CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Forestry and Wood Sciences

Department of Forest Protection and Entomology

Global Changes Forestry

Climate change impact on physiological and chemical responses of mature Norway spruce (*Picea abies*) in relation to bark beetle (*Ips typographus*) attack susceptibility

Dissertation thesis

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CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Forestry and Wood Sciences

Ph.D. THESIS ASSIGNMENT

Ing. Barbora Stříbrská

Global Change Forestry

Thesis title

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Objectives of thesis

In central Europe, conifer forests experience variable significances related to climate change such as increased temperature conditions and drought periods. Nowadays, the most economically important consequence of these disturbances in central Europe are severe damages of Norway spruce (Picea abies) monocultures caused by bark beetles' outbreaks. Comprehensive understanding of bark beetle-tree interactions in a changing climate are still restricted by substantial knowledge gaps.

The aim of this thesis is studying the response of Norway spruce to drought episodes with regard to tree physiology as well as constitutive and induced defence reactions of mature trees against bark beetle attack. The particular goals will be comparison of tree physiological data, defence chemistry profile and susceptibility to bark beetle attack on adult Norway spruce trees, which will be subjected to stresses resulting from water regime modification and sudden sun exposition on freshly created forest edges.

Methodology

The field experiments will be carried out at the experimental plots established in the mature (60 – 100 years old) Norway spruce (Picea abies) forest localities in the Kostelec n CL, Czech Republic. In several plots will be created a freshly cut forest edge in the spring months to expose trees to high temperature and sun radiation. In the other plots will be modified water regime by building roofs eventually by artificial irrigation.

The physiological parameters of monitored trees as sap flow, alterations in stem diameter and surface temperature as well as plot meteorological conditions and soil water potential will be measured by specialized sensors. On the base of these data will be evaluated drought stress level of the targeted trees. Furthermore, these features will be compared with the composition and quantity of secondary defence chemical metabolites raised after induction by local elicitor or as effect of stress. The susceptibility of such treated trees for bark beetle attack will be studied with assessment of semi-choice bioassay with living beetles in attack boxes and non-choice bioassay with beetles in individual chambers.

The proposed extent of the thesis

70-100

Keywords

drought stress, forest edge, climate change, Ips typographus, Norway spruce, Picea abies

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Recommended information sources

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Certificate of Authorship

I hereby confirm that this Ph.D. thesis “Climate change impact on physiological and chemical responses of mature Norway spruce (*Picea abies*) in relation to bark beetle (*Ips typographus*) attack susceptibility“ was elaborated independently with the usage of quoted literature and based on consultations and the recommendations of my supervisor. I agree with publishing this Ph.D. thesis according to Czech law n. 111/1998 Sb. about the universities in its current valid wording. This agreement is independent from the results of defence.

December 31th 2023, Prague

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Abstract

Eurasian Spruce Bark Beetle (*Ips typographus*, L. 1758 (Coleoptera: Scolytinae)) outbreaks have a severe impact on Norway spruce forests in Central Europe. Forest damage is exacerbated by abiotic disturbances, logging, and forest fragmentation, increasing the vulnerability of spruce forest stands. This thesis explores how Norway spruce trees react to sudden exposure on a newly formed forest edge and on infestation by bark beetles, examining physiology, defence, and parameters related to bark beetle acceptance and attraction. The goal is to provide ecological insights into these processes and propose innovative methods for managing bark beetle infestations. The first study investigates the immediate effects of forest edge establishment on critical parameters, including air and bark temperatures, soil water potential, sap flow, resin flow, bark defence compounds, and olfactometric cues for bark beetles. Observations in newly cut forest edges during spring reveal elevated bark temperatures and volatile organic compound (VOC) concentrations. Despite similar monoterpene levels in the bark, VOC concentrations were highest in May and June, coinciding with increased *Ips typographus* mobility, indicating that microclimatic conditions and olfactory signals contribute to beetle swarming. The study demonstrates that fresh forest edges are not disproportionately susceptible shortly after establishment, emphasizing the role of microclimatic conditions, induced tree defence and olfactory cues in spruce bark beetle infestation patterns. Furthermore, two publications focus on the physiological and defence responses of Norway spruce to bark beetle infestation. Besides studying these alterations, these publications also propose novel methods for early detection of *Ips typographus* infestations. Monitoring infested and non-infested Norway spruce trees reveals post-attack reductions in sap flow, terminated tree stem increment, increased bark temperature, and a surge in monoterpene emissions. Promising early detection approaches encompass the surveillance of tree bark temperature during specific meteorological conditions, analysis of monoterpene emissions, and the observation of elevated captures in passive traps. The analytical chemistry part of the study focuses on detecting volatile organic compounds, especially *α*-pinene, emitted by infested trees. Lab trials were performed to optimize *α*-pinene detection by dynamic absorption and solid-phase microextraction (SPME), revealing variable abundance in forest trials. Three-dimensional cloud mapping of *α*-pinene distribution suggests the potential for large-scale bark beetle detection, paving the way for alternative pest management methods using scanning technologies.

Keywords: *α*-pinene, early detection, forest edge, *Ips typographus*, *Picea abies,* sun irradiation, tree susceptibility, VOCs

Abstrakt

Výskyt lýkožrouta smrkového (*Ips typographus*, L. 1758 (Coleoptera: Scolytinae)) má vážné dopady na smrkové porosty ve střední Evropě. Poškození lesa zhoršují abiotické disturbance, těžba dřeva a fragmentace lesa, což zvyšuje zranitelnost smrkových porostů. Tato studie zkoumá okamžité účinky vytváření porostových stěn na kritické parametry, zejména teplotu vzduchu a kůry, půdní vodní potenciál, průtok mízy, průtok pryskyřice a látek pro obranu a olfaktorické signály pro kůrovce. Pozorování na čerstvě vykácených porostových stěnách v průběhu jara odhalují zvýšené teploty kůry a zvýšené koncentrace organických sloučenin ve vzduchu (VOC). Navzdory podobné úrovni monoterpenů v kůře byly koncentrace organických sloučenin (VOC) nejvyšší v květnu a červnu, což se shodovalo se zvýšenou mobilitou kůrovce. To naznačuje, že mikroklimatické podmínky a čichové signály přispívají k rojení brouků. Studie dále ukazuje, že čerstvé porostové stěny nejsou krátce po vytvoření neúměrně náchylné k napadení kůrovcem, což zdůrazňuje roli mikroklimatických podmínek, indukované obrany stromů a olfaktometrických signálů během infestace kůrovcem smrkovým. Práci doplňují i ekologické poznatky zjištěné prostřednictvím inovativních metod detekce napadení kůrovcem. Monitorování napadených a nenapadených stromů smrku odhaluje snížení toku mízy krátce po napadení, zastavený přírůst kmene stromů, zvýšenou teplotu kůry a prudký nárůst emisí monoterpenů. Slibné přístupy k časné detekci zahrnují sledování teploty kůry stromů během specifických meteorologických podmínek, analýzu emisí monoterpenů a pozorování zvýšených odchytů v pasivních pastech. Analyticko-chemická část studie se zaměřuje na detekci těkavých organických látek, zejména *α*-pinenu, emitovaných napadenými stromy. Za účelem optimalizace detekce *α*-pinenu byly provedeny laboratorní pokusy pomocí dynamické absorpce a mikroextrakcí na tuhé fázi (SPME), které odhalily variabilní množství α-pinenu během terénních pokusů. Trojrozměrné mapování distribuce *α*-pinenu naznačuje potenciál pro detekci kůrovce ve velkém měřítku, otevírajíc cestu pro alternativní metody ochrany proti škůdcům pomocí skenovacích technologií.

Klíčová slova: *α*-pinene, včasná detekce, porostová stěna, *Ips typographus*, *Picea abies,* sluneční záření, náchylnost stromů, VOCs

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I. **Stříbrská, B**., Hradecký, J., Čepl, J., Tomášková, I., Jakuš, R., Modlinger, R., Netherer, S. & Jirošová, A. (2022). Forest margins provide favourable microclimatic niches to swarming bark beetles, but Norway spruce trees were not attacked by *Ips typographus* shortly after edge creation in a field experiment. Forest Ecology and Management, 506, 119950; doi:10.1016/j.foreco.2021.119950.

II. **Stříbrská, B**., Hradecký, J., Čepl, J., Modlinger, R., Tomášková, I., & Jirošová, A. Physiological and Biochemical Indicators in Norway Spruces Freshly Infested by *Ips typographus*: Potential for Early Detection Methods. Frontiers in Forests and Global Change, 6, 1197229; doi: 10.3389/ffgc.2023.1197229.

III. **Stříbrská B**, Moliterno AAC, Hüttnerová T, Leiner M, Surový P, Jirošová A. Pilot Study of 3D Spatial Distribution of *α*-Pinene Emitted by Norway Spruce (L.) Karst Recently Infested by *Ips typographus* (L. 1758) (Coleoptera: Scolytinae). Forests. 2024; 15(1):10; doi:10.3390/f15010010.

Listo of abbreviation

ABA – Abscisic acid

BB - Bark beetle

CO_2 - Carbon dioxide

FE – Forest edge

GC-MS – Gas Chromatography-Mass Spectrometry

IF - Inner Forest

IT - *Ips typographus*

MeJA – Methyl jasmonate

MTs – M*on*oterpene hydrocarbons

MT-Ox - Oxygenated monoterpenes

NSC - Non-structural carbohydrates

PAR - Photosynthetically Active Radiation

PP – Polyphenolic parenchyma

SAR – Systemic acquired resistance

SPME – Solid-phase microextraction

UAV - Unmanned aerial vehicle

VOC – Volatile organic compound

VPD - Vapor Pressure Deficit

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Introduction

In recent decades, natural disturbances in forests have led to significant economic losses and global ecological imbalances, such as the intensification of disturbance regimes, unprecedented levels of bark beetle damage, and an increase in the frequency and severity of wildfires and windthrows (Seidl et al., 2016, 2014). These trends are expected to continue due to ongoing climatic changes, posing challenges to the sustainable provision of ecosystem services and the maintenance of biodiversity in forest ecosystems (Allen et al., 2015; Seidl et al., 2016; Reed and Hood, 2021).

In Central Europe, coniferous forests have been facing various environmental stressors due to the climate change, including increasing temperatures and periods of drought (Netherer and Schopf, 2010; Seidl et al., 2014). Between 1950 and 2000, abiotic factors, such as drought, floods, heavy winds, and forest fires, led to the destruction of 35 million m^3 of forests, a loss associated to an expanse constituting 8% of the entire Europe forests. Storms with heavy winds were the primary cost, accounting for 53% of the damage, followed by fire at 16% (Schelhaas et al., 2003).

In subsequent years (2003–2007), drought became the primary factor, linked to climate patterns evolving from global warming (Schröter et al., 2005). This trend affected the entire Central European region, including the Czech Republic, leading to reduced soil moisture and precipitation-induced physiological stress in forest trees. Extreme conditions were particularly notable in 2015 and 2018, with 2018 experiencing above-average temperatures (+2.1 °C deviation) and below-average precipitation (77% of normal precipitation) (Lubojacký et al., 2019), which caused exponential increases in outbreaks of the Eurasian spruce bark beetle *Ips typographus* (L. 1758) (Coleoptera: Scolytinae) peaking in 2020 (Lubojácký et al., 2023).

Knowledge about the population dynamics of *Ips typographus* and resistance of Norway spruce *Pices abies* (L.) Karst to bark beetle attack is fundamental for predicting mass outbreaks. Yet, a comprehensive understanding of bark beetle-host tree interactions in a changing climate is still restricted by substantial knowledge gaps (Biedermann et al., 2019; Huang et al., 2019). The response of individual trees to external stressors such as drought, solar irradiation exposure, or temperature fluctuations are a key factor in Norway spruce´s stress biochemistry and physiology. These factors influence both constitutive and induced defence mechanisms of the trees. The intricate interactions between bark beetles and trees are very complex and new advancements in this research field will enhance our knowledge on *I. typographus* attraction to susceptible host trees and colonization success (Stříbrská et al., 2023a).

1. Aims and overview of the dissertation

In Central Europe, conifer forests experience significant changes related to climate change such as increased temperature and drought periods. Nowadays, the most economically important consequence of these disturbances in Central Europe are severe damages to Norway spruce (*Picea abies*) monocultures caused by outbreaks of the spruce bark beetle, *Ips typographus*. Substantial knowledge gaps still restrict a comprehensive understanding of spruce bark beetle-spruce tree interactions in a changing climate.

This thesis hypothesizes that discernible differences in physiological, biochemical, and spruce bark beetle (BB) behaviour can be seen between the stressed or naturally attacked trees and the intact ones. The hypothesis suggests that these distinctions will be readily measurable, contributing to a deeper understanding of the intricate interactions between trees and bark beetles. Furthermore, it is hypothesized that the identified changes can be leveraged as valuable tools for developing innovative "BB attack early detection methods." This conjecture anticipates that the insights gained from observing these variations will pave the way for novel and effective strategies to identify bark beetle attacks, ultimately enhancing forest management practices and tree protection.

This thesis aims to study the response of Norway spruce to stress episodes regarding tree physiology as well as constitutive and induced defence reactions of mature trees against bark beetle attacks. The specific objectives were:

1) to evaluate the existence of measurable differences in physiological, biochemical, and bark beetle (BB) behaviour between stressed or naturally attacked trees and their intact counterparts (Chapter 4.1);

2) to study the features of mature Norway spruce trees under stress conditions based on tree physiological data and defence chemistry profile (Chapter 4.1);

3) to establish the susceptibility of mature spruces to bark beetle attack on adult Norway spruce trees (Chapter 4.2), which will be subjected to stresses resulting from water regime modification and sudden sun exposition on freshly created forest edges (Chapter 4.1);

4) to evaluate that these changes can be utilized as tools for the development of 'BB attack early detection methods (Chapter 4.2, 4.3).

2. Literature review

2.1. Implications of climatic changes on forest vulnerability to bark beetle outbreaks

The anticipated global temperature increase of 2-4 °C compared to pre-industrial times is expected to result in more frequent and severe droughts (Allen et al., 2010; Liu et al., 2018). These evolving environmental conditions are likely to impact the distribution of tree species and other plants and affect their vulnerability to both abiotic and biotic stressors. In the recent decades, there has been a global increase in tree mortality, partly attributed to the climate change, contributing to the escalation of insect outbreaks and the proliferation of pests (Allen et al., 2010; Ayres and Lombardero, 2000; Hartmann et al., 2022).

The most significant economic consequence of forest disturbances in Central Europe has been the extensive damage to Norway spruce (*Picea abies*) monocultures caused by bark beetle outbreaks since the beginning of the 21st century (Marini et al., 2017; Seidl et al., 2016; Stadelmann et al., 2014). These outbreaks have thrived on weakened tree defences induced by the main triggers such as storm throw, snow or ice damage (Schelhaas et al., 2003), but recently the most important factor is drought stress (Fernandez-Carrillo et al., 2020; Hlásny et al., 2021a). The colonisation of trees by bark beetles together with the introduction of associated fungi have resulted in extensive tree mortality, involving the deterioration of wood quality and considerable economic losses (Hlásny et al., 2019; Wermelinger, 2004).

In the Czech Republic, the European spruce bark beetle (*Ips typographus*) is the primary aggressive insect pest attacking the Norway spruce (*Picea abies*). In the past ten years its population has exceeded epidemic levels in numerous regions of the Czech Republic (Lubojácký et al., 2023). Across the Czech Republic, there have been reported cases of dead and rotting spruce trees on thousands of hectares, culminating in 2020 at an estimated loss of 23 million m^3 of wood (Fernandez-Carrillo et al., 2020; Hlásny et al., 2021b). Furthermore, in the years 2019 and 2020, the salvage logging reached its peak (approximately 15 million m^3), gradually decreasing to 5.5 million m^3 in the last years (Lubojácký et al., 2023).

Historic data (from year 1945) show periodic fluctuations in bark beetle outbreaks with recurrence every 20 to 30 years (Hlásny et al., 2021a).

2.2. Abiotic disturbances

Abiotic factors represent non-biotic, environmental factors that can affect the susceptibility of tree species or whole forest stands to bark beetle attack. Drought and wind are most important abiotic disturbance agents that can affect spruce stands (Schroeder, 2010). Abiotic stress factors such as solar radiation, high temperature, and drought profoundly impact tree physiology and cause shifts in carbon balances, with effects on primary and secondary metabolism. Consequently, these alterations play a role for tree growth and defence and thus, in the attraction of bark beetles, their host acceptance and development (Erbilgin et al., 2021; Netherer et al., 2021). Despite studies demonstrating increased susceptibility to biotic attacks under abiotic stress (Huang et al., 2020; Netherer et al., 2015), several aspects of climate-plant-insect interactions remain unexplored.

For example, according to Lindner et al., (2010), site conditions in northern Europe are often poor, involving low fertility, terrain steepness, and high soil erosion risk. Consequently, these challenging site conditions in northern Europe may lead to increased difficulty in establishing and maintaining forests, potentially impacting timber yield, ecosystem stability, and overall forest health. Additionally, such conditions may necessitate specialized forest management practices to mitigate the identified challenges.

In a complexity of natural systems, it is almost unexpected to find a single stress that occurs on its own (Caldeira, 2019). The interplay of abiotic stresses poses a crucial selective pressure on the adaptive traits of long-lived organisms, particularly woody plants. Despite the significance of this phenomenon, comprehensive patterns and mechanisms outlining how woody plants adapt to withstand various abiotic stressors, are still lacking (McDowell et al., 2022).

2.2.1. Wind

In summary, heavy windstorms are key contributors to the spread of bark beetle infestations in forests. The escalating damage from disturbances, particularly storms, is driven by changes in forest structure, such as the expanding coniferous forest area and a significant rise in coniferous growing stock. As Europe experiences continuous growth in forest volume, area, and stand age, effective forest management emerges as a crucial factor in curbing the escalating risks associated with these disturbances.(Schelhaas et al., 2003). Storms contribute to an abundance of suitable host material, which is colonised by bark beetles rapidly, as wind-thrown trees lack vital defences (Schroeder, 2010).

Prolonged drought, accompanied by windfalls or other abiotic disturbance events, can further escalate the bark beetle population size beyond the density threshold required for the colonization of vital trees. The windfalls usually become the basis for an extensive and rapid colonization by *I. typographus* populations. Consequently, high attack pressure from the emerging generation leads to the demise of robust and healthy trees or triggers the beetle outbreaks by diminishing tree resistance (Christiansen and Bakke, 1988).

2.2.2. Drought stress

With ongoing climate change, drought (water and temperature stress) is forthcoming as the new main abiotic factor triggering bark beetle outbreaks. There is a hydrologically based theory contemplating a relationship between tree carbon balance and resistance against biotic invaders, which permits the development and examination of hypotheses regarding tree survival and mortality during drought within a systematic study approach (McDowell et al., 2008). Changes in the tree's physiological, biochemical and structural parameters are positively correlated with current and past drought periods (Hossain et al., 2018). The tree mortality is further elevated as the insect pests or pathogen diseases increase in incidence during prolonged drought (Caldeira, 2019). However, the direct implications of drought on tree either tree survival or mortality have been controversially discussed in the literature (Hajek et al., 2022; McDowell et al., 2008). Increasing frequency of warmer temperatures and drought periods are considered as important factors affecting directly insect population dynamics and indirectly the host plant growth and defence (Hart et al., 2014; Raffa et al., 2015). Conifer species differ greatly in their ability to manage the drought, but species lacking the ability to cope with different water regimes are more likely susceptible to bark beetle attack, e.g., Ponderosa pine and Norway spruce. Different water regimes, such as drought periods, may impact water potential management by iso-/aniso-hydric strategy, therefore increasing the susceptivity to bark beetle attack (Christiansen et al., 1987). The combination of drought events and warmer temperatures leading to a reduction in the trees' water supply was shown to increase the susceptibility of trees to *I. typographus* attacks (Netherer et al., 2015). Tree physiological responses to drought can be contradictory, for example, a decrease in biochemical defence (Caldeira, 2019; Rohrs-Richey et al., 2011) but pathogen infection generally increases such tree defences (Caldeira, 2019; Oliva et al., 2014).

Severe drought stress, exemplified by natural conditions in the Czech Republic during 2017 and 2018, caused transpiration deficits in Norway spruce indicated by reduced sap flow rates (Gebhardt et al., 2023; Střelcová et al., 2013) and significantly reduced the annual stem radial increment of trees (Krejza et al., 2021). The resultant acute and accumulated transpiration deficits can be linked to a decline in Norway spruce defence mechanisms, including resin flow. This vulnerability is represented by increased successful attacks by *I. typographus* in field bioassays (Marini et al., 2017).

The plasticity in secondary metabolism is explained by the growth-differentiation balance hypothesis (GDBH), initially proposed by Loomis, (1932), and extended by Lorio Jr, (1986), as further developed by Herms and Mattson, (1992). According to this theory, a physiological trade-off exists between growth and secondary metabolism due to developmental constraints in growing cells and competition between primary and secondary metabolic pathways in mature cells. GDBH integrates this trade-off with the responses of net assimilation rate (NAR) and relative growth rate (RGR) to resource availability, predicting a parabolic effect of nutrient (or water) availability on secondary metabolite concentration (Fig. 1; Herms and Mattson, 1992).

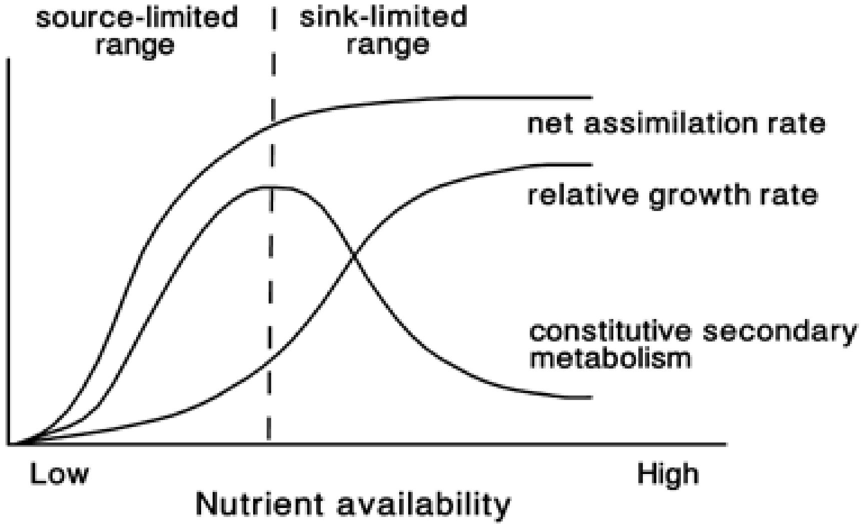


Figure 1 The growth–differentiation balance hypothesis predicts the responses of relative growth rate, net assimilation rate, and constitutive secondary metabolism along a nutrient availability gradient. In plants limited by sources, a positive correlation between growth and secondary metabolism is anticipated, whereas in sink-limited plants, the correlation is predicted to be negative (Herms and Mattson, 1992).

This theory underwent thorough testing, with additional insights provided by Luxmoore et al., (1995). Among their findings, Luxmoore et al., (1995) noted that conifer trees tend to increase carbohydrate storage during periods of minimal growth activity in conditions of moderate stress. Due to the surplus of carbohydrates invested to secondary metabolites, it is proposed that environmental conditions can influence constitutive secondary metabolism, potentially enhancing it in moderate drought conditions. It has been observed to increase tree resistance, as shown in previous studies on loblolly pine, where constitutive resin flow increased, and host suitability for bark beetles decreased under moderate water deficits (Dunn and Lorio, 1993; Reeve et al., 1995). However, extreme water deficits can lead to a collapse in the carbon pool, resulting in decreased secondary metabolism (Herms and Mattson, 1992; Lorio Jr, 1986). This decrease not only compromises tree defence capabilities but also renders the environment less nutritionally beneficial for beetles. (Lombardero et al., 2000).

Gaining insight into these interactions in the specific system Norway spruce-*Ips typographus* is vital in the context of climate change for a more comprehensive understanding of ecological impacts and possible effective mitigation.

2.2.3. Acute stress from sun irradiation

Extensive sun irradiation in a forest edge and thereby resulting in acute stress is increasingly relevant in a warming climate. Key determinants of tree susceptibility to bark beetle attacks include the effects of wind and elevated temperature conditions in the vicinity of edge trees, particularly in sun-exposed areas along the trunk (Marešová et al., 2020). Forest edges can provide favourable habitats, especially for insects favoured by sun exposure (Müller et al., 2007). Norway spruce trees on forest edges are more attractive for a subsequent attack by bark beetles due to the following reasons:

As the forest edge is less dense, the trees are impacted by high solar radiation exposure; therefore, sun stress increases the susceptibility to attacks while trees in the interior of forests are better protected.

I. typographus prefers exposed trees for breeding, such as edge trees or weakened individuals (Schroeder and Lindelöw, 2002). Furthermore, Kautz et al., (2013) found out that the exposition of the forest edges to the sun increases the probability of bark beetle infestations, even though there is no breeding site close.

In Stříbrská et al., 2022 (Chapter 4.1), we investigated the acute stress-induced changes in tree physiology and metabolism, particularly at freshly established forest edges. We also examined whether these factors make spruce trees more susceptible to bark beetle attack. The study revealed changes in microclimatic conditions at forest gaps and edges, represented by increased solar radiation and temperature. These conditions were favourable for swarming beetles and challenged the defence ability of the edge trees, potentially making them more attractive to bark beetle infestations. However, the relationship between stress states of Norway spruce trees and their susceptibility *to Ips typographus* attack is presumably non-linear. Immediately after their establishment, the fresh forest edges were not subjected to bark beetle attacks. By the end of summer, increased temperature and mild drought conditions triggered minor physiological stress in forest edge trees along with a higher number of bark beetle attacks. The study suggested that the timing of clear-cuts could impact the likelihood of natural attacks in the first or second year after edge establishment.

2.3. Eurasian spruce bark beetle (*Ips typographus*)

<u>Taxonomic Tree:</u>

Domain: Eukaryota
Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Hexapoda
Class: Insecta
Order: Coleoptera
Family: Scolytidae
Subfamily: Scolytinae
Genus: *Ips*
Species: *typographus*

Figure 2 Female adult spruce bark beetle (*Ips typographus*) (Gilles San Martin,2019).

The Eurasian or European spruce bark beetle (*Ips typographus* L. 1758; Fig. 2) is a pest of Norway spruce (*Picea abies*). The *I. typographus* adults are around 4.2 - 5.5 mm long. They have a cylindrical shape with hair and shiny dark-brown colouring. The frontal part of the pronotum is obliquely cut, dentate and squamate, and the back part is stippled. There are rows of depressed points on the glossy elytra with spaces between them. The posterior edges of the elytra form a characteristic collar shape with dents on both sides. Four teeth are on these edges, and the third is capitated. The rear side of the elytral declivity is greasy and shiny (when the insect is viewed from the rear). Females lay eggs (size is <1mm) individually in niches along both sides of the maternal gallery. One female can lay 30-80 eggs. Larvae are white, legless, with small, brown, chitinous head and brown mandibles (Zumr, 1995).

Bark beetles like *I. typographus* substantially change forest structure, composition, and function (Raffa et al., 2008). They attack weakened trees or trees that are stressed by other abiotic or biotic factors (Stříbrská et al., 2023a). Bark beetle attacks facilitate tree mortality, their decomposition and eventually, nutrient cycling. In specific conditions, bark beetle population densities can significantly rise. Such exponential increase in beetle abundance constitutes an epidemy that can turn into a bark beetle calamity from forestry perspective, where bark beetles attack even healthy trees. The most significant damages of Norway spruce (*Picea abies*) are often seen in monocultures affected by spruce bark beetle calamities, due to luck of biodiversity, limited natural resistance, uniform susceptibility and homogeneous environmental conditions (Raffa et al., 2008).

Global change including climate warming significantly affects spruce bark beetle phenology. Increasing temperatures lead to earlier spring swarming and faster evolution from egg to adult, which supports viable populations even at high elevations and latitudes (Jönsson et al., 2009). In response to increasing temperature conditions, the generation development also accelerates, allowing for a higher number of terminated generations per year. Multivoltinism is further promoted by prolonged late summer periods with high temperatures. These reasons might be a consequence of why Central Europe's lowland areas may already have three generations of *I. typographus* per year (Netherer et al., 2019). The three generations have lately occurred quite often.

Host selection of *Ips typographus* involves a sequence of behaviours (Fig. 3a-d), starting with dispersal through the landscape (Fig. 3a) and culminating in the directed search for suitable host trees. Despite the beetle's ability to devastate entire forest stands, identifying scattered individuals or groups of suitable hosts poses a challenge for this small insect, leading to high mortality rates during the dispersal phase (Coulson et al., 1980). Olfactory cues play a crucial role in guiding bark beetles at the habitat level (Fig. 3b), keeping them away from non-host trees within forest stands (Zhang and Schlyter, 2004). Spruce bark beetles, *I. typographus,* rely on a combination of positive and negative cues for host selection (Fig. 3c). Positive cues include aggregation pheromones, host foliage monoterpene hydrocarbons, and microbial volatiles. Negative cues, such as heterospecific pheromones, non-host volatiles, and microbial volatiles, deter beetles from unsuitable hosts. The avoidance of non-host volatiles, including specific compounds, such as green-leaf alcohols, bark alcohols, and a ketal (Erbilgin et al., 2007; Kandasamy et al., 2019; Lehmanski et al., 2023; Netherer et al., 2021; Raffa et al., 2016; Zhang and Schlyter, 2004), underlines the beetle's adaptability to mixed forest landscapes (Lindgren and Raffa, 2013). The complexities of beetle orientation and host choice based on stress levels underscore the need for further research in this area (Netherer et al., 2021).

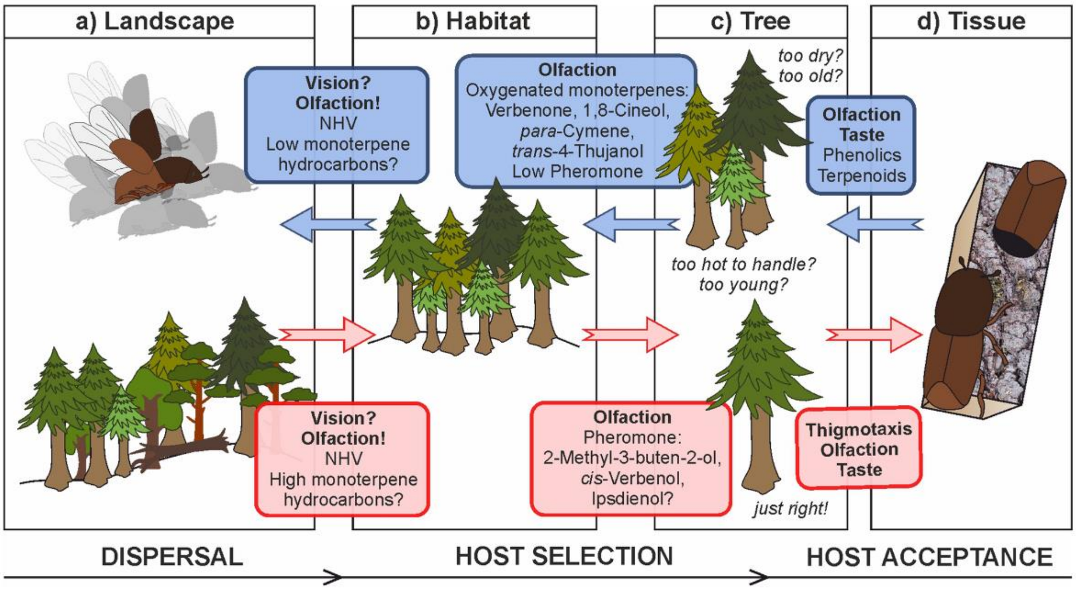


Figure 3 The behavioural sequence and mechanisms for *Ips typographus* in finding suitable host trees include olfactory cues, a) in a landscape involving the dispersal, b) in a habitat includes host selection, c) in a tree encompasses both host selection and host acceptance, and d) in a tissue. The focus is specifically on the pioneering male beetles, whose promptly emitted pheromone signals play a critical role in directing the majority of both males and females to aggregate. The individual beetle adheres to a sequence of steps guided by visual, chemo-sensory, and thigmotactic cues involving both positive (depicted by fair blue arrows and boxes) and negative cues (indicated by red arrows and boxes) (Netherer et al., 2021).

The dynamics of attacks is mediated through chemical communication (Fig.4). The process begins with male pioneer beetles selecting a host tree based on the compounds emitted by potential host trees and by non-host trees. Once a suitable host is identified and pioneer beetles start boring, trees emit terpenoid volatiles such as *α*-pinene, which is again used by the beetles for the production of aggregation pheromones (Lehmanski et al., 2023). As a result of the first boring attempts, a cloud of volatile organic compounds (VOC) consisting of host tree compounds, a combination of VOC from fungi (Jirošová et al., 2022b), and the pheromone (2-methyl-3buten-2-ol with (*S*)-*cis*-verbenol) is emitted. A mass attack ensues upon colonization, accompanied by the same VOCs, which in the second phase are enriched by ipsdienol. Ipsdienol functions as a key communication tool for bark beetles, serving both as an aggregation pheromone to coordinate mass attacks on host trees and as a signal for mate attraction. The subsequent phase involves the next generation breeding and the increasing production of the anti-attractant verbenone when trees are more and more colonised (Ramakrishnan et al., 2022). This phase continues to lead to the depletion of a tree’s defences, when resin flow ceases and the emission of VOCs from terminal fungi along with ipsenol increases. Additionally, this phase may be accompanied by sister breeding (Byers, 1989; Modlinger, 2018; Schlyter and Anderbrant, 1989).

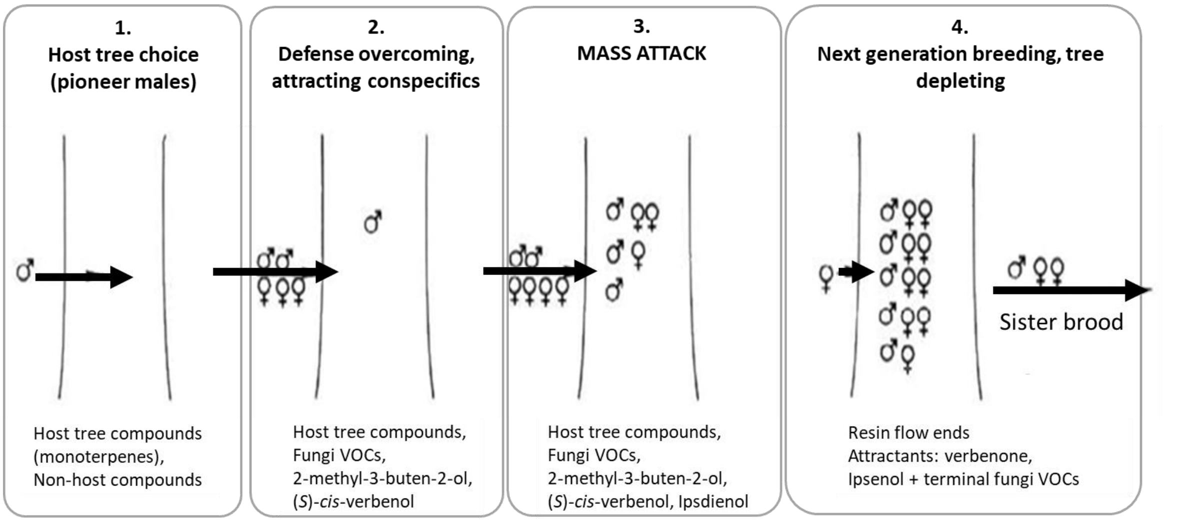


Figure 4 Chemical communication between *Ips typographus* and Norway spruce in the course of tree colonisation (Modlinger, 2018).

Distribution of *Ips typographus*

Ips typographus occupies a massive area in Europe and Asia (Fig. 5A). Originally, it was a frequent species in northern Europe and also in mountains, a species that adapted for life in the lower altitudes. It can be found in the temperate region (Skuhravý, 2002). This species is also abundantly represented in Central Europe, including the Czech Republic.

In general, the bark beetles are generally found wherever spruce or other suitable tree species for their nourishment are present (Fig. 5B).

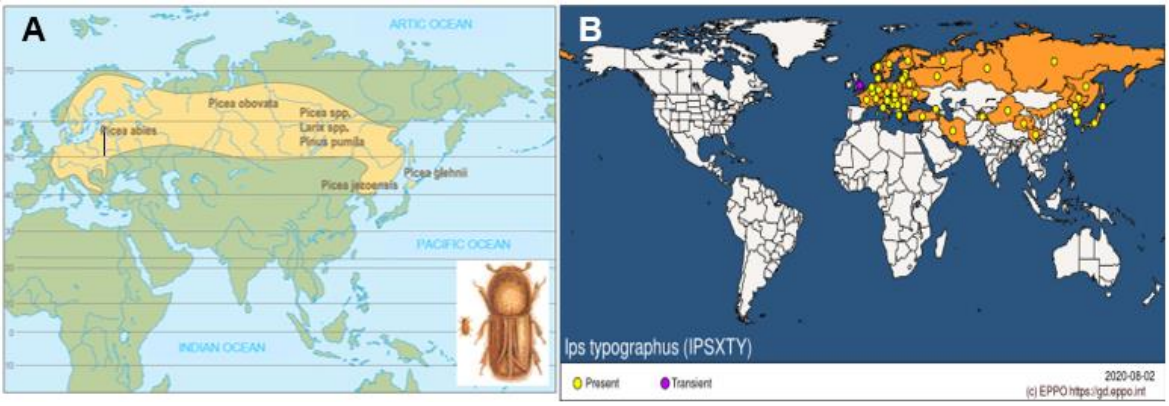


Figure 5 A) Extension of *Ips typographus* in dependency of tree species in Europe and Asia region (Skuhravý, 2002). B) Global distribution of *Ips typographus* (EPPO, 2020).

Figure 6 reports about the swarming activity of bark beetle in Czech Republic in 2023.

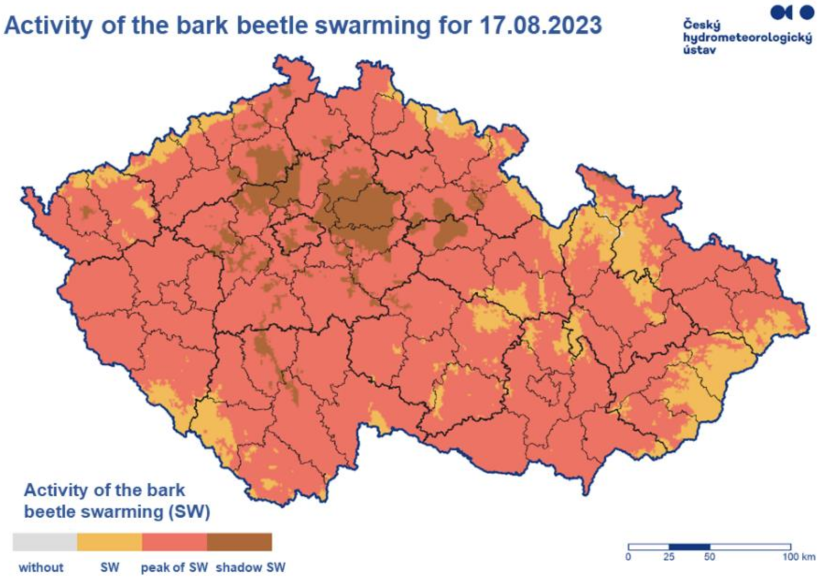


Figure 6 Swarming activity of bark beetle in Czech republic in summer 2023 (ČHMU, 2023).

2.4. Early attack detection

Early attack detection is a crucial aspect of managing bark beetle outbreaks, particularly in the case of pests like *Ips typographus* (Stříbrská et al., 2023a, 2023b). Traditional methods, such as visual inspection of the trees for boring dust at the base of the trunk (Kautz et al., 2023), have limitations due to the vastness of forest stands and the difficulty of personal inspection for each tree. Modern tools and procedures, including remote sensing methods, show promise but face challenges in processing captured records and detecting signs with accuracy (Huo et al., 2021; Hüttnerová et al., 2023; Paczkowski et al., 2021).

Alternative methods, such as chemical communication between bark beetles, have been explored. Specially trained dogs have demonstrated success in detecting infested trees (Johansson et al., 2019) more efficiently than human experts (Vošvrdová et al., 2023). Research involving an artificial nose for detecting substances in the forest environment is also underway (Hüttnerová et al., 2023).

2.5. Tree physiology – measurable characteristics

As the interaction of the tree physiology with abiotic stressors was discussed before, this section explains the processes inside the tree, including physiological parameters and responses to stress, such as the production of secondary metabolites and induction of defence mechanisms.

The intricate field of tree physiology encompasses many processes and factors crucial to the growth and well-being of trees. Examining key elements such as sap flow, Photosynthetically Active Radiation (PAR), Vapor Pressure Deficit (VPD), and tree increment provides valuable insights into the mechanisms governing a tree's vitality (Stříbrská et al., 2022). Sap flow, influenced by environmental variables, facilitates the essential transport of water and nutrients from roots to leaves for vital metabolic processes (Rabbel et al., 2018; Taiz and Zeiger, 2002). PAR is a parameter representing the light spectrum beneficial to photosynthesis and it plays a pivotal role in a tree's overall growth and health (Acosta et al., 2011; Taiz and Zeiger, 2002; Tomášková, 2000). Another physiological indicator is the VPD, which measures atmospheric moisture, it is a key parameter for assessing a tree's water balance and susceptibility to water stress (Stříbrská et al., 2022; Taiz and Zeiger, 2002). Lastly, the tree increment, measured through annual growth rings or by a dendrometer as a change in trunk circumference (Stříbrská et al., 2023a), serves as a substantial indicator of a tree's health, reflecting its response to environmental conditions, nutrient availability and water supply (Clark et al., 2007). A thorough knowledge of these physiological aspects is essential for effective forest management and the preservation of tree ecosystems. These parameters are crucial in physiological assessments for determining tree stress.

Climatic parameters, soil water availability and solar radiation influence tree net photosynthesis and thus the supply of non-structural carbohydrates (NSC), which mainly include low-molecular-weight sugars and starch (Adams et al., 2013; Hoch et al., 2003). Conifers can adapt well to extreme conditions, for example, stomatal closure maintains the water supply of tree organs (Sevanto et al., 2014). The acute drought stress in conifers significantly impacts the availability and storage of non-structural carbohydrates (NSCs) and compounds like mannitol crucial for osmotic regulation and antioxidative functions (Huang et al., 2020; Krasensky and

Jonak, 2012). Carbohydrate distribution in tree organs is intricate, influenced by abiotic stressors, genetic factors, and the tree's past stress experiences, often displaying non-linear relationships (Hartmann et al., 2018; Huang et al., 2020). Changes under drought conditions concerning attainability and allocation of NSC to defence compounds have been proven as non-linear. This relation firmly depends on various factors such as tree characteristics, class of compound, plant organ, presence of biotic agents, and, in particular, timing and intensity of stress (Huang et al., 2019). Under moderate drought stress, conifers could accumulate NSCs to sustain protective measures against biotic attacks (Ferrenberg et al., 2015; Jacquet et al., 2014). In case of source restriction, NSCs are used to repair stress-induced damages as well as for secondary metabolites biosynthesis (Hartmann et al., 2018; Huang et al., 2019). Reduced growth may result in increased NSC pools, with a preference for carbon allocation to secondary metabolites like phenolic and terpenoid compounds (Huang et al., 2021). Trees with lower stem increments showed higher carbohydrate contents (unpublished results), suggesting potential storage of unused carbohydrates for rapid post-stress recovery (Huang et al., 2019; Muller et al., 2011). However, prolonged stress exposure is expected to deplete carbohydrate supplies, especially in roots (Hartmann et al., 2013).

The secondary metabolism of conifers is the most effective mechanism of protection against environmental stressors, as well as fungal and herbivore attacks These mechanisms evolved under a complex of climatic parameters, biotic and abiotic selection pressures and variables between and within tree species like genotype, phenotype, and ontogeny (Berini et al., 2018; Ferrenberg et al., 2017).

2.5.1 Tree metabolism under the stress

Environmental stress factors influence the physiological and biochemical characteristics of trees, producing notable effects on transpiration, sap flow, and both constitutive and induced defence mechanisms, such as resin flow. Particularly, the interactions between abiotic and biotic stressors can induce changes in the composition of bark compounds, affecting defence proteins and secondary metabolites, encompassing terpenoids, phenolic substances, and alkaloids (Franceschi et al., 2005; Huang et al., 2020; Netherer et al., 2021). From the biochemical perspective, the response of trees to drought stress is a relatively complex mechanism involving some universal signs in all plants and specific reaction chains in individual orders (de María et al., 2020).

Some other biochemical and physiological tree characteristics are regarded to be reliable indicators of tree stress, such as photosynthetic ability (e.g., stomatal conductance, chlorophyll fluorescence), volume of plant stress hormones (abscisic acid (ABA), salicylic acid, jasmonic acid, and ethylene) (Jackson et al., 1995; Tan and Blake, 1993), content of key proteins and changes in proteome, volatile organic compounds (VOC) emissions (Niinemets, 2010) and fluctuations in monoterpenes (MTs) contents (Kainulainen et al., 1992).

Continued exposure to high temperatures and drought leads to a progressing decrease in water tension within the xylem. Stress factors like heat and drought activate stress hormones and proteins, leading to stomatal closure mediated by ABA (Moran et al., 2017). Decreased CO_2 in chloroplasts due to low stomatal conductance activates oxidative and photo-oxidative stress. Stomata closure, coupled with reduced photosynthetic activity, initiates a series of biochemical reactions in the tree, such as the disturbed redox state of cells which leads to a damage in cell components by free radicals and peroxides (Felicijan et al., 2015). Trees start to synthesise antioxidants (carotenoids and vitamin E) (Tausz et al., 2001) and specialised metabolites from non-structural carbohydrates (Netherer et al., 2021).

Compounds in the tree bark, such as defence compounds and various VOCs like monoterpene hydrocarbons and oxygenated monoterpenes, in addition to pheromone components, play a vital role as sensory signals for bark beetles to locate and initiate attacks on their host. Ramakrishnan et al., (2022) suggested that *α*-pinene, a major host tree compound (Jaakkola et al., 2022), may be used in the *de novo* pheromone biosynthesis pathway in *I. typographus* aggregation pheromone. Additionally, symbiotic fungi such as *Endoconidiophora polonica* and *Grosmannia penicillata* are also pivotal in detoxifying compounds and attracting beetles to the tree (Netherer et al., 2021). All interactions among Norway spruce (*Picea abies*), *Ips typographus* and symbiotic ophiostomatoid fungi during drought conditions are shown in Figure 7.

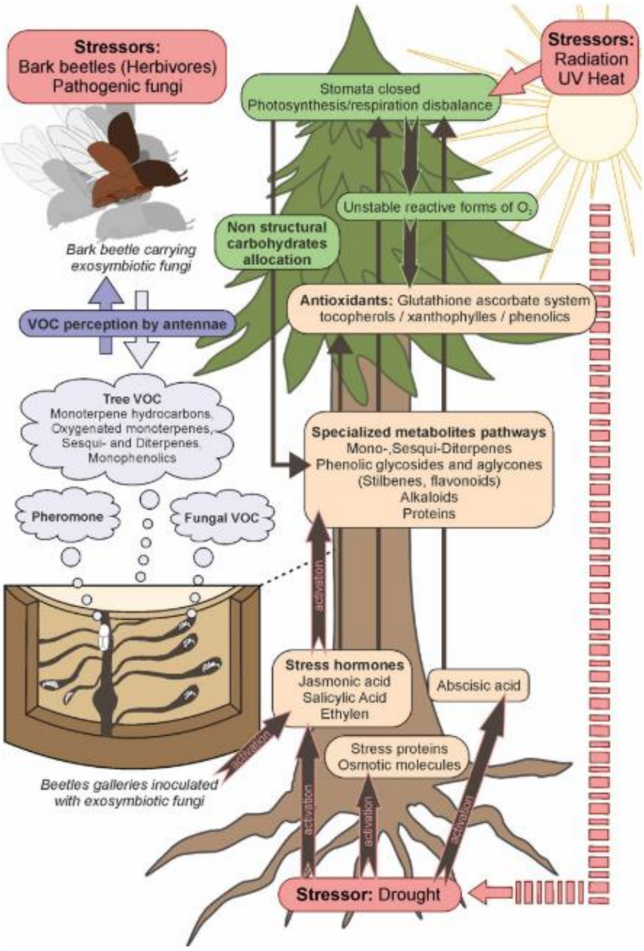


Figure 7 Schematic representation of interactions among Norway spruce (*Picea abies*), *Ips typographus* and symbiotic ophiostomatoid fungi during drought conditions. Brown arrows depict the activation of hormones, proteins, and biochemical reactions. Defence compounds in the bark and volatile organic compounds (VOCs), including monoterpene hydrocarbons and oxygenated monoterpenes, along with pheromone components (light blue clouds), serve as crucial gustatory and olfactory signals for host location and attack by bark beetles (Netherer et al., 2021).

The main pathway for eliminating reactive oxygen species is the ascorbate-glutathione cycle, and one of the main compounds in the cycle, ascorbate (ascorbic acid) is the most water-soluble antioxidant in plant cells. Glutathione is a primary component for the regeneration of ascorbate (Krajnc et al., 2014). Norway spruce needles under mild drought stress have shown elevated levels of ascorbate and glutathione together with a change in the ratio of reduced and oxidised glutathione (Tausz et al., 2004, 2001). Also, it was observed that in response to salicylic acid application to the bark region of Norway spruce, glutathione and cysteine levels increased, resulting in shorter maternal gallery lengths in comparison to untreated controls and eventually leading to a reduced number of spruce bark beetle entrance holes and activation of systemic acquired resistance (SAR) (Krajnc et al., 2011). Glutathione plays multiple roles in the plant metabolism, anti-oxidative defence, and redox control and it is also involved in the response and resistance to pathogens (Tausz et al., 2004).

The ascorbate-glutathione cycle, in combination with the storage and the synthesis of phenolics in polyphenolic parenchyma cells, has been increasingly recognized as a key system for tree response to both abiotic and biotic stress (Felicijan et al., 2015). Certain phenol families and phenolic compounds, such as stilbene glucosides, flavonoids and lignans, show clear effects in the defence against *I. typographus* and associated blue-stain fungi (Zhao et al., 2019).

Drought, cold temperatures, frost, or elevated salinity have been linked to the expression of dehydrin genes (Eldhuset et al., 2013). It has been elucidated that dehydrins play a role in preserving or emulating aqueous conditions within a dehydrated cell (Graether and Boddington, 2014). These proteins serve as a protective mechanism to alleviate the negative consequences of dehydration. While previous research (Sena et al., 2018; Velasco-conde et al., 2012) has primarily focused on genetically determined variations in response to controlled environments, the study of Čepl et al., (2020) explores such differences among individuals and populations in natural settings. The study emphasizes the role of dehydrins, proteins associated with mitigating the adverse effects of dehydration, in response to environmental conditions such as drought, low temperatures, frost, or high salinity. The research goes beyond controlled conditions, exploring natural ecotypes, and reveals significant differences in dehydrin expression among these ecotypes. Moreover, the study establishes correlations between dehydrin gene expression and key climatic variables, including precipitation, temperature, and day length. These findings contribute to a better understanding of how genetically determined variations in dehydrin expression respond to specific environmental factors in natural settings, shedding light on the adaptive mechanisms employed by different ecotypes to cope with dehydration-related stress (Čepl et al., 2020).

2.5.2 Secondary defence metabolites in Norway spruce

The defence mechanisms rely on both pre-existing (constitutive) and newly generated (inducible) defences generated in response to biotic attack, incorporating mechanical and chemical barriers in a series of one to four successive stages (Celedon and Bohlmann, 2019; Franceschi et al., 2005). The initial defence stage aims to repel and inhibit the initial attack through constitutive mechanisms, including bark anatomical structures that are challenging for insects to bore and consume, as well as chemical compounds like oleoresin, which can flush, repel, and seal entry points for bark beetles. If the constitutive defences prove insufficient, a second stage activates inducible defences to either kill or compartmentalize beetles that have successfully penetrated the host tree. After an attack, trees enhance the concentration of semiochemicals with inhibitory and toxic effects on attacking beetles and associated fungi. Moreover, there are anatomical changes in the cell structure around the injured tissue, resulting in the creation of a necrotic area impregnated with defensive compounds such as terpenoids and phenolic substances to restrict infestation and impede the development of beetle galleries and associated fungi (Christiansen et al., 1987). In the third stage, damaged tissues are sealed and repaired to curtail opportunistic or subsequent infestations (Franceschi et al., 2005). Finally, upon identification of an invading organism, a fourth stage of acquired or systemic defence can be triggered, involving the synthesis of more specialized defence responses, such as gene-for-gene interactions, to prevent future attacks (Eyles et al., 2010; Franceschi et al., 2005).

Only a limited number of studies address the impact of drought on mature and healthy trees. Analyses of terpene contents were mostly focused on pine species, needles more than phloem, and trees of various age classes (Branco et al., 2010). In Norway spruce, bark is composed of terpenoids which are strongly influenced by several aspects, such as environmental conditions, tissue type, and tree genotype (Schönwitz et al., 1990).

The ability of conifer trees to activate defence mechanisms becomes crucial in the presence of additional stress factors. This was exemplified in a study exploring the responses of *Pinus banksiana* and *Pinus ponderosae* to drought, which synthesized lower amounts of monoterpenes in fungal lesions compared to well-watered seedlings and trees (Hussain et al., 2020; Lusebrink et al., 2016). Interestingly, despite changes in stress-mediated responses of biosynthetic pathways and trade-offs between defence compounds, the total amounts of secondary metabolites measured in the bark often remain unaffected by drought, making them unreliable indicators of stress (Koricheva et al., 1998). In the context of drought, the synthesis and emission of volatile terpenoid compounds was observed to increase with rising water stress but eventually decrease under severe drought conditions (Holopainen et al., 2018; Lieutier, 2004).

2.5.3 Effect of stress on tree terpenes

The carbon-based secondary defence compounds serve as an essential chemical defence against biotic attacks (Franceschi et al., 2005). The terpenoid substances, synthesized from the C5 blocks in the epithelial cells of resin ducts, give rise to the mono-, di-, and sesquiterpenes (Celedon and Bohlmann, 2019). The effect of physiological stress consists of the formation of traumatic resin ducts (Gaylord et al., 2013; Netherer et al., 2021; Rudinsky et al., 1971), increased bark permeability, enhanced emission rates of volatile organic compounds, mainly terpenes and changes in concentrations of secondary defence compounds in tissues (Hietz et al., 2005; Mattson and Haack, 1987). Finding connections between tree disposition, bark beetle attack, and volatile blends is demanding due to the high heterogeneity among tree individuals and further intensification in temperature, light, and humidity (Hietz et al., 2005; Zhang and Schlyter, 2004). Anyway, there is a strong correlation between VOC emissions of spruce trees and temperature (Baier and Bader, 1997a; Hietz et al., 2005; Kautz et al., 2013; Laffineur et al., 2011; Stříbrská et al., 2022). Stříbrská et al., (2022) mainly reports on the effects of stress in relation to analyses of terpene contents from phloem and air collection around the tree bark in mature trees.

Constitutive defence, terpene compound content in Norway spruce under physiological conditions

To evaluate possible drought impacts on Norway spruce disposition to *I. typographus* attack, a closer look at the induced changes in the course of experimentally controlled stress events and at constitutive blends of terpenes in the bark is required. Due to the long life of conifers, the measured concentration of secondary compounds in the bark of uninfested and unscathed trees, despite the possibility of past stress events, can be interpreted as constitutive defence states (Witzell and Martín, 2008).

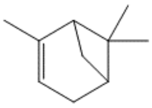
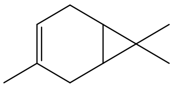
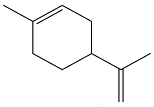
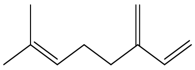
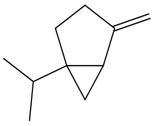
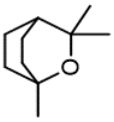
The measured contents and composition of terpenoid compounds emitted by the conifers to air (volatiles) and their content in phloem and wood are different. The variations observed can be attributed to diverse terpene collection methods, including air sampling through headspace techniques with quantification after each extraction utilizing aerated headspace columns, direct absorption of emitted monoterpenes (MTs) via solid microextraction, or extraction from the phloem using specific organic solvents (Hietz et al., 2005).

The differences are, to some extent, caused by the physical features of the compounds and possibly also by active transport between the surface and deeper layers of the bark (Baier and Bader, 1997b). The release of volatiles from spruce logs strongly intensifies at temperatures above 30°C (Baier and Bader, 1997a; Hietz et al., 2005; Kautz et al., 2013; Laffineur et al., 2011; Stříbrská et al., 2022).

The spruce phloem extractions consist of a variety of monoterpenoid hydrocarbons predominant in the low molecular fraction (Baier et al., 2002; Silvestrini et al., 2004), involving *β*-pinene (25-58%); *α*-pinene (23-39%); *β* -phellandrene (5-19%); myrcene (1.6-3.4%); limonene (1.5-4%); Δ-caren (0.6-1.1%); camphene (0.2-1.1%) (Netherer et al., 2021). Spruce phloem extracts also consist of several percent of oxygenated monoterpenes. Sesquiterpenes, which are less volatile, occur in much smaller fractions than MTs and additionally, phloem contains diterpenes and oxygenated diterpenes with high viscosity (Schiebe et al., 2012; Zeneli et al., 2006; Zhao et al., 2011b). The constitutive composition of terpenoid substances in the bark of standing trees is subjected to seasonal changes, with an increasing proportion of oxygenated compounds in older trees (Schönwitz et al., 1990).

The contents of terpenoid compounds also vary in different tissue of the tree. In seedlings and older Norway spruce trees, terpenes were extracted from needles, phloem, and sapwood and were found of differential composition and concentration. Some compounds found in the bark of Norway spruce have been identified to be of high ecological relevance for *I. typographus* (Table 1; Suppl. Table 1 on pages 132-135).

Table 1 Main terpene compounds in the Norway spruce with relation to *Ips typographus* (IT). Monoterpene hydrocarbons (MTs), oxygenated monoterpenes (MT-Ox), methyl jasmonate (MeJA) (Netherer et al., 2021).

Compound		**Bark beetle (*Ips typographus*)**
Tree / MT		**Ecological relevance**
α -pinene		Host recognition and attack success; IT pheromone precursor, higher enantiomeric proportion in more attractive (?) but lower in killed trees (?)
3-carene (Δ-3-carene)		↓abs in IT killed trees (MeJA) (+)-3-carene suggested as chemical marker of tree resistance
limonene		IT antifeedant
myrcene		↓abs in IT killed trees treated with MeJA
sabinene (4(10)-thujene)		↓abs in IT killed trees treated with MeJA
Tree / MT-Ox		
1,8-cineole (eucalyptol)		Antiattractant:↑abs emission in felled IT attacked trees, ↑abs in bark of IT surviving trees, ↓abs in IT killed trees True resistance marker for tree survival!
trans-4-thujanol (sabinene hydrate)		Antiattractant; ↓abs with tree age, ↑abs emission in felled IT attacked trees

An important feature is the terpenic enantiomeric composition, which is highly diverse among and within geographic areas (Persson et al., 1996) and also between trees of different genetic origin (Erbilgin et al., 2017). Different enantiomers have a different ecological relevance related to *Ips typographus*, e.g. (+) and (-)-*α*-pinene or (+) and (-)-limonene (Netherer et al., 2021).

Total concentrations of constitutive or induced terpenoid defences, or concentrations of specific compounds, play a decisive role in determining whether trees are attacked or not during an outbreak or when baited with pheromones, such as 1,8-cineole (Schiebe et al., 2012; Zhao et al., 2011a). Similar phenomena have been observed in other conifer-bark beetle systems, such as *Pinus ponderosa* and mountain pine beetle, *Dendroctonus ponderosae* (Erbilgin et al., 2017). Vigorous trees are expected to be provided with high constitutive protection skills based on terpenes stored in channels and reservoir cells and either water-soluble or polyphenolic compounds produced and stored in polyphenolic parenchyma cells (Franceschi et al., 2005).

Changes in terpene contents induced by drought and biotic stressors

Only a few studies report on the effects of drought on mature and previously healthy trees. Analyses of terpene contents were mostly focused on pine species, needles more than phloem, and trees of various age classes (Branco et al., 2010). The effect of drought on terpene concentrations and emissions were not found to be consistent in all Pinaceae (Holopainen et al., 2018; Lewinsohn et al., 1993). In the needles of seedlings and saplings of severe drought-stressed Scots pine and Norway spruce, the total and individual amounts of MTs (e.g., *α*-pinene, limonene, tricyclene, and camphene) and sesquiterpenes increased significantly. At the same time, diterpene resin acids showed a decreasing trend (Sancho-Knapik et al., 2017). In another study in the context of drought, the emission of volatile compounds and terpene synthesis have been observed to increase with rising water stress but eventually decrease under severe drought conditions (Holopainen et al., 2018; Lieutier, 2004).

Young trees were found to invest in terpenoid defence even under strong resource limitations (Huang et al., 2019; Kelsey et al., 2014). However, in studies exploring the response of *Pinus banksiana* and *Pinus ponderosae* to drought, trees synthesized lower amounts of MTs in fungal lesions compared to well-watered seedlings and trees (Hussain et al., 2020; Lusebrink et al., 2016).

Induced tree defences by bark beetle feeding, MeJA, and fungus interaction

When a herbivore insect attacks the tree, a biochemical process is triggered, activating stress hormones like jasmonic acid, which facilitates terpene synthesis. During the early stages of a bark beetle infestation, constitutive terpene storages are opened, leading to an increased emission (20-100 times) of main terpenic volatile substances in the surrounding area of the infested tree (Ghimire et al., 2016; Jaakkola et al., 2022).

The capacity of trees to induce defences against bark beetles, whether through the beetles' feeding or their symbiotic fungi inoculation, can be evaluated using either fungal inoculation or treatment with methyl jasmonate (MeJA), an analogue of the stress hormone jasmonic acid (Zhao et al., 2010). External application of MeJA triggers the biosynthesis of mono- and diterpenes by affecting the biosynthetic pathway of these compounds (Martin et al., 2002) by enhancing the terpene synthase activity, which regulates the *de-novo* synthesis of terpenes in Norway spruce needles (Martin et al., 2003).

Methyl jasmonate is used to evoke tree defence accumulation of terpenes (Sancho-Knapik et al., 2017), including the increased oxygenation of MTs (Novak et al., 2014; Schiebe et al., 2019).

Oxygenated monoterpenes

Oxygenated forms of monoterpenes are essential compounds for the communication between Norway spruce under stress condition and bark beetles as well as their predators (Moliterno 2023).

Spruce trees emit oxygenated monoterpenes, including 1,8-cineole, *α*-terpineol, camphor, carvone, terpinen-4-ol, isopinocamphone and pinocamphone. In a healthy tree, the oxygenated MTs are represented only in trace amounts, produced by microbial organisms or derived from autoxidation (Kandasamy et al., 2023). It has been documented that oxygenated MTs are accumulated via induction of tree defence and over time in degrading tissue of felled and attacked Norway spruce (Leufvén and Birgersson, 1987; Pettersson and Boland, 2003; Schiebe et al., 2019) or after infestation by bark beetles or ophiostomatoid fungi (Kandasamy et al., 2023). The importance of oxygenated MTs in conifer bark was underestimated for a long time despite their role in the pheromone biosynthesis of numerous bark beetle species (Francke and Vité, 1983). However, these compounds trigger a significant electrophysiological response in the antennae of *I. typographus* (Kalinová et al., 2014), with beetles possessing specialized sensilla for their detection (Andersson et al., 2009). Furthermore, the oxygenated monoterpenes play an attractive or repulsive role for bark beetles in the field, which was found in trapping experiments. For example, the *trans*-4-thujanol and 1,8-cineol are potent repellents for bark beetles (Jirošová et al., 2022a), and conversely, isopinocamphone tested in high doses revealed a robust signal for both bark beetles and their predators (Moliterno et al., 2023; Petterson & Boland, 2003).

Several studies on stress-related biochemical regulation of oxygenated MTs (Leufvén and Birgersson, 1987), inner transformation of verbenol by yeasts growing in a bark beetle brood gallery (Davis, 2015) and emission of pheromones by blue-stain fungi (Cale et al., 2019) revealed the role of interaction between these compounds in beetle communication and induced tree defence (Leufvén and Birgersson, 1987).

2.5.4 The role of phenolics in tree defence against bark beetles

Phenolic substances have been recognized for their potential to protect trees against oxidative or water stress (Rosemann et al., 1991; Song et al., 2016) and fungi associated with bark beetles (Brignolas et al., 1998; Hammerbacher et al., 2011). Polyphenols are crucial for the prevention of DNA damage in stressed trees (Hammerbacher et al., 2014; Urbanek Krajnc et al., 2014), but the influence of drought on biosynthetic pathways is still poorly understood. The phenolic substances, like flavonoids and stilbenes, originate from the polyphenolic parenchyma (PP) cells, which persist even in 100-year-old trees (Li et al., 2012). The biotic stressors can induce the activation of PP cells, leading to acquired/induced systemic resistance of trees (Mageroy et al., 2020).

The defence of trees against bark beetles based on phenolic compounds was studied on several levels. In feeding bioassays using artificial diets, the impact of phenolic compounds on male and female *I. typographus* were found to variable. Faccoli and Schlyter, (2007) reported low anti-feedant effects, even at high catechin doses, while Hammerbacher et al., (2019) demonstrated a significant reduction in tunnelling lengths in substrates enriched with taxifolin or a taxifolin-catechin mixture. In a recent field study, the application of phenolic estragole, (4-allylanisole) in high doses decreased the attraction of pheromone traps to *I. typographus* (Moliterno et al., 2023). However, *E. polonica*, a common fungal associate of the spruce bark beetle, metabolizes taxifolin and catechin (Wadke et al., 2016). Other ophiostomatoid fungi, like *G. penicillata*, degrade stilbene glucosides, benefiting bark beetles by depleting tree defences and potentially enhancing larval breeding substrates (Hammerbacher et al., 2013).

3. Methods

This chapter describes the study areas, the experimental setup and the methods used for statistical analyses in the three publications that are the basis for my dissertation thesis.

3.1 Study areas

The fieldwork was mainly carried out at the experimental plots established in the school forest enterprise, situated near Kostelec nad Černými lesy and maintained by researchers of EXTEMIT-K. The experimental sites are located in mature Norway spruce forests (90 - 110 years old) and are not connected to each other.

For the first publication (Chapter 4.1), two plots (number 3 and 4) were used close to the village Stříbrná skalice (49.913 N, 14.863 E). Every plot was divided into four subplots (A,B,C and D) with different treatments, two freshly cut forest edges (FE – A and B), and two inner forest controls (IF – C and D) (Fig. 8). In every subplot, sensors were installed to measure several physiological and meteorological conditions.

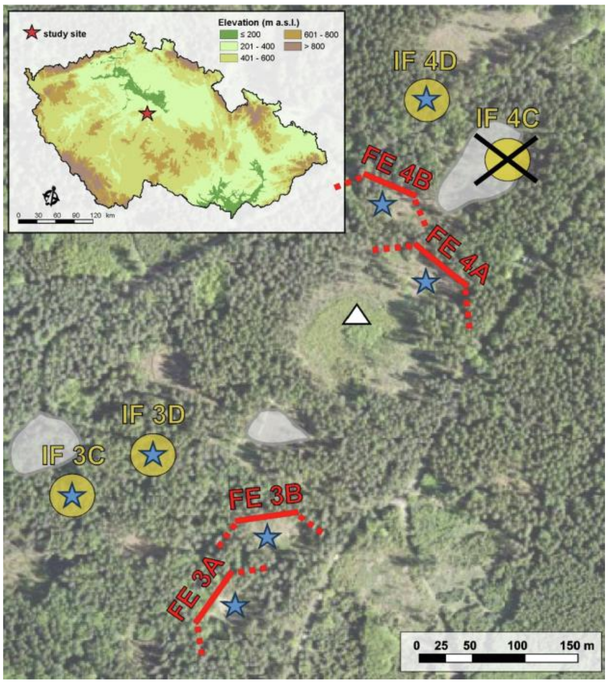


Figure 8 First publication study area (Chapter 4.1). **Arrangement of experimental subplots and forest edges** (Photo by P. Surovy). Red lines mark the positions where fresh FEs were established next to subplots A and B; yellow circles represent the IF subplots, which correspond to subplots C and D. Subplot 4C in IF was infested by *Ips typographus* and salvaged, and its data was not included in the analyses. The white triangle marks the meteorological station. FE, forest edge; IF, inner forest (Stříbrská et al., 2022).

The study for the second publication (Chapter 4.2) was also conducted close to the village of Stříbrná skalice (49.913 N, 14.863 E). Four plots in the inner forest (A-D, IF) were used for the experiment, from which one was naturally attacked on the 16th of June 2020 (Plot C, Fig. 9). Again, meteorological and physiological data was recorded.

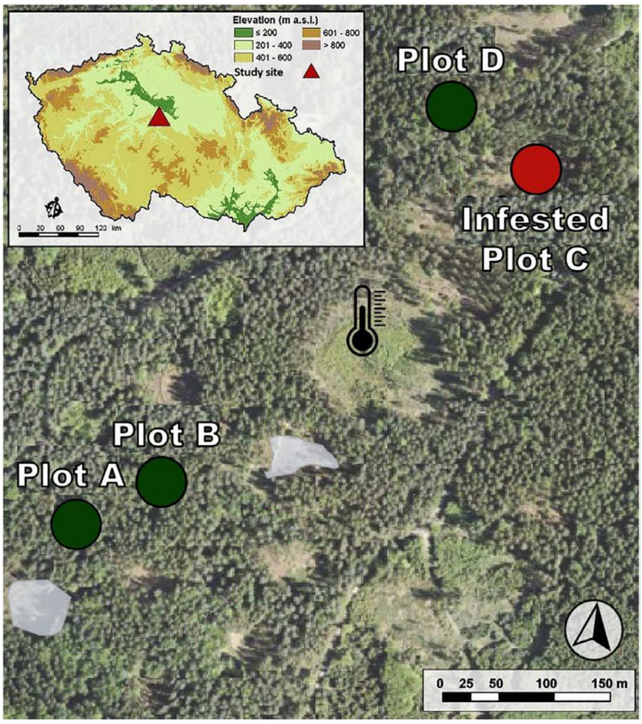


Figure 9 Second publication study area (Chapter 4.2). **Arrangement of study plots with the naturally attacked plot**. Green circles represent the inner forest plots (non-attacked trees), corresponding to plots A, B, and D. Plot C was infested by *I. typographus* on June 16th and salvaged in July (red circle). The grey spots are areas of previous natural bark beetle attack. The black thermometer marks the meteorological station (Stříbrská et al., 2023a).

The third study (Chapter 4.3) described observations at two experimental trees. Both trees were in the early attack stadium, when male beetles are excavating nuptial chambers . The first tree (Fig.10) was located close to the plots near Stříbrná skalice (49.913 N, 14.863 E), and the second tree (Fig.11) was situated close to the village Vyžlovka (49.981 N, 14.801E).

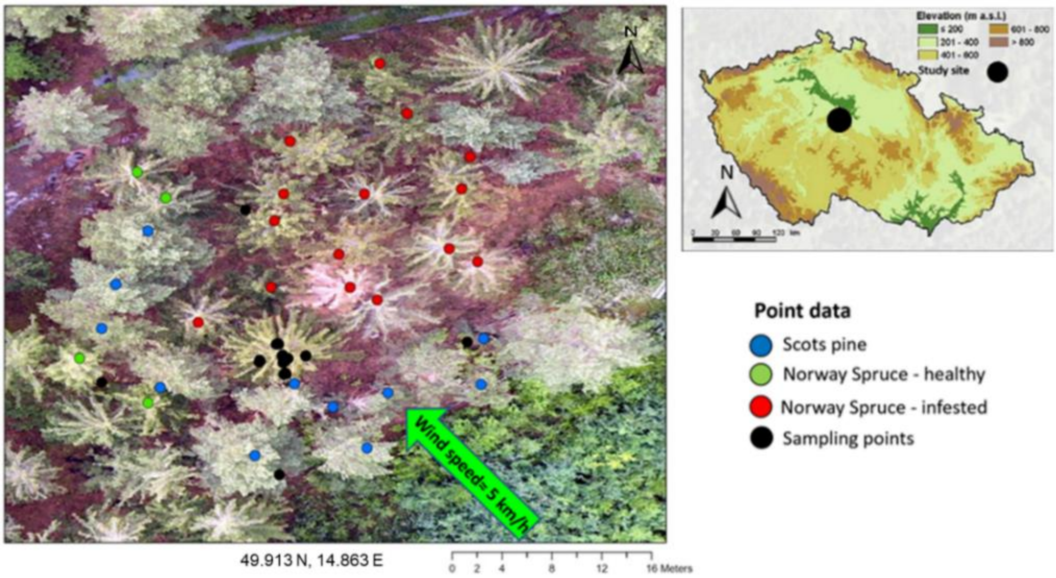


Figure 10 Third publication study area (Chapter 4.3). **The study site for the first forest spatial VOC measurement around the infested tree (June 30th 2022), Stříbrná Skalice**. Black points – sampling points around the sampled bark beetle infested tree, in nuptial chamber infestation stadia; Red points – Norway spruce infested trees in later stadia of infestation or dead; Green – healthy Norway Spruce trees; Blue points – Scot pines (Stříbrská et al., 2023b).

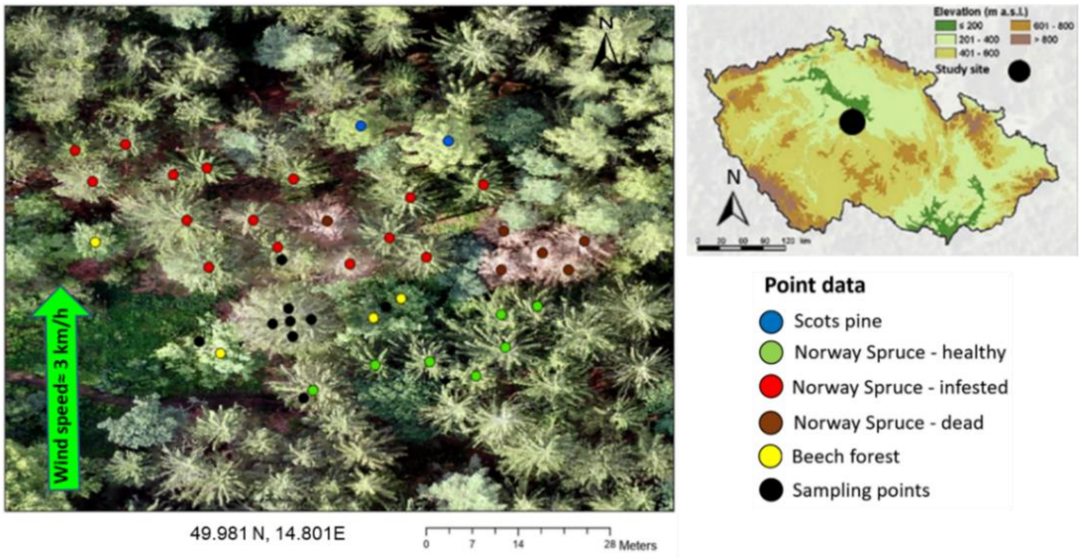


Figure 11 Third publication study area (Chapter 4.3). **The study site for the second forest spatial VOC measurement around the infested tree (August 24th 2022), Vyžlovka.** Black points – sampling points; Red points – Norway spruce infested trees; Green – healthy Norway Spruce trees; Brown points – dead Norway Spruce trees; Blue points – Scot pines and Yellow points – beech trees (Stříbrská et al., 2023b).

3.2 Experimental setup

This chapter describes the methods used for clarification of the questions based on the aims of this thesis, such as meteorological conditions and physiological changes in study trees, tree defence characteristics and beetle host acceptance bioassays.

3.2.1. Meteorological conditions and physiological changes

For publications 1 and 2 (Chapters 4.1 and 4.2), we recorded the meteorological parameters at the study sites and physiological data of individual experimental trees. Meteorological data were recorded by a meteorological station near the experimental plots, and soil water potential was measured by hexagonal sensors (EMS Brno) buried 20 cm below the surface. Five sensors were installed at each subplot, and data were recorded hourly. Sensors were installed to measure the tree diameter changes in sap flow, and thermometers were installed to measure bark temperature from the north and south directions (Fig. 12).

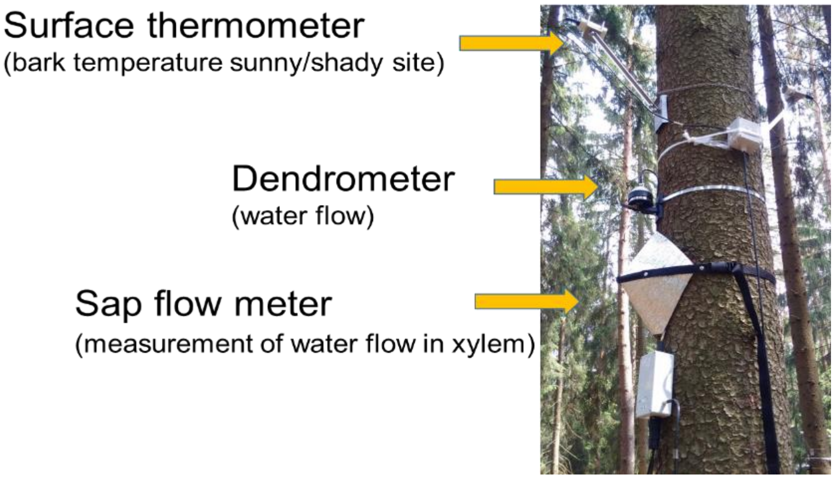


Figure 12 Physiological sensors placed on the experimental trees.

3.2.2. Tree defence characteristics

Further, resin flow and VOC’s were measured. On the same trees, resin flow measurements were made using glass sticks inserted into a hole made by a cork borer. The glass stick's position in our experiment differed from Netherer et al., (2015). We positioned the glass stick to the south side of the tree (Fig. 13), and one stick per tree, resin flow (in mm), was measured after 24 hours.

Figure 13 Collection of resin

The VOCs were measured in three ways: by headspace analyses with the use of SPME fiber, air pump analyses with cartridges, and by measuring the content of VOCs from phloem. The SPME fiber was placed in a collection chamber (aluminium box) (Stříbrská et al., 2022), which was attached to the tree trunk (Fig. 14A). After being thermally desorbed, volatile profiles were measured by GCxGC-MS/TOF.

For phloem analysis, we sampled bark plugs of 8 mm diameter. These phloem samples were stored in liquid nitrogen until further lab processing. The preparation of the samples for measuring included the grounding of the phloem sample to a powder (Fig. 14B) and then extraction for 10 min in 2 mL hexane (containing 5 µg/mL of the internal standard 1-bromododecane) in an ultrasonic bath for 10 min. Extracts were filtered into 2 mL vials for GC-MS.

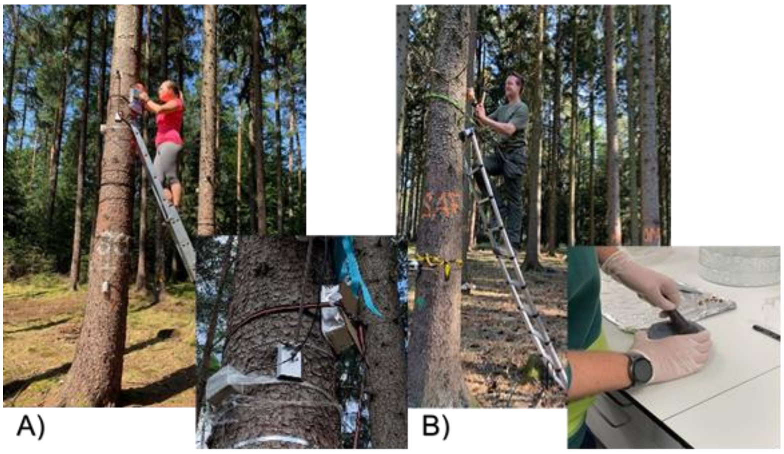


Figure 14 Collection of VOC´s. A) SPME collection; B) phloem collection

3.2.3. Beetle host acceptance and choice bioassays

In our studies, we used beetle host acceptance field bioassays. First to mention was the choice experiment, for which we used attack boxes of similar design as described by Netherer et al., (2015). After mounting of the box to the tree trunk, 20 beetles were inserted in the start bottles and beetles were left for 24 hours in the attack box system. The beetles were free to stay in the start bottle or go to into the main box. Once being in the box, beetles could either bore into the stem or remain in the box. If they liked to leave, they had to go through the hose, which was connected to the exit box, of which the lid was covered by a net to be permeable for the odours from the forest environment (Fig. 15).

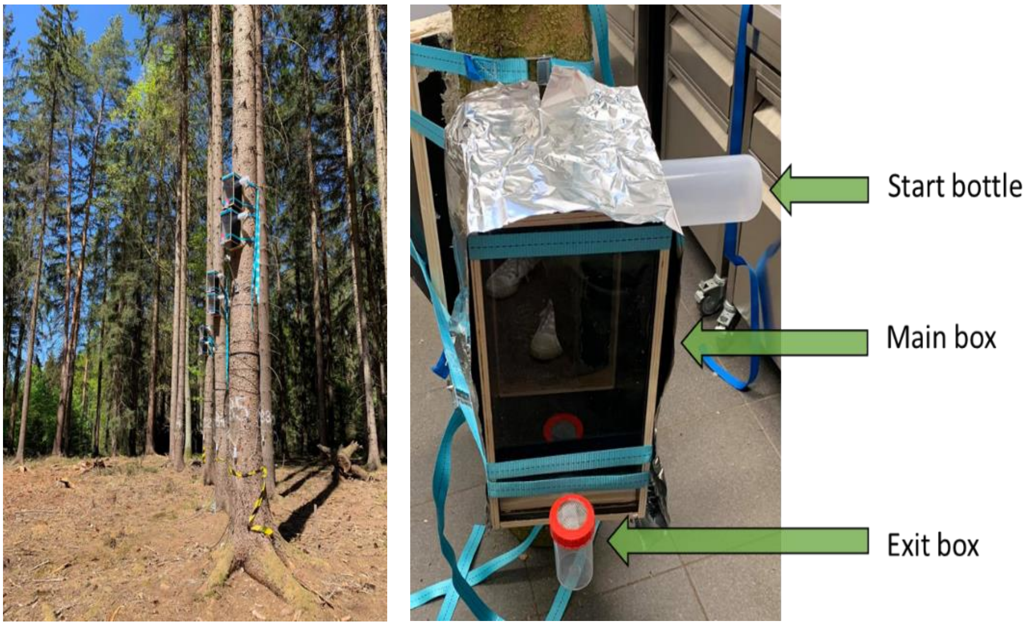


Figure 15 Choice beetle bioassays with attack box.

No-choice assays were conducted on the selected tress by introducing 10 male beetles/per tree (one beetle per Eppendorf tube) (Fig. 16), respectively, this experiment was based on research of Turčáni and Nakládal (2007). Sexing of live Eurasian spruce bark beetles (*I. typographus*) is done based on morphological characteristics by Schlyter and Cederholm (1981). Observations were recorded after 24 hours to document the number of beetles that bored into the tree bark or were prevented from boring by resin flow.

Figure 16 No-choice bioassay with male *I. typographus* in Eppendorf tubes.

Based on the research of Schlyter, Lofqvist and Byers (1987), passive traps without pheromone lures were installed on the study trees. On every measured tree a trap was attached to the southern trunk side, with a size of 40 × 60 cm and made of transparent plastic (Fig. 17). The number of beetles that were caught were counted every week from April until September.

Figure 17 Passive trap without pheromone lure.

3.3 Statistical analyses

Statistical analyses were conducted in R software (R Core Team 2021 and 2022) as described in chapters 4.1 and 4.2 and by use of Statistica software (version 14.0.0.15) as described in chapter 4.3.

Regarding the results reported in Chapter 4.1, a mixed model was used (concerning the repeated measures together with other factors), which uses restricted maximum likelihood methods to estimate variance components. The Shapiro-Wilk test was used to test normality, and the test against heteroskedasticity, the Breusch–Pagan test, was used. We got non-parametric data, so the differences between FE/IF were evaluated using the Mann–Whitney–Wilcoxon test, and Spearman’s rank correlation was counted.

Data from SPME analyses were evaluated by using PCA and partial least square-discriminant analysis (PLS-DA) in SIMCA 15 software (Chapter 4.1) and the SIMCA 17 software (Chapter 4.2).

For the analyses described in Chapter 4.2, a general linear mixed model was used. The repeated measures model was fit with the fixed effect of measurement time, bark beetle attack, and random effect of the plot. We used the gamma distribution with a log link; in cases of count data in response (number of beetles), we used the Poisson distribution. Post hoc Tukey analyses between infested and non-infested trees in the overall repeated measures model and inside each measurement separately were performed using lsmeans() function from the lsmeans package.

Based on the Shapiro-Wilk test results for testing normality, the Kruskal-Wallis tests were used to count the results in Chapter 4.3. These tests compared the individual levels and distances in attacked trees. Lately, post hoc tests have been conducted to examine differences between repetitions.

4. Results

The dissertation thesis consists of three published manuscripts. The first part evaluates tree physiology influenced by sun irradiation at freshly cut forest edges and the trees' susceptibility to bark beetle attacks (Chapter 4.1). The second part examines the differences between the tree physiological and biochemical characteristics of naturally attacked trees and trees not attacked by bark beetles (Chapter 4.2). The third part represents a pilot study on the 3D distribution of *α*-pinene emitted by Norway spruce freshly infested by *Ips typographus* (Chapter 4.3).

4.1 Forest margins provide favourable microclimatic niches to swarming bark beetles, but Norway spruce trees were not attacked by *Ips typographus* shortly after edge creation in a field experiment

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This article hypothesizes that “Norway spruce trees on forest edges exposed to sudden sun irradiation are more susceptible to bark beetle attacks.” The main objectives of this work are monitoring the microclimatic and soil hydrological parameters. The study comprised the measurement of tree physiological characteristics, such as sap flow, resin flow, and tree stem temperature on trees of the Forest Edge (FE) and located in the Inner Forest (IF). Moreover, we measured the concentrations and profile of terpenes in phloem and tree emissions on FE in comparison to IF trees. Bark beetle host acceptance behaviour was study by use of attack box bioassays on all study trees.

This article shows that creating forest edges alters microclimate and tree defence against *Ips typographus*. The bark surface temperatures, together with defence parameters (resin flow and VOC emissions), increased at FE trees. Considering study limitations in cool spring conditions and unexpected beetle behaviour, fresh forest edges do not appear specifically susceptible to *I. typographus* attacks shortly after establishment. While microclimatic conditions favour beetle activity, delayed attacks suggest timing and environmental attractiveness may influence infestation probability. In host acceptance bioassays, beetles moved more actively at FE trees in spring and attacked trees mainly in August.

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Forest margins provide favourable microclimatic niches to swarming bark beetles, but Norway spruce trees were not attacked by *Ips typographus* shortly after edge creation in a field experiment

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ABSTRACT

Mass outbreaks of the European spruce bark beetle, *Ips typographus*, have caused devastating damage to Norway spruce-dominated forests in Central Europe. Biotic and abiotic natural disturbances as well as logging activities promote the fragmentation of forest stands, further increasing their susceptibility to damaging events. The creation of forest edges abruptly alters microclimatic conditions, such as trees suddenly being exposed to the sun. A common hypothesis is that trees remaining at the margins of clear cuts are initiation spots for spruce bark beetle infestation. This study comprehensively investigates the direct effects of forest edge establishment in spring on air and bark temperatures, soil water potential, tree sap flow, resin flow, bark defence compounds, and olfactometric cues for bark beetles provided by volatile organic compounds (VOCs) in proximity to trees. *Ips typographus* host acceptance was tested in limited-choice (attack box) and non-choice (Eppendorf tubes) field bioassays.

Bark surface temperatures and resin flow clearly increased in trees of the freshly cut forest edges in spring, while sap flow rates did not differ from those of shaded trees in the inner forest. Soil water potential was high throughout the study season owing to sufficient precipitation and decreased only in August. Contrary to similar amounts of monoterpenes in the phloem of inner forest and edge trees, VOC concentrations were enhanced in May and June at the forest margin, also due to the presence of fresh logging residuals. The increased bark surface temperatures and VOC concentrations near forest edge trees were observed simultaneously with enhanced moving activity of *I. typographus* in the attack box bioassays soon after edge establishment in May. The effectiveness of increased resin flow in spring as a contributor to defence of edge trees could not be evaluated in the attack box bioassays, as boring holes at both edge and inner forest trees were observed, mainly later in the season. We conclude that fresh forest edges are not more susceptible to *I. typographus* attack shortly after establishment than closed forest stands but that microclimatic conditions of forest margins and olfactory signals from standing edge trees and logging residuals favour beetle swarming. Spruce bark beetle infestation patterns in fragmented forests are likely dependent on population densities and timing of predisposing abiotic disturbance and harvesting events.

1. Introduction

The fragmentation of Central European forests, which show patterns of variably sized gaps and stand edges, is promoted by logging activities and natural disturbance events (Pöpperl and Seidl, 2021). Although abiotic and biotic disturbances occur naturally in forest ecosystems

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(Attiwill, 1994), the increasing incidences of drought and insect herbivore infestations, such as bark beetle outbreaks, have been associated with global climate change (Bentz et al., 2010; Reed and Hood, 2021). In Central Europe, the most economically important consequence of intensified disturbance regimes is the extensive mortality of Norway spruce, *Picea abies (L.)* Karst. (Pinales: Pinaceae), which is caused by outbreaks of the Eurasian spruce bark beetle, *Ips typographus* (L.) (Coleoptera: Curculionidae: Scolytinae) (Marini et al., 2017; Seidl et al., 2016; Stadelmann et al., 2014). In particular, in the Czech Republic, the total volume of trees killed by *I. typographus* increased exponentially from 1.5 million m^3 timber annually in 2003–2015 to 23 million m^3 timber in 2019 (Hlásny et al., 2021). We can conclude with certainty that Norway spruce monocultures outside their natural habitats, namely, at lower elevations and latitudes, are highly susceptible to mass outbreaks of spruce bark beetle. Yet, many aspects of the interactions between host trees and bark beetles in a changing climate are not fully understood (Biedermann et al., 2019; Huang et al., 2020; Netherer et al., 2021).

An important question is whether trees remaining at the margins of clear-cuts or storm damaged patches are common initiation spots of spruce bark beetle infestation, and if so, why this is the case. The altered microclimatic conditions of freshly created forest gaps and edges, including sudden exposure of trees to increased solar radiation, are hypothesised to impair the defence ability of edge trees and enhance their susceptibility to *I. typographus* attack (Kautz et al., 2013; Mezei et al., 2019). Increased attack of susceptible forest edges would imply that pioneer *I. typographus* males recognise and deliberately select spruce trees with weakened defences (Netherer et al., 2016; Raffa et al., 2016). Such socially suppressed or single storm-felled trees are in fact the main niche for colonisation at low spruce bark beetle population levels. In the transition phase from endemic occurrence to mass outbreaks, population build-up is often initiated by favourable climatic conditions in forest areas fragmented by wind damage, clear cutting, and salvage logging after larger storms or other biotic damage events (de Groot et al., 2018; Kärvemo et al., 2016; Kautz et al., 2013). The ample supply of breeding habitats in storm-felled trees that completely lack induced defence mechanisms facilitates mass attacks and fast population growth of bark beetles (Hroššo et al., 2020; Kärvemo et al., 2014; Schroeder, 2010). Norway spruce trees growing near newly created forest gaps and at stand edges might still be well defended; however, abrupt changes in microclimatic conditions due to the removal of neighbours can acutely stress and weaken the trees (Jakus et al., 2011; Mezei et al., 2014).

Spruce trees at freshly cut forest edges (FEs) and forest margins created by storm throw are suddenly exposed to direct solar radiation, increased temperature, and drought conditions (Brooker, 1985; Keenan and Kimmins, 1993; Mezei et al., 2019). Moreover, harvesting activities and the mechanical forces of wind and other abiotic disturbance events often result in severe root and crown damage to residual edge trees (Picchio et al., 2020; Rizzo and Harrington, 1988). External stressors influence the physiology and biochemistry of trees, with strong effects on transpiration and sap flow as well as constitutive and induced defence traits, such as resin flow. Most notably, abiotic and biotic stress can induce changes in the bark contents of defence proteins and secondary metabolites, such as terpenoid and phenolic substances, alkaloids, and of (Franceschi, et al., 2005; Huang et al., 2020; Netherer et al., 2021). Here, we pose the question whether acute stress-induced changes in primary and secondary metabolism render spruce trees at FEs more susceptible to *I. typographus* attack than trees growing in the inner forest (IF) or, quite contrary, even strengthen tree defence.

In fact, levels of stress and chemical defence reactions are not linearly related in conifer trees. Field studies in conifer forests have shown that resin flow decreased or even ceased in instances of severe water deficit but tended to increase with mild to moderate drought (Baier et al., 2002; Gaylord et al., 2013; Netherer et al., 2015). Short to medium term reactions to abiotic and biotic stress also include the creation of traumatic resin ducts (Baier et al., 2002; Netherer et al., 2021), potentially providing additional sustainable protection to trees against biotic invaders.

In what ways do the sudden changes in tree physiology and metabolism on the one hand, and the requirements and mechanisms of host selection and acceptance in *I. typographus* on the other hand, play crucial roles in the attractiveness of FEs as targets of infestation? Primary factors influencing tree susceptibility to bark beetle attack are wind and increased temperature conditions in the surroundings of edge trees and at those parts of the trunk exposed to the sun (Marešová et al., 2020). Exposed, warm stand edges can be much more favourable for swarming insects than the cooler environments of IF zones, particularly in early spring (Müller et al., 2007). Air temperatures required for spring flight of *I. typographus* occur earlier in the year outside closed forest stands, and on the bark surface and in the phloem of exposed edge trees. Furthermore, areas of sunlit bark offer spruce bark beetles optimal temperature conditions for breeding, as long as temperatures do not exceed the lower and upper thresholds for development (38.9 °C). Apart from temperature, movement of spruce bark beetles is strongly influenced by local wind regimes and structural features of the forested landscape. Swarming was found to be concentrated in the transition zones between wind-protected inner forest areas and wind-exposed clearings (Mezei et al., 2011; Netherer and Hammerbacher, 2022). In addition, stand edges are the first structural elements to be approached by bark beetles when leaving their overwintering sites outside closed forest stands and searching for host trees.

Norway spruce growing at forest edges have silhouettes and emit chemo-sensory signals that serve as visual and olfactory cues for swarming beetles (Netherer et al., 2021). Although there is no evidence for long-distance attraction of *I. typographus* by monoterpenes emitted from tree bark (Byers, 2000), exclusive random landing of pioneer beetles is unlikely, given the numerous sensory cells in spruce bark beetle antennae that are specific for monoterpene hydrocarbons (Andersson, 2012; Andersson et al., 2013; Byers, 1989, 1999; Kalinová et al., 2014; Kandasamy et al., 2019). Selection of individual trees more likely happens at close range (Saint-Germain et al., 2009, 2007), and further aggregation of males and females occurs in response to the combined, attracting effects of the monoterpene α-pinene and spruce bark beetle pheromones (Erbilgin et al., 2007). We presume that the concentrations and mixtures of odour clouds are quite different along a sun-exposed FE compared to the cooler IF zone. Increased temperature significantly affects the emission of volatile organic compounds (VOCs) from tree bark and needles and from organic material that often accumulates at forest edges, such as cutting residuals (Holopainen et al., 2018). In a recent study, higher concentrations of α-pinene and β-pinene were measured close to spruce trees growing at the FE compared to trees of the IF zone, corresponding with increased bark surface temperatures of the edge trees (Marešová et al., 2020).

In this study, we hypothesised that there is a non-linear relationship between the stress states of Norway spruce host trees in response to their sudden exposure at newly created FEs and their disposition to *I. typographus* attack. Gradual changes in microclimatic conditions, tree physiology, and tree defence capability were observed over a period of four months from the establishment of the stand edges in spring. The observation period covers the main season of *I. typographus* spring and summer flight, and development of the new generation. The experiment was conducted in a mature Norway spruce forest in central Czech Republic by cutting four clearings in April 2020 to create fresh FEs exposed to the sun. The main objectives of this study were as follows: 1) to continuously monitor microclimatic and soil hydrological parameters in IF and at FEs; 2) to study the physiological parameters, such as sap flow and resin flow, of the abruptly exposed trees at the newly created edges compared to control trees of the IF zone; 3) to examine the concentrations and profile of VOCs collected in tree bark and air near the study trees; and 4) to observe beetle host choice and host acceptance and the defence reactions of FE and IF trees to attacking bark beetles via field bioassays and observation of the natural infestation.

2. Materials and methods

2.1. Study site and experimental setup

The study was conducted at the property of the CULS Forest Enterprise in Kostelec nad Černými lesy, near Stříbrná Skalice town (Fig. 1), from 21st April to 18th August 2020. Originally eight subplots monitored for soil water potential and sap flow were established in 2018 in two 90 to 95-year-old allochthonous Norway spruce stands, at an altitude of 430 m above sea level. One of these subplots was infested by *I. typographus* in 2020 and was cut down. Distance between the subplots ranged from 60 to 120 m. Their GPS coordinates are provided in Table 1.

The potential natural vegetation of this geographic region is beech and oak forest. The terrain is quite flat, and soils supply moderate nutrients and are partially gley-type. The study region is considered a warmer and drier area, characterised by a mean annual temperature of 7–7.5 °C in the vegetation season (150 days longevity) and the mean annual sum of precipitation is 600 mm (Tolasz 2007).

The experimental site was established in 2020 and required creation of four FEs at the most appropriate original subplots. Four clearings were cut from the south-west and south-east sides on 21st April 2020 to create four FEs that were approximately 50 m long (Fig. 1). The width of the clearing areas approximately equalled the stand height (30 m), and their margins extended at a 45° angle to the front of the FEs. At each FE, three Norway spruce trees were selected for monitoring, resulting in a total of 12 FE trees that were monitored.

Table 1
GPS coordinates of experimental subplots.

Latitude	Longitude	Subplot	Treatment
49.910822	14.873961	3A	FE
49.911366	14.874597	3B	FE
49.912771	14.873291	3C	IF
49.912819	14.873778	3D	IF
49.913641	14.876792	4A	FE
49.914351	14.876360	4B	FE
49.914527	14.877892	4C	IF
49.914667	14.877344	4D	IF

Four different subplots were assigned as control stands located in the closed IF zone. Three control trees were selected in each of the control subplots. Finally, nine control trees remained of the original twelve, as subplot 4C was infested by bark beetles and cut down in June 2020.

At all four FEs and three IF subplots, particularly on 12 FE trees and 9 IF trees, bark beetle attack experiments were performed on seven dates (7–8 May; 19–20 May; 27–28 May; 16–17 Jun; 24–25 Jun; 30 June–1 July; and 11–12 Aug) during the study period 2020. Each experimental repetition involved measurements of tree traits (resin flow, air VOCs) and the performance of field bioassays on two consecutive days (see

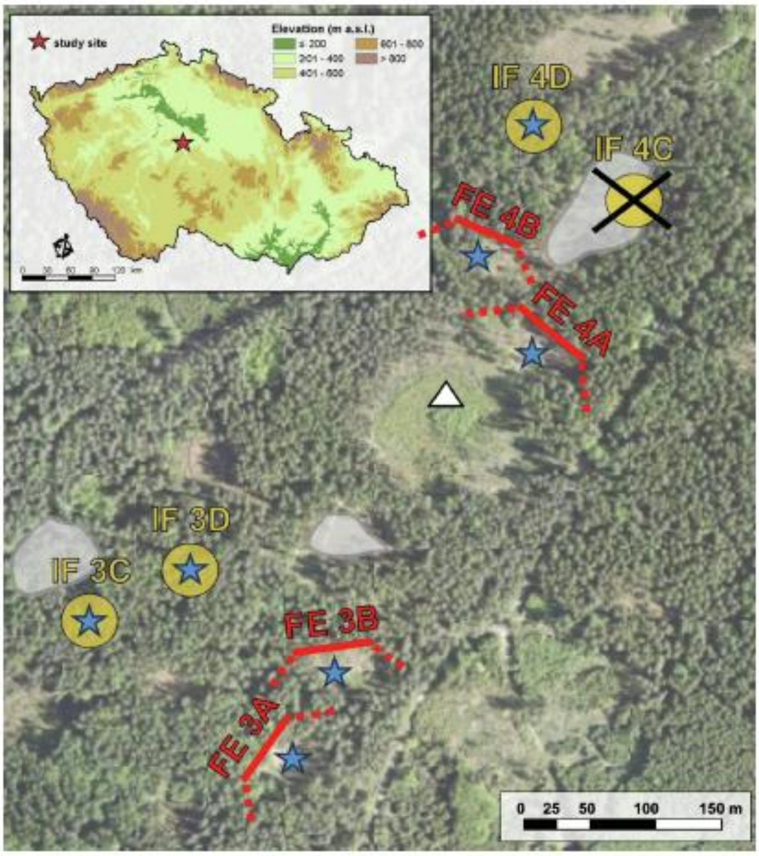


Fig. 1. **Arrangement of experimental subplots and forest edges (Photo by P. Surovy).** Red lines mark the positions where fresh FEs were established next to subplots A and B; yellow circles represent the IF subplots, which correspond to subplots C and D. Subplot 4C in IF was infested by *Ips typographus* and salvaged, and its data was not included in the analyses. The white triangle marks the meteorological station. FE, forest edge; IF, inner forest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

details below).

2.2. *Meteorological parameters*

A meteorological station was installed at a central point near the subplots (Fig. 1) to monitor air temperature and humidity (Minikin QTHi, EMS Brno, Czech Republic). Precipitation was recorded by Minikin ERi with a Pronamic Pro Rain gauge (EMS Brno). The photosynthetically active radiation (PAR) was measured by a Quantum Sensor EMS 12 (EMS Brno). The vapour pressure deficit (VPD) was calculated from the measured data using Formula (1) (Čepl et al., 2016; Goudriaan and Monteithf, 1990), as follows:

$$\mathrm{VPD} = \left(1 - \left(\frac{RH}{100}\right)\right) \times \mathrm{SVP} \tag{1}$$

$$SVP = 610.7 \times 10^{7.5\times T/(237.3+T)} \tag{2}$$

where RH is relative humidity, SVP is saturated vapor pressure, and T is the average temperature on the days of experimental repetition. The VPD, PAR, and precipitation are joint indicators of potential evapotranspiration of trees.

The 2020 vegetation season in the experimental region was characterised by warm and humid weather. The mean annual temperature was 1.6 °C above the long-term temperature average, and the aggregate annual rainfall of 620 mm exceeded the long-term average by 7% (counted from 1961). Of all the experimental dates, 7th May, 27th May, and 24th June were the coolest days, and 16th June, 30th June, and 11th August were the warmest. The detailed records of meteorological observations are listed in supplementary Table S1. The highest humidity, VPD, and PAR at the experimental site were recorded on 24th June, two days after a storm event that was accompanied by intensive rainfall (Fig. 2). Mean daily precipitation sums and air temperatures for 2020 recorded at the station of the Czech Hydrometeorological Institute in Ondřejov; 49° 54′ 54″ E; 14° 46′ 51″ N are listed in (Fig. S1A, Fig S1B).

2.3. *Bark surface temperature*

Temperature conditions at the surface of tree bark at the 12 FE and 9 IF trees were recorded by two infrared thermometers, which were installed 3 m above the ground on the south and north sides of the trunks of each monitored tree. The data were collected every hour, and daily average bark surface temperatures were calculated on each day of the experiment (24 h from the start of the field bioassays).

2.4. *Soil water potential*

Soil water potential was measured by hexagonal sensors (EMS Brno) buried 20 cm below the surface. Five sensors were installed at each subplot and data were recorded hourly. Average values for soil water potential were calculated for the week prior to the first day of the bioassays. Soil water potential was recorded at the subplots from May 2018.

2.5. *Sap flow*

Sap flow measurements were conducted at 12 FE and 9 IF trees. Measurement was based on the thermodynamic principle by heating the wood around the stainless steel electrodes, according to the trunk heat balance method (Čermák et al., 2004). The non-heated part of the wood is at a fixed distance from the heated part. The thermocouples record the temperature in the heated and non-heated parts of the trunk, and the transpiration rate is calculated based on the energy consumption (i.e. the energy needed to maintain a temperature difference of 1 K between the heated and non-heated parts of the trunk). Sap flow within the stem was recorded at 10 min intervals. During post-processing, a baseline was set to remove night-time fluxes, and sap flow rates were expressed in terms of kg per hour for all experimental days.

2.6. *Resin flow*

Resin flow of 12 FE and 9 IF trees was measured during all experimental repetitions, in parallel with the bioassays performed. For this purpose, one glass tube with an inner diameter of 3 mm, an outer diameter of 5 mm, and a length of 12 cm per tree/per repetition was inserted into holes (6 mm) drilled into the bark and phloem at breast height from the north-exposed side of the trunks. The exuded resin was collected for 24 h (start and end of experiments between 3 and 5 pm). The length of the resin column in the glass tube was measured on a millimetre scale to calculate the resin flow for 24 h per experimental day.

2.7. *Bark and VOC monoterpenes*

2.7.1. *VOC collection by solid phase microextraction (SPME)*

VOCs were collected on tree trunks of 12 FE and 9 IF trees at a height of 3.5 m using SPME fibre (PDMS/CAR/DVB, Supelco) VOC collection was performed for 60 min, from 1 to 2 pm on the sunnier and warmer day of each experimental repetition. We attached one aluminium chamber (25 cm × 5 cm × 5 cm) to the bark surface of each tree. The interface between chambers and bark was partially sealed by Teflon to minimise bark damage and SPME fibre contamination, and to reduce air interchange. Nevertheless, it was impossible to create an airtight seal because of the ragged bark surface. A pre-conditioned SPME fibre was inserted into the aluminium chamber and exposed to the air for 60 min. VOCs were additionally collected in the surrounding open space at approximately 20 m from the experimental trees in each experimental repetition. One SPME fibre per subplot (approximate positions are marked by blue stars in Fig. 1) was placed in aluminium boxes on 1.5 m high poles and exposed to the open air. Subsequently, a Thermogreen septum was placed in a closed 2 mL vial, penetrated with SPME fibre, and stored on dry ice to prevent desorption. Fibres were then transported to the laboratory, where they were stored at -25 °C before gas chromatography-mass spectrometry (GC-MS) analysis. Analysis was performed no later than 48 h after collection.

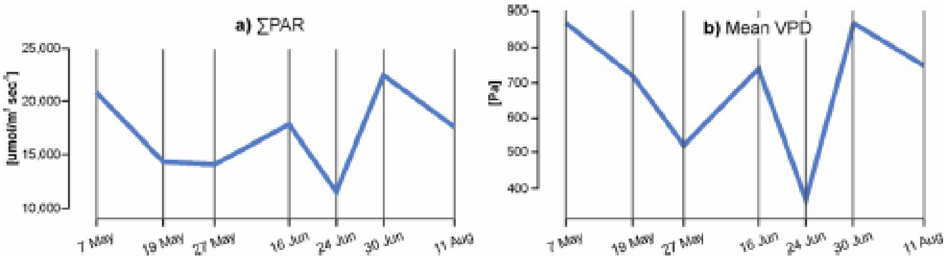


Fig. 2. **Sum of photosynthetic active radiation (∑PAR) and Average VPD** (daily average vapour pressure deficits) calculated for the times of experimental repetitions.

2.7.2. *Analysis of bark compounds by liquid extraction*

Three round phloem sections per tree with an 8 mm diameter were collected from 12 FE and 9 IF trees in the course of three experimental repetitions (27th May, 16th June, and 30th June). Sections were sampled by use of a cork borer at south sides of tree trunks in 15 cm distance from each other, stored in liquid N_2 and transported to laboratory for further processing. In the laboratory, outer bark was removed from samples, which were pooled for each tree, ground in liquid nitrogen to obtain a fine powder, and then extracted for 10 min in 2 mL hexane (containing 5 μg/mL of the internal standard 1-bromododecane) in an ultrasonic bath for 10 min. Extracts were filtered into 2 mL vials for GC–MS and stored at −80 °C before analysis.

2.7.3. *Gas chromatography–time-of-flight mass spectrometry (GC-TOF-MS)*

A gas chromatograph (Agilent 7890B; Agilent, USA) coupled to a mass spectrometer with a time-of-flight mass analyser Pegasus 4D (LECO, , USA) was used to separate, identify, and quantify the collected compounds. Separation was performed on a HP-5MS UI capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness; Agilent). A 2 min spitless period in hot PTV (265 °C) was used for SPME analysis. The temperature program of the GC oven was as follows: 40 °C for 1 min, followed by ramping at a rate of 15 °C min^{-1} to 210 °C and then at 20 °C min^{-1} to 280 °C. To analyse phloem extracts, 1 μL of extract was injected into a cold PTV injector (20 °C) in spitless mode. After injection, the inlet was heated to 265 °C at a rate of 8 °C/s. The GC oven temperature program was as follows: 40 °C for 1 min, followed by ramping at a rate of 5 °C min^{-1} to 210 °C and then at 20 °C min^{-1} to 320 °C, which was then held for 6 min. In both cases, ions (ionisation energy 70 eV) were collected in a mass range of 35–500 Da at a frequency of 10 Hz.

Automated spectral deconvolution and peak finding algorithms were carried out using ChromaTOF software (LECO). A built-in peak alignment tool, Statistical Compare, was used to align all chromatographic signals with a signal to noise ratio (S/N) higher than 50. To confirm the identity of the compounds, the retention times of the respective standards were used for comparison with mass spectra in NIST mass spectral library (The National Institute of Standards and Technology; 2017).

The amounts of the monoterpenes in the air samples were determined as the sum of areas measured on quantification masses of selected compounds, reflecting the main components of Norway spruce resin: tricyclene, α-pinene, β-pinene, myrcene, 3-carene, β-phellandrene, camphene (all m/z 93), limonene (m/z 68) and 1,8-cineol (m/z 111). Concentration of reported compounds in bark extracts was calculated based on external calibration curves.

2.8. *Monitoring of bark beetles using passive traps*

Passive traps without pheromone baits were used to obtain an impression of the natural abundance of *I. typographus* flying around at forest edges and in the interior of stands. One passive trap per tree, with a size of 40 × 60 cm and made of transparent plastic (Schlyter et al., 1987), was mounted on the bark surface on the south side of tree trunks at breast height. The traps were installed on 16 FE trees and on 16 IF trees randomly chosen within the area of experimental subplots (Fig. 1). The number of beetles caught at FE and IF trees (16 traps for each treatment) was counted every week from April until September.

2.9. *Bark beetle attack experiments*

Two different methodological approaches were followed to study the affinity of *I. typographus* for Norway spruce affected by the sudden microclimatic changes at newly created FEs: limited choice bioassays (attack box experiments, Netherer et al., 2015) and no-choice bioassays (Turčáni and Nakládal, 2007).

2.9.1. *Limited choice bioassays using attack boxes*

The 'attack box' method, described in detail by Netherer et al. (2015), was used to periodically test for (limited) host choice and acceptance of spruce bark beetles at FE and IF trees. The boxes were slightly modified by adding a ventilation hole in the upper part of the transparent plexiglass front wall. Sand was glued to the inner surface of the box to roughen it, and to facilitate walking for the spruce bark beetles. The boxes placed on FE trees were covered by aluminium foil to avoid overheating. Two boxes were installed per tree, one placed above the other at a height of 4 m on the exposed south side of the trunk and attached to a previously affixed wooden frame.

The attack box bioassays were started between 9 and 11 am on the first day of experiments by placing 20 *I. typographus* individuals not classified by sex in the starting bottle on the side of the wooden box. The individuals were sourced from our institutional rearing facility on 7th May, 19th May, 27th May, and 11th August and from pheromone traps on 16th June, 24th June, and 30th June. The attack boxes were equipped with an exit jar connected to the lower part of the front wall. Spruce bark beetles were free to choose whether to emerge from the start bottle into the attack box, roam, or feed on the exposed bark of the sample tree, attack the tree, or leave the attack box and enter the exit jar. The moving activity of the beetles was represented by how many beetles remained in the starting bottle and how many entered the exit bottle. The affinity of the beetles to a study tree was determined by observing how many beetles were hiding under the bark scales and how many beetles actually attacked the trees without being repelled by the resin.

2.9.2. *No-choice bioassays by placing individual beetles in Eppendorf tubes*

The main principle of no-choice bioassays was taken from Turčáni & Nakládal (2007). *Ips typographus* were collected from trap catches and sorted by sex and fitness (intact tarsi). Males were placed individually into 2 mL Eppendorf tubes, which had several ventilation holes and were filled with a strip of filter paper. Ten Eppendorf tubes with males (one male per tube) were attached on the south exposed side of the tree trunk at breast height and fixed by tape. The bark beetles were left in the tubes for 24 h. The number of beetles that had successfully bored, had been expelled by resin, or had tried to bore the bark but had stopped this activity were then counted. All beetles which exhibited these activities were categorised as "attacking beetles". Some beetles also remained inactively in the Eppendorf tubes ("inactive beetles"), and a few beetles managed to escape from the setup. The Eppendorf bioassay was performed four times (16th June, 24th June, 30th June, and 11th August).

2.10. *Statistical analysis*

R statistical software (R Core Team, 2021) was utilised for statistical analysis. Spearman's rank correlation coefficients and their statistical significance were assessed using the *cor.test* function, as implemented in base R. To consider repeated measures together with other factors, a linear mixed model was fitted. All traits were Box–Cox-transformed with optimal λ prior to parametric statistical analysis using MASS package. A univariate linear mixed model was fitted in the following form:

$$Y = I\mu + X_1a + X_2b + X_3ab + X_4c + Zd + e \tag{3}$$

where Y corresponds to the data vector (Box–Cox-transformed evaluated traits); X_n represents the incidence matrices for the respective fixed effect; Z represents the incidence matrices for random effect; I is a vector of ones; μ is the overall mean effect; a is the fixed vector of FE/IF; b is the fixed vector of repeated events (measurement dates); ab is a fixed vector of the interaction of FE/IF with measurement dates; c is the fixed vector of plots; d is the random vector of subplots, with

$$d \sim MVN(0, \sigma^2 I_d) \tag{4}$$

and e is the random vector of errors, with

$$e \sim MVN(0,\ I_n \otimes R) \tag{5}$$

where R is a 7 × 7 matrix of variance–covariance components for residuals defined as a heterogenous correlation among measurements for the same individual and different variances per measurement; and I_d *and* I_n represent identity matrices of the corresponding size.

Mixed models were fitted by ASReml-R v4.1 (Gilmour et al., 2015), which uses restricted maximum likelihood methods to estimate variance components. In all cases, diagnostic plots were reviewed for normality and to detect potential outliers, and residuals were checked with the Shapiro–Wilk test of normality (*shapiro.test* from base R) and the Breusch–Pagan test (*bptest* from package lmtest) against heteroskedasticity. Only cases not rejecting normality and homoskedasticity were further utilised; however, we do not ensure normality and homoskedasticity by not rejecting them.

Wald tests were used to infer the significance of the fixed factors. Subsequently, pairwise comparisons among predicted means were conducted using the *predictPlus* function in asremlPlus package (Brien, 2021) to determine the significance of FE/IF differences in each measurement.

In the case of the parameters for the inactive and attacking beetles from the no-choice experiment, normality could not be reached in residuals; thus, a non-parametric approach was utilised. For each experiment, the differences between FE/IF were evaluated using the Mann–Whitney–Wilcoxon test via the *wilcox.test* function in base R.

The chromatographic data from SPME analysis of the VOCs in the forest were cleaned, normalized (constant raw sum), and evaluated using PCA and partial least square-discriminant analysis (PLS-DA) in SIMCA 15 software (Sartorius Stedim Data Analytics AB, Malmö, Sweden).

3. Results

3.1. Bark surface temperature

Differences in the bark surface temperature between the north and south exposed sides of the stems of FE and IF trees were clearly influenced by air temperatures and VPD. Bark temperatures on the south sides of the stems were significantly higher (1–2.5 °C) at FE trees throughout the experimental season, except for 19th May (Fig. 4a, Table S2); similarly, north exposed sides of the stems were warmer (0.5–1 °C; Fig. 4b, Table S2) at FE on 27th May, 16th June, 24th June, and 11th August compared to IF trees. Average temperatures differed between the southern and northern sides of the trunk by 0.5–2 °C (at FE trees) and by 0.1–0.5 °C (at IF trees).

3.2. Soil water potential

The average soil water potential per subplot in the week prior to experiments is illustrated in Fig. 3, and per experimental day in Table S2. The soil water potential was high (>-100 kPa) throughout the season, which suggests sufficient water supply to the study trees. In August, values decreased at IF subplots (to a minimum of −200 kPa), and even more at FEs (to a minimum of −500 kPa). However, values never descended below the permanent wilting point of −1,500 kPa, at which point plants are no longer able to withdraw water from the soil, and conifers are in physiological stress stadia (Brodribb et al., 2014; Lopushinsky and Klock, 1974). Values also did not drop below the threshold value of −500 kPa, which indicates the first changes of tree physiology when cells begin to expand (Landsberg and Sands, 2011). The differences in soil water potential between FE and IF were not statistically significant throughout the season except for 11th August, when there were lower values at FE subplots. The soil water potential at individual subplots was strongly correlated between experimental dates (see Fig. 3, Spearman's correlation coefficients).

Two years before the experiment in August 2018, the soil water potential on experimental plots 3 and 4 showed values indicative of mild water stress of spruce trees (−800 to −1,400 kPa). At the end of August 2018, recordings even exceeded the wilting point for several days (−1,500 kPa). During the whole vegetation season of 2019 (one year prior to the experiments), the soil water potential constantly remained above the water stress thresholds for Norway spruce.

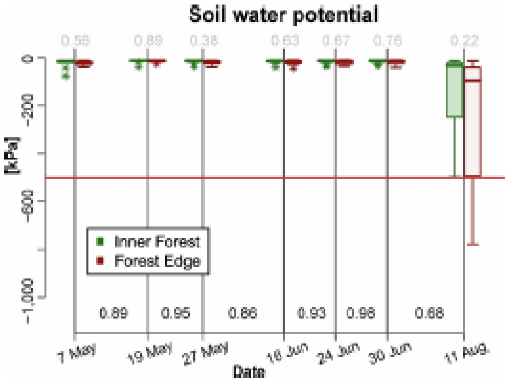


Fig. 3. Soil water potentials per subplot recorded between May 7 and Aug 11, 2020. Individual boxplots represent measurements of soil water potential one week prior the experimental days at FE (red colour)/IF (green colour). Bottom numbers, Spearman's rank correlations between experimental days; upper numbers, p-values of FE/IF differences estimated by a linear mixed model. The red line indicates the estimated value at which Norway spruce cells begin to expand. FE, forest edge; IF, inner forest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Sap flow

The mean daily sums of sap flow of FE trees were slightly higher (0.7–11.1 kg/day) than the values recorded at IF trees from May to August 2020 (Fig. 4c). IF trees showed higher values (3.7 kg/day) than the FE trees only on 11th August. Sap flow of individual trees at each subplot were clearly correlated (Fig. 4c, Spearman's correlation coefficient). All trees showed the lowest sap flow on 24th June (FE 21.8 kg/day, IF 20.6 kg/day), which corresponded with a very low VPD.

On 16th June and 30th June, low VPD and increased precipitation (161 mm) contributed to high sap flow and a soil water potential close to zero. In August, transpiration declined from 4 kg/L to 1.5 kg/L in response to the drying of the soil (soil water potential ranging between −500 and −1,500 kPa). Moderate drought stress in August was supported by high evapotranspiration demands at average temperatures of 22 °C, accompanied by low precipitation.

3.4. Resin flow

A general trend of increased resin flow of FE trees (4.5–14 mm longer resin columns than IF trees) was observed. Significantly longer resin columns (on average 32.5 mm) were measured on FE trees only once during the season, on 27th May (Fig. 4d). Resin flow varied considerably between trees; nevertheless, this parameter seems to be characteristic for individual trees, as indicated by high Spearman's rank correlation coefficients between experimental repetitions for individual trees.

3.5. Monoterpenes in phloem

Mean amounts of the chosen monoterpenes in phloem extracts did not show significant differences for the aggregated data from IF trees compared to that of FE trees on three measurement dates (27th May,

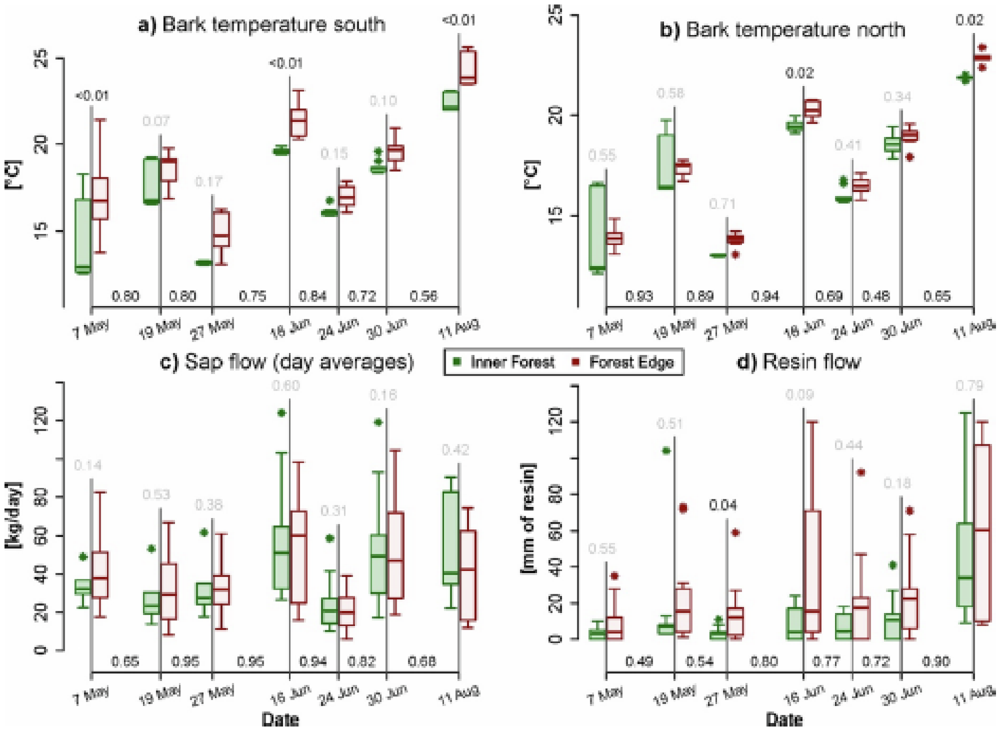


Fig. 4. Traits recorded at individual study trees on experimental days. a. Mean bark surface temperatures on the south side. **b.** Mean bark surface temperatures on the north side. **c.** Mean daily sap flow **d.** Resin flow per 24 h. Individual boxplots represent measurements on respective experimental days at FE (red colour)/IF (green colour). Bottom numbers, Spearman's rank correlations between experimental repetitions; upper numbers, p-values of FE/IF differences estimated by a linear mixed model. FE, forest edge; IF, inner forest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

16th June and 30th June). Variability of concentrations and statistical parameters are visible from Table 2. The only exception was the total amount of β-pinene and myrcene (counted from area of coeluted peaks), which were higher (Table 2) in the phloem of IF trees on 30th June. The total amount of measured monoterpenes on IF and FE trees was about twice as high on 16th June compared to that measured on 27th May or 30th June (Fig. 5c). This finding corresponded with the bark temperature maxima (Fig. 4a, b) and increased amounts of VOCs collected near the monitored trees (Fig. 5a).

3.6. Monoterpenes in VOCs

The abundance of the selected main volatile monoterpenes collected near the surface of trees was significantly higher at FEs at the beginning of the season. The most significant differences were observed on 7th May, 27th May, 16th June and, most distinctively, on 11th August (Fig. 5a). Spearman's coefficients were very low, suggesting that this trait for individual trees did not correlate between repetitions, and that variability in VOC concentrations was higher among individual trees than between the treatment and control.

VOC measurements in the open space showed a higher abundance of spruce monoterpenes in the fresh clearings than in the IF area in May and June. During August, however, values in the clearings and IF area were equally high (Fig. 5b). Initially, VOC levels measured at clearings were even higher than those collected near to the bark surface of FE trees (Fig. 5b, 7th May), showing the strong influence of cutting residuals left in the vicinity of FEs on the total amount of VOCs.

Using PCA, based on composition of VOCs data collected nearby tree surface, neither separation was observed according to the position of trees at FEs or in IF, nor on subplot location (Fig. S2A) for most measurement dates. The only exception was the August collection, when weak separation appeared (Fig. S2C). PCA explains 32% of the variability in the data. Further, weak separation was shown according to the date of sampling (Fig. S2B).

3.7. Correlations of different microclimatic, physiological, and defence traits

The correlation matrix, wherein Spearman's coefficients describe a positive or negative correlation between the compared traits, shows the extent to which individual tree traits influence each other and how they are influenced by microclimatic and meteorological conditions (Fig. 6). Resin flow was correlated with VOCs collected near the monitored trees (Spearman's rank coefficient 0.31). Resin flow, volatile monoterpenes and, most distinctively, bark temperatures were correlated with air temperature. Resin flow and VOCs did not correlate with other climatic parameters (Prec, VPD, PAR) or with sap flow of study trees.

Total sap flow on the days of the experiments was positively related to bark surface temperatures, air temperatures, VPD, and PAR. For instance, on 24th June, an extremely low VPD resulted in very low sap flow of all monitored trees (Fig. 4c). Soil water potential (average soil water potential recorded in the week prior to dates of experimental repetition) was negatively correlated with bark and air temperature and precipitation but did not show a significant correlation with traits of

Table 2
Summarised data for the content of eight chosen monoterpenes extracted from phloem of the monitored trees on given experimental days averaged for FE and IF trees.

Date	IF (mean ±SD)[1]	FE (mean ±SD)[1]	Hedge's g	Wilcoxon p	BC-LMM p	IF (mean ±SD)[1]	FE (mean ±SD)[1]	Hedge's g	Wilcoxon p	BC-LMM p
	Tricyclene					**α-Pinene**				
27 May	5.48 ± 2.77	5.56 ± 1.70	-0.03	0.94	NA	532.90 ± 239.71	476.64 ± 153.62	0.28	0.75	0.70
16 Jun	NA ± NA	NA ± NA	NA	NA	NA	942.08 ± 896.90	805.42 ± 347.44	0.21	0.55	0.80
30 Jun	1.06 ± 0.88	0.79 ± 0.30	0.43	0.80	NA	546.11 ± 291.22	510.26 ± 181.17	0.15	0.97	1.00
	Camphene					**β-Pinene + Myrcene**				
27 May	11.51 ± 5.20	9.76 ± 5.10	0.33	0.51	0.48	418.00 ± 224.79	268.13 ± 128.81	0.82	0.22	0.06
16 Jun	14.81 ± 13.9	12.5 ± 5.45	0.22	0.72	0.90	748.91 ± 292.41	688.24 ± 297.10	0.20	0.47	0.83
30 Jun	7.42 ± 2.79	7.33 ± 3.09	0.03	0.78	1.00	578.46 ± 283.54	341.79 ± 147.93	1.04	0.05	0.03
	Carene					**Limonene**				
27 May	8.73 ± 7.46	8.52 ± 7.61	0.03	1.00	0.99	52.40 ± 60.84	49.79 ± 26.57	0.06	0.51	0.52
16 Jun	18.28 ± 30.67	11.58 ± 20.85	0.25	0.26	0.55	79.08 ± 87.79	88.90 ± 69.64	-0.12	0.39	0.48
30 Jun	8.40 ± 5.01	7.07 ± 11.31	0.13	0.22	0.62	44.22 ± 56.04	49.30 ± 30.23	-0.11	0.28	0.26
	β-Phellandrene					**SUMA_MT**				
27 May	70.0 ± 47.01	46.23 ± 26.48	0.62	0.41	0.26	1090.59 ± 521.75	854.09 ± 266.06	0.58	0.42	0.24
16 Jun	147.48 ± 168.13	91.73 ± 45.08	0.47	0.70	0.40	2255.30 ± 2134.44	1697.43 ± 501.69	0.37	0.97	0.93
30 Jun	114.38 ± 70.53	77.49 ± 51.68	0.59	0.28	0.21	1296.47 ± 648.34	1046.52 ± 396.55	0.46	0.22	0.30

[1] (mean ± SD) in μg/g of dry weight; FE, forest edge; IF, inner forest; SD, standard deviation; Hedge's g, effect size of the difference between FE/IF; Wilcoxon's p, statistical significance of difference calculated by non-parametric Wilcoxon test; BC-LMM p, linear mixed model (where feasible) with Box–Cox-transformed dependent variable.

individual trees, such as resin flow, tree monoterpenes in VOCs, and sap flow.

3.8. *Passive trap catches*

Overall, the number of beetles caught in the passive traps was very low. In the traps mounted on FE trees a total number of 19 beetles landed, and in traps on IF trees only 11 beetles were caught from May to August. No more than two beetles landed in a trap per week, and in some weeks, the traps remained empty. In only 6 weeks of the study season did the catches differ from zero for at least one tree in the group (Table S3). From 28th April to 27th May, 4 weeks after FE creation, more beetles landed in traps at FE trees (13 compared to 1 at IF trees). From 27th May to 29th July, a few more beetles were caught at IF trees (10, compared to 6 beetles at FE trees).

3.9. *Bioassay using attack boxes*

The numbers of beetles remaining in the starting bottles were significantly lower in attack boxes at FE trees on 7th May and 27th May, while more beetles were found in exit boxes on the same dates. Significantly more beetles also exited the box system on 24th June and 30th June. During the August repetition of the attack box experiment, beetles were found in similarly low numbers in the start and exit boxes at trees of both treatments (Fig. 7a, b).

During all experimental repetitions from May to July, the affinity of bark beetles to the study trees was expressed by their hiding under the bark scales, but not by their boring activity (Fig. 7c). This behaviour was significantly more distinct among spruce bark beetles at FE trees, and it affected 4% of all beetles on the coolest experimental day (7th May) and 1–2% of beetles on 19th May, 30th May, 24th June, and 30th June. Actual boring activity (attack) was solely observed in August, with higher numbers of pitch tubes (7) at FE trees (4) than at IF trees (3 borings at 2 trees). Overall, the proportions of beetles initially placed in the starting bottles that finally attacked a tree did not exceed 10%. An average of 9% beetles got lost during the experiments.

3.10. *Bioassay using individual beetles in Eppendorf tubes*

Boring activity (attacking beetles) was observed during all four repetitions of the non-choice bioassays. The proportions of attacking beetles (all replications) ranged from 8% on 30th June to 40% on 11th August. *Ips typographus* males showed higher boring activity at FE than at IF on 24th June (significant difference) and on 30th June (trend). On 11th August, when overall boring activity was the highest in between performed replications, more beetles attacked IF trees (28%) than FE trees. The majority of bark beetles remained inactive in the tubes, especially in June, and some individuals were able to escape (Fig. 8).

3.11. *Correlations of beetle behaviour and environmental and tree physiological traits*

The behaviour of bark beetles observed in the bioassays was related to the various characteristics recorded at the subplots and at individual study trees (Fig. 9). In the no-choice experiment (Fig. 9A), the fraction of beetles that bored into trees (Attack_epp) was positively correlated with temperature (bark surface and air temperature), precipitation, and negatively correlated with soil water potential. In the attack box choice experiments (Fig. 9B), the number of beetles remaining in the start bottles was negatively correlated with resin flow but was not influenced by bark temperatures. The number of beetles found in exit boxes was positively influenced by south exposed bark temperatures and resin flow and negatively correlated with precipitation. Actual attack was positively correlated with air and bark temperatures as well as resin flow and negatively correlated (trend) with SWP. No correlation with any traits were found for the behaviour of beetles hiding under the bark.

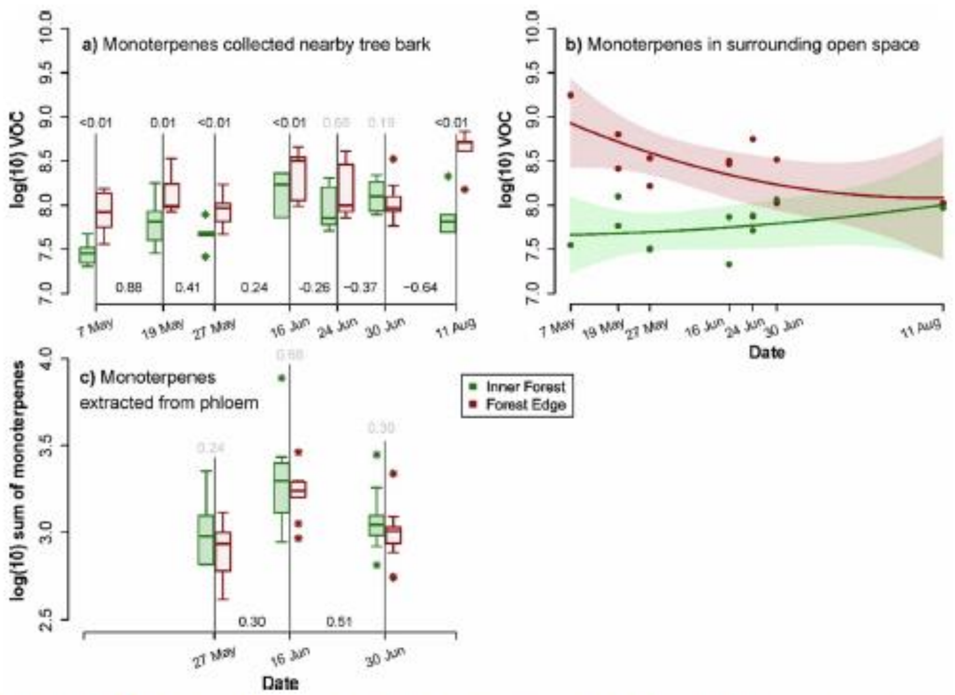


Fig. 5. Comparison of logarithmic total sum of selected monoterpenes. a. Monoterpenes collected near the monitored trees at FEs and IF throughout the season. **b.** Monoterpenes collected in open space surrounding plots (clearings). Datapoints with fitted polynomial of the second order with 95% confidence interval are indicated. **c.** Sum of selected monoterpenes extracted from the phloem collected on respective days. Individual boxplots represent measurements on respective experimental days at FE, forest edge (red colour)/IF, inner forest (green colour). Bottom numbers: Spearman's rank correlations between experimental days; upper numbers: p-values of FE/IF differences estimated by linear mixed models; y-axis shows values on a base 10 logarithmic scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Trait	RF	VOC	Bark T_N	Bark T_S	SAP	SWP	Mean air T_exp	Prec	VPD	PAR
RF	—	**0.31**	**0.41**	**0.41**	0.04	-0.10	**0.34**	-0.02	0.05	0.12
VOC	**0.31**	—	**0.49**	**0.45**	0.07	-0.30	**0.50**	-0.11	0.01	0.09
Bark T_N	**0.41**	**0.49**	—	**0.92**	**0.28**	-0.25	**0.90**	**-0.25**	0.17	**0.31**
Bark T_S	**0.41**	**0.45**	**0.92**	—	**0.25**	**-0.30**	**0.78**	**-0.31**	**0.30**	**0.41**
SAP	0.04	0.07	**0.28**	**0.25**	—	-0.14	**0.23**	-0.16	**0.36**	**0.38**
SWP	-0.10	-0.30	-0.25	**-0.30**	-0.14	—	-0.28	**-0.30**	-0.24	-0.20
Mean air T_exp	**0.34**	**0.50**	**0.90**	**0.78**	**0.23**	-0.28	—	0.06	0.04	**0.18**
Prec	-0.02	-0.11	**-0.25**	**-0.31**	-0.16	**-0.30**	0.06	—	**-0.51**	**-0.51**
VPD	0.05	0.01	0.17	**0.30**	**0.36**	-0.24	0.04	**-0.51**	—	**0.96**
PAR	0.12	0.09	**0.31**	**0.41**	**0.38**	-0.20	**0.18**	**-0.51**	**0.96**	—

Fig. 6. Spearman's rank correlations of different microclimatic, physiological, and defence traits recorded throughout experimental season. High positive and negative correlations are marked by more intense red and blue colours, respectively. Significant correlations are indicated by bold numbers. RF, resin flow; VOC, VOCs collected nearby bark of the monitored trees; Bark T_N, bark surface temperature, north side; Bark T_S, bark surface temperature, south side; SAP, total sap flow at day of experimental repetition; SWP_week, average soil water potential recorded in the week prior to dates of experimental repetition; VPD, average vapour pressure deficit on the day of experimental repetitions; PAR, average photosynthetic active radiation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

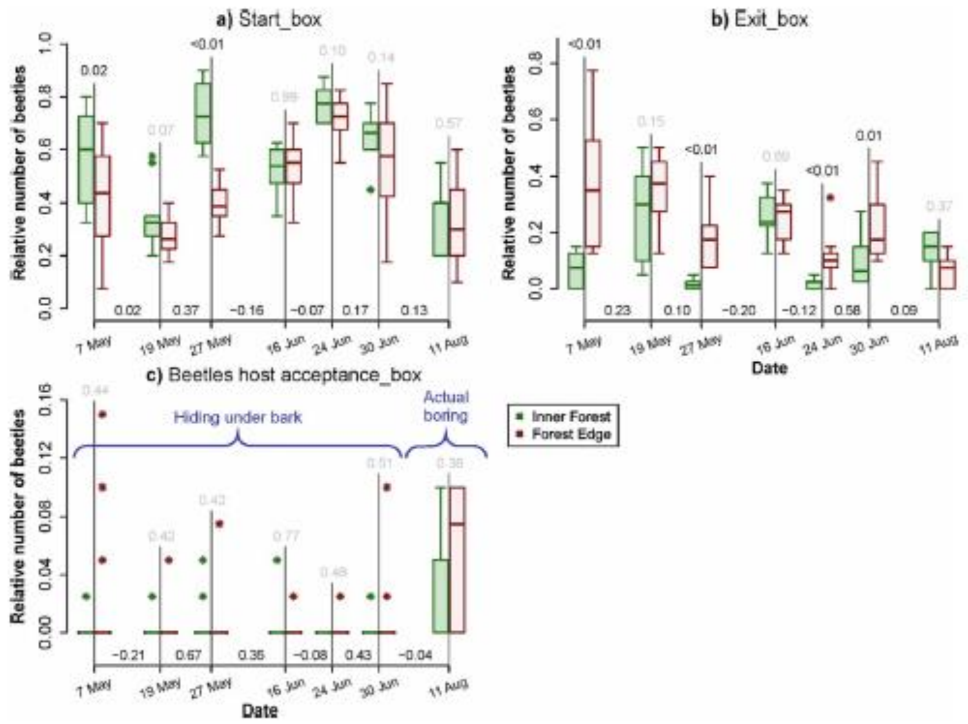


Fig. 7. Locations where beetles were found in attack box bioassays, indicating affinity to trees and host acceptance. Locations included the start box, exit box, hiding under bark, and actual boring. Individual boxplots represent findings on respective experimental days at FE (red colour)/IF (green colour). Bottom numbers: Spearman rank correlations between experimental days; upper numbers: p-values of FE/IF differences estimated by linear mixed model. FE, forest edge; IF, inner forest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

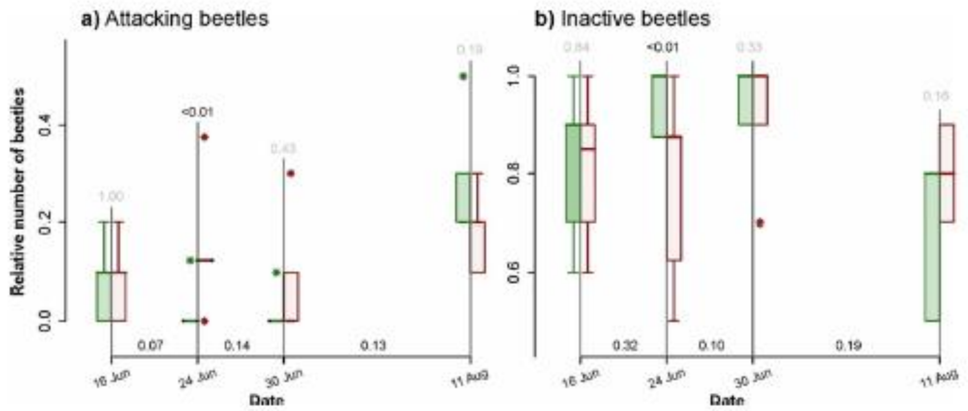
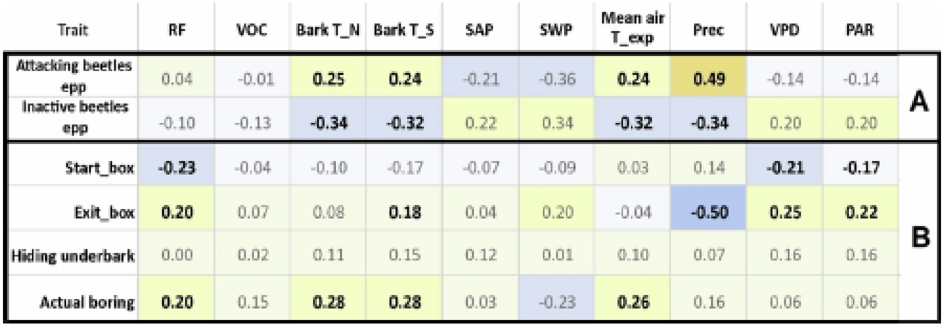


Fig. 8. Attacking and inactive bark beetles in no-choice bioassays. Attacking beetles included beetles that successfully attacked, tried to attack but stopped the activity, and unsuccessful attack when beetles were expelled by resin. Inactive beetles, no activity expressed. Individual boxplots represent measurements in respective experimental days at FE (red colour)/IF (green colour). Bottom numbers, Spearman rank correlations between experimental days; upper numbers, p-values of FE/IF differences estimated by a linear mixed model. FE, forest edge; IF, inner forest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Trait	RF	VOC	Bark T_N	Bark T_S	SAP	SWP	Mean air T_exp	Prec	VPD	PAR	
Attacking beetles epp	0.04	-0.01	**0.25**	**0.24**	-0.21	-0.36	**0.24**	**0.49**	-0.14	-0.14	A
Inactive beetles epp	-0.10	-0.13	**-0.34**	**-0.32**	0.22	0.34	**-0.32**	**-0.34**	0.20	0.20	
Start_box	**-0.23**	-0.04	-0.10	-0.17	-0.07	-0.09	0.03	0.14	**-0.21**	**-0.17**	B
Exit_box	**0.20**	0.07	0.08	**0.18**	0.04	0.20	-0.04	**-0.50**	**0.25**	**0.22**	
Hiding underbark	0.00	0.02	0.11	0.15	0.12	0.01	0.10	0.07	0.16	0.16	
Actual boring	**0.20**	0.15	**0.28**	**0.28**	0.03	-0.23	**0.26**	0.16	0.06	0.06	

Fig. 9. Spearman's rank correlations of beetle behaviour in bioassays and meteorological and tree physiological traits. A. No-choice experiment. Attack_epp describes the total number of beetles that successfully bored, attacked but were expelled by resin, or started but stopped boring activity. **B.** Choice bioassay. Actual boring, beetles that bored into bark; Hiding Underbark_box, beetles that hid under bark peel; Exit_box, beetles that entered exit bottles; Start_box, beetles that remained in start bottles; RF, resin flow; VOC, monoterpenes in volatile organic compounds emitted from tree surface; Bark T_N, bark surface temperature, north side; Bark T_S, bark surface temperature, south side; SAP, total sap flow on the day of experimental repetition; SWP_week, average soil water potential recorded in the week prior to dates of experimental repetition; VPD, average vapour pressure deficit at day of experimental repetitions; PAR, average photosynthetic active radiation. Red indicates a high correlation, blue indicates a low correlation, and significance between individual traits is expressed by bold numbers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Forest fragments and stand edges are particularly exposed to disturbance and stress factors such as wind and solar irradiation (Donis et al., 2018; Jönsson et al., 2007). This study is the first to comprehensively investigate the direct effects of forest edge establishment on microclimatic and soil hydrological parameters, physiological and defence traits of individual Norway spruce trees, and eventually, on tree predisposition to spruce bark beetle attack. The most significant changes observed at the newly established FEs were clearly increased bark surface temperatures and enhanced resin flow of sun-exposed trees, while sap flow rates did not differ significantly from those of shaded trees in the forest interior. Soil water potential was high throughout the study season due to sufficient precipitation and decreased only in August due to drier weather conditions. Contrary to unchanged concentrations of monoterpenes in the phloem of trees soon after FE creation early in the season, VOC concentrations recorded near tree bark were three times higher along the edge than in the closed forest stand. These differences became even more distinct in the course of the season, reaching a ratio of 6:1 (FE: IF) for the total amount of volatile monoterpenes collected near trees. Total amounts of VOCs in open space, originating from both standing trees and logging residuals left in the fresh clearings, were strongly increased at the forest edge. While enhanced moving activity of spruce bark beetles was observed in the attack box bioassays performed at FE trees in May and June, beetles attacked the trees mainly in August, at both FEs and IF. In the following section, the observed tree physiological changes and bark beetle behaviour in the transition zone between forest edge and inner forest are discussed with regard to biogeography, weather and characteristics of bark beetle outbreaks in the study area.

The experimental subplots were established in a sub-montane forest area outside the natural habitat of Norway spruce. The region has been strongly affected by drought in recent years (Krejza et al., 2021), and severe water deficits of Norway spruce in all parts of the forest were recorded prior to the study in 2018. In contrast, spring and early summer of the study season 2020 remained comparably humid and cool, with highest air and bark temperatures measured in August. As a consequence, soil water potential remained high in May and June at all subplots, and both IF and FE study trees were sufficiently supplied with water. Apart from slightly decreased soil water potential values at the forest edge in August, soil hydrology was not affected by the clear-cut. Earlier studies showed that the removal of trees can even result in increased soil moisture due to strong reductions in interception and evapotranspiration on the clear-cut site (Keenan and Kimmins, 1993).

An immediate effect of removing protective neighbouring trees in the small clear-cuts was the increased sun exposure of the newly created edges. Increased intensity of solar radiation and elevated air temperatures enhance bark surface temperatures on the south sides of exposed trunks (Majdák et al., 2021, Marešová et al., 2020, Mezei et al., 2019). However, as previously found by Majdák et al. (2021), the differences in average surface temperature between the south and north exposed sides of the stems were not higher than 1–2 °C for FE and 0.1–0.5 °C for IF trees. Bark surface temperatures were positively correlated with sap flow, which tended to increase at FE trees two weeks after edge creation. Higher sap flow might compensate for higher stem surface temperatures at sun-exposed trees (Marešová et al., 2020). Despite the known short response time of this physiological parameter to changes in soil water availability (Zweifel et al., 2020), lowered soil water potentials in combination with high air temperatures did not result in lower sap flow rates of FE trees in August.

While there was no indication of drought stress of edge trees in the first months of the experiment, the effects of abruptly increased air and bark temperatures on tree defence were clearly indicated by enhanced resin flow. Amounts of resin exuded by FE trees remained high 4–8 weeks after edge creation, as opposed to the constantly low resin flow of shaded IF trees in May and June. A similar increase in resin exudation caused by external stress factors was previously observed for several species of spruce and pine (Baier et al., 2002; Gaylord et al., 2007; Knebel et al., 2008; Netherer et al., 2015). Furthermore, the known high variations in resin flow among trees (Christiansen and Horntvedt, 1983; Schroeder, 1990) and comparably lower variation within individual trees (Netherer et al., 2015) were observed at all study trees. In August, 14 weeks after clearance, the short-term induced defence reactions of the exposed FE trees were followed by a (presumable) adaptation of spruces to the altered microclimatic conditions along the stand edge. Resin flow was still high in response to increased air temperatures, yet to the same extent for both FE and IF trees. The increased sun exposure of FE trees did not enhance further essential defence traits such as monoterpene content in the phloem, as earlier observed by Marešová et al., (2020). On the contrary, the reduced amount of β-pinene + myrcene recorded for FE trees in June may suggest a slightly impaired chemical defence ability against biotic invaders (Schiebe et al. 2012) of FE trees until eight weeks after edge creation.

The monoterpene content of the phloem did not correspond to the increased levels of volatile monoterpenes collected near FE trees on most sampling dates from May to August. Increased emission of volatile compounds is mainly dependent on the physical features of terpenoids Higher temperatures cause higher volatility of these compounds, whereas constitutive resin with lower viscosity is stored in resin ducts and basins (Gaylord et al., 2007). The significantly highest VOC concentrations near FE trees in August were clearly correlated with strongly elevated bark temperatures and enhanced resin flow. Although soil water potential and sap flow were reduced at that time, the data do not support an influence of tree physiological changes on emission of volatiles from the trees. Notably, VOC clouds recorded in the area surrounding trees consist not only of compounds emitted from tree bark, but also those from needles (Juráň et al., 2017). Emissions further include substances produced by microbes and fungi in the soil, which are associated with trees or with organisms living in trees, such as bark beetles. A major part of VOCs can originate from the surrounding environment, such as fresh stumps and debris on clearings. The emissions from various sources other than standing trees likely contribute or even characterise the olfactory niche of a freshly established FE. The significant accumulation of volatile monoterpenes detected in open space around FE trees shortly after edge creation strongly supports this assumption. Volatile terpenoids and other compounds emitted by trees are considered to be relevant ecological signals that enable spruce bark beetles to distinguish inappropriate habitats (deciduous forests) (Zhang and Schlyter, 2004) from suitable habitats (conifer forests), and non-host from host trees (Norway spruce) (Andersson, 2012). Yet, eventual host acceptance and successful infestation by *I. typographus* depends on the combined effects of habitat/host attractiveness, host resistance, and bark beetle abundance.

The exposed study trees at freshly created FEs were not attacked by the natural *I. typographus* population, even though an outbreak in the study area started in 2018 and peaked in 2020. Instead, one of the IF study plots was infested (4C on 16th June), and several initial attack spots appeared adjacent to clear-cuts from the previous winter or from the 2019 season. In early spring, other stand edges in the surrounding area, which were naturally created by wind during winter, were attacked. We can only speculate that aggregation pheromones produced by spruce bark beetles in these infestation spots, pictured at Fig. 1 by grey spots, guided beetles away from experimental FEs in spring and early summer. Still, the numbers of passive trap landings of *I. typographus* were slightly higher at FE than IF trees in spring, which suggests that forest edges were generally more attractive for swarming beetles than the closed forest.

Without natural attacks, the field bioassays performed allowed to some extent an evaluation of spruce bark beetle affinity to and acceptance of the study trees as hosts. Both test approaches regard short-distance selection of trees, whereby the attack box system does not enforce tree attack, while Eppendorf experiments leave beetles the decision between host acceptance or refusal. The combination of results gained in both experiments allows for several conclusions. A main finding was that test beetles were more active at FE trees in the earlier part of the experimental season (May). Beetle activity was shown by the larger number of specimens entering the exit devices of the attack boxes, indicating tendencies to fly, and by a higher number of attacks in Eppendorf tubes. Attacks in the tubes and exits from the boxes were clearly increasing with bark temperature and corresponded with periods of elevated volatile monoterpene concentrations in open space (May) and near FE trees (May, June). In contrast, beetles did not want to fly (leave the attack boxes) in the case of rain. An interesting observation was the behaviour of beetles in the attack boxes to crawl on the bark and hide behind bark scales during the majority of test runs. This behaviour of seeking protection, which was not described for the first use of attack boxes by Netherer et al. (2015), can partly be explained by adverse weather conditions, yet not all experimental days in May and June were cool and humid. As opposed to increased numbers of (repelled) attacks observed in the earlier study on less stressed and better defended trees in spring (Netherer et al., 2015; Matthews et al., 2018), the lack of boring attempts of beetles in the present study did not allow an examination of the effectiveness of increased resin flow of FE trees. However, higher amounts of resin promoted beetle activity in terms of leaving the start bottles, exiting the attack box system, or attacking the tree (in August). Spruce bark beetles were likely reacting to the olfactory stimuli of resin (Netherer et al., 2015). August attacks corresponded with the highest seasonal air and bark temperatures and the lowest soil water potential and were accompanied by enhanced VOC concentrations near FE tree bark. The many inactive beetles observed in the Eppendorf tube experiment in August were possibly due to adversely high temperature conditions in the small tubes.

5. Conclusions

Considering the limitations of the study regarding cool and humid weather conditions in spring, which had the effect of reducing tree stress at the forest edge, and the unanticipated behaviour of test beetles in the attack box bioassay (hiding under bark instead of attack attempts), we conclude that fresh forest edges are not specifically susceptible to *I. typographus* attack shortly after establishment. However, the microclimatic conditions of forest margins and olfactory signals from standing edge trees and logging residuals do favour spruce bark beetle activity. Edge trees immediately responded to altered environmental conditions by increased defence in terms of resin flow but not of monoterpene content of the bark. It was only later in the season that increased temperature and mild drought conditions triggered (minor) physiological stress of FE trees and spruce bark beetle attack. A possible explanation for this late attack is that forest edges were not present early enough in spring to be attractive for swarming beetles after overwintering. Mezei et al., (2011) observed an increased abundance (number of pheromone trap catches) of *I. typographus* at new stand edges in the years after a wind disturbance event but not in the year of edge creation.

Further research is needed to understand the gradual microclimatic, physiological, and biochemical changes at FEs and the attack patterns in fragmented forests under different *I. typographus* population densities. The timing of clear-cuts possibly influences the probability of natural attack in the first or second year after edge establishment. Follow-up studies should focus on comparing stand edges created in winter (December to February) with those freshly created in spring, which will allow the investigation of the differences in tree defence status in relation to the attractiveness of the environment for swarming beetles.

CRediT authorship contribution statement

Barbora Stříbrská: Investigation, Validation, Data curation, Funding aquisition, Writing – original draft. **Jaromír Hradecký:** Data curation, Investigation, Formal analysis. **Jaroslav Čepl:** Formal analysis, Visualisation. **Ivana Tomášková:** Methodology, Data curation. **Rastislav Jakuš:** Conceptualization, Methodology. **Roman Modlinger:** Conceptualization, Investigation. **Sigrid Netherer:** Writing – original draft, Writing – review & editing. **Anna Jirošová:** Supervision, Conceptualization, Funding acquisition, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foreco.2021.119950.

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Supplementary material: Changed microclimate and tree defence at fresh forest edges influence susceptibility of Norway spruce to bark beetle (*Ips typographus*) attack

B. Stříbrská, J. Hradecký, J Čepl, I. Tomášková, R. Jakuš, R. Modlinger, S. Netherer, A. Jirošová

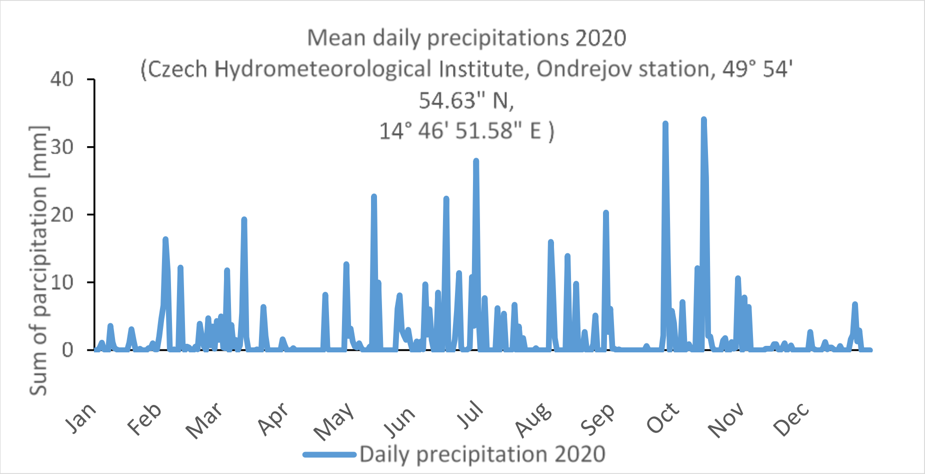

Figure S1A. Mean daily precipitations in the experimental area in 2020

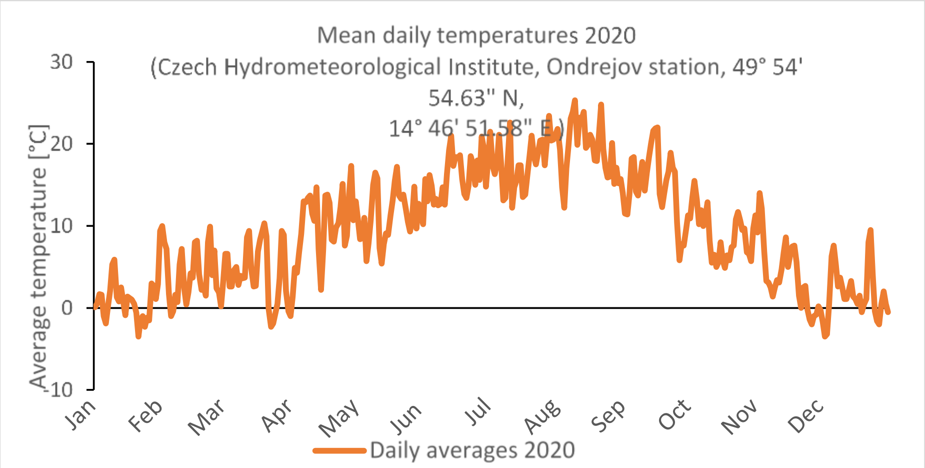

Figure S1B. Mean daily temperatures in the experimental area in 2020

Table S1. Meteorological conditions on experimental days (T = temperature; Prec = precipitation, VPD = vapour pressure deficit, PAR = photosynthetic active radiation)

No. Exp.	Date	Max T 1st day (°C)	Max T 2nd day (°C)	Min T (°C)	Average T (°C)	∑Prec (mm)	Average air humidity (%)	Average VPD (Pa)	∑ PAR (μmol/m²sec⁻¹)
1	7–8 May	19.8	22.9	3.1	13.1	0.0	59.2	860.4	20,595.8
2	19–20 May	24.4	18.0	8.2	16.3	0.0	68.0	719.8	14,407.2
3	27–28 May	20.5	14.5	9.3	13.6	3.8	71.5	524.3	13,965.1
4	16–17 Jun	24.4	23.2	14.1	19.8	0.0	71.8	731.7	17,664.8
5	24–25 Jun	20.9	23.3	11.9	16.4	0.4	84.3	367.5	11,787.1
6	30 Jun–1July	24.1	28.0	12.2	19.7	0.0	68.0	857.9	22,417.3
7	11–12 Aug	29.6	29.9	15.1	22.2	0.8	79.7	742.3	17,752.4

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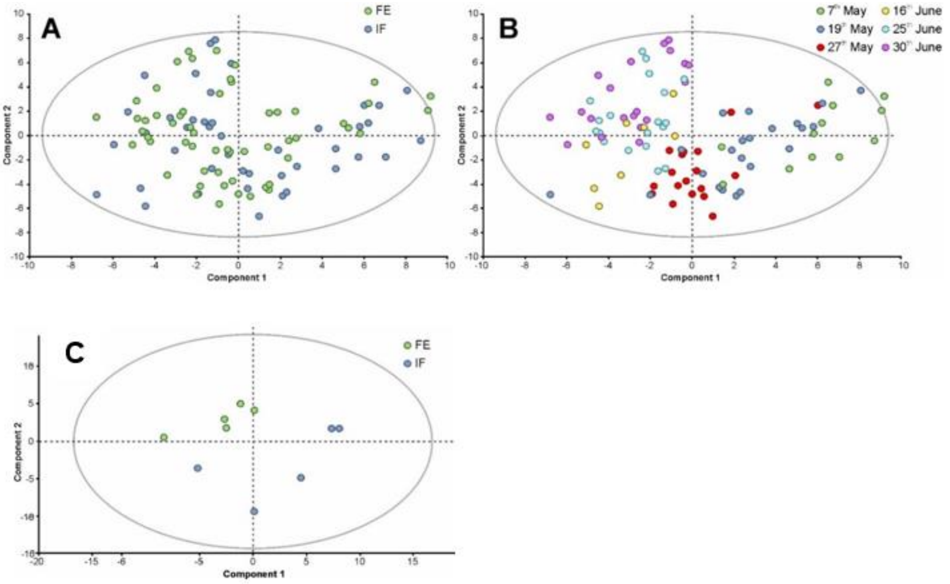

Figure S2. PCA scores plot showing separation of samples based on GC-MS records; **A** – points colored according to tree location (FE-green, IF-blue); **B** – points colored according to date of sampling; **C** - PCA scores plot showing separation of samples based on GC-MS records, collected in August (FE-green, IF-blue)

Table S2. Summarized data for each trait in given experimental days averaged for FE and IF trees with standard deviation (SD) indicated; effect size of the difference between FE/IF indicated by Hedge's *g*, statistical significance of difference calculated by non-parametric Wilcoxon test and by linear mixed model (where feasible) with Box-Cox transformed dependent variable**.**

Table S2, since its complexity, is provided as the individual Excel file.

Table S3. Cumulative numbers of beetles caught to 16 passive traps installed on trees on FE and 16 traps in IF in listed periods. In tables are only periods of collection when catches were > 0.

Periods of collection when catches >0	April 28-May 6	May 6-May 13	May 20-May 27	May 27-May 30	June 10-June 16	July 23-July 29
FE	6	2	5	4	2	0
IF	1	0	0	4	2	4

4.2 Physiological and Biochemical Indicators in Norway Spruces Freshly Infested by *Ips typographus*: Potential for Early Detection Methods

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This article has two main hypotheses: 1) “Are there any measurable differences in physiological, biochemical, and bark beetle (BB) behaviour after attack of Norway spruce?”; 2) “Can observable changes in measured parameters be used as indicators for developing “BB attack early detection methods”?”. The main objective of this work is to compare parameters of naturally BB-infested trees with non-infested control trees based on the tree physiological characteristics sap flow, resin flow, treewidth increment, the concentrations and profile of terpenes in phloem and VOCs emitted by the infested trees. The last objective is based on the beetle catches in passive traps and beetle acceptance bioassays for non-infested and infested trees.

This article concludes that there is a possible selection of characteristics for developing BB early attack detection methodology. It was found that the tree increment stopped after the first week of attack. In the initial two weeks post-infestation, the detection of infested trees is achievable through the evaluation of volatile organic compounds (VOCs) collected in close proximity to the trees. The random placing of passive traps with an alarming system can used for signalling that beetle landed on the trees.

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Physiological and biochemical indicators in Norway spruces freshly infested by *Ips typographus*: potential for early detection methods

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Intoduction: The bark beetle *Ips typographus* currently represents the primary pest of Norway spruce (*Picea abies*) in Central Europe. Early detection and timely salvage cutting of bark beetle-infested trees are functional management strategies for controlling bark beetle outbreaks. However, alternative detection methods are currently being developed, and possible indicators of bark beetle infestation can be assessed through changes in the physiological, biochemical, and beetle-acceptance characteristics of trees.

Method: This study monitored infested and non-infested Norway spruce trees before and 3 weeks after *Ips typographus* natural attack. Permanently installed sensors recorded physiological features, such as sap flow, tree stem increment, bark surface temperature, and soil water potential, to monitor water availability. Defensive metabolism characteristics, beetle host acceptance, and attractiveness to trees were monitored discretely several times per season. The forest stand that was later attacked by bark beetles had lower water availability during the 2018–2020 seasons compared to the non-attacked stands.

Results: After the attack, sap flow and tree stem increment were significantly lower in infested trees than in intact ones, and bark surface temperature moderately increased, even when measured in the inner forest stand from the shadowed side. Infested trees respond to attacks with a surge in monoterpene emissions. In addition, freshly infested trees were more accepted by males in the no-choice bioassays, and a significantly higher number of beetles were caught in passive traps in the first week of infestation.

Conclusion: The most promising characteristics for early detection methods of bark beetle-infested trees include tree bark temperature measured only in certain meteorological conditions, elevated monoterpene emissions, and significantly high catches in passive traps.

KEYWORDS

green attack, tree defence, bark beetles, *Picea abies*, tree physiology, VOC, sap flow, dendrometer

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1. Introduction

The Eurasian Spruce Bark Beetle *Ips typographus* (Linnaeus, 1758) (Coleoptera: Scolytinae) is the most devastating pest of Norway spruce [*Picea abies* (L.) Karst.] and forests in the Palearctic region (Christiansen and Bakke, 1988). When its population is in the endemic phase, it attacks weakened trees, contributing to the ecological balance in natural forests. However, the incidence of disturbance agents, such as strong winds or drought periods, lead to the transition of the *I. typographus* population to the epidemic phase (Kausrud et al., 2012). Ongoing climate change, the occurrence of spruce stands outside their natural range, and economically oriented silviculture practices have led to intense bark beetle outbreaks over the last decade (Seidl et al., 2016; Marini et al., 2017; Biedermann et al., 2019). The Central European region has been seriously affected by *I. typographus* outbreaks that started after severe drought events in 2015 and 2018 (Hlásny et al., 2021a). The scenario resulted in an exponentially growing volume of salvage logging from 2017 to 2020 (approximately 5.9 mil m^3 in 2017 to 26.2 mil m^3 in 2020 in the Czech Republic) (Hlásny et al., 2021b, 2022).

A traditional method for managing bark beetle outbreaks is the early detection of infested trees prior to the emergence of offspring generation (Hlásny et al., 2019). The presence of boring dust at the base of the trunk is considered the most reliable symptom of bark beetle infestation (Kautz et al., 2023), but its use requires personal inspection of each trunk, which is difficult to achieve. The extent of the forest stands, the intensity of the outbreaks, and the ability to distinguish infested trees were the main limits for applying this procedure. The possibility of including modern tools and procedures in the search for bark beetle-attacked trees is currently being intensively researched. Currently, the most promising and advanced methods are remote sensing methods, which include a wide range of approaches (Huo et al., 2021). Despite the large number of areas that they are able to scan, there remain problems with the demands of time and expertise involved in processing the captured records and the inaccuracy of detection owing to the considerable variability of the attack signs. A different method for detecting infested trees is based on the chemical communication between bark beetles (Raffa et al., 2016). For this purpose, specially trained dogs have recently been used (Johansson et al., 2019), which can detect trees more successfully and efficiently than human experts (Vošvrdová et al., 2023). The first study using an artificial nose to detect substances in the forest environment is currently underway (Hüttnerová et al., 2023). However, for the purpose of early detection of bark beetles, the sap flow, tree increment and content of terpenes in phloem and catches to passive traps in infested trees have not been evaluated.

Like all plants, trees infested by bark beetles rouse defense mechanisms against herbivores that have evolved over a million years (Berini et al., 2018). In conifers, it manifests itself with an immediate response as resin exudations and leads to changes in basic tree physiology, such as transpiration (Wang, 1983) and resource allocation (Franceschi et al., 2005; Boone et al., 2011). The metabolism in trees is based on the fundamental process of photosynthesis, when atmospheric carbon is sequestered, and carbohydrates are synthesized (Lawlor and Cornic, 2002). These carbon resources are distributed between primary tree metabolism (tree growth or reproduction) and secondary metabolism (constitutive defense compounds as phenolics and terpenes important in conifers) (Huang et al., 2020). Physiological characteristics that describe these processes can be measured using specific techniques. These characteristics have been previously recorded in various contexts related to tree stress and susceptibility to bark beetle attacks.

The sap flow value, which expresses a deficit in tree transpiration measured in short time intervals, is often used as a quantitative characteristic of drought stress in trees (Střelcová et al., 2013; Gebhardt et al., 2023) or stress from sudden sun radiation in fragmented forests (Özçelik et al., 2022). The acute transpiration deficit positively correlates with the lowering of the defense ability of trees against bark beetle infestation (Netherer et al., 2015; Matthews et al., 2018).

Another physiological characteristic of Norway spruce discussed in the article as an indicator of infestation is the tree stem diameter. Measuring stem increment has been reported in the literature to indicate reductions in growth rates related to drought (Ježík et al., 2015). It has also been used to evaluate wood production in different tree species or genotypes (Cocozza et al., 2016). Fluctuations in stem circumference are influenced by the dynamics of plant tissue water balance on a daily and seasonal basis, with radial growth increments depending on these fluctuations (Offenthaler et al., 2001). The stem increment is also influenced by carbohydrate distribution. In healthy trees, there is a balance between growth and defense, with carbon resources allocated to both. However, during herbivore or pathogen attacks, the allocation of carbon resources shifts toward the production of defense metabolites, limiting investment in growth, including stem increment (Huang et al., 2020).

A previous study (Majdák et al., 2021) reported a measurable increase in bark surface temperature following bark beetle infestation in sun-exposed trees on the forest edge. These trees were weakened by infestation and could not keep the optimal temperature. Generally, the temperature of the cambium and phloem in a healthy tree with sufficient water content is well-regulated. Healthy trees can maintain their internal thermal environment and prevent excessive temperature increase (Leuzinger and Korner, 2007) through evaporative cooling facilitated by the water movement within the sap flow. However, the bark surface temperature is influenced by direct radiation and air temperature (Hietz et al., 2005), and there exists some correlation between phloem temperature and surface temperature (Powell, 1967).

The most noticeable changes in tree characteristics following bark beetle infestation are chemical defense reactions. These reactions occur in two stages. The first stage is the immediate defense response, where conifers exude stored resin. The second stage, known as the induced defense response, involves the formation of traumatic resin ducts (Franceschi et al., 2005) and is triggered within the first few weeks following a bark beetle attack (Celedon and Bohlmann, 2019). The overall resin exudation of trees measured as resin flow was in several studies suggested as a marker of resistance of Norway spruce trees against the bark beetles and characteristics of conifer defense (Netherer et al., 2015). Resin flow varies from tree to tree owing to genetic variation and age (Christiansen and Horntvedt, 1983; Schroeder, 1990) and strongly depends on temperature and meteorological conditions (Baier et al., 2002; Stříbrská et al., 2022) and on phenotypic variables, and location (Zas et al., 2020).

Resin is a mixture of terpene compounds with toxic and immobilizing effects on bark beetles; however, it also has a communication function for them (Erbilgin et al., 2007). In spruce, the predominant volatile monoterpenes are alpha-pinene, beta-pinene, Δ-carene, limonene, β-phellandrene, camphene and myrcene (Borg-Karlson et al., 1993). Resin also contains sesquiterpenes in smaller quantities and higher content of diterpenes (Netherer et al., 2021). Oxidized forms of all terpenes are also present at a low level, and the content of oxygenated monoterpenes is modified by a stress reaction and tree decay (Schiebe et al., 2019) caused alternatively by inoculation by beetle's symbiotic ophiostomatoid fungi (Kandasamy et al., 2023). Even non-infested conifers emit large amounts of terpenes, mainly from the needles (Juráň et al., 2017). The emission has a diurnal rhythm and depends on the actual meteorological and physiological conditions of trees (Kopaczyk et al., 2020) as well as on genetic origin (Kännaste et al., 2013). These phytochemicals play their role in the selection of suitable bark beetle habitat (Erbilgin, 2019). When Norway spruce is attacked by bark beetles, either due to constitutive resin storage opening or induction of defense terpene biosynthesis, the content of emitted terpenes increases 10–100 fold (Ghimire et al., 2016; Jaakkola et al., 2022).

The attractivity of the freshly attacked trees for additional beetle conspecifics is modified based on olfactometric cues perceived by beetles, predominantly on aggregation pheromones (Schlyter et al., 1987a) and host volatiles (Erbilgin et al., 2007). The acceptance of host tree by attacking beetles is a function of the defense ability of trees and stadia of beetle attacks. The *I. typographus* infestation begins with the selection of a suitable host tree by pioneer males (Byers, 1989; Lehmanski et al., 2023). When males successfully overcome tree defenses, they produce potent aggregation pheromones to attract conspecifics and start mass aggregation. Pheromone consists of oxidized terpenes 2-methyl-3-buten-2-ol and *cis*-verbenol 10:1 (Birgersson et al., 1984; Ramakrishnan et al., 2022). Bark beetles can detect these highly biologically active compounds from a complex mixture of other compounds in the forest owing to their specific antennal receptors. However, pheromones are only a minor component of the total volatile emissions of infested trees.

This study aimed to identify alternative tools for the early detection of *I. typographus* attacks based on modifications in physiology, defense biochemistry, and insect-tree interaction levels. In particular, we compared changes in Norway spruce trees in the first stadia of *I. typographus* attack in terms of (i) physiological and physical parameters (sap flow, stem increment, and surface temperature); (ii) spruce defense reaction (resin flow, emission, and phloem content of defensive terpenes, including selection of compounds specific for infested trees); and (iii) beetles attraction to infested and non-infested stands by monitoring beetles using non-baited passive traps and (iv) beetle acceptance of the host tree. Furthermore, measurable characteristics that displayed significant differences in infested trees compared to non-infested trees were evaluated as potential tools for developing early attack detection methods for more efficient bark beetle management.

2. Materials and methods

2.1. Study area and sampling setup and conditions

The study was conducted from May 6th to July 2nd, 2020 at the property of the Forests CZU in Kostelec nad Černými lesy in central Czech Republic (Figure 1). The weather during the growing season of 2020 was humid and warm. The detailed recording of the meteorological conditions has been previously published (Stříbrská et al., 2022) and is attached as a supplement to this article (Supplementary Table 1).

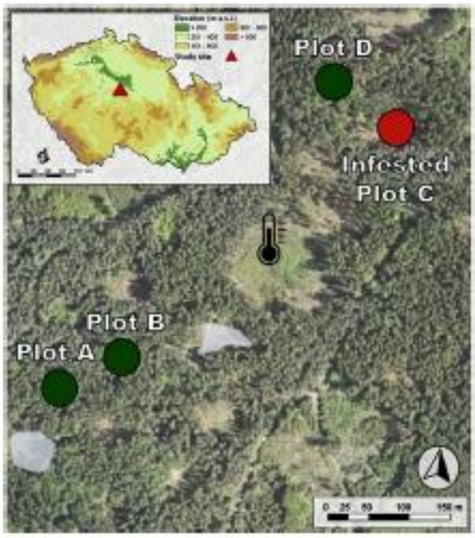

FIGURE 1
Arrangement of study plots with the naturally attacked plot. Green circles represent the inner forest plots (non-attacked trees), corresponding to plots A, B, and D. Plot C was infested by *I. typographus* on June 16th and salvaged in July (red circle). The gray spots are areas of previous natural bark beetle attack. The black thermometer marks the meteorological station.

As part of the Extemit-K project, a large study area was established in 2018 to measure physiological and dendrological characteristics in trees exposed to stress conditions such as drought and forest fragmentation in the context of bark beetle attack. Originally, eight plots with different treatments were monitored (Stříbrská et al., 2022). However, the study reported in this article focuses on four non-treated plots established within a closed, undisturbed area in Norway spruce stands (Table 1).

From 2018 to 2020, the soil water potential was monitored for all plots. In 2020, sensors for sap flow, tree stem increment, and bark surface temperature collected data from a total of eighteen trees. Four trees were monitored in plots B and D, while five trees were monitored in plots A and C. Additionally, monoterpene sampling and non-choice beetle bioassays were conducted six times per growing season in 2020 on three selected trees in each of the four studied plots (12 trees in total).

Throughout the study period, we checked the entire area within a 500 m distance from the monitored trees for bark infestation at approximately weekly intervals. However, on June 16th, 2020, a fresh infestation was detected in all five trees in plot C at the stage of the nuptial chamber building by male beetles. In next 3 weeks, from June 16th to July 2nd, these five infested trees were further monitored with sensors, along with the remaining thirteen uninfested trees. On July 2nd, the infested trees had to be cut down to prevent the emergence of new beetle generations, and the observation was concluded.

TABLE 1 Information about study area.

Studied plots	Coordinates		Altitude [m a.s.l.]	Age of Norway spruces	Monitored trees†	Tested trees††
	lat.	long.				
A	49.912771	14.873291	430	90- to 95-year-old	5	3
B	49.912819	14.873778			4	3
C	49.914527	14.877892			5	3
D	49.914667	14.877344			4	3

†Number of trees continuously monitored for sap flow, bark surface temperature, and tree increment.
††Number of trees on which resin flow, monoterpenes content in VOC, phloem, and non-choice bioassay with beetles were repeatedly tested.

In 2020, an outbreak of *I. typographus* occurred in the Norway spruce forests of the Forests CZU. Extreme droughts in the years 2015 and 2018 were the cause of the exponential increase in infested trees, which started in 2017 with 284 m^3 and culminated in 2020 with 76,113 m^3 of salvaged cut wood (Klinovský, 2021). In 2018 and 2019, the local centra (gray spots in Figure 1) of bark beetle infestation was detected in the studied area and cut down. None of them were closer than 50 m from the monitored trees (Stříbrská et al., 2022).

In each plot, the soil water potential was recorded to check the water availability of trees using five sensors (Teros 21, Meter Group, München, Germany) distributed in each plot and placed 20 cm below the surface. The whole dataset (**Supplementary Figure 1**) was taken from the 2018 to 2020 seasons to examine water stress on the trees.

2.2. Physiological characteristics of trees

The methodology for data sampling was modified from Stříbrská et al. (2022).

Briefly, sap flow, tree stem increment, and bark surface temperature were measured using sensors installed on individual trees. Data were stored in a single data logger (GreyBox N2N 3P; EMS Brno, Brno, Czech Republic) and connected to a cloud system via the GSM. To enable statistical analysis, we express the continuously recorded values of physiological characteristics as means per collection day or a specific period, which allowed us to process them together with discretely measured characteristics and show changes in the same time points.

Sap flow was measured based on the thermodynamic principle by heating the wood around stainless-steel electrodes (EMS 81; EMS Brno) using the trunk heat balance method. Data were recorded at 10-min intervals (Čermák et al., 2004; Stříbrská et al., 2022). The data were subjected to post-processing, including baseline correction, and sap flow rates were recomputed as kg/h as the sum for each sampling date.

Tree stem increment was recorded using a sensor (DR26E Band dendrometer for sap flow system; EMS 81 DR26E; EMS Brno), which was installed 3.5 m above the ground. The change in trunk circumference was measured every 5 min and stored in the data logger as a 10-min average. After cleaning up the errors caused by various influences, the data were converted to 1 h averages, and then divided by two times 3,14 (pi). The beginning of the season is determined according to the growth and the start of sap flow as the zero point of tree increment increases in a given year, for this season, it was determined on April 1st.

Bark surface temperature was measured on the north side of the tree stem to eliminate the influence of sun radiation. Infrared thermometers (Apogee Instruments, Logan, UT) were installed at a height of 3 m. Data were collected at hourly intervals, and averages for days (24 h) for the collection period were calculated (Stříbrská et al., 2022).

2.3. Defense characteristics of trees

Resin flow was measured using glass tubes (inner diameter: 3 mm; outer diameter: 5 mm; length: 12 cm). One glass tube per tree/repetition was inserted into holes (6 mm) drilled into the bark and phloem at breast height (1.3 m) from the north and south exposed sides of the trunks. The resin was collected for 24 h (start and end of collection between 3 and 5 pm), and the level of exudated resin in glass tubes was measured (Netherer et al., 2015; Stříbrská et al., 2022).

The main monoterpenes in the close vicinity of the spruce stem were collected using SPME (Solid Phase Microextraction) fiber (PDMS/CAR/DVB; Supelco, USA), which was placed in an aluminum box (25 cm × 5 cm × 5 cm) loosely fixed by rope on the tree stem surface at 3.5 m height. When collected from infested trees, the boxes were attached out of the beetle's entrance hole and frass. The collection took 1 h (from 1 pm to 2 pm) on the day of the sampling. Immediately after sampling, the fibers were sealed, stored on dry ice, and transported to the laboratory. Desorption and compound measurements by gas chromatography-electron impact-time of flight-mass spectrometry (GC-EI-TOF-MS) were performed within 3 days after collection (Stříbrská et al., 2022).

Bark samples for analysis of compounds extractable from phloem were collected on May 27th, June 16th, and June 30th. Three sections (8 mm diameter) were pinched out using a cork borer at a distance of 15 cm from each other at a height of 2 m on the south side of the tree trunks. Samples were stored in liquid N_2 and transported to the laboratory. Three sections from each tree were pooled and ground in liquid nitrogen to obtain a fine powder. The powder (200 mg) was extracted for 10 min in 2 mL of hexane (containing 5 µg/mL of the internal standard 1-bromododecane) in an ultrasonic bath. Extracts were filtered into 2-mL vials for GC-EI-TOF-MS (Stříbrská et al., 2022).

The collected monoterpenes and compounds from the phloem were analyzed using a gas chromatograph (Agilent 7890 B; Agilent, USA) coupled to a mass spectrometer with a time-of-flight mass analyzer GC-EI-TOF-MS Pegasus 4D (LECO, St. Joseph, MI, USA). The instrument was equipped with a capillary column HP-5MS UI (30 m, 0.25 mm i.d., 0.25 μm film thickness; Agilent). The analysis setups were as follows. For analysis of SPME, a hot PTV inlet (265°C) was used in a 2 min spitless period. The temperature program for the GC oven was 40°C (1 min) - 15°C/min to 210°C - 20°C/min to 280°C. To analyze phloem extracts, 1 μL of hexane extract was injected in spitless mode into the PTV inlet (programmed from 20°C–8°C/s to 265°C). The GC oven was programmed: 40°C (1 min) - 5°C/min to 210°C - 20°C/min to 320°C (6 min). The mass spectrometer setup was as follows: ionization energy in electron impact mode was 70 eV, ensuring spectra compatibility with NIST library. Full spectra were collected in mass range, 35–500 Da with speed of 10 spectra per second.

The ChromaTOF software (LECO) was used for chromatographic data processing. Compound identification was performed using comparison of measured mass spectra and spectra in mass spectral library NIST (2017), using comparison of measured retention indexes (counted on C8-C40 saturated alkane scale) with retention indexes published in NIST. For main monoterpenes, retention times of analytical standards measured by the same methods as samples were used.

The main monoterpenes detected in Norway spruce were tricyclene, α-pinene, and β-pinene in coelution with myrcene, β-phellandrene, Δ-carene, camphene (quantification mass m/z 93) and limonene (quantification mass m/z 68). To statistically evaluate the monoterpenes collected from air by solid-phase microextraction (SPME) close by infested and non-infested trees, the sum of peak areas of quantification masses of these compounds were summed and treated as a single value per tree per collection. The concentration of the sum of the same monoterpenes extracted from the phloem was quantified as μg/mg of the dry weight of material using calibration curves constructed for α-pinene, β-pinene, Δ-carene, camphene, and limonene. Peak areas were normalized by internal standard (1-bromodecane) to correct injection volume fluctuation. Furthermore, differences in the content of these eight monoterpenes individually were compared in air collected by SPME as peak areas and in the phloem extract as concentration (μg/mg of dry weight).

The peak areas of quantification masses of all detectable compounds with a sufficient threshold in GC-EI-TOF-MS chromatograms, which were recorded from the collection by SPME on June 24th, were preprocessed by aligning software and analyzed using principal component analysis (PCA) and the discriminant analysis model (PLS-DA).

2.4. Beetle catches in passive traps and beetle acceptance bioassays

Catches of beetles in passive traps made from transparent plastic (40 × 60 cm) indicated the attractivity of trees. Traps were mounted on the southern side of the tree trunk at breast height (Schlyter et al., 1987b; Stříbrská et al., 2022). Ten passive traps were mounted on trees, which were infested on June 16th, and ten passive traps on trees within the control plots. The checking of the number of beetles caught in passive traps without any additional pheromone attraction was conducted from May 1st, and the last catch collection was on June 16th.

No-choice bioassay to monitor beetle acceptance was performed by encapsulating males of *I. typographus* to Eppendorf tubes (one male per tube). Ten tubes were attached to the south-exposed side of the tree trunk at a height of 2 m and fixed with tape. The beetles were left to feed for 24 h (Turčáni and Nakládal, 2007; Stříbrská et al., 2022). Active beetles used in the statistical evaluation were assigned into three behavioral categories: beetles that bored inside the bark and stood inside the pitch tubes, beetles that bored inside but were expelled by resin, and beetles that started to feed but were interrupted. The Eppendorf bioassay was performed three times per season (June 16th, June 24th, and June 30th).

2.5. Statistical analyses

The R statistical software (R Core Team, 2022) was used for statistical analyses.

For testing the hypothesis that infested and non-infested trees differ, we utilized a general linear mixed model (glmer() function from the lme4 package; Bates et al., 2015). We split measurements into the pre-attack period and post-attack period to assess differences between those periods separately. Repeated measures model was fit with the fixed effect of measurement time and bark beetle attack and random effect of the plot. We used the gamma distribution with log link; in cases of count data in response (number of beetles), we used the Poisson distribution. *Post hoc* Tukey analyses between infested and non-infested trees in overall repeated measures model and inside each measurement separately were performed using lsmeans() function from the lsmeans package (Lenth, 2016). Histograms of residuals and residuals vs predicted values plots were inspected.

The total profile of compounds collected on SPME fiber was normalized (constant raw sum), central log transformed and pareto scaled for PCA and subsequent partial least square-discriminant analysis (PLS-DA) created in the SIMCA 17 software (Sartorius Stedim Data Analytics AB, Malmö, Sweden).

3. Results

3.1. Soil water potential

Within the vegetation season of 2020, the soil water potential did not decrease below -200 kPa in all monitored plots A, B, C, and D, which suggests sufficient water supply for all studied trees; however, in plot C, infested in June 16th, soil water potential was significantly lower than on non-infested plots ($p = 0.04$; Figure 2). The history of water supply in the study site revealed mild water stress in the trees in 2018. Soil water potential values ranged between -800 and -1400 kPa, with a short decrease below the wilting point of -1500 kPa in August 2018. In the growing season of 2019, the lowest values of soil water potential were from -400 kPa to -900 kPa. In both years preceding the study, the soil water potential in plot C was lower than that in plots A, B, and D (Supplementary Figure 1).

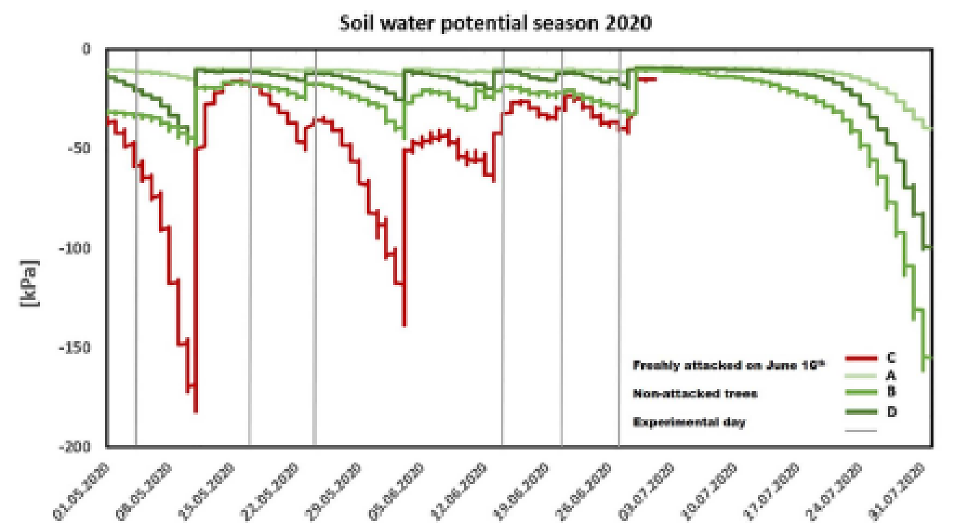

FIGURE 2
Seasonal trend of soil water potential (2020). Individual lines represent measurements through the season in freshly attacked plot C (red color)/the non-attacked plots A, B, and D (green color). The gray color (vertical line) represents individual experiments.

3.2. Physiological characteristics

The sap flow of trees in the monitored area (quantified as the sum of sap flow in kg per data sampling day (Figure 3A) did not show a significant difference in data sampling days from May to the date of beetle attack detection in plot C. On June 16th, the first week of infestation in plot C, sap flow significantly decreased in attacked trees ($p = 0.035$), and this difference was even more prominent in the next 2 weeks of advance infestation ($p = 0.019$ on June 24th and $p = 0.010$ on June 30th).

Tree stem increment counted for the week prior to the data sampling days was lower for trees in plot C from the beginning of the season. After the second week of the bark beetle attack on June 24th, there was a significantly lower increment in infested trees than in non-infested, with a continuing trend in the following weeks (Figure 3B).

The bark surface temperatures measured on the north side of tree stems before the attack was detected, were equal on all monitored plots on May 19th and May 27th. Only on May 7th were temperature higher for plots A, B, and D, which later remained non-infested. On the monitoring day of June 16th, in the first week of the attack, the temperature of infested trees in plot C significantly increased (but the difference between infested and non-infested trees was only + 0.4°C) (Figure 3C). The average temperature of plot C in the period after infestation was 18.2°C, whereas that of the non-infested control was 17.8°C.

All the statistical details are listed in Supplementary Table 2.

3.3. Tree defense characteristics

The abundance of the sum of eight main spruce monoterpenes in the headspace close to the tree was similar in all monitored plots at the beginning of the season; however, after the bark beetle infestation of plot C on June 16th, their emission significantly increased in the vicinity of the affected trees and was significantly higher than in the non-infested trees for all three sampling days (Figure 4A). Nevertheless, monoterpene emissions declined during the third week of the attack. The progress of individual monoterpene emissions was similar to the progress of their sum (Supplementary Figure 2).

The total content of monoterpenes extracted from the phloem of infested and non-infested trees did not differ significantly during the first and third weeks of bark beetle attack on June 16th and June 30th (Figure 4B). When focusing on individual monoterpenes, in the third week of the attack, statistically significant differences were found only in the content of Δ-carene, which increased, and camphene, which decreased in infested trees. The other monitored monoterpenes were less abundant in plot C, even before bark beetle infestation, and maintained the same trend after infestation on June 16th, but not significantly lower in infested plot compared to non-infested (Supplementary Figure 3).

The resin flow values recorded for individual trees showed a large variability; therefore, after the bark beetle attack, no significant differences were observed between infested and non-infested trees (Figure 4C). In the second week after attack detection (June 24th) was resin flow higher in infested trees, but this difference was not significant.

PCA score plots were created from metabolomic profiles recorded via GC-EI-TOF-MS from SPME-sampled volatiles on June 24th. The PCA (Figure 5A) explained 51% of the variance in data. A clear separation of two (from three) infested trees is shown. Following PLS-DA (Figure 5B) had parameters R2X(cum) = 0.48, R2Y(cum) = 0.97, Q2(cum) = 0.74, and connected variable importance plot revealed a higher abundance of terpinolene, α- and β-pinene, campholenal, limonene, sabinol, pinocamphone,

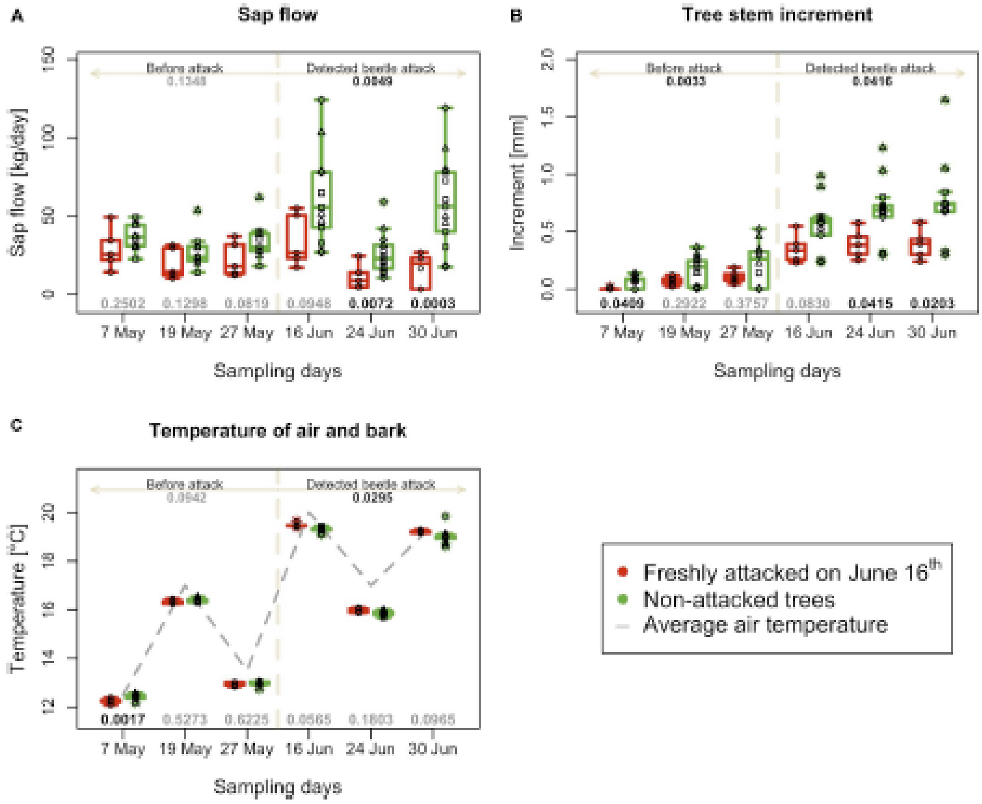

FIGURE 3
Physiological and physical characteristics of individual trees during the experimental period. **(A)** Mean daily sap flow. **(B)** Mean tree increment 1 week before sampling. **(C)** Mean bark surface temperatures on the north side of individual trees; boxplots summarize measurements on respective experimental days at the freshly attacked subplot C (red color) and control subplots A, B, and D (green color). Individual measurements are represented by circles (subplot C) and squares, triangles and diamonds for subplot A, B and C. Numbers on the top are *p*-values of differences between attacked and non-attacked plots calculated by *post hoc* Tukey analyses based on repeated measures GLMM separately for pre-attack and attack period; bottom numbers are *p*-values of differences between attacked and non-attacked plots estimated by *post hoc* Tukey analyses in the given sampling time. The dashed line represents the period when the bark beetle attack was detected (from June 16th).

and myrtenal, which had the highest importance for separation between the two classes of samples. Compounds of *I. typographus* aggregation pheromone, *cis*-verbenol, and 2-methyl-3-but-2-enol were not detected under the setup conditions on any experimental day.

All the statistical details are listed in Supplementary Table 2.

3.4. Beetle catches in passive traps and beetle acceptance bioassays

The total number of beetles caught in passive traps was low for most of the observational period. Before the detection of the beetle attack, none or only few beetles (no more than one) were recorded. In the first week of the attack (June 16th), the number of beetles caught in traps mounted in the infested plot C increased steeply. The difference in the number of beetles caught in traps in the non-infested control plots A, B, and D was significant ($p = 0.001$) (Figure 6A).

The non-choice bioassay in Eppendorf tubes was performed three times per season after the detection of a beetle attack on June 16th. Therefore, all data from plot C were collected from infested trees and compared with non-infested trees in control plots A, B, and D. The number of beetles actively boring in the infested trees was significantly higher ($p = 0.002$) than that in non-infested trees in the first week of attack (Figure 6B). In the second and third weeks, bark beetle behavior was similar in both studied groups of trees. All the statistical details are listed in Supplementary Table 2.

4. Discussion

Previous studies have explored the connection between transpiration deficit (expressed as changes in sap flow) and tree defense ability in relation to bark beetle attack (Kirisits and Offenthaler, 2002; Matthews et al., 2018). Our study focuses on the characteristics of infested Norway spruce stands and reports a significant decrease in sap flow during the development of

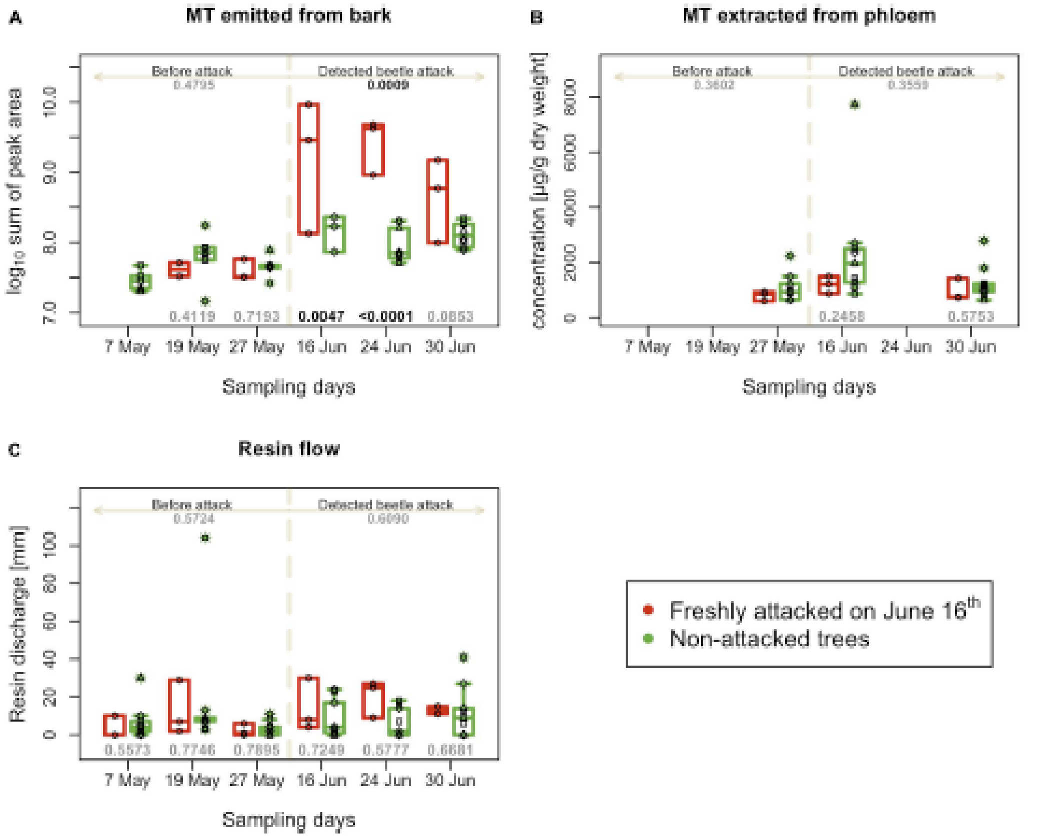

FIGURE 4

Tree defense characteristics recorded on individual trees on different experimental days. **(A)** Sum of peak areas of selected monoterpenes emitted by bark **(B)** Sum of peak selected monoterpenes extracted from phloem - performed three times per season; **(C)** resin flow per 24 h; boxplots summarize measurements on respective experimental days at the freshly attacked subplot C (red color) and control subplots A, B, and D (green color). Individual measurements are represented by circles (subplot C) and squares, triangles and diamonds for subplot A, B and C. Numbers on the top are *p*-values for differences between attacked and non-attacked plots calculated by *post hoc* Tukey analyses based on repeated measures GLMM separately for pre-attack and attack period; bottom numbers are *p*-values for differences between attacked and non-attacked plots estimated by *post hoc* Tukey analyses in the given sampling time. The dashed line represents the period when the bark beetle attack was detected (from June 16th).

I. typographus infestation, consistent with previous findings (Wang, 1983). However, sap flow changes are variable and influenced by factors such as diurnal or seasonal rhythm (Nehemy et al., 2023), selective tree cutting (Özçelik et al., 2022), terrain shape, and soil properties affecting water supply (Netherer et al., 2015). Direct measurement of sap flow changes using the heat balance method (Čermák et al., 2004) for early detection of beetle attack is impractical due to technical, cost, and knowledge requirements. Further research is needed to develop more easily measurable indicators of sap flow changes.

Before infestation, the tree stem increment measured in plot C was lower compared to other plots, likely due to lower soil water potential recorded not only in the study season 2020 but also in the two previous seasons, 2018-2019. This relationship between water availability and stem increment in conifers has been previously reported (Ježík et al., 2015). However, we did not conclude that lower water availability in plot C caused the later bark beetle attack since the trees were not exposed to real drought stress because the soil water potential did not decrease below −1500 kPa (Lopushinsky and Klock, 1974; Brodribb et al., 2014). After infestation was detected, the growth of infested trees stopped while the healthy trees continued to grow, as the affected trees saved carbon resources for defense by limiting growth investment (Hartmann et al., 2013). Although tree stems increment changed significantly in infested trees, it is challenging to measure such small alterations in diameter (less than 1 mm) using basic dendrometry methods on the scale of a large forest area on the individual trees. Terrestrial laser scanning could provide a solution but is currently capable of monitoring seasonal increments greater than 1 mm in diameter (Yrttimaa et al., 2023).

The higher bark temperature recorded in plots A, B, and D before infestation most likely indicates that these plots were located in areas within the inner forests that received more sunlight. The bark surface temperature correlated with the air temperature

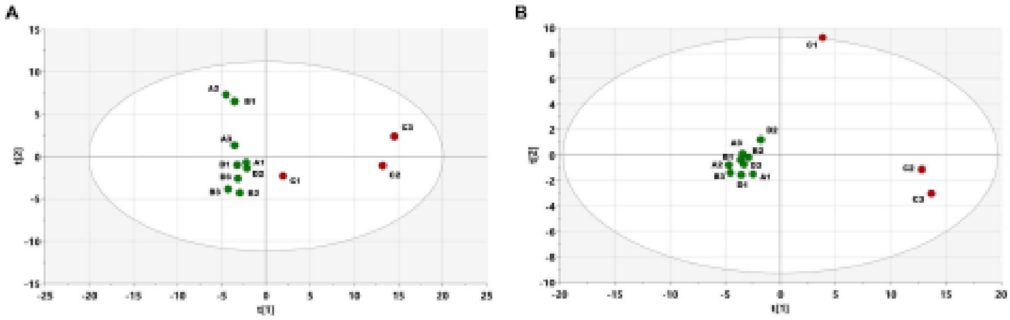

FIGURE 5

(A) PCA scores plot, showing the distribution of samples, according to the whole collected profile of volatiles, measured using GC-EI-TOF-MS. Individual points represent measurements of trees on June 24th in freshly attacked plot C (red color) with three trees/Non-attacked plots A- three trees, B-three trees, D-three trees (green color). Hoteling ellipse with significance level 0.05, t1 and t2 stand for the two most important components, together explaining 51% of the variance in data **(B)** PLS-DA scores plot showing the distribution of samples in the discriminant analysis model, using attacked vs. non-attacked as classes.

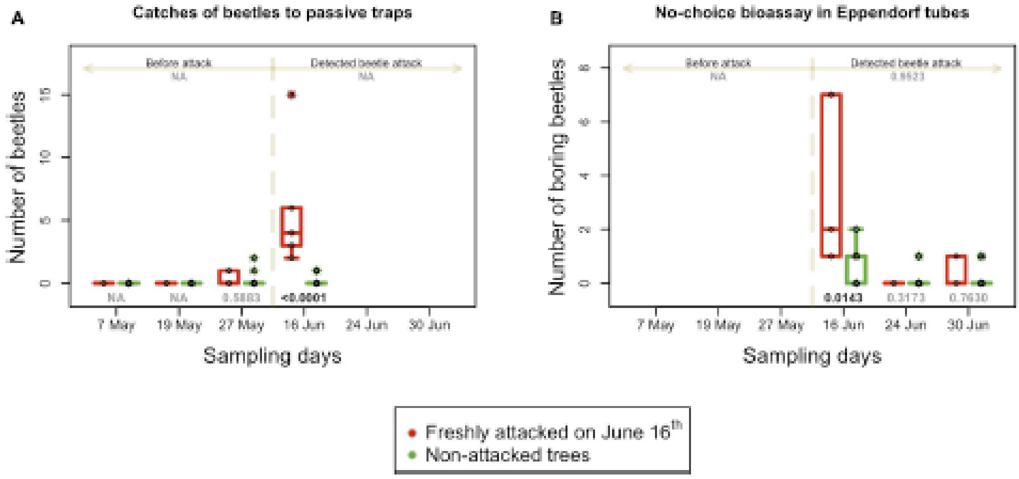

FIGURE 6

Absolute numbers of active *I. typographus* in the field. **(A)** Number of beetles caught in passive traps in the long-distance attraction bioassay. **(B)** Number of beetles that attacked trees in Eppendorf tubes in the no-choice bioassay; boxplots summarize measurements on respective experimental days at the freshly attacked subplot C (red color) and control subplots A, B, and D (green color). Individual measurements are represented by circles (subplot C) and squares, triangles and diamonds for subplot A, B, and C. Numbers on the top are *p*-values of differences between attacked and non-attacked plots calculated by *post hoc* Tukey analyses based on repeated measures GLMM separately for pre-attack and attack period; bottom numbers are *p*-values of differences between attacked and non-attacked plots estimated by *post hoc* Tukey analyses in the given sampling time. The dashed line represents the period when the bark beetle attack was detected (from June 16th).

overall (Stříbrská et al., 2022), but it increased significantly in affected trees after infestation. However, the average difference compared to the intact trees was only about 0.4°C, which is below the sensitivity threshold of the sensors. A study by Majdák et al. (2021) reported using an infrared-based thermo-camera to distinguish infested trees on forest edges. They found a significant difference in bark surface temperature (reaching tens of°C) only on the sun-exposed side of infested trees on days when air temperature reached 34°C and bark surface temperature was nearly 60°C. The temperature difference on the shaded side was lower, and on colder days (maximum air temperature of 24°C), it was not significant, which corresponds with our findings. Still, scanning the bark surface temperature from a distance using a Thermo camera can be considered a promising method for detecting infested trees. However, the methodology needs optimization, including using cameras with sufficient resolution, and measurements should be taken only in certain conditions on the sunny sides of stems on warm days with high sun radiation.

Resin exudation in the conifers, expressed as a measurement of resin flow, has been reported several times as a factor of the defense ability of trees against wood-boring insects (Turtola et al., 2003; Boone et al., 2011). However, resin flow varies significantly within a tree, limiting its usefulness as a defense marker. In our study, plot C had insignificantly higher resin flow after infestation, suggesting induced tree defense. However, the differences in resin flow between infested and intact trees were non-significant, making it an unreliable early attack detection marker. These findings align with the low incidence of resin flow as a visual symptom of *I. typographus* infestation (Kautz et al., 2023).

In volatiles collected close by infested and non-infested Norway spruce were the most abundant monoterpene fraction of resin, which was likely due to opening of constitutive resin storage. The main monoterpenes were α- and β-pinene in coelution with myrcene, Δ-carene, limonene, β-phellandrene and camphene. The summary emission of these compounds steeply increased in 1 week after infestation and remained higher till the end of the study similarly to previous findings by Ghimire et al. (2016) and Jaakkola et al. (2022). Notably, *I. typographus* pheromone 2-methyl-3-buten-2-ol and *cis*-verbenol were not detected in the overall headspace, probably because its content was below the detection limit of the used technique.

A detailed metabolomic study of the GC-EI-TOF-MS chromatograms of volatiles collected during the second week after infestation (June 24th) was conducted to identify compounds that may be significant for infestation. In addition to the major spruce monoterpenes mentioned earlier (namely terpinolene, α- and β-pinene, campholenal, and limonene), the infested trees were clustered based on a higher abundance of minor oxygenated monoterpenes (here sabinol, pinocamphone, and myrtenal) in two of the three infested trees. The increase of these compounds is a consequence of stress-related oxidations of spruce monoterpenes caused by the beetle attack and ophiostomatoid fungi infestation (Schiebe et al., 2019; Kandasamy et al., 2023). Even though internal cross-validation of the PLS-DA model provided a predictive power of 74%, owing to the lack of natural replication, the relative abundance of these compounds is only mentioned as a possible marker of tree infestation.

Despite the significant increase in the amount of emitted monoterpenes by infested trees, the content of the same monoterpenes extracted from the phloem does not increase significantly after infestation. This impairing has been previously reported by Hietz et al. (2005) and Střibrská et al. (2022). It can be explained by the fact that while the immediate emission after tree infestation is due to the opening of constitutive resin storage, the tree response by the biosynthesis of defense compounds against herbivore attack in phloem requires a longer time. The content of major monoterpenes in the phloem, except Δ-carene, was found to be higher in intact trees even before the attack when these compounds were individually analyzed. This corresponds with the theory that higher content of toxic monoterpenes can make trees more resistant in certain circumstances (Raffa et al., 2016).

Based on these findings, there are two possible approaches for using volatile compound emissions as markers for early detection of tree infestation. The first approach involves using non-specific sensors that react to the concentration gradient of the most abundant compounds emitted by an infested tree. Pilot studies have already been carried out using an electronic nose with sensors based on metal oxidation or a change in electrical conductivity, showing promising potential for application in broad-scale volatile organic compound (VOC) scanning, including mounting on unmanned aerial vehicle (UAV) carriers (Paczkowski et al., 2021; Hüttnerová et al., 2023).

The second principle involves focusing on compounds specific to infested trees, such as oxygenated monoterpenes and *I. typographus* pheromones. However, the detection of these compounds requires sensitive and specific methods, such as a special mass detector (Juráň et al., 2017) or a special sampling device (Křůmal et al., 2016), which are not available for field testing yet.

Our observations from the non-choice assay align with research on bark beetle attack dynamics (Byers, 1989). According to this theory, male beetles show a preference for boring into trees that have already been attacked, particularly in the initial week of infestation when their conspecifics have already overcome the trees' defense abilities. However, as the infestation progresses and the resources of the trees are depleted, and the previously attacking beetles enter the mating and egg-laying stages, the beetles no longer prefer the previously infested trees. In the context of bark beetle management, the acceptance of trees determined in a non-choice assay is not suitable. However, it can still provide insights into the dynamics of bark beetle attacks.

On the other hand, passive traps have been shown to be potentially useful for early attack detection, as catching beetles significantly increases when trees are infested. The use of unbaited traps for bark beetle research is rather uncommon. Changes in the biodiversity of saproxylic beetles (including bark beetles) with respect to the age of the windblown trees were evaluated using a window trap by Wermelinger et al. (2002). The same type of trap as in our research was used by Schlyter et al. (1987a) for a behavioral study on *I. typographus*. The sensitivity of the trap and the fast-signaling of changes in the aggregation activity of *I. typographus* evidenced the suitability of this method for early attack detection research.

The present study had a limitation due to the small number of *I. typographus* attacked Norway spruce trees, which were monitored. However, despite this limitation, this study observed significant differences in physiological, defense biochemistry, and bark beetle acceptance characteristics between infested and non-infested trees.

We evaluated the potential use of the measured characteristics as a supporting research tool for developing early bark beetle attack detection methods through the following three approaches. The first is scanning the bark temperature of infested trees using a high-resolution thermal camera when used on days with high temperatures and sun irradiation. The second is monitoring the abrupt increase in the emission of defense monoterpenes using non-specific sensors, such as an electronic nose, with the possibility of carrying it on the UAV. Specific detection of spruce stress and *I. typographus* aggregation pheromone compounds can also be considered. The third is installing passive traps for the automatic detection of trapped or attacking beetles.

The determination of certain measurable characteristics of freshly infested trees can provide opportunities for developing alternative methods of early attack detection as a complement to the traditional and functional approach of early detection of infested trees through personal inspection of boring dust and entrance holes. Follow-up studies should focus on a deeper understanding of the physiological and defense mechanisms in relation to the detailed stage of bark beetle infestation. Future research is required to obtain practical outcomes that would lead to improved management of bark beetle outbreaks, especially the technical development of the solutions suggested in the present study.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

BS: experimental work, data curation, and writing draft. JH: GC-EI-TOF-MS analysis, statistical data processing, and editing. JČ: formal data analysis and statistical data processing. IT: physiological data collecting and handling. RM: conceptualization and investigation. AJ: conceptualization, data sampling, formal analysis, writing, editing, and supervision. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2023.1197229/full#supplementary-material

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Supplementary Material

Physiological and biochemical changes in Norway spruces freshly infested by *Ips typographus* as tools for early detection methodologies

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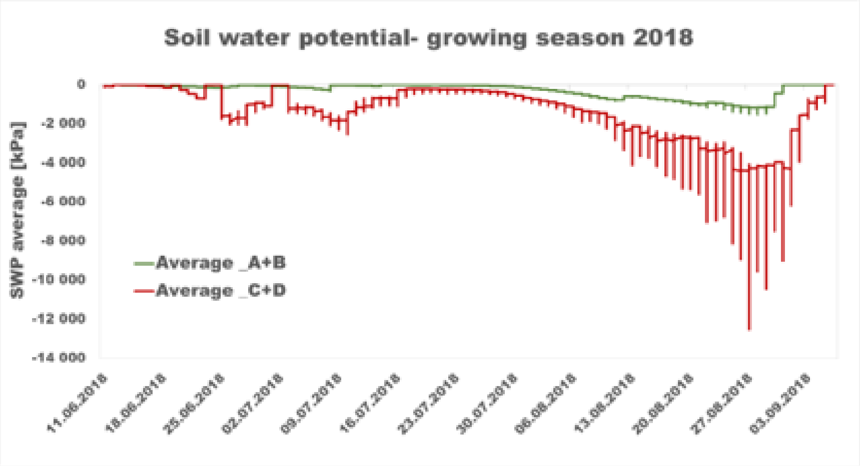
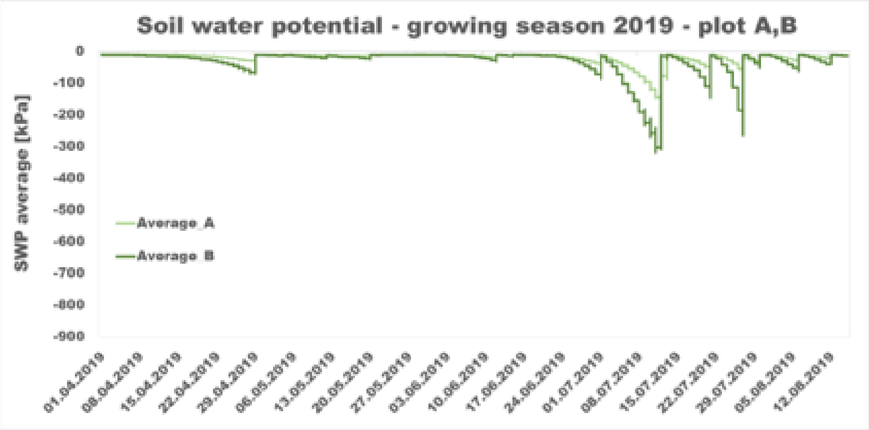
Supplementary Table 1. Meteorological conditions on experimental days (T = temperature; Prec = precipitation, VPD = vapour pressure deficit, PAR = photosynthetic active radiation)

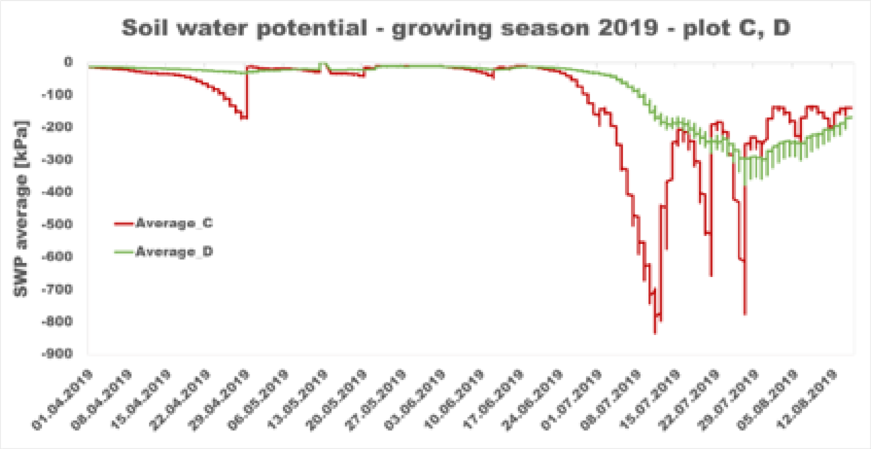
No. Exp.	Date	Max T 1st day (°C)	Max T 2nd day (°C)	Min T (°C)	Average T (°C)	∑Prec (mm)	Average air humidity (%)	Average VPD (Pa)	∑ PAR (μmol/m^{2}sec^{-1})
1	7–8 May	19.8	22.9	3.1	13.1	0.0	59.2	860.4	20,595.8
2	19–20 May	24.4	18.0	8.2	16.3	0.0	68.0	719.8	14,407.2
3	27–28 May	20.5	14.5	9.3	13.6	3.8	71.5	524.3	13,965.1
4	16–17 Jun	24.4	23.2	14.1	19.8	0.0	71.8	731.7	17,664.8
5	24–25 Jun	20.9	23.3	11.9	16.4	0.4	84.3	367.5	11,787.1
6	30 Jun–1 July	24.1	28.0	12.2	19.7	0.0	68.0	857.9	22,417.3

Supplementary Table 2. Summarized statistical data for each Anova results for each Figure seperetly.

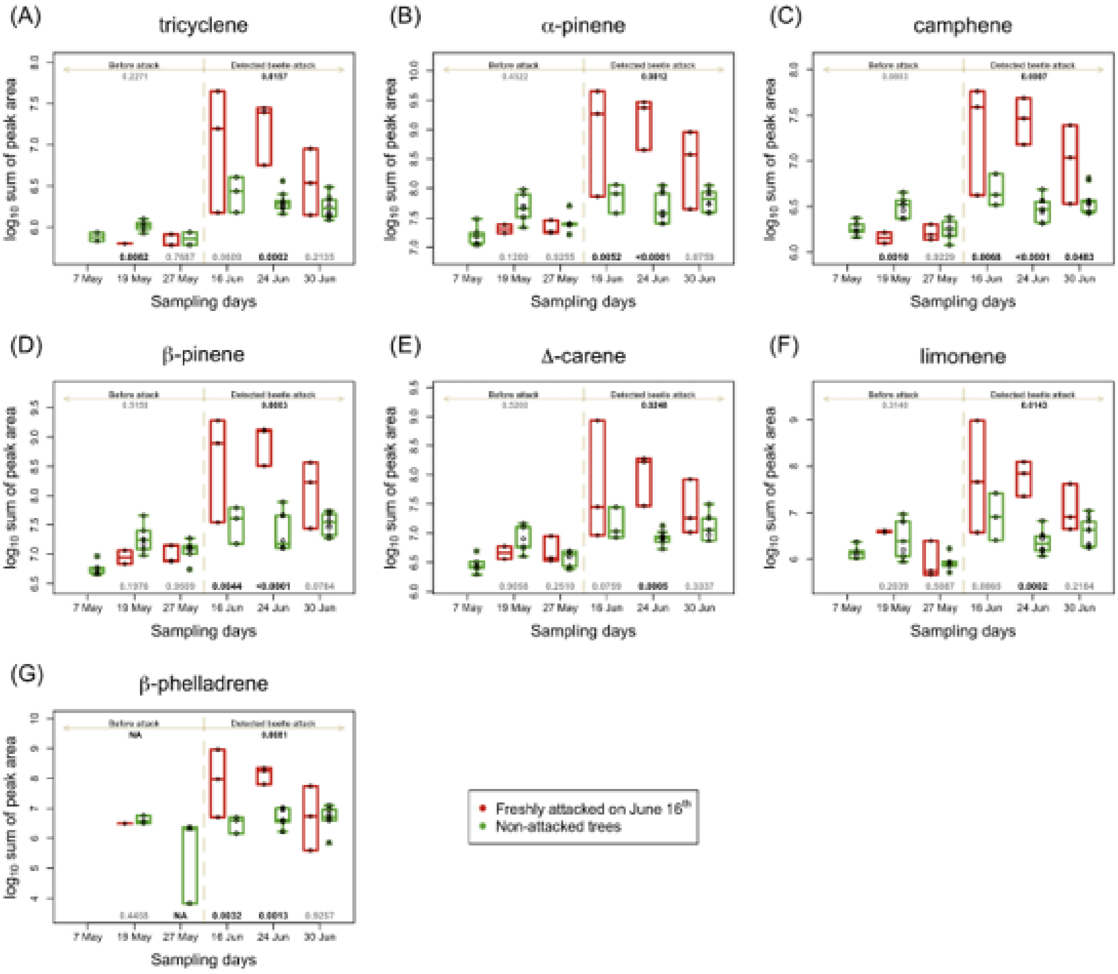
Supp_Table2_Anova_tables, since its complexity, is provided as the individual Excel file.


Soil water potential- growing season 2018
SWP average [kPa]
0
-2 000
-4 000
-6 000
-8 000
-10 000
-12 000
-14 000
Average _A+B
Average _C+D
11.06.2018
18.06.2018
25.06.2018
02.07.2018
09.07.2018
16.07.2018
23.07.2018
30.07.2018
06.08.2018
13.08.2018
20.08.2018
27.08.2018
03.09.2018

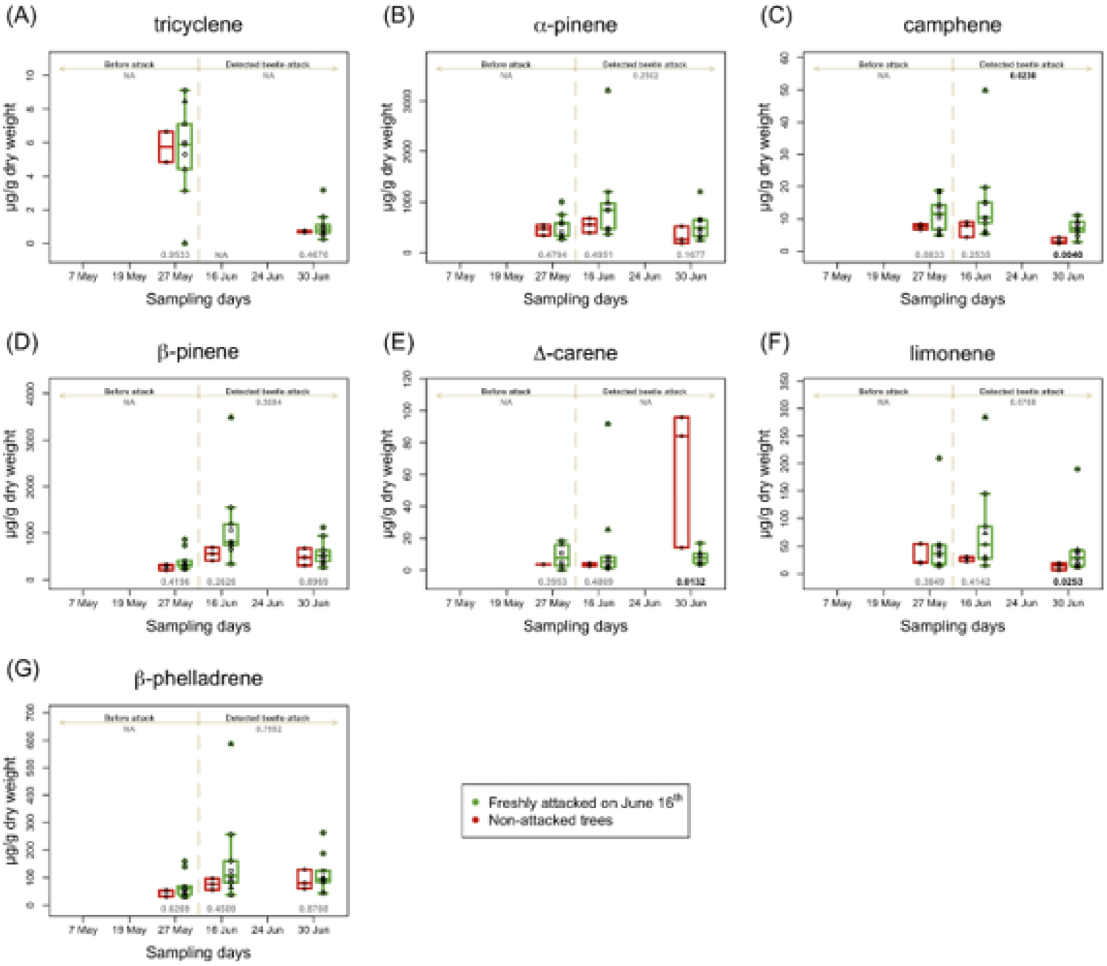

Soil water potential - growing season 2019 - plot A,B
SWP average [kPa]
0
-100
-200
-300
-400
-500
-600
-700
-800
-900
Average_A
Average_B
01.04.2019
08.04.2019
15.04.2019
22.04.2019
29.04.2019
06.05.2019
13.05.2019
20.05.2019
27.05.2019
03.06.2019
10.06.2019
17.06.2019
24.06.2019
01.07.2019
08.07.2019
15.07.2019
22.07.2019
29.07.2019
05.08.2019
12.08.2019


Soil water potential - growing season 2019 - plot C, D
SWP average [kPa]
0
-100
-200
-300
-400
-500
-600
-700
-800
-900
Average_C
Average_D
01.04.2019
08.04.2019
15.04.2019
22.04.2019
29.04.2019
06.05.2019
13.05.2019
20.05.2019
27.05.2019
03.06.2019
10.06.2019
17.06.2019
24.06.2019
01.07.2019
08.07.2019
15.07.2019
22.07.2019
29.07.2019
05.08.2019
12.08.2019

Supplementary Figure 1. Seasonal trend of Soil water potential (growing season 2018 and 2019). Individual lines represent measurements through the season in Freshly attacked plot C (red color)/non-attacked plots A, B, and D (green color).

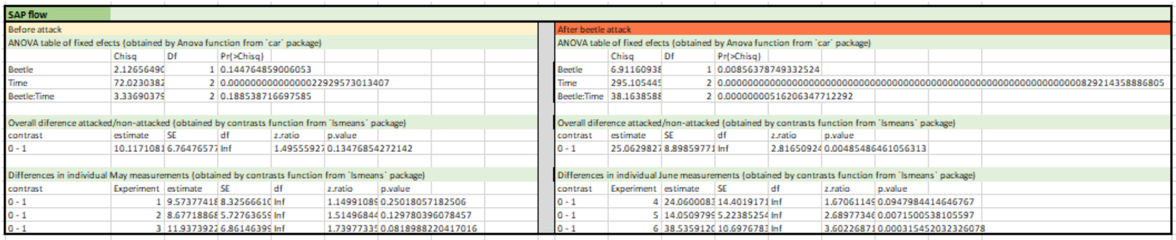


Supplementary Figure 2. Quant. masses peak areas of selected monoterpenes emitted by bark collected by SPME fiber. Boxplots summarize measurements performed five times per season at the freshly attacked subplot C (red color) and control subplots A, B, and D (green color). Individual measurements are represented by circles (subplot C) and squares, triangles and diamonds for subplot A, B and C. Numbers on the top are p-values of differences between attacked and non-attacked plots calculated by post hoc Tukey analyses based on repeated measures GLMM separately for pre-attack and attack period; bottom numbers are p-values of differences between attacked and non-attacked plots estimated by post hoc Tukey analyses in the given sampling time. The dashed line represents the period when the bark beetle attack was detected (from June 16th).



Supplementary Figure 3. The concentration of selected monoterpenes (ug/mg of dry weight in the phloem of eight selected monoterpenes extracted from phloem. Boxplots summarize measurements which was performed three times per season at the freshly attacked subplot C (red color) and control subplots A, B, and D (green color). Individual measurements are represented by circles (subplot C) and squares, triangles and diamonds for subplot A, B and C. Numbers on the top are p-values of differences between attacked and non-attacked plots calculated by post hoc Tukey analyses based on repeated measures GLMM separately for pre-attack and attack period; bottom numbers are p-values of differences between attacked and non-attacked plots estimated by post hoc Tukey analyses in the given sampling time. The dashed line represents the period when the bark beetle attack was detected (from June 16th).

Supplementary materials_Data sheet_Fig.3 Physiological parametres



SAP flow

Before attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

	Chisq	Df	Pr(>Chisq)
Beetle	2.12656490	1	0.144764859006053
Time	72.0230382	2	0.00000000000000022929573013407
Beetle:Time	3.33690379	2	0.188538716697585

Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	10.1171081	6.76476577	Inf	1.49555927	0.13476854272142

Differences in individual May measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	1	9.57377418	8.32566610	Inf	1.14991089	0.25018057182506
0 - 1	2	8.67718868	5.72763659	Inf	1.51496844	0.129780396078457
0 - 1	3	11.9373922	6.86146399	Inf	1.73977335	0.0818988220417016

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

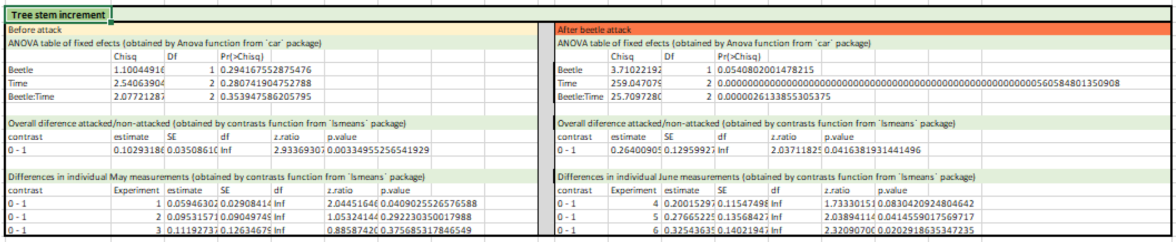
	Chisq	Df	Pr(>Chisq)
Beetle	6.91160938	1	0.00856378749332524
Time	295.105445	2	0.000000000000000000000000000000000000000000000000000000000000000829214358886805
Beetle:Time	38.1638588	2	0.00000000516206347712292

Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	25.0629827	8.89859771	Inf	2.81650924	0.00485486461056313

Differences in individual June measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	24.0600083	14.4019171	Inf	1.67061149	0.0947984414646767
0 - 1	5	14.0509799	5.22385254	Inf	2.68977346	0.0071500538105597
0 - 1	6	38.5359120	10.6976783	Inf	3.60226871	0.000315452032326078



Tree stem increment

Before attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

	Chisq	Df	Pr(>Chisq)
Beetle	1.10044916	1	0.294167552875476
Time	2.54063904	2	0.280741904752788
Beetle:Time	2.07721287	2	0.353947586205795

Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	0.10293186	0.03508610	Inf	2.93369307	0.00334955256541929

Differences in individual May measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	1	0.05946302	0.02908414	Inf	2.04451646	0.0409025526576588
0 - 1	2	0.09531571	0.09049749	Inf	1.05324144	0.292230350017988
0 - 1	3	0.11192737	0.12634679	Inf	0.88587420	0.375685317846549

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

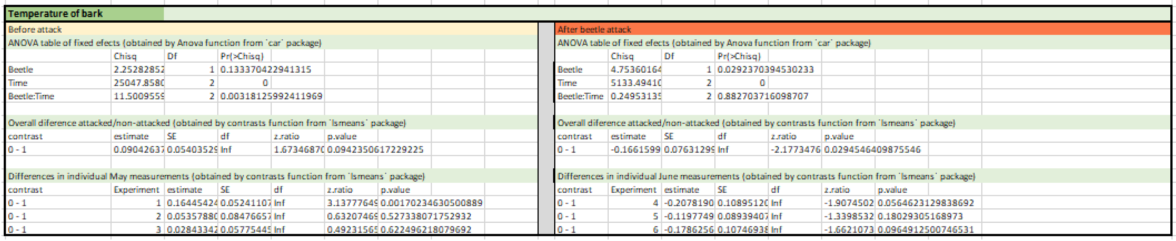
	Chisq	Df	Pr(>Chisq)
Beetle	3.71022192	1	0.0540802001478215
Time	259.047079	2	0.0000000000000000000000000000000000000000000000000000000560584801350908
Beetle:Time	25.7097280	2	0.0000026133855305375

Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	0.26400905	0.12959927	Inf	2.03711825	0.0416381931441496

Differences in individual June measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	0.20015297	0.11547498	Inf	1.73330151	0.0830420924804642
0 - 1	5	0.27665225	0.13568427	Inf	2.03894114	0.0414559017569717
0 - 1	6	0.32543635	0.14021947	Inf	2.32090700	0.0202918635347235



Temperature of bark

Before attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

	Chisq	Df	Pr(>Chisq)
Beetle	2.25282852	1	0.133370422941315
Time	25047.8580	2	0
Beetle:Time	11.5009559	2	0.00318125992411969

Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	0.09042637	0.05403529	Inf	1.67346870	0.0942350617229225

Differences in individual May measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	1	0.16445424	0.05241107	Inf	3.13777649	0.00170234630500889
0 - 1	2	0.05357880	0.08476657	Inf	0.63207469	0.527338071752932
0 - 1	3	0.02843342	0.05775445	Inf	0.49231565	0.622496218079692

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

	Chisq	Df	Pr(>Chisq)
Beetle	4.75360164	1	0.0292370394530233
Time	5133.49410	2	0
Beetle:Time	0.24953135	2	0.882703716098707

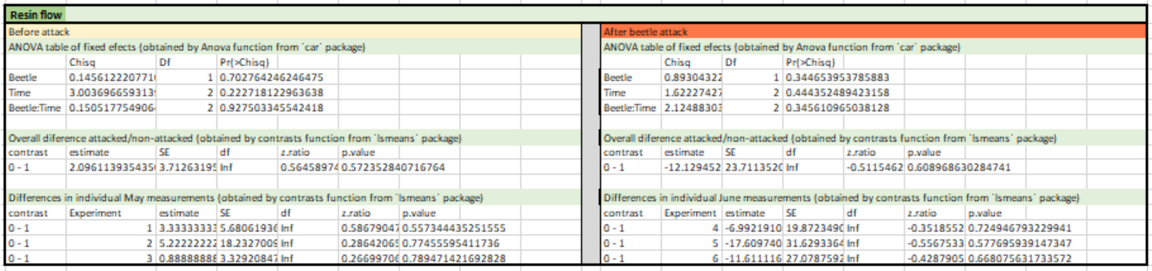
Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.1661599	0.07631299	Inf	-2.1773476	0.0294546409875546

Differences in individual June measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	-0.2078190	0.10895120	Inf	-1.9074502	0.0564623129838692
0 - 1	5	-0.1197749	0.08939407	Inf	-1.3398532	0.18029305168973
0 - 1	6	-0.1786256	0.10746938	Inf	-1.6621073	0.0964912500746531

Supplementary materials_Data sheet_Fig.4_ Defence characteristics



Resin flow

Before attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.1456122207710	1	0.702764246246475
Time	3.0036966593139	2	0.222718122963638
Beetle:Time	0.1505177549064	2	0.927503345542418

Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	2.0961139354350	3.71263195	Inf	0.56458974	0.572352840716764

Differences in individual May measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	1	3.33333333	5.68061936	Inf	0.58679047	0.557344435251555
0 - 1	2	5.22222222	18.2327009	Inf	0.28642065	0.77455595411736
0 - 1	3	0.88888888	3.32920847	Inf	0.26699706	0.789471421692828

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

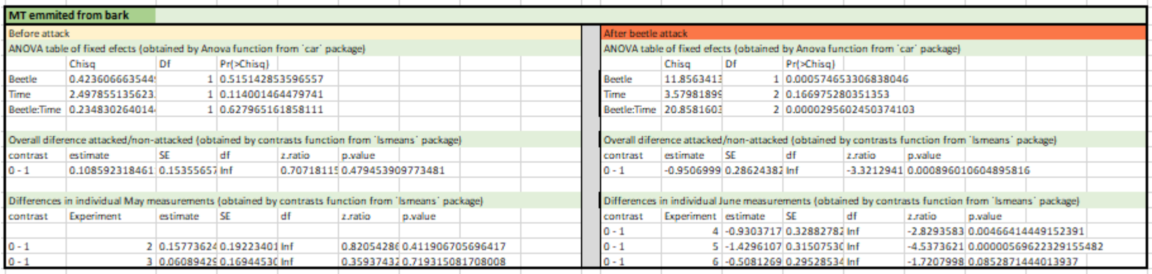
	Chisq	Df	Pr(>Chisq)
Beetle	0.89304322	1	0.344653953785883
Time	1.62227427	2	0.444352489423158
Beetle:Time	2.12488303	2	0.345610965038128

Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-12.129452	23.7113520	Inf	-0.5115462	0.608968630284741

Differences in individual June measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	-6.9921910	19.8723490	Inf	-0.3518552	0.724946793229941
0 - 1	5	-17.609740	31.6293364	Inf	-0.5567533	0.577695939147347
0 - 1	6	-11.611116	27.0787592	Inf	-0.4287905	0.668075631733572



MT emmited from bark

Before attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.4236066635449	1	0.515142853596557
Time	2.4978551356231	1	0.114001464479741
Beetle:Time	0.2348302640144	1	0.627965161858111

Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	0.1085923184611	0.15355657	Inf	0.70718115	0.479453909773481

Differences in individual May measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	2	0.15773624	0.19223401	Inf	0.82054286	0.411906705696417
0 - 1	3	0.06089429	0.16944530	Inf	0.35937432	0.719315081708008

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

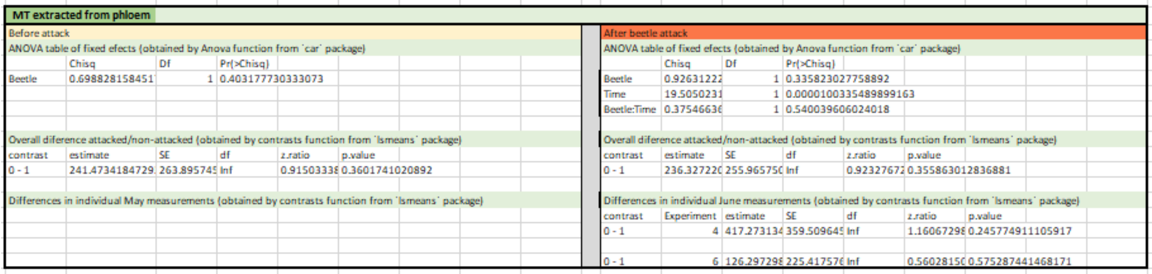
	Chisq	Df	Pr(>Chisq)
Beetle	11.8563413	1	0.000574653306838046
Time	3.57981899	2	0.166975280351353
Beetle:Time	20.8581603	2	0.0000295602450374103

Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.9506999	0.28624382	Inf	-3.3212941	0.000896010604895816

Differences in individual June measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	-0.9303717	0.32882782	Inf	-2.8293583	0.00466414449152391
0 - 1	5	-1.4296107	0.31507530	Inf	-4.5373621	0.00000569622329155482
0 - 1	6	-0.5081269	0.29528534	Inf	-1.7207998	0.0852871444013937



MT extracted from phloem

Before attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.6988281584517	1	0.403177730333073

Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	241.4734184729	263.895745	Inf	0.91503338	0.3601741020892

Differences in individual May measurements (obtained by contrasts function from ´lsmeans´ package)

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from ´car´ package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.92631222	1	0.335823027758892
Time	19.5050231	1	0.0000100335489899163
Beetle:Time	0.37546636	1	0.540039606024018

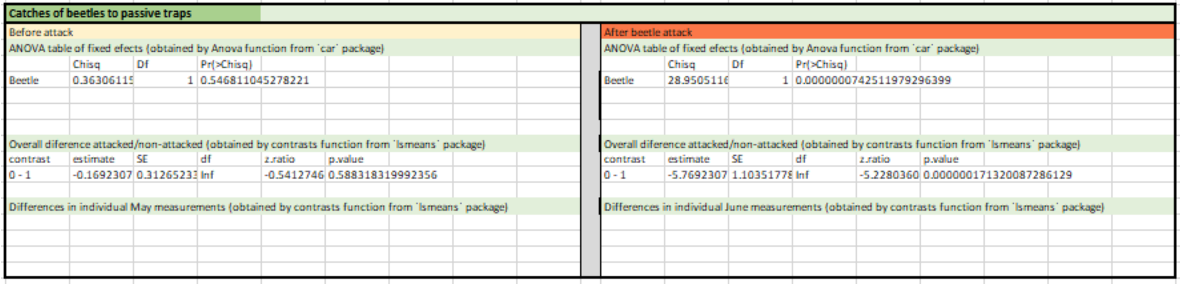
Overall diference attacked/non-attacked (obtained by contrasts function from ´lsmeans´ package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	236.327220	255.965750	Inf	0.92327672	0.355863012836881

Differences in individual June measurements (obtained by contrasts function from ´lsmeans´ package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	417.273134	359.509645	Inf	1.16067298	0.245774911105917
0 - 1	6	126.297298	225.417576	Inf	0.56028150	0.575287441468171

Supplementary materials_Data sheet_Fig.6_ Beetles



Catches of beetles to passive traps

Before attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.36306115	1	0.546811045278221

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.1692307	0.3126523	Inf	-0.5412746	0.588318319992356

Differences in individual May measurements (obtained by contrasts function from 'lsmeans' package)

After beetle attack

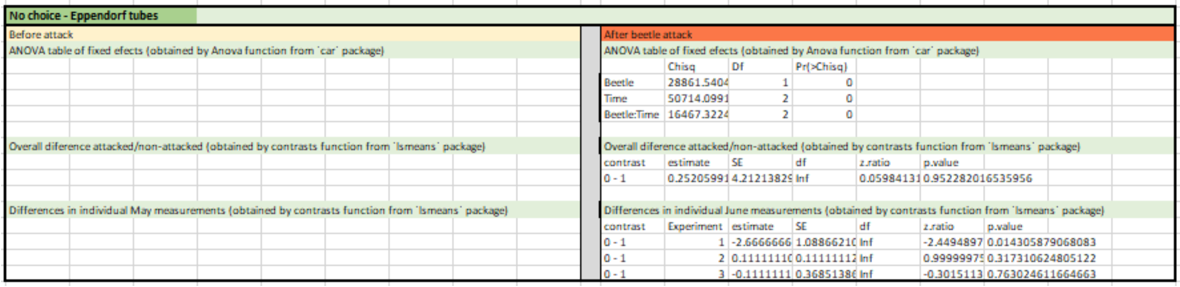
ANOVA table of fixed efects (obtained by Anova function from 'car' package)

	Chisq	Df	Pr(>Chisq)
Beetle	28.9505116	1	0.0000000742511979296399

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-5.7692307	1.1035177	Inf	-5.2280360	0.000000171320087286129

Differences in individual June measurements (obtained by contrasts function from 'lsmeans' package)



No choice - Eppendorf tubes

Before attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

Differences in individual May measurements (obtained by contrasts function from 'lsmeans' package)

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

	Chisq	Df	Pr(>Chisq)
Beetle	28861.5404	1	0
Time	50714.0991	2	0
Beetle:Time	16467.3224	2	0

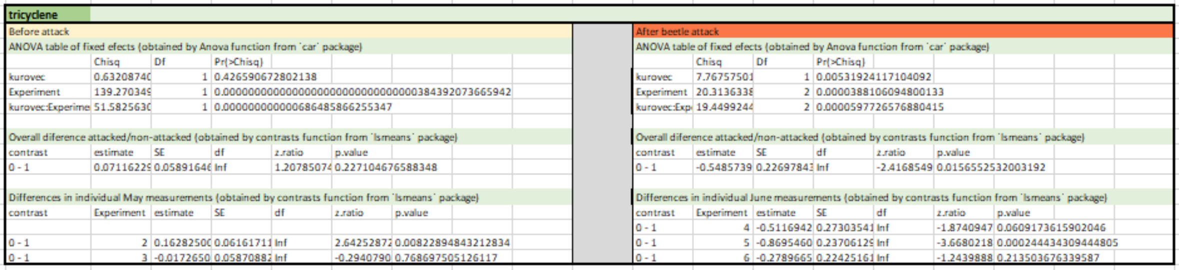
Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	0.25205991	4.2121382	Inf	0.05984131	0.952282016535956

Differences in individual June measurements (obtained by contrasts function from 'lsmeans' package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	1	-2.6666666	1.0886621	Inf	-2.4494897	0.014305879068083
0 - 1	2	0.1111111	0.1111111	Inf	0.9999997	0.317310624805122
0 - 1	3	-0.1111111	0.3685138	Inf	-0.3015113	0.763024611664663

Supplementary materials_Data sheet_Supplementary Fig.2_ SPME collections



tricyclene

Before attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

	Chisq	Df	Pr(>Chisq)
kurovec	0.6320874	1	0.426590672802138
Experiment	139.270349	1	0.0000000000000000000000000000000384392073665942
kurovec:Experime	51.582563	1	0.000000000000686485866255347

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	0.0711622	0.0589164	Inf	1.2078507	0.227104676588348

Differences in individual May measurements (obtained by contrasts function from 'lsmeans' package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	2	0.1628250	0.0616171	Inf	2.6425287	0.00822894843212834
0 - 1	3	-0.0172650	0.0587088	Inf	-0.2940790	0.768697505126117

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

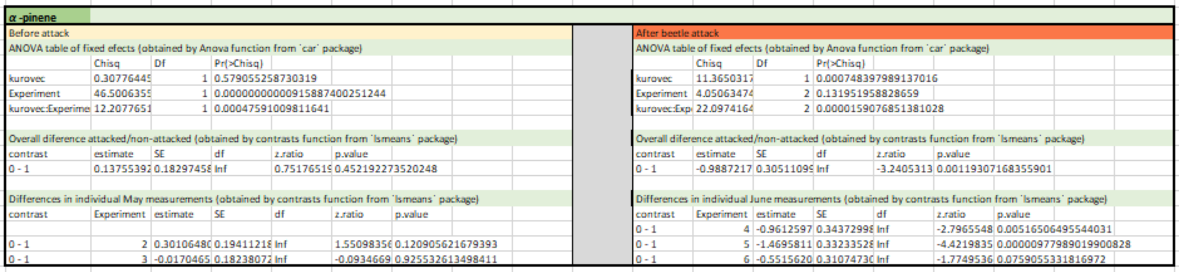
	Chisq	Df	Pr(>Chisq)
kurovec	7.76757501	1	0.00531924117104092
Experiment	20.313633	2	0.0000388106094800133
kurovec:Exp	19.449924	2	0.0000597726576880415

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.5485739	0.2269784	Inf	-2.4168549	0.015655253200319

Differences in individual June measurements (obtained by contrasts function from 'lsmeans' package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	-0.5116942	0.27303541	Inf	-1.8740947	0.0609173615902046
0 - 1	5	-0.8695460	0.2370612	Inf	-3.6680218	0.000244434309444805
0 - 1	6	-0.2789665	0.22425161	Inf	-1.2439888	0.213503676339587



α -pinene

Before attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

	Chisq	Df	Pr(>Chisq)
kurovec	0.3077644	1	0.579055258730319
Experiment	46.500635	1	0.00000000000915887400251244
kurovec:Experime	12.2077651	1	0.00047591009811641

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	0.1375539	0.1829745	Inf	0.7517651	0.452192273520248

Differences in individual May measurements (obtained by contrasts function from 'lsmeans' package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	2	0.3010648	0.1941121	Inf	1.5509835	0.120905621679393
0 - 1	3	-0.0170465	0.1823807	Inf	-0.0934669	0.925532613498411

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

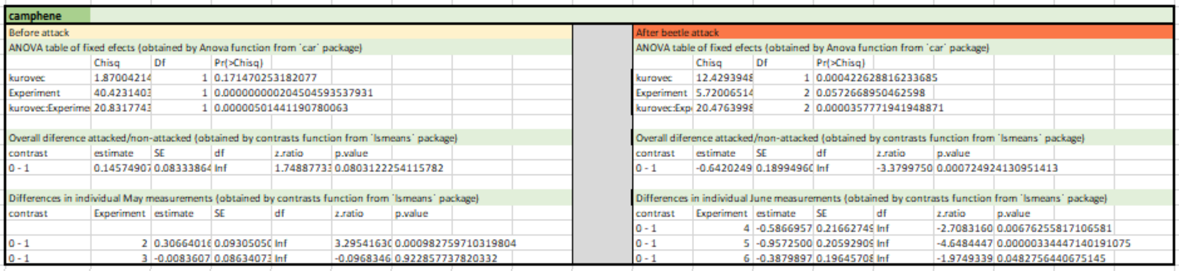
	Chisq	Df	Pr(>Chisq)
kurovec	11.3650317	1	0.000748397989137016
Experiment	4.0506347	2	0.131951958828659
kurovec:Exp	22.0974164	2	0.0000159076851381028

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.9887217	0.3051109	Inf	-3.2405313	0.00119307168355901

Differences in individual June measurements (obtained by contrasts function from 'lsmeans' package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	-0.9612597	0.3437299	Inf	-2.7965548	0.00516506495544031
0 - 1	5	-1.4695811	0.3323352	Inf	-4.4219835	0.00000977989019900828
0 - 1	6	-0.5515620	0.3107473	Inf	-1.7749536	0.0759055331816972



camphene

Before attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

	Chisq	Df	Pr(>Chisq)
kurovec	1.87004214	1	0.171470253182077
Experiment	40.423140	1	0.00000000020450459353793 1
kurovec:Experime	20.831774	1	0.00000501441190780063

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	0.1457490	0.08333864	Inf	1.7488773	0.0803122254115782

Differences in individual May measurements (obtained by contrasts function from 'lsmeans' package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	2	0.3066401	0.0930505	Inf	3.2954163	0.000982759710319804
0 - 1	3	-0.0083607	0.0863407	Inf	-0.0968346	0.922857737820332

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

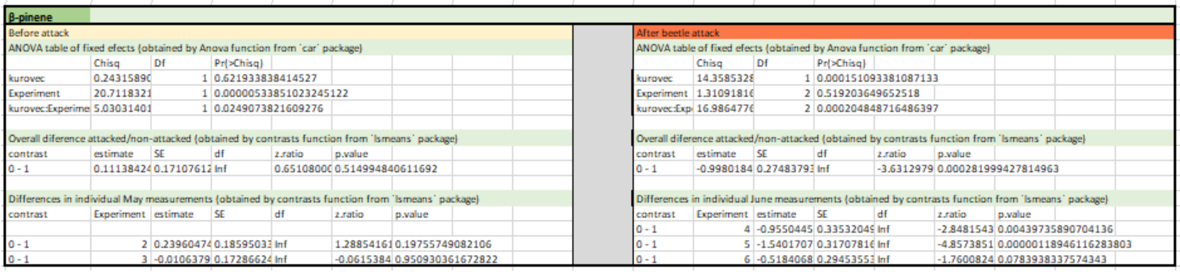
	Chisq	Df	Pr(>Chisq)
kurovec	12.429394	1	0.000422628816233685
Experiment	5.7200651	2	0.0572668950462598
kurovec:Exp	20.476399	2	0.0000357771941948871

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.6420249	0.1899496	Inf	-3.3799750	0.000724924130951413

Differences in individual June measurements (obtained by contrasts function from 'lsmeans' package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	-0.5866957	0.2166274	Inf	-2.7083160	0.00676255817106581
0 - 1	5	-0.9572500	0.2059290	Inf	-4.6484447	0.00000334447140191075
0 - 1	6	-0.3879897	0.1964570	Inf	-1.9749339	0.0482756440675145



β-pinene

Before attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

	Chisq	Df	Pr(>Chisq)
kurovec	0.2431589	1	0.621933838414527
Experiment	20.7118321	1	0.00000533851023245122
kurovec:Experime	5.03031401	1	0.0249073821609276

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	0.11138424	0.1710761	Inf	0.6510800	0.51499484061169 2

Differences in individual May measurements (obtained by contrasts function from 'lsmeans' package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	2	0.23960474	0.1859503	Inf	1.28854161	0.19755749082106
0 - 1	3	-0.0106379	0.17286624	Inf	-0.0615384	0.950930361672822

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from 'car' package)

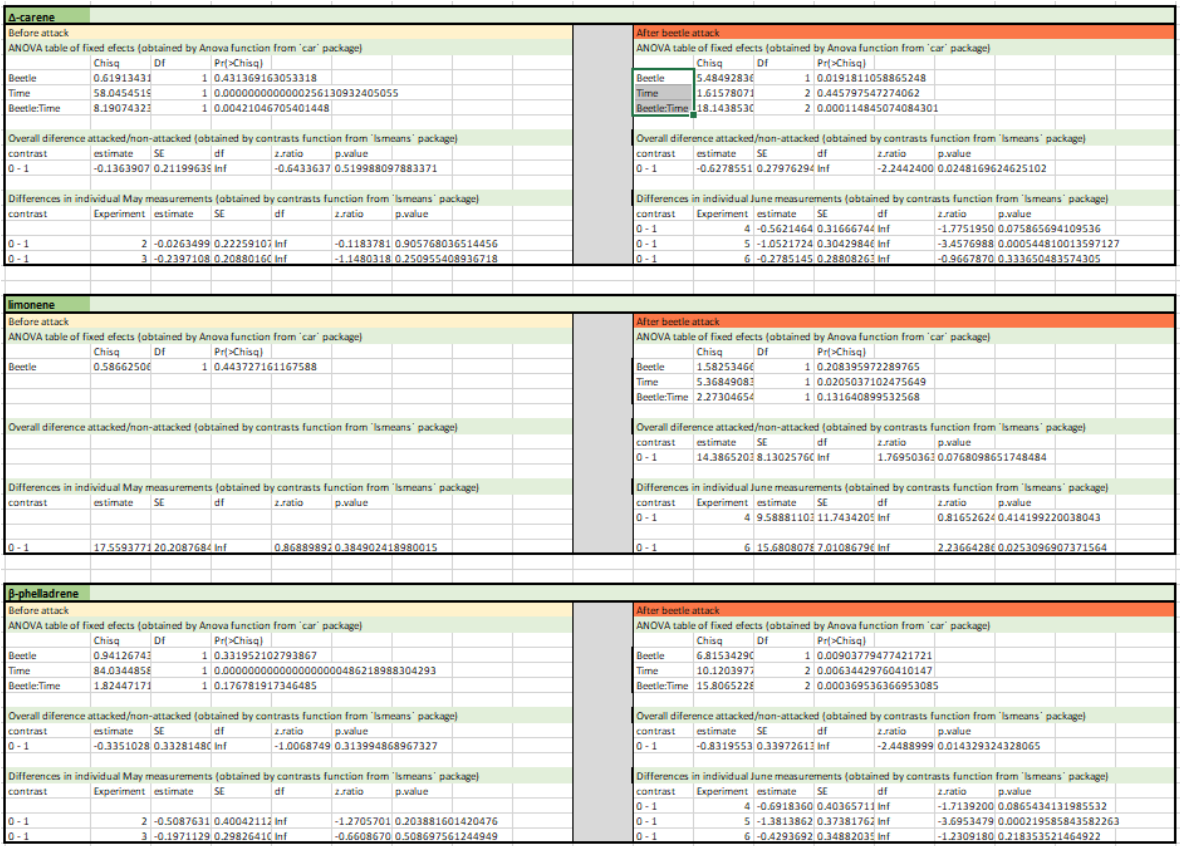
	Chisq	Df	Pr(>Chisq)
kurovec	14.358532	1	0.000151093381087133
Experiment	1.3109181	2	0.519203649652518
kurovec:Exp	16.986477	2	0.000204848716486397

Overall diference attacked/non-attacked (obtained by contrasts function from 'lsmeans' package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.9980184	0.2748379	Inf	-3.6312979	0.000281999427814963

Differences in individual June measurements (obtained by contrasts function from 'lsmeans' package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	-0.9550445	0.3353204	Inf	-2.8481543	0.00439735890704136
0 - 1	5	-1.5401707	0.3170781	Inf	-4.8573851	0.00000118946116283803
0 - 1	6	-0.5184068	0.2945355	Inf	-1.7600824	0.0783938337574343



Δ-carene

Before attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.61913431	1	0.431369163053318
Time	58.0454519	1	0.00000000000000256130932405055
Beetle:Time	8.19074323	1	0.00421046705401448

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.1363907	0.21199639	Inf	-0.6433637	0.519988097883371

Differences in individual May measurements (obtained by contrasts function from `lsmeans` package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	2	-0.0263499	0.22259107	Inf	-0.1183781	0.905768036514456
0 - 1	3	-0.2397108	0.20880160	Inf	-1.1480318	0.250955408936718

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	5.48492836	1	0.0191811058865248
Time	1.61578071	2	0.445797547274062
Beetle:Time	18.1438530	2	0.000114845074084301

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.6278551	0.27976294	Inf	-2.2442400	0.0248169624625102

Differences in individual June measurements (obtained by contrasts function from `lsmeans` package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	-0.5621464	0.31666744	Inf	-1.7751950	0.075865694109536
0 - 1	5	-1.0521724	0.30429846	Inf	-3.4576988	0.000544810013597127
0 - 1	6	-0.2785145	0.28808263	Inf	-0.9667870	0.333650483574305

limonene

Before attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.58662506	1	0.443727161167588

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

Differences in individual May measurements (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	17.5593771	20.2087684	Inf	0.86889892	0.384902418980015

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	1.58253466	1	0.208395972289765
Time	5.36849083	1	0.0205037102475649
Beetle:Time	2.27304654	1	0.131640899532568

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	14.3865203	8.13025760	Inf	1.76950363	0.0768098651748484

Differences in individual June measurements (obtained by contrasts function from `lsmeans` package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	9.58881103	11.7434205	Inf	0.81652624	0.414199220038043
0 - 1	6	15.6808078	7.01086796	Inf	2.23664286	0.0253096907371564

β-phelladrene

Before attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.94126743	1	0.331952102793867
Time	84.0344858	1	0.0000000000000000000486218988304293
Beetle:Time	1.82447171	1	0.176781917346485

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.3351028	0.33281480	Inf	-1.0068749	0.313994868967327

Differences in individual May measurements (obtained by contrasts function from `lsmeans` package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	2	-0.5087631	0.40042112	Inf	-1.2705701	0.203881601420476
0 - 1	3	-0.1971129	0.29826410	Inf	-0.6608670	0.508697561244949

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	6.81534290	1	0.00903779477421721
Time	10.1203977	2	0.00634429760410147
Beetle:Time	15.8065228	2	0.000369536366953085

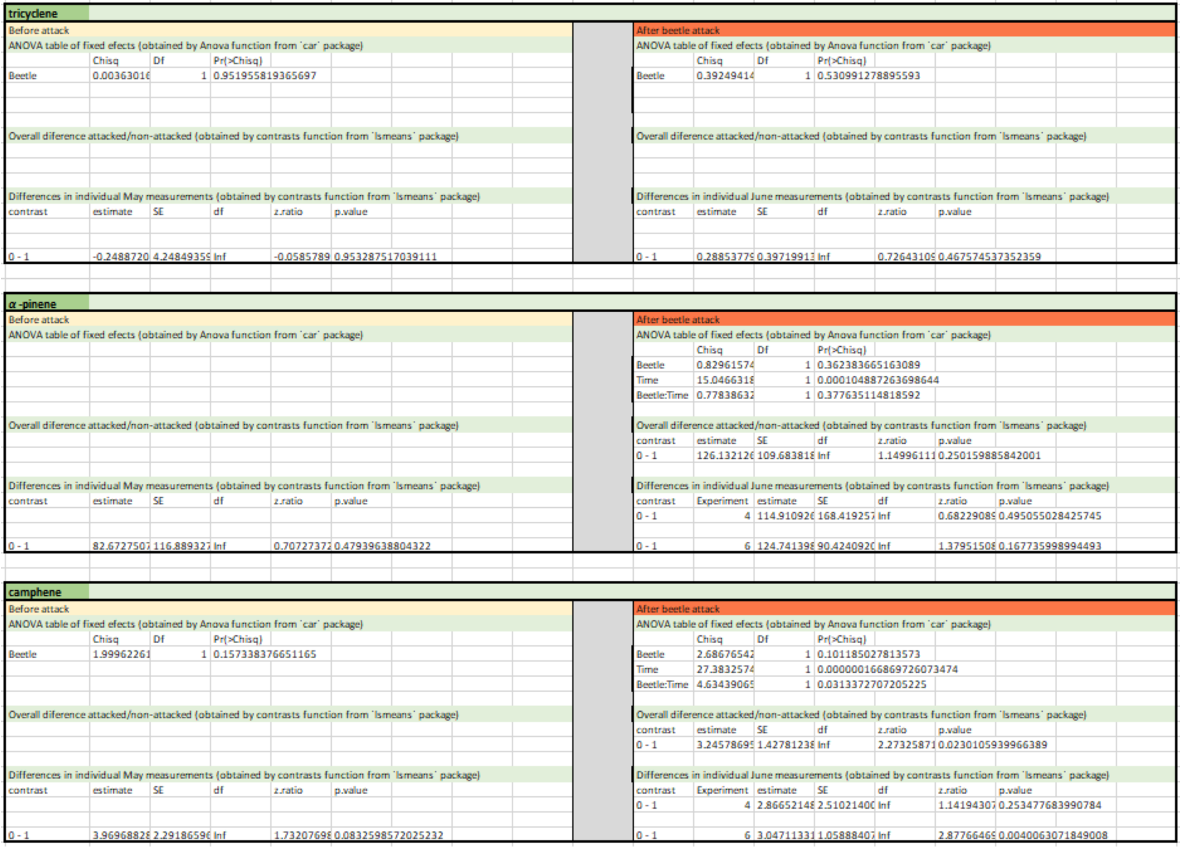
Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.8319553	0.33972613	Inf	-2.4488999	0.014329324328065

Differences in individual June measurements (obtained by contrasts function from `lsmeans` package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	-0.6918360	0.40365711	Inf	-1.7139200	0.0865434131985532
0 - 1	5	-1.3813862	0.37381762	Inf	-3.6953479	0.000219585843582263
0 - 1	6	-0.4293692	0.34882035	Inf	-1.2309180	0.218353521464922

Supplementary materials_Data sheet_Supplementary Fig.3_ phloem collections



tricyclene

Before attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.00363016	1	0.951955819365697

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

Differences in individual May measurements (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	-0.2488720	4.24849359	Inf	-0.0585789	0.953287517039111

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.39249414	1	0.530991278895593

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

Differences in individual June measurements (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	0.28853779	0.39719913	Inf	0.72643109	0.467574537352359

α -pinene

Before attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

Differences in individual May measurements (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	82.6727507	116.889327	Inf	0.70727372	0.47939638804322

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.82961574	1	0.362383665163089
Time	15.0466318	1	0.000104887263698644
Beetle:Time	0.77838632	1	0.377635114818592

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	126.132126	109.683818	Inf	1.14996111	0.250159885842001

Differences in individual June measurements (obtained by contrasts function from `lsmeans` package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	114.910926	168.419257	Inf	0.68229089	0.495055028425745
0 - 1	6	124.741398	90.4240920	Inf	1.37951508	0.167735998994493

camphene

Before attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	1.99962261	1	0.157338376651165

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

Differences in individual May measurements (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	3.96968828	2.29186596	Inf	1.73207698	0.0832598572025232

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

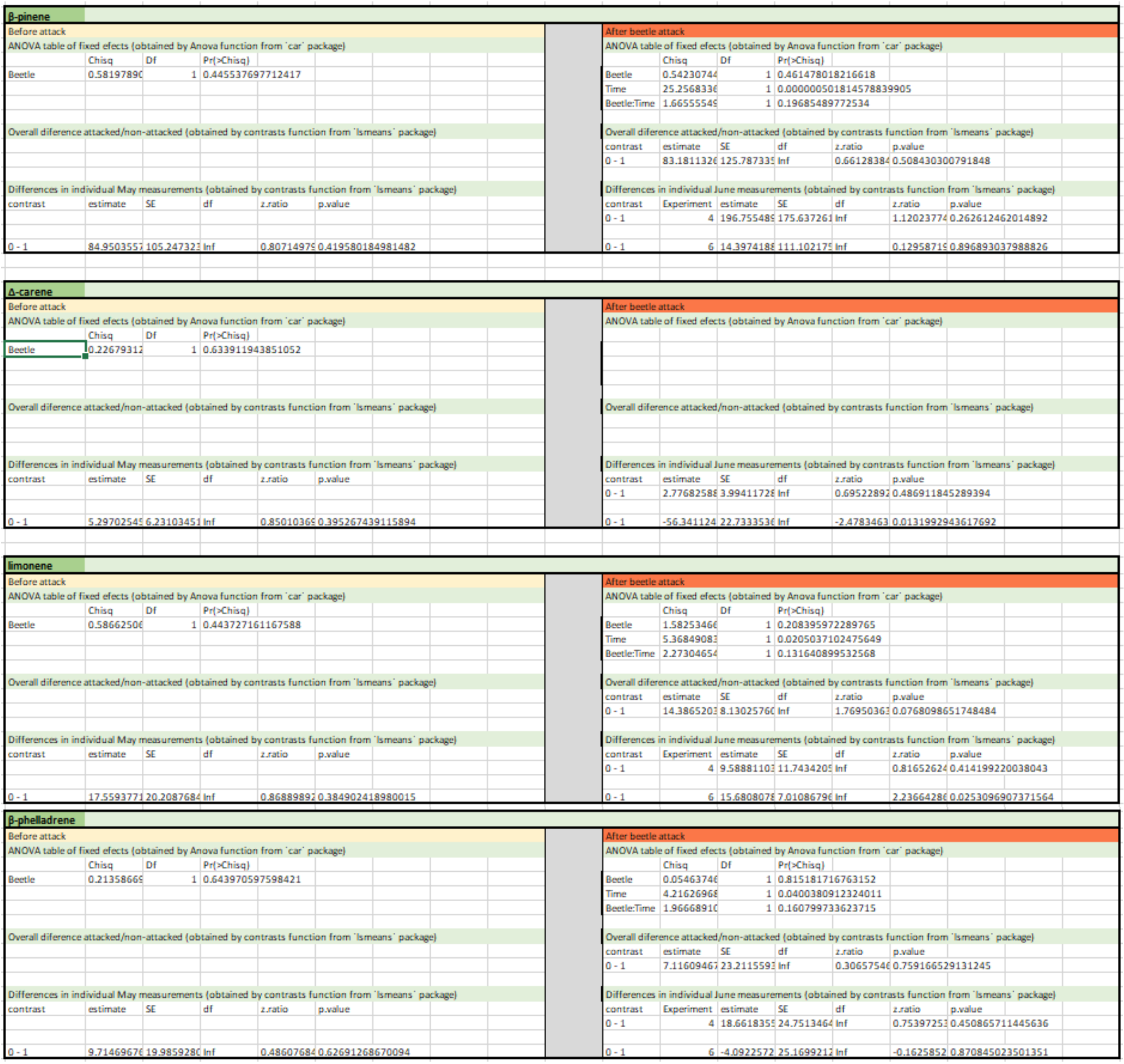
	Chisq	Df	Pr(>Chisq)
Beetle	2.68676542	1	0.101185027813573
Time	27.3832574	1	0.000000166869726073474
Beetle:Time	4.63439065	1	0.0313372707205225

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	3.24578695	1.42781238	Inf	2.27325871	0.0230105939966389

Differences in individual June measurements (obtained by contrasts function from `lsmeans` package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	2.86652148	2.51021400	Inf	1.14194307	0.253477683990784
0 - 1	6	3.04711331	1.05888407	Inf	2.87766469	0.0040063071849008



β-pinene

Before attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.58197890	1	0.445537697712417

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

Differences in individual May measurements (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	84.9503557	105.247323	Inf	0.80714979	0.419580184981482

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.54230744	1	0.461478018216618
Time	25.2568336	1	0.000000501814578839905
Beetle:Time	1.66555549	1	0.19685489772534

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	83.1811326	125.787335	Inf	0.66128384	0.508430300791848

Differences in individual June measurements (obtained by contrasts function from `lsmeans` package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	196.755489	175.637261	Inf	1.12023774	0.262612462014892
0 - 1	6	14.3974188	111.102175	Inf	0.12958719	0.896893037988826

Δ-carene

Before attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.22679312	1	0.633911943851052

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

Differences in individual May measurements (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	5.29702545	6.23103451	Inf	0.85010369	0.395267439115894

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

Differences in individual June measurements (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	2.77682588	3.99411728	Inf	0.69522892	0.486911845289394
0 - 1	-56.341124	22.7333536	Inf	-2.4783463	0.0131992943617692

limonene

Before attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.58662506	1	0.443727161167588

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

Differences in individual May measurements (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	17.5593771	20.2087684	Inf	0.86889892	0.384902418980015

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	1.58253466	1	0.208395972289765
Time	5.36849083	1	0.0205037102475649
Beetle:Time	2.27304654	1	0.131640899532568

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	14.3865203	8.13025760	Inf	1.76950363	0.0768098651748484

Differences in individual June measurements (obtained by contrasts function from `lsmeans` package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	9.58881103	11.7434205	Inf	0.81652624	0.414199220038043
0 - 1	6	15.6808078	7.01086796	Inf	2.23664286	0.0253096907371564

β-phelladrene

Before attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.21358669	1	0.643970597598421

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

Differences in individual May measurements (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	9.71469676	19.9859280	Inf	0.48607684	0.62691268670094

After beetle attack

ANOVA table of fixed efects (obtained by Anova function from `car` package)

	Chisq	Df	Pr(>Chisq)
Beetle	0.05463746	1	0.815181716763152
Time	4.21626968	1	0.0400380912324011
Beetle:Time	1.96668910	1	0.160799733623715

Overall diference attacked/non-attacked (obtained by contrasts function from `lsmeans` package)

contrast	estimate	SE	df	z.ratio	p.value
0 - 1	7.11609467	23.2115593	Inf	0.30657546	0.759166529131245

Differences in individual June measurements (obtained by contrasts function from `lsmeans` package)

contrast	Experiment	estimate	SE	df	z.ratio	p.value
0 - 1	4	18.6618355	24.7513464	Inf	0.75397253	0.450865711445636
0 - 1	6	-4.0922572	25.1699212	Inf	-0.1625852	0.870845023501351

4.3 Pilot Study of 3D Spatial Distribution of *α*-Pinene Emitted by Norway Spruce (L.) Karst Recently Infested by *Ips typographus* (L. 1758) (Coleoptera: Scolytinae)

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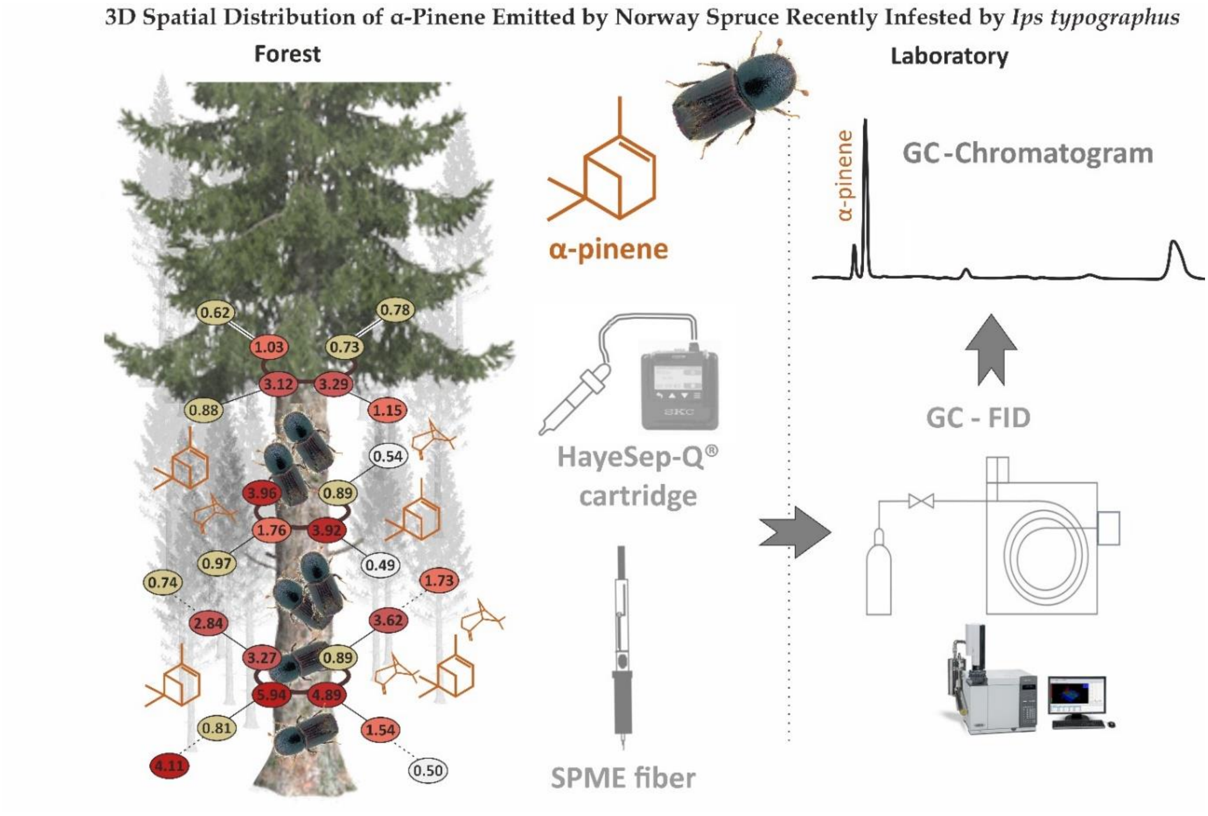
This article hypothesizes that “Volatile organic compounds (VOCs) emitted by BB freshly infested trees can be utilized as markers to detect BB attack in early stadia.” The main objective of this work is to create the methodology for detecting the space distribution of VOCs emitted by bark beetle-infested trees in real conditions via SPME fiber collection, collection of sorbent cartridges with using air pumps, and the preparation for using drones with special sensors.

This article concludes that the VOCs emitted from freshly attacked trees highly depend on environmental conditions (temperature, humidity, wind speed, and surrounding environment). The distribution of accurate data provides a solid base for scanning techniques for early attack detection.

Graphical abstract



forests

Article

Pilot Study of 3D Spatial Distribution of α-Pinene Emitted by Norway Spruce (L.) Karst Recently Infested by *Ips typographus* (L. 1758) (Coleoptera: Scolytinae)

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Abstract: The Eurasian Spruce Bark Beetle (*Ips typographus*) (L. 1758) (Coleoptera: Scolytinae) poses a significant threat to Eurasia's Norway spruce (*Picea abies*) (L.) Karst, forests. Early detection of infested trees is crucial to control beetle outbreaks and allow salvage logging before the next generation emerges. Besides traditional methods, new approaches focus on monitoring volatile organic compounds, mainly monoterpenes, emitted by infested trees. Using analytical chemistry, we studied the distribution of these compounds, particularly α-pinene, around infested trees. In lab trials, we optimized α-pinene detection using dynamic absorption and solid-phase microextraction (SPME), analyzed by gas chromatography with flame ionization detection (GC-FID). We conducted forest trials, revealing varying α-pinene abundance due to changing conditions. However, consistent trends emerged: levels were highest near the infested tree stem and 1.3 m above ground in the first trial and at a 1 m distance from the infested stem in the second. We generated a three-dimensional cloud depicting the distribution of α-pinene around infested trees in their natural habitat. These findings open avenues for detecting bark beetles on a large scale by mapping elevated concentrations of volatile organic compounds emitted by infested trees, potentially leading to alternative pest management methods. Scanning methods, such as electronic sensors combined with remote sensing, hold promise for this application.

Keywords: early attack detection; bark beetle; VOC; α-pinene; *Picea abies*; SPME; Eurasian Spruce Bark Beetle

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1. Introduction

The Eurasian Spruce Bark Beetle *Ips typographus* (L. 1758) (Coleoptera: Scolytinae) is the main pest of Norway spruce, *Picea abies* (L.) Karst. forests in the Central European region. Over the past decade, the combination of ongoing climate change, economically driven silvicultural practices, and the presence of spruce stands in areas beyond their natural range have weakened the natural defense mechanisms of trees and resulted in the occurrence of severe bark beetle spreading [1]. In the Czech Republic, outbreaks started after severe drought events in 2015 and 2018 [2] and led to an exponential increase in salvage logging volume from 2017 to 2020, with the volume rising from approximately 5.9 million m^3 in 2017 to 26.2 million m^3 in 2020 [3,4] (Figure S1).

The initial step in managing a bark beetle outbreak is early detection of newly infested trees to enable timely salvage before the emergence of offspring [4]. Forest keepers typically rely on traditional methods, which involve personally observing the boring dust produced by infesting beetles [5]. However, during bark beetle outbreaks in large, forested areas, this approach has severe limitations, often resulting in the exponential spread of the beetles.

Hence, an alternative method for early detection on a large scale is needed. Remote sensing techniques have been extensively investigated, using the detection of different indicators from spectral features to temperature [6] and recently also involving chemical substances (Sentinel SP) [7].

Recent research by [8] has proposed measuring the emission of volatile organic compounds (VOCs) from infested spruce as an indicator of bark beetle attacks. Furthermore, various methods for detecting these VOCs at different developmental stages have been introduced. Current research is investigating the utilization of an electronic nose equipped with nonspecific sensors for VOC detection [9]. Likewise, nonspecific metal oxide sensors have been mounted on UAVs to assess the concentration of α-pinene in forest environments [10]. Notably, natural olfaction systems of dogs trained to detect bark beetle pheromones have proven more effective in finding infested trees in cooperation with their dog handler compared to human experts only [11,12].

The VOC emitted by conifer mainly consists of hemi- and monoterpenes, which are produced as defense secondary metabolites. The conifer's immediate defense mechanism against wood-boring insects is the exudation of constitutive resin, which has a toxic and immobilizing effect on beetles [13]. In the later stages of a bark beetle attack, the production of resin is induced in the newly formed resin ducts in the phloem, xylem, and bark [14]. The resin is a mixture of terpenic compounds. The monoterpenes are volatile and form the main content of VOCs emitted by conifers. In spruce, α-pinene, β-pinene, Δ-carene, limonene, β-phellandrene, camphene, and myrcene dominate [15] but resin also contains sesquiterpenes and a high content of highly viscous diterpenes [16,17]. Oxidized forms of all terpenes are also present.

In addition to resin emissions from the stem, volatile terpenes are also emitted from the needles in the canopy of conifers [18,19]. The emission rate of volatile terpenes from healthy trees is influenced by various macro- and microclimatic conditions, such as temperature and humidity [17,20]. Different temperatures, and consequently varying VOC emissions, are observed in clearings and forest edges within fragmented forests [17,21]. Furthermore, VOC emissions in conifer forests exhibit vertical variations [22] and follow a diurnal rhythm dependent on tree physiological processes [19]. The terpenes emissions from conifer forests are widely discussed in the context of terpenes as a free radical source in the atmosphere [23–25], because hemi and monoterpenes are photochemically reactive compounds that affect ozone and carbon monoxide concentrations and their oxidation products can participate in the formation of secondary organic aerosol and cloud condensation nuclei [26].

When Norway spruces are attacked by bark beetles, the content of emitted terpenes from the stem significantly increases during the first two weeks of infestation. This growth is primarily attributed to the opening of constitutive resin storage and is quantified in the close vicinity of the stem. Different methods used for quantification have yielded a wide range of results, ranging from a 10 to 100-fold upturn [8,27,28]. The dominant compound in emissions was always α-pinene, representing the time and spatial distribution of the other main monoterpenes [8]. The bouquet of infested trees also includes the aggregation pheromone produced by bark beetles. Beetles use this scent for navigation to aggregate, allowing them to overcome the tree's defense during the infestation [29]. The content of bark beetle pheromones is several orders of magnitude lower than that of α-pinene in forests [30]. However, beetles are capable of discerning this signal from the background of host odors thanks to specialized receptors on their antennae, the organs responsible for perceiving smells [31]. Furthermore, beetles may orient themselves by detecting host compounds, primarily terpenes, when choosing a suitable host tree or habitat [32]. They also have specialized receptors for host compounds [33].

Numerous studies have investigated monoterpenes emitted by conifers in both laboratory and natural conditions. In the laboratory, collection systems can be readily optimized, as detailed in a comprehensive review [34]. In field conditions, VOC collection is more complex, and various techniques have been employed to address specific research questions [35].

The most common approach involves dynamic headspace sampling with compound collection onto sorbents, followed by extraction into solvents or thermal desorption. Additionally, solid-phase microextraction methods have been utilized (as shown in Table 1) [36,37].

Table 1. Methods of VOC collection from conifers in forests.

Tree Species/ Stress Occasions	Sampling Specification	Compound (Unit)	Technical Parameters (Sorbent; Amount; Flow Rate)	Time of Sorption	Analytical Method	Source
Picea abies/ intact forest	Stem (not specify) 5 m above the ground; stainless steel TD tubes	Individual monoterpenes α-pinene 3.07 ± 0.25 ppbv	Tenax TA, (35/60) 200 mg; 200 mL/min	30 min	GC-MS	[38]
Picea abies/ attacked trees	Stem 1.3 m above the ground; surrounded PET (25 × 38 cm) encloser	Individual monoterpenes α-pinene 62.8 ± 23.6 μg h^{-1} m^{-2} bark area	Tenax-TA a Carbopack-B, (60/80) 100/100 mg; 200 mL/min	60 min	GC-MS	[28]
Picea engelmannii / attacked trees	Stem 0.5 to 1.5 m above the ground; the trunk by dynamic sampling <1 cm from stem (sorbent trap)	Individual monoterpenes α-pinene 8.5 ± 2.1 ng L^{-1}	Porapak Q 110 mg; 400 mL/min	120 min	GC-MS	[39]
Picea abies/ attacked trees on forest edge	Stem 3 h 1–2 m above the ground; sanitized T gllas tube	Verbenone (ng/3 h): α-pinene (μg/3 h) 0.6 (ng/3 h): (μg/3 h)	Porapak Q, (80/100) 70 mg; 20 mL/min	180 min	GC-MS	[40]
Pseudotsuga menziesii/ attacked trees	Branch 1.5 m above the ground; Teflon bag (50 × 75 cm)	Individual VOCs α-pinene 813.9 ± 482.29 ng h^{-1} g^{-1} fresh weight	HayeSep-Q 30 mg; 500 mL/min	30 min	GC-MS	[41]
Pinus rigida and *Pinus koraiensis*/intact forest	Branch 20 L Tedlar bag	Total monoterpenes emission (μgC gdw^{-1} h^{-1}) *Pinus rigida* 0.9 μgC gdw^{-1} h^{-1} *Pinus koraiensis* 0.4 μgC gdw^{-1} h^{-1}	Tenax TA, (60/80) and Carbotrap, (20/40) 200 mg; 100–200 mL/min	15–60 min	GC-MS GC-FID	[42]
Pinus sylvestris/ intact forest	Branch canopy height; (FEP) copolymer foil (50 μm thickness) mounted in cylindrical frames	Individual monoterpenes α-pinene 917 ± 58 ng h^{-1} g^{-1} (April) α-pinene 75 ± 12 ng h^{-1} g^{-1} (July)	Tenax TA, (60/80) and Carbotrap, (20/40) 50–100 mg; 100 mL/min	60 min	GC-MS GC-FID	[43]
Pinus sylvestris and *Picea abies*/intact forest	Branch 18 L all-Teflon chamber made of 0.05 mm transparent FEP-Teflon film enclosing a 20–30 cm branch segment	Acetone and α-pinene (ng gdw^{-1} h^{-1}) α-pinene 80 ng gdw^{-1} h^{-1} Monoterpene emission 900 ± 640 ng C gdw^{-1} h^{-1}	Tenax TA 200 mg; 100 mL/min	12–40 min	GC-MS GC-FID	[44]
Picea abies/ attacked trees	Stem 1.3 m above the ground; tree trunk chamber connected with PTFE tubing	Individual VOCs (μg m^{-2} h^{-1}) α-pinene 911.14 μg m^{-2} h^{-1}	Tenax TA and Carbograph 1TD 200 mL/min	30 min	GC-MS	[27]
Picea abies/stress from sun irradiation	Stem 3.5 m above the ground; aluminum chamber	Individual monoterpenes sum of eight main MT 8.5 log10 VOC	SPME	60 min (from 1 to 2 p.m.)	GC-MS	[17]
Picea abies/attacked trees	Stem 3.5 m above the ground; aluminum chamber	Individual monoterpenes α-pinene 9.5 log_{10} sum peak area	SPME	60 min (from 1 to 2 p.m.)	GC-MS	[8]
Lerosa NP forest/conifer forest; 6 different plots	open air	Individual VOCs 894 abundance relative to hexanal (%)	SPME	300 min (from 10 a.m. to 3 p.m.)	GC-MS	[45]

To study emissions on an ecosystem scale, researchers have employed various instrumental techniques, such as proton-transfer-reaction-time-of-flight (PTR-TOF) [46–48] mass spectrometry for quantifying monoterpene fluxes [19] or specialized gas chromatographs installed in situ at collection sites. However, these instruments can be expensive and lack portability, restricting data collection to a limited number of sites [49].

Hypothesis and objectives: This study is founded on a previously proposed concept suggesting that the number of volatile organic compounds emitted by trees, particularly monoterpenes, significantly increases within two weeks of bark beetle infestation [8]. This increase can serve as a measurable characteristic upon which the foundation of a newly developed alternative early bark beetle detection method may be based.

Our objectives were to optimize the collection of α-pinene emissions from spruce logs subjected to a controlled simulative bark beetle infestation in the laboratory and consequently detect the outdoor 3D dispersion of monoterpenes, represented by α-pinene, in the surroundings of freshly infested spruce trees at horizontal and vertical distances. We employed analytical chemistry methods for collecting VOCs, which are not typically used for VOC collection in open environments.

Particular objectives were:

1. Assess the distribution of α-pinene at different distances from and heights within a simulatively infested log pile in the laboratory, considering specific temperature conditions.
2. Validate the feasibility of measuring the distribution of α-pinene under actual field conditions, specifically focusing on naturally *Ips typographus*-infested trees within a forest environment.
3. Consider other influencing factors, such as temperature, wind speed, and the immediate surroundings of the forest.
4. Compare the effectiveness of the Solid-Phase Microextraction (SPME) method with HayeSep-Q® sorbent cartridges, which involve drawing air through them using air pumps.

2. Materials and Methods

2.1. *Optimization of VOC Collection from Simulatively Infested Spruce Logs in a Laboratory*

The experiments were conducted under laboratory conditions (25 ± 1 °C, humidity 60% ± 10%) using three fresh-cut logs (height of 40, diameter of 25 cm) of *Picea abies* previously stored in a cold room (−5 °C). The experiment was repeated three times at one-week intervals. Prior of all repetitions, logs were acclimated for 24 h before volatile collection in the conditions described above. Logs were arranged vertically in piles from the floor. The lowest log represented level 1—L1 (20 cm), the middle log level 2—L2 (60 cm), and the upper log level 3—L3 (100 cm) (Figure 1a). To prevent unwanted VOC emissions from fresh-cut logs, the upper and lower exposed surfaces were covered with PE plastic film.

The bark beetle attack was simulated by drilling holes (Ø 2 mm × 0.3 cm deep) into the bark and phloem on the surface of the logs (100 holes per log, spaced in 5 cm spin) to mimic the production of VOC emissions during bark beetle infestation.

VOCs were collected from each of the three levels (L1, L2, and L3) at two distances, 5 cm and 30 cm from the bark surface, using Solid-Phase Microextraction (SPME) fibers (PDMS/CAR/DVB, Supelco, PA, USA) as shown in Figure 1b. The SPME fibers were fixed in protective hard plastic cylindrical chambers hung in an open space (7.2 cm high, Ø 5.2 cm, material PP, Merci, CZE), with an open bottom and a hole covered by septa in the upper lid to hold the fiber. To follow the distance and cardinal directions described above, the chambers in the experimental apparatus were fixed by metal wires.

SPME collection was conducted for 15 min, beginning 2 h after drilling, under controlled conditions (25 ± 1 °C, humidity 60% ± 10%). A total of 24 samples were taken, with 8 samples per level (n = 24).

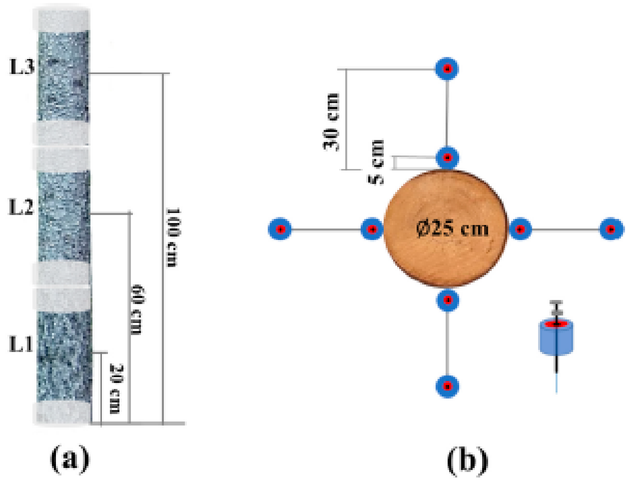

Figure 1. Volatile collection of α-pinene under laboratory conditions from fresh drilled logs of *Picea abies*. (**a**) The collection of α-pinene occurred across three height levels, namely L1, L2, and L3. (**b**) Top view of the log pile; black dots indicate distances between the bark surface and the point of collection of α-pinene for each level.

2.2. *Field Collection of Distributed VOCs Emitted by the Ips typographus Naturally Infested Picea abies*

We conducted two collections of VOCs from naturally infested trees in the field at different locations and during different periods of the 2022 growing season. In this study, we did not measure VOCs from non-infested trees as controls. This decision stems from the continuation of our prior study [8] and existing literature reports (Table 1), which have consistently demonstrated several-fold increases in emissions from infested trees using similar techniques. Both collections took place on the Forests ČZU property near Kostelec nad Černými lesy, the Czech Republic. The area is characterized by a mature forest primarily composed of a 90-year-old Norway spruce (*P. abies*) plantation, situated at an altitude of 400–450 m above sea level (Figures 2 and 3).

The distributed VOCs were collected within 900 m around the freshly *Ips typographus*-infested individual Norway spruces. The infestation status was found by the occurrence of fresh frass at the stem's base, and infestation stadia were specified by assessing the beetle attack density exhibited by the sampled tree. Both sampled trees were in the nuptial chamber building attack stadia, approximately two weeks from the beginning of the mass attack.

The VOCs were collected from infested trees at three different height levels from the ground (1.3 m, 2.6 m, and 4 m). Collection chambers were placed at three different distances from the tree trunk (5 cm, 100 cm, and 900 cm away from the tree) (Figure 3). The row of collection chambers was oriented in four cardinal directions: north (N), east (E), south (S), and west (W) (see Figures 2–4 for reference). Dataloggers were used throughout the experiment to measure the temperature and humidity (Tables S1 and S2), and an anemometer was used to measure the wind speed (Figures 2 and 3).

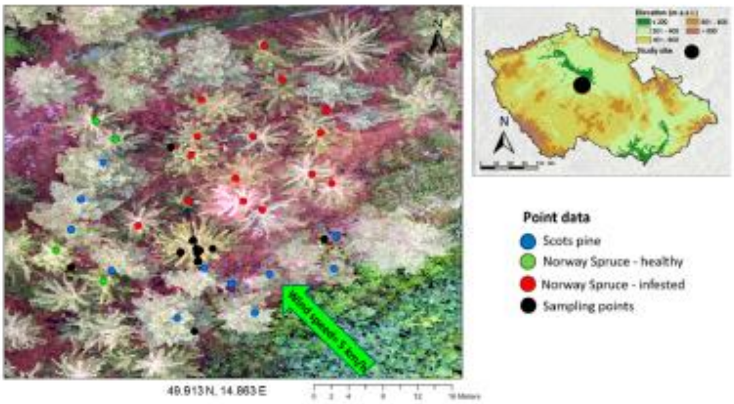

Figure 2. Study site for the first forest spatial VOC measurement around the infested tree (30 June 2022). Locality Forests ČZU close to Stříbrná Skalice, the Czech Republic (49.913 N, 14.863 E). Black points—sampling points around the sampled bark beetle-infested tree, in nuptial chamber infestation stadia; red points—Norway spruce infested trees in later stadia of infestation or dead; green—healthy Norway Spruce trees; blue points—Scot pines.

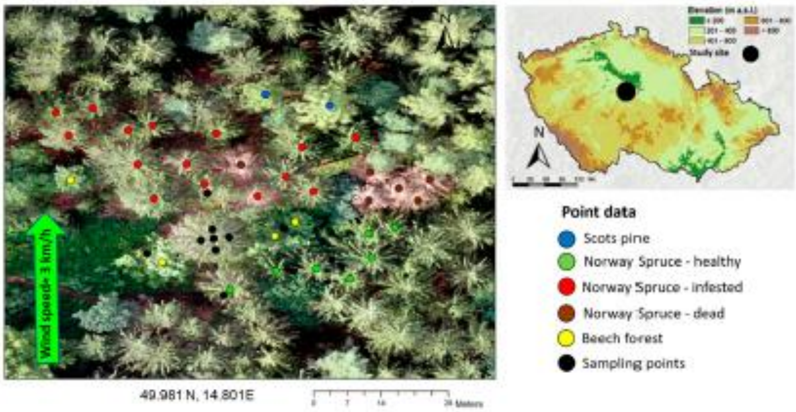

Figure 3. Study site for the second forest spatial VOC measurement around the infested tree (24 August 2022) for data in the ČZU Forests close to Vyžlovka, the Czech Republic (49.981 N, 14.801 E). Black points—sampling points; red points—Norway spruce infested trees; green—healthy Norway Spruce trees; brown points—dead Norway Spruce trees; blue points—Scot pines; yellow points—beech trees.

Two analytical approaches were employed to collect VOCs from infested trees in natural forest conditions [27,28,45]:

Sorption onto SPME fibers (PDMS/CAR/DVB, Supelco, PA, USA): Eight SPMEs fixed in protective chambers were positioned at three height levels: L1 (130 cm), L2 (240 cm), and L3 (400 cm). They were placed at two distances from the tree (5 cm and 100 cm) in the immediate proximity of the tree in four cardinal directions: north, south, west, and east. Additionally, four fibers were positioned 900 cm away from the tree in the same direction. These SPMEs were exposed for 60 min to collect volatile organic compounds (VOCs) from the forest air. After collection, the fibers were sealed in vials with septa in the same manner as in the laboratory. They were then placed on dry ice and subsequently stored in a freezer at −18 °C before undergoing measurement using Gas Chromatography-Flame Ionization Detection GC-FID, Agilent 8890 (Agilent, CA, USA).

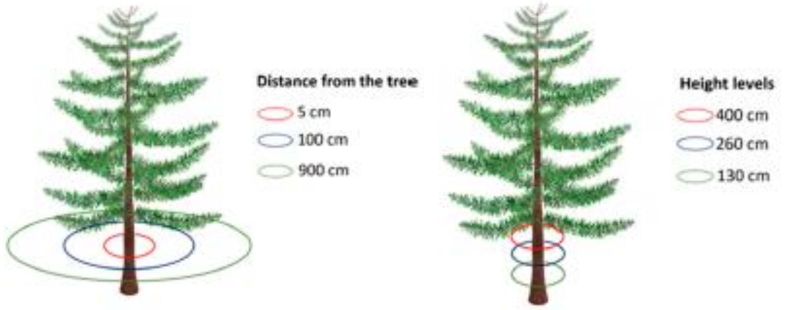

Figure 4. Established collection directions for VOCs around trees newly infested by bark beetles.

Sorption using cartridges (inner Ø 3 mm) filled with HayeSep-Q® sorbent (30 mg, Sigma-Aldrich, St. Louis, MO, USA,): This approach involved filtering the surrounding air through sorbent in cartridges sucked by sampling pumps (Pocket Pump Touch; Serie 220–1000, Eighty Four, PA, USA). The pumps were calibrated to operate at a flow rate of 100 mL/min for 60 min. Of the 12 air pumps, the chosen measurements were established along the first height level (130 cm from the ground) in all cardinal directions and different distance levels, and two cartridges were set up at the second height level in north and south directions at a 5 cm distance from the bark surface.

2.3. *Chemical Analyses of SMPE Fiber and Cartridges via Gas Chromatography-Flame Ionization Detection (GC-FID)*

Cartridges were washed with 1 mL of GC-grade hexane (GC-capillary grade; Avantor, PA, USA) and stored at −18 °C for further chemical analysis.

The SPME and cartridge analyses were carried out via Gas Chromatography-Flame Ionization Detection (GC-FID) Agilent 8890 (Agilent, CA, USA). The GC-FID was equipped with a DB-WAX capillary column (30 m × 320 µm × 0.25 µm film thickness; Agilent, CA, USA). The GC oven program followed a temperature profile of the initial temperature at 40 °C for 2 min, followed by a ramping rate of 10 °C per minute to reach 230 °C, where it was held for 2 min. The carrier gas He flow was 1.5 $\text{mL}\cdot\text{min}^{-1}$. The inlet operated in spitless mode and the inlet temperature was 220 °C. For the desorption of SPME fibers, an SPME liner was used (n°5190-4048, 78.5 × 0.75 mm id, Agilent, CA, USA). The extracts in hexane (1 µL) from the cartridge collection were analyzed in spitless mode.

2.4. *Determination of Relative Quantities of α-Pinene via SPME and Absolute Quantities Sorbed to Cartridges*

On the chromatogram, peaks of the main spruce monoterpenes and other volatile organic compounds (VOCs) from the forest air near *P. abies* were observed. However, to describe their 3D distribution, only the most abundant α-pinene was chosen, as it adequately represents the trend of the other main monoterpenes [8].

In both collection methods, α-pinene's peak identity was confirmed by comparing its retention time with the commercial standard (α-pinene, Sigma-Aldrich, St. Louis, MO, USA).

The abundance of α-pinene collected via SPME fiber was determined by measuring the peak area of α-pinene divided by the sum of areas of the peaks of the five main monoterpenes chosen (α-pinene, β-pinene, Δ-carene, limonene, camphene, and myrcene, 1.8 cineole) comparing it across individual samples. The quantification of α-pinene in the cartridge extracts was based on a calibration curve (Figure S2) constructed using the commercial standard of α-pinene diluted in hexane at 0.1, 0.5, 1, 10, 25, 50, and 100 µg/mL. The amount of α-pinene in one cartridge, expressed here as µg/mL, means µg/(6 L of air) in the vicinity of an infested tree.

2.5. Statistical Analyses

Statistical analyses were performed using Statistica (version 14.0.0.15). The normality assumption was tested using the Shapiro–Wilk test, and in each case, the null hypothesis (H0) was rejected, indicating the need for nonparametric testing. The Kruskal–Wallis test was used to compare individual levels, distances, and cardinal directions. When the test showed statistical differences, post hoc tests were conducted to examine differences between repetitions.

For SPME analysis, the dependent variable was the relative peak area of α-pinene collected. For cartridges, the amount of α-pinene quantified by the calibration curve was the dependent variable. In both analyses, the independent variables were the distance and height measurements.

3. Results

3.1. Optimization of VOC Collection to SPME in a Laboratory

The three VOC collections in the laboratory, taken at different times, were considered three repetitions since they were kept under the same experimental conditions (temperature and method of log drilling). Statistical analysis was conducted on all of them together ($n = 72$).

The abundance of α-pinene, the main monoterpene representing trends of other MT, was statistically highest at a 5 cm distance from the drilled stems compared to a 30 cm distance from the drilled stems ($p = 0.0362$) (Figure 5a).

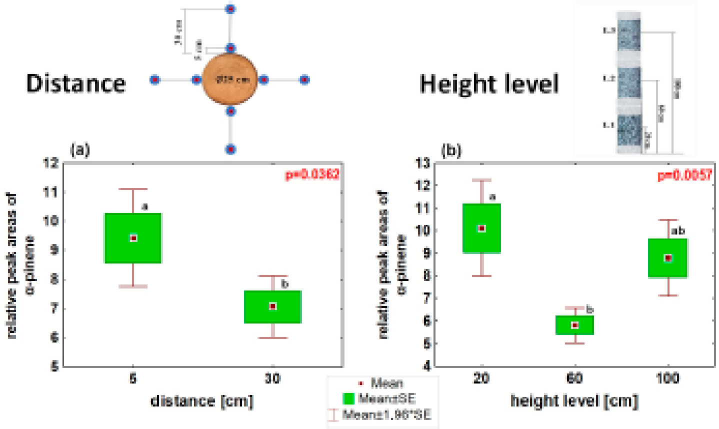

Figure 5. SPME laboratory data collections of α-pinene, three replications. (**a**) Abundance of α-pinene for lab experiment at different distances from drilled stem (5 cm and 30 cm); (**b**) abundance of α-pinene for lab experiment at different height levels from the ground (L1—20 cm; L2—60 cm; L3—100 cm). Small red squares—Means; green boxes—Means ± SE; Whiskers—Means ±1.96*SE. Lowercase letters above columns indicate significant differences between different distance or different height level. The *p*-values result from Kruskal–Wallis test; $n = 72$.

In the vertical direction, the distribution of α-pinene was statistically different ($p = 0.006$). The lowest abundance was observed at the medium level L2 (60 cm from the ground), and it was significantly different from the abundance at the bottom level L1 (20 cm from the ground) (post-hoc; $p = 0.007$) (Figure 5b).

3.2. α-Pinene Spatial Distribution around Norway Spruce Infested by *Ips typographus* for Two Weeks

The conditions in the first forest spatial VOC measurement conducted on 30 June 2022 on two-week naturally infested trees were an average temperature of 25.1 °C, average humidity of 72.6%, wind speed of 5 km/h, wind direction from the SE, and sunshine. The abundance of α-pinene emitted from the naturally infested tree was upregulated at a 5 cm distance from the stem. This upregulation was detected by both collection methods, with a significant increase observed using SPME ($p = 0.036$), where 5 cm and 100 cm distances significantly differed ($p = 0.036$ post hoc) (Figure 6a) and a non-significant increase was observed using sorption to cartridges ($p = 0.0585$). A trend of a decreasing α-pinene concentration at a 900 cm distance from the infested tree was observed in the collection involving cartridges (Figure 6d), but not in the collection using SPME (Figure 6a).

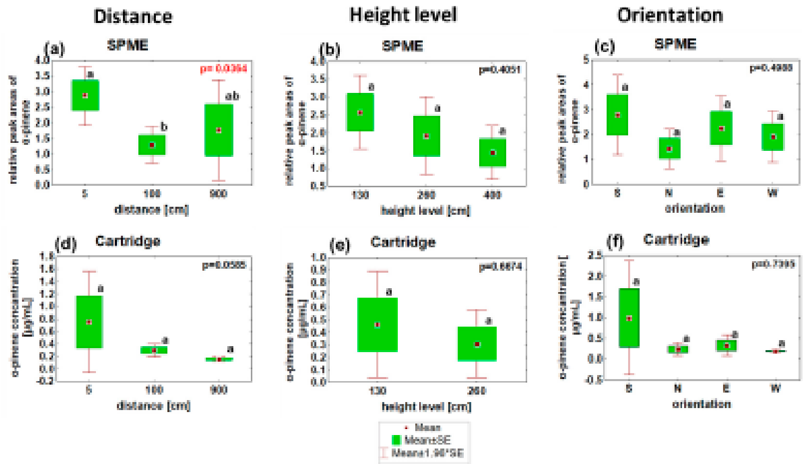

Figure 6. First field detection of α-pinene distribution around *Ips typographus*-infested spruce (30 June 2022). (**a**) Abundance of α-pinene collected by SPME in different distances from stem (5 cm; 100 cm; 900 cm); (**b**) abundance of α-pinene collected by SPME at different height levels from ground (130 cm; 260 cm; 400 cm); (**c**) abundance of α-pinene collected by SPME in cardinal directions (S—south; N—north; E—east; W—west); (**d**) abundance of α-pinene collected by HayeSep-Q® cartridges in different distances from stem (5 cm; 100 cm; 900 cm); (**e**) abundance of α-pinene collected by HayeSep-Q® cartridges at different height levels from ground (130 cm; 260 cm); (**f**) abundance of α-pinene collected by HayeSep-Q® cartridges in cardinal directions (S—south; N—north; E—east; W—west). Small red squares—Means; green boxes—Means ± SE; Whiskers—Means ±1.96*SE. Lowercase letters above columns indicate significant differences between different distance different height level, and different orientation. The *p*-values result from Kruskal–Wallis test. ($n = 27$ SPME samples) (cartridges $n = 12$).

In the vertical direction, a weak, non-significant trend was observed in the accumulation of α-pinene in the bottom level of the stem at 130 cm above the ground, decreasing with altitude up to 400 cm above the ground. This trend was consistent across both collection methods (Figure 6b,e).

Regarding cardinal orientation, there was no significant accumulation of α-pinene on any of the measured sides. However, considering the high variability of the data, there was a trend of increased α-pinene abundance on the south side of the infested stem, as observed with both SPME fiber and cartridge collection methods (Figure 6c,f).

A 3D depiction of the α-pinene collection using SPME fiber on 30 June 2022 is shown in Figure 7. Increased color saturation in the visualization corresponds to higher accumulated α-pinene levels. The accumulation aligns with Figure 6, indicating elevated concentrations at the first level (130 cm). In terms of distance, there is a notable increase at 5 cm from the stem. Beyond 900 cm, there is considerable variation in α-pinene amounts, making it difficult to observe a clear trend. Regarding the orientation, slightly higher concentrations are observed on the south and east sides, though are not significantly dominant.

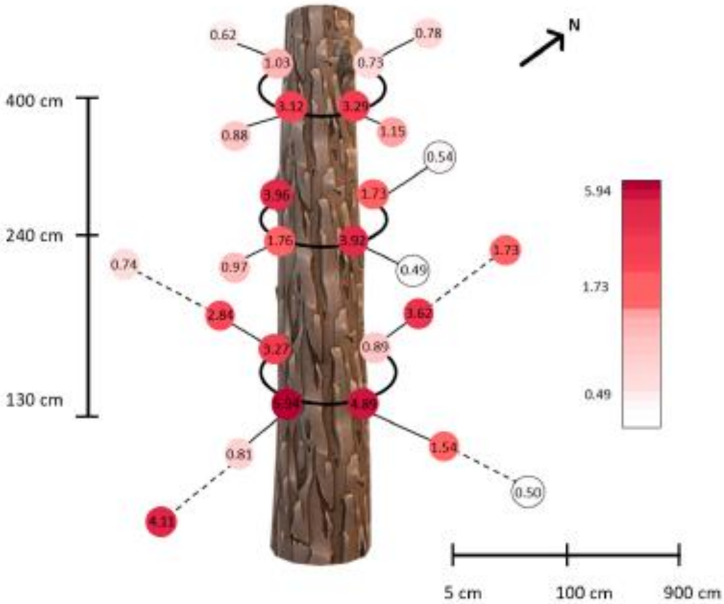

Figure 7. 3D distribution of α-pinene collected by SPME in first field data collection (30 June 2022). More saturated color means a higher abundance of α-pinene. The numbers in circles are relative peak areas of α-pinene.

The conditions for the second VOC measurement in the forest open space around a naturally infested tree, conducted on 24 August 2022, differed from the first collection in terms of location and environmental factors (average temperature of 20.7 °C, average humidity of 78.8%, wind speed of 3 km/h, wind direction from S, and sunshine).

α-pinene abundance significantly increased at a 100 cm distance from the stem, as detected by SPME ($p = 0.0037$) (Figure 8a) and sorption to cartridges ($p = 0.0244$) (Figure 8d), compared to both the 5 cm and 900 cm distances considered as controls.

A non-significant, very weak trend was observed for the accumulation of α-pinene at a height of 260 cm from the ground measured by SPME (Figure 8b). No significant differences were found in α-pinene accumulation in any cardinal direction (Figure 8c,e).

The 3D depiction of α-pinene collection using SPME fiber during the second field collection on 24 August 2022 is shown in Figure 9. Notably, there is an increase 100 cm from the stem in terms of distance and higher accumulation at the second level, 260 cm from the ground. This aligns with Figure 8.

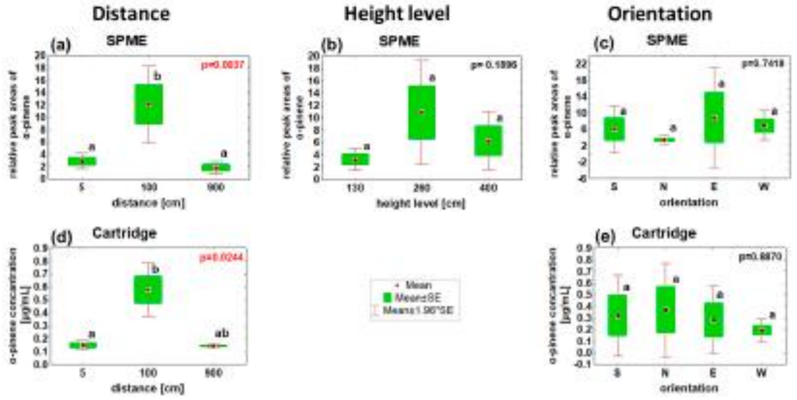

Figure 8. The second field detection of α-pinene distribution around *Ips typographus*-infested spruce (24 August 2022). (**a**) Abundance of α-pinene collected by SPME at different distances from stem (5 cm; 100 cm; 900 cm); (**b**) abundance of α-pinene collected by SPME at different height levels from ground (130 cm; 260 cm; 400 cm); (**c**) abundance of α-pinene collected by SPME in cardinal directions (S—south; N—north; E—east; W—west); (**d**) abundance of α-pinene collected by HayeSep-Q® cartridges at different distances from stem (5 cm; 100 cm; 900 cm); (**e**) abundance of α-pinene collected by HayeSep-Q® cartridges at different height levels from ground (130 cm; 260 cm), abundance of α-pinene collected by HayeSep-Q® cartridges in cardinal directions (S—south; N—north; E—east; W—west). Small red squares—Means; green boxes—Means ± SE; Whiskers—Means ±1.96*SE. Lowercase letters above columns indicate significant differences between different distance, different height level, and different orientation. The *p*-values result from Kruskal–Wallis test. (n = 22 SPME samples) (cartridges n = 12).

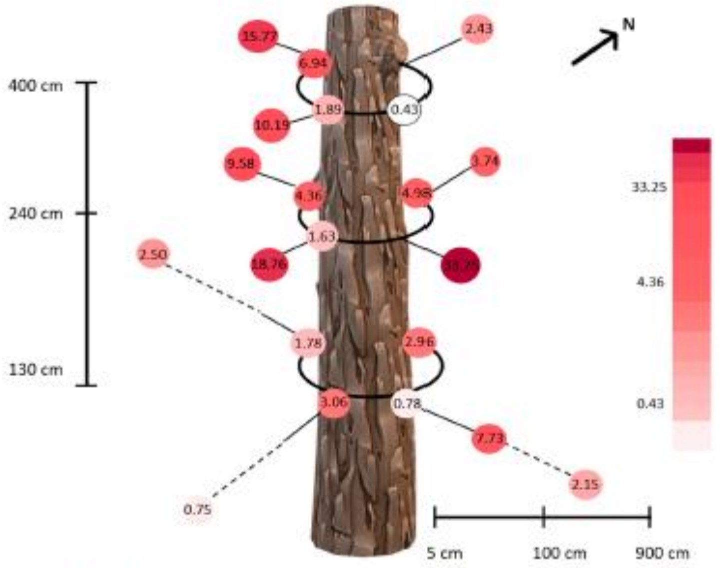

Figure 9. 3D distribution of α-pinene collected by SPME in second field data collection (24 August 2022). More saturated color means a higher amount of collected α-pinene. The numbers in circles are relative peak areas of α-pinene.

4. Discussion

The volatile compounds emitted by infested trees have been studied in two main contexts: atmospheric chemistry processes, with a focus on reactive particle formation [50], and ecological roles related to bark beetles and their predators [27,28,32,51]. Most reports in atmospheric chemistry measured volatiles at the canopy or twig level [52]. Emission measurements of biogenic volatile organic compounds (BVOC) from infested trees appeared in only a few studies investigating the potential use of these emissions as early attack detection markers [9,10,39]. Our study is unique as it examines emissions within a distance of 10 m of the infested tree, specifically at ground level and up to 5 m, where α-pinene accumulates at an average infestation season temperature of 20 to 25 °C in central European forests [53]. This novel approach contributes new insights to our understanding of volatiles emitted by bark beetle-infested trees.

Primarily, in our study, we employed laboratory optimization techniques to collect volatiles from freshly infested trees. The main emitted compound, α-pinene, was selected as a representative of the primary monoterpenes. It reflects the trends observed in other monoterpenes emitted by spruce trees infested by bark beetles [54].

In the laboratory, where the temperature was 25 °C, we observed a higher abundance of α-pinene in the open space in the closest proximity to logs, particularly in the lowest and highest positions. This increase was likely caused by the proximity of open log cuts and exposed bark edges, despite our attempts to prevent them by covering them with PE foil.

The first detection of α-pinene in the surroundings of a naturally infested tree in the field was conducted approximately two weeks after the beginning of a mass attack on the tree, corresponding with the time the monoterpene emission peaks [8,50]. This detection also took place at a temperature of 25 °C, when, expectedly, α-pinene vapors are heavier than air (with a vapor pressure of 4.9 mm Hg at 27 °C) [55].

Similar to our laboratory findings and reports from the literature [27,28], we observed the highest abundance of α-pinene in the immediate vicinity of the infested tree. Vertically, α-pinene accumulated at the lowest level, approximately 1.3 m above the ground, which was confirmed by two different collection methods.

The lower-level accumulation of the monoterpenes under similar conditions was previously observed as the output of the continual measuring campaign of BVOC in the forest. It has been reported that monoterpenes are emitted by healthy trees during the daytime, but they are also more susceptible to degradation in the atmosphere during the same period [53]. Monoterpenes are known to be unstable in the atmosphere, with a relatively short lifetime, often lasting only hours or even minutes, forming atmospheric secondary organic aerosol particles, followed by reactions with ozone and radicals like NO, OH, and NO_3 [56]. As a result of their decomposition, methanol and acetone, both of which are highly abundant in the forest atmosphere, are produced, alongside other products. Additionally, at higher elevations in the forest, strong air streams are present, resulting in monoterpenes having a higher 'mixing ratio,' which implies a lower concentration. The monoterpenes accumulate at lower altitudes in the forest, particularly during the nighttime [57].

The abundance of α-pinene in the controls placed at a further distance from the tree (9 m) exhibited greater variability [10].

This variability was most likely influenced by the surrounding trees. There were spruces, some of them in more progressed infestation stadia, or even dead and intact pines. In naturally infested trees, we also assessed an α-pinene orientation related to the infested stem's cardinal direction. During the first field collection, we noticed a non-significant trend of higher α-pinene levels on the south side of the stem, despite the prevailing wind coming from the opposite direction. This was attributed to the tree's location near the forest edge, allowing for direct afternoon sunlight exposure during collection [17,21].

The second collection, conducted later in the season, took place at a lower temperature of 22 °C. Interestingly, the highest abundance of monoterpenes was not observed at the lowest position, but rather at a height of 2.6 m, Additionally, it was not closest to the tree stem, but rather 1 m away. Results were confirmed again by two collection methods, Solid-Phase Microextraction (SPME) and HayeSep-Q® Cartridge sampling, conducted at two different time intervals. This sampled tree, despite being in a similar stage of infestation as the first tree, was fully surrounded by other trees, including some broadleaf varieties in the vicinity, resulting in no direct irradiation of the stem.

During this collection, we did not observe any significant influence of sunlight exposure or wind speed on the orientation of increased α-pinene abundance.

The temperature and humidity differences in altitude were recorded on the same experimental plot [9]. The highest temperatures were observed approximately 1 m above the ground with an upward gradient. This temperature variation may impact the higher abundance of α-pinene at an altitude of 2.6 m compared to ground level due to the physical properties of pinene vapors.

The variation in outcomes between the first and second field collections was attributed to differences in the surroundings of the measured trees, distinct parts of the season, and, primarily, variations in temperature and humidity conditions [36,37].

In forest practices, our 3D distribution data of α-pinene emitted by freshly infested spruces may serve as a foundation for scanning techniques, demonstrating promising potential for managing bark beetle populations by facilitating early attack detection.

The most advanced technique for volatile scanning in situ can be based on field and portable Gas Chromatography-Mass Spectrometry (GC-MS) instruments, which are possible to mount on UAV. These instruments have the capability to separate, identify, and quantify VOC compounds in situ and collect data at short intervals. Existing instruments on the market have been tested for various analytes, primarily for military applications [58]. However, reliable devices for detecting terpenes and other infestation marker VOCs are still lacking.

To specifically select markers of infestation by *Ips typographus* and exclude infestations of different herbivores (as relevant bark species or defoliators) from the complex odor environment of a natural forest, such as bark beetle pheromones and spruce host compounds, an olfactory perception system of the bark beetles, antennae can be used as a specific detector working via electroantennography principles [59,60]. Ongoing research is exploring the application of this method in insect pest detection, with future directions aiming to target insect protein odorant receptors for specific compounds indicative of infestation [31].

However, the most potentially promising novel device is a non-specific electronic nose, designed for real-time chemical substance detection (e.g., detecting dangerous gas leaks or measuring concentrations near landfills, monitoring volcanic activity, etc.). We previously conducted tests to explore the feasibility of early-stage stress detection in forest stands using an electronic nose, specifically the Sniffer4D [9].

Due to limitations in measuring VOCs with non-specific sensors in forests, especially in areas with higher concentrations of compounds from fresh clearings, debris, and broken trees, an ideal approach would involve integrating environmental VOC scanning with other data collected through different scanning methods. This integration could be achieved, ideally using UAV vehicles equipped with various sensors for high-resolution data collection [61]. The map of VOC abundance can be automatically overlaid with aerial maps of the area, changes in reflectance, dedicated vegetation indices [6], or temperature fluctuations [62].

Limitation of the Study

The reported study is considered a pilot trial investigating the distribution of monoterpenes around infested trees at ground level and up to 5 m. To enhance the study, future research employing classical collection and analytical techniques should result in confirmation of the quantitative monoterpene distribution. The expanded study will involve a large-scale tree group, multiple replications for robust statistical analyses, and a focus on genetically relevant trees. To ensure control, infested and non-infested trees will be monitored simultaneously, maintaining consistent bark beetle infestation density. Standardized environmental conditions will be implemented to minimize variability, with the potential to explore environmental conditions as a variable.

5. Conclusions

In summary of this pilot study, VOCs, represented here by α-pinene, emitted by freshly attacked trees may act as detectable markers for timely infestation. The spatial distribution of their concentration in an open space follows a gradient pattern that can be analyzed using various collection techniques. However, this distribution is notably influenced by environmental factors. With further optimization and integration with other scanning methods, these VOCs emitted by freshly infested trees can be used to develop effective "early detection methods" for bark beetles. Real-time data distribution provides a strong foundation for implementing crucial scanning techniques in early attack detection strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15010010/s1, Table S1. The averages of the temperature and humidity in the first data collection 30 June 2022 in two periods of collections (SPME and cartridge collection). Table S2. The averages of the temperature and humidity in the second data collection 24 August 2022 in two periods of collections (SPME and cartridge collection). Figure S1. The map of development of Norway spruce logging due to bark beetle infestation in the Czech Republic in the period 9/2021–7/2022. Figure S2. Calibration curve of α-pinene. Constructed using commercial standard of α-pinene diluted in hexane to 0.1; 0.5; 1; 10; 25; 50 and 100 µg/mL.

Author Contributions: B.S.: experimental work, data curation, statistical data processing, and writing draft. A.A.C.M.: experimental work, GC-FID analysis, and writing draft and editing. T.H.: experimental work, figures processing. M.L.: experimental work and attack expertise. P.S.: editing manuscript and supervision. A.J.: conceptualization, data sampling, formal analysis, writing, editing, and supervision. All authors contributed to the article and approved the submitted version. All authors have read and agreed to the published version of the manuscript.

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Conflicts 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 Materials

Table S1 The averages of the temperature and humidity in the first data collection June 30th 2022 in a two times collections (SPME and cartridge collection).

First field trip 30.6.2022		**Sampling SPME (14:00-15:00)**	**Sampling Cartridges (15:30-16:30)**
tree 1.3m	Temp [°C]	26.3	26.0
	Humidity [%]	67.6	67.5
tree 4m	Temp [°C]	25.2	25.7
	Humidity [%]	69.8	71.8
S_control	Temp [°C]	25.3	25.3
	Humidity [%]	71.3	70.4
W_control	Temp [°C]	24.7	25.1
	Humidity [%]	72.7	71.2
SE_control	Temp [°C]	24.8	25.1
	Humidity [%]	69.2	69.2

Table S2 The averages of the temperature and humidity in the second data collection August 24th 2022 in a two times collections (SPME and cartridge collection).

Second field trip 24.8.2022		**SPME sampling (12:10-12:25)**	**Cartridges sampling (16:00-16:15)**
tree 1.3m	Temp [°C]	20.0	21.0
	Humidity [%]	85.8	61.5
tree 4m	Temp [°C]	19.6	21.0
	Humidity [%]	84.0	61.5
W_control	Temp [°C]	20.4	22.1
	Humidity [%]	81.8	82.3
N_control	Temp [°C]	20.1	21.3
	Humidity [%]	83.3	90.3

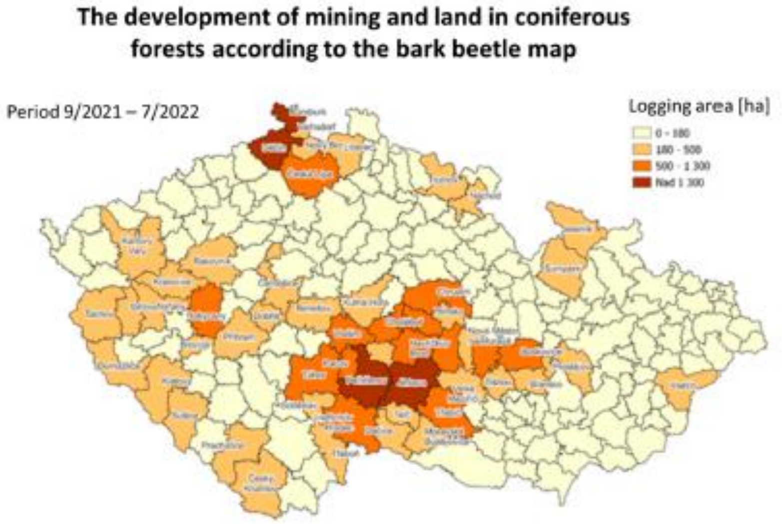

Figure S1 **The development of mining of conifer forrest according to bark beetle calamity map in Czech Republic period 9/2021 – 7/2022.**

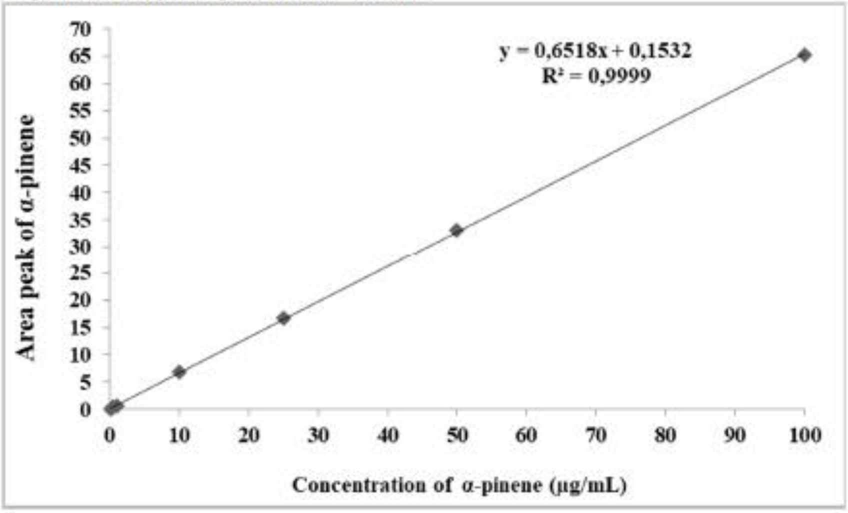

Figure S2 Calibration curve of α-pinene. Constructed using commercial standard of α-pinene diluted in hexane at 0.1; 0.5; 1; 10; 25; 50 and 100 μg/mL.

5. Discussion

The bark beetle *Ips typographus* currently represents the primary pest of Norway spruce (*Picea abies*) in Central Europe posing a critical challenge to the region's forestry. This bark beetle species is able to kill whole spruce trees in short time, endangering the function and resilience of forests and often devastating large forest areas. It leads to an ecological imbalance, emphasizing the need for proactive measures and innovative strategies to safeguard vital forest ecosystems. This thesis presents a detailed study of physiological and biochemical responses of Norway spruce exposed to acute stress from sudden sun radiation at the forest edge, and from attack by bark beetles. In the thesis the methods for early attack detection of bark beetles are discussed based on the studied changes in tree physiology and profile of volatile secondary metabolites produced in the course of induced defence reactions of in response to attack.

Study 1, as presented in chapter 4.1, was designed to explore the hypothesis that "Norway spruce trees on forest edges exposed to sudden sun irradiation will alter in physiology and primary and secondary metabolism, making them more susceptible to bark beetle attacks". This study found that physiological changes in trees, such as increased resin flow, and bark beetle behaviour, including enhanced moving activity and attack patterns, are influenced by microclimatic conditions and olfactory signals from trees and the surrounding environment at the forest edges. However, fresh forest edges were found to be not specifically more susceptible to bark beetle attack shortly after their establishment compared to closed forest stands. Prior studies on characteristics of forest fragments and stand edges pointed to the increased vulnerability of trees exposed to by external stressors, notably wind and solar irradiation (Donis et al., 2018; Jönsson et al., 2007). The current study, being a pioneering effort to examine the direct consequences of forest edge creation, revealed notable changes at newly established forest edges (FEs). The observed increased bark surface temperatures were also reported by Majdák et al., (2021); Marešová et al., (2020); and Mezei et al., (2019). Enhanced resin flow in sun-exposed trees were also reported by Knebel et al., (2008) and Netherer et al., (2015). While sap flow rates in shaded trees within the forest interior did not differ from those of the edge trees, Özçelik et al., (2022) showed a 16% increase in sap flow for trees positioned near the new edge. Despite sufficient soil water availability, distinct variations in the concentrations of volatile organic compounds (VOCs), particularly monoterpenes, were observed close to the bark of the study trees, with concentrations three times higher along the edge than in the closed forest stand. However, the implications of sun exposure of forest edges did not involve the enhancement of tree defence traits, such as monoterpene content in the phloem. In this regard, our results contradict previous observations by Marešová et al., (2020). Instead, we found reduced β-pinene + myrcene in forest edge trees, which may influence the trees' chemical defence against invaders in the long run post edge creation (Schiebe et al., 2012).

The study also highlighted a significant increase in total VOCs in open space at the forest edge in the month after cutting, which can be additional cues for the orientation of bark beetles to trees at the forest edge. In accordance with the present results, previous studies have demonstrated that the swarming activity of spruce bark beetles in spring are related to higher temperatures (Jakoby et al., 2019) at forest edges. Indeed, the observed attacks occurred predominantly in August, both at FEs and in the forest interior, during periods of high mean temperature recorded at the bark surface.

By studying physiological changes in trees and bark beetle behaviour in the transition zone between the forest edge and inner forest. We can conclude from the current study that factors, such as biogeography, weather, and characteristics of bark beetle outbreaks (e.g., progradation and retrogradation phases), that in the study area played important role which influenced the bark beetle niche. These findings are somewhat surprising given the fact that the experiments were conducted in a sub-montane forest area, outside the natural habitat of Norway spruce, at 650 - 1150 m above sea level (Röder et al., 2010), in an area which had experienced severe drought in recent years (Krejza et al., 2021). However, the study season 2020 remained humid and cool, with high soil water potential. These hydrological conditions can be an explanation for the observations that the trees at the forest edge did not experience any natural attack in the experimental season even though the population density in the area was still high. In addition, the complex interplay between microclimatic conditions and tree responses in fragmented forest ecosystems also played a key role. Previous studies have demonstrated that drought-stressed trees change the chemical composition of their bark, leading to increased host acceptance of bark beetles (Basile et al., 2024, unpuslided data). Drought stress can further lead to changes in water potential and reduced resistance to fungal infection, which in turn attracts *I. typographus* (Netherer et al., 2016, 2015).

Chapters 4.2 and 4.3 contribute valuable insights into understanding the complex interactions between Norway spruce, *Ips typographus*, and their environment. The first study in Chapter 4.2 emphasizes the importance of physiological changes in trees after bark beetle attack and the challenges in early detection of bark beetle-infested trees. In contrast, the second study in Chapter 4.3 provides innovative approaches for studying volatile emissions and their application in early attack detection methods. These findings collectively contribute to the ongoing efforts to manage and mitigate the impact of bark beetle outbreaks on forests. Yet, in chapter 4.2 we investigated not only the changes in tree biochemistry, but also the insect-tree interactions. Physiological and biochemical data can clearly indicate bark beetle infestation of trees, such as significantly decreased sap flow rates, reduced stem increment as well as increased bark surface temperatures and changes in monoterpene emissions. We see these results as key findings with respect to the advancement of early detection methods of bark beetle-infested trees. Our finding increased bark surface temperature is a result of bark beetle infestation which was also described by Hais and Kučera, (2008) and Mezei et al., (2017). Consistent with the literature, the potential of bark temperature scanning can be used as a method for early detection. Reductions in diameter growth may also be indicative for early attack, although there are challenges associated with measuring minor alterations in tree stem increment (Yrttimaa et al., 2023). Our study also highlights the need for further research into developing of readily measurable indicators of sap flow changes. It emphasizes the importance of understanding the physiological mechanisms related to bark beetle-induced mortality.

Chapter 4.3 focused on the volatile compounds emitted by infested trees and their potential use in early attack detection. This work offered a unique opportunity to profile VOCs by examining emissions within a close distance of infested trees, specifically at ground level and up to 5 meters. The study identified *α*-pinene as a key compound emitted by infested trees and discusses its accumulation patterns. The findings suggest the potential use of 3D distribution data of *α*-pinene by scanning techniques of VOCs to manage bark beetle populations through early attack detection, as pointed out by Hüttnerová et al., (2023) and Paczkowski et al., (2021). In addition, it is important to mention that the abiotic factors (e.g., sunlight, wind speed) did not influence the *α*-pinene abundance in the field experiments, which generally exhibited high variability in this compound.

A possible explanation for this might be connected to upregulation (such as, temperature, irradiation and mechanical injury) and downregulation (e.g., drought) regulations are influenced by long and short response periods (days and seconds respectively); this is leading to increased or decreased production of enzymes related to terpene emission (Loreto and Schnitzler, 2010). In addition, solar radiation in field experiments can influence the collection of *α*-pinene as reported by Stříbrská et al., (2022).

Even though the results related to the abundance of *α*-pinene were not significantly influenced by the cardinal direction, the alternative collection methods, SPME fibers and cartridges, both proved to be effective in collecting samples in an open space scenario. Prior studies have emphasized the significance of volatile collection techniques and devices, such as electronic noses, SPME fibers and Sniffer4D, for detecting and analysing volatiles in forests (Feijó Barreira et al., 2018; Hüttnerová et al., 2023; Jaakkola et al., 2022).

5.1. Limitations of the study

The study about the forest margins (Chapter 4.1) recognizes limitations related to cool and humid spring conditions, which mitigated tree stress at the forest edge and resulted in somewhat unexpected behaviour of test beetles in the attack box bioassay. The conclusion is that fresh forest edges aren't specifically prone to *I. typographus* attacks shortly after establishment. However, microclimatic conditions and olfactory signals from standing edge trees favour spruce bark beetle activity which is also related to the research of (Blackwell, 2011). Further research is required to comprehend the gradual microclimatic, physiological, and biochemical changes at forest edges over time, also considering differences between endemic and epidemic population densities of *I. typographus*. Clear-cuts' timing may impact the likelihood of natural attacks in the first or second year after edge establishment.

The study outlined in chapter 4.2 of physiological and biochemical indicators of bark beetle attack had limitations regarding the very low number (2) of monitored Norway spruce trees attacked by *I. typographus*. However, this study identified significant differences in tree physiology and defence biochemistry as well as in bark beetle landing rates between infested and non-infested trees.

Finally, the study presented in chapter 4.3 is considered a pilot trial exploring the distribution of monoterpenes around infested trees at ground level up to 5 m in opening space. Future research should adopt classical volatile collection methods and analytical techniques to enhance the study, involving a larger sampling group with multiple replications for robust statistical analyses. Ensuring consistent bark beetle infestation density and implementing standardized environmental conditions will be crucial to minimize variabilities and explore environmental conditions as additional variables. A future limitation of this study is the possibility of incorporating the sensor into a UAV machine. Although the sensor can quantify volatiles, potential constraints on repeated measurements may arise. Moreover, the downward airflow generated by the UAV could affect the precision and accuracy of the measurements.

6. Conclusion and recommendations

This chapter serves a dual purpose. First, it summarises the comprehensive research done in this thesis to draw conclusions going beyond the study questions. Second, this section addresses remaining questions and unresolved issues deriving from the initial research objectives, the knowledge produced, and the limitations inherent in the approaches employed throughout this thesis. In light of these considerations, the chapter recommends the practical implementation of the discovered insights and suggests avenues for future research.

6.1 Conclusion

The findings of this thesis contribute to the scientific knowledge about the impact of climate characteristics and forest management measures such as edge establishment as well as of fresh attack by *I. typographus* on the physiological and chemical responses of mature Norway spruce. The thesis studies the features of mature Norway spruce trees, under stress conditions, based on 1) records of physiological data of Norway spruce trees (*Picea abies*), 2) tree defence chemistry, and 3) establishment susceptibility of stressed mature spruces to bark beetle attack.

The main results of the studies are the clear effects of sudden sun exposure as well as of colonisation of trees by bark beetles on physiological and biochemical parameters and how these changes influence host tree - beetle interactions.

In conclusion, study 1 provides a comprehensive examination of the direct effects of forest edge establishment on various parameters, including microclimatic conditions, soil hydrology, physiological and defence traits of Norway spruce trees, and predisposition of trees to spruce bark beetle attack. Forest edges exhibited increased bark surface temperatures and enhanced resin flow in sun-exposed trees. Despite differences in volatile organic compound (VOC) concentrations near tree bark, actual attacks by spruce bark beetles occurred mainly later in the season (August), both at trees of the forest edge and in the inner forest. The study emphasizes the importance of microclimatic conditions and olfactory signals for spruce bark beetle activity. While fresh forest edges did not show specific susceptibility shortly after establishment, increased physiological stress and bark beetle attacks were observed later in the season. The study outcomes point to the need of further research on the predisposing characteristics of fragmented forests and the timing of clear-cuts in relation to bark beetle population dynamics.

Measurable changes in physiological and defence traits of Norway spruce trees freshly infested by bark beetles, were suggested as indicators in a spruce bark beetle early attack detection framework in study 2. It was found that higher amounts of volatiles can be detected in freshly attacked trees than in non-infested control trees. The VOCs that were collected near the infested trees were detectable in the first two weeks of infestation. Passive traps with an alarming system randomly placed on the trunks signalled the landing of beetles on the trees. The changes in physiological and biochemical parameters of acutely stressed Norway spruces correlate with their increased disposition to further bark beetle attacks.

The pilot study 3 regarding the 3D distribution of *α*-pinene was the first to explore the emission of VOCs, particularly *α*-pinene, from bark beetle-infested trees measured in the natural forest environment. The investigation comprised the measurement of VOCs from ground level up to 5 m stem height. Laboratory optimization techniques revealed higher *α*-pinene abundance in the open space near logs, especially at the lowest and highest positions. Field collections confirmed proximity-based accumulation of *α*-pinene, which corresponds with known patterns from literature (Hüttnerová et al., 2023; Paczkowski et al., 2021). The study highlights the potential of VOCs, especially of *α*-pinene, as detectable markers for early bark beetle infestation, which can improve early attack detection strategies. Integrating these findings into emerging technologies, such as UAV-mounted Gas Chromatography-Mass Spectrometry (GC-MS) instruments, electronic noses, and environmental sensors, holds promise for developing effective and real-time scanning methods for bark beetle infestations.

6.2 Practical applications of the findings

Forest edges initially show resilience against bark beetle attacks but become more susceptible over time. During the early stages of an attack a statistically significant increase in VOCs can be measured. Tree growth ceases after an attack. All these findings can help foresters in identifying high risk areas and fresh infestation spots. Utilizing real-time data distribution derived from effective scanning techniques is crucial for early attack detection strategies.

Identifying distinct measurable attributes of recently infested trees offers possibilities for alternative techniques for early attack detection. VOC measurements may serve as a supplementary approach to the conventional method of detecting infested trees early through manual inspection of boring dust and bark beetle entry points. The potential utility of the identified characteristics for early detection of bark beetle attacks was assessed in this thesis through three approaches. First, by employing a high-resolution thermal camera to scan the bark temperature of infested trees on days with elevated temperatures and sun irradiation. Second, by monitoring the sudden increases in the emission of defensive monoterpenes using non-specific sensors, such as an electronic nose, potentially deployable on UAVs. Additionally, *I. typographus* aggregation pheromone compounds might be considered to be specifically detected. The third approach involves the installation of passive traps for the automatic detection of landing or attacking beetles.

6.3 Recommendation for future research

Further investigations are crucial to yield tangible outcomes that can enhance the management of bark beetle outbreaks, with a particular focus on the technical development of proposed solutions in this thesis. To achieve statistically significant results, it is imperative to replicate the studies on a larger scale. The first study (Chapter 4.1) delves into the implications of early forest edge creation for the probability of bark beetle attack. As bark beetle swarming intensifies during warm and dry spring periods, with heightened activity through the emergence of new generations as temperatures rise, a time-series evaluation involving several dates of edge creation from winter to spring, would help to assess the attractiveness of trees to bark beetle infestation depending on time of exposure to the changed environmental conditions. Additional research on vegetation edges is essential, covering different outbreak phases as well as forest stands located at various altitudes, reflecting the scope of natural spruce habitat. Recommendations for future research include several ideas, such as: (1) Evaluating the influence of gradual interference of microclimatic, physiological, and biochemical changes at forest edges. (2) Investigating the different levels of bark beetle populations and their population dynamics in relation to microclimatic conditions, host tree physiology, and biochemical changes and responses. (3) Assessing the effects of clear-cuts over time and their impact on bark beetle population dynamics and attack.

To enhance the study approaches described in chapters 4.2 and 4.3, future research should employ classical collection and analytical techniques to confirm the quantitative distribution of monoterpenes. The expanded research should include larger tree groups, multiple replications for robust statistical analyses. Simultaneous monitoring of infested and non-infested trees should occur given ongoing bark beetle infestation. The spatial distribution of monoterpenes in an open space follows a gradient pattern, which can be analysed using various collection techniques. However, as this distribution is notably influenced by environmental factors, environmental conditions should be included as additional variables. Real-time data distribution establishes a robust foundation for implementing crucial scanning techniques in early attack detection strategies. Research for better solutions for UAV techniques and sensors placed on them in real time collection needs to be continued.

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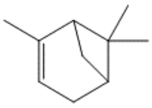
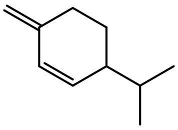
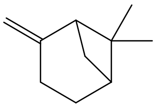
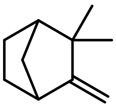
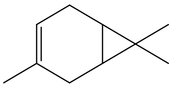
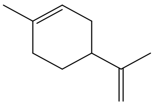
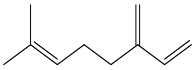
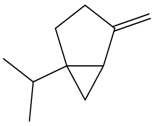
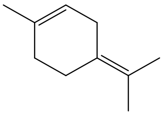
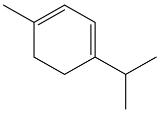
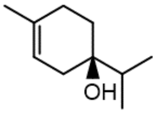
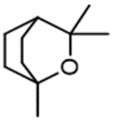
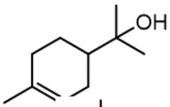
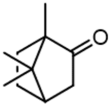
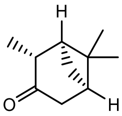
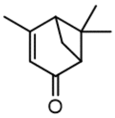
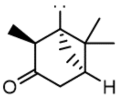
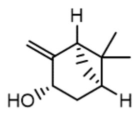
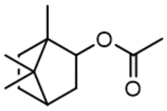
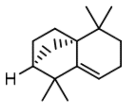
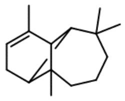
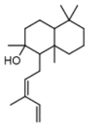
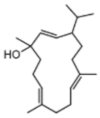
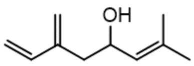
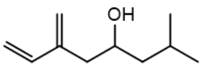
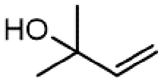
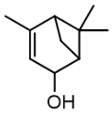
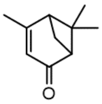
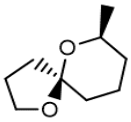
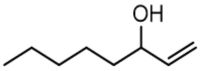
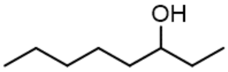
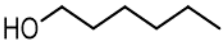
Supplementary materials

Suppl. Table 1: Compounds ecologically relevant for the Norway spruce - Ips typographus (IT) – associated ophiostomatoid fungi system: monoterpene hydrocarbons (MT-HC), oxygenated monoterpenes (MT-Ox), sesquiterpenes (ST), diterpenes (DT), semiochemical; detection via electroantennographic detection and single sensillum recording (SSR). Host tree defence: arrows indicate absolute (abs) or relative (rel) de- or increase after treatment of trees with methyl jasmonate (MeJA).

Compound		**Bark beetle (*Ips typographus*)**		
Tree / MT-HC		**Electro-Antennography**	**Ecological relevance**	**Response to MeJA**
alpha-pinene		GC-EAD, SSR	Host recognition and attack success	↑abs
(-)-alpha-pinene		GC-EAD, SSR	IT pheromone precursor, higher enantiomeric proportion in more attractive (?) but lower in killed trees (?)	↑abs↓abs
beta-phellandrene		C-EAD	?	↑abs
beta-pinene		GC-EAD, SSR	?	↑abs↓abs
camphene		GC-EAD	↓abs in IT killed trees treated with MeJA	↑abs↓abs
3-carene (delta-3-carene)		GC-EAD, SSR	↓abs in IT killed trees (MeJA) (+)-3-carene suggested as chemical marker of tree resistance	↑abs
limonene		GC-EAD	IT antifeedant	↑abs ↑rel
(-)-limonene		GC-EAD, SSR	Reduced attractiveness of trees for landing beetles: ↑abs in bark of IT less attractive and surviving trees, ↓abs in IT killed trees	↑abs
(+)-limonene		GC-EAD, SSR	↓abs emission in felled IT attacked trees, ↓abs in IT killed trees	↑abs ↑rel

myrcene		GC-EAD, SSR	↓abs in IT killed trees treated with MeJA	↑abs
sabinene (4(10)-thujene)		GC-EAD	↓abs in IT killed trees treated with MeJA	↑abs ↓abs
terpinolene		GC-EAD, SSR	Impact on tree susceptibility: ↓abs emmision in felled IT attacked trees ↓abs in IT killed trees treated with MeJA	↑abs
gamma-terpinene		GC-EAD, SSR	Impact on tree susceptibility(?): ↓abs emmision in felled IT attacked trees ↓abs in IT killed trees treated with MeJA	↑abs
Tree / MT-Ox				
(-)-terpinen-4-ol ((-)-4-terpineol)		GC-EAD, SSR		
1,8-cineole (eucalyptol)		GC-EAD, SSR inhibits response to cV when co-localised in sensillum	Antiattractant:↑abs emmision in felled IT attacked trees, ↑abs in bark of IT surviving trees, ↓abs in IT killed trees True resistance marker for tree survival!	↑abs ↑rel
alpha-terpineol (terpineol)		GC-EAD, SSR		↑rel
camphor		GC-EAD, SSR		
pinocamphone		)	↑abs emission from felled IT attacked trees	↑rel
trans-4-thujanol (sabinene hydrate)		GC-EAD, SSR	Antiattractant; ↓abs with tree age, ↑abs emission in felled IT attacked trees	
verbenone (2-Pinen-4-one)		GC-EAD, SSR	IT pheromone, antiattractant: terminates aggregation of IT, ↑abs emmision in decomposed wood	
isopinocamphone		GC-EAD		

trans-pinocarveol		GC-EAD		
bornyle acetate (borneol acetate)		GC-EAD, SSR	↑abs in bark of IT ving trees, ↓abs in IT trees const and after treatment	↑abs
Tree / ST				
isolongifolene		GC-EAD		
alpha-longipinene		GC-EAD		
Tree / DT				
abienol				↑abs
thunbergol (isocembrol)				↑abs
IT long-range semiochemicals				
ipsdienol (R)-(-)-		GC-EAD, SSR	Produced by IT males after mating with females, weakly boost of attraction to pheromone mixture MB/cV	
ipsenol, (R)-(+)-		GC-EAD, SSR	Produced by IT males after mating with females, relict of ipsdienol	
2-methyl-3-buten-2-ol		GC-EAD, SSR	Main IT pheromone component, attractant for pioneer males and females, produced by beetles de novo	
cis-verbenol		GC-EAD, SSR	IT aggregation pheromone component produced from alpha-pinene, attractant for pioneer male and female beetles	

verbenone (S)-(-)-		GC-EAD, SSR	Terminates aggregation of IT on host tree
trans-conophthorin		GC-EAD, SSR	Part of NHV anti-attractants blend; angiosperm tree bark
1-octen-3-ol		GC-EAD, SSR	Part of NHV anti-attractants blend; angiosperm tree bark
3-octanol		GC-EAD, SSR	Part of NHV anti-attractants blend; angiosperm tree bark
1-hexanol		GC-EAD, SSR	Example of GLV blend component: part of NHV anti-attractants blend, all angiosperm plants

Literature sources for table (Netherer et al., 2021): Bakke (1976); Dickens (1981); Leufvén and Birgersson (1987b); Schlyter et al. (1987); Birgersson and Bergström (1989); Borg-Karlson et al. (1993); Brignolas et al. (1995); Persson et al. (1996); Brignolas et al. (1998); Phillips and Croteau (1999); Baier et al. (2002); Martin et al. (2002); Jakuš and Blaženec (2003); Lieutier et al. (2003); Petterson and Boland (2003); Silvestrini et al. (2004); Hulcr et al. (2006); Zeneli et al. (2006); Erbilgin et al. (2007); Faccoli and Schlyter (2007); Andersson et al. (2009), Evensen et al. (2009); Andersson et al. (2010); Zhao et al. (2010); Zhao et al. (2011a); Zhao et al. (2011b); Andersson et al. (2012); Andersson (2012); Schiebe et al. (2012); Novak et al. (2013); Binyameen et al. (2014); Hammerbacher et al. (2014); Kalinová et al. (2014); Blažytė-Čereškienė et al. (2015); Zhao et al. (2015); Hammerbacher et al. (2018); Hammerbacher et al. (2019); Kandasamy et al. (2019); Schiebe et al. (2019); Zhao et al. (2019a); Zhao et al. (2019b).