\circ$ – 308°) on study days, with the sun entering the East sector at 5:00 hours and leaving the West sector around 21:10 hours. A 10 m radius buffer around each sap flow measured tree was created by overlaying the UAV imagery acquired at 20 cm spatial resolution using ArcGIS Desktop (ver. 10.8.1; ESRI 2020).

The buffer was divided into sectors (East, South, West, and North) according to the sun’s positioning. As per the SunCalc data (see Fig. 2), the solar irradiance does not strike directly from the Northern side; therefore, the northern sector was excluded. The number of trees located entirely in each sector was counted, while some trees positioned on the border of two adjacent sectors were assigned a decimal number, depending on the crown portion within each sector (see Table 2). The sap flow sum was calculated for each sector for all study days; for example, when the sun was in the East (5:00–11:10 hours, $\alpha = 52^\circ$ – 129°), South (11:10–15:10 hours, $\alpha = 129^\circ$ – 234°), and West (15:10–21:10 hours, $\alpha = 234^\circ$ – 308°). The sap flow data were recorded at 10-minute intervals; therefore, the time difference between the sun entering and leaving each sector was rounded off to align both the data uniformly.

2.5. Data cleaning and processing

The sap flow measures of selected trees were processed (by setting a baseline) to remove night-time fluxes using the system software according to the manufacturer’s recommendation (Mini32 ver. 10.2.17.0; EMS Brno, CZ). Sap flow sums calculated over the selected days for individual trees were recalculated to relative crown length

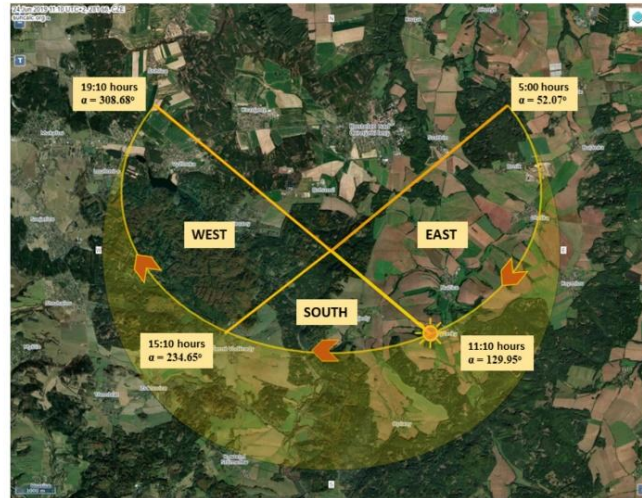


Fig. 2. Graphical representation of sun trajectory from East (05:00–11:10 hours) to South (11:10–15:10 hours) and West (15:10–19:10 hours) sectors, where the yellow arrow represents the sun path observed from the study site, α represents the azimuth angle between the beginning and end of each sector. During the study period, dawn and dusk were observed at 05:00 and 21:10 hours, respectively.

and abundance of foliage (Cudlín et al. 2015; 2017) using the following equations:

$$SF_{sector\ n} = \frac{SF_{sector\ input} \times (1 - TD)}{Crown\ length \times 1,000} \quad [2]$$

$$Total\ SF_{sector\ n} = \sum SF_n (day\ 1 + \dots + day\ 9) \quad [3]$$

where $SF_{sector\ n}$ is sap flow in a particular sector, and $1 - TD$ is the abundance of foliage (crown length – total defoliation). To compare sap flow among individual trees, it was necessary to normalize the sap flow to a unit relative to individual crown length, where all processes affecting sap flow occur. This normalized data was then used to calculate the mean sap flow per tree per day. Subsequently, we normalized SF data according to the best-performing tree (SF_{sum} as an indicator) on a particular day. The implemented equations for sap flow data normalization are as follows:

$$SF_{sum\ normalized\ n} = \frac{SF_{sum\ n}}{SF_{sum\ max} \times 100} \quad [4]$$

$$Normalized\ coefficient = \frac{SF_{sum\ normalized\ n}}{SF_{sum}} \quad [5]$$

After normalizing the data, we derived relative sap flow values (in %) for the trees on a given day. In the next step, we divided these normalized sap flow values into intervals corresponding to different sun positioning sectors – East (5:00–11:10 hours), South (11:10–15:10 hours), and West (15:10–21:10 hours). This normalization process enabled us to adjust for temporal variations in radiation

intensity throughout the selected days, ensuring that any undesired variability that could potentially skew the results of direct comparisons among individual trees over each day of the study period was minimized. The categorical values representing the number of neighboring trees (tree density) in specific directions were then standardized. Following this, categories were balanced to ensure an equal sample size, and outliers were removed, resulting in the selection of 25 trees for the analysis (Table 2). The selected 25 trees were randomly distributed across all study plots and subplots and had an average DBH of 40.2 cm (SD = 6.2 cm).

2.6. Statistical Analysis

First, the total sap flow in each sector was calculated (in kg) using equation [3] to elucidate the dynamics of sap flow in response to diurnal solar radiation. Subsequently, the sap flow data were normalized utilizing equations [4] and [5] to derive relative sap flow values in percentages. We modeled each sector's sap flow response using sap flow daily mean values of 25 selected trees and regression equations outlined in Fig. 3. The model facilitated comparison between the measured and modeled sap flow rate values and enabled quantification of the fit of the obtained regression equation. Generalized Linear models (GLMs) with θ as the natural parameter and ϕ as the dispersion parameter was used to define the relationship between tree groups and sap sums of each sector using the “glm” function from the “stats” package (R Core Team 2021). Further, Pearson's product-moment correlation test was

Table 2. Distribution of selected trees and density across sectors for balanced categories (25 trees per sector) in the Study. “Neighboring tree density (*n*)” indicates the relative tree density, while “number of selected trees in each sector” refers to the focal trees with measured sap flow chosen from each sector to ensure category balance. Foliage abundance denotes the foliage quantity utilized in sap flow calculation as per equation [2].

Neighboring tree density (<i>n</i>)	Number of selected trees in each sector		
	East	South	West
1	2	0	3
1.5	1	0	4
1.8	0	0	1
2	4	4	4
2.2	0	2	0
2.5	4	4	4
3	4	5	4
3.5	4	5	3
4	4	4	0
4.5	0	1	0
5	1	0	1
5.5	1	0	1
Weighted Average	2.96	3.02	2.47
Median	3	3	2.5
Crown length (Mean ± SE, %)	57.8 ± 1.71	59.4 ± 1.74	58.6 ± 2.21
Foliage abundance (Mean ± SE, %)	64.4 ± 0.78	64.0 ± 0.82	65.4 ± 0.76

performed using the “cor.test” function (at 0.05 type I error rate) to examine the relationship between tree density sets and sap flow (R Core Team 2021).

3. Results

3.1. Temporal dynamics of sap flow in response to solar radiation regime

Our data shows that diurnal sap flow variations in sectors differed significantly (Table 3). In the morning, the sap of trees started to flow at 05:00 hours at the onset of solar radiation; a gradual increase was seen in sap flow rates corresponding to solar radiation in the East sector. The maximum values of sap flow were reached during midday between 11:10 and 15:10 hours when the sun was at its zenith in the South sector. Equation [3] was used to calculate the total amount of flow in each sector across the study period. To better describe the changes, we used this sector as a reference in relative measure and considered the total sap flow in the South as 100%. Compared to this, there was a significantly lower mean sap flow rate in the early morning hours, reaching only 57.3% of the sap flow rates during midday. However, in the late hours, when the sun was in the West sector, the total sap flow decreased slightly by 11.8% compared to the South sector.

We modeled sap flow response using balanced categories of trees in each sector using the regression equations presented in Fig. 3 to validate the findings. The modeled sap flow values show that the regression equation used in the calculation slightly underestimates the results compared to the measured values, while the distribution between sectors remains approximately the same.

3.2. Tree density effects on sap flow

We examined the impact of tree density in individual sectors on sap flow rates, defined as the number of trees within a 10 m radius buffer around each target tree. We used the GLM model with the natural parameters θ , and the dispersion parameter ϕ to analyze the relationship between tree density and sap flow rates. Our findings indicate that sap flow rates show an inverse relationship with tree density ($p < 0.01$, Fig. 3). Specifically, denser populations showed lower sap flow rates, while sparser stands showed higher sap flow rates throughout the study period.

The relationship between sap flow rates and tree density was strongest in the West sector ($R^2 = 0.43$, $p < 0.01$, Fig. 3C). Furthermore, the sap flow response to tree density in the South sector was marginally stronger ($R^2 = 0.39$, $p < 0.01$, Fig. 3B) compared to the East sector ($R^2 = 0.32$, $p < 0.01$, Fig. 3A).

Table 3. Hourly intervals for each sector according to sun positioning and the calculated variables used in the study. The columns labeled “Sap flow measured” and “Sap flow modeled” indicate the total sap flow calculated using equation [3] and modeled, respectively, for each sector over the study period (9 selected days). The relative percentage change in sap flow in the East and West sectors is calculated by taking the South as a reference, assuming the total flow in the South equals 100%.

Sector	Hourly intervals	Azimuthal angle (α)	Sap flow measured (mean ± SE, kg)	Relative change in sap flow (%)	Sap flow modeled (mean ± SE, kg)	Relative change in sap flow (%)
East	5:00–11:10	52°–129°	45.16 ± 2.41	57.3	39.28 ± 0.98	57.8
South	11:10–15:10	129°–234°	78.78 ± 3.34	100.0	67.95 ± 1.68	100.0
West	15:10–21:10	234°–308°	69.48 ± 2.30	88.2	60.76 ± 1.51	89.4

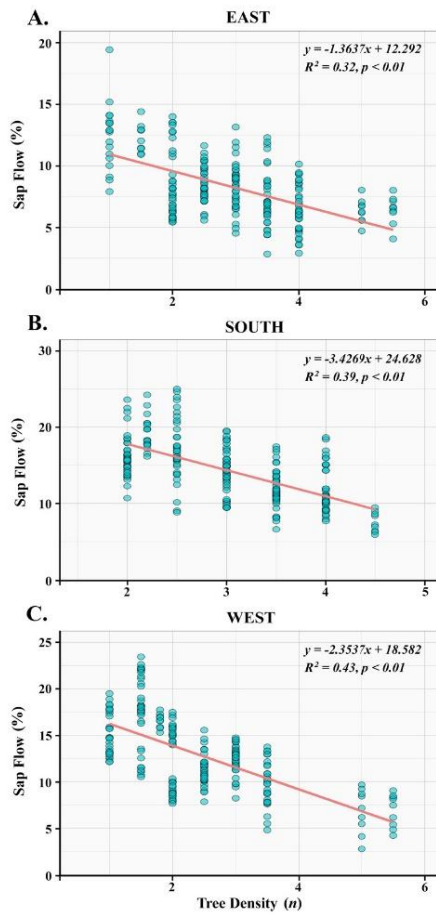


Fig. 3. Scatter plot between relative sap flow (%) from equation (5) and neighborhood tree density (n) in each sector. The significance of correlation determination (R^2) at 0.05 type I error rate (p -value) and the GLM model are provided for each E, S, and W sector.

4. Discussion

Tree density in forests can significantly affect the physiology, growth, and overall health of individual trees and the overall functioning of the forest ecosystems. Sap flow and transpiration are primarily influenced by air temperature and solar radiation, among other environmental factors. However, studies examining the effects of tree density on physiological processes are less common than dendrological measurements, likely because of the greater complexity involved in field assessments (Gabira et al. 2023). Here, we used a novel approach by calculating sap flow to relative units based on individual trees' crown lengths and defoliation parameters and by counting neighboring tree density around each target tree using high-resolution imagery. The proposed relative sap flow measure is quan-

tified per unit of available foliage and crown length as a ratio of sap flow recorded for a tree receiving the highest amount of solar radiation on a particular day, accounting for the diurnal sun position and tree-specific neighborhood competition in a stand.

4.1. Significance of data normalization relative to the crown length and defoliation

Transpiration is a vital process in plants, through which water is lost as vapor from leaf surfaces. It plays a crucial role in plant physiology, influencing various aspects such as nutrient uptake, leaf temperature regulation, and the movement of water and minerals through the xylem, ultimately impacting plant growth and productivity. Several factors influence the transpiration rate. One of the crucial factors is the amount of solar radiation intercepted by the canopy, as increased sunlight can enhance the rate of transpiration and vice-versa (Jones 2013). The physical and physiological properties of the canopy, including the canopy area and architecture (e.g., crown length and leaf area index), influence the transpiration rate (Wang & Jarvis 1990; Jia & Wang 2021), and sap flow can vary significantly between individual trees (Lagergren & Lindroth 2004). The crown length refers to the vertical extent of the tree canopy, and a longer crown length generally implies a larger surface area for transpiration. Alternatively, defoliation, or the loss of leaves, can reduce the surface area of foliage. Since the leaf area index is closely related to photosynthesis, transpiration, and productivity, reduced foliage area can limit the amount of water loss through transpiration (Sonnentag et al. 2007). Therefore, it is essential to consider crown length and defoliation attributes when analyzing and comparing data among different trees or populations to ensure a fair and robust assessment of ecological processes.

4.2. Temporal dynamics of sap flow in response to solar radiation regime

Our results show that transpiration rates were highest at midday compared to early and late hours during the day. The major driving forces of tree transpiration are soil water content and atmospheric evaporative demand (Gartner et al. 2009), and factors that determine evaporative demand are solar radiation, air temperature, wind speed, and air humidity (Allen et al. 1998; Zhang et al. 2021). The study sites had similar climatic conditions without any significant drought period across the study days; thus, the sap flow could be primarily controlled by evaporative demands (ET_0). The average air temperature and VPD values followed similar patterns to sap flow, where maximum sap flow was observed when the evaporative demands were highest at midday (refer Table 1). It has been previously shown that solar radiation and

VPD significantly affect instantaneous sap flow and total tree transpiration (Pereira et al. 2006; Oguntunde et al. 2007; Oogathoo et al. 2020; Mikita et al. 2023). Thus, the low sap flow rates during early hours were likely due to cooler air temperature and lower VPD. In contrast, a gradual increase in air temperature upregulates evaporative demands, resulting in more intensive sap flow as the sun approaches the zenith, as observed in the South sector.

Most tree species demonstrate a uniform diurnal sap flow pattern under adequate water conditions (Burgdorf 2006; Gartner et al. 2009; Deutscher et al. 2016), albeit species-specific temporal variations exist. For example, in a multi-year study by Burgdorf (2006), the highest sap flow in birch (*Betula* sp.) was consistently observed at midday. Similarly, Gartner et al. (2009) noted that in conditions where water availability was not a limiting factor, their findings aligned with ours, showing peak transpiration rates occurring around midday between 14:00–15:00 hours. However, under limited water availability, with the area receiving only 58% of the long-term average precipitation, the peak sap flow in spruce trees occurred at 10:30 hours, followed by a gradual decrease in flow rates. Likewise, another study investigated diurnal sap flow patterns in mixed forests and observed the peak flow occurrence at 14:00 hours (Deutscher et al. 2016). These findings suggest that air temperature reaches its maximum value during midday, while relative humidity values drop to their lowest point at the same time. This observation is consistent with our results, indicating that the maximum average temperature recorded during midday (31 °C in the South sector) within the study period could account for the increased transpiration rates observed.

The higher heat loads in sparser stands could negatively affect many metabolism-related functions. In trees, the stomatal opening occurs in response to red and blue light exposure, facilitating gas exchange between mesophyll tissues in leaves and the surrounding environment (Matthews et al. 2020). Gas exchange is required for the inward diffusion of CO₂, photosynthesis, and water uptake by roots (Inoue & Kinoshita 2017). As temperature increases, the mesophyll conductance improves due to decreasing water viscosity, enhancing water supply to evaporation sites (Cochard et al. 2000; Urban et al. 2017). Additionally, high temperatures can lead to reduced photosynthesis due to decreased RubisCO activity (Crous et al. 2022), but some studies suggest that this relationship may not always hold under extreme temperatures, where gas exchange may remain high despite reduced assimilation (Ameye et al. 2012; von Caemmerer & Evans 2015). Therefore, even at higher temperatures during midday, assimilation may decrease without significantly reducing transpiration. Consequently, this may affect defense-related metabolite synthesis and increase the tree's susceptibility toward biotic stressors.

4.3. Sap flow relations to tree density

Our study reveals a distinct inverse correlation between sap flow and neighboring tree density. We observed that trees within higher-density stands had lower transpiration rates, while those in sparser density exhibited higher transpiration rates. The categorical tree distribution demonstrates a high significance of density parameters ($p < 0.01$), with the regression model indicating the strongest correlation in the West sector, followed by the South and East sectors, respectively (Fig. 3).

These findings contribute to the existing evidence of intraspecific competition among neighboring trees within a stand (Canham 2004; Bhandari et al. 2021; Zabihi et al. 2023). Intraspecific competition intensifies as the distance between neighboring trees and the target tree decreases (Thorpe et al. 2010). As sessile organisms, trees are unable to relocate to more favorable environments when faced with stressful conditions. Consequently, increasing tree density heightens intraspecific competition among individual trees, limiting resource availability both above and below the ground (Zabihi et al. 2023; Thomas et al. 2024). The observed decrease in sap flow with increasing tree density likely results from intensified competition for soil water. Studies suggest that increased competition within a forest stand can exacerbate drought impacts and amplify mortality risks, especially under water-limiting conditions (Zhang et al. 2015; Young et al. 2017; Korolyova et al. 2022b). Limited resource availability can affect various metabolic and defense-related plant functions, such as resin exudation, photosynthesis, and the subsequent biosynthesis of defense metabolites (Netherer et al. 2014; Erbilgin et al. 2021), predisposing trees to various stress conditions, including pest infestations.

Conversely, fragmented forests characterized by low stand density are more susceptible to storm and windthrow disturbances and receive higher heat loads (Marcšová et al. 2020; Özçelik et al. 2022). In spruce plantations, forest fragmentation may result from natural wind/storm disturbances or management practices like thinning, sanitation felling, and salvage logging in areas affected by pest outbreaks. Generally, trees are protected against solar radiation either by their branches (individual shading) or by crowns of neighboring trees (collective shading) (Jakuš et al. 2011). The collective shading buffers trees against direct solar radiation (Mezei et al. 2014), modulating transpiration and mitigating abiotic and biotic stressors. Moreover, intact forest canopy minimizes the amount of solar radiation reaching the forest floor, lowering ground-level evaporation, while individual trees growing in open areas exhibit greater water usage driven by various factors, such as higher water availability per tree, increased radiation exposure, greater canopy roughness, and ventilation in thinned forests (Raper 1998). However, freshly fragmented forest

stands with numerous edges expose trees to sudden solar radiation changes, leading to shifts in microclimatic conditions and potentially elevating the risk of bark beetle infestations.

The shallow root system of spruce makes it susceptible to water stress, especially for trees grown in dense stands (Horgan et al. 2003; Caudullo et al. 2016); thus, mature trees need more space to access below-ground water for survival in drought conditions. Previous findings suggest that competition for below-ground resources is greater than for above-ground resources (Casper & Jackson 1997). Furthermore, the utilization of soil space is recognized as a fundamental aspect of underground competition (Casper & Jackson 1997). Despite the lack of water limitation at our study site, the reduced transpiration among denser stands could be a consequence of the morphological constraints of the root system. Although trees possess a variety of mechanisms to tolerate or withstand prolonged drought and maintain their physiological and morphological functions (Brunner et al. 2015), the root architecture might be constrained by lack of space due to adjacent trees, thereby reducing the total area available for horizontal root distribution.

The capability to occupy soil space varies from species to species and relies on several root attributes, such as fine root density, total surface area, biomass, and relative growth rate (Casper & Jackson 1997). Even though the majority of roots are present in the upper 30 cm of soil, some roots can extend to a greater depth, providing more area to explore for resources. Since different species have different spatial and temporal rooting patterns (Casper & Jackson 1997), adapting mixed forests with diverse species composition may have better overall functioning than monoculture forests. This could be due to minimal overlap between neighboring root systems, allowing for more efficient utilization of soil resources.

4.4. Disturbance processes and associated effects on spruce physiology

Previous studies suggest that trees growing in gaps or at the forest edges exhibit higher sap flow rates due to increased solar radiation exposure (Marešová et al. 2020; Özçelik et al. 2022). Higher transpiration rates are associated with better tree defense capability as transpiration is a part of constitutive and induced defenses (Özçelik et al. 2022). Reduction in living foliage volume reduces transpiration and photosynthetic rates, compromising resource-demanding induced defense mechanism against bark beetles (Herms & Mattson 1992), which select hosts with more intensive total and primary crown defoliation compared to secondarily attacked and non-attacked spruces (Korolyova et al. 2022b). However, trees in fragmented stands are likely to downregulate transpiration under water-limiting conditions. Water deficit, especially in hot summer periods, may result in

reduced transpiration rates and an ensuing decline in the cooling effect of sap flow. Marešová et al. (2020) showed that despite high transpiration levels, trees growing at sun-exposed forest edges exhibit higher bark surface temperature and volatile compound emissions than shaded trees located in stand interiors. Despite a substantial cooling effect of sap flow on cambium temperature, the maximum temperature difference between a tree with high sap flow and one without transpiration did not exceed 2.5 °C (Hietz et al. 2005). These facts indicate that higher levels of transpiration in trees located in fragmented stands may not cause sufficient cooling to avoid colonization by insect herbivores. Increased bark surface temperature and radiation may predispose trees to beetle-induced mortality (Kautz et al. 2013) through thermal effects on the *Ips typographus* L. life cycle and population development (Wermclinger & Scifert 1999). Spruce survival probability during bark beetle outbreak declines substantially with increasing proportion of stem exposed to the sun and less extensive tree crown (Jakuš et al. 2011; Korolyova et al. 2022a).

The negative relationship between tree crowding and sap flow, based on our results, supports the previous findings suggesting that aggravated resource competition significantly reduces spruce survival potential during bark beetle disturbance (Buonanaduci et al. 2020; Korolyova et al. 2022a). Light, nutrient, and water deficit impairs plant physiological functions and carbon assimilation processes. Depletion of energy pools available to produce secondary metabolites may ensue tree life history trade-offs, suggesting that carbon allocation to maintain transpiration and photosynthesis is prioritized over the synthesis of defensive compounds (Jones & Hartley 1999).

4.5. Management implications

From a practical perspective, these findings offer valuable insights for forest management strategies. Topography and inter-tree spacing significantly modulate ecosystem functions, and forest management strategies such as thinning should be tailored considering these variables to foster improved overall health and resilience of secondary forests (Thomas et al. 2024). Moreover, mixtures could be a preferable plantation strategy over monocultures (Zhang et al. 2022) because variation in canopy length and size in trees of mixed forests may provide better coverage and reduce the incoming solar radiation to the forest floor, thus lowering evaporation from the ground (Zabihi et al. 2023). Mixed forests, with their greater genetic diversity, are more adaptable to changing environmental conditions (Liu et al. 2018; Ammer 2019) and are generally more resilient to windthrow events and pest infestations than monoculture forests and can provide a broader range of ecosystem services and functions (Liu et al. 2018).

5. Conclusions

This study investigated Norway spruce sap flow dynamics during nine hot summer days in 2019, focusing on two goals. Firstly, it aimed to identify the diurnal transpiration pattern and its dependency on solar radiation, confirming that transpiration peaks at midday in June and July due to increased radiation, VPD, and ET_0 . Secondly, it explored the impact of tree density on transpiration, finding that denser stands exhibit lower transpiration rates compared to sparser stands under sufficient water availability, suggesting that tree density influences the overall water use dynamics within the forest ecosystem. In water-limited conditions, the sparser spruce stands could potentially become more susceptible to stress and bark beetle infestations, compared to their denser counterparts.

Further follow-up studies in forests with varying species composition and environmental conditions are required to understand the relationship between stand density and its subsequent effect on tree-level transpiration. For instance, hilltops are likely to experience higher solar radiation and subsequently exhibit increased transpiration rates due to lower solar incidence angles compared to hillsides during the mid-day and early afternoon in the summer season. This could potentially lead to elevated water stress conditions on hilltops, rendering them more susceptible to bark beetle infestations. Considering that tree density generally decreases with increasing elevation, it is recommended that future studies investigate the interplay between diurnal sun azimuth, transpiration rate, aspect, elevation, slope gradients, and tree density, and how these factors collectively influence forest resilience against pest infestations. The correlation between tree mortality and elevation gradients in all bark beetle outbreak stages further underscores the importance of understanding these complex dynamics.

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5.3 Robust reference gene selection in Norway spruce: essential for real-time qualitative PCR across diverse tissue, stress and developmental conditions.

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This study identified stable reference genes for quantitative PCR in Norway spruce, enhancing gene expression analysis under diverse conditions. Norway spruce seedlings were subjected to drought, heat, and pathogen infection stress treatments in the controlled greenhouse environment. Tissue samples (needle, phloem, and root) were collected from both seedlings and mature trees. Fifteen candidate reference genes were selected based on prior studies and our in-house transcriptome data, and their expression stability was assessed using five algorithms: ΔC_t , geNorm, NormFinder, BestKeeper, and RefFinder.

The results highlighted *ubiquitin-protein ligase (SPI)*, *conserved oligomeric Golgi complex (COG7)*, and *tubby-like F-box protein (TULP6)* as the most stable reference genes across various tissues and stress conditions, while *succinate dehydrogenase (SDH5)* and *heat shock protein 90 (HSP90)* showed the least stability. Notably, this is the first study to report *COG7* and *TULP6* as stable internal control genes. Pairwise variation analysis suggested that two reference genes would be sufficient for reliable normalization in gene expression studies under these experimental conditions. Validation of these findings using the drought-responsive gene *PaDhn5* confirmed that *SPI* and *COG7* provided accurate normalization, supporting their use as reliable reference genes. This comprehensive assessment offers a foundational set of stable reference genes, which are essential for accurate RT-qPCR analyses in Norway spruce. The findings of this work will facilitate a more robust exploration of stress responses and regulatory mechanisms in *P. abies*, providing valuable resources for future conifer genomics research.



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Robust reference gene selection in Norway spruce: essential for real-time quantitative PCR across different tissue, stress and developmental conditions

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Accurate gene expression analysis in Norway spruce (*Picea abies*) under diverse stress conditions requires the identification of stable reference genes for normalization. Notably, the literature lacks reports on suitable reference genes in Norway spruce. Here, we aimed to address this gap by identifying suitable reference genes for quantitative real-time PCR in Norway spruce across various stress conditions (drought, heat, pathogen infection) in seedlings, tissues (needle, phloem, root), and developmental stages (seedlings, mature trees). We evaluated the stability of 15 candidate reference genes and assessed their expression stability using five statistical algorithms (ΔC_t , geNorm, NormFinder, BestKeeper, and RefFinder). Our results highlight *ubiquitin-protein ligase (SPI1)*, *conserved oligomeric Golgi complex (COG7)*, and *tubby-like F-box protein (TULP6)* as the most stable reference genes, while *succinate dehydrogenase (SDH5)* and *heat shock protein 90 (HSP90)* were the least stable under various experimental conditions. *COG7* and *TULP6* are novel candidate reference genes reported for the first time. The expression stability of the identified reference genes was further validated using dehydrin-like protein 5 (*PaDhn5*) under drought conditions in Norway spruce. Pairwise variation analysis suggests that two reference genes were sufficient to normalize gene expression across all sample sets. This study provides a comprehensive analysis of reference gene stability under different experimental conditions and a catalog of genes for each condition, facilitating future functional genomic research in Norway spruce and related conifers.

KEYWORDS

RT-qPCR, housekeeping genes/reference genes, gene expression, developmental stages and tissues, abiotic and biotic stress

1 Introduction

Norway spruce [*Picea abies* (L.) Karst.] is one of the most ecologically and economically important coniferous tree species in Europe. Adapted to cool and wet conditions, its distribution extends from central and eastern Norway across Fennoscandia, the Baltic states, Belarus, Russia, and Central and Southeastern Europe (Danielsen et al., 2021). Over the past several decades, Norway spruce has been extensively planted in areas beyond its natural

distribution range due to its excellent growth performance and desirable wood properties for commercial forestry (Caudullo et al., 2016; Hlásny et al., 2019). However, planting it outside its niche has led to health and vitality issues, increasing its susceptibility to biotic and abiotic stresses. For instance, Norway spruce forests in the Czech Republic experienced a dramatic increase in mortality in 2019, losing an average of 118 million m² due to droughts, heatwaves, and bark beetle outbreaks (Ebner, 2020). Traditional management strategies, such as pheromone-based mass trapping, salvage logging, insecticides, and anti-attractants, have been largely ineffective in controlling mass bark beetle attacks (Dobor et al., 2020; Singh et al., 2024a). Consequently, there has been rapid exploration of functional genomics-based tools to develop effective management strategies. Notably, sequencing of the Norway spruce genome, the first among conifers (Nystedt et al., 2013), has significantly advanced gene expression studies aimed at understanding gene functions and molecular regulation under various stresses. However, the appropriate reference genes required for gene expression normalization in Norway spruce have not yet been comprehensively established. Therefore, identifying reliable reference genes is crucial for improving studies on gene expression profiling, providing novel insights into biological processes, and deepening the understanding of regulatory gene networks that contribute to spruce resistance, resilience, and survival (Wise et al., 2007).

The significance of differential gene expression analysis in understanding gene function and molecular regulation in response to various environmental factors is crucial. Real-time quantitative polymerase chain reaction (RT-qPCR) is a widely used technique for analyzing the expression of target genes owing to its high sensitivity, accuracy, specificity, reproducibility, and rapidity (Bustin, 2002; Gachon et al., 2004; Bustin et al., 2005; Takamori et al., 2017). However, the precision of RT-qPCR is highly dependent on using a reference gene as an internal control. Reference genes, also referred to as housekeeping genes, are consistently expressed in cells and are vital for fundamental cellular functions. These genes encode proteins that play crucial roles in essential cellular activities, including cell cycle regulation, DNA replication, and metabolism. An ideal reference gene maintains consistent expression levels across different experimental conditions and tissues and remains unaffected by developmental stages or the organism's genotype (Han et al., 2012; Lu et al., 2018; Sen et al., 2021).

Several studies have identified and validated reference genes in crops, commercially valuable tree species, and herbs, including *Solanum tuberosum* L. (Nicot et al., 2005), *Arabidopsis thaliana* (L.) Heynh. (Czechowski et al., 2005), *Oryza sativa* L. (Jain et al., 2006), *Triticum aestivum* L. (Paolacci et al., 2009), *Linum usitatissimum* L. (Huis et al., 2010), *Eucalyptus robusta* Sm. (de Oliveira et al., 2012), *Populus euphratica* Oliv. (Wang et al., 2014), *Solanum melongena* L. (Mogilicherla et al., 2016), *Bromus sterilis* L. (Sen et al., 2021), *Melissa officinalis* L. (Bharati et al., 2023), and *Populus tremula* L. (Pastierovič et al., 2024). Recently, efforts have been made to identify and validate reference genes in coniferous species such as Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook.) and Masson pine (*Pinus massoniana* Lamb.) (Bao et al., 2016; Mo et al., 2019; Chen et al., 2019). However, these studies were limited to specific tissues and treatment conditions. Previous research on internal control genes in *P. abies* was limited to embryogenic cell lines (e.g., Vestman et al., 2011; de Vega-Bartol et al., 2013). In maritime pine (*Pinus pinaster*),

genes such as *GADPH*, *18S rRNA*, *UBQ*, and *eIF4AII* were found unsuitable as internal controls during embryo development due to the high variability in gene expression during embryogenesis and the involvement of these genes in both basal metabolism and other functions (Gonçalves et al., 2005). More recently, a study identified *elongation factor 1-gamma*, *histone H1*, *GAPDH*, and *α-tubulin* as stable reference genes for somatic embryogenesis tissues in *Liriodendron* hybrids, while *elongation factor 1-gamma* and *actin* were most reliable for germinative organ tissues (Li et al., 2021). These findings suggest that results from embryogenic cell lines may not accurately represent seedlings or mature trees under varying stress conditions. Therefore, establishing a set of stable reference genes for Norway spruce across different developmental stages and stress conditions is crucial for improving the accuracy and reliability of gene expression analysis, enhancing our understanding of its physiological processes and stress responses.

Here, we analyzed the expression profiles of 15 candidate reference genes in different conifer species based on published research and our in-house transcriptome data (de Vega-Bartol et al., 2013; Bao et al., 2016; Mo et al., 2019; Chen et al., 2019). These genes are known for performing conserved cellular functions in gymnosperms and, therefore, are expected to show stable expression across various conditions, developmental stages, and tissues. We evaluated the expression stability of *ribosomal protein L26 (RPL26)*, *serine/threonine-protein phosphatase (PP2A2)*, *ribosomal protein L7Ae (RPL7Ae)*, *ubiquitin-like domain-containing CTD phosphatase (UBCP)*, *F-box protein (SKIP22)*, *heat shock protein (HSP90)*, *vacuolar fusion protein (CCZ1)*, *succinate dehydrogenase (SDH5)*, *18S rRNA (RID2)*, *putative lysine-specific demethylase (JM16)*, *conserved oligomeric Golgi complex (COG7)*, *ribosomal protein S10 (RSP10)*, *ubiquitin-protein ligase (SP1)*, *tubby-like F-box protein (TULP6)*, and *actin-related protein (ARP9)* across different tissues (needle, phloem and root), developmental stages (seedlings and mature trees), and under various biotic and abiotic stress conditions (drought, heat stress, and pathogen infection) in Norway spruce seedlings. The present study will enhance the accuracy of future gene expression studies and contribute to a more reliable evaluation of reference gene stability in Norway spruce. These reference genes are also expected to serve as templates for other related conifer species and should be further investigated.

2 Materials and methods

2.1 Plant materials and experimental conditions

2.1.1 Greenhouse experiment

Three-year-old containerized Norway spruce seedlings, approximately 40 cm high, were procured from a local nursery and planted in a moist peat/perlite mixture in May 2022. The seedlings were grown using seed material originating from the Bohemian-Moravian highlands (Czech Republic), characterized by 600–750 mm of annual rainfall and an altitude of 500–600 m above sea level. The seedlings were transferred to a greenhouse with a temperature range of 23 ± 2°C and 16-h/8-h light/dark photoperiod cycles at the Czech University of Life Sciences, Prague. Before any treatment, the plants were irrigated regularly with tap water for acclimation to the greenhouse conditions. A total of 16 plants were used for each treatment. The plants were

grouped into sets of four, which were pooled to form one biological replicate. Consequently, each treatment comprised four biological replicates, with each replicate consisting of four plants. **Control treatment:** This group was irrigated regularly and was not subjected to any abiotic or biotic treatment. **Water deficit treatment:** One group was subjected to drought stress by gradually interrupting the water supply until the mean plant water potential decreased below -2.1 MPa. A water potential value of -2.1 MPa in Norway spruce is considered low, indicating water stress that may lead to embolism formation and a loss of hydraulic conductivity (Rosner et al., 2019). The degree of drought stress was quantified by measuring the predawn water potential using a Scholander pressure chamber (PMS Instruments, Corvallis, Oregon, United States). **Heat stress:** To induce heat stress, the seedlings were placed in a climate chamber (FytoScope FS-SI 3400, PSI, Drásov, Czech Republic) at 35°C for 10 days with a constant relative humidity of 80% and regular irrigation. **Biotic stress:** The seedlings were inoculated with the Ophiostomatoid fungus *Ophiostoma flexuosum*, and incubated for 1 month in a controlled climate chamber mentioned above. Throughout the treatment period, the plants were carefully maintained under optimal humidity, light, and nutrient conditions.

Samples were collected from three different tissues (needle, phloem, and roots) from all treatments, including abiotic stress (drought and heat), biotic stress (pathogen infection), and control (well-watered) treatments. Tissue samples were snap-frozen in liquid nitrogen immediately after collection and stored at -80°C for further processing.

2.1.2 Field experiment

In addition to the seedlings, samples from mature trees (90–100 years) were collected from research plots of the Czech University of Life Sciences, managed by the School Forest Enterprise (ŠLP) in Kostelec nad Černými lesy (49.9940°N , 14.8592°E) in the eastern district of the Central Bohemian region of the Czech Republic (Singh et al., 2023). The climate in this region is dry and warm in summer, with a vegetation season lasting 150–160 days and an average annual temperature and precipitation of $7-7.5^{\circ}\text{C}$ and 600 mm, respectively. Norway spruce tissue samples (needle, phloem, and root) were collected from 16 trees in August 2022. The needle samples were collected by shooting branches with a shotgun (Burnett et al., 2021), while phloem and root samples were obtained using a 5 mm cork borer. The phloem samples were collected from the trunk at a height of 2 m. The cork borer was used to extract tissue cores that included both the phloem and cambium layers (Mao et al., 2019). For the root samples, lateral roots were selected from the same trees approximately 1 m from the trunk base (Kalyniukova et al., 2024). After carefully removing layers of soil and moss, the roots were exposed and sampled at 15 cm depth using the same 5 mm cork borer. All tissue samples were snap-frozen in liquid nitrogen immediately after collection and stored at -80°C for further processing. Similar to the greenhouse experiment, four biological replicates were used for each tissue type, with each replicate composed of four individual samples pooled together to reduce individual heterogeneity. The experimental design is schematically represented in Figure 1.

2.2 Selection of candidate reference genes for evaluation

A total of 15 candidate internal control genes, *RPL26*, *PP2A2*, *RPL7Ae*, *UBCP*, *SKIP22*, *HSP90*, *CCZ1*, *SDH5*, *RID2*, *JMJ16*, *COG7*,

RPS10, *SP1*, *TULP6*, and *ARP9* were selected for identifying the most suitable reference genes in Norway spruce (Table 1). The selected candidate genes were previously identified in other conifer species (de Vega-Bartol et al., 2013; Bao et al., 2016; Chen et al., 2019; Mo et al., 2019) and showed consistent expression in our in-house transcriptome data (Unpublished data). Primers for selected genes were designed through the PrimerQuest™ Tool (Integrated DNA Technologies, United States), and their primer efficiency and correlation coefficients (R^2) were calculated (Table 1). The sequence used to design primers is provided in Supplementary material S1.

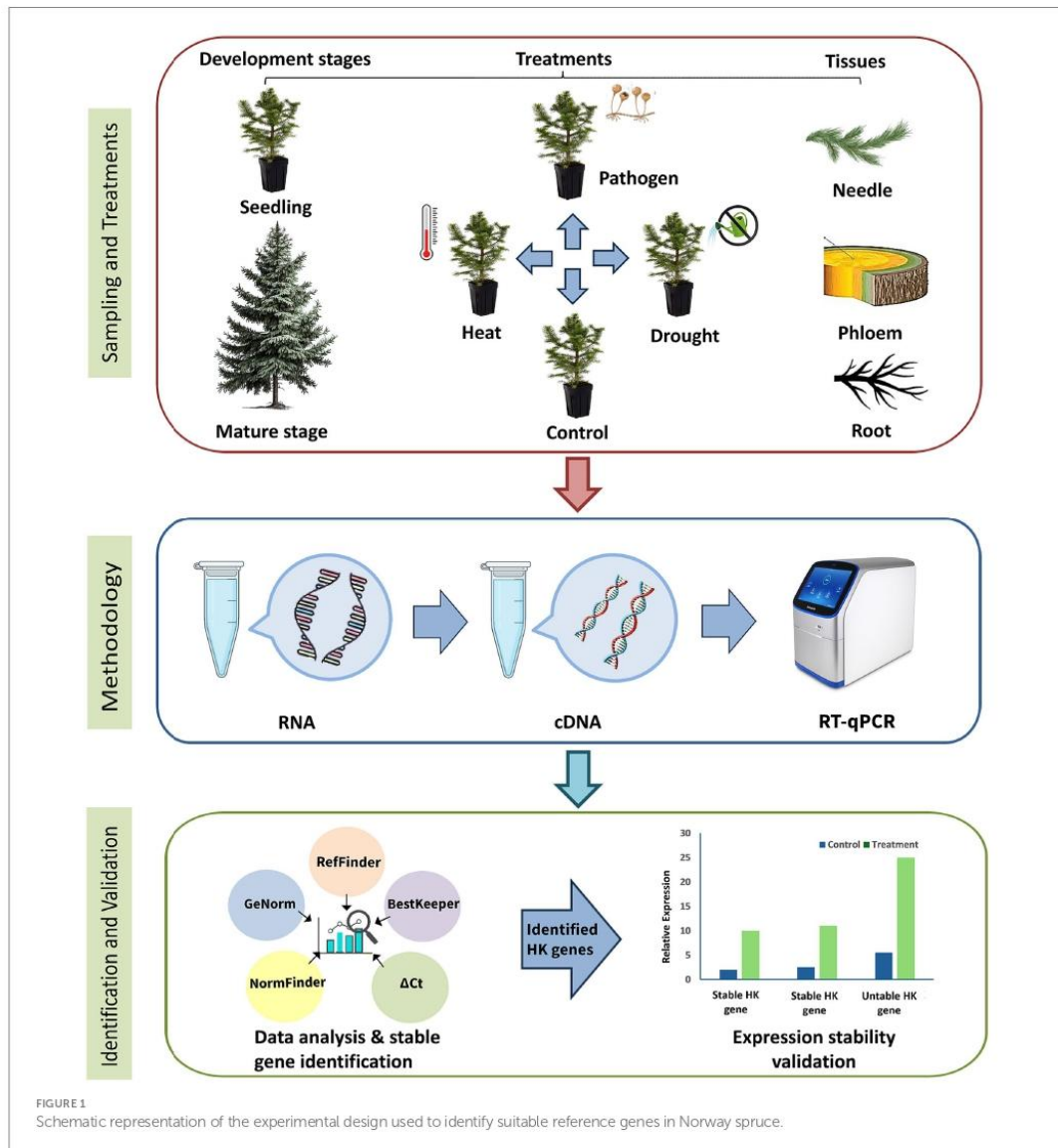
2.3 Total RNA extraction, cDNA synthesis, primer design, and quantitative RT-qPCR analysis

Total RNA from all three tissues (needle, phloem, and root) was isolated using a modified cetyltrimethylammonium bromide (CTAB) protocol described by Singh et al. (2024b). Briefly, 100 mg of homogenized tissue was added to the CTAB extraction buffer, followed by the addition of an equal amount of Chloroform:isoamyl alcohol (24:1) and subsequent centrifugation. The top aqueous layer was transferred to a new tube, and 0.5 volume of 5 M lithium chloride was added and incubated for 1 h at -20°C . Then, the mixture was centrifuged at 12,000 rpm for 10 min at 4°C , and the supernatant was discarded. The resulting RNA pellet was washed twice with 70% ethanol. The dried RNA pellet was eluted in 50 μL RNase-free water. The isolated RNA was further treated with the TURBO DNase Kit (Invitrogen, United States) to remove any genomic DNA contamination. RNA integrity and purity (260/280 and 260/230 nm absorbance ratios) were verified by electrophoresis on a 1.2% agarose gel and NanoDrop spectrophotometer (Thermo Scientific™, USA), respectively. Complementary DNA (cDNA) was synthesized from 1 μg of RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol and stored at -20°C .

Before performing RT-qPCR, the cDNA samples were diluted by a factor of five. The RT-qPCR reaction mixture consisted of 5.0 μL of SYBR® Green PCR Master Mix (Applied Biosystems, United States), 1.0 μL of cDNA from fivefold dilutions, 10 μM of forward and reverse primers, and 3.0 μL of RNase-free water (Invitrogen, USA) to make up a total volume of 10.0 μL . Amplification was conducted with initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The reactions were carried out using an Applied Biosystems™ StepOne™ Real-Time PCR System (Applied Biosystems, United States). To verify primer specificity, melting curve analysis was performed, ensuring gene-specific amplification with a gradual increase in temperature from 60 to 95°C . All RT-qPCR assays were conducted with four biological replicates per treatment and tissue, each including four technical replicates.

2.4 Statistical analyses

Five different algorithms and tools, i.e., geNorm, NormFinder, BestKeeper, ΔCt , and RefFinder were used to evaluate the gene expression stability of 15 candidate reference genes in Norway spruce. The comprehensive expression stability of candidate reference genes was evaluated using RefFinder, a web-based tool that combines the



outputs of the former four algorithms. The geNorm algorithm calculates the expression stability value (M) and conducts pairwise variation (V) comparisons, where the lowest M value suggests the most stable expression (Vandesompele et al., 2002). NormFinder ranks reference genes based on their expression stability within a given set of samples (Andersen et al., 2004). BestKeeper, another freely available algorithm, assesses the standard deviation and correlation coefficient using the C_q (quantification cycle) values of all reference genes (Pfaffl et al., 2004). The ΔCt method directly evaluates the relative expression of gene pairs within each sample (Silver et al., 2006). The mean C_q values of each reference gene from each experiment served as input

data and were processed using RefFinder, which combines widely used reference gene screening programs (geNorm, NormFinder, BestKeeper, and the ΔCt method) to calculate the geometric mean of their ranking values to provide an overall comprehensive ranking.

The optimum number of reference genes required under each experimental condition was determined by pairwise variation (V) analysis by calculating the variable value $V_n/n+1$ based on the average expression stability (M) with a cutoff of 0.15 using the geNorm algorithm. The V_n/V_{n+1} value indicates the pairwise variation between two consecutive normalization factors. When the value falls below this threshold, adding the $n+1$ reference gene becomes redundant.

TABLE 1 Primer information of the reference genes and target genes used in the study.

Gene symbol	Gene name	Primer sequence (5'-3')		Amplicon length (bp)	PCR efficiency	Regression coefficient
<i>RPL26</i>	Ribosomal protein L26	F:	CATCTAGTGTGGCGGTATT	114	96.72	0.999
		R:	CTCGTACCACCTGAACTTCATC			
<i>PP2A2</i>	Serine/threonine-protein phosphatase PP2A-2	F:	GGCCTATGTGTGATCTACTGTG	113	96.99	0.997
		R:	GTGATTGAATTGGGCTGCTATG			
<i>RPL7Ae</i>	Ribosomal protein L7Ae	F:	CCACTGTTGGCTGAGGATAAG	119	100.47	0.996
		R:	CCCTCATTGGTTGTCACAGAA			
<i>UBCP</i>	Ubiquitin-like domain-containing CTD phosphatase	F:	CCACAGAAATGGGCTTGTGATA	126	96.96	0.999
		R:	GTGGCTCAGATCATCCAGTTC			
<i>SKIP22</i>	F-box protein	F:	TTCCACAGAGCTCAAACCTG	134	91.19	0.995
		R:	CTCAGCCGCATACTTCTTCTT			
<i>HSP90</i>	Heat shock protein	F:	GGCGATCAAGATGAAGCAAAG	138	111.92	0.991
		R:	AAGCACACATGGCGAAGA			
<i>CCZ1</i>	Vacuolar fusion protein	F:	CAAACAGCAATAGCAGTGAAGG	133	93.88	0.999
		R:	ACAGAGAGCTGGCTAGTAAGA			
<i>SDH5</i>	Succinate dehydrogenase	F:	GCTCTACGGGTGCATATAAA	119	106.87	0.879
		R:	ACCAACAGCGTCACTAACC			
<i>RID2</i>	18S rRNA	F:	GAGGAGCACAGCTGTATTG	104	93.72	0.987
		R:	CACAACCAGACCACCTGAAA			
<i>JMJ16</i>	Putative lysine-specific demethylase	F:	AGCAGATGTGGAGACTAGGA	122	93.88	0.993
		R:	GGAGCCGTGCAATGTTATTAG			
<i>COG7</i>	Conserved oligomeric Golgi complex	F:	CCTCGGCTGAAGAAGACAAT	135	103.63	0.997
		R:	TTGTCCAGCACCCAATAC			
<i>RPS10</i>	ribosomal protein S10	F:	CCTCGATTGTTGACAGAGATG	110	96.72	0.999
		R:	GCCCTAAATTGAGGCTGGTATT			
<i>SP1</i>	ubiquitin-protein ligase SP1	F:	GGCACTACTCTGACTGTTGT	117	114.86	0.999
		R:	GGCAGATCTAGCCGTTTC			
<i>TULP6</i>	Tubby-like F-box protein	F:	CCTGAGCCTAATCCATCAGTTT	104	94.79	0.999
		R:	AGTGGGTAGCGATAATCCATTG			
<i>ARP9</i>	Actin-related protein 9	F:	CCTGGAAAGTGGTGTCTATT	120	93.65	0.999
		R:	AATCTCTGACTTTTCGACCCTC			
Target gene						
<i>PaDhm5</i>	Dehydrin like protein	F:	ATCAATGTGGGGTGAAG	122	120.48	0.951
		R:	ACTCCACACTGATCTGAA			

2.5 Reference gene validation

Dehydrins are a part of the group 2 late embryogenesis abundant (LEA) protein family that protects conifers against osmotic stress in response to cold and drought conditions (Stival Sena et al., 2018; Čepl et al., 2020; Krokene et al., 2023). To validate the selected reference genes, the relative expression levels of the dehydrin gene *PaDhm5* were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the most and the least stable reference genes. The data was checked for normality using the Kolmogorov–Smirnov test, and mRNA expression levels of target genes were assessed via single factor ANOVA using XLSTAT cloud (ver. 1.0), and a significance level of $\alpha = 0.05$ was used as the cutoff to indicate significant differences among the control and treatment groups.

3 Results

3.1 Amplification efficiency and specificity of candidate reference genes

Each primer pair produced a single amplicon with an expected amplicon length, as shown by agarose gel electrophoresis (Supplementary material S2). Melting curve analysis revealed a single peak, indicating no primer dimer was present (Supplementary material S3). The amplification efficiency of each primer pair ranged from 91.19 to 114.86%, and the correlation coefficient (R^2) values were greater than or equal to 0.879 (Table 1 and Supplementary material S4). The average cycle threshold (Ct) value was 28.14 (Supplementary material S5), while *SDH5*, *SP1*, *RID2*, and *ARP9* showed the highest transcript abundance across most

experimental conditions. In contrast, *RPL7Ae* and *RPL26* were the least expressed reference genes. The remaining nine reference genes exhibited moderate expression levels.

3.2 Expression stability of candidate reference genes

3.2.1 Tissuewise stability

In needle tissues, the three most stable genes were *SP1*, *JMJ16*, and *RID2* according to the comprehensive ranking by RefFinder and by Δ Ct, while *UBCP*, *HSP90*, and *SDH5* were the least stable genes (Table 2). The BestKeeper and NormFinder rankings were inconsistent with those of RefFinder and Δ Ct, suggesting that *PP2A2* and *UBCP* are the most stable genes, respectively. The geNorm ranking suggested a combination of *RID2*/*TUPL6* as the most stable gene (Supplementary material S6).

For phloem tissues, *RID2*, *SP1*, and *PP2A2* were the most stable genes according to RefFinder, while the least stable genes were *SKIP22*, *HSP90*, and *SDH5*. This ranking was consistent with that of the Δ Ct method, except that Δ Ct ranked *COG7* as the third most stable gene. The BestKeeper and NormFinder algorithms ranked *PP2A2* as the most stable gene in phloem tissues. According to geNorm, the combination of *RPL26*/*RPS10* was the most stable gene in phloem tissues (Supplementary material S6).

In root tissues, the BestKeeper, Δ Ct, and NormFinder rankings for the three most stable genes were not consistent with those of RefFinder. *UBCP*, *RPL7Ae*, and *SP1* were suggested to be the three most stable genes based on the comprehensive rankings of RefFinder (Table 2). A combination of *COG7*/*TUPL6* was the most stable reference gene in root tissues according to geNorm (Supplementary material S6). The least stable reference genes by the RefFinder comprehensive ranking were *SDH5*, *HSP90*, and *RID2*.

3.2.2 Treatmentwise stability

3.2.2.1 Control conditions

According to the comprehensive rankings of RefFinder, the three most stable genes under control conditions were *RPS10*, *RPL26*, and *SP1*, while *CCZ1*, *SDH5*, and *HSP90* were the least stable (Table 3). BestKeeper ranked *RID2* as the most stable gene, whereas NormFinder ranked *COG7* as the most stable gene. The Δ Ct value indicated that *RPS10* was the most stable gene, which is consistent with the RefFinder ranking. The best combination of reference genes according to geNorm was *PP2A2*/*TUPL6* under control conditions (Supplementary material S7). The *HSP90* gene was the least stable, as determined by geNorm analysis and the other four algorithms.

3.2.2.2 Drought stress conditions

Under drought conditions, *RID2*, *SP1*, and *COG7* were identified as the three most stable reference genes by the RefFinder algorithm, while a combination of *UBCP*/*SP1* was the most stable as per geNorm (Supplementary material S7). BestKeeper and NormFinder identified *PP2A2* and *RPL26* as the most stable genes, respectively. Both geNorm and other algorithms (Δ Ct, BestKeeper, NormFinder, and RefFinder) identified *SDH5* as the least stable reference gene under drought conditions.

3.2.2.3 Heat stress conditions

For studies under heat stress conditions, *RID2*, *SP1*, and *RPL26* were identified as the most stable genes by comprehensive ranking by the RefFinder program, whereas BestKeeper and NormFinder ranked *PP2A2* and *RPL26* as the most stable genes, respectively. A combination of *RPL26*/*ARP9* was ranked as the most stable gene according to geNorm (Supplementary material S7). Unanimously, *SDH5* was suggested as the least stable gene under heat stress conditions by geNorm and other algorithms.

3.2.2.4 Pathogen infection conditions

The topmost three stable genes after Ophiostomatoid fungus inoculation were *SP1*, *COG7*, and *RPL26* based on the rankings of the RefFinder algorithm, whereas the combination of *PP2A2*/*RPL7Ae* was the most stable according to geNorm (Table 2; Supplementary material S7). BestKeeper and NormFinder ranked *PP2A2* and *RPL26* as the most stable genes, respectively, which was not consistent with Δ Ct and RefFinder rankings. The gene *SDH5* was the least stable reference gene after fungal inoculation as per four algorithms, and geNorm ranked *RID2* as the least stable reference gene.

3.2.2.5 Mature tree

In mature tree samples, the comprehensive ranking algorithm RefFinder identified *SP1*, *COG7*, and *TUPL6* as the most stable reference genes and *SDH5* as the least stable gene. Similar to previous observations, the BestKeeper and NormFinder rankings were not consistent with that of RefFinder. BestKeeper and NormFinder ranked *PP2A2* and *HSP90*, respectively, as the most stable genes. In contrast, a combination of *RPL26*/*ARP9* was suggested to be the most stable gene and *COG7* as the least stable reference gene by geNorm analysis (Supplementary material S7).

3.3 Optimal reference gene selection for normalization

To achieve more accurate and reliable gene expression results, it is often recommended to use multiple reference genes. According to Vandesompele et al. (2002), if the V_n/V_{n+1} value is below 0.15, the addition of an additional reference gene ($n+1$) is deemed unnecessary, suggesting that the initial reference gene is sufficient for normalizing target gene expression. Using the geNorm algorithm, we determined the optimal number of reference genes for each condition. Our analysis revealed that at least two reference genes were necessary for accurate normalization in (mature trees and combined conditions), as indicated by the pairwise comparison values (Figure 2).

3.4 Validation of reference gene selection

Based on the RefFinder comprehensive analysis, *SP1* and *COG7* were identified as the most stable genes, whereas *SDH5* was identified as the least stable gene for gene expression studies in Norway spruce samples under drought conditions. To validate the reliability of the candidate reference genes, the relative expression of the target gene *PaDhm5* under drought stress was normalized with the most stable reference genes (*SP1* and *COG7*) and the least stable reference gene

TABLE 2 Tissue-wise rankings of candidate reference genes based on stability value calculated by Δ Ct, BestKeeper, NormFinder, and RefFinder. Each tissue group represent 4 biological replicates, each individually consisting of 4 plants pooled together.

Conditions	Gene names	geNorm				Δ Ct				Methods				Recommended gene		
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	BestKeeper	Stability value	Ranking	NormFinder	Stability value	Ranking		RefFinder	Stability value
Needle	SPI	0.265	2	0.71	1	0.77	3	0.150	2	1.32	1					
	JMI16	0.328	4	0.71	2	0.85	6	0.234	10	2.21	2					
	RIF2	0.231	1	0.74	3	0.77	4	0.208	5	3.66	3					
	TUL16	0.231	1	0.76	4	0.94	9	0.225	9	4.56	4					
	RPL7Ae	0.443	8	0.77	6	0.72	2	0.236	12	4.92	5					
	PP2A2	0.516	12	0.94	10	0.59	1	0.316	13	5.62	6					
	COG7	0.370	6	0.77	5	0.97	11	0.210	6	5.76	7					
	CCZ1	0.349	5	0.78	7	0.86	7	0.190	4	6.48	8					
	AFP9	0.486	11	0.82	8	0.79	5	0.224	8	7.11	9					
	RPS10	0.418	7	0.83	9	0.94	8	0.215	7	8.74	10					
	RPL26	0.477	10	1.03	11	0.97	10	0.163	3	10.98	11					
	SKP22	0.300	3	1.03	12	1.17	13	0.236	11	11.98	12					
	UBCP	0.463	9	1.12	13	1.02	12	0.146	1	12.74	13					
	HSP90	0.567	13	1.60	14	1.54	15	0.318	14	14.24	14					
	SDH5	0.684	14	1.89	15	1.31	14	0.475	15	14.74	15					
	RIF2	0.633	8	0.85	1	0.61	7	0.164	9	1.63	1					
	SPI	0.710	10	0.86	2	0.62	8	0.180	10	2.38	2					
	PP2A2	0.478	3	0.94	6	0.42	1	0.075	1	3.22	3					
	RPS10	0.316	1	0.91	4	0.46	2	0.137	6	3.56	4					
	COG7	0.796	12	0.91	3	0.64	9	0.226	12	4.82	5					
TULP6	0.601	7	0.92	5	0.56	6	0.149	8	5.33	6						
AFP9	0.504	4	0.98	7	0.46	3	0.137	7	5.86	7						
RPL26	0.316	1	0.98	8	0.46	4	0.097	2	6.51	8						
RPL7Ae	0.560	6	1.00	10	0.51	5	0.127	3	7.21	9						
JMI16	0.670	9	0.98	9	0.78	10	0.199	11	9.74	10						
CCZ1	0.887	14	1.13	11	0.79	11	0.246	13	11.47	11						
UBCP	0.532	5	1.17	13	0.88	12	0.129	4	12.22	12						
SKP22	0.841	13	1.17	12	0.98	13	0.265	15	12.24	13						
HSP90	0.753	11	2.28	14	1.51	14	0.249	14	14	14						
SDH5	0.413	2	2.70	15	2.09	15	0.135	5	15	15						
Phloem																

(Continued)

TABLE 2 (Continued)

Conditions	Gene names	Methods										Recommended gene
		geNorm		ΔCt		BestKeeper		NormFinder		RefFinder		
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	
Root	UBCP	0.449	4	1.06	3	1.30	6	0.197	1	3.22	1	
	RH7Ae	0.529	6	1.12	5	1.20	5	0.321	10	3.34	2	
	SPI	0.343	2	1.01	1	1.43	9	0.215	2	3.46	3	
	PP2A2	0.562	7	1.13	6	1.02	1	0.321	9	3.46	4	
	RPS10	0.492	5	1.14	7	1.18	4	0.304	7	3.74	5	
	COG7	0.330	1	1.04	2	1.38	8	0.218	3	4.56	6	
	RPL26	0.583	8	1.21	9	1.08	3	0.266	5	5.05	7	
	TUJF6	0.330	1	1.07	4	1.30	7	0.242	4	5.29	8	
	ARP9	0.593	9	1.28	11	1.05	2	0.311	8	5.76	9	
	SKIP22	0.622	10	1.20	8	1.50	10	0.387	12	9.21	10	
	JMI16	0.639	11	1.26	10	1.62	13	0.372	11	11.20	11	
	CZ1	0.722	13	1.51	12	1.60	12	0.525	14	12.00	12	
	RIL2	0.871	14	1.65	13	1.51	11	0.683	15	12.47	13	
	HS990	0.391	3	1.92	14	1.93	14	0.270	6	14.00	14	
	SDH5	0.677	12	2.75	15	2.18	15	0.410	13	15.00	15	

UBCP, RH7Ae, SPI

TABLE 3 Expression stability rankings of the reference genes, calculated by ΔCt , BestKeeper, NormFinder, and RefFinder across various treatment conditions in seedlings and untreated mature trees. Each treatment group represents 4 biological replicates, each individually consisting of 4 plants pooled together.

Conditions	Gene names	Methods										Recommended gene
		geNorm		ΔCt		BestKeeper		NormFinder		RefFinder		
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	
Control	<i>RPS10</i>	0.158	2	0.45	1	0.31	5	0.100	3	1.78	1	<i>RPS10, RPL26, SPI</i>
	<i>RPL26</i>	0.209	8	0.45	2	0.27	4	0.112	5	2.38	2	
	<i>SPI</i>	0.168	3	0.46	4	0.35	8	0.121	6	3.56	3	
	<i>PP2A2</i>	0.136	1	0.48	6	0.23	2	0.129	7	3.83	4	
	<i>MDJ16</i>	0.195	6	0.46	3	0.31	7	0.167	10	4.21	5	
	<i>RID2</i>	0.183	4	0.50	7	0.23	1	0.176	12	4.30	6	
	<i>COG7</i>	0.189	5	0.48	5	0.31	6	0.089	1	5.18	7	
	<i>TULP6</i>	0.136	1	0.56	9	0.26	3	0.145	8	6.98	8	
	<i>RPL7Ac</i>	0.258	11	0.56	8	0.54	12	0.175	11	9.12	9	
	<i>SKP22</i>	0.240	10	0.57	10	0.49	10	0.163	9	10.00	10	
	<i>AMP9</i>	0.201	7	0.60	11	0.59	13	0.094	2	10.91	11	
	<i>URCP</i>	0.217	9	0.68	13	0.40	9	0.101	4	11.62	12	
	<i>CCZ1</i>	0.309	12	0.67	12	0.51	11	0.202	13	11.98	13	
	<i>SIH5</i>	0.389	13	0.97	14	0.72	14	0.358	14	14.00	14	
	<i>HSP90</i>	0.471	14	1.32	15	1.04	15	0.361	15	15.00	15	
	<i>RID2</i>	0.274	7	0.85	1	0.79	5	0.102	9	2.11	1	
	Drought	<i>SPI</i>	0.169	1	0.86	2	0.80	7	0.080	4	2.30	
<i>COG7</i>		0.211	2	0.89	3	0.88	9	0.081	5	4.49	3	
<i>AMP9</i>		0.305	9	0.95	7	0.74	3	0.097	8	4.74	4	
<i>RPL26</i>		0.221	3	0.93	5	0.77	4	0.063	1	4.86	5	
<i>MDJ16</i>		0.322	10	0.95	8	0.96	10	0.130	11	5.32	6	
<i>PP2A2</i>		0.265	6	1.02	10	0.55	1	0.092	7	5.48	7	
<i>RPL7Ac</i>		0.357	12	0.97	9	0.70	2	0.137	13	5.58	8	
<i>RPS10</i>		0.342	11	0.93	4	0.79	6	0.138	14	5.63	9	
<i>TULP6</i>		0.232	4	0.93	6	0.85	8	0.077	2	6.62	10	
<i>SKP22</i>		0.293	8	1.05	11	1.06	13	0.120	10	11.47	11	
<i>URCP</i>		0.169	1	1.13	13	1.00	11	0.084	6	12.22	12	
<i>CCZ1</i>		0.253	5	1.13	12	1.01	12	0.080	3	12.24	13	
<i>HSP90</i>		0.370	13	1.94	14	1.46	14	0.137	12	14.00	14	
<i>SIH5</i>		0.384	14	3.07	15	2.46	15	0.150	15	15.00	15	

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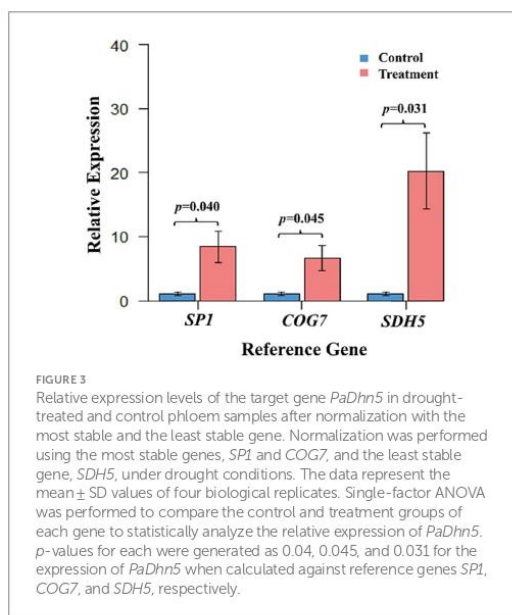
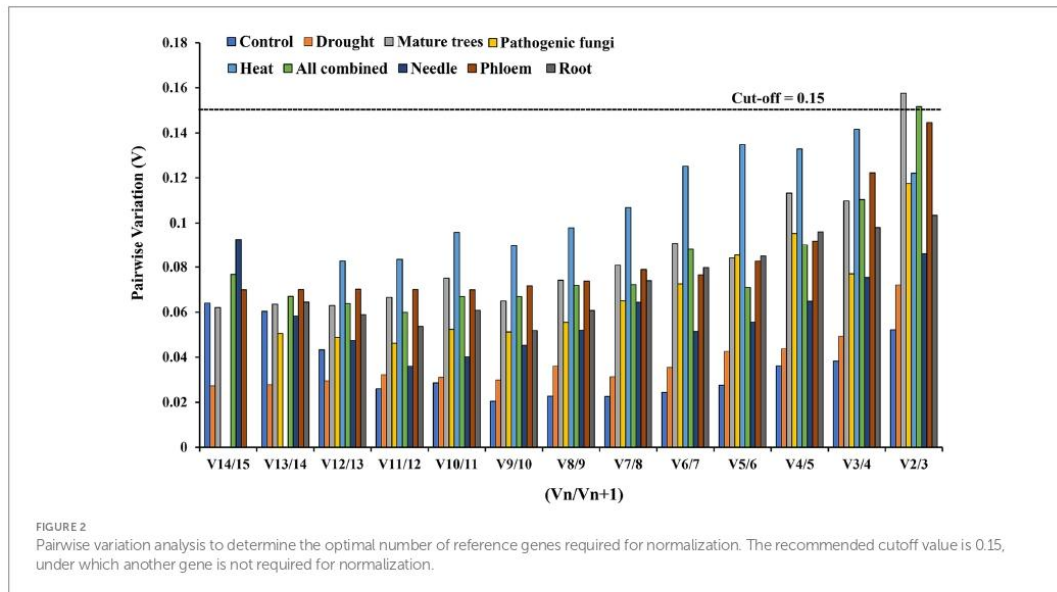
TABLE 3 (Continued)

Conditions	Gene names	Methods										Recommended gene
		geNorm		ΔCt		BestKeeper		NormFinder		RefFinder		
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	
Heat	<i>RID2</i>	0.816	7	0.92	1	1.06	6	0.249	6	1.57	1	<i>RID2, SPI, RPL26</i>
	<i>SPI</i>	0.978	11	0.94	2	1.07	7	0.264	8	3.60	2	
	<i>RPL26</i>	0.320	1	1.00	4	0.98	4	0.181	1	3.72	3	
	<i>TULP6</i>	0.767	6	1.00	5	1.17	8	0.205	2	4.23	4	
	<i>COG7</i>	0.891	9	0.96	3	1.19	9	0.290	9	4.24	5	
	<i>ARP9</i>	0.320	1	1.03	7	0.94	3	0.206	4	5.38	6	
	<i>PP2A2</i>	0.679	5	1.14	10	0.65	1	0.326	10	5.61	7	
	<i>RPL2Ac</i>	0.382	4	1.06	9	0.82	2	0.261	7	5.80	8	
	<i>RPS10</i>	0.370	2	1.02	6	1.03	5	0.244	5	5.96	9	
	<i>JMJ16</i>	0.853	8	1.04	8	1.26	10	0.339	11	7.95	10	
	<i>SKUP22</i>	0.936	10	1.16	11	1.40	12	0.384	13	10.98	11	
	<i>URCP</i>	0.484	3	1.27	12	1.29	11	0.206	3	11.98	12	
	<i>CCZ1</i>	1.107	13	1.29	13	1.41	13	0.495	14	12.74	13	
	<i>HSP90</i>	1.022	12	2.29	14	2.01	14	0.384	12	14.00	14	
	<i>SDH5</i>	1.216	14	3.01	15	2.73	15	0.496	15	15.00	15	
Pathogen infection	<i>SPI</i>	0.430	4	0.89	1	0.87	5	0.258	2	1.50	1	<i>SPI, COG7, RPL26</i>
	<i>COG7</i>	0.383	9	0.93	2	0.93	9	0.293	6	3.22	2	
	<i>RPL26</i>	0.616	12	0.97	4	0.85	3	0.311	7	3.83	3	
	<i>PP2A2</i>	0.320	1	1.01	7	0.66	1	0.260	4	4.14	4	
	<i>JMJ16</i>	0.479	5	0.98	5	0.95	10	0.389	9	4.47	5	
	<i>RPL2Ac</i>	0.320	1	1.01	8	0.79	2	0.087	1	5.03	6	
	<i>TULP6</i>	0.583	10	0.96	3	0.90	8	0.436	12	5.09	7	
	<i>RPS10</i>	0.512	6	0.98	6	0.89	7	0.262	5	5.38	8	
	<i>ARP9</i>	0.645	13	1.05	9	0.85	4	0.435	14	7.35	9	
	<i>RID2</i>	0.850	14	1.23	13	0.89	6	0.963	15	10.72	10	
	<i>SKUP22</i>	0.596	11	1.07	10	1.07	13	0.417	11	10.94	11	
	<i>URCP</i>	0.364	2	1.11	11	1.02	12	0.258	3	10.98	12	
	<i>CCZ1</i>	0.536	7	1.15	12	0.95	11	0.407	10	11.74	13	
	<i>HSP90</i>	0.363	8	2.13	14	1.79	14	0.442	13	14.00	14	
	<i>SDH5</i>	0.368	3	2.71	15	2.08	15	0.328	8	15.00	15	

(Continued)

TABLE 3 (Continued)

Conditions	Gene names	Methods															Recommended gene			
		geNorm			ΔCt			BestKeeper			NormFinder			RefFinder						
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking					
Mature trees	<i>SPI</i>	0.688	9	0.91	1	0.96	7	0.192	3	1.63	1									
	<i>COG7</i>	0.851	14	0.94	2	1.02	9	0.355	14	2.45	2									
	<i>TULP6</i>	0.638	7	0.98	3	0.96	6	0.189	2	3.57	3									
	<i>PP2A2</i>	0.443	2	1.03	6	0.67	1	0.312	10	3.66	4									
	<i>RPL7Ae</i>	0.531	4	1.02	5	0.83	3	0.334	11	4.36	5									
	<i>RPS10</i>	0.473	3	1.02	4	0.89	5	0.278	7	4.47	6									
	<i>ARP9</i>	0.333	1	1.13	9	0.83	2	0.344	12	6.00	7									
	<i>RPL26</i>	0.333	1	1.10	8	0.86	4	0.245	6	6.51	8									
	<i>JMI16</i>	0.754	11	1.07	7	1.13	12	0.312	9	8.53	9									
	<i>UBCP</i>	0.552	5	1.16	11	1.08	10	0.230	5	10.24	10									
	<i>SKIP22</i>	0.816	13	1.15	10	1.20	13	0.355	15	11.20	11									
	<i>RID2</i>	0.728	10	1.21	13	0.97	8	0.296	8	11.29	12									
	<i>CCZ1</i>	0.780	12	1.20	12	1.12	11	0.347	13	11.98	13									
	<i>HSP90</i>	0.603	6	1.95	14	1.63	14	0.168	1	14.00	14									
	<i>SDH5</i>	0.670	8	2.45	15	1.87	15	0.203	4	15.00	15									



(*SDH5*). The results indicated that the expression levels of *PaDhn5* in drought-treated samples increased by 8-fold and 6-fold (*p*-values = 0.040 and 0.045), respectively, when normalized with the stable reference genes *SPI* and *COG7*, compared to the control. In contrast, normalization with the least stable reference gene *SDH5* resulted in an almost 20-fold increase (*p* = 0.031) in expression (Figure 3).

4 Discussion

In recent years, forests worldwide have experienced climate change-driven droughts, storms, windthrows, and high-temperature disturbances, leading to extensive tree mortality. In Europe, one of the most economically important forest tree species is Norway spruce, which was widely planted outside its natural distribution area during the eighteenth and nineteenth centuries. Nevertheless, recent climatic conditions have led to a decline in its vitality, increasing susceptibility to stress and rendering the species particularly vulnerable to the tree-killing spruce bark beetle, *Ips typographus* and other *Ips* beetles (Biedermann et al., 2019; Singh et al., 2024a). These factors underscore the importance of understanding the molecular responses of Norway spruce to both abiotic and biotic stresses.

RT-qPCR is a highly effective and dependable technique for quantitatively studying the relative abundance of target genes. This method is widely employed to investigate plant-pathogen interactions and uncover underlying molecular mechanisms (Vijayakumar and Sakuntala, 2024). RT-qPCR is a relatively less expensive and faster method for exploring target genes under specific conditions. Despite its frequent use, there are concerns that researchers may not always adhere to recommended protocols, which can lead to misleading results (Bustin et al., 2009). Housekeeping genes, commonly referred to as reference genes, are a group of genes that are consistently expressed in cells and are crucial for fundamental cellular functions. They are stable throughout a species irrespective of treatments but can be specific to a particular tissue or developmental stage (Lin and Lai, 2010; Li et al., 2021). For accurate measurement of relative gene expression, it is crucial to use multiple reference genes with stable expression under specific biological conditions (Dai et al., 2018; Wang et al., 2021). While RT-qPCR is effective and reliable

for quantifying mRNA levels across different experimental conditions, several factors, such as RNA extraction, cDNA synthesis, primer design, and material handling, can impact the results (Schmittgen and Zakrajsek, 2000; Bustin et al., 2005; Huggett et al., 2005). Reliable reference genes help mitigate these variations, necessitating the evaluation of the stability of reference genes for each experimental condition to ensure precise and reliable data interpretation. Recent studies have evaluated reference genes in various coniferous species (e.g., Bao et al., 2016; Mo et al., 2019; Chen et al., 2019); however still, such studies on Norway spruce are limited to the embryonic developmental stages (e.g., Vestman et al., 2011; de Vega-Bartol et al., 2013).

Among the 15 evaluated candidates, *SP1*, *COG7*, and *TULP6* were the most stable reference genes, making them suitable for gene expression normalization in Norway spruce. The *SP1* ubiquitin-protein ligase plays a crucial role in regulating chloroplast protein import, essential for plant development (Ling and Jarvis, 2015). It interacts with the translocon at the outer envelope membrane of chloroplasts (TOC) to recognize and import client proteins. *SP1* ubiquitin-protein ligase associates with TOC complexes and mediates the ubiquitination of TOC components, leading to their degradation through the ubiquitin-proteasome system (UPS) and reorganization of the TOC machinery. Ubiquitin-protein ligase (ubiquitin-conjugating enzyme) genes have been identified as suitable reference genes in various tissues of Brazilian rubber trees (*Hevea brasiliensis*) (Li et al., 2011), in alfalfa (*Medicago sativa*) cultivars under different stress conditions (Castonguay et al., 2015), in *Arabidopsis thaliana* (Czechowski et al., 2005), *Brachypodium distachyon* (Hong et al., 2008), *Vernicia fordii* (Han et al., 2012), *Capsicum annuum* (Wan et al., 2011) and *Brachiaria brizantha* (Silveira et al., 2009). Our findings are in accordance with these studies, showing stable expression of *SP1* in various tissue types and under various environmental conditions (Tables 2–4). Nevertheless, not all ubiquitin conjugating enzymes can be used as internal controls for normalization (Wan et al., 2011).

Conserved oligomeric Golgi complex subunit 7 (*COG7*) is a crucial component of the COG complex and is essential for maintaining the normal morphology and function of the Golgi apparatus (Klink et al., 2022). Specifically, *COG7* is part of lobe B of the COG complex, which contains COGs 5–8 (Ungar et al., 2002; Blackburn et al., 2019). This protein plays a vital role in various developmental processes, including embryo development, pigmentation, cell and organ expansion, and the formation of the organized shoot apical meristem (Ishikawa et al., 2008; Vukašinović et al., 2017; Rui et al., 2020, 2021). Despite its unstable expression across different tissue types, *COG7* was ranked second in overall expression stability after *SP1*, according to RefFinder analysis. This implies that *COG7* could be a viable reference gene for gene expression normalization, particularly in experiments investigating mechanisms of resistance to drought and pathogens (Tables 3, 4). To our knowledge, no previous studies have considered *COG7* as a candidate reference gene.

Tubby-like F-box proteins (TULPs) were first discovered in mice and are highly conserved across a wide range of organisms. In plants, these Tubby-like proteins play a role in stress signaling pathways (Lai et al., 2004; Reitz et al., 2013). To date, 11 Tubby genes have been

identified in *Arabidopsis*, 14 in rice, and 11 in poplar (Yang et al., 2008; Hong et al., 2015). Most plant TULPs possess a conserved F-box domain at the N-terminus and a Tubby domain at the C-terminus (Yang et al., 2008). Although TULPs are believed to function as transcription factors, their exact mechanisms of action remain largely unknown (Yulong et al., 2016). *TULP6*, while exhibiting variable expression across treatments, was the third most stable gene after *SP1* and *COG7* according to RefFinder analysis, indicating its potential as a reference gene for normalizing gene expression, especially in studies involving developmental stages (Tables 3, 4). Notably, TULPs have not been previously considered as reference genes.

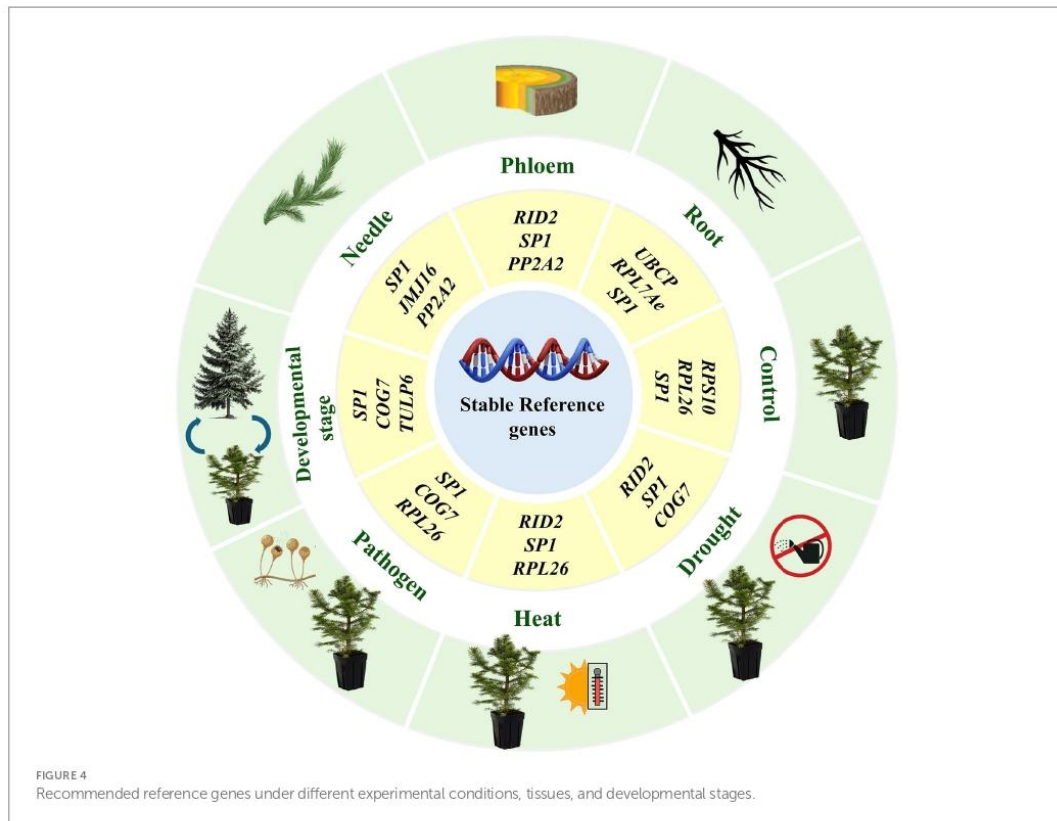
To validate the identified reference genes, we evaluated the expression of dehydrin-like protein 5 (*PaDhn5*), a stress-responsive protein, across various Norway spruce tissues, developmental stages, and stress conditions. Dehydrins, part of the late embryogenesis abundant (LEA) family, accumulate in response to abiotic stresses and play a role in cryoprotection and membrane protection from reactive oxygen species (Close, 1997; Nylander et al., 2001; Rorat et al., 2006; Sun et al., 2021). Our results revealed that using the least stable reference gene (*SDH5*) led to erroneous data, showing a 20-fold increase in *PaDhn5* expression under stress conditions. In contrast, the most stable reference genes, *SP1* and *COG7*, resulted in more consistent *PaDhn5* expression levels, with an 8-fold and 6-fold increase, respectively (Figure 3). The significant discrepancy between the most and least stable reference genes highlights the importance of using stable reference genes to prevent bias in RT-qPCR normalization. Based on our findings, we recommend using the identified stable reference genes and their combinations (Figure 4) for accurate normalization in RT-qPCR analyses of gene expression in Norway spruce, which will improve the sensitivity and reproducibility of the results.

5 Conclusion

This study provides a catalog of genes that have been reported in various conifer species and validated for Norway spruce for the first time. We evaluated 15 candidate reference genes in various tissues and developmental stages under different environmental conditions using RT-qPCR and systematically assessed their expression stability to identify the most suitable reference gene for each condition. The study identified *ubiquitin-protein ligase (SP1)*, *conserved oligomeric Golgi complex (COG7)*, and *tubby-like F-box protein (TULP6)* as the most suitable candidate reference genes, while *succinate dehydrogenase (SDH5)* was found to be the least stable. Notably, this is the first report testing *COG7* and *TULP6* as candidates for reference genes and confirming their stable expression. In addition, normalizing RT-qPCR data with both stably and unstably expressed genes showed that *PaDhn5* expression aligns with the current knowledge of Norway spruce physiology. The fluctuations in gene expression between stable and unstable gene normalizations emphasize the need for validating reference genes for reliable RT-qPCR results. In summary, the identified and recommended reference genes and their combinations for normalizing gene expression in Norway

TABLE 4 Overall expression stability rankings of the reference genes throughout the tissues across all stress treatments, tissue types, and developmental stages as calculated by geNorm, ΔCt, BestKeeper, NormFinder, and RefFinder.

Conditions	Gene names	Methods															Recommended gene		
		geNorm			ΔCt			BestKeeper			NormFinder			RefFinder					
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking				
	<i>SPI</i>	0.567	6	0.91	1	0.96	7	0.195	2	1.63	1								
	<i>COG7</i>	0.633	8	0.94	2	1.02	9	0.243	5	2.45	2								
	<i>TULP6</i>	0.597	7	0.98	3	0.96	6	0.215	4	3.57	3								
	<i>PP2A2</i>	0.487	3	1.03	6	0.67	1	0.244	7	3.66	4								
	<i>RPL7Ac</i>	0.507	4	1.02	5	0.83	3	0.265	8	4.36	5								
	<i>RPS10</i>	0.459	2	1.02	4	0.89	5	0.243	6	4.47	6								
	<i>ARP9</i>	0.389	1	1.13	9	0.83	2	0.265	9	6.00	7								
	<i>RPL26</i>	0.389	1	1.10	8	0.86	4	0.192	1	6.51	8								
	<i>JMT16</i>	0.663	9	1.07	7	1.13	12	0.285	10	8.53	9								
	<i>UBCP</i>	0.515	5	1.16	11	1.08	10	0.202	3	10.24	10								
	<i>SKP22</i>	0.698	10	1.15	10	1.20	13	0.322	12	11.20	11								
	<i>RID2</i>	0.869	14	1.21	13	0.97	8	0.347	15	11.29	12								
	<i>CCZ1</i>	0.762	12	1.20	12	1.12	11	0.330	13	11.98	13								
	<i>HSP90</i>	0.725	11	1.95	14	1.63	14	0.307	11	14.00	14								
	<i>SDH5</i>	0.806	13	2.45	15	1.87	15	0.340	14	15.00	15								
Overall																			<i>SPI, COG7, and TULP6</i>



spruce under various experimental conditions will benefit future gene expression and functional genomics studies in Norway spruce and related conifer species.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary material](#), further inquiries can be directed to the corresponding author.

Author contributions

VVS: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Resources, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing. AN: Methodology, Data curation, Formal analysis, and Writing – review & editing. GS: Methodology, Data curation, Formal analysis, Writing – review & editing. KM: Methodology, Writing – review & editing. RG: Methodology, Writing – review & editing. AR: Methodology, Resources, Writing – review & editing. RJ: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

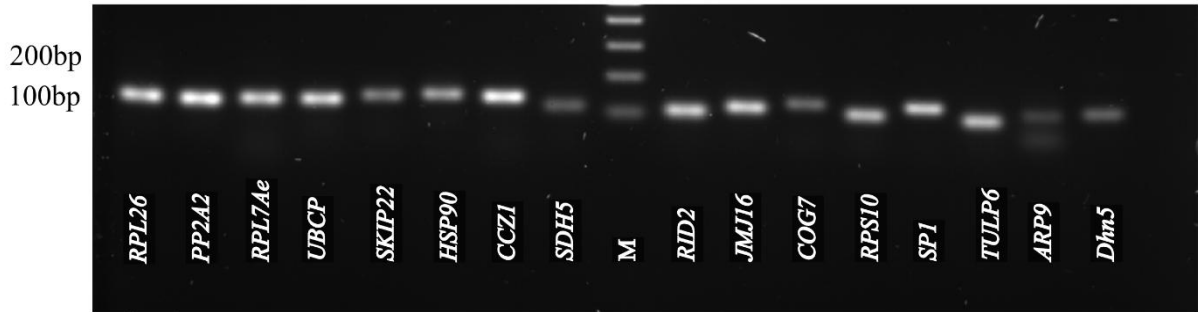
The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/ffgc.2024.1458554/full#supplementary-material>

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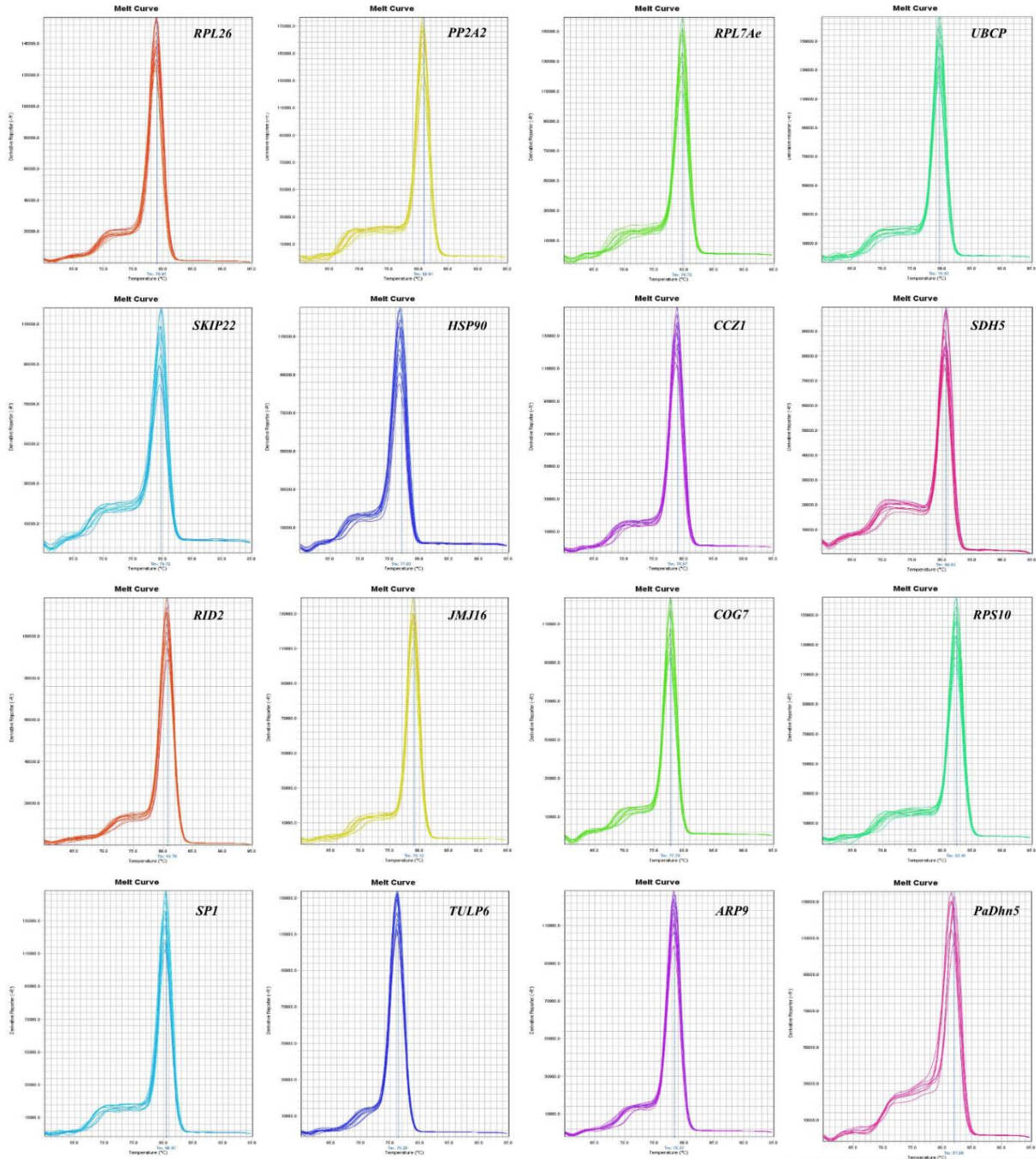
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Supplementary material 1. Agarose gel analysis of amplified PCR products of candidate reference genes and the target gene. The products were run on a 1.2% agarose gel to demonstrate the expected size of the products obtained from primer pairs. The label “M” in the center represents a 100bp DNA ladder.



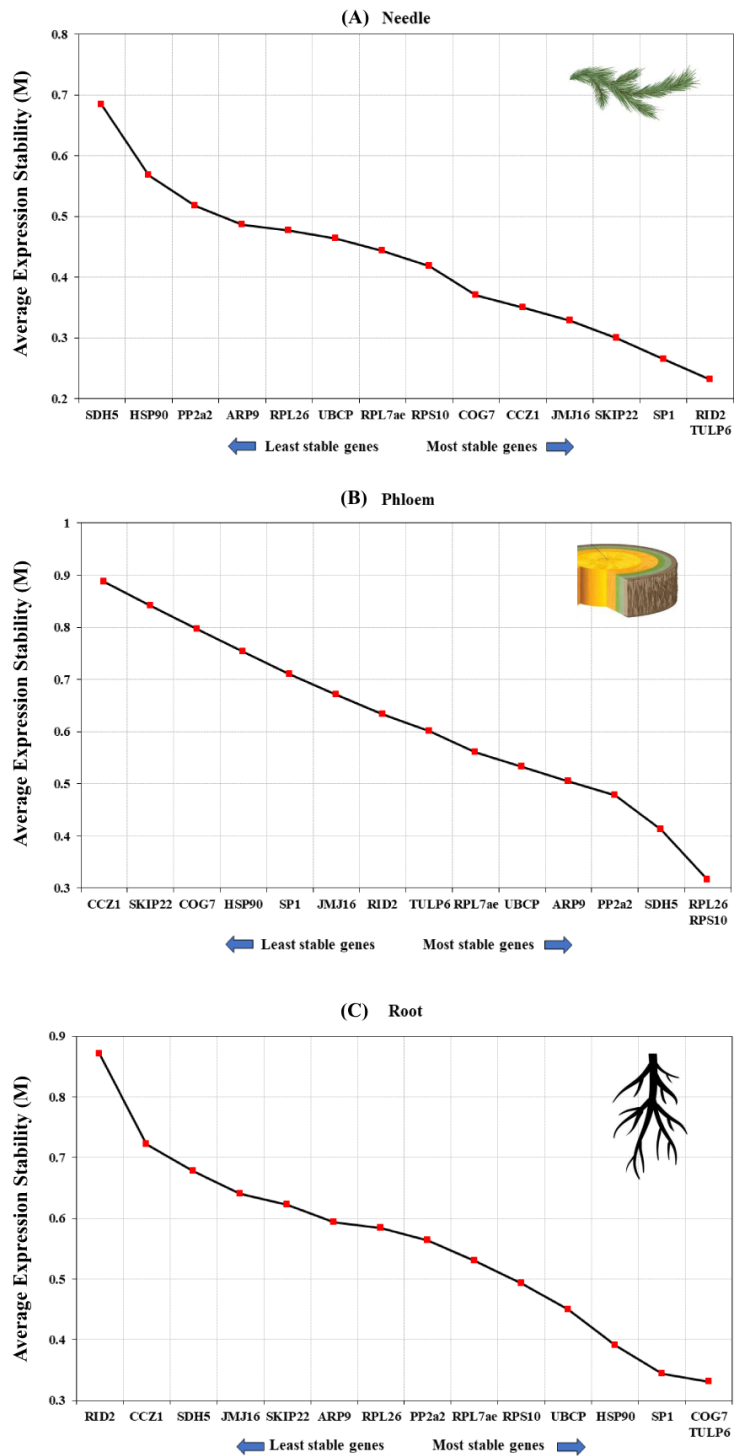
Supplementary material 2. Melting curve analysis with a single peak generated for candidate reference genes and target genes. The candidate genes include ribosomal protein L26 (*RPL26*), serine/threonine-

protein phosphatase (*PP2A2*), ribosomal protein L7Ae (*RPL7Ae*), ubiquitin-like domain-containing CTD phosphatase (*UBCP*), F-box protein (*SKIP22*), heat shock protein (*HSP90*), vacuolar fusion protein (*CCZ1*), succinate dehydrogenase (*SDH5*), 18S rRNA (*RID2*), putative lysine-specific demethylase (*JMJ16*), conserved oligomeric Golgi complex (*COG7*), ribosomal protein S10 (*RPS10*), ubiquitin-protein ligase (*SPI*), tubby-like F-box protein (*TULP6*), and actin-related protein (*ARP9*). The target gene used was dehydrin-like protein 5 (*PaDhn5*).



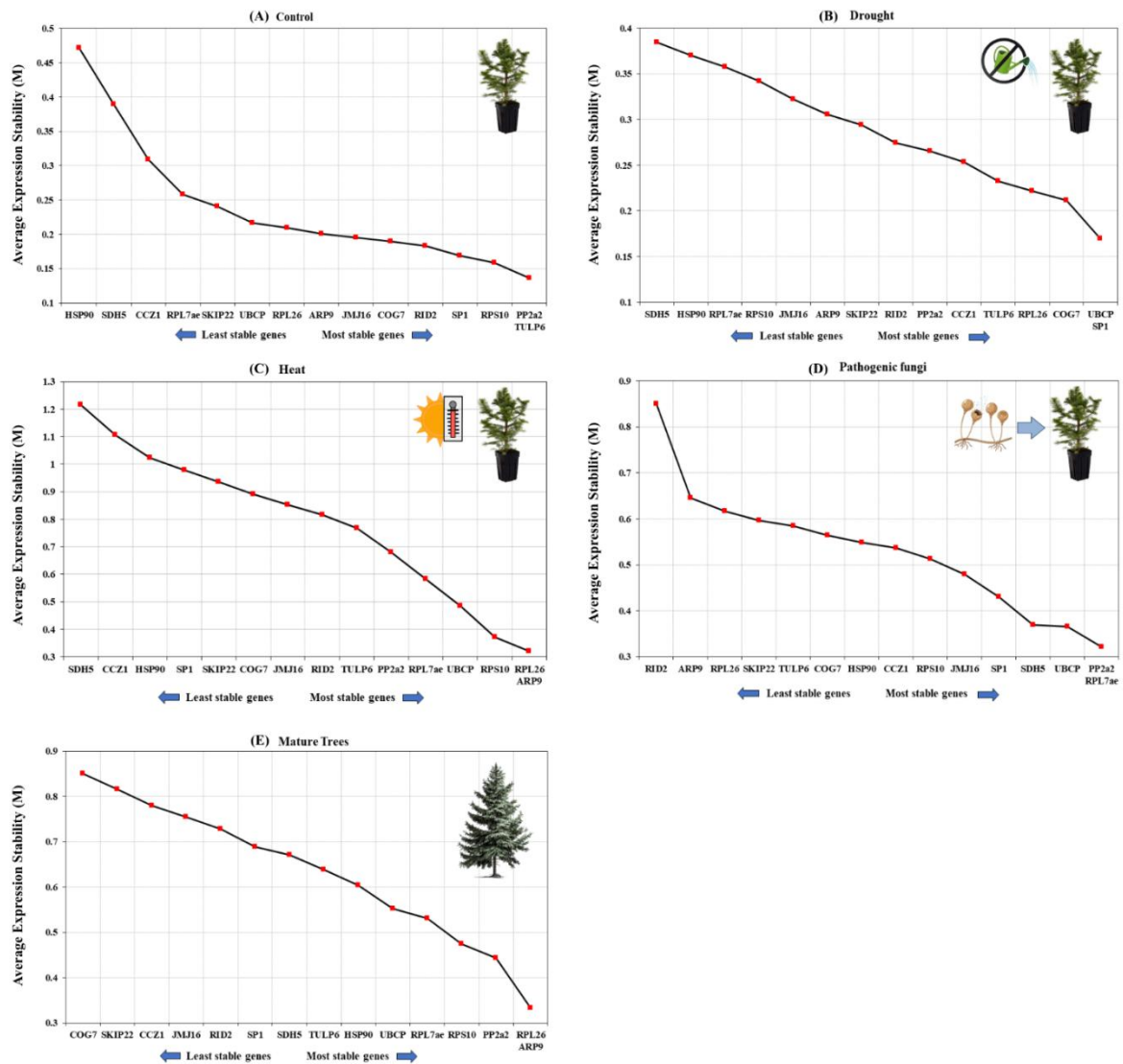
Supplementary material 3. The geNorm-calculated average expression stability values (M) for fifteen candidate reference genes in (A) needle, (B) phloem, and (C) root tissues display a range from the least

stable to the most stable genes (from left to right). Each tissue group represents 4 biological replicates, each consisting of 4 plants pooled together.



Supplementary material 4. The average expression stability values (M) of fifteen candidate reference genes under different treatment conditions—(A) control, (B) drought, (C) heat, (D) pathogenic fungi, and (E) mature trees were calculated using geNorm and indicate a range from the least stable to the most

stable genes from left to right. Each treatment group represents 4 biological replicates, each consisting of 4 plants pooled together.



The remaining Supplementary materials (Gene sequences and data files) can be found at: <https://www.frontiersin.org/articles/10.3389/ffgc.2024.1458554/full#supplementary-material>

5.4 Tissue-specific drought transcriptome atlas of Norway spruce reveals coordinated yet distinct adaptive strategies.

Based on: **Singh, V.V.**, Naseer, A., Mogilicherla, K., Jamnická, G., Blaženec, M., Jakuš, R., Gebauer, R. & Krokene, P. (2025b). Tissue-specific drought transcriptome atlas of Norway spruce reveals coordinated yet distinct adaptive strategies.

Contributions: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Resources, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

This work presents the first tissue-specific drought transcriptome atlas of Norway spruce, revealing both conserved and tissue-specific adaptive strategies. A total of 24,487 differentially expressed genes were identified, including 2,418 shared across tissues that form a core drought-responsive regulon. Needles exhibited strong repression of photosynthesis-related genes, alongside the induction of defense and structural remodeling pathways, reflecting an energy-conserving yet defense-oriented response. Phloem exhibited major shifts in carbohydrate and secondary metabolism, with pronounced upregulation of flavonoid and terpene synthases, highlighting its role in systemic signaling and chemical defense. Roots displayed the most extensive reprogramming, characterized by the induction of aquaporins, galactinol synthases, dehydrins, and genes involved in ROS detoxification, highlighting their central role in osmotic adjustment and mitigating oxidative stress. Functional enrichment analysis confirmed that photosynthesis and carbon metabolism are central processes in needles, oxidative stress responses and phenylpropanoid biosynthesis in phloem and roots, and the conserved activation of MAPK signaling, glutathione metabolism, and carbohydrate pathways across all tissues.

In total, 892 drought-responsive transcription factors were identified, dominated by AP2/ERF, MYB, NAC, WRKY, and bZIP families, with both tissue-specific patterns and a conserved subset of WRKY and GATA factors forming a systemic regulatory core. RT-qPCR validation supported the RNA-seq findings. Collectively, these results demonstrate that Norway spruce coordinates systemic and organ-specific responses to drought by downregulating energy-intensive processes while activating osmoprotective, antioxidant, and defense pathways. This transcriptome atlas offers mechanistic insights into conifer drought adaptation, identifying candidate genes and transcription factors with potential applications for breeding and conservation strategies under climate change.

Tissue-specific drought transcriptome atlas of Norway spruce reveals coordinated yet distinct adaptive strategies

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Keywords

Drought
RNA-seq
Gene expression
RT-qPCR validation
Pathway analysis
Tissue-specific response
Transcription factors

Abstract

Climate change-driven droughts pose a major threat to boreal forests. Norway spruce (*Picea abies*), as a dominant conifer of European forests, is highly susceptible to water deficits; however, the tissue-specific molecular basis underlying drought adaptation remains poorly understood. To gain a better understanding of molecular adaptation, we performed transcriptome analysis of needles, bark phloem, and fine roots of Norway spruce seedlings subjected to severe drought, supplemented by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analysis, transcription factor analysis, and RT-qPCR validation. A total of 24,487 differentially expressed genes (DEGs) were identified, including 2,418 shared across tissues, highlighting both coordinated and tissue-specific responses. Drought induced strong up-regulation of genes linked to oxidative stress defense, carbohydrate, fatty acids, amino acid metabolism, and phytohormone signaling (ABA, auxin, JA, cytokinin, and ethylene), while transcripts associated with pectin and secondary metabolite metabolism were largely down-regulated. Pathway enrichment analysis revealed common regulation of starch/sucrose metabolism and mitogen-activated protein kinase (MAPK) signaling across tissues, with carbon and glutathione metabolism enriched in needles, flavonoid biosynthesis in phloem, and galactose/pentose–glucuronate interconversions in roots. Notably, hormone signaling, phenylpropanoid biosynthesis, pathogen interaction, ABC transporters, and amino sugar metabolism were strongly induced in phloem and roots, where transcriptional reprogramming was most pronounced. Hierarchical clustering and MapMan analyses further underscored the tissue-specific regulation of stress response, kinases, secondary metabolism, and nutrient/ion transport. Physiological assays confirmed increased proline accumulation under drought, while chlorophyll and carotenoid levels remained unaffected. Validation of selected DEGs by RT-qPCR corroborated RNA-seq results. Together, this integrative transcriptome atlas reveals key transcripts encoding transcription factors, phytohormones, and pathways underlying the drought response of different tissues, providing molecular markers and targets for studying drought resilience in Norway spruce and safeguarding forest ecosystems in the face of climate change.

1. Introduction

Global climate change is projected to increase both mean temperatures and frequency and severity of droughts, resulting in more recurrent climate-driven disturbances in forests worldwide (Hlásny et al., 2021; Altman et al., 2024). Eurasian boreal forests, among the largest biomes in the Northern Hemisphere, are particularly vulnerable to these climatic extremes. In these ecosystems, drought has emerged as a dominant driver of change, reducing productivity, altering species composition, and weakening ecosystem resilience to subsequent disturbances (Price et al., 2013; Hlásny et al., 2021; Singh et al., 2024a). Drought also impairs the hydraulic integrity of trees, limiting their capacity to synthesize and mobilize defensive compounds essential for resistance against herbivores and pathogens (Netherer et al., 2015; Erbilgin et al., 2021). As a result, drought and pest outbreaks often interact, amplifying damage and contributing to large-scale forest dieback. Even moderate drought can reduce oleoresin flow and phenolic production—the key elements of conifers' constitutive and inducible defense systems (Anderegg et al., 2015; Netherer et al., 2024).

Advances in sequencing technologies now allow comprehensive transcriptome profiling, revealing how trees respond to drought through coordinated changes in signaling, ion transport, and gene regulation to maintain turgor and cellular integrity (Kizis et al. 2001; Osakabe et al. 2014; Haswell and Verslues 2015; Choat et al. 2018). High-resolution transcriptomics in non-model species reveals that drought triggers rapid ABA accumulation in roots and leaves. This in turn regulates key transcription factors (AP2/ERF, MYB, NAC), stress-related genes (dehydrins, aquaporins), and stomatal closure to drive adaptive responses (Osakabe et al. 2014; Polle et al. 2019; Haas et al. 2021; Ribeyre et al. 2025). Drought-responsive gene regulation involves both ABA-dependent signaling, where PYR/PYL/RCAR receptors activate SnRK2 kinases and bZIP

transcription factors, and ABA-independent pathways, where AP2/ERF factors like DREB/CBFs control stress-protective genes through DRE/CRT motifs (Yamaguchi-Shinozaki and Shinozaki 2006; Yoshida et al. 2010; Yoshida and Shinozaki 2014; Yao et al. 2021; Mader et al. 2023). The interplay of these pathways is further modulated by MYB, NAC, and HD-ZIP transcription factors, enabling the coordination of osmotic adjustment, antioxidant defenses, transcriptional reprogramming, and maintenance of ion homeostasis (Yao et al. 2021; Haas et al. 2021). Although conifers constitute the principal taxa of boreal forests, research on drought has predominantly focused on deciduous species. Conifers, however, possess unique morphological, physiological, and genetic adaptations to water deficit, including ABA-mediated signaling, reconfiguration of carbohydrate metabolism, induction of stress-associated proteins, and enhanced biotic defenses (Eldhuset et al. 2013; Moran et al. 2017; Krokene et al. 2023).

Norway spruce (*Picea abies* [L.] H. Karst.), a keystone conifer species of European boreal and montane forests, is both ecologically and economically significant, contributing nearly half of Europe's forest economic output. However, it is highly susceptible to climatic extremes, particularly drought and rising temperatures at the southern and low-elevation margins of its range (Kapeller et al. 2012; Trujillo-Moya et al. 2018). The recent pan-European drought of 2018–2020 exemplified these vulnerabilities, coinciding with widespread canopy dieback and increased vulnerability to bark beetle infestations and root rot. Despite the species' importance, the physiological and molecular mechanisms underpinning drought adaptation remain poorly understood, especially with respect to tissue- and organ-specific responses. Earlier transcriptomics studies have largely focused on needles alone or, in some cases, a limited combination of needles with roots or shoots (e.g., Fossdal et al. 2007; Haas et al. 2021; Bag et al. 2021; Krokene et al. 2023; Ahmad et al. 2025). Moreover,

emerging evidence suggests that their drought responses diverge from those of herbaceous models, involving distinctive regulatory elements and long-term acclimation strategies suited to their ecological niches (Haas et al. 2021). Genes related to cuticle reinforcement, secondary metabolism, and reactive oxygen species (ROS) detoxification are often upregulated, emphasizing the importance of structural defenses in these long-lived perennials (Li W et al. 2021). Transcriptomic analyses further reveal that Norway spruce exhibits pronounced transcriptional plasticity across tissues during drought, with roots activating genes involved in osmolyte synthesis and water transport, needles regulating stomatal function and antioxidative pathways, and the stem phloem mediating carbohydrate metabolism and systemic signaling (Behringer et al. 2015; Haas et al. 2021).

Addressing the tissue-specific and systemic drought response mechanisms of Norway spruce, which were previously associated with technical complexity and high sequencing costs, is essential for uncovering novel insights to inform breeding programs, as climate projections indicate intensifying water scarcity throughout much of the species' distribution (Mader et al. 2023). Hence, we conducted tissue-specific transcriptome analysis of Norway spruce seedlings subjected to severe drought stress. We aim to identify key candidate genes and reveal both tissue-specific and systemic drought response mechanisms through integrated differential gene expression, functional enrichment, pathway mapping, and RT-qPCR validation. We hypothesized that severe water deficit would trigger a conserved drought response across tissues, associated with tissue-specific transcriptional reprogramming. Linking gene expression to functional traits provides a mechanistic foundation for understanding drought tolerance in Norway spruce. As forest management strategies increasingly prioritize resilience and climate adaptation, identifying molecular markers and the genetic basis of drought response in Norway spruce will be required for breeding and conservation programs to support climate-smart forest management.

2. Materials and methods

2.1 Plant material and experimental design

The experiment was conducted using three-year-old containerized Norway spruce seedlings ($n = 48$), each approximately 40 cm in height and displaying uniform phenotypic characteristics. Seedlings were sourced from a local nursery and planted in 500 mL pots with a moist peat-perlite substrate on May 24, 2022. The seed material originated from the Bohemian-Moravian highlands with 600-750 mm of annual rainfall and 500-600 m altitude above sea level. All seedlings were acclimated to greenhouse conditions for one month at the Czech University of Life Sciences, Prague. The temperature was maintained at 23 ± 2 °C, and the photoperiod was set to 16 h light/8 h dark (Singh et al. 2024b). Before initiating treatments, seedlings were regularly irrigated with tap water. The seedlings were randomly assigned to control and drought treatment groups on June 24, 2022, with each group subdivided into four biological replicates, each consisting of six seedlings. The control group received regular watering, while the treatment group was subjected to a progressive reduction in irrigation over six weeks (until August 5, 2022) to induce a severe water deficit (Supplementary figure 1). Drought progression was monitored weekly by measuring midday shoot water potential on current-year lateral shoots using a Scholander pressure chamber (PMS Instruments, Corvallis, USA) in both control and drought-stressed seedlings. Drought treatment was maintained until the shoot water potential in the stressed group declined below -2.1 MPa, a threshold associated with severe water stress and an increased risk of xylem embolism in Norway spruce (Rosner et al. 2019). Temperature and photoperiods were kept constant for both groups throughout the experiment. Seedlings were harvested for current-year needles, stem phloem (hereafter referred to as phloem), and fine roots, which were immediately flash-frozen in liquid nitrogen and stored at -80 °C until subsequent analyses.

2.2 Spectrophotometric measurements

For each treatment (control and drought), needles from six individual seedlings were pooled to form a single biological replicate, with four biological replicates per treatment. The free proline content was estimated using the ninhydrin-based colorimetric method of Bates et al. (1973) with a cuvette UV-Vis spectrophotometer (Cintra 6010, GBC Scientific Equipment, Australia). Proline extraction was performed by homogenizing (~0.5 g FM) needle tissue in 10 mL of 3% sulfosalicylic acid. The homogenate was filtered, and 2 mL of the filtrate was mixed with 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent. The mixture was incubated in a water bath at 100°C for 1 h. Following repeated centrifugation, the mixture was heated to 95°C for 1 hour using a heat block. Once cooled, 4 mL of toluene was added and mixed for 20 s. The absorbance of the toluene phase was measured at 520 nm with toluene as the reference. Chlorophyll a (chl a), chlorophyll b (chl b), total chlorophyll (a+b), and total carotenoids (Car x+c) were determined spectrophotometrically (GBS Cintra 6010, Australia) at 470, 646, and 663 nm, respectively, following the method of Lichtenthaler (1987). For pigment analysis, homogenized spruce needles (~1 g) per biological replicate were extracted with 80% acetone, and chlorophyll and carotenoid contents were expressed on a dry mass basis ($\text{mg}\cdot\text{g}^{-1}$).

2.3 Transcriptome Analysis

2.3.1 RNA extraction

Total RNA was isolated from needle, phloem, and root tissue using a modified cetyltrimethylammonium bromide (CTAB) protocol after Singh et al. (2024c). For each tissue and treatment, RNA was extracted from four biological replicates, with each replicate consisting of pooled tissue from six individual plants to reduce individual heterogeneity and sequencing efforts. Briefly, approximately 100 mg of homogenized tissue was lysed in CTAB extraction buffer and extracted with Chloroform:isoamyl alcohol

(24:1, v/v). RNA was precipitated with 5M lithium chloride (LiCl), and the resulting pellet was washed twice with 70% ethanol, air-dried, and resuspended in 50 μL of RNase-free water. Residual genomic DNA was removed by treatment with the TURBO DNase Kit (Invitrogen, USA). RNA integrity and purity were assessed using 1.2% agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) before mRNA sequencing. Purified RNA samples were stored at -80°C for transcriptome sequencing and RT-qPCR validation.

2.3.2 Transcriptome sequencing, library preparation, and data analysis

RNA samples with RQN ≥ 7.5 and 260/280 ratio ≥ 2.0 were selected for sequencing on the Illumina NovaSeq 6000 platform (NovoGene, China), generating approximately 50 million paired-end reads (PE150) per sample. mRNA was enriched using poly-T oligo-attached magnetic beads, and cDNA libraries were prepared with random hexamer primers. Raw sequencing data (FASTQ format) were processed using NovoGene's in-house scripts to remove adapter sequences, poly-N, and low-quality reads, resulting in high-quality clean reads. Quality metrics were calculated, including Q20, Q30, and GC content (Table 1). All downstream analyses were performed using clean data. Clean reads aligned with the Norway spruce reference genome (*P. abies* v1.0; Nystedt et al. 2013) with Hisat2 v2.0.5 (Mortazavi et al. 2008). Differential gene expression analysis was conducted using the DESeq2 R package (ver. 3.22.5) (Robinson et al., 2010; Love et al., 2024). P-values were adjusted using the Benjamini-Hochberg approach to control the false discovery rate (FDR) (Benjamini and Hochberg 1995). Genes with adjusted p-values ≤ 0.05 and absolute log₂ fold change ± 1 were considered significantly differentially expressed.

Table 1 | Summary of RNA sequencing data quality across Norway spruce tissues and treatments. The table presents sequencing output for needle (ND), phloem (PH), and root (RT) samples under control (C) and drought-treated (T) conditions. For each biological replicate (1–4), the sequencing library ID, total raw and clean read numbers, corresponding base counts, sequencing error rate, percentage of bases with quality scores above Q20 and Q30, and GC content (%) are provided. All libraries displayed high quality, with >94% bases above Q30 and consistent GC content.

sample	library	Raw reads	Raw bases	Clean reads	clean bases	error rate	Q20	Q30	GC pct
PA_ND_C1	ERAS230049018-1r	1.46E+08	21.96G	1.45E+08	21.71G	0.02	98.46	95.34	46.39
PA_ND_C2	ERAS230049019-1r	1.23E+08	18.39G	1.22E+08	18.25G	0.02	98.18	94.54	45.92
PA_ND_C3	ERAS230049020-1r	1.2E+08	18.06G	1.19E+08	17.89G	0.02	98.48	95.28	46.67
PA_ND_C4	ERAS230049021-1r	1.53E+08	22.89G	1.51E+08	22.72G	0.02	98.52	95.43	46.86
PA_ND_T1	ERAS230049022-1r	1.38E+08	20.67G	1.36E+08	20.34G	0.02	98.47	95.33	45.9
PA_ND_T2	ERAS230049024-1r	1.37E+08	20.59G	1.36E+08	20.33G	0.02	98.48	95.32	45.87
PA_ND_T3	ERAS230049026-1r	1.28E+08	19.19G	1.27E+08	18.98G	0.02	98.53	95.42	46.08
PA_ND_T4	ERAS230049027-1r	1.09E+08	16.33G	1.07E+08	16.03G	0.02	98.26	94.71	45.65
PA_PH_C1	ERAS230049029-1r	1.09E+08	16.34G	1.08E+08	16.17G	0.02	98.3	94.91	45.44
PA_PH_C2	ERAS230049031-1r	1.42E+08	21.31G	1.4E+08	20.98G	0.02	98.36	95.03	45.36
PA_PH_C3	ERAS230049032-1r	1.47E+08	22.09G	1.45E+08	21.72G	0.02	98.43	95.25	45.12
PA_PH_C4	ERAS230049033-1r	1.33E+08	19.97G	1.32E+08	19.78G	0.02	98.46	95.25	45.73
PA_PH_T1	ERAS230049035-1r	1.32E+08	19.74G	1.3E+08	19.49G	0.02	98.4	95.15	45.07
PA_PH_T2	ERAS230049036-1r	1.21E+08	18.21G	1.2E+08	18.01G	0.02	98.48	95.31	45.25
PA_PH_T3	ERAS230049037-1r	1.04E+08	15.55G	1.03E+08	15.41G	0.02	98.23	94.66	45.28
PA_PH_T4	ERAS230049038-1r	1.39E+08	20.87G	1.38E+08	20.7G	0.02	98.34	94.9	44.97
PA_RT_C1	ERAS230049041-1r	1.36E+08	20.37G	1.34E+08	20.06G	0.02	98.26	94.69	44.98
PA_RT_C2	ERAS230049043-1r	1.41E+08	21.16G	1.39E+08	20.84G	0.02	98.41	95.15	45.24
PA_RT_C3	ERAS230049044-1r	1.32E+08	19.74G	1.3E+08	19.55G	0.02	98.42	95.23	45.65
PA_RT_C4	ERAS230049045-1r	1.49E+08	22.35G	1.48E+08	22.15G	0.02	98.44	95.24	45.76
PA_RT_T1	ERAS230049046-1r	1.39E+08	20.8G	1.37E+08	20.59G	0.02	98.49	95.36	45.86
PA_RT_T2	ERAS230049049-1r	1.52E+08	22.84G	1.49E+08	22.39G	0.02	98.38	95.09	45.17
PA_RT_T3	ERAS230049050-1r	1.19E+08	17.91G	1.17E+08	17.61G	0.02	98.39	95.12	45.43
PA_RT_T4	ERAS230049051-1r	1.47E+08	22.03G	1.45E+08	21.81G	0.02	98.5	95.35	45.33

* All samples passed quality thresholds (Q30>94%).

2.4 Functional Annotation of Transcripts

Functional annotation of DEGs was performed to characterize their biological roles and provide insight into molecular responses to drought stress. Gene Ontology (GO) enrichment analysis was conducted to assign DEGs to three principal domains: biological processes (BP), cellular components (CC), and molecular functions (MF). Significantly enriched GO terms were identified using a corrected p-value threshold of < 0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted to identify metabolic and signaling pathways associated with DEGs. Both GO and KEGG

analyses were performed using the enrichGO and enrichKEGG functions in the clusterProfiler R package (ver. 4.8.3; Yu et al. 2012) referencing the GO (<http://www.geneontology.org/>) for ontological data and metabolic pathways from the KEGG database (<http://www.genome.jp/kegg/>) for metabolic pathway annotations (Kanehisa et al. 2020). Statistical enrichment of DEGs in KEGG pathways was assessed within clusterProfiler using a corrected p-value < 0.05 as a threshold, ensuring robust identification of enriched categories. Hierarchical clustering analysis (HCA) of DEGs was conducted using log2 fold change data, centroid linkage, and Euclidean distance in Cluster 3.0 (Eisen et al. 1998), generating

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dendrograms to visualize expression patterns and clustering dynamics. Transcription factors (TFs) were identified by extracting coding sequences (CDS) from DEGs and translating them into protein sequences with ESTScan 3.0, facilitating the identification of open reading frames. The resulting protein sequences were queried against PlantTFDB 4.0 (Jin et al. 2016) using an E-value cutoff of $\leq 10^{-5}$ to classify the TF families.

For functional categorization and pathway visualization, MapMan v3.6.0RC1 (Thimm et al. 2004) was used with a custom mapping file for *P. abies*. MapMan organizes gene expression data into a hierarchical tree structure of functional categories, termed “Bins,” predefined based on biological processes and pathways, facilitating a comprehensive overview of metabolic and regulatory shifts. A custom mapping file was generated using the Mercator4 online annotation platform, which assigned functional Bin terms to *P. abies* sequences from a reference FASTA file, ensuring accurate gene-to-function associations for this non-model species. DEGs uniquely regulated in each tissue and common across all tissues were identified based on a false discovery rate (FDR) < 0.05 and an absolute log₂ fold change of ± 1 . Annotated DEGs were then mapped onto functional Bins and visualized in MapMan to assess metabolic and regulatory pathways, supporting tissue-specific and systemic regulation evaluation. All pathway diagrams were systematically examined to identify regulatory patterns and adaptive responses across tissues. Statistical enrichment of pathways in MapMan was determined by the Wilcoxon rank-sum test, comparing the distribution of expression values for genes within each Bin versus those outside; Bins with $p < 0.05$ were considered functionally enriched.

2.5 RT-qPCR analysis

The expression levels of selected genes in each sample and tissue type were analyzed by RT-qPCR using relative quantification according to the MIQE guidelines (Bustin et al. 2009). First-strand cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) from 1 μ g of RNA, following the manufacturer's

protocol, and stored at -20°C . Gene-specific primers were designed using PrimerQuest™ Tool (Integrated DNA Technologies, United States), with default parameters for an amplicon length of 120 bp to 180 bp, GC content of 50 ± 5 , and optimum annealing temperature of $60 \pm 2^{\circ}\text{C}$. The list of used primers is presented in [Supplementary Material 1](#). The RT-qPCR reaction mixture consisted of 5.0 μL of SYBR® Green PCR Master Mix (Thermo Fisher Scientific, United States), 1.0 μL of cDNA from fivefold dilutions, 0.5 μL of forward and reverse primers each of concentration 10 μM , and 3.0 μL of RNase-free water (Invitrogen, USA) to make up a total volume of 10.0 μL . Amplification was conducted with initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The reactions were conducted using an Applied Biosystems™ StepOne™ Real-Time PCR System (Applied Biosystems, United States), and three biological replicates per treatment and tissue were used. To verify primer specificity, melt curve analysis was conducted under default conditions, gradually increasing the temperature from 60°C to 95°C . The relative expression of each gene was calculated using $2^{-\Delta\Delta\text{Ct}}$ (Livak and Schmittgen 2001), and 18S rRNA was selected as the reference gene for expression normalization (Singh et al. 2024b).

2.6 Statistical analysis

The spectrophotometric and RT-qPCR data for the treatment and control groups were analyzed for normality using the Shapiro–Wilk test, followed by an assessment of variance homogeneity between the control and treatment groups with Levene's test. Subsequently, an independent t-test was conducted, applying either the Student's t-test for equal variances (if Levene's $p > 0.05$) or Welch's t-test for unequal variances (if Levene's $p < 0.05$). The p-values indicating significant differences between the control and treatment groups at a 95% confidence interval were calculated using RStudio (version 4.2.3). Differential expression and functional enrichment analyses for RNA-seq data were performed as described above (see sections 2.3 and 2.4).

3. Results

3.1 Higher proline accumulation but stable pigment pool under drought

Proline content in needles differed significantly between the control and the drought-treated seedlings ($p < 0.01$; Figure 1A). Drought-treated samples accumulated approximately tenfold more proline ($15.3 \pm 2.0 \text{ mg.L}^{-1}$) than controls ($1.5 \pm 0.1 \text{ mg.L}^{-1}$). By contrast, chlorophyll and carotenoid levels

showed no significant differences between control and drought-treated samples ($p > 0.05$; Figure 1B). Mean chlorophyll content was slightly higher in drought-stressed ($6.55 \pm 0.54 \text{ mg.g}^{-1}$) than in control needles ($6.30 \pm 0.16 \text{ mg.g}^{-1}$), although this difference was not statistically significant. Similarly, the carotenoid content was marginally higher under drought ($1.31 \pm 0.10 \text{ mg.g}^{-1}$) than in control ($1.23 \pm 0.02 \text{ mg.g}^{-1}$), reflecting a slight, non-significant increase.

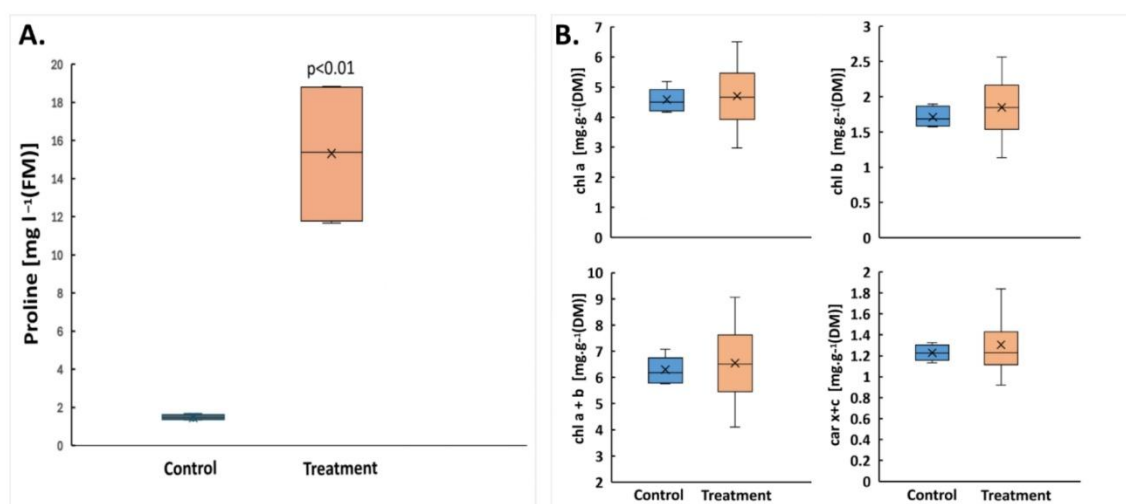


Figure 1 | Proline and photosynthetic pigment concentrations in the current-year needles of Norway spruce saplings growing under control and drought conditions. **A.** Proline concentration (mg.L^{-1}) and **B.** photosynthetic pigment concentrations (chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids; $\text{mg.g}^{-1} \text{DM}$). Boxes represent the interquartile range; the horizontal line indicates the median, and the “x” denotes the mean. Whiskers extend to the minimum and maximum values (outliers excluded).

3.2 Needle exhibits a distinct expression profile compared with phloem and root

A total of approximately 3.20 billion raw reads were generated, yielding 3.16 billion clean reads following quality filtering. Of these, 1.05 billion clean reads were derived from needle samples, 1.03 billion from phloem, and 1.11 billion from root tissues. High read quality was confirmed with 98.4% of bases exhibiting a Phred score above 20. Principal component analysis (PCA) was performed using FPKM values to assess variation within and between tissue types. PCA revealed distinct clustering by tissue, with phloem and root samples clustering together more closely than needles (Figure 2A). Volcano plots were

generated for each tissue to visualize DEGs between control and treatments ($\text{FDR} \leq 0.05$; $\text{Log}_2\text{FC} \geq 1$; Figure 2B-D), and Venn diagrams illustrated tissue-specific and overlapping DEGs among needle, phloem, and root (Figure 2E, F).

In needles, 3,125 genes were upregulated and 3,004 downregulated in response to drought (total 6,129; $\text{log}_2\text{FC} \geq 1$, $\text{FDR} \leq 0.05$). Major DEG categories included 85 cytochrome P450s (including abscisic acid 8'-hydroxylase 1, ~7-fold up), 55 transferases, 50 UDP-glycosyltransferases (UGTs), 42 ABC transporters, 24 glycosyl transferases, 23 glutathione S-transferases (GSTs; GSTU23, ~7-fold up), 20 late embryogenesis abundant (LEA) proteins,

19 pathogenesis-related thaumatin-like proteins (TLPs), 17 heat shock proteins (HSPs), 11 dehydrins (DHNs), 10 ABA/WDS-induced proteins, 10 cysteine-rich transmembrane (CYSTM) genes, 16 MAP kinases (MAPKs), 8 aquaporins (AQPs), 4 LURP one-related, 3 HOTHEADs, and 2 PAR1 genes (Supplementary Material S2.1). Genes involved in ABA biosynthesis and signaling included 28 protein phosphatases 2C (PP2Cs), 5 PYL ABA receptors, 2 NCEs (9-cis-epoxycarotenoid dioxygenases), and 182 serine/threonine-protein kinases. Among these, key drought-responsive kinases such as CIPK9, CIPK20,

SRK2E/SnRK2.6 (OST1), RBK2, CTR1, and D6PK/D6PKL2 were identified, which are known to regulate ion transport, ABA signaling, auxin transport, ethylene signaling, stomatal closure, and ROS responses. Importantly, one gibberellin 2-beta-dioxygenase (GA2ox) and two leucoanthocyanidin reductase (LAR) genes (~6-fold up) were also upregulated. In addition, 15 genes belonging to the terpene synthase (TPS) family, including pinene synthase, myrcene synthase, and alpha-terpineol synthase, were identified in needles. Among these, only gamma-humulene synthase (MA_737780g0010)

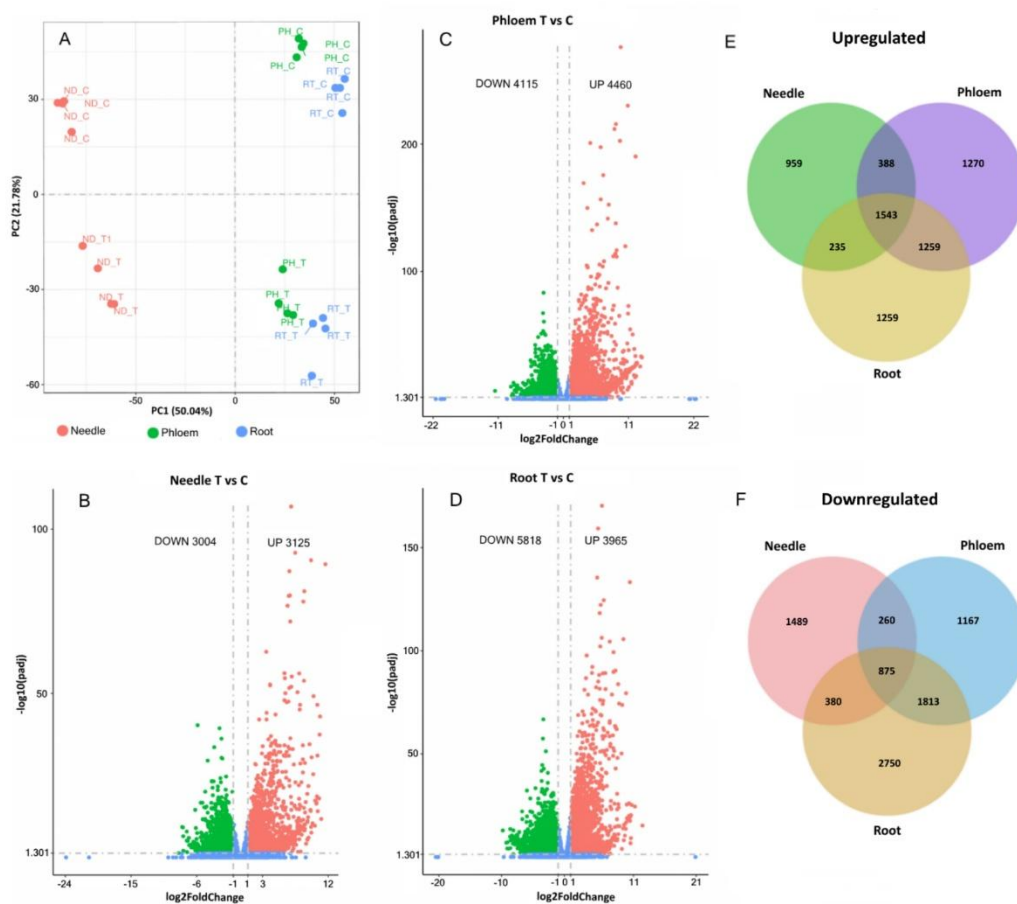


Figure 2 | Transcriptome overview of drought-responsive DEGs. (A) PCA of gene expression (FPKM) showing distinct clustering of samples by tissue (needles-ND, phloem-PH, roots-RT), with separate clusters indicating tissue-specific expression profiles under control (C) and drought treatment (T). (B–D) Volcano plots for needles (B), phloem (C), and roots (D) illustrate the up- and down-regulation of DEGs between drought-treated versus control samples (thresholds: $FDR \leq 0.05$, $\log_2FC \pm 1$). (E, F) Venn diagrams show the overlap of upregulated (E) and downregulated (F) DEGs among the three tissues, highlighting both shared and tissue-specific responses.

and alpha-farnesene synthase (MA_8061716g0010) were upregulated 4- and 1.6-fold, respectively. Furthermore, nine genes associated with flavonoid and stilbene biosynthesis, originating from the chalcone and stilbene synthase enzyme families, were detected, of which only two exhibited upregulation under water stress.

Phloem showed 4,460 upregulated and 4,115 downregulated genes in response to water deficit. Highly regulated categories included 129 cytochrome P450s, 78 transferases, 75 ABC transporters, 63 UGTs, 24 GSTs, 19 LEA proteins, and 17 each for AQPs and MAPKs. Additional regulated groups included 15 HSPs, 14 dehydrins, 13 ABA/WDS-induced proteins, 13 CYSTM genes, 12 LURP, 3 PAR1, and 3 HOTHEAD genes were also identified with 20 TLPs differentially expressed ([Supplementary Material S2.2](#)). ABA-related genes included 27 PP2Cs, 11 PYL ABA receptors, 3 NCEDs, and 174 serine/threonine-protein kinases. Among highly upregulated genes, GA2ox showed ~11-fold change, and four LARs were upregulated (two transcripts with 10- and 13-fold increase). In addition, nearly 30 terpene synthase (TPS) genes were identified, with five displaying significant upregulation in phloem, including gamma-humulene synthase (7.5-fold), two alpha-farnesene synthases (5.6- and 4.9-fold), alpha-bisabolene synthase (6.5-fold), and pinene synthase (5.4-fold). Furthermore, 11 genes associated with flavonoid and stilbene biosynthesis were identified, among which 3-ketoacyl-CoA synthase 1 exhibited eightfold upregulation.

Root showed the largest number of DEGs, with 3,965 upregulated and 5,818 downregulated genes. A total of 190 cytochrome P450s, 102 ABC transporters, 87 transferases, 76 UGTs, 43 HSPs, 39 GSTs, 27 LEA proteins, and 21 MAPKs were differentially expressed ([Supplementary Material S2.3](#)). Other major groups included 20 aquaporins, 18 ABA/WDS-induced proteins, 17 TLPs, 15 galactinol synthases, 15 dehydrins, 13 LURP, 10 CYSTM genes, 6 PAR1, and 2 HOTHEAD genes. Several ABA biosynthesis and signaling genes (25 PP2Cs, 11 PYL ABA receptors, 3 NCEDs, 235 serine/threonine kinases), one GA2ox, and four LARs were also

identified. A total of 28 TPS genes were detected in the roots, all of which were downregulated. Additionally, 12 genes associated with flavonoid and stilbene biosynthesis were present, with only one showing 3-fold upregulation (3-ketoacyl-CoA synthase 1).

Across all tissues, 1,543 genes were upregulated and 875 downregulated under drought, representing core drought-responsive pathways ([Figure 2E, 2F; Supplementary Material S2.4](#)). These shared DEGs included 33 cytochrome P450s, 23 UGTs, 23 ABC transporters, 12 galactinol synthases (all upregulated), 11 dehydrins, 10 LEA proteins, 10 ABA/WDS-induced proteins, 9 TLPs, 8 PP2Cs, and 8 GSTs. Additional conserved DEGs included 5 RADIALIS-like proteins (all downregulated), 3 aquaporins, 2 LURP genes (with 9 to 11-fold upregulation), 2 PAR1, and 1 HOTHEAD gene (MA_10434427g0010; ~10-fold up in needle and phloem, ~2-fold in roots). We also observed that early light-induced proteins (ELIPs) were upregulated and expressed ubiquitously across all tissues. Multiple expansin (EXP) superfamily members, including expansin-like proteins, were also differentially expressed. Notably, expansin-like B1 was strongly upregulated in needle and phloem, while expansin-like A1 and A2 were upregulated in roots. Four of each TPS, flavonoid & stilbene biosynthesis genes were found to be commonly expressed across all tissues, and all were downregulated.

3.3 Hierarchical Clustering analysis (HCA) reveals distinct tissue-specific regulation and functional enrichment of DEGs

To focus on the most responsive DEGs, HCA was restricted to 1,626 genes with large expression changes ($\log_2FC \geq 2$). These were subsequently examined for enrichment of specific functional categories ([Figure 3](#)). Clustering grouped DEGs into eleven distinct clusters (labeled A through K) based on expression similarities ([Supplementary Material S3](#)). Clustering analysis revealed distinct tissue-specific expression patterns, with clusters A–D comprising only downregulated genes, while clusters E–K consisted entirely of upregulated genes. Clusters A

(12/12) and B (129/129) showed downregulation in phloem and root, but not needles. Cluster C (96/96) was mainly downregulated in needles, with partial downregulation in phloem, and cluster D (243/243) contained genes uniformly downregulated across all tissues. Genes related to signal transduction & kinases, stress response & defence, and secondary metabolism were predominantly enriched in downregulated clusters A–C, while cluster D was mainly enriched with stress & defence-associated genes. Clusters E (104/105), F (73/73), G (211/211), H (136/136), I (387/387), and J (134/141) were largely

upregulated across tissues, with only a few isolated downregulated genes, mainly in needles or roots, associated with metabolic, stress-related, or uncharacterized functions. Overall, these patterns highlight strong tissue-specific upregulation with only minimal and functionally distinct downregulation events. Upregulated clusters E, H, I, G, and F were primarily enriched for stress-response & defence mechanisms, signal-transduction & kinase, and secondary-metabolism genes, while clusters J and K showed strong enrichment for nutrient & ion transport functions.

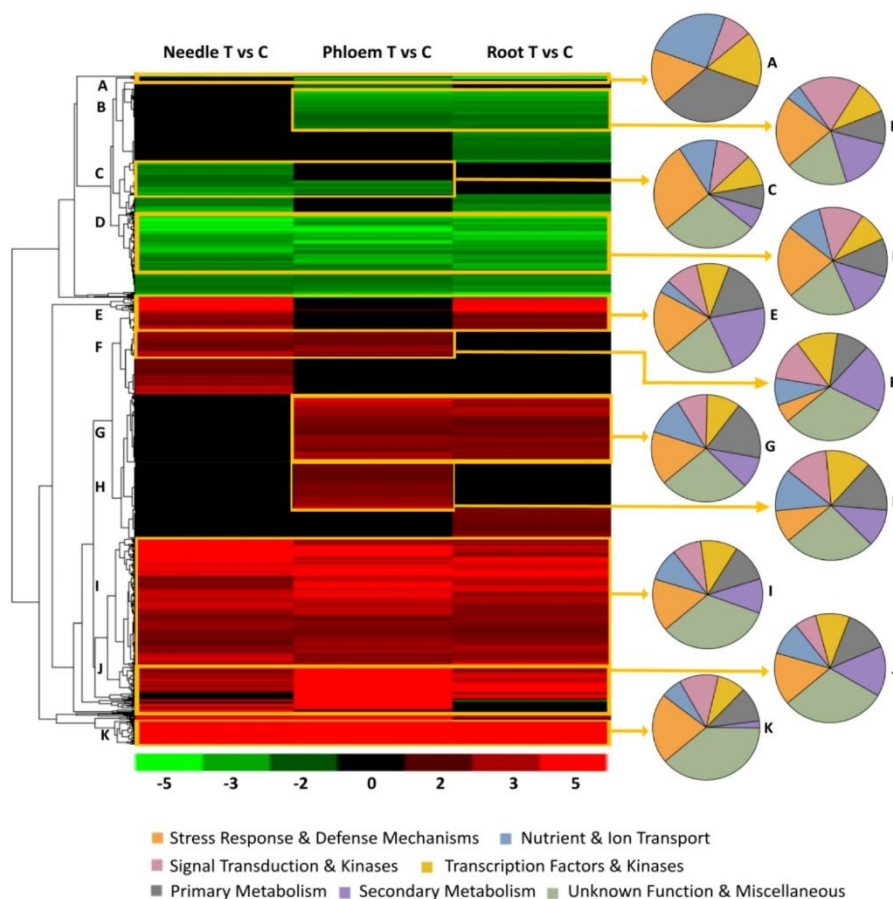


Figure 3 | Hierarchical clustering analysis (HCA) of drought-responsive DEGs. The dendrogram (left) is divided into 11 major clusters (A–K) based on similar expression patterns (including DEGs with $\log_2FC \pm 1$, drought vs. control). Pie charts for each cluster display the functional categories of strongly regulated transcripts ($\log_2FC \pm 2$) within that cluster, highlighting distinct biological processes enriched among upregulated (red sectors) and downregulated (green sectors) genes.

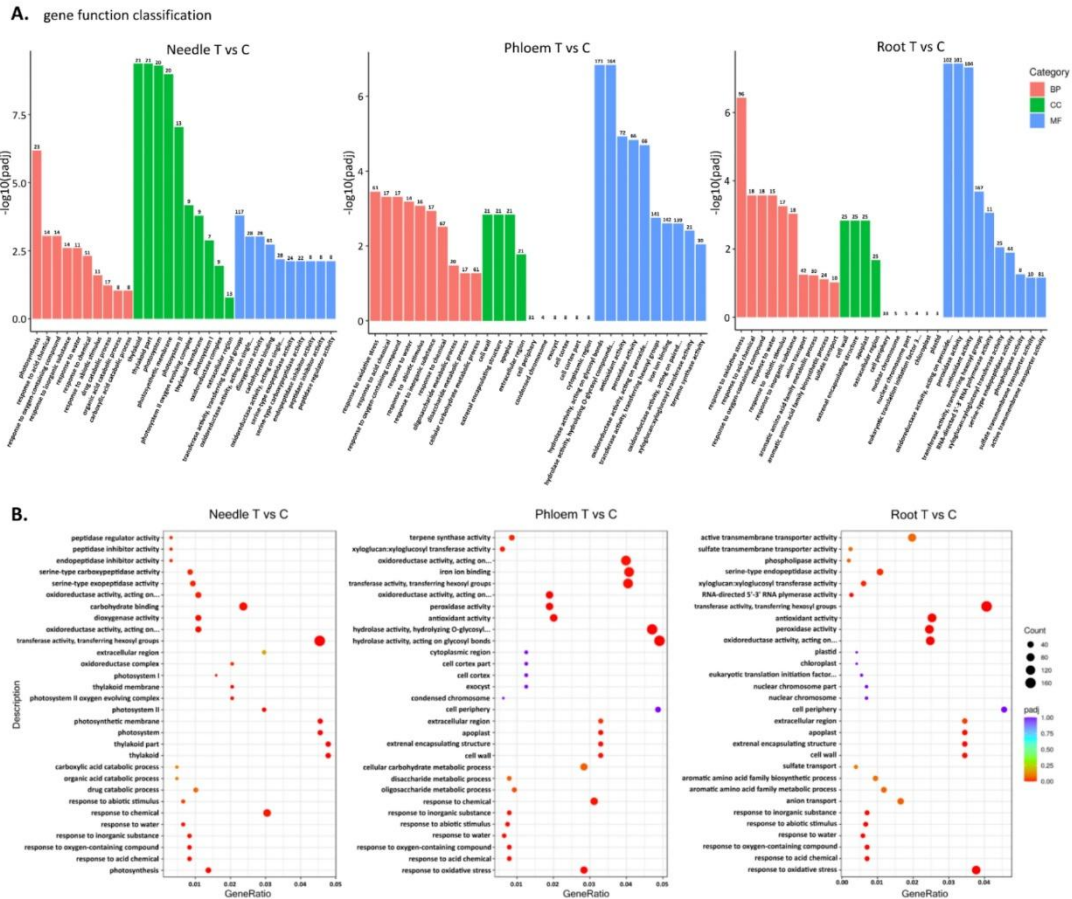


Figure 4 | Gene Ontology (GO) enrichment analysis of drought-responsive DEGs. (A) GO annotations of DEGs across three domains: biological process (BP), molecular function (MF), and cellular component (CC). The top 30 significantly enriched GO terms overall are shown. (B) Bubble plot of the top 30 enriched GO terms in needles, phloem, and roots under drought. Terms are ranked by significance ($p < 0.05$), highlighting key processes like photosynthesis (needles), oxidative stress response (phloem and roots), cell wall and apoplast components (phloem/roots), and various metabolic and catalytic activities.

3.4 Different GO terms were enriched in needles but not in phloem and root

Gene Ontology (GO) enrichment analysis was performed for the significant DEGs ($P < 0.05$) and categorized into three GO domains: biological process (BP), cellular component (CC), and molecular function (MF) for the top 30 terms (Figure 4A). In needles, *photosynthesis* (GO:0015979) was the most significantly enriched BP term; *thylakoid* (GO:0009579) and *thylakoid part* (GO:0044436) were most enriched in the CC domain; and *transferase*

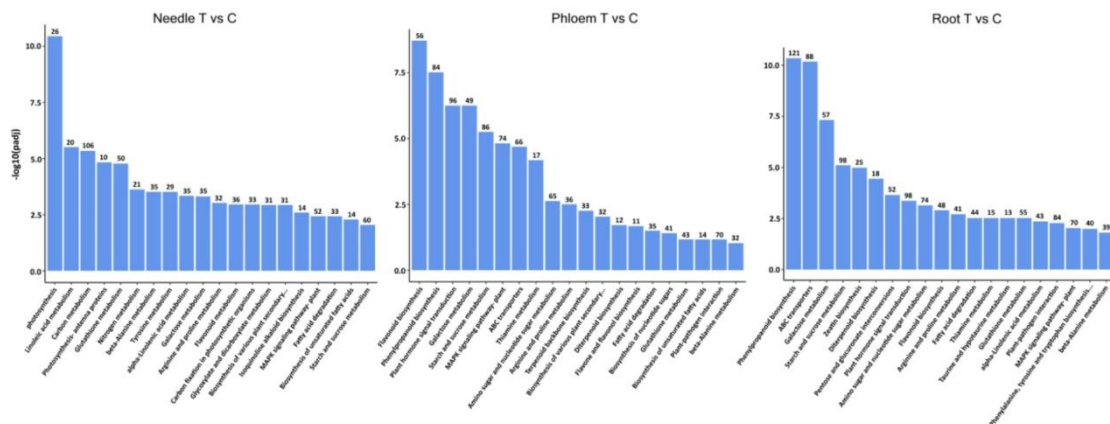
activity (GO:0016758) was the top MF term (Figure 4B). In phloem, *response to oxidative stress* (GO:0006979) was the most enriched BP term; the CC terms *cell wall* (GO:0005618), *external encapsulating structure* (GO:0005576), and *apoplast* (GO:0048046) were also highly represented, while *hydrolase activity* (GO:0016798) was the most enriched MF term (Figure 4B). In roots, *response to oxidative stress* (GO:0006979) was likewise the most significantly enriched BP term and CC enrichment in the root mirrored that of the phloem, with *cell wall* (GO:0005618), *external encapsulating structure*

(GO:0005576), and *apoplast* (GO:0048046) as the most overrepresented terms (Figure 4B). In roots, *Oxidoreductase activity* (GO:0016684) was the most significantly enriched for the MF domain (Figure 4B).

In addition to GO analysis, KEGG pathway enrichment analysis was conducted to further explore the metabolic and signaling pathways associated with drought-responsive DEGs across tissues (Figure 5A, 5B). In needles, the most significantly enriched pathways were photosynthesis (ko00195), linoleic acid metabolism (ko00591), carbon metabolism (ko01200), photosynthesis – antenna proteins (ko00196), and glutathione metabolism (ko00480). In

phloem, the top enriched pathways included flavonoid biosynthesis (ko00941), phenylpropanoid biosynthesis (ko00940), plant hormone signal transduction (ko04075), galactose metabolism (ko00052), and starch and sucrose metabolism (ko00500). Root tissues showed significant enrichment in phenylpropanoid biosynthesis (ko00940), ABC transporters (ko02010), galactose metabolism (ko00052), starch and sucrose metabolism (ko00500), and zeatin biosynthesis (ko00908). Notably, some pathways were consistently enriched across all tissues, such as the MAPK signaling pathway – plant (ko04016), glutathione metabolism

A. KEGG enrichment analysis histogram



B.

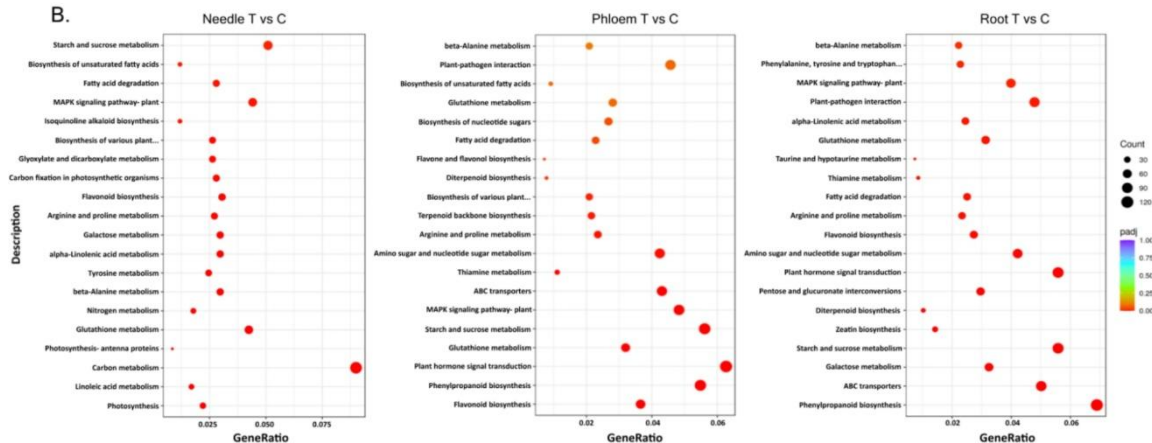


Figure 5 | KEGG pathway enrichment analysis of drought-responsive DEGs. (A) Histogram of the 20 most significantly enriched pathways across tissues, with bar length indicating significance levels. (B) Bubble plot showing enriched pathways in needles, phloem, and roots under drought. Each bubble's size corresponds to the number of annotated genes in the pathway, and bubble color (red to purple) indicates enrichment significance.

(ko00480), galactose metabolism (ko00052), flavonoid biosynthesis (ko00941), and starch and sucrose metabolism (ko00500).

3.5 Drought-related transcription factors

Transcription factors (TFs) are crucial regulators of gene expression that modulate stress responses by binding to specific cis-regulatory elements in gene promoters. Using PlantTFDB v4.0, we identified 892 differentially expressed transcription factors (DE-TFs) out of 24,487 DEGs. The most abundantly represented TF families among these DE-TFs were the

AP2/ERF superfamily (172 members), MYB (117 classical MYB and 83 MYB-related), bHLH (73), NAC (63), WRKY (37), bZIP (27), and C2H2-type zinc finger proteins (14). Of the 892 DE-TFs, 258 were strongly upregulated ($\log_2FC > 2$) and 225 were strongly downregulated ($\log_2FC < -2$) under drought. Across tissues, 94 DE-TFs were found to be commonly regulated in all three tissues, of which 65 were upregulated and 29 were downregulated (Figure 6; Supplementary Material S4.4). This shared set spanned prominent TF families such as MYB (18), ERF (18), MYB-related (15), NAC (10), bHLH (4), GATA (3), WRKY (3), and bZIP (2) families,

as well as several less-represented families such as SBP (2), NF-YC (2), WOX (2), GeBP (1), HD-ZIP (1), and ZF-HD (1).

Tissue-specific profiling identified 196, 279, and 302 DE-TFs in needle, phloem, and roots, respectively (Figure 7; Supplementary material S4.1-4.3). In needles, almost 60% of DE-TFs were upregulated, predominantly belonging to the MYB (53), AP2/ERF (42), and NAC (24) families. Phloem showed a similar expression pattern (59.5% upregulated, 39.5% downregulated), with dominant families including MYB (68), AP2/ERF (66), bHLH (24), and NAC (18). Roots had the greatest number of DE-TFs, with ~46% up- and 54% downregulated, enriched in the MYB (78 members), AP2/ERF (58), bHLH (36), and NAC (21) families. TALE (Three-Amino-acid-Loop-Extension) homeobox TFs were also moderately regulated in phloem and root.

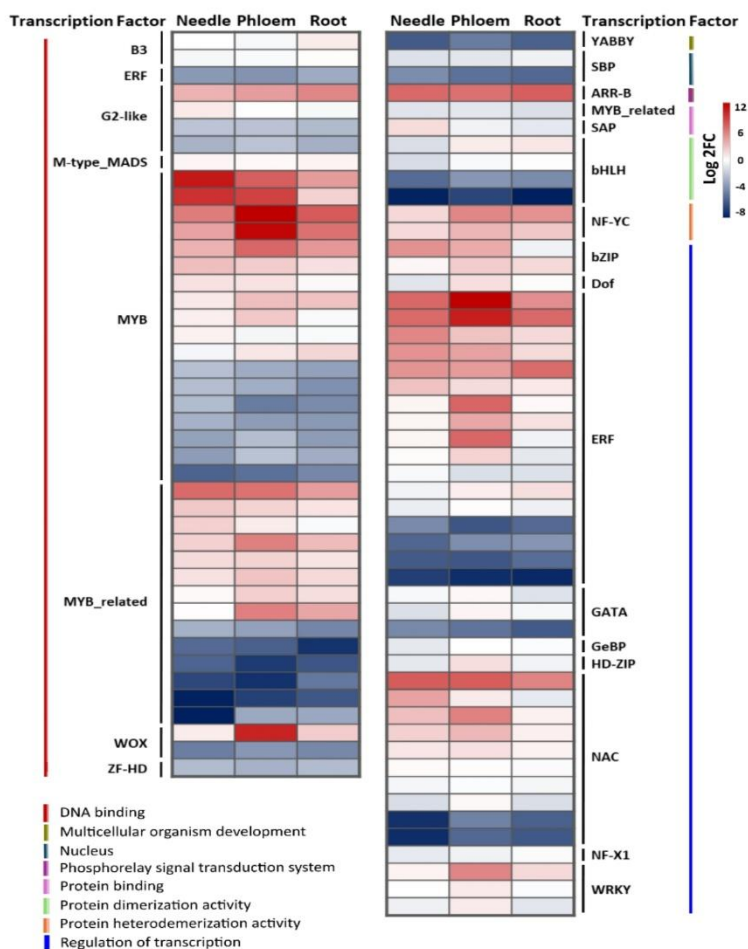


Figure 6 Heatmap of differentially expressed transcription factors (TFs) that are commonly regulated in needles, phloem, and roots under drought stress. Red indicates upregulation and blue indicates downregulation (relative to controls) for each TF across the three tissues.

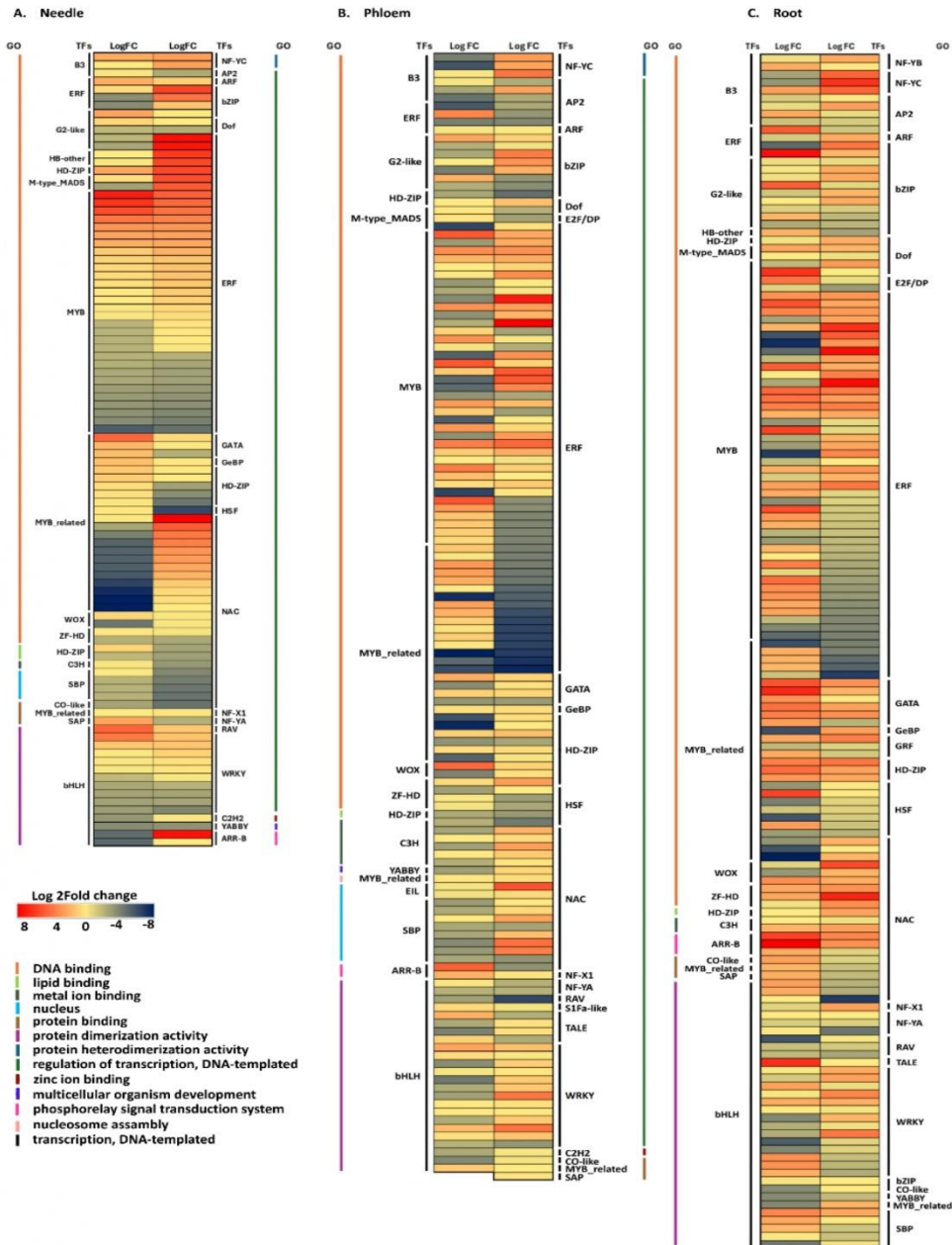


Figure 7 | Heatmaps of tissue-specific differentially expressed transcription factors under drought. Each panel shows TF expression profiles in one tissue (needles, phloem, or roots), with red indicating upregulation and blue indicating downregulation (drought vs. control). The patterns highlight distinct transcriptional responses: roots exhibit the largest number of TF changes (both up and down), whereas needles and phloem show comparatively fewer or more moderate TF expression changes.

3.6 MapMan metabolic pathway analysis

As a complement to the GO and KEGG analyses, MapMan was used to classify DEGs into functional “Bins” (categories) and to visualize their involvement in key biological pathways affected by drought. Separate MapMan analyses were conducted for tissue-specific DEGs and 2,418 DEGs common across the three tissues. Among the common DEGs, the Metabolism Overview panel indicated broad changes in primary and specialized metabolism. The Photosynthesis and Abiotic Stress & Redox panels revealed a consistent downregulation of photosynthetic components (light-harvesting

complexes, Calvin cycle enzymes) and antioxidant genes (e.g., thioredoxins, glutathione S-transferases, ascorbate peroxidases).

Analysis of the common DEGs revealed enrichment in functional categories related to cell wall organization (86 genes), lipid metabolism (39), amino acid metabolism (25), and carbohydrate metabolism (21). Additional shared bins included redox homeostasis (19), cytoskeleton organization (9), nucleotide metabolism (8), photosynthesis (6), RNA homeostasis (5), nutrient uptake (5), cellular respiration (5), and coenzyme metabolism (1) (Figure 8; Supplementary Material S5.1; S5.5).

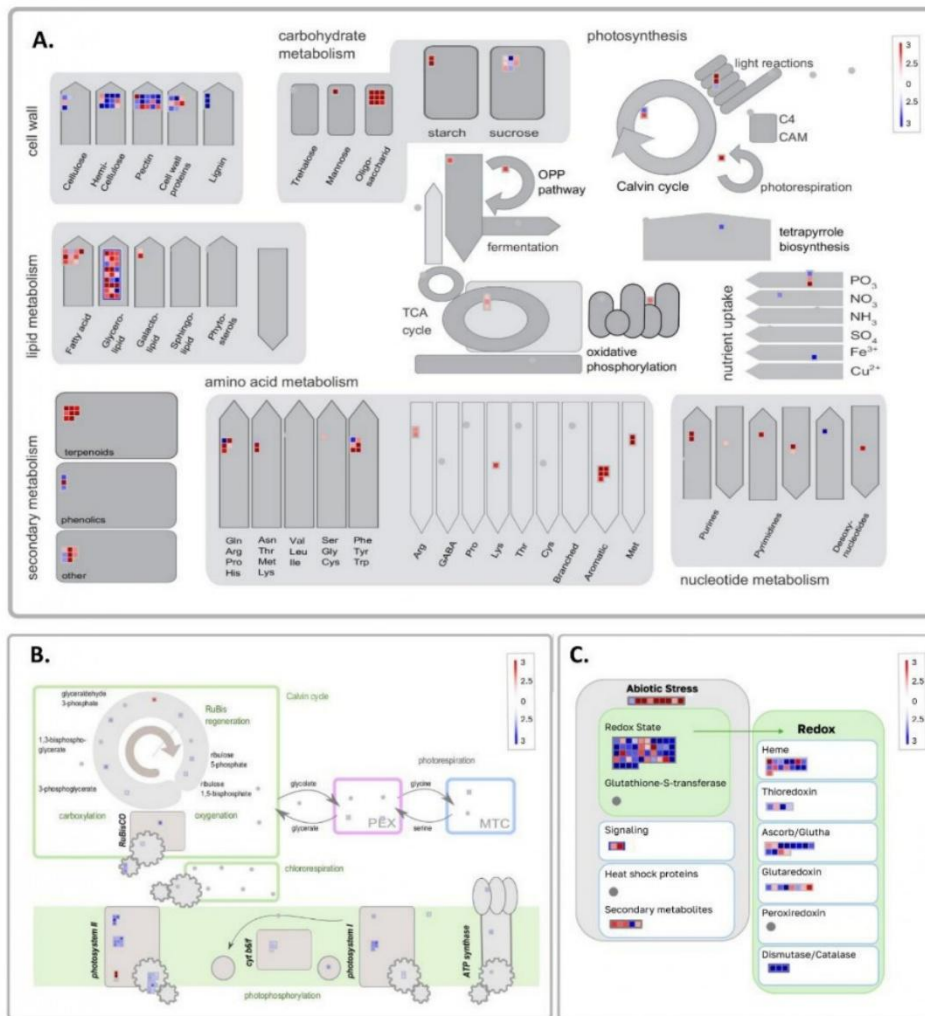


Figure 8 | MapMan pathway visualization for DEGs common to all tissues under drought. (A) Metabolism overview: highlights changes in primary and secondary metabolic pathways. (B) Photosynthesis: shows widespread downregulation of photosynthetic components (blue squares). (C) Abiotic stress and redox homeostasis: illustrates changes in stress-related pathways (red = upregulated genes, blue = downregulated genes). Each square represents a DEG mapped to a functional Bin. Panel C was customized after Ribeyre et al. (2025) by merging relevant MapMan pathways to specifically depict drought stress responses (modified from biotic stress and cellular response pathways).

MapMan visualizations showed broad shifts in both primary and secondary metabolism, with consistent downregulation of photosynthesis-related genes (e.g., light-harvesting complexes, Calvin cycle enzymes) and ROS-detoxifying enzymes (e.g., thioredoxins, glutathione S-transferases, ascorbate peroxidases).

In needles, a total of 476 DEGs, of which 51% were upregulated, were assigned to 13 functional MapMan bins. The most represented categories were photosynthesis (77 genes), cell wall organization (69), amino acid metabolism (55), lipid metabolism (54), and nutrient uptake (54). Additional bins included carbohydrate metabolism (45), redox homeostasis (38), cytoskeleton organization (21), RNA homeostasis (19), and nucleotide metabolism (16) (Supplementary Figure 2; Supplementary Material S5.2). Genes upregulated in needles were primarily involved in structural and metabolic functions such as cell wall modification, carbohydrate metabolism, lipid turnover, and amino acid metabolism, whereas genes associated with photosynthesis (photosystem I/II components, ATP synthase, and chlorophyll biosynthesis) and nutrient acquisition were largely downregulated.

In phloem, 712 DEGs (59% upregulated) were mapped to MapMan bins. The most enriched functional categories were cell wall organization (281 genes), lipid metabolism (118), amino acid metabolism (65), carbohydrate metabolism (59), and redox homeostasis (45). Other significant bins included RNA homeostasis (40), nutrient uptake (30), photosynthesis (30), and nucleotide metabolism (15) (Supplementary Figure 3; Supplementary Material S5.3). Upregulated genes in the phloem were predominantly linked to lipid and carbohydrate metabolism, Redox homeostasis, while genes associated with cell wall organization showed widespread downregulation.

DEGs were assigned to 13 functional bins in roots, with 47% being upregulated and 53% being downregulated. The largest bins comprised cell wall organization (349), lipid metabolism (102), amino acid metabolism (68), carbohydrate metabolism (66), and

redox homeostasis (56). Additional functional categories were photosynthesis (36), cytoskeleton organization (34), RNA homeostasis (33), nutrient uptake (31), nucleotide metabolism (15), and cellular respiration (13) (Supplementary Figure 4; Supplementary Material S5.4). Notably, several photosystem II repair and reassembly-related genes in the photosynthesis bin were upregulated, and many DEGs associated with cell wall organization were broadly downregulated in roots. DEGs were assigned to 13 functional bins in the roots, with 47% being upregulated and 53% being downregulated. The largest bins comprised cell wall organization (349), lipid metabolism (102), amino acid metabolism (68), carbohydrate metabolism (66), and redox homeostasis (56). Additional functional categories were photosynthesis (36), cytoskeleton organization (34), RNA homeostasis (33), nutrient uptake (31), nucleotide metabolism (15), and cellular respiration (13) (Supplementary Figure 4; Supplementary Material S5.4). Notably, several photosystem II repair and reassembly-related genes in the photosynthesis bin were upregulated, and many DEGs associated with cell wall organization were broadly downregulated in roots.

3.7 RT-qPCR validation

RT-qPCR was performed on 13 selected genes to validate the RNA-seq expression profiles. These genes included known drought-responsive genes and transcription factors such as dehydrin (*DHN2*), late embryogenesis abundant protein (*LEA88*), ABA/WDS-induced protein (*IP3*), NAC domain-containing protein (*NAC68*), as well as defense-related genes including pinene synthase (*PTI*), glutathione S-transferases (*GSTUN*), and alpha-pinene synthase (*TPS6*). The expression profile obtained through RT-qPCR was consistent with that observed in the RNA-seq data, confirming their reliability. The analysis revealed distinct tissue-specific expression patterns of stress-responsive genes in needle, phloem, and root tissues. Several genes, including *NAC68*, *IP3*, *GPX4*, *TPS6*, *GSTUN*, *DHN5*, *BCH2*, *DHN2*, *LEA88*, and *PXG*, were

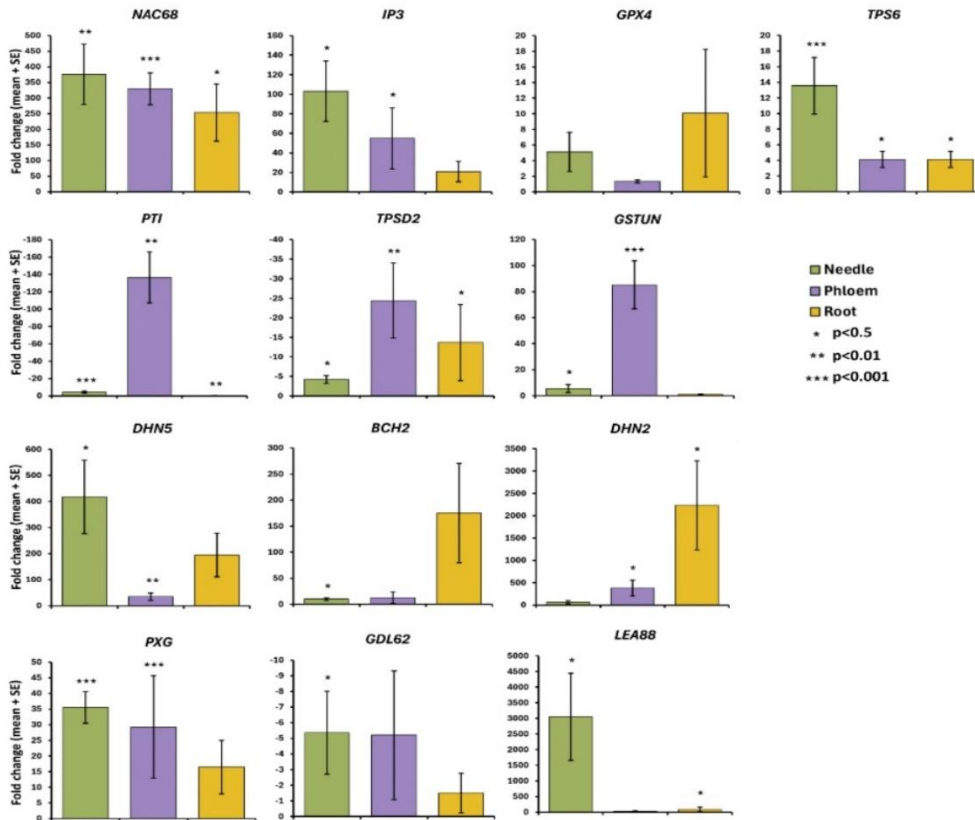


Figure 9 RT-qPCR validation of selected drought-responsive genes. The relative expression ($2^{-\Delta\Delta Ct}$) of 13 candidate genes (*NAC68*, *IP3*, *GPX4*, *TPS6*, *PTI*, *TPSD2*, *GSTUN*, *DHN5*, *BCH2*, *DHN2*, *PXG*, *GDL62*, and *LEA88*) is shown for drought-treated vs. control seedlings in needles, phloem, and roots. Bars represent mean \pm SE (standard error) of three biological replicates. Asterisks denote statistically significant differences between treatment and control groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

markedly upregulated, with *NAC68*, *LEA88*, and *DHN2* showing particularly strong upregulation (Figure 9).

4. Discussion

This study advances understanding of tissue-specific Norway spruce responses to drought by integrating physiological and transcriptomic analyses, thereby identifying key drought-responsive genes and generating valuable genomic resources. Norway spruce and other conifers adapt to prolonged drought through integrated molecular, physiological, and structural strategies that support survival under water limitation (Moran et al. 2017; Polle et al. 2019). Nevertheless, knowledge of tissue-specific responses

has been limited, and our results provide new insights into how needles, phloem, and roots contribute to coordinated drought adaptation.

4.1 Physiological Mechanisms of Drought Response in Norway Spruce

Abscisic acid (ABA) serves as a central regulator of drought responses in conifers, coordinating stomatal control and transcriptional reprogramming to mitigate water deficits. Elevated ABA levels trigger stomatal closure in Norway spruce, representing an isohydric strategy that maintains tissue water potential and enables survival under prolonged drought, even at the cost of reduced photosynthesis (Brodribb et al. 2014; Brunner et al. 2015). The

regulation of stomatal dynamics involves *NCEDs*, *PYL* receptors, and *PP2Cs*, consistent with enhanced ABA biosynthesis and signaling during stress (Verslues and Zhu 2007; Cutler et al. 2010; Haas et al. 2021; Ribeyre et al. 2025). Upon drought perception, ABA signaling activates bZIP transcription factors, particularly the AREB/ABF family. These factors are phosphorylated by *SnRK2* kinases and subsequently bind to ABRE/G-box motifs, inducing the expression of genes that confer osmotic protection (Haas et al. 2021). In parallel, AP2/ERF transcription factors are induced in an ABA-independent manner through their interaction with drought-responsive elements (DREs). The co-activation of ABA-dependent AREB/ABFs and ABA-independent AP2/ERFs shows how distinct signaling modules converge to enhance stress tolerance (Soma et al. 2021; Ye et al. 2024; Ribeyre et al. 2025). The downstream targets of these pathways include LEA proteins, dehydrins, proline biosynthesis enzymes, ROS-scavenging systems, and cuticular wax synthases, which collectively provide cellular protection during water deficit (Yoshida et al. 2015; Guo et al. 2024).

Our results demonstrate extensive ABA-mediated transcriptional reprogramming across tissues, with pronounced upregulation of bZIP transcription factors in the phloem, highlighting its role as a hub for systemic stress signaling (Figures 6, 7, and 10). By contrast, most bZIPs in roots were downregulated, although both roots and needles showed strong induction of ABA-responsive genes, reinforcing ABA's central role in drought signaling and stomatal regulation. Several ABA metabolism and stress-associated genes were differentially regulated across tissues. Abscisic stress-ripening protein 1, ABA/WDS-induced protein, aldehyde dehydrogenase family 3 member 11, and NINJA-family protein AFP3 were consistently upregulated across tissues, whereas the receptor *PYL4* was repressed. Strong upregulation was also observed for multiple *PP2C* isoforms, *NCED3*, and *SnRK* gamma-1-like. Among transcription factors, bZIP53 and bZIP43 were highly induced, along with protective proteins such as *LEAs*, dehydrins, and *NDR1/HIN1*-like proteins (*NHL6*, *NHL3*), whereas *NHL10* was downregulated.

An increase in proline under drought highlights its multiple protective roles, including osmotic adjustment, antioxidant defense, and protein–membrane stabilization, making it a key biomarker of drought tolerance across angiosperms and gymnosperms (Szabados and Savouré 2010; Hayat et al. 2012). Plant cells synthesize proline mainly from glutamate via the delta-1-pyrroline-5-carboxylate synthetase (*P5CS*) and *P5C* reductase pathway. Under water deficit, both transcriptional and post-translational regulation increase the flux through this pathway (Ozturk et al. 2021). In our study, drought-exposed seedlings accumulated higher levels of proline in needles, supported by the upregulation of *P5CS*, indicating that proline acts as an adaptive osmoprotectant, stabilizing cellular structures, maintaining turgor, and scavenging reactive oxygen species under water deficit (Figure 1A; Figure 10; Supplementary Material S2). By contrast, majority of genes encoding proline-related kinases (*PERK1/2*) and transporters (*ProT1/2*) were downregulated in needles, suggesting a shift in resource allocation, where stress signaling and systemic redistribution of proline are minimized to prioritize local osmoprotection (Figure 10). This coordinated regulation reflects a tissue-specific strategy, concentrating proline in needles to maintain cellular homeostasis and enhance survival under prolonged water deficit.

Tree survival to drought is largely influenced by their hydraulic structure and function. In general, anisohydric species involve less strict control over their leaf water potential during drought, leading to lower water potential but allowing them to maintain higher photosynthetic rates for longer periods by keeping stomata open. While this allows for greater growth under mild drought, it increases their susceptibility to xylem embolism under severe drought conditions. On the other hand, isohydric species use a conservative water-use strategy, maintaining a stable leaf water potential by strictly regulating their stomata to limit water loss during drought. In our study, several genes involved in pectin metabolism, including pectinesterase 1, pectinesterase 2, polygalacturonase 1 beta-like

protein 1, pectate lyase, beta-galactosidase 1, and beta-L-arabinofuranosidase, were consistently downregulated across all tissues under drought relative to controls (Figure 10). This coordinated suppression of pectin-modifying enzymes likely weakens the integrity of xylem and leaf cell walls, thereby compromising water transport efficiency, reducing resistance to cavitation, and constraining leaf area adjustments under drought conditions (Willats et al. 2001; Mader et al. 2023; Ribeyre et al. 2025). Under drought, reductions in stomatal conductance and photosynthesis promote the reallocation of nonstructural carbohydrates (NSCs) to mitigate carbon starvation, while root and leaf architecture undergoes adaptive remodeling to regulate transpiration (Adams et al. 2013; Anderegg and Anderegg 2013; Reinhardt et al. 2015; Galiano et al. 2017). Consistent with these processes, our study revealed upregulation of several NSC metabolic enzymes, including beta-fructofuranosidase, multiple galactinol–sucrose galactosyltransferases (2, 5, and 6), trehalose biosynthesis enzymes (*TPS5*, *TPS6*, *TPP4*), glycolytic enzymes (hexokinase-1, hexokinase-2, 6-phosphofructokinase, glycerol-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase-like proteins 1 and 2), and mannitol dehydrogenase, whereas sucrose synthase 2 and 4 were downregulated (Figure 10). Collectively, these findings suggest that drought resistance is primarily determined by stomatal regulation, protective metabolites, carbohydrate metabolism, and pathogen defense mechanisms, whereas resilience relies on the recovery of photosynthetic capacity, structural persistence, and the ability to restore xylem functionality.

Although chlorophylls and carotenoids exhibited some modulation and concentration variability under severe water deficit, these changes were not significant, indicating that photosynthetic pigment pools were largely maintained (Figure 1b). This suggests that Norway spruce, like other evergreen conifers, employs protective mechanisms to preserve pigment integrity and sustain photosynthesis during short-term drought. Potential mechanisms include sustained xanthophyll cycling,

enhanced non-photochemical quenching, active ROS scavenging, and rapid repair of photosystem components (Lee and Kim 2024). Several studies have shown that during drought, stomatal closure and reductions in photochemical efficiency occur earlier and more rapidly than chlorophyll degradation, with visible pigment loss typically appearing only under prolonged or severe stress conditions (Ditmarová et al. 2010; Marešová et al. 2022). Plants can mitigate these effects by reallocating resources and adjusting their metabolic activity, such as by accumulating proline, compatible osmolytes, and antioxidants, which help maintain chloroplast integrity. Schiop et al. (2017) observed that in certain Norway spruce populations, chlorophyll content remained largely stable after 42 days of water deficit, with only minor decreases in some populations and slight increases in others, while other physiological traits, including chlorophyll fluorescence and needle water potential, were significantly affected. To assess metabolic adjustments to drought stress in two young spruce provenances, Ahmad et al. (2025) measured pigment (zeaxanthin, lutein, violaxanthin, neoxanthin, β -carotene, and chlorophyll) and tocopherol concentrations at four time points representing both early and late stages of drought exposure. Among these pigments, zeaxanthin exhibited a significant increase in drought-stressed seedlings of both provenances, lutein increased significantly in one provenance, and changes in the other pigments were not statistically significant. Similarly, Slugeňová et al. (2011) reported that in a 43-day experiment with four-year-old Norway spruce seedlings subjected to severe drought (-2.4 MPa), photosynthetic pigment concentrations were not significantly altered, except under conditions combining drought and aluminum stress.

In our data, the upregulation of several genes such as early light-induced protein 1 (*ELIPs*), Pheophorbide a oxygenase, beta-carotene hydroxylase 2, *NCED3*, and beta-D-glucosyl crocetin beta-1,6-glucosyltransferase suggests an enhanced protective and stress-induced response, including the activation of carotenoid biosynthesis and ABA-mediated signaling to mitigate oxidative damage and

regulate stomatal closure (Figure 10). *ELIPs*, specifically, are known to protect against photooxidative stress (Hutin et al. 2003). They are transient thylakoid pigment-binding proteins that protect the photosynthetic apparatus from photooxidative stress by binding free chlorophyll and stabilizing light-harvesting complexes; however, their role in conifers remains poorly studied (Hutin et al. 2003; Liu et al. 2020; Lee et al. 2020). In contrast, the downregulation of chlorophyll a-b binding protein, Thioredoxin F2, and short-chain dehydrogenase reductase 2a may reflect a controlled reduction in light-harvesting activity and photosynthetic electron transport to prevent the formation of excess reactive oxygen species under limited water availability (Figure 10). Together, these transcriptional adjustments indicate a strategic shift in the Norway spruce metabolism toward stress tolerance while minimizing photodamage under drought.

4.2 Tissue-Specificity of Drought-Induced Gene Expression

Transcriptome profiling revealed distinct tissue-specific drought responses in Norway spruce, with needles showing a unique expression profile compared to the more similar profiles of phloem and roots, as confirmed by PCA clustering. The identification of 3,125 upregulated and 3,004 downregulated genes in needles, alongside higher numbers of DEGs in phloem (8,575) and roots (9,783), reveals the complexity of drought responses across tissues. We reported several genes differentially regulated across all tissues involved in metabolism, nutrient and ion transport, signal transduction & kinases, and defense mechanisms such as cytochromes, transferases, *UGTs*, *GSTs*, ABC transporters, PR proteins, *TLPs*, and several drought-markers including dehydrins, LEA proteins, *AQPs*, *HSPs*, and ABA/WDS-induced proteins. In addition, several transcripts encoding *TPS* and flavonoid & stilbene biosynthesis were also reported. Drought triggered the suppression of photosynthesis-related genes in needles, including light-harvesting complex proteins, Calvin cycle enzymes, and chlorophyll biosynthesis enzymes, consistent with energy-saving and photoprotection strategies (Klápště et al. 2020;

Wu et al. 2022; Qiao et al. 2024). However, the strong upregulation of pathogenesis-related proteins and chitinases (e.g., MA_10211971g0010, MA_10429511g0010) may suggest that defense priming also contributes to needle-level drought responses, possibly mitigating secondary biotic threats. The upregulation of genes involved in cell wall remodeling, carbohydrate metabolism, and lipid turnover in needles indicates structural adaptations and osmotic adjustments that help minimize transpiration (Sanders and Arndt 2012; Xiao et al. 2021). The enrichment of photosynthesis-related GO terms and KEGG pathways in needles, such as *photosynthesis (ko00195)* and *carbon metabolism (ko01200)*, reflects their role as primary photosynthetic organs, where maintaining metabolic functions is critical (Xiao et al. 2021). Conversely, the enrichment of *oxidative stress response (GO:0006979)* and *phenylpropanoid biosynthesis (ko00940)* in phloem and roots suggests a stronger emphasis on ROS detoxification and secondary metabolite production, which are vital for structural integrity and defense under stress (Dixon and Paiva 1995; Xiao et al. 2021).

Several key DEGs involved in flavonoid biosynthesis (e.g., *LAR*, MA_7866760g0010) and *gibberellin 2-beta-dioxygenase (GA2ox, MA_26738g0010)* were upregulated across all tissues. *LARs* play a crucial role in the biosynthesis of flavan-3-ols, specifically in the synthesis of catechin, an essential building block of condensed tannins (also known as proanthocyanidins) in plants. *LAR* catalyzes the NADPH-dependent reduction of 2R,3S,4S-flavan-3,4-diols (leucoanthocyanidins) into 2R,3S-flavan-3-ols, such as catechin. *GA2ox* enzymes are critical in regulating plant growth during drought by deactivating bioactive gibberellins (GAs). This deactivation reduces GA levels, which in turn promote drought avoidance responses, such as stomatal closure and inhibition of shoot growth. Consistent with stress-induced growth arrest, suppression of several aquaporins and metal ion transporters suggests a slowdown of root growth and uptake functions, potentially to reduce water loss and

preserve root integrity under osmotic stress (Ksouri et al. 2016; Bhaskarla et al. 2020).

Despite the tissue-specific nuances, a core set of 2,418 DEGs was shared across all three tissues, indicating whole plant systemic regulation of drought responses. These genes are primarily associated with carbohydrate metabolism, lipid biosynthesis, amino acid metabolism, ROS detoxification, and photosynthetic downregulation. The uniform suppression of Calvin cycle and oxidative phosphorylation components reflects a whole plant shift toward metabolic quiescence, aimed at minimizing energy expenditure. Although tissue-specific responses varied, the consistent upregulation of several genes associated with antioxidant systems (e.g., ascorbate peroxidases, catalases, thioredoxins) suggests an increased demand for reactive oxygen species (ROS) detoxification under drought (Tausz et al. 2004; Netherer et al. 2021). This pattern implies that metabolic activity remains sufficient to generate oxidative stress, thereby necessitating reinforcement of antioxidant capacity. Such activation is widely interpreted as a protective mechanism that delays cellular damage and mitigates drought-induced oxidative stress. The upregulation of drought-responsive genes, such as abscisic acid (ABA) 8'-hydroxylase 1, dehydrins, LEA proteins, and glutathione S-transferases (*GSTs*), across all tissues indicates activation of core stress response pathways. In addition, ABA-related genes, including *PP2Cs*, *PYL* receptors, and *NCEs*, were consistently upregulated, which are essential in ABA-mediated signaling, regulating stomatal closure and stress gene expression (Cutler et al. 2010). While the majority of terpene synthase (*TPS*) genes were downregulated, the tissue-specific upregulation of *TPS* genes in needle and phloem (e.g., gamma-humulene synthase) suggests localized volatile organic compound production for defense against oxidative stress or herbivores under drought (Loreto and Schnitzler 2010; Krokene et al. 2023). Together, these findings demonstrate that while Norway spruce tissues deploy distinct transcriptional programs to cope with drought, a conserved core of systemic responses underpins whole-plant resilience by integrating

metabolic downregulation, antioxidant reinforcement, and ABA-mediated signaling.

4.3 Modular Clustering Reveals Conserved and Tissue-Specific Gene Expression Programs Driving Drought Resilience

Hierarchical clustering analysis (HCA) revealed two contrasting gene expression profiles under drought, where clusters E–K were strongly upregulated and enriched for genes involved in stress response and defence pathways, signal transduction and kinase activity, and secondary metabolism, which are key functional categories required for rapid perception and mitigation of drought-induced cellular damage (Zhu 2016; Li et al. 2023). In contrast, clusters A–D were consistently downregulated and primarily associated with photosynthetic electron transport, nutrient assimilation, and core metabolic processes, reflecting a strategic physiological shift from growth and resource acquisition toward stress tolerance and survival during drought. The tissue-level mapping of these expression clusters indicates that Norway spruce employs both universal and tissue-specific drought-response modules: while several clusters (e.g., F, G, I) were consistently upregulated across tissues and likely represent core systemic drought-adaptation pathways, others showed clear tissue specificity, revealing localized regulatory circuits tailored to distinct physiological functions (Haas et al. 2021; Kim et al. 2022). For instance, cluster H was exclusively upregulated in the phloem and enriched for secondary metabolism (Cinnamoyl-CoA reductase; Flavonoid 3'-monooxygenase, etc.; [Supplementary material S3](#)) and detoxification (e.g., UGTs, *GSTs*) genes, underscoring specialized vascular roles in managing oxidative and chemical stress. A notable exception within broadly upregulated cluster E was fructokinase-4 (*FRK4*), which was induced in needles but suppressed in phloem and roots, suggesting a strategic reallocation of carbon resources, enhancing hexose utilization in photosynthetic tissues while limiting metabolic expenditure in sink organs (Rolland et al. 2006; Hummel et al. 2010; Bolouri Moghaddam and Van den Ende 2012). Together, these modular patterns highlight a coordinated drought-response strategy in which conserved core modules support

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systemic resilience, while tissue-specific modules fine-tune local metabolic and defense processes to maintain survival and functional specialization.

4.4 Functional Enrichment Reveals Divergence of Needle, Phloem, and Root Strategies

Gene Ontology and KEGG pathway analyses revealed the biological processes underlying tissue-specific drought responses. Strong enrichment for photosynthesis, thylakoid organization, and transferase activity was found in needles, reflecting the importance of light energy capture and metabolic flux adjustments in the leaf's response to drought (Yuan et al. 2023; Adams et al. 2020). By contrast, phloem and roots showed enrichment for responses to oxidative stress, cell wall organization, and apoplast function, which are critical in mediating stress signal propagation, osmotic regulation, and physical defense. At the metabolic pathway level, drought induced a strong activation of secondary metabolism, including phenylpropanoid and flavonoid biosynthesis in phloem and roots, as well as galactose, starch, and sucrose metabolism, key pathways for osmolyte production and ROS detoxification (Li W et al. 2021; Li et al. 2023). Notably, MAPK signaling, glutathione metabolism, and core carbohydrate metabolism were consistently enriched across tissues, acting as central "hubs" of the spruce drought response (Zhu 2016; Haas et al. 2021). Taken together, the enrichment profiles highlight a functional divergence among tissues, whereby needles prioritize the modulation of photosynthetic capacity, while phloem and roots reinforce defense, signaling, and metabolic buffering, collectively sustaining whole-plant resilience under drought.

4.5 Transcriptional Regulatory Networks and Plasticity

A remarkable feature of the Norway spruce drought response was the large cohort of differentially expressed transcription factors (DE-TFs) (Supplementary Material S4.1–S4.4). Several major drought-responsive TF families were identified, including AP2/ERF, MYB, NAC, WRKY, bZIP, and bHLH, which are well-established as key regulators of ABA signaling, ROS detoxification, stress-responsive gene

networks, and secondary metabolism in other conifers (Fox et al. 2018; Du et al. 2018; de María et al. 2020; Li J et al. 2021; Ribeyre et al. 2025). Many of these DE-TFs coordinate responses to water deficit by activating downstream targets involved in osmotic adjustment, cell wall modification, hormone signaling, and redox homeostasis (Yamaguchi-Shinozaki and Shinozaki 2006; Baldoni et al. 2015). The presence of both shared and tissue-specific DE-TFs indicates a hierarchical regulatory architecture in which a core drought regulon is complemented by tissue-localized transcriptional circuits that enable context-specific adaptation (Behringer et al. 2015; Haas et al. 2021).

Heatmap analysis revealed tissue-specific TF expression (Figure 7), with NAC and MYB families showing predominant upregulation in needles, consistent with their known functions in stomatal regulation, flavonoid biosynthesis, and senescence control (Nakano et al. 2006; Yamaguchi et al. 2010). Phloem showed strong induction of MYB-related TFs associated with phenylpropanoid metabolism and cell wall remodeling, supporting drought-reinforced phloem defenses (Fraser and Chapple 2011). The enrichment of AP2/ERF and bHLH TFs in roots aligns with their roles in osmotic adjustment, root elongation, and ABA cross-talk under drought. At the same time, consistent upregulation of WRKY and GATA TFs across all three tissues suggests a core regulatory module coordinating systemic drought responses. WRKY TFs are known to mediate crosstalk between biotic and abiotic stress pathways, whereas GATAs participate in nitrogen and light signaling, functions that become tightly coupled under drought stress. Their concurrent activation therefore indicates a coordinated network that aligns metabolic resource allocation with stress defense, supporting an integrated whole-plant response to drought (Rushton et al. 2010; Behringer et al. 2015).

Besides these well-known transcriptional regulators, several genes encoding Type-B Arabidopsis Response Regulator (ARR-Bs) TFs were upregulated across all tissues (e.g., MA_601142g0010, MA_83273g0010, MA_114951g0010). ARR-Bs are plant-specific MYB-

domain TFs that transmit cytokinin signals influencing shoot growth and stomatal behavior. In most dicots, drought rapidly suppresses ARR-B activity through reduced transcript levels and stress-activated MAPK-mediated protein degradation, shifting signaling toward ABA dominance to promote stomatal closure and root development (Nguyen et al. 2016; Yan et al. 2021). In conifers, ARR-B TFs are recognized at the sequence and expression level, but functional characterization remains limited. Genome annotations for *P. abies*, *P. taeda*, and other gymnosperms reveal a conserved but small ARR-B clade clustering with Arabidopsis ARR1/ARR10, suggesting conservation of the cytokinin two-component pathway. Comparative transcriptomic and proteomic analyses indicate that spruce ARR-B orthologs may follow a “cytokinin-off/ABA-on” model (Laitinen et al. 2017; Haas et al. 2021). The sustained expression of ARR-Bs in our dataset may reflect tissue-specific modulation or delayed repression in response to prolonged drought in Norway spruce, necessitating further functional validation. Altogether, the transcriptional landscape reveals conserved core stress-responsive modules alongside divergent tissue-specific circuits, demonstrating the plasticity of spruce regulatory networks and identifying ARR-B transcription factors as promising but underexplored regulators of conifer drought adaptation.

4.6 Metabolic Reprogramming Under Drought: MapMan Insights

MapMan analysis showed a strong repression of photosynthesis-related DEGs in needles (Bin 1), particularly those involved in light reactions and photophosphorylation (e.g., PSI/PSII components, ATP synthase; [Supplementary Table S5.2](#)). Such downregulation aligns with drought-induced stomatal closure and the need to limit photodamage under restricted CO₂ supply (Chaves et al. 2009; Flexas et al. 2006). Concurrently, genes associated with cell wall organization (Bin 21) and carbohydrate metabolism (Bin 3) were upregulated, reflecting remodeling of cell wall structures and mobilization of stored carbohydrates to maintain osmotic balance (Tenhaken 2015; Gall et al. 2015). Among these, expansin (EXPs) transcripts (Bin 21.4.2.4) were among

the most strongly induced, consistent with their role in cell wall loosening and stress-modulated growth (Yamaguchi et al. 2010). In phloem, DEGs were enriched in secondary metabolism pathways, particularly phenylpropanoid biosynthesis (Bin 16), which produces lignin and flavonoids crucial for defense against herbivores and pathogens (Dixon and Paiva 1995; Fraser and Chapple 2011). Genes linked to cell wall modification were also activated ([Supplementary Table S5.3](#)), pointing to structural reinforcement of vascular tissues to enhance resistance against mechanical stress and biotic threats, such as bark beetles (Netherer et al. 2015). Conversely, primary metabolism pathways, including lipid metabolism and nitrogen assimilation, were suppressed, reflecting a shift in metabolic priorities from growth to survival (Baerenfaller et al. 2012). Roots exhibited profound induction of carbohydrate metabolism (Bin 3), particularly genes involved in oligosaccharide biosynthesis pathways (raffinose, trehalose), which act as osmoprotectants and ROS scavengers (Nishizawa et al. 2008; Farrant and Moore 2011; Keunen et al. 2013). Additionally, ABA-responsive genes were strongly upregulated in both roots and needles, consistent with ABA’s central role in initiating drought signaling cascades and mediating stomatal and hydraulic adjustments (Verslues and Zhu 2007; Cutler et al. 2010). Across all tissues, a shared repression of photosynthesis-related genes, including the photophosphorylation components pathway (e.g., photosystem I/II, ATP synthase; Bin 1.1.1.4.3), reflected a systemic energy-conserving strategy (Xiao et al. 2021), while common induction of cell wall remodeling and stress-related pathways pointed to a conserved program for maintaining tissue integrity and basal defense. Overall, these patterns depict a flexible yet convergent transcriptional response, aligning tissue-specific functional priorities with a core survival-oriented strategy. In summary, MapMan analysis highlights a metabolic shift from growth to survival, with tissues prioritizing structural reinforcement, osmoprotection, and stress signaling over energy-intensive primary processes. A comprehensive overview of DEGs involved in critical pathways is shown in [Figure 10](#).

Figure 10 | Diagrammatic representation of DEG analysis in the drought-exposed Norway spruce seedlings of needle, phloem, and root compared to their respective controls. The red (↑) and green (↓) arrows represent the upregulated and downregulated genes, respectively (Corrected p-value<0.05). Under drought, the downregulated transcripts are primarily associated with pectin and secondary metabolite metabolism, which may contribute to reduced cell wall plasticity, impaired xylem function, weakened defense capacity, and higher susceptibility to hydraulic failure and biotic stress. In contrast, upregulated transcripts are primarily involved in carbohydrate, fatty acid, glutathione, and phytohormone metabolism, suggesting that conifers enhance drought tolerance by protecting cellular structures, conserving water, and activating stress defense mechanisms. **Sucrose, trehalose, cellulose & hemicellulose metabolism:** SuSy: Sucrose synthase, FFase: Beta-fructofuranosidase, GSGT2: Galactinol-sucrose galactosyltransferase 2, TP55: Alpha, alpha-trehalose-phosphate synthase [UDP-forming] 5, TPP4: Trehalose-phosphate phosphatase 4, CESA: Cellulose synthase A catalytic subunit, CSI: Cellulose synthase interactive 1 & 3, XTHP: Xyloglucan endotransglucosylase/hydrolase protein 8, 9, 32, XI: Xylose isomerase, BXL4: Beta-D-xylosidase 4, TK: Transketolase. **Pectin metabolism:** PE: Pectinesterase, PG: Polygalacturonase, PL: Pectate lyase, BGAL: Beta-galactosidase 1, BABF: Beta-L-arabinofuranosidase. **Riboflavin & Thiamine metabolism:** AP1: Acid phosphatase 1, TrxR2: Thioredoxin reductase 2, TPK: Thiamine pyrophosphokinase 2, TPP: Thiamine phosphate phosphatase-like protein. **Glycolysis and Mannitol metabolism:** HK: Hexokinase, PFK: 6-phosphofructokinase, G3PDH: Glycerol-3-phosphate dehydrogenase, PGK: Phosphoglycerate kinase, PGM: Phosphoglycerate mutase-like protein 2, MDH: Mannitol dehydrogenase. **Pyruvate metabolism & TCA Cycle:** AD: Aldehyde dehydrogenase, ADH: Alcohol dehydrogenase, PEPC: Phosphoenolpyruvate carboxylase, CS: Citrate synthase, ICL: Isocitrate lyase. **Inositol-P & Jasmonic acid metabolism:** ISYNA1: Inositol-3-phosphate synthase 1, PI3P5K: 1-phosphatidylinositol-3-phosphate 5-kinase, PLC: Phospholipase C, IPP5P: Inositol polyphosphate 5-phosphatase 13, VIP2: Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase VIP2, PLA2: Phospholipase A2, 9S-LOX: Linoleate 9S-lipoxygenase 1, AD: Aldehyde dehydrogenase, OPR3: 12-oxophytodienoate reductase 3, CYP94B1: Cytochrome P450 94B1. **Amino acid metabolism:** SCP: Serine carboxypeptidase, SAP: Serine aminopeptidase, RD21B: Cysteine protease RD21B, RD19D: Cysteine protease RD19D, CRK: Cysteine-rich receptor-like protein kinase, CRTM: Cysteine-rich TM module stress tolerance. **Methionine, auxin, ethylene & polyamine metabolism:** MGL: Methionine gamma-lyase, SAMDC: S-adenosylmethionine decarboxylase proenzyme, PMT12: Methyltransferase PMT12, PMT26: Methyltransferase PMT26, IDM1: Increased DNA methylation 1, ACS7: 1-aminocyclopropane-1-carboxylate synthase 7, ACO: 1-aminocyclopropane-1-carboxylate oxidase, ERF3: Ethylene-responsive transcription factor 3, ERF110: Ethylene-responsive transcription factor ERF110, TINY: Ethylene-responsive transcription factor TINY, IAMT1: Indole-3-acetate O-methyltransferase 1, PIN1C: Auxin efflux carrier component 1c, LAX1: Auxin transporter-like protein 1, IAA7: Auxin-responsive protein IAA7, IAA8: Auxin-responsive protein IAA8, SAUR10: Protein SMALL AUXIN UP-REGULATED RNA 10, SAUR76: Auxin-responsive protein SAUR76, SAUR32: Auxin-responsive protein SAUR32, SAUR50: Auxin-responsive protein SAUR50, SAUR71: Auxin-responsive protein SAUR71, ODC: Ornithine decarboxylase, PAO1: Polyamine oxidase 1, PAO2: Polyamine oxidase 2, ADC: Arginine decarboxylase. **Glutathione & proline metabolism:** GDH: NAD-specific glutamate dehydrogenase, HSP70: Heat shock cognate 70 kDa protein, GH: Glutathione hydrolase, GST: Glutathione S-transferase, GLR: Glutamate receptor 2.8, PHGPx: Phospholipid hydroperoxide glutathione peroxidase, P5CS: Delta-1-pyrroline-5-carboxylate synthase, PERK1: Proline-rich receptor-like protein kinase PERK1, PERK2: Proline-rich receptor-like protein kinase PERK2, PAT1: Proline transporter 1, PAT2: Probable proline transporter 2. **Shikimate metabolism:** DHQD: 3-dehydroquinate dehydratase, SKD: Shikimate dehydrogenase, CS: Aminodeoxychorismate synthase. **Fatty acid metabolism:** HMGCR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1, G8H: Geraniol 8-hydroxylase, CYP76: Cytochrome P450 76T24, 7DLGT: 7-deoxyloganetin glucosyltransferase, SDR: Short-chain dehydrogenase reductase 2a, KCS: 3-ketoacyl-CoA synthase 1, OAR: 3-oxoacyl-[acyl-carrier-protein] reductase, KAT: 3-ketoacyl-CoA thiolase 2, DHNAT: 1,4-dihydroxy-2-naphthoyl-CoA thioesterase 1, BHSD: 11-beta-hydroxysteroid dehydrogenase, FAH1: Dihydroceramide fatty acyl 2-hydroxylase. **Phenylalanine & Tyrosine metabolism:** PAL: Phenylalanine ammonia-lyase, MNGT: (R)-mandelonitrile beta-glucosyltransferase, AGD: Arogenate dehydrogenase 2, T3M: Tyrosine 3-monooxygenase, TAT2: aminotransferase TAT2, HPPD: 4-hydroxyphenylpyruvate dioxygenase, HPPR: Hydroxyphenylpyruvate reductase, CSE: Caffeoylshikimate esterase, CCAMT: Caffeoyl-CoA O-methyltransferase, CCR1: Cinnamoyl-CoA reductase 1, CAD6: cinnamyl alcohol dehydrogenase 6, IRL1: Phenylcoumaran benzylic ether reductase IRL1, PT1: Phenylcoumaran benzylic ether reductase PT1, SGT: Scopoletin glucosyltransferase. **Flavonoid metabolism:** PSS1: Pinosylvin synthase 1, PSS2: Pinosylvin synthase 2, FMO1: Flavin-containing monooxygenase 1, F3H: Flavonoid 3'-monooxygenase, F3'5'H: Flavonoid 3',5'-hydroxylase, LAR: Leucoanthocyanidin reductase, ANR: Anthocyanidin reductase, UF3GT3: UDP-glucose flavonoid 3-O-glucosyltransferase 3, AN3GRT: Anthocyanidin-3-O-glucoside rhamnosyltransferase. **ABA, Cytokinin & secondary metabolites metabolism:** BCH2: Beta-carotene hydroxylase 2, NCED3: 9-cis-epoxycarotenoid dioxygenase NCED3, SDR: Short-chain dehydrogenase reductase 2a, UGT94E5: Beta-D-glucosyl crocetin beta-1,6-glucosyltransferase, PYL4: Abscisic acid receptor PYL4, ASR1: Abscisic stress-ripening protein 1, AFP3: Ninja-family protein AFP3, ALDH31: Aldehyde dehydrogenase family 3 member I1, UGT84: Gallate 1-beta-glucosyltransferase 84A23, ZOGT: Zeatin O-glucosyltransferase, CKX3: Cytokinin dehydrogenase 3, APS: (-)-alpha-pinene synthase, ATS: (-)-alpha-terpineol synthase, PS: Pinene synthase. **Abbreviations:** OGs: Oligogalacturonides; UOGs: Unsaturated oligogalacturonides; FAD: Flavin adenine dinucleotide; FMN: Flavin mononucleotide; 1D-myo-I-3P: 1D-myo-Inositol 3-phosphate; 1-P-1D-myo-I-3,5P2: 1-phosphate-1D-myo-inositol-3,5-bisphosphate; D-myo-I-4,5P2: D-myo-inositol 4,5-bisphosphate; D-myo-4P: D-myo-inositol-4-phosphate; D-myo-6P: D-myo-inositol 6-phosphate; 13-HPOT: 13-hydroperoxy-octadecatrienoic acid; OPDA: 12-oxo-phytyldienoic

acid; OPC-8:0: 3-oxo-2-(2'-pentenyl)-cyclopentane-1-octanoic acid; JA: Jasmonic acid; MeJA: Methyl Jasmonate; JA-Ile: Jasmonoyl-Isoleucine; RDGs: Reactive defense genes; G3P: Glyceraldehyde-3-phosphate; 1,3BPG: 1,3-Bisphosphoglycerate; 3PG: 3-Phosphoglycerate; 2PG: 2-Phosphoglycerate; PEP: Phosphoenolpyruvate; OAA: Oxaloacetate; α -KG: α -Ketoglutarate; Suc-CoA: Succinyl-CoA; ACD: Acetaldehyde; GM: Glutamate; GT: Glutathione; GSC: Glutathione-S-Conjugate; GDS: Glutathione Disulfide; ROSD: ROS detoxification; α -KB: α -ketobutyrate; SAM: S-adenosylmethionine; dcSAM: Decarboxylated S-adenosylmethionine; SAH: S-adenosylhomocysteine; IAA: Indole-3-acetic acid; MeIAA: Methyl-Indole-3-Acetic Acid; ACC: 1-aminocyclopropane-1-carboxylic acid; DAHP: 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHQ: 3-dehydroquinate; DHS: 3-dehydroshikimate; EPSP: 5-enolpyruvylshikimate-3-phosphate; L-ARG: L-Arogenate; TYS: Tyrosine; DOPA: L-3,4-dihydroxyphenylalanine; HPP: 4-Hydroxyphenylpyruvate; HMG: Homogentisate; HPLA: 4-Hydroxyphenyllactate; PA: Phenylalanine; CNM: Cinnamate; SPL: Scopoletin; MDL: Mandelonitrile; Cou-CoA: Coumaroyl-CoA; Caf-SM: Caffeoyl-shikimate; CFA: Caffeic acid; CF-CoA: Caffeoyl-CoA; FRL-CoA: Feruloyl-CoA; CFALD: Coniferaldehyde; CFAL: Coniferyl alcohol; 3KACA: 3-ketoacyl-CoA; 3-HACA: 3-hydroxyacyl-CoA; HMG-CoA: 3-hydroxy-3-methylglutaryl-Coenzyme A; IPP: Isopentenyl diphosphate; GPP: Geranyl diphosphate; GRL: Geraniol; 8HGO: 8-Hydroxygeraniol; NPPL: Nepetalactol; 7DLGA: 7-deoxyloganic acid; 7DLA: 7-Deoxyloganic acid; SL: Secologanin; NRGN: Naringenin; DHK: Dihydrokaempferol; DHQ: Dihydroquercetin; LCAR: Leucoanthocyanidins; ECHN: Epicatechins; ACGS: Anthocyanidin-3-O-glucoside; ACRS: Anthocyanidin-3-O-rutinoside; IPA: Isopentenyladenine; TZ: Trans-zeatin; ZOG: Zeatin-O-glucoside; BDGC: Beta-D-glucosyl crocetin; 9-cis-NX: 9-cis-Neoxanthin; ABA: Abscisic acid; ABA-S: ABA suppression; ABA-I: ABA induction.

5. Conclusion

Norway spruce, a drought-sensitive boreal conifer, faces increasing threats from climate change-induced water stress, yet its molecular adaptation mechanisms remain unclear. This study exposed three-year-old Norway spruce seedlings to six weeks of drought under controlled conditions and employed NGS-based transcriptome and physiological analyses of needles, phloem, and roots to uncover tissue-specific drought responses. Multi-tissue analysis revealed both systemic and tissue-specific transcriptional reprogramming, with stress and defence signaling, nutrient and ion transport, and secondary metabolism playing central roles. Pathway enrichment highlighted conserved regulation of starch and sucrose metabolism and MAPK signaling across tissues, alongside tissue-specific enrichment of carbon and glutathione metabolism in needles, flavonoid biosynthesis in phloem, and galactose and pentose-glucuronate interconversions in roots. Functional analyses revealed both shared and tissue-specific pathways, including the conserved upregulation of ABA signaling, ROS detoxification, and cell wall remodeling, alongside tissue-specific enrichments in photosynthetic regulation in needles, secondary metabolism in phloem, and carbohydrate metabolism in roots. Identifying core stress pathways, tissue-specific adaptations, and key regulatory networks offers a foundation for future research and applied strategies in forest conservation and climate-

smart management. Overall, this study enhances the mechanistic understanding of drought adaptation in Norway spruce and identifies candidate genes and pathways for functional validation. By integrating these findings with complementary omics approaches and field validations, we can further refine predictive modeling of boreal forest dieback under RCP8.5 scenarios, with potential applications in forestry and ecological restoration.

Declarations

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Conflict of interest

None declared.

Author contributions VVS: conceptualization, methodology, data curation, formal analysis, resources, project administration, Funding acquisition, writing – original draft, writing – review and editing. AN: methodology, data curation, formal analysis, writing – review and editing. KM: methodology, writing – review and editing, supervision. GJ: data curation, writing – review and

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editing. MB: funding acquisition, writing – review and editing, supervision. RJ: funding acquisition, writing – review and editing, supervision. RG: methodology, writing – review and editing, supervision. PK: methodology, writing – review and editing, supervision. All the authors have read the article and approved the submitted version.

Data availability statement

All the data used and analyzed are included in this article and in the Supplementary files. The raw RNASeq data will be made available upon acceptance of the article.

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Supplementary Materials

Supplementary Figure 1. Experimental setup in the greenhouse. The left panel shows Norway spruce seedlings at the beginning of the acclimation phase. The right panel shows seedlings after six weeks of drought treatment, with a drought-stressed seedling on the left and a well-watered control seedling on the right.

Supplementary Figure 2. MapMan pathway visualization for DEGs in the needle under drought. (A) Metabolism overview: highlights changes in primary and secondary metabolic pathways. (B) Photosynthesis: shows widespread downregulation of photosynthetic components (blue squares). (C) Abiotic stress and redox homeostasis: illustrates changes in stress-related pathways (red = upregulated genes, blue = downregulated genes). Each square represents a DEG mapped to a functional Bin. Panel C was customized by merging relevant MapMan pathways to specifically depict drought stress responses (modified from biotic stress and cellular response pathways).

Supplementary Figure 3. MapMan pathway visualization for DEGs in phloem under drought. (A) Metabolism overview: highlights changes in primary and secondary metabolic pathways. (B) Photosynthesis: shows widespread downregulation of photosynthetic components (blue squares). (C) Abiotic stress and redox homeostasis: illustrates changes in stress-related pathways (red = upregulated genes, blue = downregulated genes). Each square represents a DEG mapped to a functional Bin. Panel C was customized by merging relevant MapMan pathways to specifically depict drought stress responses (modified from biotic stress and cellular response pathways).

Supplementary Figure 4. MapMan pathway visualization for DEGs in the root under drought. (A) Metabolism overview: highlights changes in primary and secondary metabolic pathways. (B) Photosynthesis: shows widespread downregulation of photosynthetic components (blue squares). (C) Abiotic stress and redox homeostasis: illustrates changes in stress-related pathways (red = upregulated genes, blue = downregulated genes). Each square represents a DEG mapped to a functional Bin. Panel C was customized by merging relevant MapMan pathways to specifically depict drought stress responses (modified from biotic stress and cellular response pathways).

Supplementary Material 1. List of RT-qPCR primers.

Supplementary Material 2.

Table S2.1. Details of gene expression analysis for needle treatment vs control. Information in the table: gene ID, log₂ fold change, p-adjusted value, and gene description.

Table S2.2. Details of gene expression analysis for phloem treatment vs control. Information in the table: gene ID, log₂ fold change, p-adjusted value, and gene description.

Table S2.3. Details of gene expression analysis for root treatment vs control. Information in the table: gene ID, log₂ fold change, p-adjusted value, and gene description.

Table S2.4. Details of gene expression analysis across all treatment vs control tissues. Information in the table: gene ID, log₂ fold change for needle (ND), phloem (PH), and root (RT), and gene description.

Supplementary Material 3.

Table S3. Details of cluster analysis (A-K): Information contains cluster name, gene ID, gene expression (Log₂ fold change) across each tissue (ND=needle, PH=phloem, RT=root) compared to their respective control. The next rows contain their GO and KEGG annotations, description, and their function.

Supplementary Material 4.

Table S4.1. Summary of the differentially expressed transcription factors in needle tissue. Information in the table: the gene ID and encoding transcription factor; Log₂ fold change and associated gene ontology term.

Table S4.2. Summary of the differentially expressed transcription factors in phloem tissue. Information in the table: the gene ID and encoding transcription factor; Log 2 fold change and associated gene ontology term.

Table S4.3. Summary of the differentially expressed transcription factors in root tissue. Information in the table: the gene ID and encoding transcription factor; Log 2 fold change and associated gene ontology term.

Table S4.4. Summary of the differentially expressed transcription factors across all tissues. Information in the table: the gene ID and encoding transcription factor; Log 2 fold change og all tissues (ND=needle, PH=phloem, RT=root)and associated gene ontology term.

Supplementary Material 5.

Table S5.1. Summary of the main Bin categories in needle, phloem, root, and common Bin categories across all tissues. Information in the table: the main bin code and bin name; the number of genes per bin name.

Table S5.2. Summary of Bin categories in needle tissues. Information in the table: the main bin code, bin name, gene ID, and their expression fold change.

Table S5.3. Summary of Bin categories in phloem tissues. Information in the table: the main bin code, bin name, gene ID, and their expression fold change.

Table S5.4. Summary of Bin categories common across all three tissues. Information in the table: the main bin code, bin name, gene ID, and their expression fold change.

Table S5.5. Summary of Bin categories in root tissues. Information in the table: the main bin code, bin name, gene ID, and their expression fold change.

6. Discussion

Forests across Europe and the world are increasingly affected by drought, heat, and extreme weather driven by climate change, resulting in widespread mortality of conifers and other tree species. Among them, Norway spruce (*P. abies*) has experienced particularly severe declines in vitality and survival due to its extensive planting outside the natural range and its sensitivity to prolonged drought. As one of the continent's most economically valuable species, it plays a central role in forestry and the wood industry. However, its vulnerability to water deficit, high temperature, and subsequent bark beetle infestations threatens both ecological stability and economic sustainability (Biedermann et al., 2019; Hlásny et al., 2021; Singh et al., 2024a). This thesis aims to integrate ecological and molecular perspectives to understand how drought interacts with spruce physiology, stand structure, and gene regulation, and how these factors together determine spruce resistance and resilience. The discussion here connects results from the four research components, ranging from large-scale outbreak dynamics and forest management to gene expression and physiological adaptation.

The first study (Chapter 5.1) focused on the relationship between climate change and the dynamics of *I. typographus* outbreaks. Rising temperatures have expanded the beetle's reproductive window, allowing the formation of multiple generations per year and accelerating population buildup (Kautz et al., 2024). Although many studies have previously addressed aspects of the Norway spruce-bark beetle relationship, covering topics like host defense mechanisms (Krokene, 2015; Huang et al., 2020), ecological dynamics (Biedermann et al., 2019; Hlásny et al., 2021), drought and fungal symbiosis (Netherer et al., 2021), and early detection methods (Marvasti-Zadeh et al., 2023; Trubin et al., 2023, 2024; Kautz et al., 2024), this study integrates these elements into a unified framework. Drought exacerbates this process by reducing resin production and impairing the defensive capacity of spruce, thereby promoting beetle colonization. Under sustained water deficit, the decline in non-structural carbohydrate (NSC) reserves limits the tree's ability to sustain induced defenses, such as resin synthesis and phenolic compound production (Herms and Mattson, 1992; Netherer et al., 2021). High temperatures also enhance beetle dispersal, shorten development time, and increase overwintering survival, resulting in synchronous mass attacks that overcome even healthy trees. The combined effects of temperature, drought, and storm disturbances create conditions under which

spruce stands lose resilience and large-scale mortality follows. This study also addressed how forest structure and elevation mediate outbreak intensity. Stands at lower elevations, where drought and heat stress are more pronounced, often experience more severe infestations, indicating that both climatic and structural conditions determine beetle success. Beyond assessing these drivers, the work evaluated current management strategies and their limitations, arguing for innovative approaches including molecular biotechnologies such as RNA interference (RNAi), CRISPR-based genome editing, and sterile insect technique (SIT). These tools have the potential to enhance forest protection by providing targeted, environmentally safer control options that complement traditional logging and sanitation practices.

The second study (Chapter 5.2) built upon these ecological insights by examining the link between stand density, tree transpiration, and beetle susceptibility. Dense, even-aged spruce monocultures are especially vulnerable because competition for light, water, and nutrients limits individual tree vigor. Trees growing in canopy gaps or along forest edges generally show higher sap flow rates due to greater solar exposure (Marešová et al., 2020; Özçelik et al., 2022). Increased transpiration correlates with stronger constitutive and inducible defenses, since water flux supports resin flow, nutrient transport, and photosynthetic supply for secondary metabolism. Conversely, reduced foliage and photosynthetic capacity weaken the ability to sustain costly defensive responses against beetle attack (Herms and Mattson, 1992). Drought amplifies these vulnerabilities by reducing sap flow and the cooling capacity of transpiration, resulting in higher bark surface temperatures. These warmer microclimates accelerate beetle development and facilitate colonization (Wermelinger and Seifert, 1999; Kautz et al., 2013). Field studies have shown that mortality probability increases with the proportion of sun-exposed stem area and decreases with crown size, confirming that edge trees face higher infestation risk (Jakuš et al., 2011; Korolyova et al., 2022a). The negative association between crowding and sap flow observed here is consistent with evidence that competitive stress lowers survival during beetle outbreaks (Buonanduci et al., 2020). In addition to limiting water availability, crowding reduces light penetration and soil temperature, further restricting photosynthesis and root activity. The resulting energy limitation forces carbon allocation toward maintaining primary metabolism at the expense of secondary compound synthesis, which diminishes defense (Jones and Hartley, 1999). These results reinforce the importance of stand management that accounts for local topography and microclimate. Thinning should

be tailored to site-specific conditions to reduce resource competition and improve individual tree vigor. Mixed-species stands, with greater structural heterogeneity, buffer microclimate, reduce pest spread, and exhibit greater overall resilience to drought, windthrow, and infestation (Liu et al., 2018; Ammer, 2019; Zhang et al., 2022; Zabihi et al., 2023). The evidence supports moving away from large monocultures toward diverse, uneven-aged forest compositions.

The third study (Chapter 5.3) addressed gene expression normalization, a critical step for accurate gene expression analysis. Despite widespread use of RT-qPCR in plant stress studies, reliable reference genes for Norway spruce had not been systematically validated. We provide a catalog of genes that have been reported in various conifer species and validated for Norway spruce for the first time. We identified two novel reference genes that have not been reported elsewhere. We evaluated fifteen candidate reference genes in various tissues and at different developmental stages under various environmental conditions using RT-qPCR and systematically assessed their expression stability to identify the most suitable reference gene for each condition. RT-qPCR is a widely used and reliable method for quantifying relative transcript abundance, routinely applied to dissect plant–pathogen interactions and associated molecular processes (Vijayakumar and Sakuntala, 2024). The approach is comparatively rapid and cost-effective for analyzing target genes under defined conditions. However, inconsistent adherence to best-practice guidelines can compromise data quality and interpretation (Bustin et al., 2009). Accurate normalization is therefore essential. Reference or housekeeping genes are typically selected because they perform fundamental cellular functions and exhibit relatively constant expression, although stability may vary among tissues and developmental stages (Lin and Lai, 2010; Li et al., 2021). Best practice supports the use of multiple, stably expressed reference genes tailored to the specific biological context (Dai et al., 2018; Wang et al., 2021). Experimental steps, such as RNA extraction, cDNA synthesis, primer design, and sample handling, can each influence the outcome, which further underscores the need for validated reference genes in every study system (Schmittgen et al., 2000; Bustin et al., 2005; Huggett et al., 2005). Although several conifer studies have evaluated reference genes (Bao et al., 2016; Mo et al., 2019; Chen et al., 2019), work in Norway spruce has largely focused on embryogenic material (Vestman et al., 2011; de Vega-Bartol et al., 2013).

From a panel of 15 candidates, *SPI*, *COG7*, and *TULP6* emerged as the most stable reference genes in Norway spruce. *SPI* encodes a ubiquitin–protein ligase that regulates

chloroplast protein import by remodeling the TOC complex through ubiquitination and proteasomal turnover, thereby supporting normal plant development (Ling and Jarvis, 2015). Ubiquitin pathway genes have previously served as reliable normalizers in diverse species and conditions, including *Hevea brasiliensis*, *Medicago sativa*, *Arabidopsis thaliana*, *Brachypodium distachyon*, *Vernicia fordii*, *Capsicum annuum*, and *Brachiaria brizantha* (Czechowski et al., 2005; Hong et al., 2008; Silveira et al., 2009; Li et al., 2011; Wan et al., 2011; Han et al., 2012; Castonguay et al., 2015). Our results align with these observations, as *SPI* exhibited stable performance across various tissues and treatments. Nevertheless, not every ubiquitin conjugation gene is suitable for normalization in all contexts, which necessitates empirical validation in the system of interest (Wan et al., 2011). The second gene, *COG7*, a component of lobe B of the conserved oligomeric Golgi complex, is required for maintaining Golgi morphology and function and has roles in embryo development, pigmentation, organ and cell expansion, and shoot apical meristem organization (Ungar et al., 2002; Ishikawa et al., 2008; Blackburn et al., 2019; Vukašinović et al., 2017; Rui et al., 2020, 2021; Klink et al., 2022). Although the expression stability of *COG7* can vary among tissues, RefFinder ranked it second overall after *SPI* in our study, indicating its reliability for normalization, particularly in experiments that explore resistance mechanisms to drought and pathogens. To our knowledge, *COG7* has not previously been proposed as a reference gene. The third most stable gene, *TULP6*, belongs to the tubby-like protein family, which is conserved across eukaryotes and implicated in stress signaling in plants (Lai et al., 2004; Reitz et al., 2013). Plant *TULPs* commonly contain an N-terminal F-box and a C-terminal tubby domain, and several family members have been catalogued in *Arabidopsis*, rice, and poplar (Yang et al., 2008; Hong et al., 2015). Although the precise mechanisms of action remain unknown, *TULPs* have been suggested to act as transcriptional regulators (Yulong et al., 2016). In our analysis, *TULP6* ranked third in stability after *SPI* and *COG7*, which supports its application as a reference gene, especially for studies spanning developmental stages. *TULPs* have not previously been considered for normalization, which highlights their potential value in conifers.

To demonstrate the importance of appropriate gene expression normalization, we examined expression of *PaDhn5*, a dehydrin-like protein that accumulates during abiotic stress and contributes to cryoprotection and membrane stabilization in the presence of reactive oxygen species (Close, 1997; Nylander et al., 2001; Rorat et al., 2006; Sun et al., 2021). Normalization with the least stable candidate, *SDH5*, resulted in a 20-fold apparent

induction of *PaDhn5* under stress. In contrast, normalization with the most stable genes yielded more conservative and internally consistent estimates, approximately 8-fold with *SPI* and 6-fold with *COG7*. The divergence between unstable and stable normalizers illustrates how inappropriate reference selection can bias RT-qPCR outcomes. On this basis, we recommend using *SPI*, *COG7*, and *TULP6*, either individually where appropriate or in validated combinations, to normalize gene expression in Norway spruce. The adoption of these references is expected to enhance sensitivity, reproducibility, and interpretability in RT-qPCR analyses conducted across various tissues, developmental stages, and stress treatments in this species.

The final study (Chapter 5.4) advances understanding of drought responses by integrating physiological observations with transcriptomic profiling of needles, phloem, and roots. Abscisic acid (ABA) serves as the central regulator of drought responses in conifers by integrating stomatal control with extensive transcriptional reprogramming. In Norway spruce, elevated ABA levels during drought induce rapid stomatal closure, which reflects an isohydric water-use strategy that maintains tissue water potential and prevents desiccation (Brodribb et al., 2014; Brunner et al., 2015). The strong activation of *NCEDs*, *PYL* receptors, and *PP2Cs* confirms enhanced ABA biosynthesis and perception in response to water stress (Verslues and Zhu, 2007; Cutler et al., 2010). Through phosphorylation by SnRK2 kinases, bZIP TFs of the AREB/ABF family bind ABRE motifs to activate downstream genes involved in osmotic regulation, cell protection, and water balance (Haas et al., 2021). In parallel, AP2/ERF TFs are induced independently of ABA through DRE/CRT elements, demonstrating that both ABA-dependent and ABA-independent modules operate in concert to strengthen drought tolerance (Soma et al., 2021; Ye et al., 2024; Ribeyre et al., 2025). These transcriptional networks coordinate the production of LEA proteins, dehydrins, ROS-scavenging enzymes, proline biosynthesis genes, and wax synthases, all of which stabilize membranes and preserve cell integrity under water deficit (Yoshida et al., 2015; Guo et al., 2024). The present study revealed strong ABA-mediated regulation across all tissues, with pronounced upregulation of bZIP transcription factors in the phloem, indicating its role as a conduit for systemic stress signaling. Although most bZIPs were downregulated in roots, both roots and needles showed high expression of ABA-responsive genes, confirming a coordinated systemic response. Several ABA metabolism and stress-related genes, including abscisic stress-ripening protein 1, ABA/WDS-induced protein, aldehyde dehydrogenase 3I1, and *AFP3*,

were consistently upregulated, while *PYL4* was repressed. Enhanced expression of *PP2Cs*, *NCED3*, and *SnRKγ1-like* proteins, alongside bZIP53 and bZIP43, further supports the activation of ABA signaling. The induction of protective proteins such as *LEAs*, dehydrins, and *NDR1/HIN1-like* proteins (*NHL6*, *NHL3*) emphasizes their contribution to cellular protection, while the downregulation of *NHL10* may reflect feedback control during prolonged stress.

Metabolic adjustments were closely coupled with these signaling processes. Drought exposure led to substantial proline accumulation, confirming its multifunctional role in osmotic adjustment, antioxidant defense, and membrane stabilization (Szabados and Savouré, 2010; Hayat et al., 2012). The strong induction of *P5CS* suggests enhanced proline biosynthesis, whereas the repression of *PERK1/2* and *ProTI/2* indicates restricted long-distance transport and prioritization of local osmoprotection. Such regulation ensures that essential tissues retain osmolytes and maintain hydration. In addition to amino acid metabolism, carbohydrate metabolism underwent significant reorganization. Upregulation of β -fructofuranosidase, *TPS5*, *TPS6*, *TPP4*, and glycolytic enzymes (*HXK1/2*, *PGK*, *G3PDH*), alongside the repression of sucrose synthase 2/4, reflects carbon reallocation to sustain osmotic balance and energy production when photosynthesis is reduced (Adams et al., 2013; Galiano et al., 2017). Early in the drought period, transient root proliferation may enhance water uptake, but prolonged water limitation typically reduces root biomass and transport capacity, forming a hydraulic “memory” that affects future stomatal behavior and growth (Virlouvet and Fromm, 2015; Anderegg et al., 2015; Phillips et al., 2016; O’Brien et al., 2017). Several genes related to pectin metabolism, including pectinesterases, polygalacturonase, and pectate lyase, were downregulated across tissues, suggesting a decline in cell-wall flexibility and possible modification of xylem mechanics, which could limit water transport and leaf-area adjustment (Koepke et al., 2010; Mayr et al., 2014). Despite severe drought, chlorophyll and carotenoid pools remained largely stable, indicating effective photoprotection typical of evergreen conifers. Upregulation of *ELIPI*, Pheophorbide a oxygenase, and β -carotene hydroxylase 2 supports active carotenoid biosynthesis and photoprotective repair mechanisms that minimize photooxidative stress (Lee and Kim, 2024). *ELIPs* likely stabilize antenna complexes and bind free chlorophyll to prevent oxidative injury (Hutin et al., 2003; Liu et al., 2020). Concurrent repression of chlorophyll a/b-binding protein and thioredoxin F2 suggests a controlled downscaling of light-harvesting and electron transport to avoid the overproduction of reactive oxygen

species (ROS), allowing the plant to maintain pigment stability while reducing photochemical activity.

Transcriptome profiling confirmed that drought responses were strongly tissue-specific yet interconnected. Needles exhibited a characteristic suppression of photosynthetic and Calvin-cycle genes, accompanied by a strong induction of defense and structural genes, such as chitinases and pathogenesis-related proteins, indicating a combined photoprotective and defensive role (Klápště et al., 2020; Wu et al., 2022). Phloem and roots were enriched in phenylpropanoid and flavonoid biosynthesis, reflecting a focus on antioxidant production and structural reinforcement (Dixon and Paiva, 1995). A conserved set of approximately 2,400 drought-responsive genes was shared among all tissues and included genes involved in carbohydrate and amino acid metabolism, ROS detoxification, and the downregulation of energy-demanding processes (Tausz et al., 2004). Functional enrichment analysis revealed that needles primarily focused on modulating photosynthesis and photoprotection, whereas phloem and roots emphasized oxidative defense, cell-wall organization, and osmotic regulation (Li W et al., 2021). MAPK signaling and glutathione metabolism were enriched across all tissues, representing core pathways in drought adaptation (Zhu, 2016; Haas et al., 2021). Transcriptional regulation involved multiple transcription factor families, including AP2/ERF, MYB, NAC, WRKY, bZIP, and bHLH, which coordinate ABA signaling, secondary metabolism, and ROS detoxification (Fox et al., 2018; de María et al., 2020). NAC and MYB factors predominated in needles, consistent with their functions in stomatal regulation and flavonoid biosynthesis (Nakano et al., 2006; Yamaguchi et al., 2010), whereas MYB and AP2/ERF factors were abundant in phloem and roots, linking hormonal control with lignification and osmotic adjustment (Fraser and Chapple, 2011). WRKY and GATA factors were expressed across all tissues, representing a conserved regulatory core that integrates cross-tissue signaling (Rushton et al., 2010; Richter et al., 2010). Interestingly, several ARR-B cytokinin-response regulators remained active during drought, unlike in most angiosperms, where they are rapidly downregulated (Nguyen et al., 2016; Haas et al., 2021). Their sustained expression in spruce may represent a gymnosperm-specific strategy for maintaining cytokinin–ABA balance during long-term stress.

In conclusion, these results highlight that Norway spruce responds to drought through an integrated network of ABA-centered signaling, metabolic reorganization, and tissue-specific coordination. ABA serves as the primary signal linking environmental

perception to physiological adjustment, while proline, sugars, and carotenoids act as key metabolites that stabilize the cellular environment and sustain core functions under limited water availability. Distinct tissue roles—photosynthetic regulation in needles, vascular signaling and defense in phloem, and osmotic control in roots—demonstrate how spruce maintains functional balance during prolonged stress. The combined regulation of transcription factors, stress-protective proteins, and antioxidant systems ensures that energy resources are diverted from growth toward survival. Together, these responses provide a mechanistic basis for drought resilience in spruce, explaining how it can withstand extended water deficits while preserving the potential for recovery once favorable conditions return.

Taken together, the four studies reveal insights into Norway spruce physiology under drought and its implications for resistance to pest infestations across various scales. At the stand level, water limitations and heat reduce tree vigor, increasing the risk of bark beetles in monocultures. At the physiological and molecular levels, trees shift metabolism from productivity toward survival through hormonal control, osmotic buffering, and photoprotection. Tissue-specific transcriptomic adjustments contribute to a unified whole-plant response, while long-term structural and physiological memory influence resilience to future stress. The integration of these findings provides a mechanistic explanation for the recent decline of Norway spruce under climate stress, offering direction for mitigation through silvicultural adaptation and genetic improvement. Managing stand density and species composition can reduce physiological stress and pest vulnerability. Furthermore, identification of novel housekeeping genes would greatly enhance gene expression normalization in Norway spruce. Identifying and validating molecular targets, such as ARR-B, FRK4, and genes involved in the trehalose/raffinose pathway, can support breeding and biotechnological efforts to enhance drought tolerance in conifers.

7. Conclusion, Limitations, and Recommendations

7.1 Conclusion

This thesis provides an integrated understanding of Norway spruce's responses to biotic and abiotic stressors, with particular emphasis on bark beetle interactions, forest stand dynamics, and the molecular mechanisms underlying drought adaptation. Collectively, the

four studies demonstrate that the resilience of spruce forests is governed by the interplay of ecological processes, forest structure, and stress-responsive molecular pathways.

Chapter 5.1 describes the ecology of bark beetles, the effects of different climatic extremes on their populations, and critically discusses current management strategies while suggesting novel molecular toolbox-based approaches that could help contain outbreaks. It emphasized the importance of innovative, molecular-based methods as a complement to silvicultural practices to mitigate future risks. Building upon the stand structure effects on tree transpiration discussed in Chapter 5.1, Chapter 5.2 highlighted how stand fragmentation and solar radiation loads influence tree transpiration. By showing that tree density and canopy openness substantially regulate sap flow, this work identified structural forest attributes that can predispose stands to drought stress and subsequent bark beetle infestation. Furthermore, Chapter 5.3 addressed a critical methodological limitation in the functional genomics of Norway spruce by identifying stable reference genes for RT-qPCR. This foundational study validated novel candidate reference genes across various tissues, developmental stages, and stress conditions, providing a robust foundation for future molecular investigations in Norway spruce and other conifer species. Building upon this methodological framework, which was used further in the fourth study (Chapter 5.4). In Chapter 5.4, we generated the first multi-tissue transcriptome atlas of Norway spruce under severe drought stress. This analysis revealed coordinated yet distinct transcriptional reprogramming across needles, phloem, and roots, demonstrating systemic regulation of drought responses while also identifying tissue-specific adaptive pathways. Taken together, these studies provide a comprehensive framework that links ecological dynamics, stand-level processes, and molecular mechanisms in Norway spruce under drought conditions. By integrating knowledge across scales, from landscape to gene expression, the thesis advances our understanding of how spruce forests respond to compounded stressors. The findings underscore the need to combine adaptive forest management with molecular and genomic tools to enhance resilience in the face of ongoing climate change. Furthermore, the resources generated, including validated reference genes and the drought transcriptome atlas, offer valuable platforms for future functional genomics and breeding initiatives aimed at developing stress-resilient conifer populations.

7.2 Study Limitations

While the present thesis provides a multi-scale analysis of Norway spruce responses to abiotic stressors, several limitations should be acknowledged. First, the ecological studies were restricted to specific regions of Central Europe and therefore may not fully capture the heterogeneity of spruce ecosystems across their natural distribution range. Variability in elevation, soil type, and climatic gradients could influence stand structure and drought sensitivity in ways that extend beyond the conditions examined. This geographical limitation constrains the generalizability of the ecological findings, particularly for northern and high-altitude populations of spruce. Second, the sap flow analysis focused on a limited number of trees and short observation periods during peak summer conditions. Although the results revealed clear relationships between tree density, radiation load, and transpiration, longer-term measurements across multiple growing seasons would be necessary to account for inter-annual climatic variability and to strengthen the predictive value of the conclusions. Third, the molecular investigations were performed on seedlings under controlled greenhouse conditions. While this experimental system ensured reproducibility and allowed for high-resolution transcriptomic analyses, it does not fully replicate the complexity of natural forest environments. Mature trees growing under variable field conditions may display different physiological thresholds, defense capacities, and transcriptional plasticity compared with seedlings. Thus, extrapolation of the findings to older life stages must be approached with caution. Fourth, although the reference gene validation (chapter 5.3) improved the reliability of RT-qPCR analyses in Norway spruce, the set of genes tested remains finite. Additional candidate reference genes could further enhance normalization accuracy, particularly under combined stress conditions or in tissues not assessed here. Similarly, the transcriptome study identified candidate genes and pathways, but functional validation of these genes through knockdown or overexpression experiments remains to be carried out. Finally, while the review component of this thesis (chapter 5.1) highlighted the potential of molecular tools, such as RNA interference and CRISPR, for bark beetle management, these approaches are still at the conceptual or laboratory stage. Their efficacy, feasibility, and biosafety in operational forestry remain unresolved, and practical translation will require extensive testing under field conditions.

7.3 Practical applications

The findings presented in this thesis have several practical implications for the management and conservation of Norway spruce forests under changing climatic conditions. At the stand level, results demonstrate that tree density and canopy structure have a strong influence on sap flow and vulnerability to drought. These insights can directly inform silvicultural practices by emphasizing the importance of density regulation and careful thinning to maintain adequate resource availability and reduce physiological stress. Management strategies should therefore prioritize stand structures that minimize competition while avoiding excessive fragmentation, which can amplify solar radiation loads and increase susceptibility to pest attacks. The identification of stable reference genes for quantitative PCR provides a methodological resource that will enhance the reliability of molecular studies in Norway spruce and can be applied to other conifers. This tool enables the accurate monitoring of stress-responsive gene expression, providing a basis for the functional validation of candidate genes associated with resilience traits. Such molecular markers can be integrated into tree improvement programs, facilitating the selection of drought- and pest-tolerant genotypes. The transcriptome atlas of drought-stressed spruce tissues represents a foundational dataset for studying traits/genes for breeding and conservation. By identifying candidate transcription factors and stress-responsive pathways, the atlas provides genetic targets for the development of molecular markers, transgenic approaches, or genome-editing strategies aimed at enhancing drought tolerance. Furthermore, this information can be applied in early screening of seedlings to improve the efficiency of breeding programs, thereby accelerating the deployment of climate-resilient spruce populations in vulnerable regions.

At the broader scale, the integration of ecological and molecular perspectives offers a framework for adaptive forest management. By linking stand-level processes with gene regulation, this research supports predictive modeling of forest responses to combined biotic and abiotic stressors. Such models can guide policymakers and practitioners in designing interventions that sustain both ecological integrity and economic productivity of spruce-dominated forests.

7.4 Recommendations for Future Research

Several promising directions for future research arise from this work. To begin with, ecological studies should be expanded across different regions of the Norway spruce distribution to capture how local climate and soil conditions shape stand dynamics and susceptibility to bark beetle outbreaks. Establishing long-term monitoring networks that combine sap flow measurements, microclimate data, and remote sensing would provide the kind of datasets needed to scale these insights up to the landscape level.

Future experiments should also move beyond seedlings in controlled environments and include mature trees exposed to drought in field conditions. While greenhouse trials are valuable for isolating specific responses, field-based studies capture the complexity of natural systems, where trees simultaneously face pathogens, symbiotic interactions, and variable weather conditions. This would provide a more realistic view of how drought responses play out under actual forest conditions. Since transcriptomics provides only an overview of the gene expression landscape, and not all mRNAs are translated into proteins, another key step forward will be integrating transcriptomics with other omics approaches, including proteomics, metabolomics, and epigenomics. Such multi-layered analyses can uncover how gene expression translates into protein activity, metabolic shifts, and long-term regulatory changes. In particular, studying drought legacies and transgenerational memory will be critical, as these processes remain poorly understood in conifers but could strongly influence resilience over time. The functional testing of candidate genes identified in the transcriptome atlas is also essential. Approaches such as gene editing, transgenic validation, or association mapping in natural populations can help determine which genes truly contribute to stress tolerance. Comparative studies across related conifer species would further clarify whether these regulatory pathways are unique to Norway spruce or shared more broadly within conifers.

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