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Biotic pest disturbance - risk, evaluation, and management in forest ecosystems

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Biotic pest disturbance - risk, evaluation, and management in forest ecosystems

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Editorial: Biotic pest disturbance - risk, evaluation, and management in forest ecosystems

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Biotic pest disturbance - risk, evaluation, and management in forest ecosystems

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Introduction

Forests are complex, adaptive ecosystems whose resilience depends on their capacity to withstand and recover from disturbances. Historically, forest ecosystems have always been shaped by a diverse array of disturbance agents, from storms to fires and fungal and insect outbreaks, that are integral parts of natural forest dynamics (Seidl and Turner, 2022). However, the past few decades have witnessed a dramatic rise in the frequency and intensity of biotic pest disturbances (Patacca et al., 2022); this has overwhelmed the adaptive capacity of the forests (Forzieri et al., 2024). Invasive insects and pathogens pose a growing threat to health, productivity, biodiversity, ecosystem services, and socio-economic function of forests (Hartmann et al., 2025) and can cause extensive tree mortality (Senf et al., 2020). These biotic threats are interacting with a complexity of other environmental challenges, such as rapid climate change (Ramsfield et al., 2016), global increase in travel and trade (Fenn-Moltu et al., 2023), and monocultural plantations to reshape the structure, composition (Forrester and Bauhus, 2016), and ecosystem services of forests around the world (van Lierop et al., 2015). Windstorms, drought, fire, and human interventions exacerbate the spread and impact of insects and diseases (Seidl et al., 2017), while climate change is altering pest population dynamics, extending ranges and outbreak periods (Jactel et al., 2019), and introducing new risks such as novel disease vectors and pest-pathogen interactions (Franic et al., 2023).

This Research Topic, *Biotic Pest Disturbance - Risk, Evaluation, and Management in Forest Ecosystems*, provides a timely synthesis of current research that offers a multifaceted perspective on how science and practice can respond to these forest threats and challenges. Biotic Pest Disturbance includes all living agents that damage a forest, mainly insects and pathogens. The Research Topic of 15 studies exemplifies the diverse strategies needed to better understand, detect, and respond to biotic threats in forests. It emphasizes the need for improved risk identification, robust evaluation of the impact of biotic pest disturbance agents under changing environmental conditions, and the deployment of innovative, sustainable management solutions. Together, these contributions advance our understanding of how to maintain resilient forests in an era of multiple global change pressures, reflecting the need for a comprehensive, multidisciplinary, and innovative approach to safeguarding forests for future generations.

Below, we synthesize the accepted articles under three main sections, i.e., Risk: recognizing emerging threats from pests and pathogens, Evaluation: understanding host-pest interactions under climate change and advancing pest detection, and Management: toward innovative, integrated solutions for sustainable forest health that move beyond traditional chemical control.

Risk: recognizing emerging threats from pests and pathogens

Risk in forest pest management is the probability of an outbreak or the likelihood of damage in a particular stand, considering pest population density and stand susceptibility (Wainhouse, 2008). Early and accurate pest risk assessment is fundamental for preventing and mitigating large-scale insect and pathogen outbreaks, and it forms the cornerstone of any proactive pest management strategy. Understanding risk begins with recognizing which insects and pathogens threaten forests, and how multiple agents can act synergistically. Life history characteristics offer a general insight into the damaging potential of pests, providing a starting point for comprehensive risk assessment. The characteristic features of an outbreak vary depending on the type of pest involved. While the timing of outbreaks remains difficult to predict, estimating the risk to specific forest stands holds considerable practical value. Anticipating where outbreaks are most likely to occur enhances the likelihood of early detection during pest evaluations.

Effective forest pest management begins with understanding and anticipating risk—not only from individual agents but from multi-faceted, interacting threats under changing environmental conditions. The contributions in this section collectively underscore how pathogen complexity, pest interactions, climate-induced shifts, and human-mediated pathways are reshaping our understanding of forest health risks.

Several papers highlight the increasing relevance of multiple agents acting simultaneously. For instance, Zlatković et al. and Marković et al. both focus on pedunculate oak *Quercus robur*, a keystone species in European lowland forests, demonstrating how co-occurring foliar pathogens and insects significantly impact tree health and regeneration. Zlatković et al. reveal a complex of pathogenic fungi, such as *Tubakia* spp., *Didymella macrostoma*, and *Apiognomonia errabunda*, that contribute to anthracnose and leaf spot on *Q. robur* leaves in riparian forests. This article underscores how even well-studied species can harbor previously underrecognized pathogen complexes, raising questions about latent risk and the importance of accurate species-level diagnostics. Complementing this, Marković et al. show how multiple foliar pests, including oak powdery mildew *Erysiphe alphitoides* and oak lace bug *Corythucha arcuata*, can collectively suppress growth in young trees—especially when compounded by environmental stressors like drought and groundwater decline. Together, these studies emphasize the need for integrated risk frameworks that account for synergistic interactions and cumulative stress.

Climate change as a modifier of pest risk emerges as another critical cross-cutting issue. Macháčová et al. offer compelling evidence that elevated atmospheric CO_2-a hallmark of future climate scenarios –can influence host-pathogen interactions. Their study on *Alnus glutinosa* responses to *Phytophthora* bark infections reveals that disease outcomes vary under different CO_2 levels, suggesting that future pest dynamics may shift in non-linear, species-specific ways. These findings reinforce the necessity of integrating climate variables into risk assessments, expanding from pest virulence to also consider host physiological responses and ecosystem-level vulnerabilities.

Climate change is a driving factor for shifts in the distribution areas of many species. Gao et al. use predictive modeling to project the expansion of *Monochamus saltuarius*, a vector of Pine wilt disease in China, under current and future climate scenarios. Their results point to a marked northward and regional expansion of risk zones, offering important insights for biosecurity planning. The study reflects the growing importance of bioclimatic modeling in forecasting risk trajectories—especially invasive species—but also illustrates the uncertainty that accompanies such projections across decadal timescales.

Various preventive actions must be applied to minimize the threat of invasive species to forests. Budzyn et al. evaluate a firewood transport campaign in Michigan, revealing that campaign awareness slightly decreased between the survey years, personal firewood transport has decreased, and knowledge of invasives remains low. Their findings call attention to the behavioral dimension of forest pest risk, suggesting that outreach should be paired with stronger regulatory mechanisms to meaningfully mitigate spread.

Despite differences in taxa and regions, all four studies point to the need for early detection, cross-disciplinary approaches, and multi-agent monitoring systems. They also reveal that pest risk is no longer a static or localized concept—it is dynamic, multi-scalar, and increasingly shaped by climate, connectivity, and complexity. The emergence of underestimated pathogen complexes, the cumulative impact of mild but chronic stressors, and the interaction between human behavior and pest movement are key themes that emerge across the studies.

In sum, this section demonstrates that risk assessment must evolve toward flexible, integrative, and anticipatory models—ones that account for biological complexity, environmental change, and human activity in concert.

Evaluation: understanding host-pest interactions under climate change and advancing pest detection

Evaluation of forest pests includes characterizing the symptoms of pest infestation, developing an appropriate detection method for monitoring, and establishing specific critical thresholds. In general, two primary methods of evaluation are distinguished: population sampling and damage monitoring. For both approaches, stand-level risk rating can help identify priority areas for targeted monitoring. In the case of invasive species, presence–absence strategies are commonly employed, with detection methods requiring high sensitivity—such as pheromone traps—to provide rapid confirmation of species presence. Population data are frequently evaluated to classify pest levels as above or below critical thresholds, often using sequential assessment methods. Damage monitoring is a rapidly evolving field, driven by technological advances in unmanned aerial vehicles (UAVs), satellite imagery, remote sensors, and classification algorithms. One of the main functions of monitoring is to support decision-making in forest pest management by providing timely and actionable information.

Evaluating forest pest outbreaks is a crucial step toward timely management interventions, particularly under the pressures of climate change and increasing global trade. The studies in this section explore innovations in early detection—from physiological and biochemical responses in trees to remote sensing technologies and pheromone-based trap networks. Collectively, they reinforce that successful pest evaluation will combine early physiological signals, volatile chemical detection, and spatial monitoring tools into an integrated approach.

Several papers focus on the devastating impact of the spruce bark beetle *Ips typographus*—the most important pest in Central Europe, responsible for the loss of ca. 100 mil. m^3 of growing stock in Czechia between 2016 and 2022 (Washaya et al., 2024). Stríbrská et al. assess physiological and biochemical changes in *Picea abies*, identifying reduced sap flow, stem increment, and increased monoterpene emissions in freshly infested trees. These biological responses, along with bark temperature measurements and trap catches, could enhance early warning systems. Similarly, Hüttnerová and Surový test three electronic nose devices for their ability to detect bark beetle-induced volatile organic compounds. Their findings confirm that infestation can be detected within 1 week of attack onset, pointing to the potential of chemical sensing for rapid, non-invasive diagnostics.

In parallel, Klouček et al. explore UAV-borne multispectral imaging to distinguish between healthy and infested spruce trees at early infestation stages. Vegetation indices, particularly NDVI and BNDVI, proved more effective than individual spectral bands, and detection accuracy improved as infestation progressed. These results underscore the growing utility of remote sensing technologies for large-scale forest health evaluation, especially when integrated with on-ground physiological and chemical indicators.

While much focus is placed on *I. typographus*, Fiala and Holuša broaden the scope by proposing a national-scale monitoring network targeting invasive bark and ambrosia beetles in Czechia. They recommend 24 high-risk locations based on proximity to borders, trade hubs, airports, and botanical gardens, using ethanol-baited traps as a sensitive detection method. This proactive approach provides an early warning infrastructure aimed at intercepting invasive species before establishment, reinforcing the need for geographically targeted surveillance.

Together, these studies demonstrate that forest pest evaluation is evolving into a multi-level and multi-method discipline, bridging physiological measurements, chemical ecology, spatial modeling, and biosecurity infrastructure. They also highlight the importance of early signals, both from trees and pests, as well as the need for flexible monitoring strategies that can adapt to shifting pest dynamics in a changing climate.

The research article Modlinger et al., "*Ectomycorrhizal response to bark beetle attack: a comparison of dead and surviving trees*", contributes insights into the ecological consequences of bark beetle infestations on below-ground interactions, offering a deeper understanding of forest ecosystem responses to widespread tree mortality. In this study, the dynamics between tree root systems of the Norway spruce and ectomycorrhizal fungi in the aftermath of bark beetle-induced tree mortality were investigated. The density of vital mycorrhizal tips (VM) on living trees gradually increased, peaking in the 2nd and 3rd years after the surrounding forest decay. VM on bark beetle snags was significantly lower compared to living trees, with minimal variation over time. Most of the fine root biomass decomposes within the first half year after tree death.

Management: toward innovative, integrated solutions for sustainable pest control

Knowledge of risk from emerging pests and pathogens and advanced impact assessment and detection methods must be paired with practical strategies and new technologies to manage pest outbreaks in forests. Managing biotic disturbances demands novel, innovative solutions that are effective, sustainable, and ecologically responsible. Recent cutting-edge advances in biotechnology and biological control offer promising new avenues for managing pest outbreaks while minimizing environmental impacts. Early stages of pest detection improve the efficiency and effectiveness of management. However, in most forest areas of Europe, the key changes should lie in modifying forestry practices and mitigating the impacts of climate change in forests.

The review Sharan et al. "*Transgenic poplar for resistance against pest and pathogen attack in forests: an overview*" explores advances in genetic engineering aimed at enhancing tree resistance against multiple pests and pathogens, an approach that could potentially reduce reliance on chemical treatments and safeguard plantation productivity. It explains how *Populus* spp., a model genus for forest biotechnology, can be engineered to express resistance genes targeting key pests and pathogens and reviews transformation techniques (*Agrobacterium*-mediated, CRISPR/Cas, RNA interference). It also reminds us of the need to navigate regulatory, ecological, and ethical considerations surrounding the deployment of genetically modified trees in natural and plantation forests.

Complementing this technological approach, Gupta et al., in "*Prospects for deploying microbes against tree-killing beetles (Coleoptera) in the Anthropocene*", explore the potential and challenges of using microorganisms as nature-based biopesticides to combat devastating bark beetle outbreaks. By targeting bark beetle symbiotic bacteria and fungi and their microbial volatile organic compounds (VOCs), beneficial microbes in forest soil and plants, entomopathogenic fungi, and even symbiont-mediated RNA interference (RNAi), researchers could develop biocontrol tools that work in synergy with tree defenses. Given the destructive power of bark beetles globally, microbial biocontrol presents a sustainable alternative that can be integrated with existing forest management practices.

Finally, the perspective by Mogilicherla and Roy, "*RNAi-chitosan biopesticides for managing forest insect pests: an outlook*", presents an exciting frontier of how precision-targeted biopesticides such as RNA interference technology (RNAi) that can silence essential species-specific pest genes combined with biodegradable carriers like chitosan could revolutionize pest management by offering precision pest control with minimal off-target effects. RNA interference (RNAi) enables highly targeted pest suppression, and chitosan-based carriers improve environmental safety and application efficiency. If further developed for field applications, these innovations that offer high specificity could serve as next-generation biopesticides that align with ecological conservation and sustainability goals, offering an alternative to broad-spectrum insecticides that harm beneficial insects and forest microbiota.

Mass trapping of bark beetles is a traditional pest management approach, which has many supporters but also a lot of criticism in terms of the actual impact on the population density of the pest (Kuhn et al., 2022). One of the current scientific directions is developing an anti-attractive blend and using it to protect vulnerable forest stands. Various compounds behaviorally active for spruce bark beetle were tested in research by Moliterno et al. in "*Field effects of oxygenated monoterpenes and estragole combined with pheromone on attraction of Ips typographus and its natural enemies*". Based on the catches to the traps at low, medium, and high doses of the compounds, they found that all 1,8-cineole doses and the high estragole dose acted as anti-attractants for *I. typographus*, whereas all (+)-isopinocamphone doses enhanced attraction to the pheromone. The compounds 1,8-cineole, isopinocamphone, and estragole may play vital roles in tritrophic interactions among spruce trees and *I. typographus* and its natural enemies, and these compounds may be developed into new or enhanced semiochemical-based pest control methods.

A direct trapping and tree protection experiment with an anti-attractant blend containing 1-hexanol, 1-octen-3-ol, 3-octanol, eucalyptol, trans-thujanol, and trans-conophthorin (see Jakuš et al., 2024) was reported in an article by Korolyova et al., "*Mitigating Norway spruce mortality through the combined use of an anti-attractant for Ips typographus and an attractant for Thanasimus formicarius*". The anti-attractant blend was compared with commercial pheromone bait and attraction lure for *Thanasimus formicarius*, an important spruce bark beetle predator. Tree mortality was observed exclusively among trees treated only with *T. formicarius* attractant and in their vicinity, suggesting a unique bark beetle response to the mixture of predator's attractant and host tree kairomones, a phenomenon that was not previously reported. Application of anti-attractant and *T. formicarius* treatment effectively prevented tree mortality, demonstrating the repellent potential of anti-attractant against bark beetles.

Outlook and future directions: toward resilient, innovative, and integrated forest pest management

Taken together, the contributions in this Research Topic illustrate complex challenges of biotic pest disturbances in a rapidly changing world. They remind us that while disturbances are natural and often necessary drivers of forest renewal, their increasing frequency and severity, exacerbated by climate change, pose unprecedented risk. They also show how Risk, Evaluation, and Management of biotic disturbances must be viewed as interdependent and interconnected elements of forest resilience strategies. The growing complexity of forest threats demands interdisciplinary integrated approaches that bridge pathology, entomology, ecophysiology, microbiology, biochemistry, molecular biology and biotechnology, remote sensing, climate science, forest policy, and silviculture. To address these challenges, we must continue to expand our knowledge of disturbance agents and their complex interactions, develop advanced monitoring and evaluation tools, and pursue integrated management strategies that enhance the resilience of our forests.

Looking forward, priorities for research and practice should include the following:

- Expanding monitoring networks and early-warning systems that combine traditional field methods with remote sensing and molecular diagnostics.
- Developing predictive models that integrate climate projections, multiple disturbance agents, and forest stand dynamics.
- Advancing biotechnology and biocontrol, ranging from natural enemy enhancement, semiochemicals, and microbial biocontrol to precision biotechnology and transgenic trees while ensuring safety, efficacy, and societal acceptance.
- Promoting forest management practices that enhance species diversity, structural heterogeneity, and adaptive capacity.

The diverse articles in this Research Topic show that addressing forest pest disturbance demands research that is holistic, anticipatory, integrated, and socially accepted. Amid rapid environmental challenges, no single discipline can tackle this challenge alone. By fostering collaboration across disciplines and bridging fundamental science with technological innovations, we can better understand the role of disturbances while developing innovative tools and practices that maintain forest health and the vital ecosystem services forests provide.

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Conflict of interest

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

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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

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 (Juráò 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**).

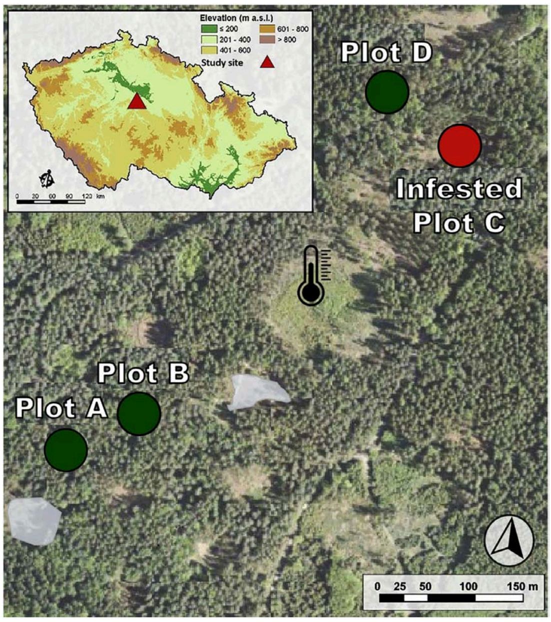

FIGURE 1
Arrangement of study plots with the naturally attacked plot. Green circles represent the inner forest plots (non-attacked trees), corresponding to plots A, B, and D. Plot C was infested by *I. typographus* on June 16th and salvaged in July (red circle). The gray spots are areas of previous natural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

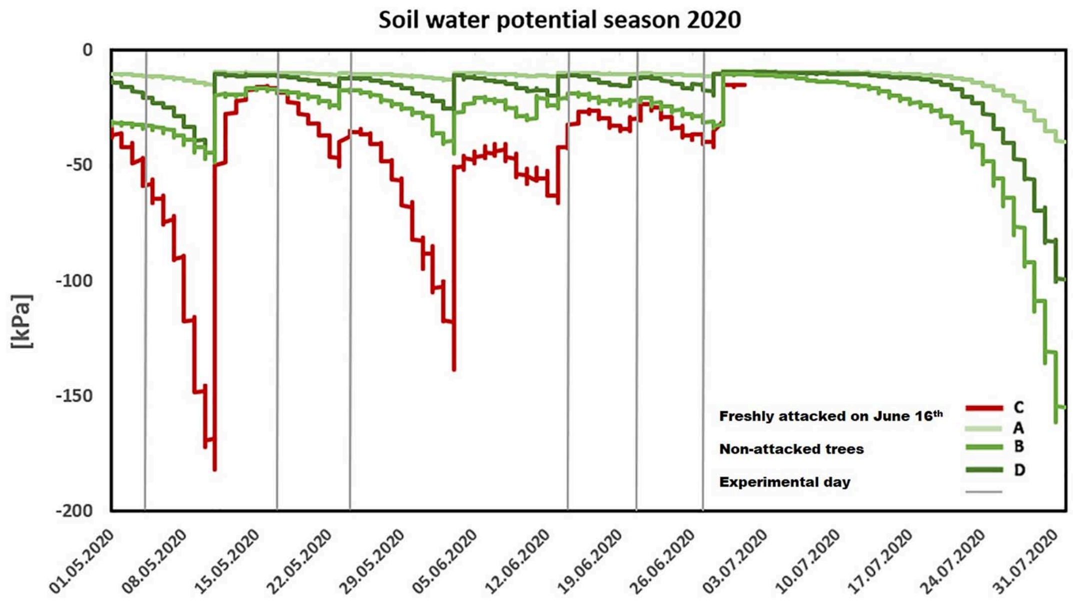

FIGURE 2
Seasonal trend of soil water potential (2020). Individual lines represent measurements through the season in freshly attacked plot C (red color)/the non-attacked plots A, B, and D (green color). The gray color (vertical line) represents individual experiments.

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,

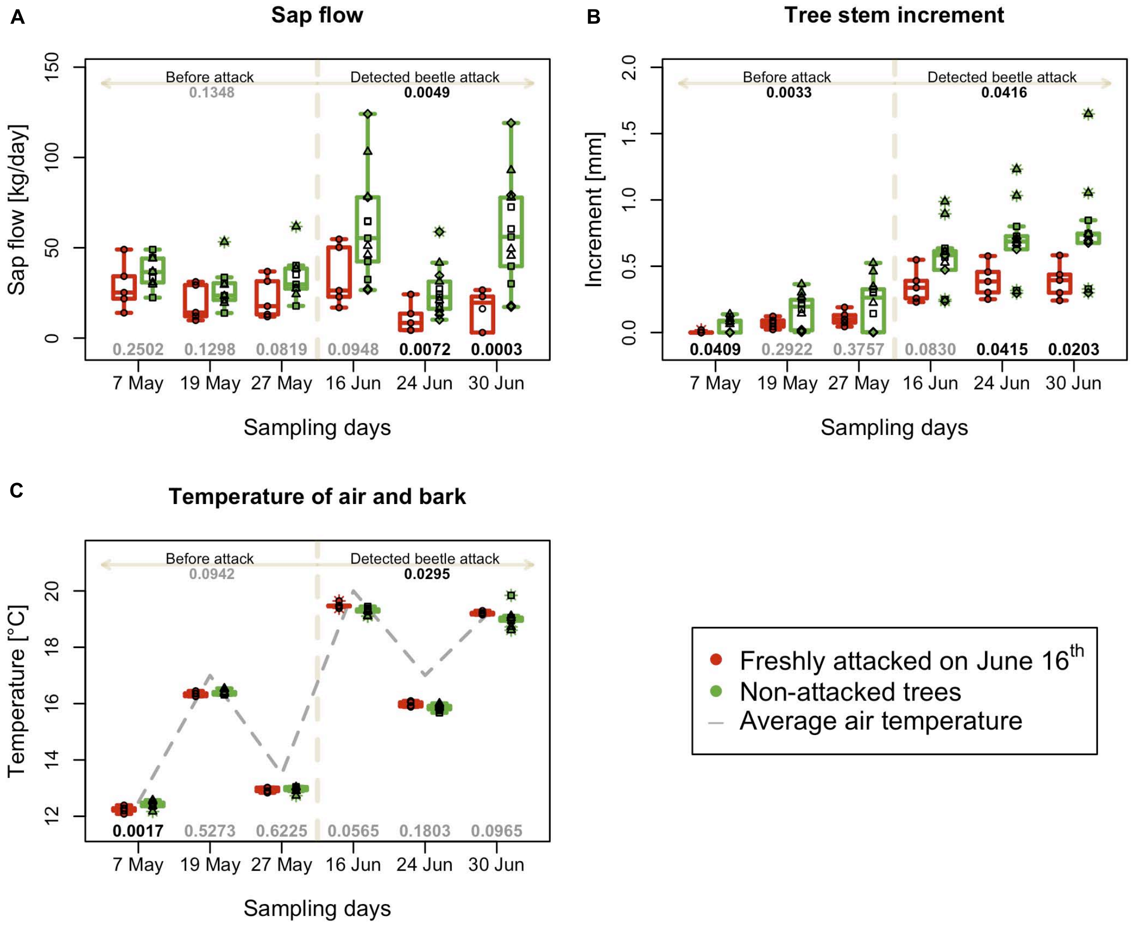

FIGURE 3

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

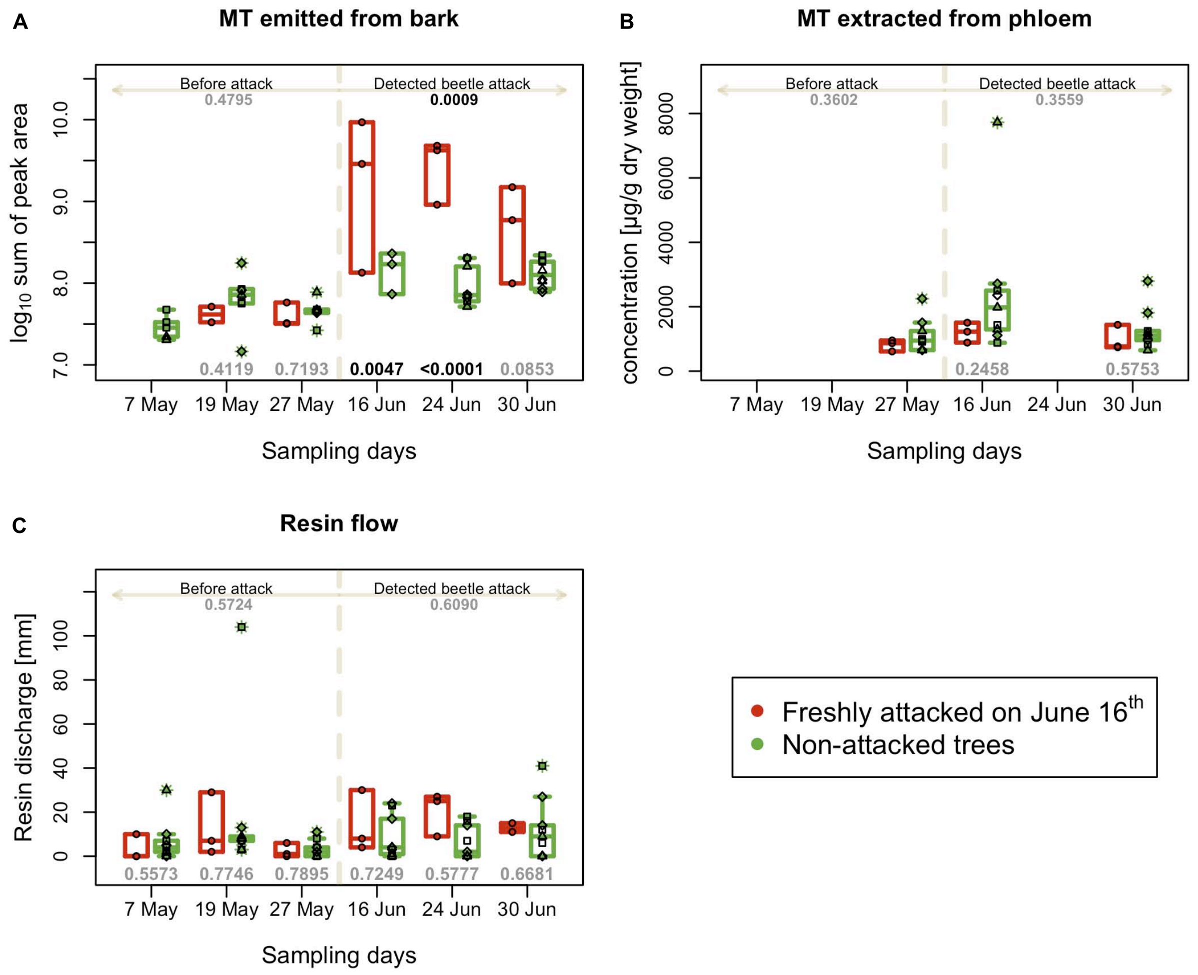

FIGURE 4
Tree defense characteristics recorded on individual trees on different experimental days. **(A)** Sum of peak areas of selected monoterpenes emitted by bark **(B)** Sum of peak selected monoterpenes extracted from phloem - performed three times per season; **(C)** resin flow per 24 h; boxplots 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

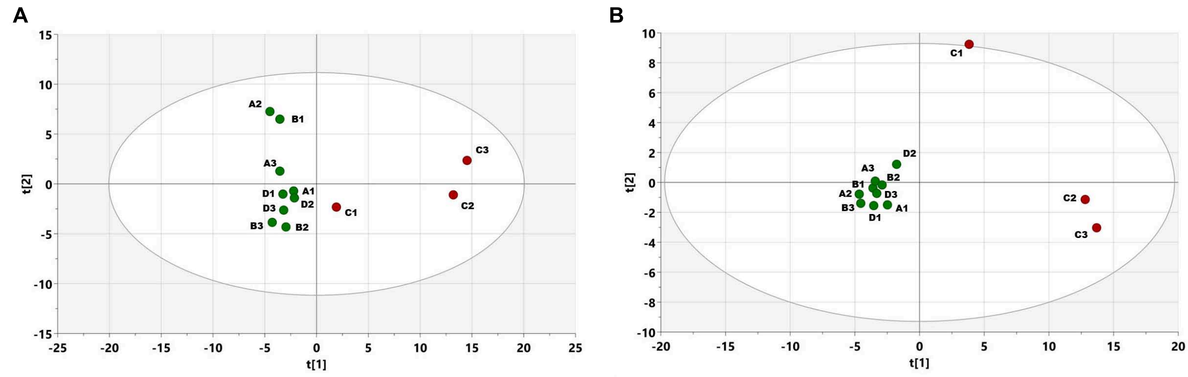

FIGURE 5

(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.

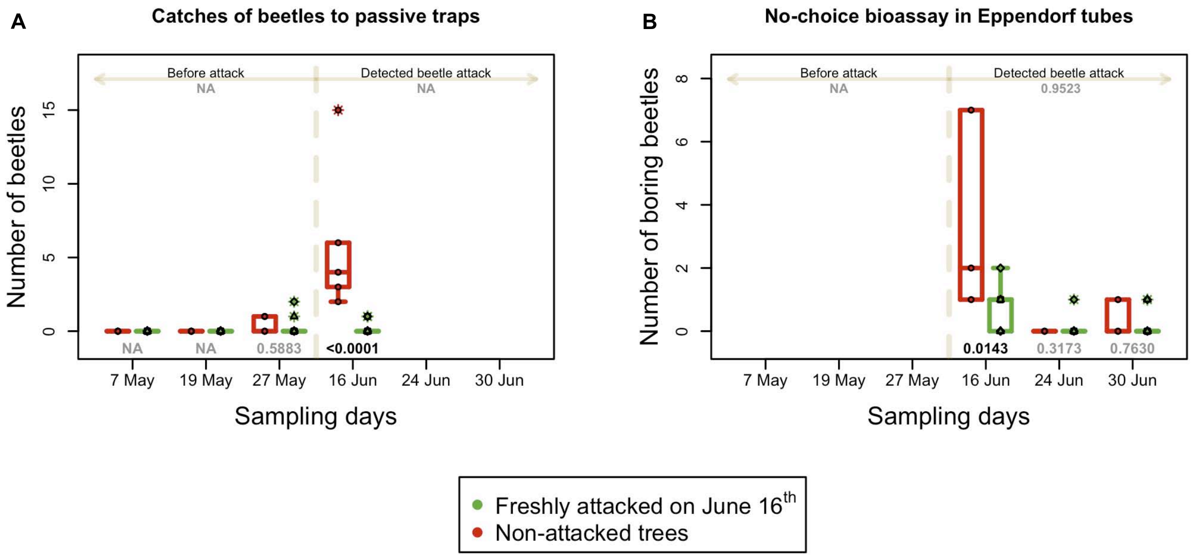

FIGURE 6

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 Stříbrská 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 (Juráò 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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Prospects for deploying microbes against tree-killing beetles (Coleoptera) in Anthropocene

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Forests form rich biodiversity hubs that act as large reservoirs of natural carbon. The spatial and temporal heterogeneity of these complex habitats of forest floors provides ecological services of immense socio-economic importance. However, these socio-economic ecological hotspots are incessantly exposed to multifarious abiotic, biotic, and anthropogenic disturbances, amongst which unpredictable forest pest (i.e., bark beetle) outbreak account for the loss of vegetation and microbiome of measurable quantum. The importance of the microbiome in forming an inseparable functional unit of every host and shaping its interaction with other partners has been well realized. Interestingly, forest pests, including bark beetles, are also reported to rely on their endosymbiotic microbial partners to manipulate tree defense machinery. In contrast, the microbiome forming the holobiont of trees also regulates the overall function and fitness of the host and significantly contributes to tackling these challenging situations. Nevertheless, how the holobiont of trees directly or indirectly influence beetle holobiont is still an enigma. The present review shall elaborate on the role of microbial tools in enhancing tree performance and fitness, which helps counter beetle damage. Besides, it shall also emphasize exploiting the role of microorganisms in acting as biocontrol agents in shielding the trees against beetle destruction. The application of endosymbiont-mediated RNA interference (RNAi) in working with two-tier specificity for controlling beetle devastations shall be discussed as new-age technological advances. All explanations are expected to put forth the potential of the microbial toolbox in offering better and more sustainable beetle management strategies in the future.

KEYWORDS

forest microbiome, bark beetles, biocontrol agents, microbial volatile organic compounds (MVOCs), coleopteran forest pest management, RNA interference (RNAi), symbiont-mediated RNAi (SMR)

1. Introduction

Forests are immensely important due to their socio-economic and ecosystem services (Pan et al., 2013). They serve the ecosystem as a large sink of atmospheric carbon, supporting wildlife habitats and timber industries. Over the years, the resilience and functionality of forests have been under constant threat due to rise in temperature, drought stress, windthrows, wildfire outbreaks, and pest infestation (Hlásny et al., 2019). Amongst them, pest infestation is reported to account for measurable losses and hence has drawn the concern of forest scientists. Climate-driven intensification in the frequency, severity, and cyclicity of coleopteran forest pest outbreaks has already been documented (Haynes et al., 2014; Hlásny et al., 2021). Besides, bark beetles have expanded their geographic range by exploiting native hosts previously unexplored due to low temperatures (Ramsfield et al., 2016; Howe et al., 2021). Such insect range expansion also causes widespread tree mortality, decreasing forest productivity and carbon storage and enhancing discharges from the decayed dead wood. Frequent pest outbreaks cause severe forest depletion triggering trajectories outside the resilience limits of forest ecosystems, causing irreversible ecosystem regime shifts (Dhar et al., 2016). With global climate change, beetles have become a formidable threat to forest health worldwide (Hlásny et al., 2021). The most common aggressive tree-killing coleopteran beetles that have caused widespread damage to millions of trees across Europe and North America include Emerald Ash Borer (EAB) [*Agrilus planipennis* (Fairmaire)] (Coleoptera: Buprestidae), Asian long-horned beetle (ALB) [*Anoplophora glabripennis* (Motschulsky)] (Coleoptera: Cerambycidae), and bark beetles (Coleoptera: Curculionidae: Scolytinae); ambrosia beetle, mountain pine beetle (*Dendroctonus ponderosae* Hopkins), Eurasian spruce bark beetle [*Ips typographus* (L.)]. Several conventional approaches, such as sanitation felling (Seidl et al., 2016), removal of wind-felled trees (Leverkus et al., 2018), and deployment of pheromone-baited and poisoned log tripod traps (Wermelinger, 2004) have been deployed for the last few decades to manage the pest population levels within endemic limits. However, the success of all these approaches remains questionable in managing the recent outbreaks (Hlásny et al., 2019). Several synthetic pesticides have been used to suppress forest beetles over the past years (Holmes and MacQuarrie, 2016; Liebhold and Kean, 2019). However, many of these compounds caused other problems, such as environmental pollution, detrimental effects on non-target organisms, and caused widespread pesticide resistance (Billings, 2011; Bras et al., 2022; Avanasi et al., 2023; Kenko et al., 2023). Therefore, questions have been raised about the effectiveness of conventional phytosanitary measures. Hence, eco-friendly alternatives for controlling these devastating tree-killing forest beetles have become the utmost priority in the Anthropocene.

Very interestingly, the associations between the forest beetles and their bacterial and fungal endosymbionts are reported to add to the host fitness by aiding them to feed on recalcitrant bark tissues, provide protection against pathogens and increase intra and intercellular communicability that helps to overpower host defense and build up pest population (Cheng et al., 2018; Chakraborty et al., 2020a,b). Besides, forest flora, predominantly comprised of trees, house a huge population of diverse microorganisms both above and belowground. These forests exhibit a wide and dynamic spatial and temporal heterogeneity ranging from short-term seasonal events to long-term standing developments, allowing diverse microorganisms to thrive in these variable habitats and form an ecosystem of rich bio-resources (Baldrian, 2017). Soil microbiome and plant-associated above and below-ground microbiome, also called the "plant holobiont," are believed to directly influence plant metabolism and defense that ultimately demarcate the expanses and limitations of an ecosystem (Soldan et al., 2022). Soil microbial profiles are often similar to gut microbial profiles of herbivorous insect pests suggesting a direct link between soil and insect pest microbiome, which are often independent of tree host microbiome (Hannula et al., 2019). On the contrary, studies conducted on forests and woodlands have shown spruce beetle infestation to modify soil microbial assemblage by altering edaphic properties (Custer et al., 2020). Such resemblance of microbial composition across several microbiomes indicates bottom-up and top-down connectivity amongst food webs. However, how these microbial assemblies add to the functionality of diverse holobionts is still an enigma. Research on the role of plant and pest holobionts and their microbial exchange is increasing (Pirttilä et al., 2023). Few knowledge bases are being translated from laboratory benchtops to agricultural fields. However, owing to the vastness of forests, comparative analyses and similar studies conducted on forests are notably less (**Figure 1**). Research on agriculture is prioritized as it meets the growing population's demand and ensures food security. Hence, forest research is trailing behind (**Figure 1**), but it has recently gained more interest. The beneficial impact of a less polluted environment is vivid after COVID-19 pandemic. Forests play a major role in our environment, and saving forest ecosystemss from insect pests are crucial. However, limited studies have been conducted on forest pests and their microbiomes (**Figure 1**). There are a handful of forest pest genomes published so far. Besides, how the edaphic and other environmental cues alter the soil biodiversity and biogeochemical performance in forest ecosystems is also understudied (Chen et al., 2019). The present review shall look into the different aspects of tree-beetle interaction, emphasizing the role of forest beetles and their associated microorganisms that directly or indirectly influence the host-beetle-microbiome tripartite relationships. Using such knowledge for future forest beetle management is also debated (**Figure 2**).

2. Coleopteran insect pests: a constant threat to forest flora

Forest beetles, such as Asian longhorn beetles, EAB, bark beetles, etc., have been reported to have taken advantage of current climate change and caused substantial damage to the forests worldwide (Aukema et al., 2011; Meng et al., 2015; Hlásny et al., 2019). EAB is a tree-killing forest insect pest from northeastern Asia that recently entered North America via solid wood packaging material (Poland and McCullough, 2006). EAB has killed millions of North American Ash (*Fraxinus* sp.) and is recognized as one of the costliest insect pest invaders in American history (Aukema et al., 2011). EAB larvae disrupt the translocation of water and essential nutrients in the attacked plants during phloem and cambium feeding (cambio-xylophages), killing Ash trees within 3–4 years of infestation (Haack et al., 2002). Like EAB, the Asian longhorn beetle (*A. glabripennis*), native to China and Korea, is a globally known invader with the potential to thrive on more than 100 species of trees (Meng et al., 2015). If uncontrolled, the ALB population is believed to cause tremendous economic losses (in billions of dollars) (Pedlar et al., 2020). Fascinatingly, the trunk injection of imidacloprid (systemic insecticide) was documented to be effective against ALB infestation. However,

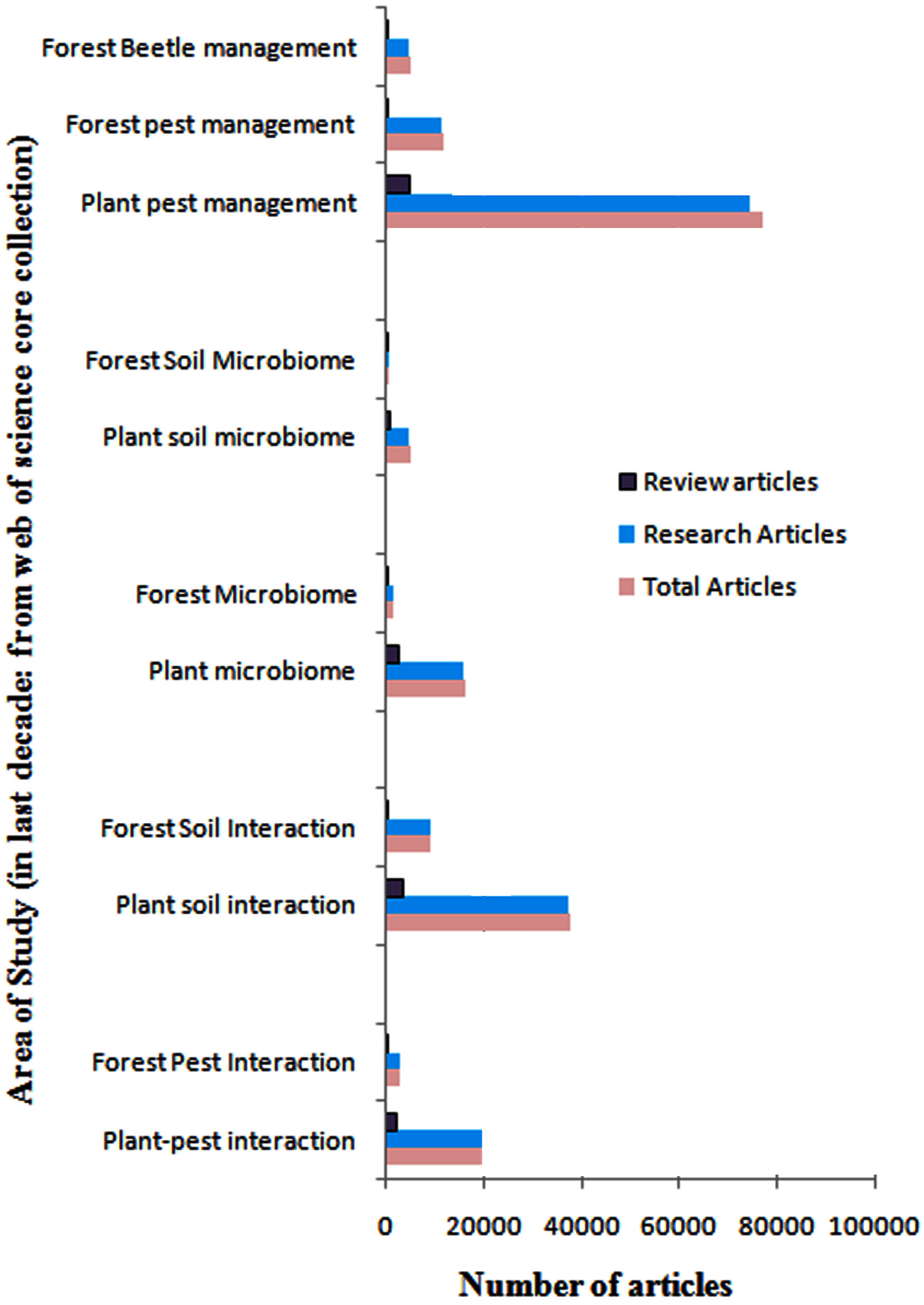

FIGURE 1
Comparative analyses of numerical data of studies on plant-microbe-pest interaction of typical habitats versus forest areas over the past decade. The source of publications is core collections indexed in the Web of Science (January 2023).

the cost and environmental impact of using chemical pesticides jeopardize such strategies, which demand better eco-friendly alternatives for ALB management (Avanasi et al., 2023).

The forest beetles, primarily bark beetles (Coleoptera: Curculionidae: Scolytinae), feed as larvae and adults in the phloem of trees and woody shrubs (Coulson, 1979), have caused widespread coniferous tree mortality and severe economic losses around the globe (Sun et al., 2013). Bark beetles are thought to destroy more trees than all other natural factors combined (Franceschi et al., 2005). Furthermore, these insects are enormous profiteers of global climate change, as increasing temperatures have contributed to historically unprecedented beetle outbreaks, killing hundreds of millions of conifer trees (Raffa et al., 2008). One important tree-killing species is *I. typographus*, which has

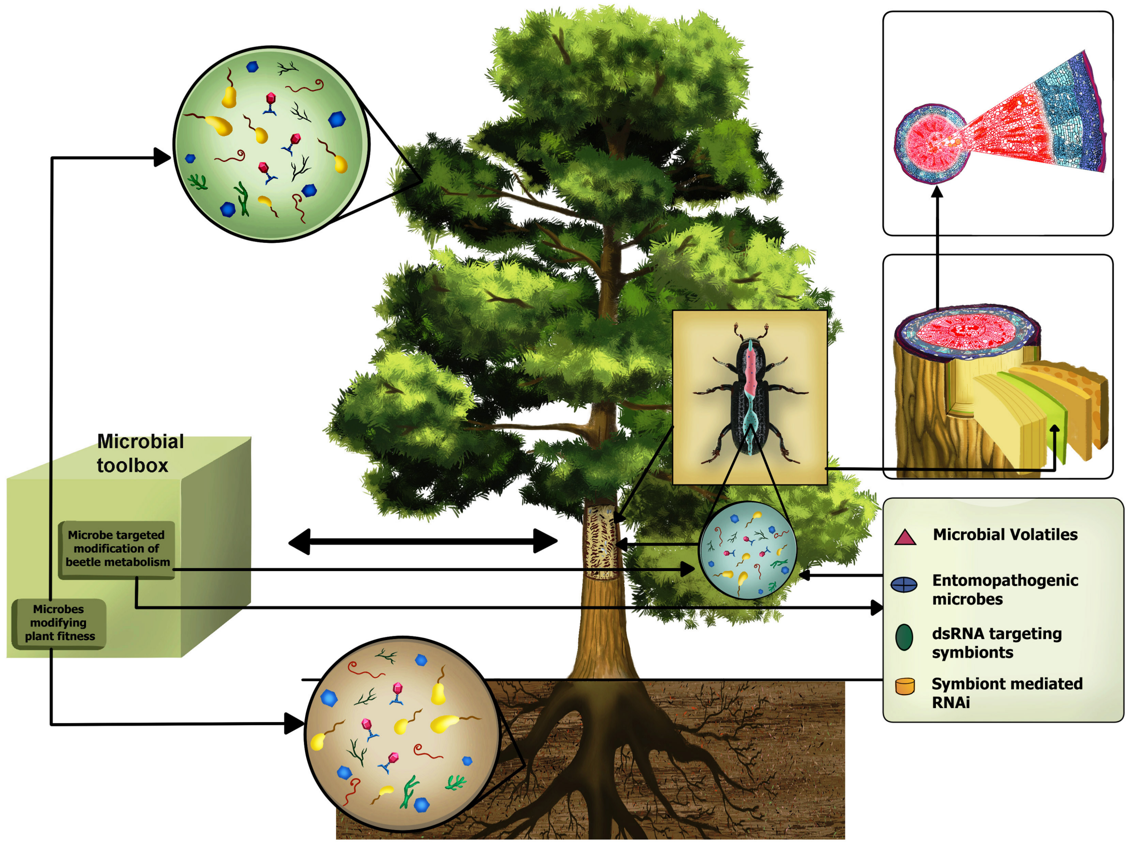

FIGURE 2
Schematic illustration projecting the probable roles of the microbial toolbox in developing a direct or indirect defense of forest trees against beetle infestation.

decimated more than 170 million m^3 of spruce in Europe over the last 65 years (Økland et al., 2016). During outbreaks, the beetle population surpasses a critical threshold and overwhelms the defensive capacity of individual trees in pheromone-mediated mass-attacks (Wermelinger, 2004). Males are the pioneering sex that selects trees, tunnels into the bark to make a mating chamber and produces aggregation pheromones while detoxifying fierce spruce *defenses*. Females are attracted to the pheromones and ingest large quantities of well-defended spruce tissues as they chew out ∼10 cm long tunnels in the inner bark where they deposit their eggs (Wermelinger, 2004; Schebeck et al., 2023). Despite their enormous economic and ecological importance, the exact mechanisms by which these tiny insects can overwhelm the *defenses* of giant conifer trees are still unknown (Krokene, 2015). Only limited studies provided molecular insights into bark beetle adaptive strategies (Liu et al., 2022; Naseer et al., 2023; Tholl et al., 2023; Zaman et al., 2023).

3. Microbial symbiosis: ecological benefits

The recipe for such adaptive success intended for thriving under the bark is often hinged on the association with microbial mutualists that benefit its host by feeding on recalcitrant plant material, protecting against pathogens, and enhancing the inter-and intraspecific communications (Chakraborty and Roy, 2021; Singh et al., 2021; Picciotti et al., 2023). Interestingly, microorganisms exhibit extremely short generation times and thus possess rapid evolutionary rates compared to multicellular organisms. Hence, they can evolve various metabolic pathways and adapt to diverse ecological niches. Multiple studies suggest mutualistic associations with microbes in coleopteran forest pests; however, rigorous experimental validation remains lacking. Thus, the exact mechanisms or the molecular *dialogue* during symbiosis enabling the spectacular destructiveness of tree-killing forest beetles is still unknown (García-Fraile, 2018). However, it is already established that microorganisms could give their host a substantial advantage over non-symbiotic competitors in the severe interspecies competition. Insects, including forest beetles, cannot survive without their associated microbes. Hence, such microorganisms can be alternative targets for controlling forest beetles (García-Fraile, 2018; Rupawate et al., 2023). Although, in most cases, these symbiotic associations are species-specific and fine-tuned on the ecological requirements of their host, targeting symbionts for pest management reduces the chances of non-target effects (Douglas, 2007; Chung et al., 2018).

The advent of high throughput omics technology such as genomics, metagenomics, meta-transcriptome, meta-proteomics, and metabolomics has provided exciting insights into the function of the microbiome in forest beetle ecology and evolution. It has been possible to characterize the full spectrum of biological diversity of previously hidden habitats, including inside plants and insects. This has opened up fascinating new research fields, such as the study of phytobiomes, i.e., all microbial diversity in and around plants (Ledford, 2015), and endomicrobiomes, i.e., primarily gut symbionts of different animals (Adams et al., 2013). Many studies suggest that microbes in and around an insect's body are more critical to its success than was once believed (Picciotti et al., 2023). Symbiotic bacteria and fungi have been found to play essential roles in many processes, including the breakdown of food (Scully et al., 2014; Lee et al., 2015), protection against plant defenses (Ceja-Navarro et al., 2015; Howe and Herde, 2015) and fungal pathogens (Cardoza et al., 2006), and even affecting insect reproduction and development (Vavre and Kremer, 2014). For instance, in some North American bark beetle species, the endomicrobiome is critical for the digestion of plant tissues, breakdown of plant defenses (especially terpenes), and parts of pheromone production (Adams et al., 2013; Boone et al., 2014). Adams et al. (2013) conducted a shotgun metagenomic sequencing analysis of microbiota in the mountain pine beetle, a severe pest of pine, and detected enrichment in bacterial genes involved in diterpene degradation. Also, most of these genes were derived from *Gamma-proteobacteria* and *Beta-proteobacteria* (genus *Burkholderia*) (Adams et al., 2013). Coupled with this culture-independent analysis, they isolated terpenoid-degrading bacteria from mountain pine beetle gut belonging to the bacterial genera *Serratia*, *Rahnella*, and *Brevundimonas*.

Moreover, symbiont-mediated terpenoid degradation in the large pine weevil, *Hylobius abietis* (L.) (Coleoptera: Curculionidae), *in vitro* and *in vivo* analyses is already reported (Berasategui et al., 2017). In conjunction with physiological confirmations, the metagenome and metatranscriptome investigations have identified microbial genes involved in the detoxifying symbiosis in beetle systems (Itoh et al., 2018). This opens up the opportunities to search for essential microbial genes within tree-killing beetles, give vital survival services, detoxify terpenes, and aggregate pheromone production in EAB, ALB, and other targets beetles that pose a constant threat to forests globally under the influence of climatic changes. Such genes of microbial origin can be targeted to formulate eco-friendly strategies to control the beetles via symbiont management (Rupawate et al., 2023). For instance, targeting horizontally transferred genes to manage whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Wang and Luan, 2023). The work on genetic engineering of honeybee gut microbiota shows the feasibility of such an approach (Leonard et al., 2018; Chhun et al., 2023). Although there is increasing scientific literature on the importance of symbiotic microorganisms in forest beetles that evolved from our perspective of viewing them as a holobiont, the idea of decoding such knowledge into developing forest pest management tools is still limited.

4. Tree defense against coleopteran forest beetles

Statistical records suggest that tree mortality is caused by the sporadic outbreaks of the beetle population, which under natural environmental conditions, remain confined within endemic limits (Raffa et al., 2008). Such restrain in the beetle population is attributed to the intrinsic surveillance and defense system of the host trees (Krokene et al., 2013; Whitehill and Bohlmann, 2019). Minor fractions of aggressive beetles that usually thrive on dead and decayed wood tissues are known to succeed in overwhelming host defense and colonizing tree trunks under conducive conditions, while semi-aggressive and facultative parasitic members complete their life cycle primarily on weak and/or dead trees (Krokene et al., 2013). Beetles are reported to entrap the host immune system by employing pheromone-mediated mass attack. Symbiotic microbes residing in the beetle gut collaborate with the host beetle in devastating tree defense (Six and Wingfield, 2011). Beetle mass outbreaks are reported predominantly in coniferous forests where colonization is predominantly found on the bark tissues. Successful establishment of the beetles on its prospective hosts is achieved in a phase-dependent manner where the first stage is to inhibit the pest by the preformed defenses. The second stage is to compartmentalize the attackers by inducible defenses. The third stage means sealing damaged areas, while the last stage imparts systemic acquired resistance to tackle consecutive outbreaks (Eyles et al., 2010). Heavy impregnation of lignin and suberin polymers in the periderm of bark regions are reported to provide preformed constitutive defense against invasion and establishment of beetle pests, while the inducible defense is offered by chemicals such as tree metabolites, proteins and enzymes (Franceschi et al., 2005; Keeling and Bohlmann, 2006). The conifer stem anatomy offers a highly structured natural barrier for the invading beetles (**Figure 3**). The bark is the primary site of conifer defense. The bark tissue is known to impart mechanical support and also supply photosynthates. Besides, the specialized cells of the inner secondary phloem named polyphenolic parenchyma cells (PP), lignified sclerenchyma cells, cells with calcium oxalate crystals, resin ducts and the connecting radial cells, contribute to providing resistance against beetle aggression (Franceschi et al., 2005). The sap wood also contains a few resin ducts that help act as sources of inducible chemical defenses (Krokene et al., 2013; Whitehill and Bohlmann, 2019). The defenseless vascular cambium plays a pivotal role in tissue differentiation and supplying needful cells to outer bark region and inner sapwood region. Hence, damage of the said region due to beetle infestation leads to irreversible loss and ultimately death of the host conifer (Franceschi et al., 2005).

Chemical signaling has been known to impact almost all facets of species-species interaction. Amongst several chemicals, terpenoids, and phenolics stored in resin ducts, PP cells and secondary phloem are considered to modulate beetle-tree interaction (Keeling and Bohlmann, 2006). Terpenoids are reported to have mutually exclusive bidirectional roles of acting as flavoring and venomous agents for beetles from time to time. On one hand, they assist the beetles in locating compromised hosts and exhaust their immune system, while on the other hand, they form a formidable barrier against their mass colonization and attract their predators and competitors (Gitau et al., 2013; Zhang et al., 2013). Besides terpenoids, tree phenolics are also known to induce immune responses upon beetle infestation (Krokene et al., 2013). Different secondary phenolic compounds, such as soluble stilbenes and flavonoids, condensed tannins, polyphenols, and structural lignin polymers, modulate host defense. Majority of these secondary metabolites are produced via the shikimic acid pathway, where carbohydrate metabolism is connected to producing the

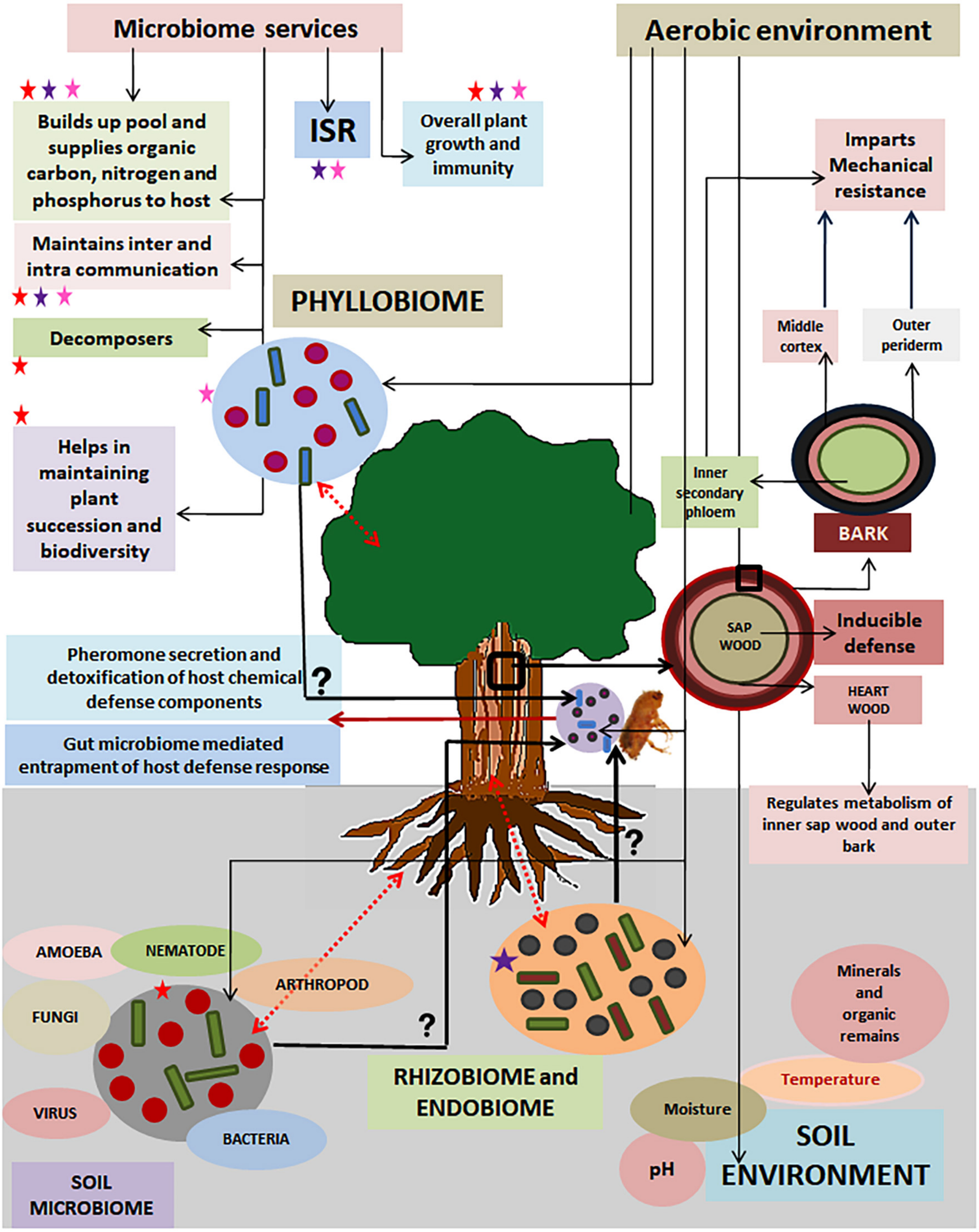

FIGURE 3
A schematic graphic illustration shows tree defense against forest beetle destruction and the role of soil microbiome and tree holobiont in providing indirect host resistance in a forest ecosystem. The unidirectional dashed arrows show the unidirectional regulation of the aerobic environment with its different biotic and abiotic components of soil, the tree host, tree holobiont, and beetle holobiont. The bidirectional arrows show bidirectional regulation between the soil microbiome tree holobiont and the host tree. The interrogation sign shows unknown roles and regulations of the tree holobiont and soil microbiome with beetle endosymbiont. Red, purple, and pink stars indicate soil, rhizosphere/endosphere, and phyllosphere microbiome, respectively. These star indicators have been used to mark the microbiome services they provide in a forest ecosystem. The small square-marked boxes of tree trunks have been enlarged to highlight the different layers of tree trunk sections. The roles of different layers of bark in providing defense against attacking beetles are elaborated in brief.

primary precursor aromatic amino acid of all secondary phenolics, the phenyl alanine (Ralph et al., 2006).

Over the past two decades, research has defined different types of acquired resistance mechanisms in forest trees, especially conifers, that have made them resistant to consecutive attacks (Whitehill and Bohlmann, 2019; Mageroy et al., 2020). Two such defense mechanisms are reported in conifer hosts, one induced upon fungal attack while the other upregulated following treatment with the phytohormone methyl jasmonate (MeJA) (Wilkinson et al., 2019). Although defense priming has provided experimental evidence in sufficiently equipping the immunogenic memory of a host plant, similar studies showing comparable results in real-world ecological contexts of forest regions are still not adequate. Besides, the long generation time of forest trees makes it hard to quantify the fitness versus allocation costs (Neilson et al., 2013). Moreover, the priming-induced decline in a symbiotic relationship that otherwise imposes additional fitness costs on trees by increasing their vulnerability toward pathogenic attack also questions the sustainability of priming as a defense measure in forest backdrops (Martinez-Medina et al., 2016). Despite of highly structured defense system of forest trees, the devastating bark beetles manage to bring on mass killings mainly by exploiting their own bacterial and fungal symbionts (Krokene, 2015; Zaman et al., 2023). In contrast, tree hosts are also shelter-providers to a large number of microorganisms referred to as the "tree holobiont"(Soldan et al., 2022). These microorganisms are also supposed to significantly contribute to shaping "tree health" and "immunity."

5. Role of microbiome in boosting tree fitness: indirect protection against beetle damage

The role of microbiome in shaping the entire biological world and its communication with all other biotic entities have been well established (Berg et al., 2020; Singh et al., 2021). Plants depend on beneficial microorganisms to enhance their stress tolerance and pathogen resistance. The discovery and refinement of technological tools as well as continuing upgradation of understanding has provided re-definitions of "microbiome" and "microbiota" where any and every eukaryote is considered to have an inseparable unit referred to as "second genome" or "holobiont" or "meta-organism," and likewise, death of any and every organism is considered as "dysbiosis" and loss of biodiversity comprising of its entire meta-organism (Lakshmanan et al., 2014; Stegen et al., 2018; Berg et al., 2020). Hence, the addition and/or alteration of microbial entities could result in significant alterations in the immune signaling cascade and lead to a paradigm shift in the health and productivity of the primary host (Bello et al., 2018). The following sections shall elaborate on the potential roles of the microbiome in developing tree resistance against beetle destruction.

5.1. The role of the below-ground microbiome and its potential for forest insect pests management

Plants being stationary, are predominantly dependent on their roots for their development from the onset of the seed emergence stage. These roots are exposed to a plethora of microorganisms both externally (by soil and rhizospheric organisms) as well as internally (by endophytic organisms) (Leach et al., 2017). Among the external members, soil microbes influence almost all spheres of below and aboveground ecosystems directly by altering the plant performance and/or indirectly by modulating the mutualistic or pathogenic interactions which ultimately impact host fitness (Pieterse et al., 2016).

The belowground microbiome of a forest comprises biotic entities present in various habitats ranging from soil, in close proximity to tree roots and within their rhizosphere, in litter, in deadwood, on the rock surfaces, inside rock clefts, etc. (Baldrian, 2017). Amongst all habitats, forest soils are known to regulate microbial behavior to a large extent. The spatial diversity of microorganisms of forest top soils is controlled by primarily two driving factors (1) soil and litter chemistry that regulate the primary metabolism of soil bacteria and fungi; and (2) the attributes of above-ground vegetation that alter the performance and efficiency of soil microorganisms (Tedersoo et al., 2016). Spatial variation and the relative effects of the above-mentioned two driving factors cause wide diversity in microbial abundance and activity, often restricting them to form "activity hotspots" comprising of the rhizosphere, the detritusphere and biopores which altogether regulate the soil ecosystem of vast expanses (Baldrian, 2014; Kuzyakov and Blagodatskaya, 2015). Soil microbiota comprises predominantly fungal and bacterial members. Fungal members belong to the ectomycorrhizal class (EMF), which forms almost one-third of the soil microbial biomass and adds to 50% of dissolved organic carbon (Ekblad et al., 2013). These EMFs not only supply nitrogen and phosphorus from the dead organic remains to their symbiotic hosts but also form communication networks with different tiers of vegetation. Besides, they also form mats and establish interaction with other saprophytic fungi, endobacteria belonging to the Alphaproteobacteria group, amoeba, nematodes, microarthropods, enchytraeids, etc., that influence the mycelial growth to a large extent (Bertaux et al., 2005; Anderson et al., 2014). Archaebacteria of class Thaumarchaeota, known to recycle nitrogen, are dominantly found in acidic soils with low ammonia (Levy-Booth et al., 2014). However, soil microorganisms exhibit variation across soil profiles, natural seasonal variation, as well as variation due to abrupt climatic eruptions referred to as "hot moments" of climatology and also variation due to anthropogenic disturbances (Kuzyakov and Blagodatskaya, 2015; Žifčáková et al., 2016). The saprophytic basidiomycetes solely or in combination with ascomycetes microfungi and bacterial members belonging to Proteobacteria and Bacteroidetes act as decomposers of the recalcitrant plant remains (Eichlerová et al., 2015). Deadwood, rock surfaces and rock clefts harbor cord-forming saprophytic basidiomycete decomposers, low pH tolerant bacteria, macrophages, commensal bacterial population, lignicolous lichens, and EMF members. These microorganisms aid in the decomposition and/or weathering process of organic matter or rock surfaces and deliver essential mineral nutrients to the host trees (Johnston et al., 2016). Metagenomic data of the forest soil samples from the Lipova region of the Czech Republic showed a predominance of Ectomycorrhiza (ECM), fungal basidiomycetes population, and acidophilic bacterial members are known to control nitrogen-supply mediated metabolical processes of aboveground vegetation (Chakraborty et al., 2023). However, the assemblage and activity of microorganisms of bulk soil and/or soil adjoining specialized habitats are exposed to constant modification during soil degradation, changing climatic conditions and throughout anthropogenic disturbances to provide resilience against any form of opposing selection pressure (Liu et al., 2019). On the contrary, the climatic alteration cause changes in above ground vegetation which in turn modify their root exudations of antimicrobials and/or semiochemicals to attract suitable microbes of the soil toward their rhizospheric zones and/or beyond to the endospermic regions to help the host trees tackle the stressful situation by triggering induced systemic responses (ISR) and recruiting antagonistic/parasitic approaches against pathogens and pests (Rolfe et al., 2019).

Apart from soil microbes, rhizospheric and endophytic microbes significantly promote plant growth and immunity to above-ground vegetation. Besides, they are known for their crucial role in manipulating belowground microbial assembly, building up "activity hotspots" and controlling ecological parameters of vast expanses (Baldrian, 2014; Kuzyakov and Blagodatskaya, 2015). Comparative studies carried out with temperate, boreal and Mediterranean forest trees revealed that apart from fungal members belonging to EM class, Ascomycetes and saprophytic Basidiomycetes, bacterial members belonging to Actinobacteria, Acidobacteria, Firmicutes, Bacteroidetes, Proteobacteria, etc. were found in the rhizosphere, endosphere, and root adjoining soil samples. All of this microbiota, in a concerted manner, played essential roles in nutrient acquisition, proper allocation, and recycling within host trees, thus helping maintain their vigor and vitality (Pettifor and McDonald, 2021). Detailed analyses conducted on the belowground microbiota and its effect on tree health using both conventional (community-based physiological fingerprinting methods) and advanced molecular approaches (epifluorescence-based assays and meta-omics techniques), explained multidimensional beneficial roles of belowground microorganisms (Mercado-Blanco et al., 2018). The mycorrhizal fungi [EMF and arbuscular mycorrhiza fungi (AMF)], the endophytic bacterial and fungal members and diazotrophs secrete different volatile organic compounds (VOCs), which altogether alter soil attributes for promoting soil-water-tree growth relations, increase soluble phosphate and availability of iron by synthesizing siderophores, increase root absorbing surface area by hundred to thousand manifold, control the invasion of pathogens and alien species, increase hormonally mediated tree tolerance toward both abiotic and biotic stressors, manipulate internal ROS levels to decrease self-decay, influencing primary and secondary metabolic signaling to balance optimum growth of host trees, etc. Besides, these microorganisms also account for properly restoring destroyed forest lands, upholding plant succession and maintaining above and belowground biodiversity (Rasmann et al., 2017; Mercado-Blanco et al., 2018). In addition, the belowground microbiome is also known to contribute to induced priming and inheritance of above-ground host plants (Tiwari et al., 2022). However, similar generalized interpretations in the case of forest trees are complex since plant-soil-microbe inter-chemistry is still not very clearly understood in different forest ecosystems. Moreover, studies on *Arabidopsis* spp. and other uncultivated species showed that belowground microbiomes controlled above-ground herbivores and cell-feeding pests through complex plant-soil-feedback (PSF) (Pineda et al., 2020). However, such PSF studies in forest backgrounds are still challenging due to the unavailability of growth and survival models of complex and bio-diverse microbial populations that are perpetually exposed to dynamic environmental factors. Considering the established role of the belowground microbiome, including soil, rhizosphere, and endospermic members in well-studied agricultural backdrops, it is time to think about how this information can aid in boosting tree resistance against biotic stresses, including beetle attacks. Preparation of beneficial soil microbial formulations for boosting tree health and stress resistance and testing them in the experimental forest can be a good starting point, and inspirations can be taken from agricultural set-ups (Naik et al., 2020; Tyagi et al., 2023). However, the primary task will be ensuring the survival and establishment of soil microbial inoculants in the forest (Kaminsky et al., 2019).

5.2. The role of above-ground microbiome and its potential for forest insect pests management

In the forest ecosystem, above ground microbiome comprises of foliar parts, stem and bark regions, and reproductive parts (Sieber, 2019). The foliar microbiome consists of beneficial and pathogenic fungi (epiphytic and endophytic), bacteria (pathogenic, commensal, and symbiotic) and invertebrates. All these organisms are known to help in hormone-mediated plant growth, nitrogen uptake, plant protection through antibiosis and control secretion of organic volatiles, thus regulating inter and intra-communication signaling and overall host metabolism. Studies on forest regions of temperate zones suggest that epiphytic fungi belonging to Ascomycetes and Basidiomycetes classes mainly colonize on foliar surfaces. These epiphytic fungi depend on host exudations, spores, pollens, organic aerosols, and honeydew released from sucking pests for their nutrition (Menkis et al., 2019). Besides, endophytic and pathogenic fungi also comprise a large portion of the foliar microbiome. However, the segregation between the endophyte and pathogen often becomes inconspicuous as many pathogenic fungi (for example, rust, mildew, and snow mold-forming fungi) remain as harmless endophytes for long periods, while many defined pathogens restrict themselves as local endophytes under unfavorable conditions (Schlegel et al., 2018; Sieber, 2019). The climatic conditions, location, precipitation amounts, host specificity, site specificity, host, and tissue age, interaction with other symbiotic and/or pathogenic entities and anthropogenic hindrances define the etiology of the endophyte/pathogen (Sieber, 2021).

In forests, the phyllosphere is inhabited by many bacterial members who reside in phylloplane and inside the phyllotelma region (Doan and Leveau, 2015). They belong to class Proteobacteria, Firmicutes, Acidobacteria, Actinobacteria, Firmicutes, Saccharibacteria, etc. (Doan and Leveau, 2015). The phyllosphere forms an extreme habitat for the bacterial population because of its variable phylloplane topology, trichome physiology, uneven nutrient distribution within its internal segments, and direct exposure to extreme environmental factors. The phyllosphere bacteria depend on minimal amounts of carbon, nitrogen, volatiles, etc., for nutrition (Vorholt, 2012). Some bacteria may be transitory, while others may be permanent residents (Suslow, 2002). However, the biodiversity of the bacterial population supporting selection or drift is driven by co-evolutionary parameters of both the phyllobiome and the host (Vacher et al., 2016). The phyllobacteria are known to influence host metabolism and fitness either by producing or by altering the production of host phytohormones IAA (indole acetic acid), cytokinins, ABA (abscisic acid), etc., which control host growth, transportation of nitrogen, and regulate the opening and closure of stomata thus controlling the entry of pest and pathogens (Holland, 2011; Munemasa et al., 2015; Romero et al., 2016). On the other hand, *Pseudomonas syringae* and many other epiphytic bacteria are known to emit bio-surfactants that form biofilm and help in their uniform distribution on the host phylloplane. Besides, they alter cuticle permeability and increase water and sugar availability, supporting their growth (van der Wal and Leveau, 2011). Besides, the phyllobacteria are also known to enhance the abiotic stress tolerance of the host, prime the host against biotic stressors and promote ISR-mediated resistance response (Schlechter et al., 2019). However, research regarding how these phyllofungi and phyllobacteria and their community dynamics shape host health is still being extensively performed (Gong and Xin, 2021).

In addition to the above, the microorganisms (fungi and bacteria) associated with reproductive structures are also known to contribute remarkably to transgenerational microbiome transfer (Ren et al., 2021). The microbiome (mainly endophytes) associated with reproductive structure may result from vertical transfer from the maternal parent or environment through horizontal transfer (Perlmutter and Bordenstein, 2020). Maternal effects of the reproductive microbiome are reported to impact offspring physiology and its relation with other abiotic and biotic factors, especially in choosing pollinators (Vannette and Fukami, 2016). It also helps in nullifying the ill effects of climatic change (Burgess and Marshall, 2014). Interestingly, the reproductive microbiome inherited through vertical transfer is also known to bring about epigenetic modifications such as histone methylation and modulate the expression of many stress-induced host tree genes (Vivas et al., 2015). In the case of horizontal microbiome transfer, the role of soil in transferring microbes to seeds, the role of sap-sucking and non-sap sucking insects and animal pollinators in introducing and/or relocating microbes to visiting floral parts as well as agents (wind, water, human interference, etc.) influencing the pollination appears to be very important in building up of tree holobiont (Ren et al., 2021).

Experimental studies involving multitrophic interaction comprising microbial pathogens, symbionts, higher-order pests, and predators showed that complex, stressful situations induced localized and systemic defensive responses in tree hosts and enhanced their metabolic performance compared to trees exposed to symbionts only (Saleem et al., 2017). This view was further supported by dysbiosis of *Pseudomonas* population during insect herbivory, which enhanced immune responses in *Cardamine hirsuta* (Humphrey and Whiteman, 2020). Such information at the level of above-ground microbial interactions with trees can be used to formulate strategies to boost tree resistance against pests and pathogens using microbes (Ajayi and Olufolaji, 2023). For instance, priming inducible defenses can protect Norway spruce (fungus-inoculated trees) against tree-killing bark beetles (Mageroy et al., 2020). However, more research endeavors toward delineating the role of microbes at tree holobiont in various forest ecosystems define the feasibility of such a management approach. A better understanding of the defense priming of forest trees and its impact on establishing beneficial microbial communities and *vice versa* will be an essential starting point for assessing the ecological implication of forest tree defense priming. Such knowledge is vital to employ defense priming as an effective pest management strategy in forestry.

6. Biological control of coleopteran forest pests: exploring the microbial toolbox

6.1. Entomopathogenic control of coleopteran beetles

Coleopteran forest insect pests cause economic damage and impact the environment globally. Chemical pesticides have been a primary measure for pest control for several decades. Extensive use of these pesticides and the constant evolutionary dynamics of insects have introduced pesticide resistance in target species and have killed several non-target beneficial insects (Usta, 2013; Bras et al., 2022). Therefore, integrated pest management (IPM) strategies have emerged to address these issues and promote environmentally friendly, economically viable, socially acceptable, and sustainable alternatives to managing insect pests (Dara, 2019). Classical biological control involves the introduction of living organisms such as parasitoids, predators, and entomopathogenic microbes to reduce pest populations (Eilenberg et al., 2001; Heimpel and Mills, 2017). Entomopathogenic microbes are the natural regulators that significantly control insect populations (Roy and Cottrell, 2008). Entomopathogens as pest regulators controlling insect pest populations efficiently and are environmentally safe for non-target organisms (Hajek and Bauer, 2009). Several invasive forest pests belonging to the order Coleoptera, Hemiptera, Hymenoptera, and Lepidoptera have been a target for management practices, including using microbes as a control agent.

Over the years, several entomopathogenic microbes, including viruses, bacteria, fungi, and nematodes, have been commercially produced and used augmentatively as microbial pesticides (Lacey et al., 2015). Baculoviridae has been commercially developed significantly among the different viral pesticides (Moscardi et al., 2011). For instance, the naturally occurring host-specific virus *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV) successfully controls the outbreak gypsy moth (*L. dispar* L.) population in the United States, Canada, and Germany (Boukouvala et al., 2022). Similarly, other nucleopolyhedroviruses are used to control forest pests, including balsam fir sawfly (*Neodiprion abietis* Harris), pine sawfly (*Neodiprion sertifer* Geoffroy), and Douglas-fir tussock moth (*Orgyia pseudotsugata* McDunnough) (Moscardi, 1999). Compared to viral pesticides, entomopathogenic bacteria are commercially less available (Jurat-Fuentes and Jackson, 2012). For example, *Bacillus thuringiensis* var. *kurstaki* (Btk) is commercially used worldwide to suppress defoliate forest insects and crop pest outbreaks (Hajek and Van Frankenhuyzen, 2017). However, suppressing the pest population is temporary and requires repeated application of Btk during the outbreak event (Hajek and Van Frankenhuyzen, 2017). Btk is extensively used to control forest pests, including spruce budworm (*Choristoneura fumiferana* Clemens); gypsy moth (*L. dispar*), and other lepidopteran forest defoliators (Polanczyk et al., 2017).

The phytophagous beetle *Chrysomela tremulae* Paykull is also susceptible to Btk, but large-scale field trials have not yet been conducted against these forest pests (Génissel et al., 2003). Other commercially used entomopathogenic bacteria include *Serratia entomophila*, *Paenibacillus* spp., and *Chromobacterium subtsugae* (Lacey et al., 2015). Besides entomopathogenic viruses and bacteria, fungal entomopathogens or mycoinsecticidal products from entomopathogenic fungi are widely used as microbial control agents for managing insect populations, including many bark beetle species belonging to genera *Dendroctonus*, *Ips*, and *Polygraphus* (Mann and Davis, 2021). Several fungal species, including *Beauveria bassiana, Metarhizium anisopliae, Hirsutella guignardii, Isaria farinosa,* and *Lecanicillium lecanii* are applied to manage bark beetle populations (Mann and Davis, 2021). These fungi species are considered generalist pathogens that are virulent against arthropods that occupy trees, vegetation, and forest soil. For instance, *Metarhizium brunneum* is deployed against Asian longhorn beetles in field trials in China and North America (Hajek and Bauer, 2009). Similarly, the isolate of *B. bassiana* is virulent against the EAB (Hajek and Bauer, 2009). These entomopathogenic fungi can be isolated from different sources and cultured to produce a large number of spores for potential commercial applications. In contrast to other entomopathogenic organisms that kill the insects after their entry through wounds or after ingestion, the entomopathogenic fungi infect the insect host by penetrating directly into their exoskeleton (Lacey et al., 2015). Furthermore, host-associated microbes from genera *Wolbachia* spp., *Arsenophonus* spp., *Cardinium* spp., *Rickettsia* spp., and *Spiroplasma* spp. often influence host reproduction by inducing cytoplasmic incompatibility parthenogenesis, feminizing genetic males, or male-killing (Lv et al., 2021). Using such reproductive manipulators may be an alternative strategy for controlling insect pests (Chakraborty and Roy, 2021).

Despite extensive research on entomopathogenic microbes as biological control agents, their large-scale commercial field application is limiting due to several abiotic and biotic factors, including susceptibility to ultraviolet light, low moisture, temperature, plant secondary metabolites, and competition with other microorganisms that impact their pathogenicity (Mann and Davis, 2021). The colonization of entomopathogens is achieved mainly by foliar spraying, injections, seed soaking, and soil drenching (Peña-Peña et al., 2015; Qayyum et al., 2015; Jaber and Araj, 2018; Rondot and Reineke, 2018). Direct spraying with entomopathogens or their products to the affected area is the most common and straightforward application practice that requires less equipment. Nevertheless, such an approach is not consistently successful in the field application against wood-boring insects due to their susceptibility to environmental conditions. Hence, adding protectants such as polymeric matrix, plant oils, clays, and humic acid increases the viability of the entomopathogenic microbes under laboratory and field conditions without compromising their pathogenic efficiency (Barta et al., 2018; Kaiser et al., 2019). However, spraying an entire forest to control pest infestation is not feasible. Alternatively, using an auto dissemination device to control pests is a promising strategy. The semiochemical baited traps connected to an auto-inoculative device attract insect pests and then contaminate them with the entomopathogen before returning to the environment to infect other pests horizontally or vertically (Dimbi et al., 2003; Ekesi et al., 2007). Such a dissemination approach can protect entomopathogens from UV exposure and rainfall (Srei et al., 2017). For instance, *B. bassiana* is introduced into a spruce bark beetle, *I. typographus* population, via an auto-inoculative device (Kreutz et al., 2004). Although this approach is reported to be successful against bark beetles, the studies are done on relatively smaller areas and for a shorter period. Hence, large-scale field applications need to be done to assess the feasibility of such a strategy further. Additionally, the formulations used should effectively kill all the developmental stages of the bark beetle. The formulation targeting only adults will not effectively control the beetle population unless they die before reproduction (Mann and Davis, 2021). Another approach is establishing entomopathogenic fungi as plant endophytes through colonization through seed and root coatings (Brownbridge et al., 2012). For example, studies reported that soil fungi, *B. bassiana*, colonize the pine trees via root and are pathogenic against invasive bark beetle species (*Hylastes ater* Paykull and *Hylurgus ligniperda* Fabricius) in New Zealand (Brownbridge et al., 2012).

Until recently, the identification of potential pathogens was limited to microscopy-based methods. With the advancement in genome sequencing and other molecular tools like RNA interference (RNAi), the construction of recombinant entomopathogenic microbial strains with enhanced virulence can be achieved (Chen et al., 2015; Wang et al., 2020). The advent of "omics" technologies can provide new insights into interactions among entomopathogens, host insects, and their host trees (Akinola and Babalola, 2020). Identifying new diverse host-specific entomopathogens and improvements in mass production, formulation, and their application will increase the commercial application of entomopathogens for efficient and sustainable use in pest management. Furthermore, deploying entomopathogens in combination with inorganic insecticides will help mitigate the risk of resistance and reduce environmental pollution caused due to excessive use of chemical pesticides (Al-Ani et al., 2021). An alternative strategy is integrating entomopathogens with semiochemicals and other natural enemies of insect pests that successfully manage insects (Sharma et al., 2019). A similar concept was recently used to protect agricultural and forest crops from the Arvicolinae rodents in Sweden (Villalobos et al., 2022). Although the application of entomopathogens or their products serves as a promising alternative to chemical pesticides, more research is still needed in this field to delve into their services thoroughly. Moreover, the policy and regulations for applying entomopathogens must be addressed apart from the laboratory and field trials.

6.2. Microbial volatile organic compounds as biocontrol agents

Like plants and animals, microbes also emit a plethora of VOCs in their metabolic processes (Bitas et al., 2013; Binyameen et al., 2021). These microbial volatile organic compounds (MVOCs) belong to different chemical classes, including alkenes, alcohols, ketones, benzenoids, pyrazines, sulfides, and terpenes (Kanchiswamy et al., 2015; Schmidt et al., 2015; Lemfack et al., 2018). The volatile compounds are perceived as chemical signals to communicate with each other, partaking in innumerable interactions and contributing significantly to multitrophic interaction (Schulz-Bohm et al., 2017). Insect chemoreception of microbial emissions plays a major role in insect-microbe interactions. Apart from the gustatory, tactile, and visual cues, insects have evolved complex chemosensory systems influencing their behavior. Insects are primarily sensitive to odors that endorse nutrient resources, predators, competitors, potential mates, and suitable habitats (Price et al., 2011; Davis et al., 2013). Although the ability of odor to influence animal behavior is long studied, relatively little is known about MVOCs as behavioral cues. MVOCs have been shown to influence insect behavior, such as stimulating insect oviposition, signaling food sources, and attracting or repelling insects (Davis et al., 2011). Furthermore, insects possess pheromones that are emitted by or associated with microbial symbionts (Tillman et al., 1999; Wertheim et al., 2005). Studies reported that gut symbionts could produce compounds used as pheromones by their hosts (Hunt and Borden, 1990; Zhao et al., 2015). For instance, yeasts, *Hansenula capsulata* Wickerham, and *Pichia pini* (Holst) Phaff are associated with the mountain pine beetle, *D. ponderosae*. They can convert *cis* and *trans*-verbenol to the anti-aggregation pheromone verbenone in female beetles (Frühbrodt et al., 2023). The anti-aggregation pheromone signals subcortical population density and terminates aggregation and mass attack on host trees (Hunt and Borden, 1990). Mycangial fungi and yeast *Candida nitrophila* play a similar role in the southern pine beetle and spruce beetle, respectively (Brand et al., 1976; Leufvén et al., 1984). Nevertheless, the mutualistic fungus, *Grosmannia clavigera*, associated with *D. ponderosae* produces sterols that act as a precursor for the biosynthesis of aggregation pheromones by the females (Bentz and Six, 2006). Interestingly, fungal volatiles also increased the attractiveness of host-mimic volatiles to the invasive ambrosia beetle, serving as insect lures. Despite the importance of microbes in insect life, combining chemical, and microbial ecology in entomological research is rarely explored. Integrating these approaches in entomological research, harnessing the MVOCs, and targeting these candidates is a promising avenue for controlling insect pests (Baig et al., 2023). Currently, less than 10% of MVOCs have been functionally described (Lemfack et al., 2018), suggesting the immense potential of MVOCs in sustainable pest management.

Furthermore, bark beetle-associated ophiostomatoid fungi release many volatile compounds such as terpenoids, aliphatic alcohols, fusel alcohols, and aromatic compounds, which positively or negatively influence the behavior of bark beetle, *I. typographus* (Kanchiswamy et al., 2015; Kandasamy et al., 2016; Tanin et al., 2021). Hence, they can potentially be used in bark beetle control (**Table 1**). For instance, studies revealed that the symbiotic fungi (ophiostomatoid fungi such as *Grosmannia europhioides, Endoconidiophora polonica*, *Grosmannia penicillata*, *Ophiostoma bicolor*, and *Ophiostoma piceae*) of *I. typographus* modify the spruce bark volatile profiles by converting the major spruce monoterpenes into a blend of oxygenated derivatives that are more attractive to bark beetles (Kandasamy et al., 2023). Other interesting fungal associates of bark beetles from the genera *Geosmithia* spp. (e.g., *Geosmithia morbida*), and *Fusarium* spp. (e.g., *Fusarium circinatum)*, might serve as a nutrient source for the beetles and exhibit antimicrobial activity, but their role in ecological implications is unknown (Teetor-Barsch and Roberts, 1983; Bezos et al., 2018; Zhang et al., 2022). Interestingly, bark beetle species vector primarily either *Geosmithia* spp. or *ophiostomatoid* fungi, but not both. The less water content of the tree substrate facilitates *Geosmithia* association (Williams and Ginzel, 2021). Several independent studies have proven *Geosmithia* species as stable and dominant symbionts of many bark beetles worldwide, as described thoroughly in a recent comprehensive review article (Kolaøík and Hulcr, 2023). Interestingly, *Geosmithia* is an ecological complement to the *ophiostomatoid* fungi and co-evolved with bark beetle vectors (Kirschner, 2001). However, the molecular basis underlying *Geosmithia*–bark beetle symbiosis and the potential of volatile compounds (MVOCs, **Table 1**) identified from *Geosmithia* for bark beetle management demands further investigation (Blood et al., 2018; Kolaøík and Hulcr, 2023).

The MVOCs, recognized by beetles to aid in host finding, are currently being tested along with aggregation pheromones in the forests in Czechia. In this study, the fungal volatile and aggregation pheromone used in the dispensers worked synergistically to attract *I. typographus* (**Figure 4**; Jirošová et al., 2022). However, such preliminary observations must be further experimentally validated by performing similar field experiments across different forest locations under different forest management regimes. It is worth mentioning here that beetle symbiosis is very complex, and the behavior of the bark beetles varies depending on the bacterial or fungal species, host tree, and stage of the beetle life cycle (Popa et al., 2012; Briones-Roblero et al., 2017; Netherer et al., 2021). Further research on the bark beetle symbiosis by taking advantage of affordable omics and electrophysiological methodologies will facilitate the deployment of MVOCs as management tools. Identifying and characterizing next-generation semiochemicals from microbial origin, their emission pattern, and the influence of target forest pests, including bark beetles, will ensure the sustainability of the pest management products based on MVOCs. It is interesting to point out that the influence of such MVOCs on the natural enemies of the coleopteran insect pests needs to be evaluated for their greater exploitation for safeguarding conifer and other broadleaved forests. Nevertheless, local laws and the government must approve the ecological consequences and environmental risks of introducing fungal or bacterial cultures and other potential pathogenic microbes into the forest ecosystem via traps and dispensers. Deploying fungal MVOCs, in general, may avoid these issues as they can be used in pheromone dispensers similar to the field trials in Czechia. Such deployments of MVOCs as attractants or deterrents (i.e., push and pull strategy) alone or in combination with other types of existing semiochemicals could substantially improve our chances against forest pests.

7. Molecular control of coleopteran insect pests: a new age technological development

7.1. Targeting coleopteran forest insect pest symbionts using RNAi

A surge of recent studies highlighted the contribution of the symbiotic microbiome underlying insect adaptations, ecological niche expansion, and invasion. However, symbionts

TABLE 1 A list of fungal volatile organic compounds (VOCs)* from different coleopteran beetles that can be applied for management practices.

Coleopteran pest	Fungal associates	Name of the substances	References
Family: Nitidulidae			
Carpophilus humeralis (Fabricius)	*Candida shehatae, Candida guilliermondii*	Ethanol, acetaldehyde, 2-methyl-1-propanol, 1-propanol, ethyl acetate, 3-methyl-1-butanol, 2-methyl-1-butanol, 3-hydroxybutanone	Nout and Bartelt, 1998
Carpophilus hemipterus (Linnaeus), *Carpophilus humeralis* (Fabricius), *Carpophilus lugubris* (Murray)	*Saccharomyces cerevisiae*	Acetaldehyde, ethanol, 1-butanol, 1-propanol, 2-methyl-1-propanol, 2,3-butanediol, 2-methyl-1-butanol, 3-methyl-1-butanol, ethyl hexanoate, acetoin, ethyl octanoate, acetic acid, ethyldecanoate, 2-phenylethanol, ethyl acetate, isopentyl acetate	Lin and Phelan, 1991; Phelan and Lin, 1991; Nout and Bartelt, 1998
Carpophilus lugubris (Murray)	*Candida krusei*	Not determined	Blackmer and Phelan, 1991
Family: Ciidae			
Cis bilamellatus (Wood), *Cis boleti* (Scopoli), *Cis nitidus* (Fabricius), *Octotemnus glabriculus* (Gyllenhal)	*Coriolus versicolor, Ganoderma adspersum, Piptoporus betulinus*	1-Octen-3-ol, (R)-curcumen, α-cedrene	Guevara et al., 2000
Family: Cantharidae			
Malthodes fuscus (Waltl)	*Fomes fomentarius, Fomitopsis pinicola*	Rac-1-octen-3-ol	Jonsell and Nordlander, 1995
Family: Curculionidae			
Dendroctonus frontalis (Zimmermann)	*Endoconidiophora polonica, Grosmannia clavigera, Grosmannia penicillata, Grosmannia europhioides, Ophiostoma bicolor, Ophiostoma piceae, Ophiostoma minus, Ophiostoma ips, Leptographium abietinum*	Isoamyl alcohol, isoamyl acetate, 2-phenylethanol, 2-phenylethyl acetate	Brand et al., 1977; Sullivan et al., 2007
Dendroctonus frontalis (Zimmermann), *Dendroctonus ponderosae* (Hopkins), *Dendroctonus rufipennis* (Kirby), *Ips typographus* (Linnaeus)	*Grosmannia europhioides, Ophiostoma bicolor, Ophiostoma piceae, Ophiostoma minus*	1-Hexanol	Dickens et al., 1992; Borden et al., 1998; Poland et al., 1998; Zhang et al., 1999
Dendroctonus brevicomis (LeConte), *Dendroctonus ponderosae* (Hopkins), *Dendroctonus rufipennis* (Kirby), *Ips pini* (Say)	*Grosmannia penicillata, Ophiostoma piceae, Ophiostoma ips*	Benzyl alcohol	Borden et al., 1998; Huber et al., 2001
Dendroctonus ponderosae (Hopkins)	*Hansenula capsulata, Pichia pinus*	Verbenone	Hunt and Borden, 1990
Dendroctonus frontalis (Zimmermann)	*Hansenula holstii*	3-Methyl-1-butanol; 3-methylbutyl ethanoate, 2-phenylethanol, 2-phenylethyl acetate	Brand et al., 1977
Dendroctonus armandi (Tsai & Li), *Pityogenes bidentatus* (Herbst)	*Grosmannia penicillata*	(E)-β-Caryophyllene	Byers et al., 2004; Zhang et al., 2010
Dendroctonus ponderosae (Hopkins), *Dendroctonus rufipennis* (Kirby), *Ips pini* (Say)	*Ophiostoma ips*	Acetophenone	Pureswaran et al., 2000; Pureswaran and Borden, 2004
Dendroctonus ponderosae (Hopkins)	*Endoconidiophora polonica, Grosmannia clavigera, Grosmannia penicillata, Grosmannia europhioides, Ophiostoma bicolor, Ophiostoma piceae, Ophiostoma minus, Ophiostoma ips, Leptographium abietinum*	2-Phenylethanol	Pureswaran et al., 2000

(Continued)

TABLE 1 (Continued)

Coleopteran pest	Fungal associates	Name of the substances	References
Dendroctonus brevicomis (LeConte), *Dendroctonus frontalis* (Zimmermann), *Dendroctonus pseudotsugae* (Hopkins)	*Ophiostoma ips*	Acetophenone	Pureswaran and Borden, 2004; Erbilgin et al., 2008
Ips typographus (Linnaeus)	*Endoconidiophora rufipennis*	Isoamyl acetate, sulcatone, 2-phenethyl acetate, geranyl acetone, geranyl acetate, citronellyl acetate, (*R*)- and (*S*)-sulcatol, (*R*)-sulcatol acetate	Lindmark et al., 2023
	Endoconidiophora polonica	3-Methyl-1-butyl acetate, 2-methyl-1-butyl acetate, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-phenylethanol, 2-phenylethyl acetate	Kandasamy et al., 2019
	Grosmannia penicillate, Grosmannia europhioides	2-Methyl-3-buten-2-ol	Zhao et al., 2015
Ips subelongatus (Motschulsky)	*Endoconidiophora polonica, Ophiostoma bicolor, Grosmannia clavigera*	Geranyl acetone	Zhang et al., 2007
Xyleborus ferrugineus (Fabricius), *Xyleborus glabratus* (Eichhoff)	*Ambrosiozyma* sp.	Not determined	Hulcr et al., 2011
Xyleborus glabratus (Eichhoff)	*Raffaelea lauricola*	4,5-Dimethyl-1-hexene, 7 ethylidenebicyclo [4.2.1] nona-2,4-diene, 4-ethenylidene-6,6-dimethylbicyclo [3.1.1]heptane, 8-methylene bicyclo[4.2.0]oct-2-ene	Simon et al., 2017
Xylosandrus crassiusculus (Motschulsky), *Xyleborinus saxesenii* (Ratzeburg)	*Ambrosiella xylebori, Raffaelea lauricola*	Not determined	Hulcr et al., 2011
	Grosmannia clavigera, Ophiostoma ips, Ophiostoma montium, Leptographium longiclavatum	Acetoin	Cale et al., 2016
	Ophiostoma ips	Ethyl cinnamate, ethyl benzoate, 2,3-dihydrobenzofuran, methyl cinnamate	Kandasamy et al., 2016
	Grosmannia clavigera, Ophiostoma ips	Verbenone	Cale et al., 2019
Hylobius abietis (Linnaeus)	*Penicillium expansum*	3-Methylanisole	Azeem et al., 2013
Pityophthorus juglandis (Blackman)	*Geosmithia morbida*	Not determined	Blood et al., 2018

*More information on VOCs was summarized in the following reviews: Davis et al. (2013), Kandasamy et al. (2016), and Cale et al. (2019).

can constrain the evolutionary potential by fostering niche specialization; for instance, symbiont-aided detoxification of caffeine by the gut bacteria (*Pseudomonas fulva*) enables coffee berry borer beetles (*Hypothenemus hampei* Ferrari) (Coleoptera: Curculionidae: Scolytinae) to thrive on unique caffeine-rich coffee beans (Ceja-Navarro et al., 2015). There are copious examples where symbionts aided their insect hosts to detoxify entomotoxic compounds from plant origin from different insect orders (Hosokawa et al., 2007; Xia et al., 2017; Cheng et al., 2018). It is also documented that detoxifying symbionts confer pesticide resistance in pest insects (Kikuchi et al., 2012; Cheng et al., 2017; Bras et al., 2022). In the case of forest insect pests (Coleoptera), symbiont-mediated detoxification of monoterpenoids such as limonene or diterpene acids is also reported. Mountain pine beetle (Coleoptera: Curculionidae), causing catastrophic damage to pine forests (*Pinus contorta* and hybrid *P. contorta* × *Pinus banksiana*), showed high expression of genes from the microbial origin involved in diterpene degradation after shotgun DNA sequencing (Adams et al., 2013). Most of these genes for diterpene degradation originated from bacterial genera *Serratia*, *Erwinia*, *Rahnella*, and *Pseudomonas* under Gammaproteobacteria and genus *Burkholderia* under Betaproteobacteria (Adams et al., 2013). Symbionts-mediated terpenoid degradation was also documented in the *H. abietis* (L) (Coleoptera: Curculionidae) based on *in vitro* and *in vivo* investigations (Berasategui et al., 2016, 2017). *Serratia, Erwinia*, and *Rahnella* were found within the core bacterial community in *H. abietis* (L). Interestingly, a gut bacteriome study on conifer-feeding bark beetles from the genus *Ips* (Coleoptera; Curculionidae; Scolytinae) also documented the same bacteria (*Serratia, Erwinia*, and *Rahnella*) within the core microbial community (Chakraborty et al., 2020a). Hence, it can be predicted that these symbiotic microbes perform a conserved function, i.e., detoxification of host allelochemicals, in conifer-feeding insect pests.

FIGURE 4
Using MVOCs in bark beetle (Coleoptera: Curculionidae: Scolytinae) management. A typical way of trapping bark beetles in the forests by using pheromone traps comprising fungal VOCs with or without beetle pheromones. Using MVOCs could enhance bark beetle capture substantially.

There are also reported symbionts offering defensive functions for their insect hosts by protecting against pathogens. For instance, filamentous actinobacteria are documented as defensive symbionts in many insects due to their capacity to produce secondary metabolites with antibiotic properties (Kaltenpoth, 2009; Seipke et al., 2012; Qin et al., 2017; Rupawate et al., 2023). Symbiotic bacteria (*Streptomyces* sp.) in southern pine beetle (*Dendroctonus frontalis* Zimmermann) (Coleoptera, Curculionidae) produce antifungal compounds against antagonistic fungi, *Ophiostoma minus* (Scott et al., 2008). Furthermore, microbial symbionts can protect their insects even against natural enemies (Flórez et al., 2017; Wang and Rozen, 2018; Muhammad et al., 2019).

With the increase in the information about detoxifying and defensive symbionts in pest insects, including forest insect pests (Coleoptera), it is necessary to use that information to formulate novel species-specific pest management opportunities. With increasing genomes available for major forest pests (Powell et al., 2021; Gagalova et al., 2022; Keeling et al., 2022) and microbial genome sequencing advancements, the feasibilities for conducting in-depth symbiosis studies, i.e., metatranscriptomic and metaproteomic level, increased manifold. Such advanced studies can delineate the metabolic exchange between beetles and their symbionts (Liu et al., 2022; Salem and Kaltenpoth, 2022) and offer exciting targets for future forest insect pest management via molecular tools such as RNAi, a post-transcriptional gene silencing mechanism suppressing protein formation by introducing environmental RNA (Montgomery and Fire, 1998). Technically, dsRNA can knock down key functional genes from symbionts to intrude symbiosis, making the target pest more susceptible to plant toxins or natural enemies (Chung et al., 2018). RNAi targeting symbiont genes is an intriguing idea worth dedicated research efforts (Wang and Luan, 2023). However, there is a significant concern about the environmental stability and biosafety of RNAi-based wood-boring pest control products (Joga et al., 2021; Mogilicherla et al., 2023; Singewar and Fladung, 2023). Although the contribution of symbionts in the evolution of bark beetles is unquestionable, targeting the symbionts inside the beetles, completing most of their life underneath the bark, is challenging and needs some intelligent strategy. Developing a sophisticated, eco-friendly, species-specific delivery system for dsRNA to reach its target will be crucial. Protection of dsRNA can be obtained by coating it inside nanostructures such as Chitosan, Carbon Quantum Dot, Silica nanoparticles, BioClay, etc. (Das et al., 2015; Pugsley et al., 2021; Jain et al., 2022; Sandal et al., 2023). Recent studies also documented the development of catechin, poly-L-lysine, for optimizing dsRNA delivery against target pests (Dhandapani et al., 2021; Yan et al., 2021). Such biodegradable nano formulations open a possibility of safe and efficient delivery of dsRNA to targets and often give protection against dsRNases (i.e., cationic polymers) inside the pest. However, spraying the nanoparticle-coated dsRNA is undoubtedly not the most favorable option in forestry applications at the landscape level.

Interestingly, using microbes (i.e., bacterial minicells) as dsRNA carriers and for mass production seems a promising alternative to pesticides (Guan et al., 2021; Whitten et al., 2023; Xue et al., 2023). Unfortunately, investigation on these vesicles for dsRNA delivery is limited; however, specific companies, e.g., AgroSpheres,[1] delve into such technology for dsRNA or siRNA delivery for agricultural field applications. Another attractive option might be the co-expression of viral-like particles (VLPs) (Xue et al., 2023) and dsRNA inside the bacterial cell to use in the forest or isolate dsRNA-containing particles using the capacity of VLPs to self-assemble *in vitro*. Replicating engineered viruses permit superior cellular uptake and protection of the dsRNA in the extracellular environments in the forest (Kroemer et al., 2015; Sun, 2015; Xue et al., 2023), but after the COVID-19 pandemic, the approval of such alternatives might encounter hurdles to receive societal acceptance.

7.2. Symbiont-mediated RNAi: two-tier specificity for enhanced coleopteran forest insect pests control

A novel way to exploit the symbiotic associations for managing forest pests (Coleoptera) is symbiont-mediated RNAi (SMR) (Whitten et al., 2016). Strategies like feeding and injections are labor-intensive, posing a significant limitation for practical application. Alternatively, expressing dsRNA targeting specific pest insects inside recombinant bacteria or yeast lacking RNase III activity is a rewarding alternative when applied to the food source (Tian et al., 2009; Zhang et al., 2019; Ma et al., 2023). SMR is an advancement of this approach using symbiotic microbes as a carrier for the species-specific dsRNA delivery against target pests (Whitten et al., 2016; Xue et al., 2023). SMR has selective advantages over other strategies. Recombinant bacteria can fail to establish themselves after entering the target pest as they are alien inside the insect body (i.e., gut), and the possibility of inducing an immune response is also high. This limits the sustainable application as a minimum bacterial cell number is required to stimulate a decent RNAi response inside target pests. SMR technology will not have such issues. After establishment, it helps continuous dsRNA synthesis inside the pest, eliminating the necessity for repeated application for longer living pest insects because the amplification of interfering RNA is not possible due to the lack of RNA-dependent RNA polymerases (RdRP) in an insect leading to transient RNAi (Whitten and Dyson, 2017). It also facilitates the escape from dsRNA degrading enzymes expressed in saliva and gut of many pest insects based on the localization of SMR bacteria, or the dsRNA produced continuously by symbionts can overwhelm the activities of dsRNA degrading enzymes. There are already documented successes for SMR against short and long-lived insects (Whitten et al., 2016; Whitten and Dyson, 2017). Furthermore, SMR can be transmitted horizontally to other members, i.e., engineered *Rhodococcus rhodnii* expressing dsRNA transmitted horizontally to other kissing bugs via ingestion of feces (Whitten and Dyson, 2017).

Species specificity of RNAi is one of the key advantages of RNAi over other pest control strategies. SMR technology further complements it as it enhances the specificity of RNAi. SMR introduces two-tier specificity for RNAi; the first tier is from the species-specific target dsRNA design, and the second is by selecting the symbiont specific to the target pest. The inefficiency of true symbionts outside the environment also minimizes the concern for biosafety issues and their effect on non-target organisms. Although SMR is intriguing, some key issues must be alleviated before taking it to forest application (Elston et al., 2023; Whitten et al., 2023). Understanding the true nature of insect-symbiont relationships is fundamental for the success of SMR, as the optimal selection of the symbiont for delivery of dsRNA is the key to success. The information about the symbiont's colonization potential (Engel and Moran, 2013), cell number per insect and association with different insect life stages is cardinal for the success of SMR technology. It is worth mentioning that holometabolous insects (e.g., coleopterans, dipterans, and lepidopterans) undergo a metamorphosis, implying the loss and re-acquisition of symbionts that can limit the success of SMR. However, with coleopteran forest pests, this might not be an issue. The issue here might be the lack of studies on forest pest symbiosis. For instance, only a few studies revealed the gut microbial assemblage of bark beetles (Chakraborty et al., 2020a,b; Cheng et al., 2023; Moussa et al., 2023). More in-depth studies are necessary at the functional level, such as metatranscriptomics or metaproteomics, to understand the molecular basis of symbiosis within forest pests (Salem and Kaltenpoth, 2022). Identifying symbionts with the possibility of *in vitro* culturing (Liu et al., 2022) and manipulation is fundamental. The advent of genome sequence data for a wide range of pest insects and their symbionts undoubtedly facilitates in-depth studies on symbiosis. However, more genome sequencing efforts for forest pests (Powell et al., 2021; Gagalova et al., 2022; Keeling et al., 2022) and their symbionts (Liu et al., 2022; Cheng et al., 2023) are required to

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[1] https://www.agrospheres.com/

develop SMR as a forest pest management tool in future. Another major limitation of SMR is that often true symbionts are due to genome reduction as a consequence of symbiotic tie-ups with their insect host (McCutcheon and Moran, 2012; Boscaro et al., 2017; Noh, 2021); hence they are not culturable and cannot be engineered to express dsRNA. The technological development in this area is impending and crucial for SMR against forest pests.

8. Understanding of holistic tree-beetle-microbe dialogue for futuristic IPM

The rapid advancement of sequencing methodologies has enormously increased microbiology research in the past few decades. However, a vast number of the studies (approximately 50%) lack adequate details of experimental design (information source: ESA 2020 conference presentation, titled: A synthesis of insect-microbiome research). Thus, the requirement for standard norms or protocols of metagenomic, metatranscriptomics, and metaproteomic studies that can ensure the reliability and reusability of the sequencing data is crucial. Besides, depositing the obtained data in the appropriate public data repository is also essential. Above all in-depth understanding of the plant holobiont, beetle holobiont, and/or "plant-beetle-microbiome-environment" interaction pyramid for protecting and improving plant performance and managing beetle destruction is undoubtedly critical. In addition, analytical evaluations of factors that are likely to cause variation in data generation, such as nucleic acid extraction method, sequencing platform used, primer pairs used (i.e., 16S, ITS, and 18S studies) in microbial omics study, insect handling procedure and material (whole body/gut, surface-sterilized or not) and ecological factors such as phylogeny, sex, life stage, diet, treatment, origin, etc., that are known to influence the microbial community of insects, need to be sufficiently described. Hence, mentioning the above information in the published report is a prerequisite for accurately understanding the symbiosis mechanism and using the information for management purposes. The lack of good-quality bacterial and fungal databases for microbial studies at a functional level is also considered a void in forest-beetle-microbiome research.

Technological innovations such as SMR for the environmental application of RNAi using symbiotic microbes as carriers (Zhang et al., 2019) appear promising. Hence, bio-engineered microbes could be applied in the forest for pest control and tree health development in the near future. While we are at the onset of such intriguing possibilities, the biosafety aspect for the short- and long-term environmental impact of deploying such genetically engineered microbes must be formulated under a defined regulatory framework and evaluated in forest habitation. Although risk assessment protocols exist for RNAi-based GM crops or RNAi-based biocontrol products (De Schutter et al., 2022), no published risk assessment protocol for GM microorganisms obtained through targeted delivery of dsRNA has been reported. Thus, certified regulatory agencies must formulate appropriate risk assessment protocols for forest microbiome research. Such protocols should also incorporate the short and long-term impact of GM microbes on the forest ecosystem.

9. Conclusion

Even though safer GM microbes hold an excellent perspective of the application instead of utilizing synthetic pesticides in agricultural ecosystems, translation of the knowledge base from agricultural fields to the forest is challenging because of landscape enormity. Besides, knowledge of plant-soil feedbacks that largely shape the soil and host microbiome in the forest is lacking. Steering soil microorganisms in forest floors to assemble all good things may be practically attainable only after extensive in-depth experimentation. Thus, exploring and exploiting the role of holobionts of forest tree hosts and their associated beetle pests and implementing targeted bioengineering of beetle-associated symbiotic microorganisms within the regulated legal framework could help devise better management stratagem for tackling the problem of Coleopteran insect pests-mediated forest destruction in future.

Author contributions

AR and AC: conceptualization. SG, AC, and AR: manuscript writing and figure preparation. All authors approved the final version of the manuscript.

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Conflict of interest

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

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A monitoring network for the detection of invasive ambrosia and bark beetles in the Czech Republic: principles and proposed design

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Invasive bark beetles pose a threat to native biodiversity and to functional ecosystems and the economic productivity of forests, parks, and orchards. In the Czech Republic, there are six species of invasive ambrosia and bark beetles with a stable natural population, and it can be assumed that other invasive species that will be found. In the Czech Republic, there are no guidelines or methods for the early detection of invasive ambrosia and bark beetles. We propose monitoring at a total of 24 locations considering the following: (i) monitoring approaches used in other countries; (ii) identified entrance gates of invasive ambrosia and bark beetles found in the Czech Republic; (iii) presumed invasive species that occur in surrounding countries and are expanding their range; (iv) substances attractive to all the above mentioned species; (v) commonly available traps; and (vi) minimization of operating costs. Most of the chosen locations are located on the state borders and in river valleys, which are probably the entrance gates to the Czech Republic for invasive ambrosia and bark beetles. In addition, two large timber warehouses where international trade takes place, all international airports and three botanical gardens with tropical greenhouses were selected. Three Theysohn or Ecotrap impact traps should be installed every year at all locations. Traps should be baited with ethanol and exposed from mid-April to the end of July and should be checked every 2 weeks.

KEYWORDS

Cyclorhipidion bodoanum*, *Dryocoetes himalayensis*, *Gnathotrichus materiarius*, *Phloeosinus aubei*, *Xyleborinus attenuatus*, *Xylosandrus germanus

1. Introduction

Invasive ambrosia and bark beetles (further BB) represent a threat to biodiversity, functional ecosystems, and the economic productivity of forestry (Brockerhoff et al., 2006; Aukema et al., 2011; Gohli et al., 2016), as well as to parks and orchards (Francardi et al., 2017; Branco et al., 2019; Fiala et al., 2022). BB are important vectors of fungal diseases that cause massive tree death. The simultaneous effect of several invasive species, their symbiotic fungi, and the subsequent interaction with climate change creates a situation in which it is difficult to predict the future impact of ambrosia and bark beetles on the environment (Lovett et al., 2013). Early detection is key to controlling BB because only then can a real integrated pest management (IPM) strategy be developed (Brockerhoff et al., 2006, 2010; Douglas et al., 2009; Samons, 2022).

Bark beetles spread in several ways, the most common being the global trade in wood material (treated and untreated wood), wooden packaging, and fruits or live seedlings of various non-native trees (Mathew, 1987; Meissner et al., 2008; Pombo et al., 2010; Augustin et al., 2012; Brockerhoff and Liebhold, 2017; Meurisse et al., 2019). It has also been confirmed that they can be introduced with wooden material that has been treated according to the international standard ISPM 15 (Haack and Petrice, 2009; Haack et al., 2014). In Europe, ports on the Atlantic and Mediterranean coasts are most often the gateway (Hagedorn, 1910; Hoffmann, 1942; Schedl, 1962; Cola, 1971, 1973; Faccoli, 2008; Moraal, 2010; Inghilesi et al., 2013; Rassati et al., 2015; Binazzi et al., 2019; Branco et al., 2019; Barnouin et al., 2020). Another entry point is botanical gardens, where non-native ambrosia and bark beetles may be introduced when expanding collections of exotic trees (Chobaut, 1897; Merkl and Tusnádi, 1992; Schuler et al., 2023).

Due to climate change, the host tree species are spreading northwards into areas where they did not originally occur (Ge et al., 2017). Even ambrosia and bark beetles, which are only found in southern Europe, may spread north; e.g., the bark beetle *Phloeosinus aubei* Perris, 1855 has spread to colder areas in Central Europe (Fiala and Holuša, 2019). Ambrosia and bark beetles not only spread through global trade but also naturally, as some are good flyers (Nilssen, 1984; Jones et al., 2019). Dry summers contribute to the appearance of ambrosia and bark beetles in alpine locations, even though they do not normally ascend to high altitudes, also (Marini et al., 2012).

However, the influence of humans on the spread of BB is far greater than the influence of climate (Gohli et al., 2016; Ward et al., 2019). Establishing plantations of non-native trees increases the risk of introducing non-native ambrosia and bark beetles (Lantschner et al., 2017). In Central Europe, this mainly concerns the cultivation of black pine (*Pinus nigra*) and bark beetles, which feed on it; *Pityogenes bistridentatus* Eichhoff, 1878 and *Orthotomicus robustus* Knotek, 1899 are found in several areas in the Czech Republic (Pfeffer and Knížek, 1996; Urban, 2000; Knížek, 2006; Knížek and Mertelík, 2017; Fiala et al., 2022). Climate change may help the maintenance of populations of BB on continents (Rassati et al., 2016a).

Most ambrosia and bark beetles are native to temperate and subtropical forests, so they represent the greatest danger for southern Europe due to a similar climate; hence, damage is most concentrated here (Pennacchio et al., 2004, 2012; Alfaro et al., 2007; Francardi et al., 2017; Leza et al., 2020). In the more northern countries of Europe, only damage by the ambrosia beetle *Xylosandrus germanus* Blandford, 1894 has been recorded (Maksymov, 1987; Graf and Manser, 2000; Galko et al., 2019).

Due to the economic and ecological damage caused by ambrosia and bark beetles, some governments perform regular monitoring of BB in their territory. This is helpful for identifying risk in a timely manner. There have been several monitoring attempts, of which baited traps are the most effective and least expensive method (Poland and Rassati, 2019).

Since BB are spreading increasingly around the world, there have also been efforts to introduce global monitoring. Observations were made on several continents at the same time to determine the abundance of ambrosia and bark beetles in the affected regions. The following semiochemicals were used in the traps: α-pinene + ethanol and α-pinene + ethanol + ipsdienol + ipsenol + Z-verbenol. The study is the first step toward the development of an international monitoring protocol based on trapping in traps baited with different types of substances (Faccoli et al., 2020).

There are six species of BB in the Czech Republic with a stable population in the wild (Knížek, 1988; Procházka et al., 2018; Fiala and Holuša, 2019; Fiala et al., 2020, 2021), and other species can be expected to occur in this territory (Gebhardt, 2014; Gebhardt and Doerfler, 2018). In the Czech Republic, there are no guidelines or methods for the early detection of BB. In addition, approximately half of the records of new species of ambrosia and bark beetles for the Czech Republic were accidental; the species were caught by amateur entomologists, and there was a delay of approximately 1–3 years between detection and publication (cf. Knížek, 2009a,b, 2011; Knížek and Kopecký, 2021). An extreme example is a report published 18 years after the species *Pityophthorus balcanicus* Pfeffer, 1940 was captured (Knížek and Liška, 2015). Therefore, it is necessary to create a stable network of traps for monitoring invasive species of ambrosia and bark beetles. To determine the methodology, several experiments were carried out in the Czech Republic, providing basic knowledge about the spread of BB and their bionomics in the Czech Republic (Fiala and Holuša, 2019, 2020; Fiala et al., 2020; Holuša et al., 2021; Fiala et al., 2023).

The aim of this work is to propose a methodology for monitoring BB based on the following:

(i) monitoring approaches in other countries;
(ii) the entrance gates of the existing species of BB found in the Czech Republic;
(iii) presumed species that occur in surrounding countries and are expanding their range;
(iv) substances attractive to all of the above;
(v) commonly available traps;
(vi) minimization of operating costs.

1.1. Monitoring methods in North America

In Canada, the first attempts to detect BB were made at the end of the 1990s in the vicinity of Vancouver. The following substances were used for trapping: ethanol, α pinene, and attractants (*cis*-verbenol, ipsdienol, and methylbutenol) for *Ips typographus* Linnaeus, 1758 (Humble, 2001). Ethanol and α-pinene are kairomons for many ambrosia and bark beetles (Schroeder and Lindelöw, 1989). After that, long-term monitoring began, and was carried out in the period from 2000 to 2021. Each year between 2000 and 2011, six Lindgren funnel traps were installed at each of 63–80 locations (ports, industrial zones, and wood processing industries). Traps at each location included three baited with ethanol + α-pinene and *cis*-verbenol + ipsdienol + methylbutenol and three baited with ethanol alone. Since 2012, another trap baited with ethanol + C6-ketol + C8-ketol as aggregation pheromones have been added to longhorned beetles (see Hanks et al., 2019). Since 2015, traps for longhorned beetles have been baited with the combination of racemic (E,Z)-fuscumol + racemic

(E,Z)-fuscumol acetate + ethanol and the combination of ipsenol + monochamol + α-pinene + ethanol. During the experiment, seven species of BB were captured, of which three species were new to Canada (Thurston et al., 2022).

The most sophisticated system of regular monitoring is carried out in the US, where monitoring has been ongoing for 20 years (Rabaglia et al., 2008). Even before the start of this program, BB were caught in ports and airports in the US (Rabaglia and Cavey, 1994; Haack, 2001, 2006; Mudge et al., 2001). The American system is based on a dense network of Lindgren funnel traps lured with ethanol, α-pinene + ethanol, and ipsdienol + cis-verbenol + methylbutenol, each separately. Traps are located mainly along both ocean coasts but also in the interior of the US. The US territory is divided into three parts, and each part is monitored once every 3 years. Even connected overseas territories such as Puerto Rico or Guam regularly participate in monitoring, where other volatile substances are also used for captures, such as manuka oil or ethanol + cubeb oil. Traps are located at seaports or at companies in the wood processing industry (Rabaglia et al., 2019). Data from this monitoring are used to determine the behavior of BB and to model their spread in the US (Rassati et al., 2016a). During the evaluation of this program (Rabaglia et al., 2019), ethanol was found to be the most suitable for trapping BB, while trapping with *Ips* lures was not effective for BB. Specific substances can be used to target selected BB (Hartshom et al., 2021).

1.2. Monitoring methods in Australia and New Zealand

Efforts to detect BB has also taken place in New Zealand. The first attempts to develop invasive species monitoring were in the 1980s (Hosking and Gadgil, 1987; Carter, 1989). Lindgren funnel traps with baits of α-pinene + ethanol, β-pinene + ethanol, frontalin + ethanol, and ipsdienol were also used in ports, international airports, and forests near these locations. This monitoring model has been proven to be successful in the early detection of BB, and it has, therefore, a good chance of eliminating these ambrosia and bark beetles (Brockerhoff et al., 2006). There was also an experimental trial to detect damage by invasive pests using field observations (car and walking) in New Zealand. Virtually no difference in results was found between these two methods (Bulman et al., 1999).

The monitoring of invasive species in Australia was broader; Lepidoptera was also caught. In sticky traps, Lindgren and Ecotrap. Ethanol, cineole, α-pinene, phellandrene, and a mixture of pinene, phellandrene, cineole, terpene, and cymene were used as bait. Traps were placed near ports and airports, and others were placed in a zone within 5 km of ports and airports (Bashford, 2012). The following baits were also tested in Brisbane harbor from 2006 to 2007: ipsenol, ipsdienol, frontalin, exo-brevicomin, and a combination of ethanol and α-pinene; a total of 29 species of ambrosia and bark beetles were caught (Wylie et al., 2008). In Tasmania, a method of static traps baited with a combination of α-pinene and ethanol was developed to monitor BB in *Pinus radiata* plantations (Bashford, 2008). These attempts subsequently developed into massive permanent monitoring throughout Australia (Carnegie et al., 2018, 2022; Carnegie and Nahrung, 2019).

1.3. Monitoring methods in Asia

In China, an IPM plan has been created and monitoring is carried out in designated areas using various methods, from baited traps with different types of semiochemicals to light traps to simply patrolling the area (Anonymus, 2009). At the same time, ambrosia and bark beetles are caught in ports (Lin et al., 2021). China also has an IPM standard for *P. aubei*, which causes serious damage to cypress trees there (Anonymus, 2017).

Other maritime countries also monitor BB in ports. In Japan, BB have been monitored in ports since the 1950s (Murayama, 1957; Schedl, 1966, 1969, 1970; Browne, 1980a,b; Ohno, 1989). In South Korea, BB were also monitored in harbors as early as the late 1970s (Choo et al., 1981; Choo and Woo, 1983; Choi et al., 2003).

1.4. Monitoring methods in Europe

In Italy, BB have long been monitored in ports (Cola, 1971, 1973). In total, 15 international ports and their adjacent forest stands are monitored; for trapping, Lindgren funnel traps and semiochemicals similar to those in the USA, ethanol, α pinene + ethanol, and ipsdienol + ipsenol + methylbutenol, are applied. Three traps were placed in the harbor, and three traps were placed in the adjacent forests. More species were found in deciduous forests than in coniferous stands. Invasive species richness was higher in forests than in harbors. The ambrosia and bark beetles were caught in the harbors, and were not yet able to establish a permanent population in the surrounding forests (Rassati et al., 2015). At Malpensa International Airport, the capture of invasive beetles in PET bottles was successfully tested using the following baits: apple cider vinegar, red wine, and 80% ethanol (Ruzzier et al., 2021).

Monitoring of invasive longhorned beetles (Cerambycidae) was launched in France, where they also tested trapping with α pinene + ethanol in Ecotrap traps. The traps were placed in natural forests and then in ports, airports, and orchards (Fan et al., 2019).

In Lithuania, as part of prevention, the bark beetle *Dendroctonus rufipennis* Kirby, 1837 was monitored in 2000 in the port of Klaipeda, near the Vaidotai railway station and along forest roads. *D. rufipennis* was not detected (Ostrauskas and Ferenca, 2010). In the period from 2002 to 2005, further monitoring was carried out at the borders, again in the port of Klaipeda, and at temporary wood warehouses, but no BB were caught. Lures α-pinene, myrcene, and *cis*-verbenol were used in Lindgren funnel traps (Ostrauskas and Tamutis, 2012).

Extensive monitoring of invasive species took place in Great Britain between 2013 and 2017. Lindgren funnel traps and cross-vane panel traps were placed in different types of forests near the ports. Ethanol and ethanol + α-pinene were used as bait. A total of three species of BB, *Cyclorhipidion bodoanum*, *Gnathotrichus materiarius*, and *X. germanus*, were captured (Inward, 2020).

2. Invasive species of ambrosia and bark beetles in the Czech Republic and expected invasive species

In the Czech Republic, there are six species of BB with a stable natural population: *C. bodoanum* Reitter, 1913, *Dryocoetes himalayensis* Strohmeyer, 1908, *G. materiarius* Fitch, 1858, *P. aubei*, *Xyleborinus attenuatus* Blandford, 1894, and *X. germanus* (Knížek, 1988; Procházka et al., 2018; Fiala and Holuša, 2019; Fiala et al., 2020, 2021, 2023). Furthermore, several introduced species that could not form a stable population due to an unfavorable climate or absence of host plants were found in the territory of the Czech Republic: *Coccotrypes dactyliperda* Fabricius, 1801, *Hypothenemus areccae* Hornung, 1842, *Hypothenemus hampei* Ferrari, 1867, *Hypothenemus setosus* Eichhoff, 1868, *Xyleborus affinis* Eichhoff, 1868, *Xyleborus volvulus* Fabricius, 1794, and *Xylosandrus morigerus* Blandford, 1894 (Reitter, 1913; Fleischer, 1927–1930; Pfeffer and Knížek, 1989).

New invasive species of ambrosia and bark beetles which are already present in Germany may be expected to invade the Czech Republic. These include, *Xyloterinus politus* Say, 1826, which was detected in Bavaria in 2014 (Gebhardt and Doerfler, 2018), and *Cyclorhipidion pelliculosum* Eichhoff, 1878, which was found in Baden-Württemberg in 2013 (Gebhardt, 2014). The greatest economic danger to tree species in the Czech Republic is the bark beetle *Pityophthorus juglandis* Blackman, 1928, which has been spreading in Italy since 2013 and is a carrier of the serious fungal disease, thousand cankers disease (Montecchio and Faccoli, 2014). From the east, we can expect an invasion of the bark beetle *Polygraphus proximus* Blandford, 1894, which spreads from Siberia toward the west, and its harmfulness is comparable to that of *I. typographus* (Peña et al., 2020). Therefore, a pest risk analysis was developed for both species (EPPO, 2014, 2015).

The MaxEnt algorithm can be used to model the spread of invasive species around the world. For the invasive ambrosia beetle *Xylosandrus compactus* Eichhoff, 1876, which occurs in southern Europe (Pennacchio et al., 2012; Barnouin et al., 2020; Leza et al., 2020; Riba-Flinch et al., 2021), with the continuation of average climatic values from 1970 to 2000, *X. compactus* is predicted to find suitable ecological conditions for development in southern Moravia (which is the warmest region of the Czech Republic) by 2050 (Urvois et al., 2021).

2.1. Basic points for determining the monitoring methodology of invasive ambrosia and bark beetles in the Czech Republic

Since 2020, efforts have been underway to determine the possible entry gates and directions of expansions of BB in the Czech Republic (**Figure 1**; Fiala and Holuša, 2019; Fiala et al., 2020, 2021, 2022, 2023). Potential types of volatile substances that could be used for monitoring were compared to find the simplest monitoring method (Fiala and Holuša, 2020; Fiala et al., 2023).

The Czech Republic has no seaports, but has five international airports (Prague, Brno, Ostrava, Pardubice, and Karlovy Vary; **Table 1**) and many road and rail border crossings with foreign countries. Therefore, global trade is a possible reason for the flight activity of individual invasive species when entering the Czech Republic. In 2022, 302,640 tons of wood materials with a size larger than 6 mm were imported from all over the world into the Czech Republic, of which 4,993 tons were tropical wood of all kinds (ČSÚ, 2023).

The invasive ambrosia beetle *X. germanus* in the middle of the Czech Republic in 2007 (Knížek, 2009a) was first found near the largest wood warehouse of Stora Enso in Ždírec nad Doubravou, similar to the invasive sawfly *Urocerus albicornis* Fabricius, 1781, was found on the grounds of the Kronospan wood processing plant in Jihlava (Háva and Holuša, 2019). The occurrence in botanical gardens through the importation of live exotic plants has only been demonstrated once in the Czech Republic, in the case of *X. morigerus* (Reitter, 1913); however, this does not mean that other introductions have not occurred and escaped notice. The ambrosia beetle *G. materiarius* was first found through flight monitoring near the border with Bavaria in western Bohemia (Knížek, 2009a). Likewise, the spreading of *X. germanus* in northern Bohemia and southern Moravia (Fiala et al., 2020) or *D. himalayensis* in southern Moravia (Procházka et al., 2018) is a result flight of beetles.

Most of the BB were found near the borders with Germany and Austria (cf. Fiala et al., 2021; **Figure 1**). This is logical because most of the BB in Europe have been detected near seaports in western and southern Europe. The main entry points were clearly identified as river valleys and border crossings (Fiala et al., 2020, 2023).

2.2. Results of case studies in the Czech Republic

In 2021, two experiments were conducted to detect BB: (i) the capture of ambrosia and bark beetles at a warehouse of tropical wood imported from Central Africa in Pilsen – Doubravka town[1] and (ii) the capture of ambrosia and bark beetles in the Botanical Garden in Prague – Troja with a tropical greenhouse, where tropical trees are brought in every year. This botanical garden is the largest in the Czech Republic, and its tropical greenhouse offers vegetation of dry tropics and subtropics, lowland rainforest, and tropical forests of high mountains.[2]

No invasive bark beetle was caught near Pilsen (**Appendix Table 1**); only the bark beetle *Lymantor coryli* Perris, 1855, which is rarely found throughout Europe, was detected (Fiala, 2021). No bark beetles were caught in the tropical greenhouse, but the two BB, *X. germanus* and *D. himalayensis*, were caught at the edge of oak forests (**Appendix Table 2**).

At the same time, at the end of 2021, 13 companies involved in the coffee trade in the Czech Republic were asked to cooperate to detect the occurrence of introduced species of ambrosia and bark beetles damaging coffee beans. Several samples of damaged beans were obtained, and the bark beetle *H. hampei* (**Figure 2**) from Brazil, Colombia, and India (**Appendix Table 3**) was detected

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[1] www.exoticke-drevo.com

[2] https://www.botanicka.cz/en

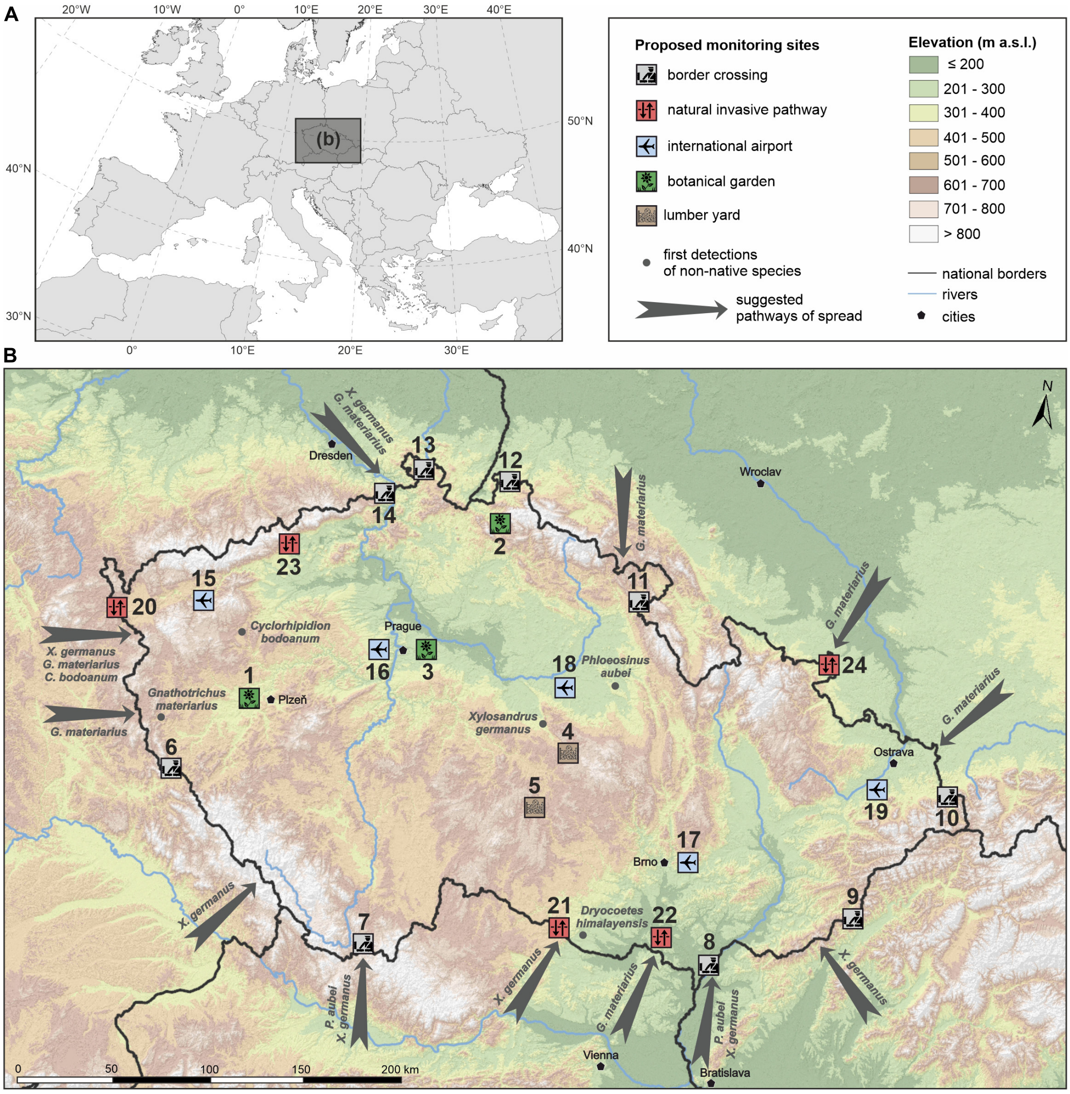

FIGURE 1

The position of the Czech Republic in Europe **(A)** and the possible entry gates, places of first detections, and a proposal for monitoring locations for invasive ambrosia and bark beetles in the Czech Republic **(B)**.

by the occurrence several dead individuals in the Czech Republic. However, *H. hampei* does not pose a danger, even to undamaged coffee stocks, as its stages do not survive the Central European climate (Jaramillo et al., 2009). It can be speculated that beetles may, however, introduce various fungal and bacterial infections into uninfected beans (Damon, 2000; Jaramillo et al., 2006).

3. Proposal of a methodology for the detection of invasive species of ambrosia and bark beetles in the Czech Republic

The selection of locations is based on possible entry points such as border crossings, border river valleys, international airports, large timber warehouses, and botanical gardens; at the same time, these points will be used to monitor already established species whose abundance is still very low (Procházka et al., 2018; Fiala and Holuša, 2019, 2020; Fiala et al., 2020, 2021, 2022; Holuša et al., 2021). For the purposes of regular and permanent monitoring of BB, we therefore propose the following locations (**Table 1** and **Figure 1**). A quarter of the locations are in protected areas; there is sufficient dead wood, and there are overgrown stands that provide a suitable environment for the development of ambrosia and bark beetles (Lee et al., 2019; Fiala et al., 2021).

Some invasive bark beetles are polyphagous, such as *X. germanus* (Weber and McPherson, 1983) and *X. politus* (MacLean and Giese, 1967), and can attack both coniferous and deciduous trees; some attack only deciduous trees, such as *X. attenuatus* (Kvamme et al., 2020), or only conifers, such as *G. materiarius* (Kamp, 1970). The representation of tree species is not significant for ambrosia and bark beetle monitoring because the type of forest has no effect on the abundance of beetles (Bouget et al., 2008). Therefore, the type of forest in which the trap is placed is not important, although a mixed forest with different tree

FIGURE 2
Dead individual of bark beetle *H. hampei* found in damaged coffee bean introduced to the Czech Republic.

species is preferable. We prefer oak forests, in the vicinity of which there are also conifers. In the Czech Republic, almost all forests are cultural, and conifers grow even at low altitudes. Therefore, choosing a combination of forests at the different locations was straightforward (**Table 1**).

Most BB in Europe are ambrosia species (Alonso-Zarazaga et al., 2023), and in our study in oak forests in western Bohemia, we found that ambrosia beetles had a higher abundance with a greater canopy cover, due to the wetter microclimate and greater amount of dead wood (Holuša et al., 2021). The influence of the close canopy on the abundance of ambrosia and bark beetles was also confirmed by Menocal et al. (2022). Therefore, forests with close canopy is generally preferred, although we are aware that *C. bodoanum* seems to prefer open forests (Fiala et al., 2021).

We also tested substances suitable for trapping BB. Factory-produced pheromones were suitable for trapping ambrosia and bark beetles of the genus *Trypodendron*; we found one specimen of *X. germanus* (Fiala and Holuša, 2020). Among volatile substances, we found the best combination of ethanol and juniper twigs suitable for trapping bark beetles *P. aubei* (Fiala et al., 2023). We found ethanol to be the most suitable for *G. materiarius* (Fiala et al., 2023). Likewise, *C. bodoanum* was captured in ethanol (Fiala et al., 2021), and although *D. himalayensis* and *X. germanus* were captured in impact traps as such, they were also captured in ethanol (Procházka et al., 2018; Hauptman et al., 2019a; Fiala et al., 2020; **Appendix Table 2**). *X. attenuatus*, like the ambrosia bark beetle, was attracted to ethanol (Galko et al., 2014).

Although sulcatol, which is considered a potential aggregation pheromone of *G. materiarius*, was expected to be successful (Flechtmann and Berisford, 2003), it was not the best lure tested in Central European conditions. The combination of sulcatol and ethanol resulted in the capture of a significantly greater number of beetles of *Gnathotrichus* sp. (McLean and Borden, 1977). However, in our case, ethanol alone captured more beetles than the combination of baits. Ethanol also significantly attracted other invasive ambrosia beetles, *C. bodoanum*, *X. germanus*,

TABLE 1 Proposed localities for permanent monitoring of invasive ambrosia and bark beetles (types of protected areas of the Czech Republic: NP, National Park; NPR, National Nature Reserve; PP, Nature Monument; PR, Nature Reserve).

No.	Monitoring locations	GPS	Reason for location selection and inclusion
1	Zoologická a botanická zahrada Plzeň	49.7595N, 13.3598E	Botanic garden
2	Botanická zahrada Liberec	50.7768N, 15.0768E	Botanic garden
3	Pražská botanická zahrada	50.1224N, 14.4138E	Botanic garden
4	Ždírec	49.7022N, 15.8088E	Wood storage
5	Jihlava	49.4219N, 15.6050E	Wood storage
6	Česká Kubice	49.3643N, 12.8522E	Border crossing
7	PP Horní Malše	48.6553N, 14.4575E	Border crossing
8	Tvrdonice	48.7504N, 17.0210E	Border crossing
9	PP Okrouhlá	49.0466N, 18.0576E	Border crossing
10	Třinec	49.6795N, 18.6930E	Border crossing
11	Hronov	50.4776N, 16.2129E	Border crossing
12	PR Meandry Smědé	50.9808N, 15.0345E	Border crossing
13	Velký Šenov	50.9960N, 14.4053E	Border crossing
14	Hřensko	50.8730N, 14.2392E	Border crossing
15	Karlovy Vary	50.1998N, 12.9028E	International airport
16	Praha Ruzyně	50.1244N, 14.3054E	International airport
17	Brno	49.1606N, 16.6602E	International airport
18	Pardubice	50.0203N, 15.7153E	International airport
19	Ostrava	49.6981N, 18.1397E	International airport
20	PR Rathsam	50.1013N, 12.2485E	Assumed migration path
21	NP Podyjí	48.8495N, 15.8835E	Assumed migration path
22	NPR Děvín	48.8587N, 16.6511E	Assumed migration path
23	NPR Jezerka	50.5433N, 13.4844E	Assumed migration path
24	PP Osoblažský výběžek	50.3032N, 17.7005E	Assumed migration path

X. attenuatus, and other species of native ambrosia and bark beetles. Ethanol attracts both ambrosia and bark beetles *X. politus* and *C. pelliculosum*, which are already present in Germany (Ranger et al., 2011, 2014). Ethanol generally has a better capture ratio of invasive ambrosia beetles than the other substances (Fiala et al., 2023). Ethanol has long been known to be the main volatile substance on ambrosia and bark beetles (Kelsey and Joseph, 2003; Ranger et al., 2013, 2019).

For capturing and monitoring the dangerous invasive species *P. juglandis*, ethanol is also a suitable substance (Roling and Kearby, 1975). However, in acute situations, the monitoring network can be extended by adding a trap with the aggregation pheromone prenol, which was detected in this bark beetle (Seybold et al., 2015). Ethanol can also be used to detect *P. proximus*, although the beetles will most likely be caught in small quantities, as it reacts mainly to *cis*-verbenol, ipsdienol, and ipsenol (EPPO, 2014), like *I. typographus* (Schlyter et al., 1987). If the occurrence of *P. proximus* in the vicinity of the Czech Republic has already been predicted, the monitoring network can be expanded by adding another trap to the monitoring location with one of the industrial attractants containing *cis*-verbenol.

We propose total of 24 monitoring locations. Most of them are located at the border crossings of the Czech Republic and in river valleys, which are probably the entrance gates to the Czech Republic of BB (**Figure 1**). In addition, two large timber warehouses in which international trade takes place were selected (Žemlička, 2012), along with all international airports and three botanical gardens with tropical greenhouses. The latter locations cover a variety of modes of invasion by ambrosia and bark beetles: natural dispersal by the flight abilities of ambrosia and bark beetles and spread by global trade (**Table 1**).

We designed specific locations so that they were easily accessible in forests and were warmer locations of southern exposures. We selected overgrown forests near state borders or places that represent a "steppingstone," as in the case of point 22, NPR Děvín (a woven area in an agricultural landscape), and point 23, NPR Jezerka (located on the migration route along the Ohøe River valley). From airports and large timber warehouses, we assume that bark beetles will fly to the nearest forest stands. Botanical gardens have the character of open forests and are mostly surrounded by forests, so localities in the territory of the garden have been suggested.

Three traps at each location is sufficient (Rassati et al., 2015; Thurston et al., 2022). In the Czech Republic, two types of impact traps are used; both are inexpensive and commonly available. They are easy to install and do not catch large numbers of non-target insects (Lubojacký and Holuša, 2014; Galko et al., 2016). The traps can be a Theysohn slot type, which is the most widely used in forestry in the Czech Republic (Zahradník and Zahradníková, 2016), or impact type Ecotrap, from which it is easier to extract the caught beetles. They can be disassembled after each season and stored in a much smaller space than the Theysohn traps.

These types of traps are primarily intended for catching economically important bark beetles that are attracted by specific pheromones (Flechtmann et al., 2000; Šramel et al., 2021); however, they can also be used to capture invasive species without any

TABLE 2 Basic costs of operating the proposed monitoring network of invasive species of ambrosia and bark beetles in the Czech Republic (prices for the year 2023 in €) [energy costs (freezer), human fieldwork and labor costs, and determination costs are not included].

Numbers of traps	Cost per trap	At total for all traps	Number of ethanol lures	Cost per lure	At total for all lures	The total postage for all locations	At total
72	60[1]/22[2]	4,320[1]/1,584[2]	144	10.20	1,469	150	5,939[1]/3,203[2]

Additional years can be calculated without the cost of traps.

[1] Theysohn trap.

[2] Ecotrap.

problems (Holuša et al., 2021; Fiala et al., 2023). Different species of ambrosia and bark beetles are found to prefer different types of traps. *Dryoxylon onoharaense* Murayama, 1934, an invasive species also found in Europe (Marchioro et al., 2022), or *G. materiarius* prefer the Ecotrap type. In contrast, bark beetles *X. affinis* and *Premnobius cavipennis* Eichhoff, 1878 prefer the Theysohn type (Flechtmann et al., 2000; Dodds et al., 2010; Miller and Crowe, 2011).

Each trap is baited with ethanol, which is universal for catching ambrosia and bark beetles (Rassati et al., 2016b; Chen et al., 2021). Traps should be placed between 30 and 50 m apart (Niemeyer, 1997; Rassati et al., 2014). Ethanol is also partly attractive to common species of ambrosia and bark beetles that live on conifers (Fiala et al., 2023). Traps should be operated from mid-April to the end of July, as the flight activity of ambrosia and bark beetles decreases in August (Fiala et al., 2023). Traps are checked once every 14 days, and the collected samples are then stored in the freezer for later determination. Ethanol should be changed in early June since the evaporators are active for approximately 60 days.[3]

In total, there are only 72 traps (e.g., three traps at 24 locations), which represent 144 ethanol lures per year (**Appendix 4**). Given that the Czech Republic is a small country, the number of locations is small, and monitoring should be carried out annually. Since most of the locations are forested, we suggest, if agreeable, partnering with the local forest administration of Forest of the Czech Republic (LČR, s.p., in Czech), a company that manages more than 50% of the Czech Republic's forest stands and has cooperation with the Forest Advisory Service (Lesní ochranná služba in Czech) of Forestry and Game Management Research Institute (FGMRI, VÚLHM in Czech) Jíloviště at Prague, capital of the Czech Republic. In total, the LČR manages thousands of trappers throughout the country every year. The traps that we suggest, slightly more than 70 traps, are not difficult to manage because foresters move around the forests every day. Similarly, workers at the botanical gardens and timber warehouses move around daily and can send samples for determination. The average catch per trap in the world varies between 200 and 500 specimens, similarly in the Czech Republic it is between 50 and 500 specimens (**Appendix Table 5**).

The entire organization of monitoring corresponds to the activity and assignment of the Forest Advisory Service. The Forest Advisory Service deals with research, expert, and monitoring activities in forest protection against biotic pests. It monitors the occurrence of the bark beetle *Ips duplicatus* Sahlberg, 1836, every year. This monitoring has been ongoing for a total of 25 years, and during this period, a total of approximately 400 traps baited with *I. duplicatus* were placed around the country (Holuša et al., 2010; Knížek and Liška, 2022). The traps were checked by foresters, and beetles were collected and sent to FGMRI for determination. In Central Europe, other forest research institutes have also been involved in monitoring BB, e.g., in Slovenia (see Hauptman et al., 2019a), Slovakia (see Galko et al., 2014), and Latvia (see Ostrauskas and Tamutis, 2012); however, these were one-time events.

Our proposed monitoring of BB can be easily merged with the existing monitoring of *I. duplicatus*. It involves incorporating only 72 traps. The Forest Advisory Service would purchase ethanol vaporizers for cooperating entities and provide basic operator training; however, it is also possible to use a recorded instructional video. The total volume of all samples from the three traps does not exceed 1 dm^3, so workers can place it in closed cans in any freezer where the insects will be frozen. It is necessary to determine the entire material of beetles into species by a specialist because data will be obtained on several species of ambrosia and bark beetles, especially rare ones (Fiala, 2021; Holuša et al., 2021; Fiala and Nakládal, 2022; Fiala et al., 2023).

Due to the importance of early detection of invasive species of ambrosia and bark beetles, the economic costs are minimal (**Table 2**) compared to the damage that can occur. In the US, the annual loss associated with all invasive species is estimated at $120 billion (Pimentel et al., 2005). In Europe, the loss caused by all invasive species is estimated to be hundreds of millions of € per year (Vilà et al., 2010); e.g., for invasive longhorned beetles of the genus *Anoplophora*, the cost of eliminating one infested hectare of vegetation is $25,000 (Anonymus, 2014). Estimated economic loss to landowners exceeded hundreds of dollars per hectare for invasive pests in *Pinus taeda* Linnaeus, 1753 stands in the southern US when no monitoring was performed (Susaeta et al., 2016). When carrying out integrated protection, the cost is less than the loss of value of the wood (Franjević et al., 2016). At the same time, lures require smaller financial expenditure than the human labor associated with the control of traps (Šramel et al., 2021).

4. Conclusion

The proposed monitoring method based on commonly used traps in selected locations (entrance gates at borders, wood warehouses, tropical greenhouses, and airports) is necessary because we BB have already been detected in the Czech Republic. Therefore, it is necessary to monitor these species and be able to detect new ones. Ethanol is effective for capturing the species that have already been detected, and the method is inexpensive. The method can be implemented by the research institute for monitoring pests. The monitoring results can inform the professional actions of the Central Institute for Supervising and

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[3] www.e-econex.net

Testing in Agriculture and for the targeted eradication of invasive species, as required by EU regulations.

Author contributions

TF and JH contributed to the conception and design of the study and wrote the first draft of the manuscript. Both authors contributed to manuscript revision, read, 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

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Appendix

APPENDIX TABLE 1 Detection of ambrosia and bark beetles according to the type of bait at a tropical wood warehouse in Pilsen.

Species	Ethanol	Ethanol + α-pinen	Ethanol + E-conophthorin
Anisandrus dispar Fabricius, 1792	1		
Hylastes attenuatus Erichson, 1836	1	1	
Hylesinus varius Fabricius, 1775	1		
Lymantor coryli Perris, 1853	1		
Scolytus rugulosus P.W.J. Müller, 1818	1		
Tomicus piniperda Linnaeus, 1758		3	
Xyleborinus saxesenii Ratzeburg, 1837	2		1

In Plzeò – Doubravka (GPS 49.7622N, 13.4095E), three Lindgren funnel traps with wet capture and ethanol, ethanol + α-pinene and ethanol + E-conophthorin were used as bait. Trapping took place from mid-April to mid-July, and beetles were collected once a month (det. T. Fiala, M. Knížek).

APPENDIX TABLE 2 Detected species of ambrosia and bark beetles in the Prague-Troja Botanical Garden (GPS 50.1224N, 14.4139E).

Species	Number of specimens
Anisandrus dispar Fabricius, 1792	599
Dryocoetes himalayensis Strohmeyer, 1908	**1**
Dryocoetes villosus Fabricius, 1792	12
Ernoporus tiliae Panzer, 1793	1
Pityogenes chalcographus Linnaeus, 1761	1
Polygraphus grandiclava C.G. Thomson, 1886	4
Scolytus rugulosus P.W.J. Müller, 1818	5
Xyleborinus saxesenii Ratzeburg, 1837	367
Xyleborus dryographus Ratzeburg, 1837	70
Xyleborus monographus Fabricius, 1792	44
Xylocleptes bispinus Duftschmid, 1825	1
Xylosandrus germanus Blandford, 1894	**1**

Theysohn traps baited with ethanol were used at the Troy Botanical Garden. Ten traps were placed in nature near the tropical greenhouse, and two traps were placed inside the tropical greenhouse. Trapping was performed from mid-April to mid-August, and beetles were collected at 2-week intervals (det. T. Fiala, M. Knížek). Invasive species are in bold.

APPENDIX TABLE 3 The presence of feeding and the detected numbers of *Hypothenemus hampei* Ferrari, 1867 in samples of ten coffee beans imported to the Czech Republic from seven countries in 2021–2022 (det. T. Fiala).

Country of origin	Brazil	Brazil, region São Paulo	Colombia	Ethiopia, region Yirgacheffe	Ethiopia, region Guji	India, region Tamil Nadu	Salvador
Presence of feeding	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Numbers of beetles	1	0	2	0	0	1	0

Appendix 4 | Basic monitoring design.

- Twenty-four localities
- Three traps per locality, 30–50 m each other
- Each trap baited with ethanol
- Traps checked once every 14 days

APPENDIX TABLE 5 Overview of the number of scolytines caught by trap in the Czech Republic and in the world.

Country	Year	Traps/Sites	Lures	Total *Scolytinae*	Numbers of invasive species/Specimens	References
United States	2001–2005	1,240/310	Variable	250,000+	24/?	Rabaglia et al., 2008
	1985–2000	?/97	Variable	6,825	67/2,737	Haack, 2001
	2007–2016	4,320/1,440	Variable	840,000+	28/456,000+	Rabaglia et al., 2019
Italy	2009–2011	72/4	Variable	1,043	4/30	Rassati et al., 2014
	2012	90/15	Variable	40,473	11/406	Rassati et al., 2015
Czech Republic	2020	10/10	Ethanol	4,179	3/24	Holuša et al., 2021
	2022	20/4	Ethanol	1,176	4/186	Fiala et al., 2020
	2018	1/1	Ethanol	124	0/0	Fiala, 2019
Slovenia	2017	19/19	Ethanol	94,104	3/67,605	Hauptman et al., 2019b
Slovakia	2010–2012	53/1	Ethanol mixture	24,705	2/561	Galko et al., 2014

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RNAi-chitosan biopesticides for managing forest insect pests: an outlook

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The expanding world population demands superior forest protection to fulfil feasible environmental certainty. The persistent pest infestations negatively influence forest health and cause substantial economic losses. In contrast, the traditional use of conventional pesticides results in a loss of soil microbial biodiversity, a drop in the population of pollinators, and adverse effects on other non-target organisms, including humans. Global forestry is looking for solutions to reduce the adverse environmental effects of current chemical pesticides. RNAi-nanotechnology has recently drawn much attention for its use in pest management. The advantages of engineered RNAi-chitosan nano-formulations in terms of simple digestion and dissolution, non-toxicity, high adsorption power, potential biodegradation in nature, and widespread availability and cost-effectiveness, have been well documented for pest management in agroecosystems. However, deploying such control strategies in forest ecosystems is still pending and demands further research. Hence, we highlight the putative uses of RNAi-chitosan biopesticides and their preparation, characterization, and putative application methods for forest pest management. We also discussed potential environmental risks and plausible mitigation strategies.

KEYWORDS

forestry, RNA interference, nanotechnology, chitosan-RNAi biopesticides, forest insect pest management, forest protection

Introduction

Given the ongoing increase in global population, many countries have lost forests and facing climate change (Ritchie and Roser, 2021).[1] Forestry is a crucial industry in many developing countries, and it can produce food and gross income as a domestic product for both people and animals, as well as contributes to balancing the environmental conditions,

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Abbreviations: *A. aegypti*, *Aedes aegypti*; AchE, *acetylcholine esterase*; AMN, *Aminopeptidase*; AMY, *Alpha-amylase*; *A. gambiae*, *Anopheles gambiae*; *A. solani*, *Alternaria solani*; BMI, bacterial metabolic infiltrates; CAD, *Cadheri*; CHS, *Chitin synthase*; CHS1, *chitin synthase 1*; CHS2, *chitin synthase 2*; CPB, Colorado potato beetle; *D. melanogaster*, *Drosophila melanogaster*; DCDA, degree of chitosan deacetylation; dsRNA, double-stranded RNA; *E. vittella*, *Earias vittella*; *H. armigera*, *Helicoverpa armigera*; IAP1, *Inhibitor of apoptosis 1*; JHAMT, *juvenile hormone methyltransferase*; mRNA, complementary messenger RNA; *N. lugens*, *Nilaparvata lugens*; *P. grisea*, *Pyricularia grisea*; PEC, polyelectrolyte complex; PRR, pattern recognization receptor; PSTV, Potato spindle tuber virus; RCNPs, RNAi-chitosan nanopesticides; RISC, RNA-induced silencing complex; Sec23, *Sec23 homolog A*; SNF7, *ESCRT-III subunit protein SNF7*; SRC, *SRC proto-oncogene*; *S. frugiperda*, *Spodoptera frugiperda*; *S.litura*, *Spodoptera litura*; *S. lycopersicum*, *Solanum lycopersicum*; siRNA, small interfering RNA; TBSV, bean/tomato bushy stunt virus; TNV, tobacco necrosis virus; Vg, *vestigial*; V-ATPase, *V-type proton ATPase*; V-ATPase B, *Vacuolar-type ATPase B*; SMR, symbiont mediated RNAi.

[1] https://research.wri.org/gfr/latest-analysis-deforestation-trend

respectively. However, a variety of biotic factors like insect pests (i.e., bark beetles, weevils, chewing, sucking, and foliage-feeding insects) and diseases caused by pathogens (i.e., tree leaves diseases, pine needle diseases, hardwood leaf diseases, tree bark diseases, and tree root diseases) limit forest growth and tend to get worse with a growing human population (Kan et al., 2023). Consequently, to address pest-related issues, pesticides (insecticides, fungicides, herbicides, etc.) have been overused and often misused, which has had fatal short and long-term effects on humans and other life forms (Chhipa, 2017). Pesticide resistance is common in pest insects, and their preexisting adaptive capabilities facilitate quick resistance in field conditions (Bras et al., 2022). With the accessibility of new technologies, superior approaches to controlling insect pests and disease-caused pathogens can be considered. RNA interference (RNAi) technology and nanotechnology have recently captured the interest and imagination of scientists and researchers due to recent advancements in the discipline. Delivering RNAi biopesticides with the use of nanotechnology in the forestry sector is a quick, innovative, and promising field (Shang et al., 2019; Joga et al., 2021; Silver et al., 2021; Mogilicherla et al., 2022).

Polymeric nanoparticles are non-toxic, economical, environmentally friendly, and most significant controlled-release formulations, so researchers are interested in the feasibility of their application in different sectors (Prajapati et al., 2022). Nonetheless, employing some polymeric nanoparticles at higher concentrations demonstrates a phytotoxicity effect on plants, and it depends on initial material selection, nanoparticle preparation methods, and the impact varies according to plant species (Jogaiah et al., 2021). Remarkably, no instances of phytotoxicity have been reported concerning RNAi-polymeric nanoparticles. Chitin is the second-most common natural polymer after cellulose and is obtained mainly from shrimps, crabs, lobsters, and crawfish by-products (**Figure 1**; Faqir et al., 2021). Chitin is a linear, poly-(1,4)-N-acetyl-D glucosamine that appears in nature as organized crystalline microfibrils called α-chitin, β-chitin, and γ-chitin (Vani and Stanley, 2013). Chitosan is a partly deacetylated polymer of N-acetyl glucosamine produced by the alkaline deacetylation of chitin (**Figure 1**). Chitosan has several unique features due to the amine and hydroxyl groups, making it useful in many contexts and accessible for chemical reactions (Chouhan and Mandal, 2021). Since it may produce safe and non-toxic complexes through electrostatic interaction with its positive cationic group and the negative anionic group of the RNAi molecules (dsRNA/siRNA), it enhances the stability of RNAi molecules (Gurusamy et al., 2020a; Sandal et al., 2023). A natural process of RNAi converts dsRNA into 21-25-nucleotide-long siRNAs, which are then recruited to the RNA-induced silencing complex (RISC), which then finds and degrades the mRNA (Fire et al., 1998; Agrawal et al., 2003; Yu et al., 2013). RNAi has demonstrated considerable potential for formulating new pest control practices because of its species specificity and high efficacy (Zhu and Palli, 2020; Joga et al., 2021). However, it is underexploited in the forestry sector (Joga et al., 2021; Mogilicherla et al., 2022).

Variable RNAi efficiency among insects has been linked to several mechanisms, including dsRNA degradation in the hemolymph and midgut lumen, decreased dsRNA uptake by cells, decreased induction of RNAi components upon exposure to dsRNA, missing components in the RNAi pathway, and accumulation of dsRNA in endosomes (Katoch et al., 2013; Shukla et al., 2016; Singh et al., 2017; Yoon et al., 2017; Cooper et al., 2019). The last 10 years have spotted the development and implementation of a chitosan-based dsRNA delivery method that boosts the possibility of RNAi applications in insect pest management (**Table 1**; Zhang et al., 2010; Das et al., 2015; Gurusamy et al., 2020a; Kolge et al., 2021). In order to prevent insect pests and diseases, chitosan-RNAi is utilized in the field of agriculture (Reglinski et al., 2004; Fitza et al., 2013; Bharani et al., 2014; Sahab et al., 2015; Silva-Castro et al., 2018; Ingle et al., 2022) and can also be used for forest protection (Joga et al., 2021; Mogilicherla et al., 2022). This succinct perspective discusses the synthesis of RNAi-chitosan nanopesticides (RCNPs) and characterization, as well as the evaluation of their effectiveness and biocompatibility against insect pests and microbes from a forest insect pest management and forest health point of view (**Figure 1**).

RNAi-chitosan biopesticides synthesis methods

Chitosan is a polycationic polysaccharide that occurs naturally and is produced when chitin is partially deacetylated (**Figure 1**). Chitosan has several physicochemical characteristics, including molecular weight, viscosity, degree of deacetylation, and crystallinity (Kas, 1997; Riseh et al., 2022). A primary amine group with a pKa value of around 6.5 is present in every deacetylated subunit of chitosan; as a result, chitosan is soluble in acidic pH, like acetic acid but insoluble in neutral and alkaline pH. The amount of chitosan© deacetylation, molecular weight, ionic strength of the solution, and pH significantly impact its solubility (Mao et al., 2010). Chitosan dissolved in acetic acid and spontaneous mechanical churning at room temperature leads to caused nanoparticles. In addition, adjusting the chitosan-to-stabilizer ratio altered the particle size and surface charge (Hosseini et al., 2015). Several methods have been described for synthesizing RCNPs, such as electrostatic interaction, encapsulation, and adsorption (**Figure 1**). When chitosan is dissolved in acidic circumstances, the degree of chitosan deacetylation (DCDA) value influences the positive charge density; more DCDA results in an enhanced positive charge, allowing a better dsRNA/siRNA binding capacity (Liu et al., 2007; Mao et al., 2010). The ionotropic gelation method uses the electrostatic contact between a negatively charged group of nucleotides (e.g., in dsRNA) and the amine group of chitosan and self-assembled to form the polyelectrolyte complex (PEC) as a result of a decrease in hydrophilicity caused by charge neutralization between the cationic polymer and dsRNA. When dsRNA is added to chitosan (in acetic acid) solution and with continuous stirring at room temperature, the RCNPs can be produced spontaneously (**Figure 1**). Also, chitosan© molecular weight affects the physicochemical characteristics of RCNPs, including their size, zeta potential, shape, and complex stability (Mao et al., 2010). The surface charge of the RCNPs is dependent on the molar ratio of chitosan nitrogen (N) to dsRNA phosphate (P) (N/P ratio), which affects the particle capacity to efficiently condense dsRNA and interact with negatively charged cells, which in turn affects the transfection efficiency (Köping-Höggård et al., 2001; Huang et al., 2005; Jeong et al., 2007; Nafee et al., 2007).

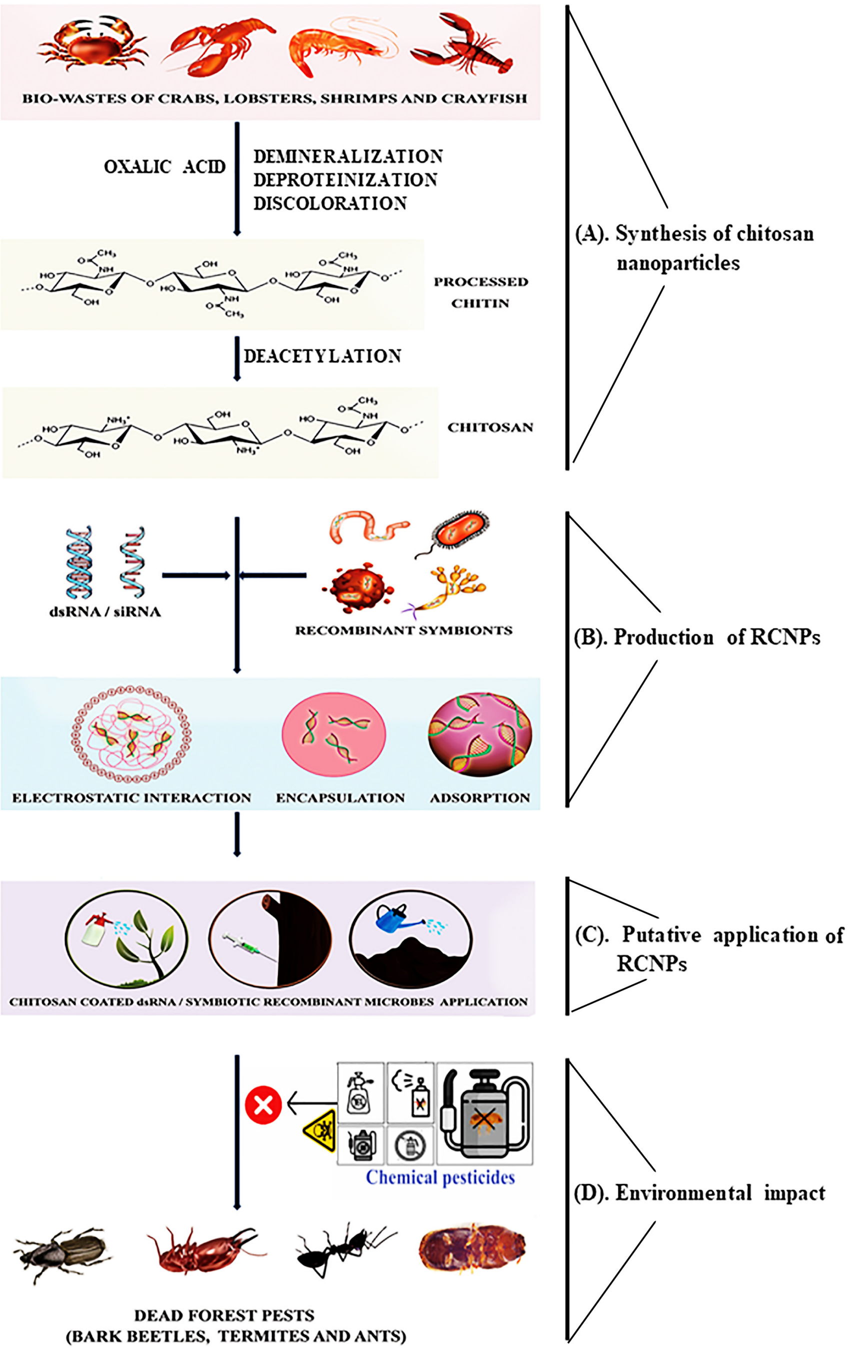

FIGURE 1
Scheme illustrating the RNAi-chitosan biopesticides formulations and their applications for forest insect pest management. **(A)** Synthesis of chitosan nanoparticles: marine by-products will produce chitin, which has been partially deacetylated and yields chitosan. Chitosan nanoparticles produced by chitosan dissolved in acetic acid under spontaneous mechanical churning at room temperature. **(B)** Production of RCNPs: RCNPs can be created via the adsorption, encapsulation, and electrostatic interaction approaches. Also, chitosan can be used as a coating material for dsRNA-expressed symbiotic microbes. RCNPs can be characterized in terms of size, zeta potential, and shape. **(C)** Putative application of RNCPs: RCNPs can be applied to forests to control forest pests and diseases using the foliar application, trunk injection, and soil drenching approaches leading to the species-specific killing of forest insect pests (bark beetles, termites, ants). **(D)** Environmental impact: deploying RNCPs will reduce the application of commercial pesticides.

The chitosan salt form also impacted the RCNPs, such as chitosan glutamate, which had a larger molecular weight, created smaller complexes with dsRNA/siRNA, and had a higher siRNA loading efficiency than chitosan hydrochloride (Katas and Alpar, 2006). The amount of dsRNA at a certain point within the RCNPs plays a fundamental role in host cell transfection efficiency, whereas more concentration of dsRNA will increase the diameter of the particles and form an aggregation, and will decline the transfection (MacLaughlin et al., 1998; Romøren et al., 2003; Zhao et al., 2006; Mao et al., 2010). Chitosan can be employed as a dsRNA-chitosan

TABLE 1 RNAi-chitosan biopesticides: current status against pest insects.

Insect species	Target gene	Nanomaterial	dsRNA/siRNA/miRNA	Delivery method	References
Anopheles gambiae	*Chitin synthase 1* and *Chitin synthase 2*	Chitosan	dsRNA	Feeding by diet	Zhang et al., 2010; Zhang et al., 2015
Aedes aegypti	*Semaphorin-1a*	Chitosan	siRNA	Feeding by diet	Mysore et al., 2013
Aedes aegypti	*Vacuolar-sorting protein SNF7* and *SRC proton-oncogene*	Chitosan	dsRNA	Feeding by diet	Das et al., 2015
Aedes aegypti	*Vestigial (vg)*	Chitosan	dsRNA	Feeding by diet	Kumar et al., 2016
Aedes aegypti	*Inhibitor of apoptosis*	Chitosan-sodium tripolyphosphate	dsRNA	Feeding by diet	Dhandapani et al., 2019
Spodoptera frugiperda	*Inhibitor of apoptosis*	Chitosan	dsRNA	Feeding by diet	Gurusamy et al., 2020a
Ostrinia nubilalis	lethal giant larvae protein (OnLgl; MT467568)	Chitosan	dsRNA	Feeding by diet	Cooper et al., 2020
Chilo suppressalis	*Glyceraldehyde-3-phosphate dehydrogenase*	Chitosan	dsRNA	Feeding by oral drinking	Wang et al., 2020
Helicoverpa armigera	*Acetylcholinesterase (AChE)*	Chitosan	dsRNA	Feeding by topical spray	Kolge et al., 2021
Helicoverpa armigera	*Lipase* and *chitinase*	Chitosan	dsRNA	Feeding by diet and leaf	Kolge et al., 2023
Nilaparvata lugens	*Chitin synthase A*	Rosin-modified PEG and chitosan	dsRNA	Feeding by topical application	Lyu et al., 2023

complex as well as a coating material for symbiotic microbes that express dsRNA to provide a flexible technology platform for the management of forest insect pests (**Figure 1**; Mao et al., 2010; Joga et al., 2021; Riseh et al., 2022).

RNAi-chitosan biopesticides: current status

RNAi-chitosan nanopesticides extend to precision use due to their minuscule dimensions, high surface area, enhanced permeability, thermal stability, dispersion, and biodegradability to improve forest yield and to control target action based on insect pests or microbes infection (**Figure 1**; Adisa et al., 2019; Kumar et al., 2019). For applying RCNPs in forestry, several methods like foliar application, trunk injection, and soil drenching can be considered (**Figure 1**; Joga et al., 2021; Mogilicherla et al., 2022). Chitosan nanoparticle-mediated RNAi has been developed over the last 10 years as an alternative to traditional pest control methods (**Table 1**).

The formulations of RCNPs have significant potential to control the attack of several common pests like aphids, moths, and beetles (Sahab et al., 2015; Gurusamy et al., 2020a). Silencing of the *CHS1*, *CHS2*, *semaphorin-1a*, and *vestigial* (*vg*) genes by feeding chitosan-dsRNA nanoparticles to mosquitoes (*Anopheles gambiae* and *Aedes aegypti*) showed more pesticide-susceptible (Zhang et al., 2010, 2015; Mysore et al., 2013; Kumar et al., 2016). Our group and colleagues successfully knocked down the target genes (CAD, AMN, CHS, JHAMT, AMY, V-ATPase, IAP1, V-ATPase B, Sec23, SNF7, and SRC) using chitosan-dsRNA nanoparticles and observed decent mortality in *A. aegypti* and *Spodoptera frugiperda* (Das et al., 2015; Gurusamy et al., 2020a). Also, the complexes of chitosan-sodium tripolyphosphate-dsRNA (CS-TPP-dsRNA) showed improved mortality in *A. aegypti* (Dhandapani et al., 2019). In another study, the chitosan-dsRNA nanopesticides showed good stability, cellular uptake, and mortality in *Chilo suppressalis* (Wang et al., 2020). *Helicoverpa armigera* was significantly controlled when RCNPs were applied topically to chickpea plants (Kolge et al., 2021). Additionally, RCNPs were stable for 5 days on leaf surfaces, effectively protected from nuclease degradation and insect gut pH, and efficiently knocked down the targeted genes (*JHAMT* and *AChE*), resulting in 100% insect mortality, whereas the non-targeted insects like *Spodoptera litura* and *Drosophila melanogaster* were unaffected and showed no signs of toxicity (Kolge et al., 2021, 2023). A recent study demonstrated that topically applying dsRNA-coated with rosin-modified PEG and chitosan (dsRNA/ROPE@C) to *Nilaparvata lugens* (Brown plant hopper) causes excellent gene knockdown and mortality (Lyu et al., 2023). Recently, our team created chitosan-dsRNA nanopesticides, fed them to bollworms (*Earias vittella*), and observed considerable target gene knockdown and mortality (Sandal et al., 2023). Additionally, the price drop from $12500 to $2 for 1 g of dsRNA has increased the likelihood that RNAi technology will be applied in the field (Zotti et al., 2018). Our colleagues successfully applied bacterially expressed dsRNA in a tropical setting and observed a significant reduction in Colorado potato beetle (CPB) infection (Máximo et al., 2020; Petek et al., 2020). Most recently, researchers developed an RNAi-based biopesticide known as "ledprona" against the CPB, which inhibits enzyme expression, facilitates protein breakdown, and ultimately causes mortality (Pallis et al., 2023). These investigations could pave the path for creating and using RCNPs as a safe, effective, and novel way to protect crops and forest trees.

Furthermore, researchers used the chitosan domain to encapsulate metal-based nanoparticles (Ag, Au, Fe, Co, Cu, TiO_2, ZnO, SiO_2, and $CaCO_3$) to increase plant resilience to salt, drought, and heavy metal environments (Souri et al., 2017;

Behboudi et al., 2019; Sen et al., 2020; Ali et al., 2021; Sheikhalipour et al., 2021) and improve their health for protecting themselves from other biotic stresses (Naidu et al., 2023). The previous studies successfully used double-layered hydroxide (LDH), carbon quantum dots (CQD), branched amphiphilic peptide capsules (BAPCs), and lipid nanoparticle-based dsRNA formulations to address biotic stress caused by insects (Mitter et al., 2017; Christiaens et al., 2020; Gurusamy et al., 2020b; Kaur et al., 2020). Such findings encourage researchers to adopt similar approaches to improve forest health. However, the above-mentioned nanomaterials have some limitations, i.e., manufacturing synthetic nanomaterials is expensive, and excessive nanoparticle concentrations may negatively impact forest soil health and microfauna. Dedicated studies can evaluate the feasibility of these nanoparticles in forest protection.

Chitosan encapsulated microbes: new hope against forest insect pests

Chitosan is frequently utilized as a carrier for encasing microbial agents because of its ability to take the form of particles, films, capsules, gels, fibres, and porous forms and its unquestionable success in field applications (Lakkis, 2016; Saberi Riseh et al., 2021). Three potential methods (diffusion, osmotic burst, and erosion or breakdown) will work separately or together and release the microbial substances from chitosan encapsulations. Encapsulating chitosan-microbes (chitosan-ATCC393 and chitosan-139S1) can protect against several environmental challenges (Li et al., 2011; Vejan et al., 2019). Moreover, the Harpinpss-chitosan, BMI-chitosan, *B. thuringiensis*-chitosan, *B. cereus*-chitosan, *E. fergusonii*-chitosan, *B. thuringiensis*-chitosan, and *Pseudomonas*-chitosan encapsulations tested on tomato, soybean, cotton, tobacco, bean, corn, and *Hyaloptera peroni* plants showed a reduction in egg-laying in female insects, thereby reducing the population and insect damage (Badawy and El-Aswad, 2012; Zeng et al., 2012; Chandrashekharaiah et al., 2015; Sahab et al., 2015; Badawy and Rabea, 2016; Kitherian, 2017; Ureña Saborío et al., 2017; Nadendla et al., 2018; De Oliveira et al., 2021). Based on the aforementioned findings, RNAi molecules expressed in microbes that can be encapsulated with chitosan are a viable technology and can be used as RNAi-biopesticides in forest pest management (**Figure 1**). However, such potential demands further dedicated studies and pilot field experiments.

Chitosan-symbiont-mediated RNAi (CSMR): an appealing idea

SMR is a potent tool, and researchers have developed endogenous symbionts to express target dsRNAs for insect control (Chen et al., 2015; Hu and Wu, 2016; Whitten et al., 2016; Hu and Xia, 2019). Recent research identified the bacterial symbionts, used them to express dsRNA effectively, and controlled the two evolutionarily divergent insect species (*R. prolixus* and *F. occidentalis*) (Whitten et al., 2016). Additionally, entomopathogenic fungi were identified and used to induce fungal-induced gene silencing (FIGS) in the insects *B. tabaci* and *L. migratoria* (Chen et al., 2015; Hu and Xia, 2019). Our colleagues from the United States are deploying fungal-induced gene silencing (FIGS) technology to manage bark beetles, i.e., genetically modifying the bark beetle-associated yeast *Ogataea pini*, to generate specific dsRNA molecules that target *Ips calligraphus* (information based on personal communication). Our team has also successfully identified and isolated insect-symbiotic bacteria and fungi (Chakraborty et al., 2020a,b, 2023b) and may use them as a CSMR for tropical application to control the bark beetles and termites (Gupta et al., 2023). Recently, our group identified 69 core bacterial genera and 19 fungal genera among six bark beetles (*Ips typographus*, *Ips duplicatus*, *Ips cembrae*; *Ips sexdentatus*, *Ips acuminatus*, and *Polygraphus poligraphus*). Notably, the most abundant bacterial genera were *Erwinia*, *Sodalis*, *Serratia*, *Tyzzerella*, *Raoultella*, *Rahnella*, *Wolbachia*, *Spiroplasma*, *Vibrio*, and *Pseudoxanthomonas* whereas the most abundant fungal genera belong to the phylum *Ascomycota* (Chakraborty et al., 2020a,b, 2023a). Further, our group focused on exploring how varying ages of Norway spruce wood and different terpene concentrations affect the microbial compositions associated with two termite species, *Reticulitermes flavipes* and *Microcerotermes biroi* (Chakraborty et al., 2023b). In termite-infested wood samples, the relative abundance of bacterial genera like *Pseudomonas*, *Massilia*, and *Rhizobium* was high, and *Spirochaeta* and *Treponema* revealed notable changes in relative abundance between these two species. Moreover, within termite-infested wood, fungal communities affiliated with the *Eurotiales*, *Sordariales*, *Hypocreales*, *Trichospornales*, and *Ophiostomatales* orders were identified, notably, the fungal genera *Apiotrichum*, *Fusarium*, *Hawksworthiomyces*, *Lasiodiplodia*, *Sporothrix*, *Trichosporon*, and *Trichoderma* displayed substantial prevalence in the termite-infested wood. As described thoroughly in our recent review, some identified microbial associates of bark beetle or termites can be good candidates for Symbiont-mediated RNAi or SMR (Gupta et al., 2023). Nevertheless, SMR technology can be considered for its potential in forest conservation; additional refinements are necessary before applications.

Environmental risks and regulatory status of RNAi-chitosan biopesticides

In order to increase forest production and health, RCNPs will be utilized more frequently in forestry and dispersed into the environment. Due to their biodegradable nature, these substances may not harm non-target organisms; they may not bioaccumulate and not interact with other environmental contaminants and dissolved organic matter, which means they will not harm the environment as well as humans and animals (Chandy and Sharma, 1990; Aspden et al., 1997; Rao and Sharma, 1997). RNAi-biopesticides made of chitosan are easily dissolved in nature and unable to accumulate in the food chain, stunt plant growth, or potentially harm people and animals. Although studies have shown that nanoparticles pose a risk to the environment, they have also sparked much interest in environmental cleanup (Roy et al., 2021). Therefore, more research is necessary to comprehend RCNPs and their relationship to the environment thoroughly. Understanding the characteristics of various RNAi-chitosan bioformulation is crucial, as is making comparisons between pure active ingredients and both nanoformulations and traditional formulations to see how the behaviour of the active components changes (Kah et al., 2018).

Future of RCNPs in forestry: a long way to go

Applications for RCNP in the forest have numerous obstacles, including developing different delivery strategies for various microbes and insect pests, facilitating plant uptake and *in planta* systemic movement of RCNPs, looking for synergistic effects, such as dsRNAs targeting multiple genes and combining RNAi with other pest control methods, and establishing a congruent confluence, building a regulatory framework that is widely accepted for the commercialization, therefore lowering the price for their widespread use. Recently, RCNPs were used in the field, and the result demonstrated their compact size, cationic charge, effective loading, resistance to degradation, effective cellular uptake, stability, and adhesion to leaf surfaces (Petek et al., 2020; Kolge et al., 2021, 2023). Few RNAi-based insecticides have so far been licensed and will soon be available on the market (Li et al., 2023; Pallis et al., 2023).

The advancement of RNAi-nanotechnology has been beneficial to forestry. However, strict controls are in place for forests to ensure the security of feed and food sources, possible risks to human and animal health, non-target organisms and beneficial microbes, and the long-term environmental effects of the deliberate release of RNAi-nanomaterials (Kumar et al., 2019; Gilbertson et al., 2020; Hofmann et al., 2020; Mogilicherla et al., 2022). The European Union is developing regulatory rules for engineered RNAi-nanomaterials in forestry food safety (Lowry et al., 2019). Preparing regulatory guidelines for RNAi-nanomaterials is more difficult due to several factors, including the difficulty in defining nanomaterials, tracing their sources and transport pathways, quantifying them in environmental samples, assessing their bioavailability, and interpreting their toxicity (Lai et al., 2018; Hofmann et al., 2020; Gottardo et al., 2021). Under such circumstances, creating cutting-edge analytical methods for regulatory purposes is necessary.

The capacity of researchers and scientists to develop forest pest-specific dsRNAs will increase as more omics data for forest insects, helpful microorganisms, and non-target organisms become accessible and help to reduce possible risks. Fortunately, our group and CZU colleagues (CZU, Prague) have recently involved forest insects (bark beetles and termites) genome and transcriptome sequencing and their symbiotic microbes transcriptome sequencing, which along with other excellent efforts from colleagues worldwide, will significantly enhance sequence information on forest insect pests and facilitate future species-specific RNAi-based biopesticides development.

Conclusion

RCNPs may replace currently used pesticides since they are biodegradable, biocompatible, and low toxicity (**Figure 1**). Chitosan nanoparticles can encapsulate different RNAi molecules (dsRNA/siRNA) and RNAi-symbiotic microbes and form RCNPs. They are more effective and have better bioavailability, a longer half-life, and a higher surface-to-volume ratio and act as a bio-stimulant used to combat microbial illnesses and insect pests in forest management. RCNPs can be applied in forests using various techniques, including foliar application, trunk injection, and soil drenching. Based on current findings, using RCNPs can also increase forest productivity, protect forests from insect pests, and extend their commercialization. However, research on product development and technique optimization is required before commercial manufacture and environmental application. Nevertheless, this perspective will provide new direction to the research community working on forest protection and enhance their interest in using alternative approaches, such as deploying molecular toolboxes against forest insect pests.

Data availability statement

The original contributions presented in this study are included in the article, further inquiries can be directed to the corresponding author.

Author contributions

AR contributed to the conceptualization. KM wrote the first draft. Both authors contributed to the figure preparation, reviewing, and finalizing the draft.

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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

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Predicting potential distributions of *Monochamus saltuarius*, a novel insect vector of pine wilt disease in China

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Monochamus saltuarius Gebler was first identified as a new vector of pine wilt disease in Northeast China in 2018, and monitoring of *M. saltuarius* has become a key strategy to prevent and control the disease in this region. However, the potential distributions of *M. saltuarius* in China are unclear. In this study, we clarified bioclimatic environmental variables affecting the distribution of *M. saltuarius*, predicted the geographically suitable habitats of *M. saltuarius* in current and future climate conditions, and determined changes in the spatial pattern of a suitable distribution area of *M. saltuarius* under current and future climate conditions. This is the first study to use the optimized maximum entropy model and ArcGIS to accurately predict suitable geographical areas for *M. saltuarius* based on different climatic conditions in China. and the average area under the receiver operating characteristic curve reached 0.954 ± 0.0024. Of the 32 bioclimatic variables, temperature seasonality, precipitation of wettest month, precipitation seasonality, maximum temperature of the warmest month, and elevation played significant roles in determining the potential distribution of *M. saltuarius*, with contribution rates to the model of 32.1, 31.8, 11.5, 7.5, and 6.5%, respectively. Under the current climate scenario, the predicted suitable areas for *M. saltuarius* were mainly at latitudes north of 33° in China, and larger suitable areas were mainly distributed in Northeast China and North China, with areas of 87.04×10^4 and 73.15×10^4 km^2, respectively. Using future climatic scenarios SSP126 and SSP585, the predicted suitable areas of *M. saltuarius* will continue to expand from that of 2040, 2070, and 2100, with highly and moderately suitable areas showing larger increasing trends but low suitable distribution areas will decrease to varying degrees. The potential suitable areas of *M. saltuarius* may increase greatly in Northwest, Central, and Eastern China. This study provides important scientific theoretical knowledge for effectively controlling and preventing *M. saltuarius* and pine wilt disease in northern China.

KEYWORDS

pine wilt disease, *Monochamus saltuarius*, *Bursaphelenchus xylophilus*, MaxEnt, climate change

1. Introduction

Bursaphelenchus xylophilus (Steiner and Buhrer) Nickle can cause pine wilt disease (PWD), which systematically infects and causes wilting of healthy pines (Sun, 1982; Mamiya, 1983). *Bursaphelenchus xylophilus* leads to high mortality of conifer trees, including of *Pinus* plants and non-*Pinus* plants such as Picea, Larix, Abies, and Cedrus (Nunes et al., 2013; Foit et al., 2019). PWD was first discovered in 1982 in *Pinus thunbergii* Parl. in Nanjing, Jiangsu province of China (Sun, 1982). Since then, it has become one of the most destructive forest quarantine pests, causing very large economic and ecological losses (Gao et al., 2017; Ye and Wu, 2022). As of February 2023, PWD had spread to 700 county-level administrative regions in 19 provinces of China (**Figure 1**) and continuously spread to the northern and western parts of China (Li et al., 2022; Ye and Wu, 2022; Zhang et al., 2022; Zong and Bi, 2022).

As a plant parasitic nematode, *B. xylophilus* lives in the xylem of host pines, and its natural transmission relies on insect vectors that are transported from infected host plants to healthy plants (Aikawa, 2008; Li M. et al., 2021). Currently, the main vector insects that can carry *B. xylophilus* are beetles in the genus *Monochamus* (Cerambycidae) (Linit et al., 1983; Kobayashi et al., 1984; Linit, 1988; Li et al., 2020; Li M. et al., 2021). The 4th dispersal juveniles of *B. xylophilus* attach to the surface respiratory trachea and reproductive systems of the vector insect before eclosion (Pan et al., 2020). When vector insects eclose from pines killed by *B. xylophilus*, they feed on a new healthy host plant and release *B. xylophilus*, which then infects the healthy host tree (Balestrini et al., 2009; Zhao et al., 2013; Zhang et al., 2020). Therefore, vector insects are an important link in the PWD infection system, and efficient control of vector insects is the most important measure for preventing PWD (Linit et al., 1983; Kobayashi et al., 1984; Linit, 1988; Li et al., 2020, 2022; Li M. et al., 2021 Ye and Wu, 2022).

Monochamus saltuarius is among the main vector insects of *B. xylophilus* in Southeast Asia, including China, South Korea, and Japan (Takizawa and Shoji, 1982; Sato et al., 1987; Jikumaru and Togashi, 1995; Kim et al., 2006; Koo et al., 2013; Han et al., 2016; Yu and Wu, 2018; Li et al., 2020; Li M. et al., 2021). Before 2016, *Monochamus alternatus* was the only insect vector of *B. xylophilus* in China and was widely distributed south of the Yellow River; its northern boundary was Dalian, Liaoning province (Li et al., 2007; Wu et al., 2013; Gao et al., 2023). However, in recent years, *M. saltuarius* has been considered as a novel vector of *B. xylophilus* in PWD-infected pines in high-latitude and newly invaded areas of China, including in Jilin and Liaoning provinces (Yu and Wu, 2018; Li et al., 2020; Li M. et al., 2021). *Monochamus saltuarius* plays a key role in carrying, spreading, and assisting the pathogen *B. xylophilus* to enter its host, and in its transmission efficiency and harmful effects (Ye, 2019; Li et al., 2022; Ye and Wu, 2022).

Monochamus saltuarius is a native species that is widespread in northern China and was a common pest in Shanxi, Inner Mongolia, Liaoning, Jilin, and Heilongjiang provinces before being identified as a new vector insect of *B. xylophilus* in these areas (Ye, 2019; Li M. et al., 2021; **Figure 2**). Moreover, by feeding on host plants, *M. saltuarius* can enable full-scale invasion of *B. xylophilus* and directly threaten healthy host trees in northern China (Chen et al., 1959; Wang, 2014; Yu et al., 2019). However, studies predicting *M. saltuarius* invasion in China have not been

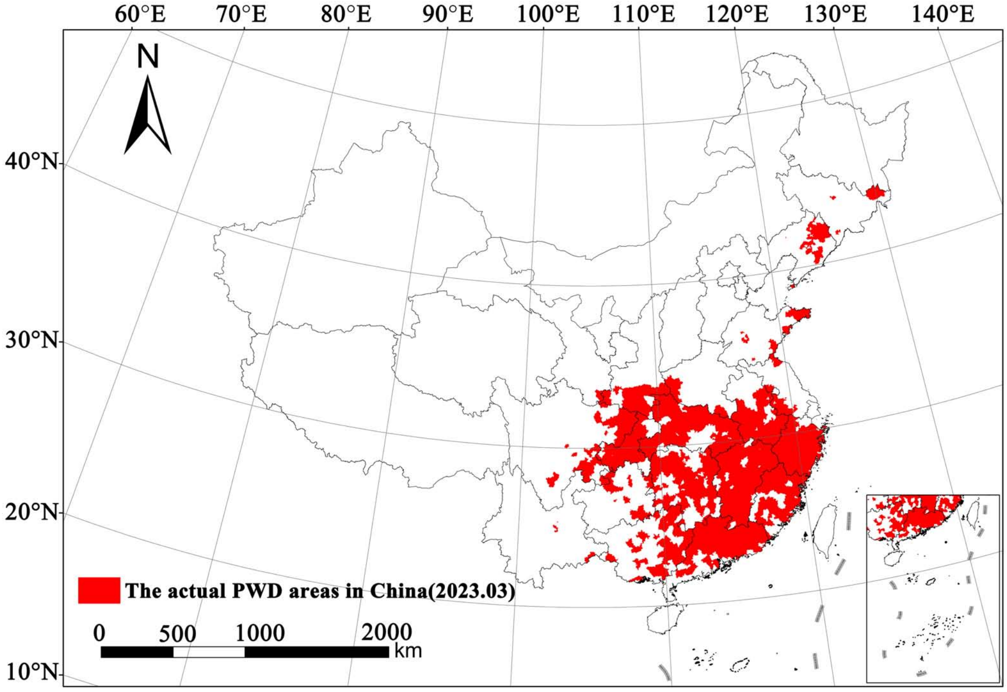

FIGURE 1
Actual epidemic distribution areas of pine wilt disease in China by March 2023 (Data obtained from the No. 7 bulletin of National Forestry and Grassland Administration [NFGA], 2023).

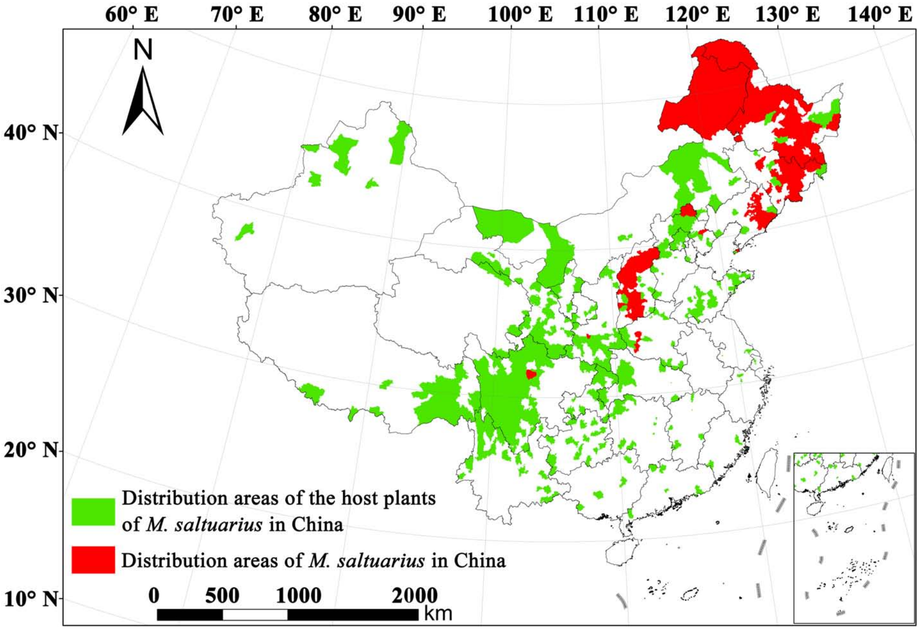

FIGURE 2
Distribution areas of *Monochamus saltuarius* in China.

TABLE 1 The contribution rates of selected variables affecting the geographical distribution of *M. saltuarius*.

Code	Variables	Contribution rate/%
Bio4	Temperature seasonality (°C)	32.1
Bio13	Precipitation of wettest month (mm)	31.8
Bio15	Precipitation seasonality (mm)	11.5
Bio5	Max temperature of warmest month (°C)	7.5
Elev	Elevation (m)	6.5
Bio9	Mean temperature of driest quarter (°C)	6.0
Wind3	Wind speed in March (m/s)	2.8
Wind9	Wind speed in September (m/s)	1.8

TABLE 2 The performance of MaxEnt model under default and optimized settings.

	Default	Optimization
RM	1.0	0.5
FC	LQHP	LQHP
Mean AUC	0.904	0.906
AUC_{DIFF}	0.058	0.054
OR_{MTP}	0.076	0.076
OR_{10}	0.355	0.360
ΔAICc	39.274	0

RM means regularization multiplier, FC means feature combination, AUC means area under the ROC curve, AUC_{DIFF} means the difference between the training AUC and the test AUC, OR_{MTP} means the "Minimum Training Presence" omission rate, OR_{10} means the 10% training omission rate, ΔAICc means Akaike information criterion.

performed, preventing accurate prevention and control measures for *M. saltuarius* and PWD.

There is an urgent need to carry out the research on the distribution and change of the potential suitable areas of *M. saltuarius* in China. An optimized MaxEnt model based on the 175 latest county-level geographical distribution points was used to predict a suitable distribution of *M. saltuarius* in China during different climatic conditions. The main objectives of this study were to (1) clarify the related bioclimatic environmental variables affecting the distribution of *M. saltuarius*, (2) predict geographically suitable areas for *M. saltuarius* under different climate conditions, and (3) determine changes in the spatial pattern of *M. saltuarius* in different climate conditions. This study provides important scientific theoretical knowledge for the effective control and prevention of *M. saltuarius* and PWD in northern China.

2. Materials and methods

2.1. Data collection

A total of 175 county-level geographical distribution points of *M. saltuarius* was obtained from four sources. First, 58 distribution points were obtained from field surveys of the distribution of *M. saltuarius* in different regions of China. Second, 69 distribution points were obtained from published references. Third, seven distribution points of *M. saltuarius* were obtained from the National Animal Specimen Database.[1] Fourth, 41 distribution points were obtained from the relevant databases and official websites. To remove the influence of spatial autocorrelation and

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[1] http://museum.ioz.ac.cn/index.html

sampling bias, the distribution point data of *M. saltuarius* were imported into ArcGIS10.7, and the buffer analysis function in the software was used for sparse processing of distribution points to ensure that there was one *M. saltuarius* distribution point within areas of 25 km^2.

2.2. Selection of variables

A total of 19 bioclimatic variables, 12 monthly average values about the wind speed as historical weather data and terrain elevation were downloaded from the WorldClim website (**Supplementary Table 1**). Future climate data were simulated using SSP126 and SSP585 of Beijing Climate Central Climate System Model 2 Medium Resolution (BCC-CSM2-MR), and the simulated time periods included the years 2050, 2070, and 2100. To avoid autocorrelation between bioclimatic variables and overfitting (Sillero, 2011; Fotheringham and Oshan, 2016; Li X. et al., 2021; Gao et al., 2023), the 32 variables were screened and removed when the MaxEnt selected variables affecting the distribution of *M. saltuarius*. This step was performed to control the impact of redundant information

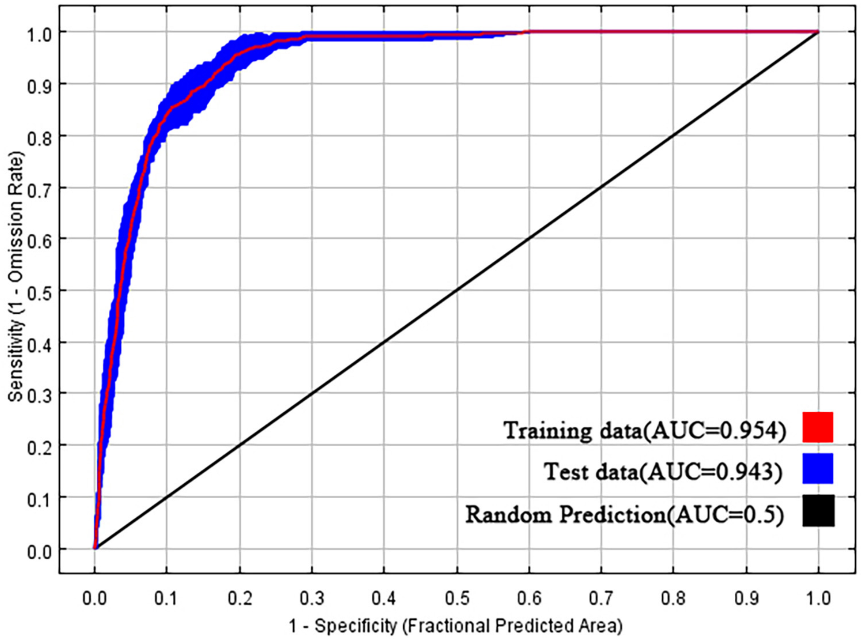


FIGURE 3
Receiver operating characteristic curve and areas under the curve for the optimized MaxEnt model.

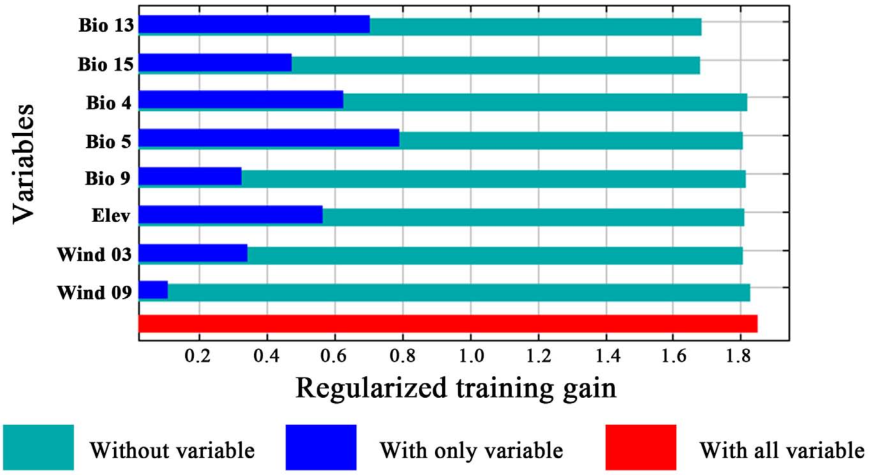


FIGURE 4
Importance of key bioclimatic variables in MaxEnt model for the distribution of *Monochamus saltuarius* determined using the Jackknife test. "Without variable" represents the regularized training gain of the model without this variable, "With only variable" represents the regularized training gain of the model with only this variable, "With all variable" represents the regularized training gain of the model with all variables.

on the simulation results and retain the environmental variables that limited the distribution of *M. saltuarius*. Initially, the MaxEnt was used to model the 32 variables, and the contribution rate of each variable was calculated. We continued to process bioclimatic variables using Pearson correlation analysis and removed variables with correlation coefficients higher than 0.8 (**Supplementary Figure 1**). Finally, eight variables were selected from 32 bioclimatic variables to predict the potential geographical distribution of *M. saltuarius* (**Table 1**).

2.3. Model setting and analysis

The feature combinations and regularized multipliers of the model were optimized using the "ENMeval" package in R to screen the optimal combination and reduce the model complexity, and then randomly selected 75% of the data for model training and 25% for model testing. The regularization multiplier value of the MaxEnt model was set to change from 0.5 to 4 with an increase of 0.5 each time. Moreover, the accuracy of the MaxEnt was evaluated as the area under the receiver operating characteristic curve (AUC). A larger AUC value indicates higher model accuracy, and the evaluation criteria of the model were as follows: failure, poor, fair, good, and excellent. Finally, we used the Jenks' natural breaks method (Qi et al., 2015; Ge et al., 2021) to reclassify the predicted suitable habitats for *M. saltuarius* into four categories, namely non-suitable (0–0.09), low suitable (0.09–0.0.28), moderately suitable (0.28–0.5), and highly suitable (0.5–1.00).

3. Results

3.1. Optimization and evaluation

The optimized MaxEnt parameters were adjusted to feature combination = LQHP, regularization multiplier = 0.5, ΔAICc = 0, and AUC_{DIFF} = 0.054 (**Table 2**). Evaluation of the optimized MaxEnt model showed that the average AUC (0.954 ± 0.0024) of the simulation run results of the MaxEnt model repeated 10 times was higher than 0.9 (**Figure 3**), demonstrating that the prediction results reached an high standard. Therefore, the MaxEnt model, which was set to optimize parameters.

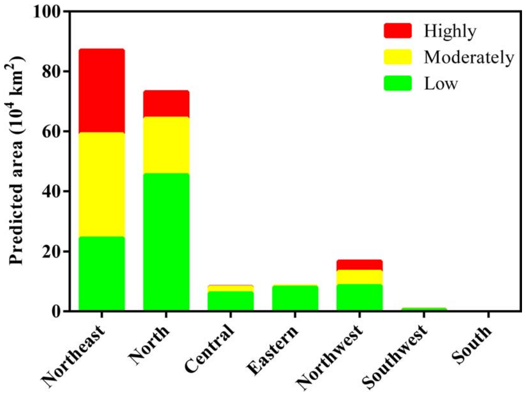


FIGURE 6
Predicted suitable distribution areas of *Monochamus saltuarius* in different regions of China under current climate conditions.

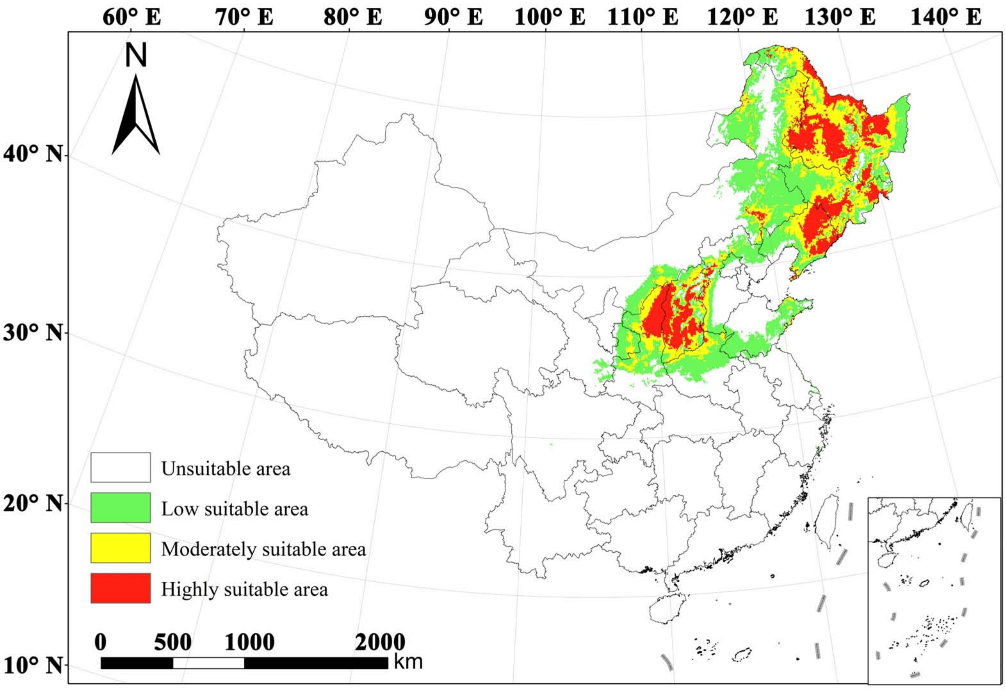
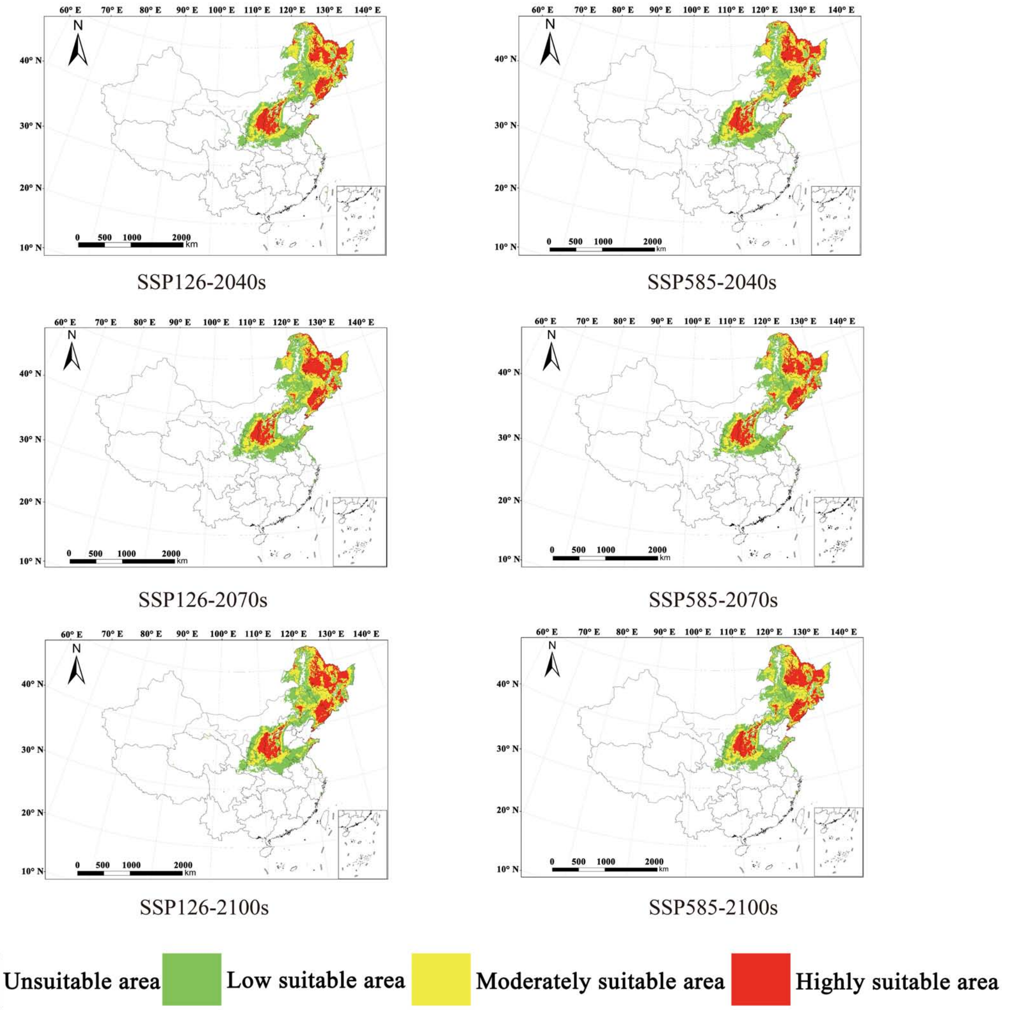


FIGURE 5
Predicted map of suitable distribution areas of *Monochamus saltuarius* in China under current climate conditions.



SSP126-2040s

SSP585-2040s

SSP126-2070s

SSP585-2070s

SSP126-2100s

SSP585-2100s

FIGURE 7
Predicted map of potential suitable distribution areas of *Monochamus saltuarius* in China under the future climate conditions of SSP126 and SSP585.

3.2. Key bioclimatic environmental variables

Eight variables were screened to predict the potential geographical distribution of *M. saltuarius*. Among them, the bioclimatic variables Bio4, Bio13, and Bio15 showed higher contribution rates, with a cumulative contribution rate of 75.4% (**Table 2**). Furthermore, the importance results of the selected bioclimatic variables using the Jackknife test showed that Bio4, Bio5, Bio13 and Elev were the variables with higher regularized training gains were when only one bioclimatic variable was used (**Figure 4**). Therefore, the key bioclimatic variables restricting the distribution of *M. saltuarius* were Bio4, Bio5, Bio13, Bio15, and Elev.

3.3. Current risk areas

Based on the historical climatic data and current distribution data, a suitable area for *M. saltuarius* was predicted using the current climate scenario (**Figure 5**), and the predicted total area was approximately 193.59 × 10^4 km^2, accounting for approximately 20.10% of the total land area of China. The predicted areas of high, moderate, and low suitability for *M. saltuarius* were 40.26 × 10^4, 60.76 × 10^4, and 92.57 × 10^4 km^2, accounting for 20.8, 31.38, and 47.82% of the total predicted suitable areas, respectively. The predicted suitable habitats of *M. saltuarius* were mainly at latitudes north of 33° in China, and the larger suitable areas were mainly distributed in Northeast and North China, with areas of 87.04 × 10^4 and 73.15 × 10^4 km^2, respectively (**Figure 6**). There were some suitable distribution areas for *M. saltuarius* in Central, East, and Northwest China, with almost no predicted areas of *M. saltuarius* in Southwest and South China.

3.4. Future risk areas

We predicted the potentially suitable areas of *M. saltuarius* in 2040, 2070, and 2100 using future climatic scenarios (**Figure 7**). The predicted potentially suitable areas of *M. saltuarius* continued to expand in future climatic scenarios (**Figure 8**). Moreover, the center point of the suitable distribution area at different

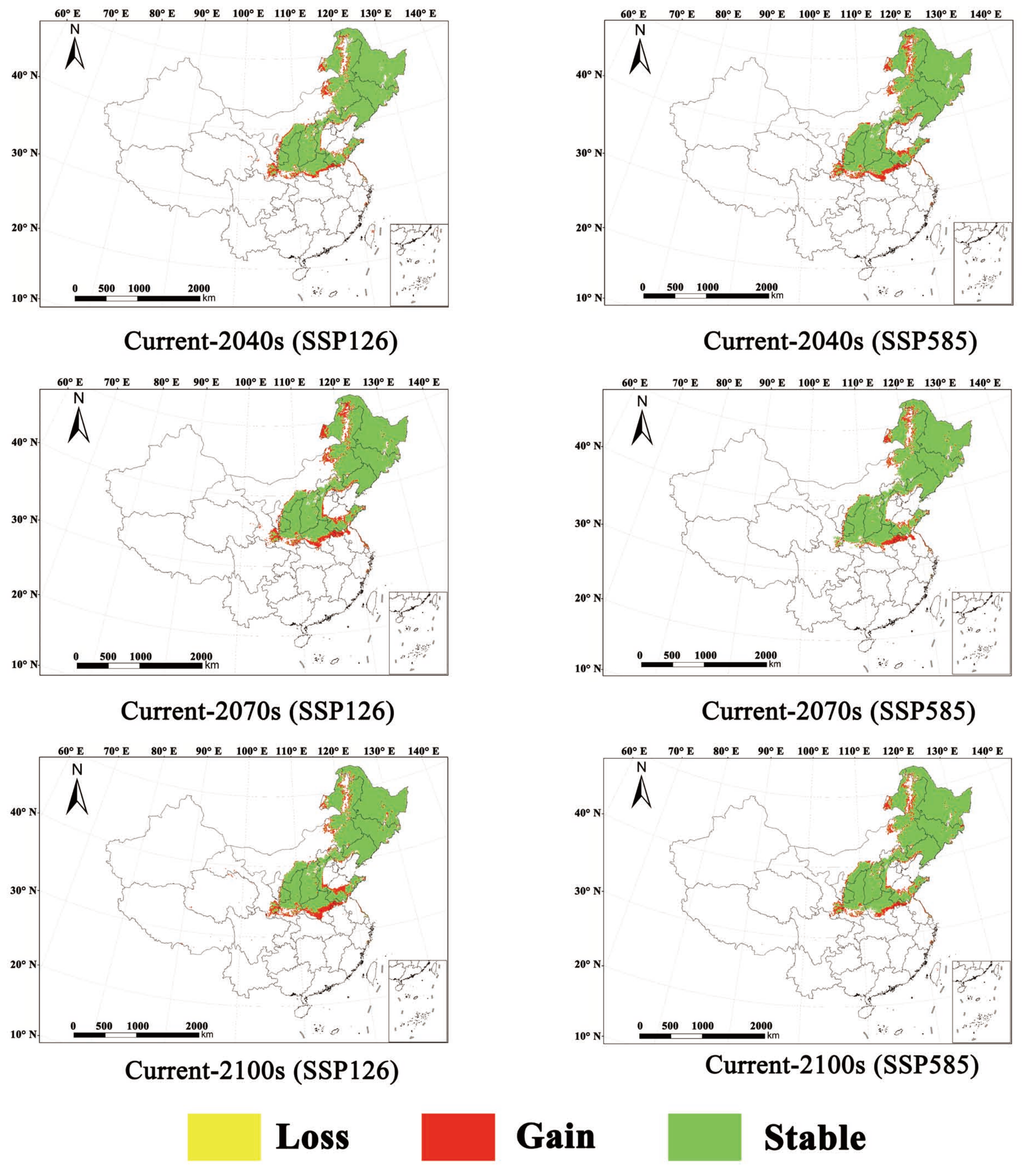


FIGURE 8
Changes in potential suitable areas of *Monochamus saltuarius* in different future climate conditions compared with the current areas.

times of *M. saltuarius* were predicted to shift with future climate change, showing an obvious spreading trend to the south and west (**Figure 9**). The range of centroid shifting occurred in Inner Mongolia. Moreover, highly and moderately suitable areas showed a larger increasing trend, but areas with low suitability distributions decreased to varying degrees (**Table 3**).

Similar to the current climate, the predicted areas of *M. saltuarius* under future climatic conditions are concentrated in Northeast and North China (**Figure 10**). In Northeast China, highly suitable areas for *M. saltuarius* showed an increasing trend in future climatic conditions, whereas moderately and low-suitability areas generally showed a decreasing trend. In North China, highly suitable and moderately suitable areas for *M. saltuarius* showed a rising trend for future climatic conditions but low-suitability areas showed an overall decreasing trend. Moreover, there was a large increasing trend in the suitable distribution areas of *M. saltuarius* in Northwest, Central, and Eastern China (**Table 4**); however, there was almost no distribution area of *M. saltuarius* in Southwest and South China.

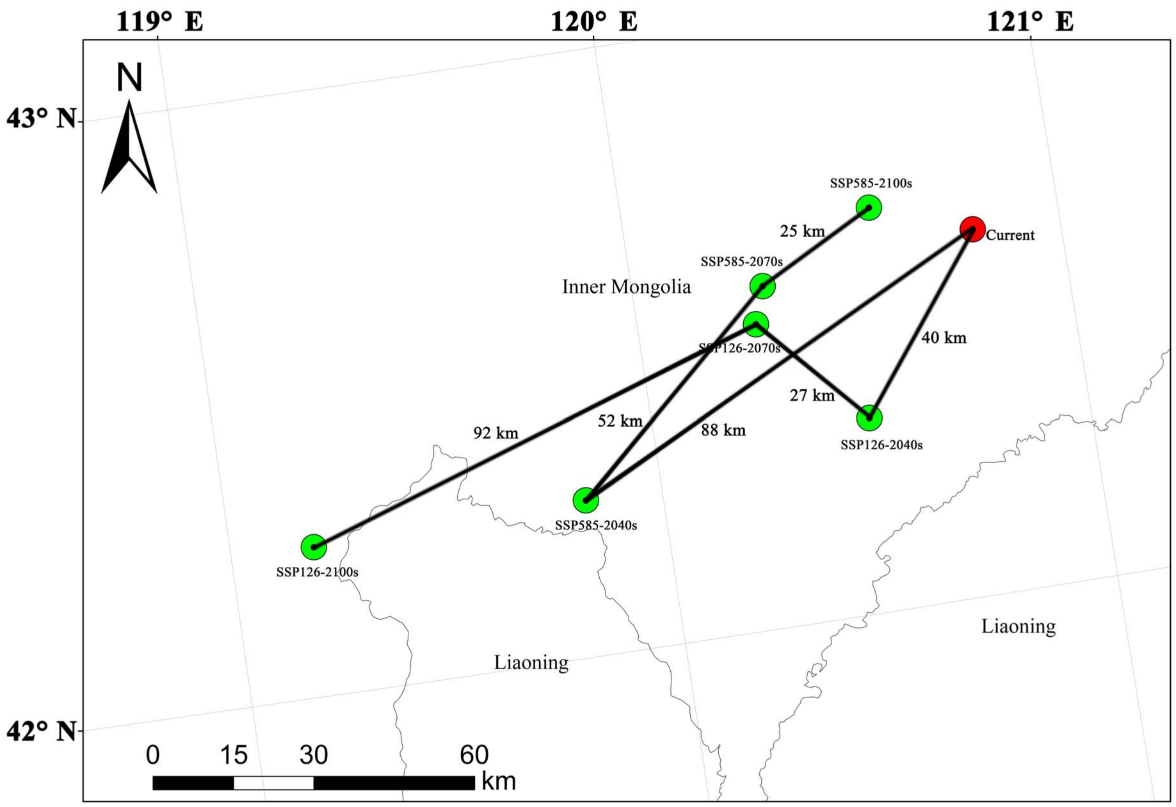


FIGURE 9
Changes in geographical centers of potential distribution areas of *Monochamus saltuarius* in different periods.

TABLE 3 The difference in potential suitable areas for *M. saltuarius* under current and future climate scenarios.

Decade	Scenarios	Total	Predicted area (10^4 km^2)			Comparison with current (%)		
			Highly	Moderately	Low	Highly	Moderately	Low
Current	–	193.59	40.26	60.76	92.57			
2040s	ssp-126	216.10	54.60	70.73	90.77	35.62	16.41	−1.94
	ssp-585	221.58	58.14	77.79	85.65	44.41	28.03	−7.48
2070s	ssp-126	222.47	57.81	73.05	91.61	43.59	20.23	−1.04
	ssp-585	213.85	56.19	69.27	88.39	39.57	14.01	−4.52
2100s	ssp-126	218.78	58.12	73.22	87.44	44.36	20.51	−5.54
	ssp-585	216.60	58.12	71.39	87.09	44.36	17.50	−5.92

4. Discussion

Monochamus saltuarius Gebler was first identified as a insect vector of PWD in Northeast China in 2018 (Yu and Wu, 2018; Li et al., 2020, Li M. et al., 2021). Monitoring *M. saltuarius* has become a key strategy for the prevention and control of PWD in this region (Ye, 2019; Li M. et al., 2021, Li et al., 2022; Ye and Wu, 2022). Therefore, understanding the potential distribution of *M. saltuarius* can facilitate the prevention and control of *M. saltuarius* and PWD.

The MaxEnt model is a widely used species distribution model to predict the geographic spatial area of target species and exhibits highly accurate prediction when only the "existence only" distribution data of the target species are used (Ge et al., 2018; Raffini et al., 2020; Lee et al., 2021; Ramasamy et al., 2022; Gao et al., 2023). The maximum entropy distribution of a species under environmental constraints in a specific area can be estimated using the accurate geographical location of species occurrence and related biological environmental variables (Jackson and Robertson, 2011; Mitchell et al., 2016; Cao et al., 2021). However, predicting species distribution may be limited when using the MaxEnt model. Setting the default parameters of the MaxEnt model may lead to excessive model fitting, (Warren and Seifert, 2011; Muscarella et al., 2014; Jin et al., 2022). Hence, the "ENMeval" packet in R software was used to optimize the default parameters of the MaxEnt model to reduce the fit degree of the model (Warren and Seifert, 2011; Muscarella et al., 2014; Porfirio et al., 2014; Yan et al., 2021). The MaxEnt model was optimized through ENMeval package, and it was found that when the feature combination = LQHP and the

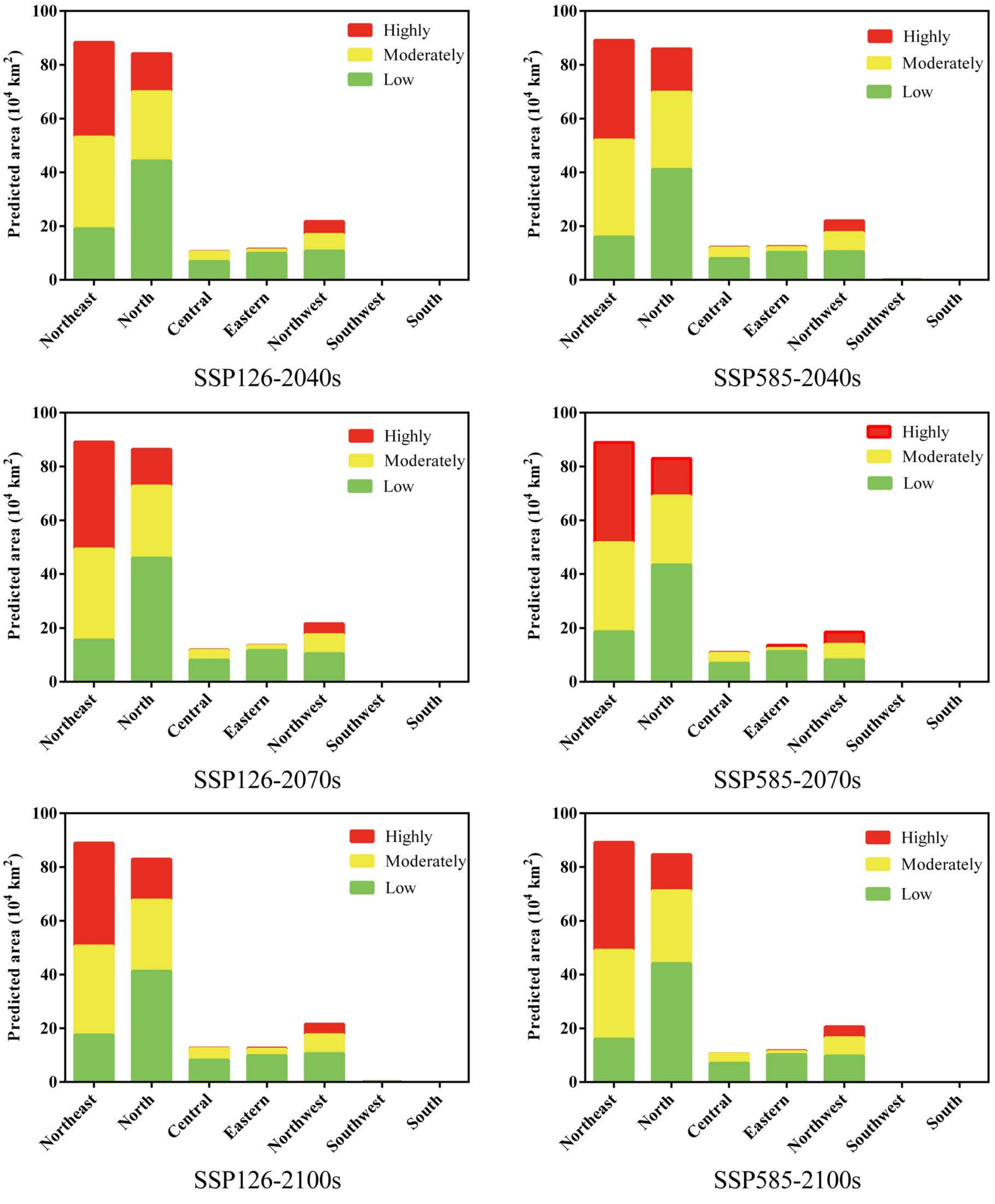


FIGURE 10
Predicted suitable distribution areas of *Monochamus saltuarius* in different regions of China under future climatic conditions.

regularization multiplier = 0.5, the model was the optimal model, and the predicted range of the suitable area of *Pinus sylvestris* var. *mongolica* was basically consistent with the actual distribution (Zhang et al., 2023).

In typical ectotherms, environmental variables can significantly affect the diversity, richness, and geographical distribution of insect species (Austin, 2002; Kreft and Jetz, 2007; Tang et al., 2021). The selected eight key bioclimatic variables in this study may significantly affect the distribution of *M. saltuarius*. The results showed that Bio4, Bio5, Bio13, Bio15, and Elev played important roles in restricting the suitable distribution of *M. saltuarius*. In addition, Bio4 and Bio13 contributed relatively more to the distribution of *M. saltuarius*, indicating that *M. saltuarius* is highly sensitive to fluctuations in precipitation and temperature. The change in temperature is an important driving factor affecting the growth, development, and diffusion of *M. saltuarius*, and changes in temperature due to global warming will inevitably affect

TABLE 4 The difference in potential suitable areas for *M. saltuarius* in different regions of China under future climate scenarios compared with the current.

Decade	Scenarios	Northeast (%)			North (%)			Central (%)			Eastern (%)			Northwest (%)		
		Highly	Moderately	Low	Highly	Moderately	Low	Highly	Moderately	Low	Highly	Moderately	Low	Highly	Moderately	Low
2040s	ssp-126	26.27	−2.07	−21.84	59.82	36.49	−2.62	85.71	78.89	11.24	3,300	223.68	23.07	39.31	28.93	25.41
	ssp-585	32.94	3.94	−34.75	83.20	52.12	−9.61	242.86	96.98	29.32	3,000	394.74	27.06	25.43	50.31	22.13
2070s	ssp-126	42.62	−2.88	−36.27	54.71	42.06	0.97	142.86	81.91	30.94	1,100	368.42	45.01	18.50	48.22	22.25
	ssp-585	33.58	−4.92	−23.77	58.46	35.70	−4.49	257.14	86.93	12.54	11,300	200.00	40.52	29.48	34.17	6.56
2100s	ssp-126	37.17	−4.84	−28.11	72.99	40.10	−9.24	171.43	115.58	33.06	4,100	536.84	22.19	15.03	46.33	24.00
	ssp-585	43.91	−5.01	−34.34	52.67	43.43	−3.06	57.14	72.86	14.01	3,000	194.74	27.43	18.21	41.51	13.82

its distribution region (Cornelissen et al., 2019; Daniel et al., 2020; Jin et al., 2022). In addition, the emergence period of adults of *M. saltuarius* is concentrated from May to August (Ochi, 1969; Jikumaru and Togashi, 1996; Han et al., 2007, 2009). This species is mainly active in the daytime, and its flight distance is generally not more than a few hundred meters (Ciesla, 2021); therefore, precipitation during this period affects the flight and dispersal of *M. saltuarius* (Gao et al., 2019; Zhao et al., 2021). Elevation was an important variable that significantly affected the potential distribution of *M. saltuarius*, possibly because of the close relationship between the host plant distribution and altitude, which also greatly affected the feeding and oviposition preferences of *M. saltuarius* (Zhao et al., 2021).

To predict the potential distribution areas of *M. saltuarius*, we previously obtained 175 distribution points of *M. saltuarius* from published references, authoritative databases, and official websites, and combined these data with field survey data. Each distribution point had accurate data sources. The prediction results of the MaxEnt model indicated that the potential distribution area of *M. saltuarius* under current climatic conditions included actual geographical distribution points, indicating that the optimized model was highly reliable and accurate (Lee et al., 2021; Gao et al., 2023).

Studies are urgently needed to accurately analyze and predict the natural distribution patterns and potential distribution areas of *M. saltuarius* in China. Under current climatic conditions, the predicted suitable habitats of *M. saltuarius* were mainly at latitudes north of 33°N in China, and the larger suitable areas were mainly distributed in Northeast China and North China. Moreover, in areas with large numbers of geographical distribution points of *M. saltuarius*, such as in Shanxi province, Heilongjiang province, Jilin province, Liaoning, and Inner Mongolia, the suitable areas for *M. saltuarius* will be further expanded under future climate conditions. Furthermore, the highly and moderately suitable areas of *M. saltuarius* have spread to the eastern Gansu province, northeastern Shaanxi province, northern Henan province, and southeastern Shandong province; currently, there is almost no geographical distribution of *M. saltuarius* in these areas. Therefore, local forestry departments should increase their monitoring efforts to prevent *M. saltuarius* from invading these areas. Our results also show that there are almost no suitable distribution areas for *M. saltuarius* in Southwest China and South China; however, *M. saltuarius* may occur in these areas in the future. Furthermore, the impacts of human activities and natural enemies on *M. saltuarius* should be considered when predicting potentially suitable distribution areas (Choi et al., 2017; Takahashi and Park, 2020; Gao et al., 2023).

As an insect vector of PWD in northern China, *M. saltuarius* can cause serious harm to the ecological environment and economic production in invaded areas (Yu and Wu, 2018; Li et al., 2020, Li M. et al., 2021). Previous studies showed that the effective control of *M. saltuarius* and other vector insects is among the most useful measures for preventing the occurrence of PWD (Linit et al., 1983; Kobayashi et al., 1984; Linit, 1988; Li et al., 2020, 2022; Li M. et al., 2021; Ye and Wu, 2022). Particularly, in areas where PWD is not currently occurring, *M. saltuarius* is a common insect, such as Shanxi, Heilongjiang, Jilin, and Liaoning provinces, and PWD monitoring should be a focus when evaluating vector insects.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

RG: conceptualization and writing-original draft preparation. LL, RL, SF, and JD: data curation. RG and LL: formal analysis. RG and LZ: writing-review and editing. All authors have read and agreed to the published version of the manuscript.

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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

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Field effects of oxygenated monoterpenes and estragole combined with pheromone on attraction of *Ips typographus* and its natural enemies

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Introduction: Central European Norway spruce monocultures face *Ips typographus* outbreaks due to decreasing resistance. These beetles use volatile compounds to communicate and select suitable host trees. Spruce trees, beetles, and their symbiotic ophiostomatoid fungi emit oxygenated monoterpenes, including 1,8-cineole, α-terpineol, camphor, carvone, terpinen-4-ol, isopinocamphone, and pinocamphone, and the phenylpropanoid estragole, particularly in the infestation phase. These compounds trigger strong responses in *I. typographus* antennae, motivating our field study.

Objective: This study aimed to assess how adding these compounds to the aggregation pheromone of *Ips typographus* modulates the attraction of this bark beetle and its natural enemies.

Methods: In combination with *I. typographus* pheromone, estragole, 1,8-cineole, (±)-camphor, (−)-carvone, *alpha*-terpineol, (−)-terpinen-4-ol, (+)-pinocamphone, and (+)-isopinocamphone at low, medium, and high doses were tested in pheromone traps at two sites in the Czech Republic.

Results: All 1,8-cineole doses and the high estragole dose acted as anti-attractants for *I. typographus*, whereas all (+)-isopinocamphone doses enhanced their attraction to pheromone. Catches of natural enemies, the Staphylinidae and Pteromalidae, varied by location.

Conclusion: 1,8-cineole, isopinocamphone, and estragole may play vital roles in tritrophic interactions among spruce trees, and *I. typographus* and its natural enemies, and these compounds may be developed into new or enhanced semiochemical-based pest control methods.

KEYWORDS

Eurasian spruce bark beetle, host compounds, Pteromalidae, Staphylinidae, Norway spruce, *Picea abies*, spruce kairomone, pheromone traps

1 Introduction

In Central Europe, Norway spruce (*Picea abies*) (L.) Karst. (Pinales: Pinaceae) has been severely affected by infestations of the spruce bark beetle, *Ips typographus* (L.) (Coleoptera: Curculionidae: Scolytinae), that in the Czech Republic have resulted in timber losses of 5.9 mil. m^3 in 2017 and 26.2 mil. m^3 in 2020 (Hlásny et al., 2022). In outbreak regions, managing bark beetles often involves applying insecticides to *P. abies* trunks or stored timber to eliminate the emerging beetles (Fettig and Hilszczański, 2015). However, the use of pesticides can negatively impact the forest ecosystem, including beneficial bark beetle predator species (Hlásny et al., 2019). The development of alternative, eco-friendly strategies in forestry is a logical progression. One such strategy involves utilizing semiochemicals, compounds that mediate the interactions of beetles with each other and other organisms. These signals enable beetles to locate a mate or host tree by providing intraspecific and interspecific chemical information (Bergström, 2007). Aggregation pheromone components, produced by male beetles after successful colonization (Birgersson et al., 1984; Ramakrishnan et al., 2022), have been employed for monitoring and controlling *I. typographus* populations (Heber et al., 2021). Furthermore, recent research has focused on the management potential of kairomones, compounds originating from both host spruce trees and non-host trees, e.g., broadleaf trees (Zhang and Schlyter, 2004; Jakuš et al., 2022). Bark beetles possess a sophisticated olfactory system that enables them to detect and distinguish the chemical composition and quantity of these odors (Andersson, 2012).

The principal olfactory stimulants for *I. typographus* emitted by *P. abies* are primarily composed of high-abundance monoterpenes such as α-pinene (23–39%), β-pinene (25–58%), β-phellandrene (5–19%), limonene (1.5–4%), myrcene (1.6–3.4%), Δ-3-carene (0.6–1.1%), and camphene (0.2–1.1%; Netherer et al., 2021). However, recent comparative analysis utilizing *I. typographus* antennae as biological detectors (gas chromatography coupled with electroantennography, GC-EAD) has identified several novel compounds present in relatively small amounts but exhibiting high activity with the beetles' antennae (Kalinová et al., 2014; Schiebe et al., 2019). These compounds include oxygenated monoterpenes, 1,8-cineole (eucalyptol), *trans*-4-thujanol (sabinene hydrate), camphor, pinocarvone, pinocamphone, isopinocamphone, terpinen-4-ol, alpha-terpineol, carvone, and phenylpropanoid estragole (4-allylanisole and methyl chavicol). In single-cell electrophysiological studies, researchers identified 24 classes of olfactory sensory neurons (OSN) within olfactory sensillae for *I. typographus* (Hallberg, 1982). Plant odor-responding OSNs exhibit a variety of response specificities from broadly tuned OSNs for host monoterpene hydrocarbons to several highly specific OSN classes responding mainly to oxygenated monoterpenes (1,8-cineole, isopinocamphone, *trans*-4-thujanol, or verbenone; Andersson et al., 2009; Schiebe et al., 2019; Kandasamy et al., 2023).

In Norway spruce, oxygenated monoterpenes are minor compounds (∼1% representation), and their content is influenced by tree health and stress levels (Netherer et al., 2021). The production of oxygenated monoterpenes in trees naturally occurs through the cytochrome P450-catalyzed oxidation of monoterpene hydrocarbons or by cyclization of oxygenated intermediates (Celedon and Bohlmann, 2019). The release rate of oxygenated monoterpenes, including 1,8-cineol, camphor, pinocarvone, terpinen-4-ol, and α-terpineol, from healthy trees at 24°C ranges from 0.1 to 7 $\mu g/m^2/h$ of stem surface area (Ghimire et al., 2016). In infested trees, these rates increased 10–100 times (Jaakkola et al., 2022), and in cut trees, they increased 10 times (Schiebe et al., 2019).

Bark beetle symbiotic ophiostomatoid fungi generate oxygenated terpenes in laboratory conditions when they are inoculated onto a wood substrate (Kandasamy et al., 2023). In the forest, fungi may assist beetles in colonizing healthy trees by being involved in detoxifying host defense terpenes (Krokene, 2015; Kandasamy et al., 2021). Additionally, the beetles themselves generate oxygenated monoterpenes, as they metabolize toxic terpenes while feeding on the spruce tree's phloem (Blomquist et al., 2021). The detoxification process of terpenes involves a series of steps. In the first step, a hydroxyl group is introduced to a terpene molecule by cytochrome P450 catalysis. This modification increases the molecule's polarity and solubility in water, enabling beetles to eliminate it (Blomquist et al., 2021). In subsequent steps, the resulting terpenic alcohols are either excreted from the body or bound to detoxification conjugative molecules, such as fatty acid esters (Chiu et al., 2018) or glycosylates (Dai et al., 2021). This mechanism was studied in *Dendroctonus ponderosae* (Chiu, 2018; Chiu et al., 2019), *Dendroctonus armandi* (Dai et al., 2021), as well as in *Ips* species (Blomquist et al., 2021; Ramakrishnan et al., 2022) that feed on conifer trees, as these trees possess terpenes as a defense trait. This adaptation allows the beetles to overcome the tree's defenses and successfully colonize it. It is theorized that during evolution, some of these detoxification products, such as *cis*-verbenol in *I. typographus*, started to serve as aggregation pheromones for the bark beetles (Blomquist et al., 2021; Schebeck et al., 2023).

The exact behavioral role of all host-produced oxygenated monoterpene semiochemicals in bark beetles is not fully understood. However, according to the primary attraction theory proposed by Lehmanski et al. (2023), these compounds may play a role in helping male bark beetles detect weakened host trees, thereby facilitating successful colonization. Other compounds, e.g., 1,8-cineole and *trans*-4-thujanol, have proved to be potent anti-attractive compounds inhibiting beetle attraction to their pheromone (Jirošová et al., 2022b). 1,8-cineole has been identified as a potential predictor of bark beetle-resistant trees, along with several other specialized metabolites (Schiebe et al., 2012). Higher levels of *trans*-4-thujanol were detected in younger Norway spruces. Given that this compound has demonstrated repellency in high doses in laboratory olfactometer studies, it provides a potential explanation for the reduced attraction of *I. typographus* to trees below the age of 60 years (BlaŽyte-Cereškiene et al., 2016). The activity of 1,8-cineole and *trans*-4-thujanol for *I. typographus* has been evaluated in a field trapping experiment using different doses in combination with pheromones. Both compounds demonstrated a similar level of dose-dependent anti-attractant activity, with *trans*-4-thujanol inhibiting more the captures of females than males (Jirošová et al., 2022b). These compounds have been tested in combination with other anti-attractants for the protection of spruce trees in various forest environments, such as fresh forest

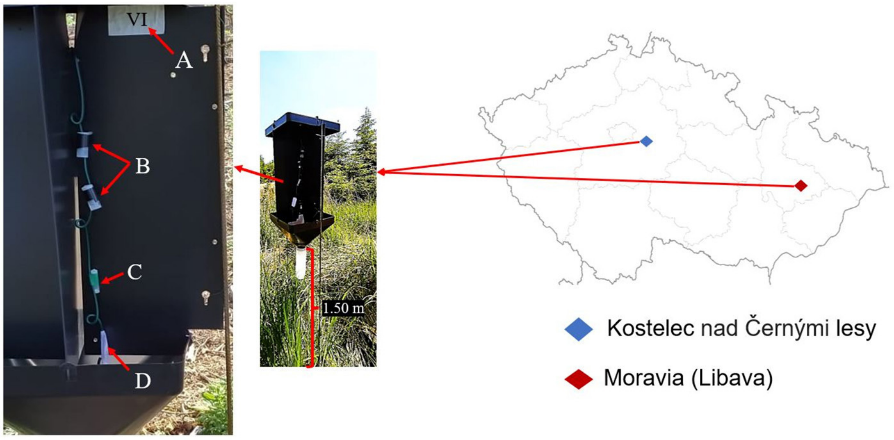

FIGURE 1
Field experiment sites and cross-panel field traps in the Czech Republic: **(A)** Trap label, **(B)** synthetic aggregation pheromone dispensers, **(C)** synthetic host tree odor dispenser, and **(D)** bait label for treatment identification.

edges or fragmented forests (Jakuš et al., 2022; Jirošová et al., 2022a). Furthermore, individual compounds *trans*-4-thujanol, (+)-isopinocamphone, camphor, and terpinen-4-ol were tested in different doses in two-choice Petri dish walking laboratory tests to assess their attractiveness to *I. typographus*. The activity of these compounds was largely insignificant, and only *trans*-4-thujanol and camphor at a high dose were attractive. In contrast, a more complex mixture of oxygenated monoterpenes, generated through the metabolization of (−)-β-pinene and (−)-bornyl acetate by the ophiostomatoid fungus *Grosmania polonica*, exhibited dose-dependent attractivity in the test (Kandasamy et al., 2023).

In multi-trophic interactions, the bark beetle predator-prey relationships are influenced by qualitative and quantitative aspects of host tree compounds and prey pheromones (Erbilgin and Raffa, 2001; Netherer et al., 2021). The natural enemies associated with *I. typographus* include Hymenoptera: Pteromalidae (*Rhopalicus* spp.), Diptera: Dolichopodidae (*Medetera* spp.), Coleoptera: Cleridae (*Thanasimus* spp.), and Coleoptera: Staphylinidae (*Nudobius* sp. and *Quedius* sp.; Wegensteiner et al., 2015). Laboratory bioassays demonstrated the strong attraction of female *Rhopalicus* spp. to infested logs, with antennal responses to oxygenated monoterpenes including pinocamphone, pinocarvone, and the phenylpropanoid estragole (Pettersson, 2001; Pettersson and Boland, 2003). Similarly, camphor, pinocamphone, and terpinen-4-ol derived from bark beetle-associated microorganisms in infested spruce logs exhibited strong attraction with *Medetera signaticornis* Loew (Sousa et al., 2023). Staphylinidae feeds on a wide range of prey, and some species are hypothesized to be associated with bark beetles (Pelto-Arvo, 2020). However, their specific association with host tree volatiles, such as oxygenated monoterpenes and *I. typographus* pheromone, remains unexplored.

The foraging strategies of some *I. typographus* predators, *Medetera* spp. and *Thanasimus* spp., could be governed by a more complete blend of the aggregation pheromone and host volatiles (represented by oxygenated monoterpenes; Hulcr et al., 2006). The hemiterpene pheromone component 2-methyl-3-buten-2-ol by itself does not increase predator catch, while the minor component ipsdienol does (Bakke and Kvamme, 1981; Hulcr et al., 2006; Raffa, 2014).

Further research is necessary to achieve a more comprehensive understanding of the mechanisms and functions of oxygenated monoterpenes and estragole in the behavior of bark beetles and their predators.

In this study, we aimed to address the following specific questions:

1. Can individual host tree-oxygenated monoterpenes and estragole enhance or reduce the attractiveness of *I. typographus* pheromones in field traps for capturing bark beetles?
2. If so, which of the three tested decadic steps in release rates, namely, low (representing conditions closest to natural levels), medium, and high (representing doses used in management), exhibits the highest efficacy?
3. What is the species composition and abundance of predatory insects attracted to the tested compounds using the methodology described?
4. Can we propose a specific role for the tested compounds in bark beetle ecology?

We conducted a field trapping experiment to investigate the activity of eight synthetic host tree compounds (estragole, 1,8-cineole, (±)-camphor, (−)-carvone, (−)-α-terpineol, (−)-terpinen-4-ol, (+)-pinocamphone, and (+)-isopinocamphone) for *I. typographus* and some of its natural enemies. The compounds were tested at low, medium, and high release rates, in combination with components in pheromone barrier traps.

2 Materials and methods

2.1 Experimental area and design

For field experiments, we chose two locations in the Czech Republic. The first location was in North Moravia in Libavá

TABLE 1 **The gravimetric establishment of released rates of tested compounds performed in the laboratory and field.**

Compounds	Sources	Purity (%)	Doses	Release rates (mg/day)			Dispenser design
				Nominal	Lab. ± SEM $N = 3$	Field ± SEM $N = 3$	
Estragole	Sigma-Aldrich	98	L M H	0.1	0.13 ± 0.04	0.14 ± 017	Glass vial (2 ml), lid hole (1 mm)
				1	1.19 ± 0.17	1.72 ± 0.94	†Foil sachet: hole of 2 mm
				10	8.78 ± 1.71	1.86 ± 0.4	†Foil sachet: hole of 9 mm
1,8-Cineol	Sigma-Aldrich	98	L M H	0.1	0.06 ± 0.07	0.11 ± 0.01	††PE-vial (Kartell 731), without hole with paraffin oil (1 ml)
				1	0.66 ± 0.12	0.92 ± 0.12	Glass vial (2 ml), lid hole (1 mm)
				10	5.20 ± 0.30	5.70 ± 6.7	††Kartell 730 with hole (2 mm)
(−)-terpinen-4-ol	Sigma-Aldrich	98	L M H	0.1	0.07 ± 0.04	0.35 ± 0.06	Glass vial (2 ml), lid hole (1 mm)
				1	0.41 ± 0.21	0.52 ± 0.49	††Kartell 731 without hole
				10	9 ± 2.34	7.8 ± 10.16	†Foil sachet: hole of 18 mm
(−)-carvone	Sigma-Aldrich	95	L M H	0.1	0.13 ± 0.05	0.27 ± 0.13	Glass vial (2 ml), lid hole (1 mm)
				1	0.66 ± 0.28	0.22 ± 0.16	††Kartell 730 without hole
				10	9.1 ± 2	4.92 ± 3.45	†Foil sachet: hole of 18 mm
(±)-camphor	Alfa Aesar	95	L M H	0.1	0.09 ± 0.05	0.14 ± 0.9	Glass vial (2 ml), lid hole (1 mm)
				1	0.58 ± 0.06	1.71 ± 0.77	††Kartell 730: hole of 2 mm
				10	7.33 ± 0.94	0.67 ± 10	PE-sachet without hole

(Continued)

TABLE 1 (Continued)

Compounds	Sources	Purity (%)	Doses	Release rates (mg/day)			Dispenser design
				Nominal	Lab. ± SEM $N = 3$	Field ± SEM $N = 3$	
(-)-α-terpineol	Sigma-Aldrich	90	L M H	0.1	0.11 ± 0.05	0.05 ± 0.02	[††]Kartell 730 without hole
				1	1.37 ± 0.27	0.14 ± 0.07	[†]Foil sachet: hole of 9 mm
				10	4.01 ± 0.4	1.11 ± 0.48	PE-sachet without hole
(+)-isopinocamphone	[†††]	99	L M H	0.1	0.37 ± 0.40	0.40 ± 0.11	[†]Foil sachet: hole of 1 mm
				1	1.47 ± 0.08	1.87-0.67	[††]Kartell 730 without hole
				10	8.39 ± 0.98	8.21 ± 1.41	[††]Kartell 731, lid hole (2 mm)
(+)-pinocamphone	[†††]	[††††]	L M H	0.1	0.4 ± 0.11	0.3 ± 0.6	[†]Foil sachet: hole of 1 mm
				1	1.01 ± 0.34	1.32 ± 0.83	[††]Kartell 730 without hole
				10	9.1 ± 0.59	7.91-2.14	[††]Kartell 731, lid hole (2 mm)
2-methyl-3-buten-2-ol	Across	97	H	50	32.2 ± 20.5	9.10 ± 16.1	[††]Kartell 731, lid hole (1 mm)
(*S*)-*cis*-Verbenol	Sigma-Aldrich	95	H	1	1.53 ± 0.15	0.85 ± 1.34	[††]Kartell 731, lid hole (9 mm)

[†] Cellulose sponge square 7.5 × 3.5 × 0.25 cm sealed in PE foil thickness 0.1 mm, loaded with 200 µl of compounds, finally sealed in an outer layer made of aluminum/PE foil with the hole with a given diameter in the middle of one side of the dispenser.

[††] PE vials Kartell (Labware-Italy) size 731 and 730.

[†††] Compounds synthesized in Unelius laboratory (Ganji et al., 2020).

[††††] The (+)-pinocamphone contained 29% (+)-isopinocamphone.

(Military Forests, latitude 49°38′49 "N, longitude 017°33′50" E, 350 m above sea level). It consisted of a 40-year-old Norway spruce forest that has been heavily impacted by a bark beetle outbreak since 2015 (Brázdil et al., 2022). The experiment in Moravia was conducted from 18 May to 3 June 2022. The second location, Kostelec nad Cerným Lesy (Forests CZU; latitude: 49°55′57 "N, longitude: 014°55′13" E, 600 m above sea level), consisted of a 70–90-year-old Norway spruce forest. Traps were placed in a 2-year-old clearing measuring ∼200 m × 300 m. The experiment was carried out from 3 June to 28 July 2022. The experiment was designed identically in both locations (Figure 1).

The activity of estragole, 1,8-cineole, (±)-camphor, (-)-carvone, (-)-α-terpineol, (−)-terpinen-4-ol, (+)-pinocamphone, and (+)-isopinocamphone was tested at three different doses, represented by their release rates evaporated/sublimated from the dispenser (nominal 0.1, 1, and 10 mg/day, Table 1). The doses were determined based on the published releases of oxygenated monoterpenes from healthy trees at 24°C, which varied from 0.1 to 7 µg/m²/h of stem surface (Ghimire et al., 2016). When considering a tree stem with a 50 cm diameter and an exposed surface area of ∼24 m^2 (representing 15 m of stem height vulnerable to bark beetle attack), the estimated daily release rate of these oxygenated monoterpenes over a 24-h period would be ∼0.5–4 mg/day. We used the pure enantiomers of (−)-carvone, (−)-terpinen-4-ol, (+)-pinocamphone, and (+)-isopinocamphone, which triggered a higher response on the *I. typographus* antennae (Schiebe et al., 2012; Hou et al., 2021; Kandasamy et al., 2023) and were commercially available or synthesized in the laboratory (Ganji et al., 2020). Experimental dispensers were designed in the laboratory, and their exact laboratory and field release rates were established using the gravimetric method and measured six times (Jirošová et al., 2022b) in a laboratory fume hood (temperature 25°C and airflow 0.5 m/s) and in the field under the same weather conditions as the experiments (Table 1).

In the field, the intercept pheromone traps (Ecotrap/Fytofarm, Ltd., Bratislava; Slovakia) were mounted on poles 1.5 m above the ground in rows >30 m from any forest edge. The distance between traps was >15 m (Supplementary Figure 1). In each field location, 32 intercept pheromone traps were baited with dispensers with *I. typographus* pheromone (2-methyl-3-buten-2-ol at 9.1 mg/day and (*S*)-*cis*-verbenol at 0.9 mg/day). In 24 of these traps, an additional dispenser was placed with one of the eight test compounds in one of three doses.

For each compound, one block represented four traps arranged in a row: three traps with different doses in combination with pheromone and one trap with pheromone-only (Control). The position of the tested baits among these four traps was changed four times according to the randomization scheme in a Latin square design (Evans et al., 2020). These four rotations were repeated twice for each compound, resulting in a total of eight collections of catches for each treatment. Insects collected during the field experiment in both localities were preserved in ethanol for further analysis. Predators and parasitoids were sorted by family and identified at the genus level. The identification of Pteromalidae wasps followed the methods described by Peck et al. (1964), Graham (1969), and Bouček and Rasplus (1991). For Staphylinidae (rove beetles), the identification followed the guidelines provided by Arnett and Thomas (2000) and Navarrete and Newton (2003).

TABLE 2 Testing of relative catches of *I. typographus* in the tested compounds in different doses.

Compound	Model family	AIC	Pr(>Chi)		*t*-test contrast comparison to pheromone-only					
					Low		Medium		High	
Estragole	genpois	521.63	0.003476	**	0.4973		0.2991		0.0206	*
1,8-Cineol	genpois	557.89	8.03E-08	***	0.0003	***	0.0051	**	3.58E-09	***
(−)-terpinen-4-ol	nbinom2	532.27	0.2849		0.106		0.667		0.81	
(−)-carvone	nbinom2	509.67	0.8852		0.869		0.64		0.782	
(±)-camphor	nbinom2	537.5	0.5298		0.19		0.345		0.221	
(−)-α-terpineol	nbinom2	526.26	0.8826		0.655		0.903		0.721	
(+)-isopinocamphone	genpois	550.17	0.003014	**	0.0785	.	0.00079	***	0.003142	**
(+)-pinocamphone	genpois	521.03	3.32E-07	***	0.924		1.3E-06	***	0.483	

Asterisks mark a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) of the dose from pheromone alone.

2.2 Statistical analysis

For the evaluation of the effects of each individual compound, a separate regression model was fitted with the relative number of *I. typographus* as the dependent variable. The relative number was expressed as the number of insects of a single taxon captured by a treatment within a block divided by the total catches by the block for a single catch collection. Due to the experimental design, we utilized a mixed-effects model approach (Zuur et al., 2009). The random part of the model was, in all cases, trap-nested in the locality. During model building and validation, an appropriate distribution function was selected by minimizing the Akaike information criterion (AIC), and the significance of the model was tested by the likelihood ratio test (χ^2). Between the best models, only two distribution functions were selected (Table 2): generalized poison distribution (Joe and Zhu, 2005) and negative binomial distribution in quadratic parameterization, according to Hardin and Hilbe (2007). We used a *t*-test to compare the response to each compound dose against the pheromone-only control. The model formulation was performed in R version 4.3.1 (R Core Team, 2023) in the package *glmmTMB* following the procedures described by Brooks et al. (2017).

3 Results

3.1 *Ips typographus* response to tested compounds in combination with aggregation pheromone

During field experiments, a total of 39,650 *I. typographus* adults were caught. The number of adults captured in Kostelec ($N = 28{,}931$) was 2.7 times higher than in Moravia ($N = 10{,}719$). However, the pattern of catches for the tested compounds was almost the same. Compounds with significantly different catches in the treatments: estragole (χ^2; $p < 0.01$), 1,8-cineole (χ^2; $p < 0.001$), (+)-pinocamphone (χ^2; $p < 0.01$), and (+)-isopinocamphone (χ^2; $p < 0.01$; Table 1, Figure 2). For 1,8-cineol, all three doses in combination with pheromones resulted in significantly fewer beetles caught than the pheromone-only control, with stronger effects observed for the high dose (*t*-test; $p < 0.001$, $p < 0.01$, $p < 0.001$; Table 2, Figure 2). For estragole, the inhibitory effect was significant only for the high dose (*t*-test; $p < 0.05$), while for pinocamphone, it was observed at the medium dose (*t*-test; $p < 0.001$). However, regarding the medium dose of pinocamphone, there were different catch rates in Libava and Kostelec (Figure 2), suggesting a potential problem with the dispenser used in Kostelec. Conversely, isopinocamphone resulted in statistically higher catch rates at both high and medium doses (*t*-test; $p < 0.01$ and $p < 0.001$, respectively) compared to the pheromone-only treatment (Figure 2). A low dose of isopinocamphone showed the same nearly significant trend (*t*-test; $p = 0.08$; Table 2). Catches of beetles of remaining tested compounds in combination with pheromone, including (±)-camphor, (–)-carvone, (-)-α-terpineol, (-)-terpinen-4-ol, and pheromone-only, did not exhibit significant differences (Table 2). Additionally, there were no significant differences in catches between the individual doses and the pheromone-only control group for any of these compounds (Supplementary Figure 2).

For each compound, a separate model (GLMM) was created with the formula: *Relative count of I. typographus* ~ *Compound dose* + *(1|Locality)*. The appropriate distribution function (model family: genpois—Generalized poison; nbinom2—Negative binomial), Akaike information criterion (AIC), and significance test (χ^2) are stated for each compound. The results of the t-test contrast comparison given for each compound and dose combination against pheromone-alone (control). Asterisks mark a significant difference ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***) of the dose from pheromone alone.

3.2 Predatory insect response to tested compounds in combination with aggregation pheromone

The catch of natural enemies was three times higher in Kostelec compared to Moravia, which corresponds to a higher number of bark beetle catches in Kostelec. Four families of natural enemies of bark beetles were identified with a prevalence of Pteromalidae and Staphylinidae (Supplementary Tables 1, 2). In Kostelec, there were 93 specimens of parasitoid wasps belonging to the genus *Rhopalicus* sp. (Hymenoptera: Pteromalidae), while in Moravia, there were only 13 specimens. Additionally, in Kostelec, 19 specimens of rove beetles (Coleoptera: Staphylinidae) were caught, and in Moravia, 23 specimens (Table 3).

The ratio between these two groups appeared different between the two locations, with Kostelec having a higher proportion of Pteromalid wasps compared to Staphylinids, while in Moravia, we observed the opposite trend. There were a few catches recorded for *Medetera* sp. (Diptera: Dolichopodidae), with five specimens in Kostelec and three specimens in Moravia. There were four catches of *Lonchaea* sp. (Diptera: Lonchaeidae), three in Kostelec, and one in Moravia. For *Thanasimus* sp. (Coleoptera: Cleridae), there were two catches in Kostelec and three catches in Moravia.

The catches of *Rhopalicus* sp. wasps did not show significant differences between compounds and their doses. The number of caught specimens in Moravia and Kostelec is listed in the Supplementary Tables 1, 2.

4 Discussion

4.1 Response of *Ips typographus* to tested compounds

The variability in total catches of *I. typographus* and its predators, as well as their varying distributions across different treatments in the experimental locations of Kostelec and Moravia, could be attributed to the unique weather conditions experienced during each field experiment, as described in Supplementary Figure 3. Furthermore, this variation may have been influenced by the season impacting the flight activity of the beetles. The variation between catches in blocks of traps may have been due to different locations of blocks within the clearing, in terms of their distance to the forest edge and wind speed and

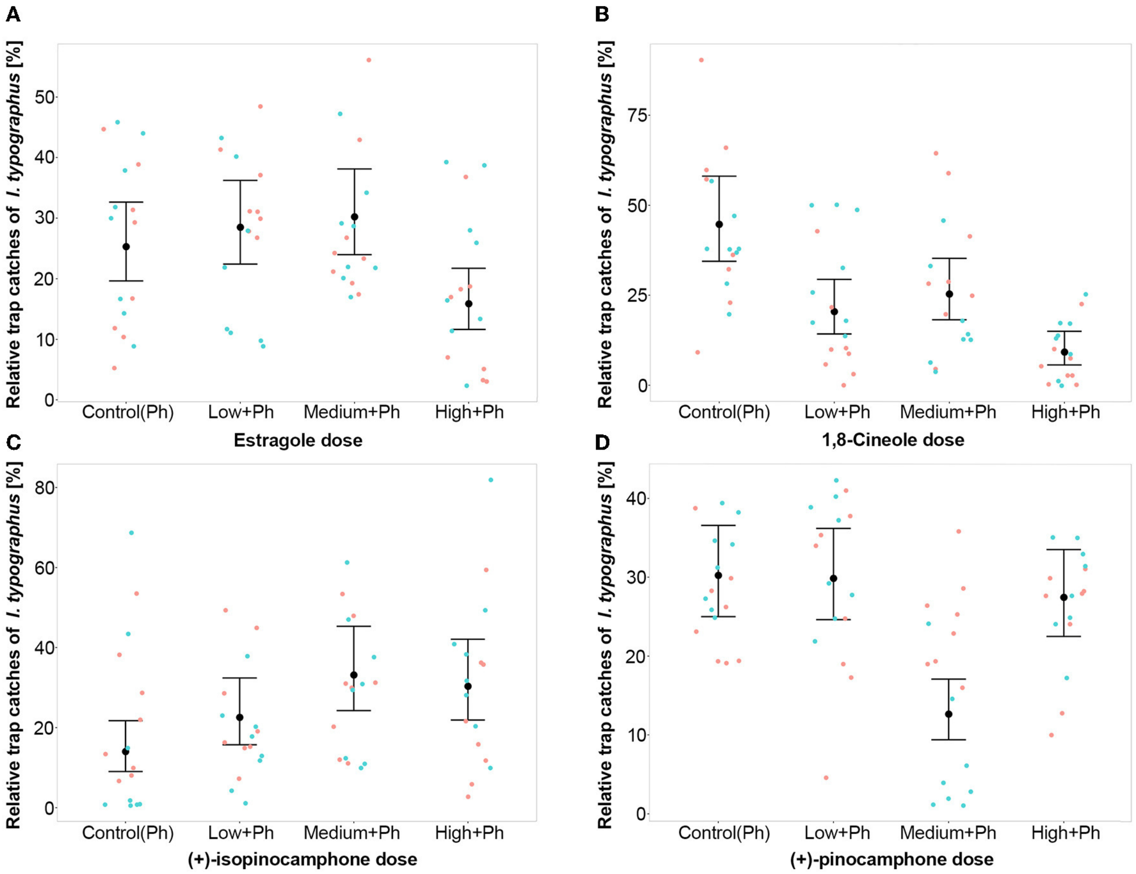

FIGURE 2
Relative number of *I. typographus* captured in traps baited with pheromone attractant (Ph) alone or attractant with host volatiles **(A)** estragole, **(B)** 1,8-cineole, **(C)** (+)-pinocamphone, and **(D)** (+)-isopinocamphone in a low-medium, high-release dose combined with pheromone (Ph) and control pheromone-only. The bigger black dot in the middle of the vertical line is a posterior mean value and whiskers. Error bars represent the 95% confidence interval, and colored points represent the raw catches. The smaller dots are original values: blue Kostelec and red Libavá.

direction. The trap catches could also have been influenced by different ages of spruce stands near the experiments.

In many cases, the beetles exhibited weak differences in response to the tested compounds in combination with the pheromone compared to the pheromone alone, and the observed effects were only marginally significant. However, there was a noticeable anti-attractive effect observed with 1,8-cineole, a compound for which similar findings and similar trends have been reported previously (Andersson et al., 2010; Binyameen et al., 2014; Jirošová et al., 2022b).

New findings were the anti-attractive effect of a high dose of estragole and the effect of (+)-isopinocamphone on enhancing the attractive activity of the pheromone for *I. typographus.* There was also a less clear inhibition effect of a medium dose of pinocamphone, probably caused by a defective test dispenser for a medium dose at Kostelec. In contrast, when α-terpineol, camphor, carvone, or terpinen-4-ol were added to the pheromone, they did not alter its attractiveness, despite these compounds eliciting strong responses from *I. typographus* antennae (Kalinová et al., 2014; Schiebe et al., 2019).

The ecological role of estragole, 1,8-cineole, and isopinocamphone in the interaction between *I. typographus* and Norway spruce trees was investigated by Schiebe (2012, 2019). The amount of these compounds, along with other oxygenated monoterpenes found in spruce, increased in felled trees and in standing trees after the application of the plant hormone analogue methyl jasmonate. The quantity of these compounds was negatively correlated with the density of bark beetle attacks when the beetles infested the felled trees, and the standing trees that exhibited a higher induction of these compounds were able to survive a natural bark beetle infestation.

The effect of estragole has been tested on several bark beetle species (Coleoptera: Curculionidae: Scolytinae), including *Dendroctonus brevicomis* LeConte (Hayes and Strom, 1994), *Ips pini* (Say), *Ips latidens* (Leconte) (Joseph et al., 2001), and *Tomicus piniperda* (Curculionidae: Scolytinae) (Haack et al., 2004). Its inclusion in their host odor blend resulted in reduced trap catches. Estragole was also reported to interrupt the responses of bark beetle species *Dedroctonus simplex* (Le Conte) and *D. ruffipenis* (Kirby) to their attractive pheromone components (Werner, 1995).

TABLE 3 Natural enemies of bark beetles (Staphylinidae and Pteromalidae) captured in synthetic host tree compounds: estragole, 1,8 cineole, camphor, carvone, alpha-terpineol, terpinene-4-ol, (+)-pinocamphone, and (+)-isopinocamphone, in combination with pheromone and pheromone-only controls.

Doses/ location	Estragole+Ph	1,8-cineole+Ph	a-terpineol+Ph	Camphor+Ph	Carvone +Ph	terpinen-4-ol+Ph	(+)-isopinocamphone +Ph	(+)-pinocamphone +Ph	Pheromone-only Ph
Doses/Moravia **Pteromalidae**	*Rhopalicus*	*Rhopalicus* (N = 4)	*Rhopalicus* (N = 2)	*Rhopalicus*	0	*Rhopalicus*	*Rhopalicus* (N = 3)	0	*Rhopalicus*
Doses/Moravia **Staphylinidae**	*Gyrohypnus* (N = 2)	*Nudobius* (N = 4)	*Gambrinus*	*Bisnius Nudobius* *Anotylus*	*Bisnius* (N = 2) *Anotylus*	*Nudobius* *Gabrius*	*Nudobius*	*Bisnius; Gyrohypnus*	*Nudobius* (N = 2); *Gyrohypnus* *Anotylus Rugilus*
Doses/Kostelec **Pteromalidae**	*Rhopalicus* (N = 16)	*Rhopalicus* (N = 9)	*Rhopalicus* (N = 8)	*Rhopalicus* (N = 2)	*Rhopalicus* (N = 10)	*Rhopalicus* (N = 5)	*Rhopalicus* (N = 14)	*Rhopalicus* (N = 7)	*Rhopalicus* (N = 22)
Doses/Kostelec **Staphylinidae**	*Nudobius* *Anotylus* *Gyrohypnus*	*Nudobius* (N = 2); *Stenus* *Gyrohypnus* *Heterothops*	*Rugilus Quedius*	*Aleochara* *Nudobius* *Gyrohypnus*	*Bisnius Nudobius*	*Aleochara*	*Nudobius*	*Nudobius*	*Anotylus*

Doses: The sum of total captures in all three tested release rates of compounds or pheromone-only controls summed together for both localities, Kostelec and Moravia.

However, recent research has revealed that the addition of estragole increased catches of both *Dendroctonus frontalis* (Zimmermann) and *D. terebrans* (Olivier) on their pheromone lures (Munro et al., 2020). Based on these findings, we suggest that this semiochemical has variable ecological roles for these different species.

In our study, (+)-isopinocamphone caused a synergistic increase in beetle catches when added to the pheromone. This observation, combined with the fact that bark beetles possess specialized sensilla on their antennae (Hou et al., 2021) to detect it, suggests the potential role of (+)-isopinocamphone in the selection of host trees. Kandasamy et al. (2023) tested in a short-range two-choice test in a Petri dish synthetically prepared (+)-isopinocamphone added as a solution in mineral oil to spruce bark agar, which did not exhibit significant attractivity for *I. typographus* bark beetles in tested doses. This further indicates that the effect of (+)-isopinocamphone we see in trap catches may be a long-range attraction (in accordance with the known long-range attraction of *cis*-verbenol; Schlyter and Birgersson, 1999).

4.2 Response of bark beetle insect natural enemies to tested compounds

The anticipated captures of the common predatory beetle *Thanasimus* sp. were relatively low. This could be attributed to the fact that our pheromone bait only contained the two major pheromone components, 2-methyl-3-buten-2-ol and (*S*)-*cis*-verbenol, and not ipsdienol, an *I. typographus* pheromone component emitted in smaller amounts in the later attack states (Birgersson et al., 1984; Hulcr et al., 2006). Furthermore, we observed only a few captures of *Medetera* sp. and *Lonchaea* sp. flies, likely due to the use of a trap optimized for Coleoptera that lacked sticky surfaces.

Although there is limited information on Staphylinidae predators using host tree volatiles for locating bark beetles (Wegensteiner et al., 2015), it has been reported that they are attracted to pheromone traps used for monitoring *Ips typographus* (El-Sayed, 2023). Additionally, commercial pheromone traps tested in combination with host tree logs (*P. abies*) caught ~38% more predatory Staphylinidae than traps without logs, in comparison to a 32% increase in catches of *Thanasimus formicarius* (L.) (Zumr, 1983). Hence, host tree compounds may mediate staphylinid prey location.

The pteromalid parasitoid wasp *Rhopalicus* sp. was the most abundant among the captured bark beetle natural enemies, but there were no significant preferences for any of the tested compounds due to the low number of caught specimens. In the literature, an electroantennographic study of bark beetle gallery smell was tested on the antennae of *Rhopalicus tutela* (Walker) females. The antennae showed sensitivity to oxygenated monoterpenes and estragole (Pettersson, 2001). Additionally, the olfactory response to estragole was reported in other species of parasitoid wasps, *Spathius pallidus* Ashmead, 1893, and *Roptrocerus xylophagorum* (Hymenoptera: Pteromalidae; Sullivan et al., 1997).

5 Conclusion

The effect of 1,8-cineole, estragole, and (+)-isopinocamphone, as observed in our field experiments, provides evidence that these oxygenated monoterpenes and estragole can exhibit biological activity for *I. typographus* and their natural enemies when combined with *I. typographus* aggregation pheromone. This suggests that their long-range activity is not solely dependent on a complex mixture, such as that emitted by symbiotic fungi inoculated on wood substrates (Kandasamy et al., 2023).

The discovery of new attraction inhibitors or adjuvants for attractants can be applied to the development of integrated pest management methods for controlling *I. typographus*. Anti-attractants, a term broadly used for attraction inhibitors, have already been tested to deter various pest bark beetles, such as *Dendroctonus ponderosae*, *D. rufipennis*, *D. pseudotsugae*, *Ips pini*, and *Dryocoetes confusus*, from attraction to their pheromone or to the host tree (Schlyter, 2012). These anti-attractants can originate from host trees, non-host trees, associated microorganisms, or the beetles themselves (Borden et al., 2000; Munro et al., 2020).

In the protection of Norway spruce trees against *I. typographus*, verbenone, a well-established repellent for bark beetles, was tested with varying success (Jakuš et al., 2003; Frühbrodt et al., 2023). In nature, verbenone signals an old and over-exploited host. The synergistic blend effect of verbenone mixed with green leaf volatiles (C6 alcohols) and C8 alcohols (3-octanol and 1-octen-3-ol) and the angiosperm and fungal spiroacetal conophthorin (Zhang and Schlyter, 2004) was also evaluated for tree protection against *I. typographus* (Schiebe et al., 2011), resulting in a reduction of tree killing ranging from 35 to 76% in protected areas.

The recently tested anti-attractant mixture also includes, besides the 3-octanol, 1-octen-3-ol, hexanol and conophthorin, 1,8-cineole and *trans*-4-thujanol from spruce and excludes verbenone (Jirošová et al., 2022a). Anti-attractant blends offer partial protection for standing trees but are ineffective for windfallen trees. Adding new anti-attractants, e.g., geranyl acetone (Lindmark et al., 2023), to the mixture may enhance tree protection effects.

A comprehensive approach to semiochemical tree protection against *I. typographus* attacks could employ the push-pull strategy. Trees are protected by anti-attractants, and repelled beetles are caught in pheromone traps baited with attractive *I. typographus* pheromones. Both the push and pull might be enhanced by the addition of new semiochemicals (Jakuš et al., 2022; Deganutti et al., 2023). The addition of (+)-isopinocamphone to the trapping lure could increase beetle attraction away from trees while simultaneously protecting them from an estragole-enhanced beetle repellant.

Data availability statement

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

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because Ethical review and approval were not required for the study on animals in accordance with the local legislation and institutional requirements. We have performed all beetle experiments that comply with the ARRIVE guidelines and were carried out in accordance with (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

Author contributions

AACM: Data curation, Formal analysis, Investigation, Validation, Writing—original draft. RJ: Data curation, Investigation, Writing—review & editing. RM: Formal analysis, Writing—review & editing. CRU: Methodology, Writing—review & editing. FS: Conceptualization, Methodology, Supervision, Validation, Writing—review & editing. AJ: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2023.1292581/full#supplementary-material

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The effect of the oak powdery mildew, oak lace bug, and other foliofagous insects on the growth of young pedunculate oak trees

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Pedunculate oak (*Quercus robur* L., 1753) is one of the widely distributed oak species in Europe. A large number of organisms develop on its leaves. To determine the extent to which the oak powdery mildew, oak lace bug, and other foliofagous insects affect the growth of young oak trees, three experimental fields were selected in a 10-year-old pedunculate oak stand. In each of them, 50 trees were randomly selected, and their height was measured at the beginning of the vegetative season. The first experimental field was treated with a systemic insecticide, the second with a systemic fungicide, and the third, a comparison area, with water, during the entire vegetative season. At the end of the vegetative season, 25 plants with one apical branch were selected in each experimental field. Their height was measured, and 20 leaves were taken from each plant to determine the extent of the damage on them at the end of the experiment. After processing the obtained data, it was determined that: 1. Both foliofagous insects and oak leaf inhabiting fungi affect the growth of the oak trees significantly; 2. The oak lace bug did not influence the growth of the young trees significantly, as its abundance was low in all of the experimental areas; 3. The greatest damage on the leaves was caused by defoliator insects, which is why they contributed the most to the decrease in growth caused by insects; 4. The influence of the foliofagous insects on the growth of the trees was not significantly different from the influence of fungi; 5. Suppression of oak powdery mildew and foliofagous insects on young trees is useful as it positively influences the vitality and growth of those trees, and contributes to economic and ecological gain.

KEYWORDS

foliofagous insects, chemical control, *Corythucha arcuata*, defoliators, *Erysiphe alphitoides*, *Quercus robur*, height growth

1 Introduction

Pedunculate oak (*Quercus robur* L., 1753) is one of the most widely distributed oak species in Europe (Bobinac et al., 2012; Puchałka et al., 2017). Its range covers most of Europe, excluding its most southern and northern parts (Eaton et al., 2016). This species always had significant meaning for the people, as it provided construction and fuel material, food for livestock, and bark for tanning (Eaton et al., 2016). Because of its impressive appearance and longevity, it has a symbolic role in many cultures in Europe (Askeyev et al., 2005; Mills, 2013; Eaton et al., 2016). Its forests have a significant ecological value as they provide high biodiversity (Mölder et al., 2019). Due to its high quality of wood, it is one of the most important species in managed forests in Europe (Eaton et al., 2016).

A large number of organisms develop on pedunculate oak leaves (Karadžić, 2010; Dobrosavljević et al., 2020; Ermolaev et al., 2021; Mladenović et al., 2021; Marković, 2022). Among them, the oak lace bug - *Corythucha arcuata* (Say, 1832) (Hemiptera: Tingidae) and oak powdery mildew, which is most frequently caused by *Erysiphe alphitoides* (Griffon and Maubl). Braun and Takam (Erysiphales: Erysiphaceae), present the most problematic ones in southeastern Europe (Glavaš, 2011; Pap et al., 2013; Simov et al., 2018; Drekić et al., 2019; Bălăcenoiu et al., 2021a; Franjević et al., 2023). Defoliators such as *Lymantria dispar* Linnaeus, 1758 (Lepidoptera: Erebidae), *Tortrix viridana* Linnaeus, 1758 (Lepidoptera: Tortricidae), *Erannis defoliaria* (Clerck, 1759) and *Operophtera brumata* (Linnaeus, 1758) (Lepidoptera: Geometridae) can also cause significant damage as their outbreaks can spread over large areas (Marović et al., 1998; Harapin and Jurc, 2000; Pernek et al., 2008; Tomescu and Netoiu, 2008).

Corythucha arcuata and *Erysiphe alphitoides* are invasive species' (Marçais and Desprez-Loustau, 2014; Bălăcenoiu et al., 2021a). The first one originates from North America (Csóka et al., 2020), while the origin of second one is most probably from Asia (Desprez-Loustau et al., 2017). The first finding of *C. arcuata* in Europe happened in Italy in 2000 (Bernardinelli and Zandigiacomo, 2000), while *E. alphitoides* was first found in France in 1907 (Hariot, 1907). These two species are now one of the most widely distributed oak leaf-inhabiting pest organisms in Europe (Marçais and Desprez-Loustau, 2014; Csóka et al., 2020). *C. arcuata* causes significant damage during each vegetative season. Severe outbreaks of this species have been reported in many European countries (Paulin et al., 2020). Its larvae and adults damage the leaves by sucking the sap on the underside of the leaf. Necroses which their feeding causes can cover the entire leaf area in the case of high abundance. That is why decolorization, lower photosynthetic activity, transpiration, and stomatal conductance occur on those plants (Nikolic et al., 2019; Paulin et al., 2020; Bălăcenoiu et al., 2021a). All of these effects can consequentially lead to a decrease in growth, premature leaf abscission, and a decrease in the size of the acorn (Tomescu et al., 2018; Drekić et al., 2019; Paulin et al., 2020). *E. alphitoides* is constantly present in oak forests. This obligate parasite creates an epiphyte mycelium on the leaf, which takes nutrients from the host and covers the leaf surface (Karadžić and Milijašević, 2005). All this consequentially causes a reduction in photosynthetic activity and transpiration (Pap et al., 2014b). That causes a decrease in growth and can cause dieback of younger plants (Karadžić and Milijašević, 2005; Bert et al., 2016). The dieback of young oak trees which this fungus causes is a significant problem (Karadžić and Milijašević, 2005; Pap et al., 2012). That is why the control of this pathogen is conducted during forest regeneration (Bobinac and Karadžić, 1994; Glavaš, 2011; Pap et al., 2012). *E. alphitoides* causes problems even in older forests in the cases of defoliation, when it can significantly diminish the vitality of oak trees (Pap et al., 2014b).

As *E. alphitoides* has been present in Europe for more than 100 years, a lot is known about it (Desprez-Loustau et al., 2011; Marçais and Desprez-Loustau, 2014; Lonsdale, 2015; Kebert et al., 2022; Mieslerová et al., 2022). *L. dispar*, *T. viridana*, *E. defoliaria,* and *O. brumata* have also been a topic of many studies (Ivashov et al., 2002; Tikkanen and Julkunen-Tiitto, 2003; Glavendekić, 2010; Milanović et al., 2020a,b, 2022). *C. arcuata* is still a new species for Europe so it is currently intensively studied (Bernardinelli, 2006; Franjević et al., 2018; Drekić et al., 2019; Nikolic et al., 2019; Csóka et al., 2020; Kern et al., 2021; Marković et al., 2021a; Bălăcenoiu et al., 2021b; Paulin et al., 2023; Stancă-Moise et al., 2023; Valdés-Correcher et al., 2023). As pedunculate oak is one of the most significant European oaks (Eaton et al., 2016; Mölder et al., 2019) we conducted a study to determine: how *C. arcuata* and other foliophagous insects affect the growth of young pedunculate oak trees; which type of foliofagous insect damage is dominant on the leaves; how oak powdery mildew affects the growth of young trees; and does the influence of foliofagous insect on the growth of young oak trees differ from the influence of oak powdery mildew.

2 Materials and methods

2.1 Study area

The study was conducted in 2022 in a 10-year-old regenerated pedunculate oak stand[1] (44° 45′ 2.88″ N and 19° 59′ 45.88″ E). It is located in a plane, at an altitude of 74 m. The average tree height was 2.4 m and the average diameter at root collar was 2.8 cm. The studied area is located on an alluvial deposit of clay and sand, where the soil is eutric cambisol. The average annual temperature is about 11°C, while the annual precipitation is 569.6 mm. The climate is characterized as continental with some features of the Pannonian-steppe temperate continental climate. The plants in the investigated stand use only atmospheric water and underground water in spring since the area is not flood-prone.

2.2 Experimental design

Three experimental areas, measuring 20 × 10 m, separated by a distance of 100 m, were selected in the stand. At each of them, 50 randomly selected trees were singled out at the beginning of the study (on April 1st). They were labeled and their heights were measured by a tape measure with a precision of 1 cm. The first study area was treated with the systemic insecticide Tonus (active substance Acetamiprid 200 g/kg) in a concentration of 0.25 g per liter of water to prevent and suppress insect damage. The second area was treated with the systemic fungicide Falcon 460-EC (Tebuconazole 167 g/L + Triadimenol 43 g/L + Spiroxamine 250 g/L, in a concentration of 0.35 mL) per liter of water to prevent and suppress the harmful fungi. The third area was the comparison area which

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[1] All the data except the plant dimensions were gathered from the Public company "Vojvodinašume" which manages the forests in which the experiment was conducted.

was treated only with water. These pesticides were selected because they have a broad spectrum of effect and were already successfully used or they gave satisfactory results in similar experiments (Pap et al., 2015; Drekić et al., 2021). The pesticides were applied by spraying from the ground with a backpack sprayer. Each experimental area was treated with 10 liters of the listed formulation prepared with water (500 L/ha). All of the areas were treated simultaneously, every 15 days starting from April 1 to October 1 (entire vegetation). After that, 25 plants with a single apex and similar initial heights (± 25 cm) were selected in each experimental field to isolate extreme values, as some plants were broken while some formed multiple apical branches. Their heights were measured on the 15th of October when the experiment was finished. Twenty leaves were then randomly selected from each of the plants to assess the amount of damage caused by the analyzed organisms. They were packed in plastic bags and brought to the laboratory of the University of Belgrade Faculty of Forestry, where they were kept in the refrigerator for 2 days until the analyses were done. Damages were divided into the following groups: defoliators, miners, gallers, sucking insects, and oak powdery mildew (Figure 1). The assessment of the damage by category was done visually by the naked eye. The damaged area was estimated as the share of leaf area covered by mycelia, mines, galls, discoloration or simply eaten (missing) in relation to the total surface area of the leaf. The damaged area was measured in percentages as a relative measure because the leaf size differed significantly between and within each tree.

2.3 Leaf damaging organisms

Before each treatment and at the end of the experiment, leaves from randomly selected plants in the comparison area were analyzed to identify the foliofagous insect fauna on them. The noted species were identified on the site as they are common for the area in which the study was conducted. As oak powdery mildew can be caused by multiple fungi (Karadžić and Milijašević, 2005) of which *E. alphitoides* is listed as the most important one in the studied area, the damage on the leaves was labeled only as oak powdery mildew.

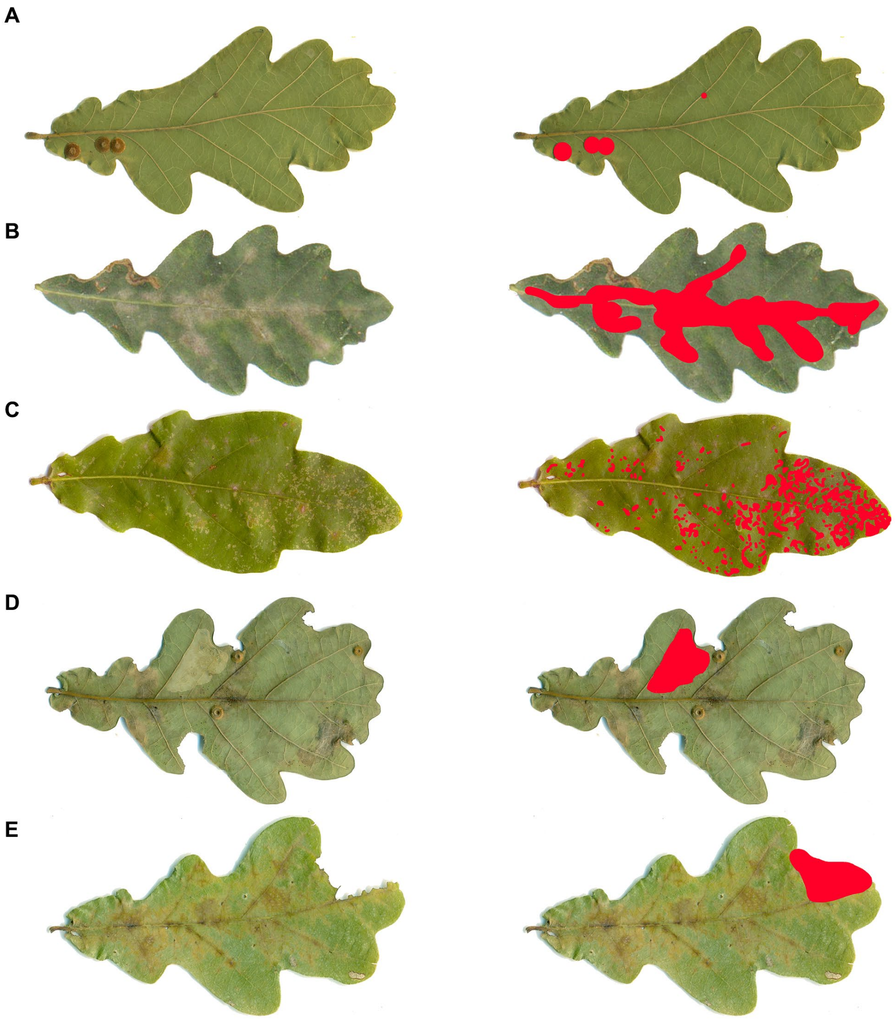

FIGURE 1
Illustration of how the different damage types were identified and estimated: **(A)** galls (Cynipidae sp.); **(B)** oak powdery mildew; **(C)** oak lace bug (*Corythucha arcuata*); **(D)** mines (*Phyllonorycter* sp.); **(E)** defoliation.

2.4 Statistical analysis

As the distribution of the analyzed parameters did not fit any of the standardized distributions (Kolmogorov–Smirnov test), nonparametric tests were used for further analysis. Kruskal-Wallis ANOVA by Ranks was used to determine the influence of the treatments on the growth of the analyzed trees and the influence of the treatments on the damage caused by different insect groups. Mann–Whitney U test was used as a post-hoc test, to determine the differences between individual treatments where the Kruskal-Wallis ANOVA showed significant differences. Mann–Whitney U test was also used to determine the differences between the leaf areas damaged by different insect groups in each treatment. All the data were analyzed at the tree level, at the level of significance 0.05. All of the statistical analyses were performed using Statistica 8.0 (StatSoft, Inc., Tulsa, OK, United States).

3 Results

Among the insects observed on the experimental areas, the most significant sucking species was *C. arcuata*; defoliators *L. dispar*, *E. defoliaria*, *O. brumata T. viridana* and *Periclista* sp. (Hymenoptera, Tenthredinidae); gallers *Andricus curvator* Hartig, 1840, *Neuroterus numismalis* (Fourcroy, 1785) and *N. quercusbaccarum* (Linnaeus, 1758) (Hymenoptera, Cynipidae); miners *Profenusa pygmaea* (Klug, 1816) (Hymenoptera, Tenthredinidae), *Phyllonorycter harrisella* (Linnaeus, 1761), *Ph. roboris* (Lepidoptera, Gracillariidae), and *Tischeria ekebladella* (Bjerkander, 1795) (Lepidoptera, Tischeriidae). The dominant fungal damage on the leaves was caused by oak powdery mildew.

Statistically significant differences were identified between the treated and the comparison area in the intensity of the damage caused by oak powdery mildew, defoliator insects, sucking insects, and leaf miners (Figure 2; Table 1). No significant differences were observed in the damage caused by gallicolous insects. In the area treated with fungicide, the intensity of the damage caused by leaf miners and sucking insects was significantly higher compared to the comparison area.

Statistically significant differences in growth were identified between each of the treated and the comparison area (Insecticide - Zadj = 4.590, $p = 0.000$; Fungicide - Zadj = 4.910, $p = 0.000$). There were no significant differences between the treated areas (Zadj = −1.136, $p = 0.256$). The greatest increase in growth was detected in the area treated with fungicide, less with insecticide and the lowest was in the comparison area (Figure 3). The growth on the area treated with fungicide was on average 62.3% higher, and on the surface treated with insecticide 50.5% higher in respect to the comparison area.

4 Discussion

Pedunculate oak hosts a large number of insects and fungi (Županić et al., 2009; Marković and Stojanović, 2011; Wrzesińska, 2017; Demeter et al., 2021; Ermolaev et al., 2021; Milanović et al., 2021; Jankowiak et al., 2022; Pilipović et al., 2022; Marković and Dobrosavljević, 2023). Many of them can be effectively suppressed by using insecticides and fungicides (Mihajlović and Glavendekić, 2006; Margaletić et al., 2007; Glavaš, 2011; Pap et al., 2012, 2014a; Pajnik et al., 2017; Drekić et al., 2021). The results of our study showed that the insecticide applied in the experiment can be successfully used for the control of sucking insects, defoliators, and leaf-mining insects, as the leaf area damaged by these groups was significantly lower in the insecticide-treated area. It should not be used to control gallicolous insects because it is not very effective. The applied fungicide can be used to efficiently control the oak powdery mildew as the damage caused by it was significantly lower in the fungicide-treated area.

Among the fungi, oak powdery mildew caused the greatest damage on the observed leaves on the area treated with insecticide, and the comparison area. This was expected because oak powdery mildew is one of the biggest problems on the leaves of young pedunculate oak trees in Southeastern Europe (Karadžić and Milijašević, 2005; Glavaš, 2011; Pap et al., 2013). The plants treated with fungicide showed lower oak mildew damage in comparison to other plots, and also the greatest height increment. This height increment is most likely connected to the lower share of damaged leaf area and subsequentially more available leaf area for photosynthesis

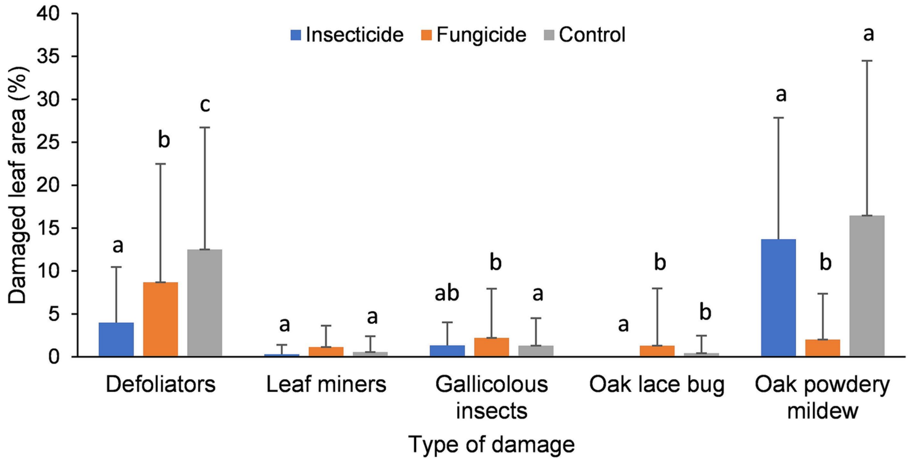

FIGURE 2
Damaged leaf area per type of damage (%) + SD, per each treatment, with letters above indicating the significant differences.

TABLE 1 **Results of the Mann–Whitney *U* test between the damaged area of the leaf per category.**

	Insecticide - control		Fungicide - control		Insecticide - fungicide	
	Z_{adj}	p	Z_{adj}	p	Z_{adj}	p
Defoliators	−12.52	0.00	−6.40	0.00	−6.14	0
Leaf miners	−1.37	0.17	5.29	0.00	−6.74	0
Gallicolous insects	1.24	0.21	2.21	0.03	−1.02	0.31
Sucking insects	−5.75	0.00	0.99	0.32	−6.37	0
Oak powdery mildew	−1.58	0.11	−19.03	0.00	18.13	0

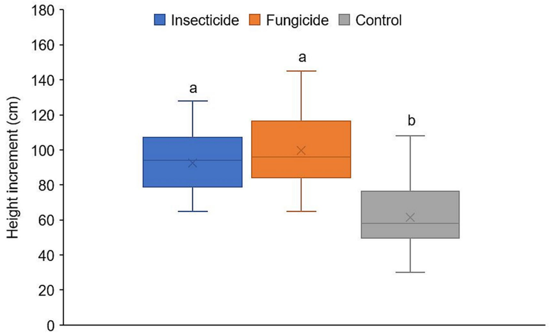

FIGURE 3
Influence of pesticide treatment on the height growth of the analyzed trees (cm) ± SD, with letters above indicating the significant differences.

(Nikolic et al., 2019; Paulin et al., 2020; Bălăcenoiu et al., 2021a). Of the insect groups, defoliators damaged the greatest leaf area. Damage from sucking insects, leaf miners, and gallicolous insects was negligible on all three plots. The fact that the damage from the sucking insects was small is a real surprise because, among the foliophagous insects in the old pedunculate oak forest near the location where the study was carried out, significant discoloration in the leaves caused by *C. arcuata* was noticed. Since the discoloration of the leaves in the old forest was higher, *C. arcuata* may prefer older trees, as it is already known that insect community and abundance change with the forest ages (Nagy et al., 2016; Marković et al., 2021b). The reason for this may be the fact that the allocation of defense chemicals is highest in young trees. On the other side, mature trees require resources for flower and seed production, they are frequently water deficient and have unfavorable photosynthesis/respiration, and saplings need the energy for the production of more aboveground biomass and increase of photosynthetic area, so they have a significantly lower amount of defense chemicals (Boege and Marquis, 2005; Barton and Hanley, 2013). In the forest where the research was carried out, areas with young trees of pedunculate oak, Turkey oak (*Q. cerris* L.), and Hungarian oak (*Q. frainetto* Ten.) of similar age were observed. The damage caused by *C. arcuata* was significantly greater on Turkey and Hungarian oak than on pedunculate oak. The pedunculate oak may be a less favorable host for *C. arcuata* compared to other oak species (Marković et al., 2021a).

There were no significant differences in tree growth between the treated areas. This shows that insects and fungi have a similar effect on their growth. This result is a novelty since the literature only mentions the effect of the oak powdery mildew (Bobinac and Karadžić, 1994; Karadžić and Milijašević, 2005; Glavaš, 2011; Pap et al., 2013; Rađević et al., 2020). In the area treated with the fungicide, the damage from sucking insects and leaf miners was higher than in the comparison area. Since oak powdery mildew was suppressed on it, this higher abundance indicates that there are competitive relationships between them. Such a relationship between the oak powdery mildew and insects is already known (Zargaran et al., 2012; Marković et al., 2021a).

The results of this study show that during the growing season, under the influence of fungi, the height growth of 10-year-old pedunculate oak trees decreases by 62.3%, and under the influence of insects by 50.5%. The real growth decrease is greater since the pesticides used did not achieve complete protection of the leaves from insects and fungi. The influence caused by these organisms is significant, which is why the need to suppress them arises. In plants up to 2 years of age, the control of oak powdery mildew should be carried out, as it is one of the limiting factors of the plants' development (Glavaš, 2011; Pap et al., 2012). Pesticide treatment of young trees older than 2 years is also useful as it positively influences their height growth and vitality. However, the treatment of older trees is complicated because of the characteristics of those stands (high density and height of trees), so the question of cost to benefit arises. The benefits of increased growth contribute both to the ecological functions such as sequestration of carbon dioxide, and economic functions such as the production of more wood. On the other side, any pesticide treatment affects other, non-targeted organisms, so the balance between these two needs to be found. The only place where the suppression of pest organisms on older trees should be carried out is in parks, gardens, and other areas where it does not require the use of expensive techniques and does not cause serious non-target effects.

Pedunculate oak is one of the dominant forest-forming species throughout Europe (Eaton et al., 2016). The restoration of its forests encounters many problems (Rumiantsev et al., 2018; Axer et al., 2023). To assist it, it is important to have a broader knowledge of the factors that can negatively affect those forests. When talking about the influence of insects and fungi on the growth of its young trees, based on the results of this study, it can be concluded that: 1. Both foliofagous insects and fungi significantly affect the height growth of the pedunculate oak, as the trees treated with pesticides had less damaged leaf area and grew significantly higher than trees in the comparison area; 2. *C. arcuata* did not influence the growth of the young trees significantly, as its abundance was low in all of the experimental areas; 3. The greatest damage on the leaves was caused by defoliator insects, which is why they contributed the most to the decrease in growth caused by insects; 4. The influence of the foliofagous insects on the growth of the trees was not significantly different from the influence of the fungi; 5. Suppression of oak powdery mildew and foliofagous insects on young trees is useful as it positively influences the vitality and growth of those trees, and contributes to economic and ecological gain; 6. As pedunculate oak is a less favorable host for *C. arcuata* compared to other oak species, it would be useful to determine whether there are differences between it and other oak species in terms of the influence of oak lace bug on the growth of young trees.

Data availability statement

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

Ethics statement

The animal study was approved by the Ethics board of Faculty of Forestry, University of Belgrade. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ČM: Conceptualization, Investigation, Resources, Supervision, Writing – original draft, Writing – review & editing. BK: Data curation, Investigation, Resources, Writing – review & editing. UP: Investigation, Writing – review & editing. JD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Response of *Alnus glutinosa* to *Phytophthora* bark infections at ambient and elevated CO_2 levels

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Introduction: Mortality of the riparian alder population caused by Phytophthora pathogens has been studied for over 20 years throughout Europe, recently gaining more importance in the context of evident climate change. The main objective of this study was to examine the pathogenicity of species from the "*Phytophthora alni complex*" present in the Czech Republic (*P. ×alni* and *P. uniformis*) and *P. plurivora* to *Alnus glutinosa* seedlings grown at ambient and elevated CO_2 concentration.

Methods: An underbark inoculation test was performed with seedlings grown from seeds collected from two Czech alder populations, one suffering from severe *Phytophthora* decline and the other disease-free.

Results: The results showed significant differences in lesion development and seedling mortality. After a 13-week experimental period, at both CO_2 levels *P. ×alni* and *P. uniformis* showed high aggressiveness to *A. glutinosa* seedlings causing lesions of variable sizes and mortality of 33.3%, and 45.8% of plants, respectively. In contrast, *P. plurivora* did not cause mortality to any plant, and lesion sizes did not differ significantly from those in control plants. Physiological measurements did not reveal any significant differences between *Phytophthora* species except for plants inoculated with *P. plurivora* showing increased values in specific physiological parameters 4 weeks post-inoculation. Net photosynthesis decreased over the measurement period in all treatments with significant differences found between measurements conducted 2 and 4 weeks after the inoculation. Transpiration showed a decreasing trend in all inoculated plants with no significant differences between *Phytophthora* species at both CO_2 levels. Chemical analyses of root samples showed high variability in sugars and phenolic compounds related to the plant's health status.

Discussion: This is the first study to examine the response of alder seedlings to *Phytophthora* pathogens at different CO_2 levels. The findings demonstrate high aggressiveness of *P. ×alni* and P. uniformis and weaker aggressiveness of *P. plurivora* to alder seedlings regardless of the CO_2 level.

KEYWORDS

alder dieback, underbark inoculation, elevated CO_2 concentration, sugars, phenolics, photosynthesis rate

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Introduction

Phytophthora species are fungal-like organisms within the kingdom Stramenopiles/Chromista and SAR supergroup (Beakes et al., 2015). More than 260 species are currently described (Brasier et al., 2022; Chen et al., 2022; Jung et al., 2022, 2024; Abad et al., 2023) and most of them are pathogens causing numerous diseases and devastating epidemics of agricultural crops, ornamental plants, and natural ecosystems worldwide (Erwin and Ribeiro, 1996; Jung et al., 2018; Brasier et al., 2022; Abad et al., 2023). The *Phytophthora* genus contains both generalist pathogens like the notorious *P. cinnamomi* Rands and *P. ramorum* Werres, De Cock & Man in 't Veld with particularly wide host ranges (Grünwald et al., 2012; Hardham and Blackman, 2018; Jung et al., 2021) and specialists like the oak-specific *P. quercina* (Jung et al., 1999) or the species from the "*Phytophthora alni* complex" which are exclusively pathogenic to alder (*Alnus* spp.) trees (Brasier and Kirk, 2001; Jung et al., 2018).

Alder stands are ecologically very important and alder trees are key nitrogen-fixing riparian species due to the symbiosis with the actinomycete *Frankia alni* (Vor.) Von Tub., which enables them to colonize extreme sites (Claessens, 2003). Most alder species grow in riparian ecosystems stabilizing riverbanks, acting as riparian buffers and windbreaks, and reduce erosion (Bjelke et al., 2016). Although showing fast growth and strong vegetative regeneration alder trees may suffer from multiple pathogens and pests with potentially devastating effects to riparian forests. Large-scale decline of alder stands across Europe, known as "alder dieback," is attributed mainly to root and collar infections by species from the "*P. alni* complex" (Brasier et al., 2004; Jung and Blaschke, 2004; Černý and Strnadová, 2010; Jung et al., 2013; Bjelke et al., 2016). All European alder species are affected by this epidemic which has caused devastating mortality of alder trees in riparian and forest stands across Europe (Gibbs et al., 1999; Streito et al., 2002; Jung and Blaschke, 2004; Jung et al., 2013, 2018; Husson et al., 2015; Kanoun-Boulé et al., 2016; Corcobado et al., 2023). The causal pathogen was first described as *P. alni* by Brasier et al. (2004) with three subspecies, i.e. *P. alni* subsp. *alni*, *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis*, which were later described as three distinct species, i.e., hybrid species *P.* ×*alni* (PAA) and *P.* ×*multiformis* (PAM) and the non-hybrid species *P. uniformis* (PAU) (Husson et al., 2015). It was shown that *P.* ×*alni* originated from the hybridization between *P. uniformis* and *P.* ×*multiformis* (Ioos et al., 2006; Husson et al., 2015). *Phytophthora uniformis* may have been introduced to Europe from North America (Aguayo et al., 2013), whereas the origin of *P.* ×*multiformis* is still unknown. Besides the "*P. alni* complex," *P. plurivora* Jung and Burgess (PLU) was frequently isolated from alder trees, including *Alnus glutinosa* L. and *A. viridis* (Chaix.) DC (Jung and Blaschke, 2004; Májek et al., 2019; Corcobado et al., 2023), indicating that the pathogen may also contribute to alder decline. This was also indicated by several pathogenicity tests on *A. glutinosa* (Jung and Nechwatal, 2008; Rytkönen et al., 2012; Mrázková et al., 2013; Haque et al., 2015).

Surveys in several European countries demonstrated that PAA is the most frequently isolated species, whereas PAU and especially PAM are comparatively rare (Nagy et al., 2003; Jung and Blaschke, 2004; Aguayo et al., 2013). Accordingly, pathogenicity tests revealed significant differences between species within the "*P. alni* complex" with PAA being more aggressive to *A. glutinosa* than PAU when infecting black alder (*Alnus glutinosa*) (Brasier and Kirk, 2001; Santini et al., 2003). Interestingly, PAU was shown to play an increasing role in alder dieback with increasing latitude in Scandinavia and increasing altitude in the Alps which is related to the lower frost tolerance of PAA as compared to PAU (Schumacher et al., 2006; Černý and Strnadová, 2010; Redondo et al., 2015; Corcobado et al., 2023). This is particularly important in the context of ongoing climatic changes and potential spread of the aggressive PAA strains to the currently unfavorable climatic regions.

Climatic changes are characterized by shifts in annual rain patterns, increased frequencies of extreme weather events, and the increase of average annual temperatures and atmospheric CO_2 concentration (Moore et al., 2015). The most rapid increase of CO_2 concentration was recorded during the second half of the 20th century and the beginning of the 21st century (Belmecheri and Lavergne, 2020). According to the Intergovernmental Panel on Climate Change (IPCC) the projected concentration of CO_2 in the year 2100 will range in different scenarios from 540 to 970 ppm (IPCC, 2001). Pessimistic scenarios predict an increase of CO_2 levels to 900–1,000 ppm by the end of the 21st century (Van Vuuren et al., 2011).

Elevated CO_2 levels can have a significant impact on host-pathogen interactions (Percy et al., 2002; McElrone et al., 2005). Generally, a stronger resistance against plant pathogens is predicted in relation to elevated CO_2 level in the atmosphere, as the excess of carbon can promote the production of plant defensive secondary chemicals (Kazan, 2018). However, plants grown at elevated CO_2 can often alter constitutive and induced defense-related phytohormone levels, reflecting complex and dynamic biochemical interactions (Bazinet et al., 2022). So far, existing studies reported positive, negative, and neutral effects of elevated CO_2 level on plant immune responses to fungal pathogen infection (Smith and Luna, 2023). The majority of studies on plant-biotic interaction investigate above-ground plant parts and related fungal pathogens (Kazan, 2018). However, only a limited number of studies have been performed on *Phytophthora* pathogens and underbark or root infections. These studies showed both positive and negative effects of elevated CO_2 level on disease incidence depending on the *Phytophthora* and host species and the length of the experiment (Jwa and Walling, 2001; Fleischmann et al., 2010; Tkaczyk et al., 2014; Oszako et al., 2016; Milanović et al., 2020).

Phytophthora-induced damage diminishes transpiration and nitrogen allocation in leaves, affecting starch allocation (Clemenz et al., 2008; Osswald et al., 2014). Before transpiration decline, reduced stomatal conductance and assimilation are observed (Clemenz et al., 2008). Water relations are more affected than photosynthesis, with rapid hydraulic conductance decrease when the pathogen colonizes up to 15% of the root system. The swift response involves down-regulated cytokinins, up-regulated ABA, and 1-aminocyclopropane-1-carboxylic acid (Osswald et al., 2014). ABA and ethylene act as stress markers, closing stomata and increasing antioxidant activity (Müller, 2021). Maximal photosynthetic rate decreases with the extent of cortex destruction from 12 to 4 $\mu mol\ m^{-2}\ s^{-1}$, accompanied by a decrease of PSII electron quantum yield (Osswald et al., 2014). Successful tree defense is suppressed, as *Phytophthora* deactivates salicylic-acid response genes, resulting in the reduction of lignin, phenols, and PR proteins in infected roots compared to healthy ones (Osswald et al., 2014).

Changes in physiological parameters are reflected in saccharides translocation. For example, in beech seedlings inoculated by PLU, the content of total saccharides decreased significantly compared to the control plants (Corcobado et al., 2022). Even though the sugar alcohols were even higher in the roots of control plants, the differences between infected and control seedlings became less pronounced after three weeks of inoculation (Corcobado et al., 2022). There is a different response to *Phytophthora* attack in leaves and roots at the biochemical level (Corcobado et al., 2022).

While the influence of temperature on the activity of different species from the "*P. alni* complex" was discussed within previous studies (Schumacher et al., 2006; Černý and Strnadová, 2010; Redondo et al., 2015) suggesting possible further spreading due to climatic changes, studies on *Phytophthora-Alnus* interaction at elevated CO_2 levels are, however, still missing. This study aimed to compare the responses of *A. glutinosa* plants to infections by PAA, PAU, and PLU at ambient and elevated CO_2 levels.

Prior to the experiment, the following hypotheses were raised: (i) the survival of alders is influenced by the interaction between *Phytophthora* species inoculation and CO_2 level, and (ii) variations in physiological and biochemical responses are expected in inoculated alders depending on the *Phytophthora* species and the CO_2 level.

Materials and methods

Plant material and growth conditions

Alder (*A. glutinosa*) tree cones were collected in autumn 2020 in the Czech Republic. To achieve higher variability of alder genotypes, two different riparian populations were selected: one showing typical alder dieback and collar rot symptoms (Jung and Blaschke, 2004; Jung et al., 2018), and the second being asymptomatic (Table 1). The seedlings were grown in a ready-mixed substrate containing peat and perlite (Forestina, Czech Republic) in individual 2-liter containers. The plants were watered to field capacity with tap water once per week. Plants were fertilized 2 months before the inoculation (Osmocote, ICL Specialty Fertilizers). No fungicides were applied during the experiment.

All plants were grown in Walk-In growth chambers (PSI Ltd., Drásov, Czech Republic) with the following conditions: 14/10 h photoperiod, 20/15°C, 65 % relative humidity, light spectrum 250 μmol m^{-2} s^{-1}. Two chambers each were used for the ambient and elevated CO_2 levels, respectively. The mean global atmospheric CO_2 concentration of the year 2020 (=415 ppm; https://gml.noaa.gov/webdata/ccgg/trends/co2/co2_annmean_mlo.txt) was chosen as ambient CO_2 level whereas the elevated CO_2 level was twice as high (830 ppm).

Underbark inoculation test

Isolates of three *Phytophthora* species, PAA, PAU, and PLU were sourced from the CZU/MM Oomycetes collection (Table 2). Their identity was previously confirmed by sequence analysis of the internal transcribed spacer (ITS1-5.8S-ITS2) region of the ribosomal DNA according to Jung et al. (2019), for the PAU isolate the β-tubulin gene was also sequenced using primers TUBUF2 and TUBUR1 (Kroon et al., 2004) (Table 2). Underbark inoculation was performed in June 2021 according to Milenković et al. (2018). The bark surface of the alder saplings at a distance of 10–15 cm from the collar was cleaned with 70% ethanol. Plants were wounded using a sterilized 7-mm metal cork borer. Same-sized plugs cut from the edges of 5 to 7-day-old *Phytophthora* colonies grown on V8-agar medium (V8A) (Jung et al., 1996) were placed with the mycelial side onto the exposed wood. The agar plugs were covered with the removed piece of bark and cotton moistened with sterile distilled water and sealed with Parafilm and aluminum foil.

In total, 12 six-months-old plants per *Phytophthora* species and CO_2 level were inoculated, including six plants originating from populations 1 and 2, respectively (Table 1). The control treatment also had 12 plants, but they were mock-inoculated with sterile V8A plugs and sealed in the same way. After the inoculation the plants were moved back to their growth chambers. The plants were inspected every 2 weeks for the appearance of symptoms, such as seedling mortality, vertical length of stem lesions and extent of girdling (Table 3). Girdling was visually evaluated on a five-point scoring system, according to Zamora-Ballesteros et al. (2017).

Thirteen weeks after inoculation, when 50% of plants in one treatment died, the experiment was finished and evaluated. Necrosis lengths were measured using a precise ruler, while necrosis widths were measured using a flexible measurement tape. Re-isolations were made from all inoculated and control plants by plating small pieces from the upper and lower margins of necrotic lesions or, in the absence of necroses, from the margins of the inoculation points onto PARPNH selective agar medium (Jung et al., 1996). Pathogen identification was confirmed by comparing the colony morphologies on V8A with those of the original isolates.

Biomass of above- and below-ground plant tissues was weighed and expressed as the above/below-ground dry biomass ratio. The aerial tissues of each seedling were divided into stems and leaves and dried in the oven at 65°C for 48 hr. Roots were dried after thoroughly washing to remove adhering soil.

Physiological and chemical analysis

Physiological analysis

Six plants from each treatment (*Phytophthora* species/CO_2 level) were randomly selected for the physiological measurements. These were performed six times during the experiment in 2-week intervals, always using the same plants: one measurement prior to the inoculation and five measurements after the inoculation. Physiological measurements included chlorophyll a fluorescence and gas exchange measurements.

Chlorophyll a fluorescence was measured using FluorPen FP 110 (Photon Systems Instruments, Brno, Czech Republic).

TABLE 1 Origin of the *Alnus glutinosa* seeds used in this study.

No	Location	River	Coordinates	Altitude (m a.s.l.)	Age of alder trees (years)	Health status of alder trees
1	Vltava River basin	Litavka	49.8235244N 13.9740708E 49.8729619N 13.9963381E	260–340	70	Dieback, collar rots, mortality; *P. ×alni* isolated
2	Beskydy Mountains	Morávka	49.6372400N 18.4426000E 49.6020831N 18.5187214E	380–440	30	No symptoms of *Phytophthora* infection

TABLE 2 Origin of the *Phytophthora* isolates used in this study and GenBank accessions of their ITS [(a)] and β-tubulin [(b)] sequences.

Phytophthora species	Isolate code	Year of isolation	Host/origin	Locality (CZ)	GenBank accession no.
P. ×alni	MM123	2020	*Alnus glutinosa*/bark sample	Příbram, Litavka river	OQ703915[a]
P. uniformis	MM078	2020	*A. glutinosa*/bark sample	Příbram, Litavka river	OQ703916[a] PP210887[b]
P. plurivora	MM033	2020	*A. glutinosa* /rhizosphere soil	Kožlany (Plaská pahorkatina), Javornice river	OQ703917[a]

TABLE 3 Girdling evaluation scoring system.

Score	Explanation
0	No necrosis
1	One-quarter of stem girdled
2	Two-quarters of stem girdled
3	Three-quarters of stem girdled
4	Totally girdled

Measurements were performed on adaxial surfaces of 3–4 leaves per plant. Before the fluorescence measurement, dark acclimation of the three selected leaves was assured by special leaf clips with a retractable metal plate. After 20 min of dark adaptation, the instrument was mounted to the clip and the actinic light (3,000 µmol m^{-2} s^{-1}) was applied to the leaves. For statistical analysis, the following indexes were selected to estimate plant health and performance (according to Živčák et al., 2008): (a) maximum quantum yield of primary PSII photochemistry (1) and (b) performance index expressing energy conservation from absorption of light by an antenna (2). Its expressions are detailed below:

$$\Phi_{PSII} = \frac{F_V}{F_M} \tag{1}$$

Φ_{PSII} – maximum quantum yield of primary PSII photochemistry

F_V – variable fluorescence

F_M – maximum fluorescence

$$\mathrm{PI_{ABS}} = 1 - \frac{\frac{\mathrm{F_0}}{\mathrm{F_M}}}{\frac{\mathrm{M_0}}{\mathrm{V_j}}} * \frac{\mathrm{F_M} - \mathrm{F_0}}{\mathrm{F_0}} * \frac{1 - \mathrm{V_j}}{\mathrm{V_j}} \tag{2}$$

F_0 – fluorescence intensity at 50 µs

F_j – fluorescence intensity at the J step (at 2 ms)

F_M – maximal fluorescence intensity

V_j – relative variable fluorescence at 2 ms

M_0 – initial slope of fluorescence kinetics.

An open portable photosynthesis system with infrared gas analyser LI-6400 XT (LICOR, Lincoln, NE, USA) was used for *in situ* gas exchange measurements. Net photosynthetic rate (PN), transpiration (E), stomatal conductance (Gs), and internal-to-ambient CO_2 concentration ratio (Ci/Ca) were measured at photosynthetic photon flux density of 1,500 ± 1 µmol m^{-2} s^{-1} and an ambient CO_2 concentration of 415/830 ± 1 µmol mol^{-1} (ambient/elevated CO_2 treatments) which was above the saturation point measured in advance (350–400 µmol m^{-2} s^{-1}). A standard leaf chamber with a red/blue LED light source was used. The samples were taken between 9 am and 5 pm. Measurements were performed on 2 leaves per plant.

Chemical analysis

Three root samples per treatment (*Phytophthora* species/CO_2 level) were taken for chemical analysis during the final assessment. Selected plants were gently removed from the planting pots, and the root systems washed in distilled water to remove all substrate particles. Immediately after cleaning the samples were frozen and kept at −32°C until further processing. After freeze-drying and homogenization, approximately 0.03 g per sample were used for the extraction of selected sugars and phenolic compounds. The detailed method is given in Supplementary Table 1.

Data analysis

Lesion development and mortality, plant biomass

The assessment of normality within each group was conducted using the Shapiro-Wilk test. Given the observed deviation from the assumptions of normal data distribution, differences in various parameters, including lesion length and width, lesion area, above-/below-ground biomass ratio, percentage of lesion length from plant height, final plant height, and collar diameter, were subsequently analyzed using the paired samples Wilcoxon test. Significances of pairwise differences were computed by the pairwise Wilcoxon test implemented in "pairwise.wilcox.test" base R statistical software (R development CORE team, 2021) with *p*-value adjustment set as BH—i.e., false discovery rate adjustment (Benjamini and Hochberg, 1995). Differences between groups were investigated at the significance level α = 0.05. Hedge's g effect sizes were computed using "cohens.d" function from "effsize" package with "hedges.correction" parameter set to TRUE.

For girdling analysis, the Area Under Disease Progress Curve (AUDPC) was used for the disease assessment. The AUDPC was measured using the trapezoidal integration method, according to Madden et al. (2007), using the expression:

$$AUDPC = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i)$$

where: y_i: initial score of girdling; y_{i+1}: final score of girdling; t_{i+1}: final time; t_i: initial time.

AUDPC values were analyzed using ANOVA and Fisher's LSD test performed in STATISTICA 13.0 software.

Survival curves describing the relationship between the mortality rate and time after the infection were generated using the Kaplan–Meier Estimates method in STATISTICA 13.0 software. Statistical significance of survival differences among groups was assessed employing the Chi-Square Test. Pairwise comparisons were conducted using the Log Rank test with a significance threshold set at α = 0.05.

Mortality rate was evaluated using the Chi-Square Test. Correlations between mortality and selected parameters (lesion area, percentage of lesion length from plant height, final plant height, and above-/below-ground biomass ratio) were analyzed using Spearman Rank Order Correlations because of the inherent characteristics of the data. Relationships between stem girth and girdling/mortality were analyzed using either linear regression models or Welsh test, employing the R software. The data used for the analysis of the correlation between stem girth and lesion girdling are originated from an equally spaced five-point scoring system of lesion girdling, hence suitable for any kind of analysis including linear regression models.

Physiological and chemical analyses

For physiological parameters, the R software was used. Since the measurements were conducted six times, a repeated measurements approach was adopted with each plant serving as the measurement unit. The experimental design encompassed treatment groups with four levels (control, PAA, PAU, and PLU), CO_2 concentration at two levels (415/830 ppm). The evaluation of physiological traits involved the application of a mixed linear model, accounting for the repeated measurements.

PAA- and PAU-inoculated plants showed gradual mortality, leading to a decrease in the number of measured plants; therefore, the measurements performed at 6, 8, and 10 weeks post inoculation (p.i.) were unsuitable for statistical analyses of gas exchange measurements.

The model was structured to assess the influence of time-varying factors and their interactions on the response variable y. The fixed effects in our model encompassed Time, Treatment, and CO_2, as well as their interactions with Time, formulated as:

$$\begin{aligned} y \sim \mathrm{Time} + \mathrm{Treatment} + \mathrm{CO_2} + \mathrm{Time:Treatment} \\ + \mathrm{Time:CO_2} + \mathrm{Time:Box} \end{aligned}$$

To adequately capture the within-subject variation typical in repeated measures designs, the model controlled variation among replicated boxes by including random effect formulated as Time:Box. Furthermore, to address the autocorrelation typically present in repeated measures data, the residual structure of the model was specified as: id(ID):cor(Time), allowing for correlations between time of the same experimental unit.

All mixed models were fitted with ASReml-R v4.1 (Butler et al., 2017), which uses restricted maximum likelihood methods to estimate variance components. In all cases, diagnostic plots were reviewed for normality and to detect potential outliers.

In the statistical analysis of chemical parameters (sugars and phenolic compounds), the Kruskal-Wallis ANOVA was used to examine intergroup differences. Prior to analysis, the normality of the data distribution was assessed, revealing a deviation from the assumption of normality. To further test significant variations among groups, a *post hoc* Dunn's test was conducted (significance level α = 0.05).

Results

Lesion development and survival analysis

Representative symptoms 13 weeks post inoculation (p.i.) are shown in Figure 1 and explained below. Lesions started to appear within the first 2 weeks p.i.. The lesion size varied between the *Phytophthora* species and among the plants within the same treatment. Lesions developed rapidly in plants inoculated with PAU and PAA. In most plants the lesions reached their final length within 4 weeks p.i. with mean lesion lengths ranging from 7.8 to 8.7 cm in PAA, 7.7 to 11.4 cm in PAU and 1.8 to 1.9 cm in PLU (Table 4, Figure 2). For PAU a statistically non-significant trend of higher average lesion lengths was recorded in the ambient as compared to the elevated CO_2 level (11.43 ± 7.33 cm vs. 7.66 ± 6.87 cm, $p = 0.249$; Table 4, Supplementary Figure S1). For PAA a similar trend was observed (8.68 ± 7.11 cm vs. 7.76 ± 5.91 cm, $p = 0.951$; Table 4, Supplementary Figure S1). On the contrary, plants inoculated with PLU had only very small lesions that were not significantly different from the control (Table 4,

FIGURE 1
Representative symptoms of *Alnus glutinosa* seedlings caused by *Phytophthora* species 13 weeks after underbark inoculation at ambient (aCO_2) and elevated (eCO_2) levels; **(A)** control plants at aCO_2; **(B)** *P. uniformis*–inoculated plants at eCO_2; **(C)** *P. plurivora*–inoculated plants at aCO_2; **(D)** control at eCO_2; **(E)** *P. ×alni* at aCO_2; **(F)** *P. uniformis* at eCO_2; **(G)** control; **(H, I)** *P. ×alni* lesions at eCO_2 and eCO_2; **(J–L)** *P. uniformis* lesions at aCO_2 **(J, L)** and at eCO_2 **(K)**; **(M, N)** small constrained lesions caused by *P. plurivora* at eCO_2 and aCO_2.

TABLE 4 Pathogenicity of three *Phytophthora* species to *Alnus glutinosa* seedlings in the underbark inoculation test after 13 weeks.

CO_2 conc. [ppm]	*Phytophthora* species	No. of plants with lesions	Lesion length x± SD [cm]^1	Lesion width [cm] x± SD^1	Lesion area x± SD [cm^2]^1	No. of plants with dieback/ mortality	Reisolation frequency [%]
415	Control	0	0.92 ± 0.15a	0.95 ± 0.12a	0.69 ± 0.19a	0/0	0
	P. ×alni	12	8.68 ± 7.11b	1.70 ± 0.58b	13.69 ± 13.59b	5/3	100
	P. uniformis	12	11.43 ± 7.33b	1.94 ± 0.52b	18.23 ± 12.19b	8/6	100
	P. plurivora	12	1.84 ± 0.50c	1.10 ± 0.31a	1.63 ± 0.72c	0/0	100
830	Control	0	0.98 ± 0.14a	0.94 ± 0.16a	0.73 ± 0.21a	0/0	0
	P. ×alni	12	7.76 ± 5.91b	1.38 ± 0.45b	9.74 ± 9.64b	6/5	100
	P. uniformis	12	7.66 ± 6.87b	1.22 ± 0.35b	8.13 ± 7.92b	7/5	100
	P. plurivora	12	1.95 ± 0.39c	0.85 ± 0.24a	1.30 ± 0.48c	0/0	100

Twelve plants were inoculated in each treatment (CO_2/*Phytophthora* species). ^{1}Different letters indicate significant differences between values within each CO_2 level. For significance of differences between CO_2 levels see Figure 2 and Supplementary Figures S1, S2.

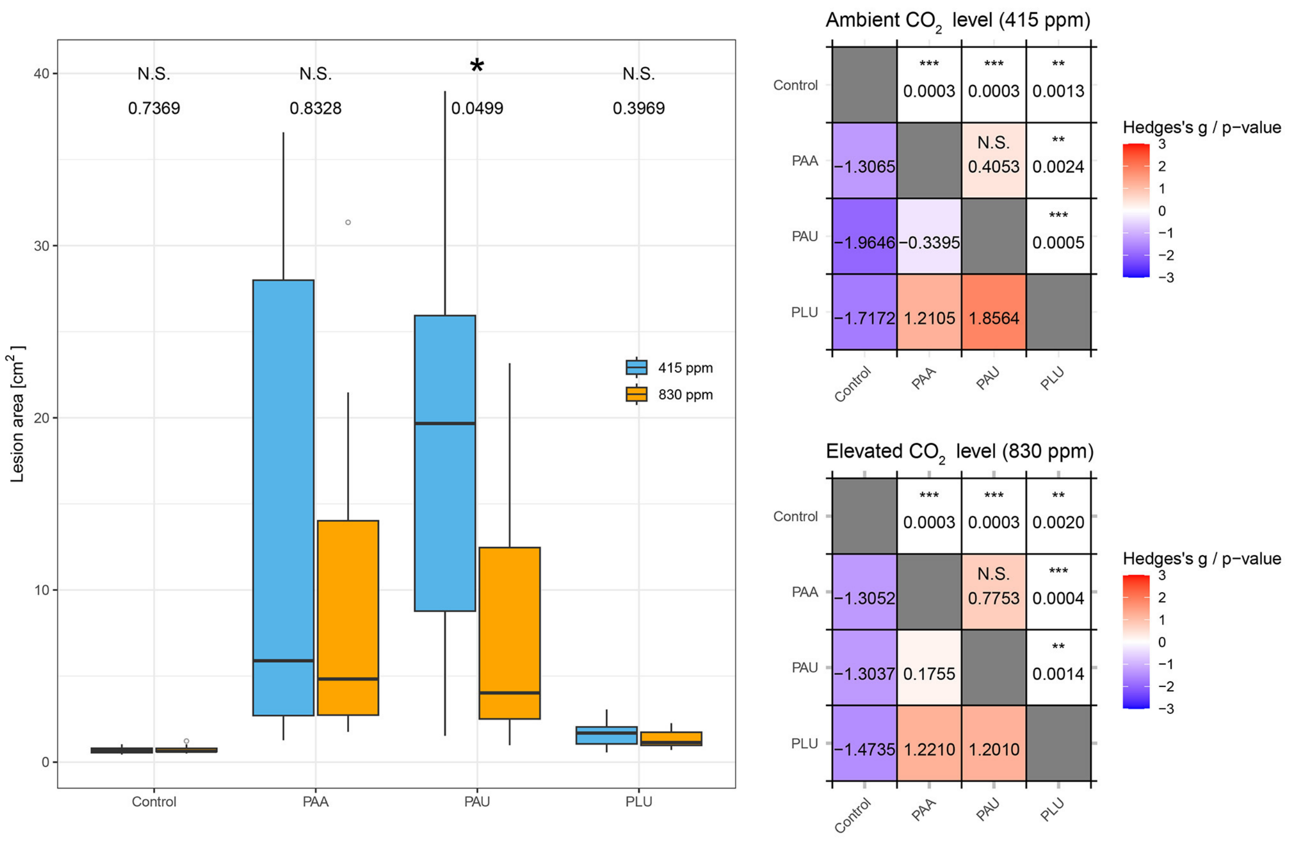

FIGURE 2
Comparison of lesion area in the stems of *Alnus glutinosa* seedlings caused by three *Phytophthora* species at two CO_2 levels (ambient 415 ppm, elevated 830 ppm). PAA, *P. ×alni*; PAU, *P. uniformis*; PLU, *P. plurivora*. The statistical significance of CO_2 pairs comparisons is indicated by N.S. (non-significant) or an asterisk ($p \leq 0.05$). The tables on the right contain all pairwise comparisons; the upper triangle represents the p-value of pairwise comparisons, the lower triangle represents the effect size of Hedges'g.

Figure 2, Supplementary Figure S1). The re-isolations confirmed the pathogen presence in all inoculated plants (Table 4).

The lesion area, calculated as the ellipse area (A = πab), showed differences between the *Phytophthora* species and CO_2 levels which were partly statistically significant (Table 4, Figure 2). At both CO_2 levels, PAA and PAU caused lesions with significantly different lesion areas compared to both the control and PLU-inoculated plants ($p \leq 0.05$; Table 4). In contrast to PAA-inoculated plants, in PAU-inoculated plants the effect of CO_2 was statistically significant ($p = 0.049$) with bigger lesions found at the ambient as compared to the elevated level (18.23 ± 12.19 cm vs. 8.13 ± 7.92 cm) (Figure 2). PLU differed significantly from the control at both CO_2 levels, but the variability was much lower and no significant difference existed between different CO_2 levels (Table 4).

PAA and PAU had significantly different lesion width compared to both the control and PLU, whereas PLU did not differ from the control at both CO_2 levels (ambient: $p = 0.276$, elevated: $p = 0.572$) (Table 4). However, the effect of CO_2 was significant only in PAU ($p = 0.007$) with bigger lesion width at ambient compared to the elevated level (1.94 ± 0.52 cm vs. 1.22 ± 0.35 cm, respectively) (Supplementary Figure S2).

For both PAA and PAU-inoculated plants a statistically significant positive correlation was found between lesion area and mortality. A larger lesion area was consistently associated with a higher mortality rate (PAA $r = 0.72$; PAU $r = 0.78$) (data not shown).

The statistical analyses revealed significant differences in girdling caused by PAA and PAU compared to the control regardless of the CO_2 level (Figure 3). The first dying plants developed lesions very quickly and complete girdling of the collar occurred within the first weeks p.i. On the contrary, PLU girdled in all plants <25% of collar circumference. Complete girdling was significantly correlated with smaller stem circumference (stem girth) in both PAA ($R^2 = 0.272$, $p = 0.0052$) and PAU-inoculated plants ($R^2 = 0.368$, $p = 0.001$; Figure 4). Complete girdling also induced the swelling of sleeping buds above the girdling lesions a strong reaction in several plants (Figures 1I, J).

Seedling mortality

The first symptoms of severe dieback and mortality appeared in PAU-inoculated plants 2 weeks p.i., followed by PAA-inoculated plants 6 weeks p.i. All control and PLU-inoculated plants survived until the end of the experiment (Figure 1, Table 4). Overall, PAA caused mortality in 33.3% (8/24 plants) and PAU in 45.8% (11/24) of the inoculated plants (Table 4). However, there was no significant effect of the CO_2 level on the mortality rates of either PAA or PAU ($p > 0.05$).

Log-rank test of equality from survival analysis revealed significant differences in survival probability among experimental groups ($\chi^2 = 29.38$, $p < 0.001$). At ambient CO_2 level, survival probabilities in PAA and PAU-inoculated plants were 70% and 45%, respectively. At elevated CO_2 level, both isolates had survival rates lower than 60%. First mortality associated with PAU was recorded 3 and 5 weeks after inoculation at elevated and ambient CO_2 levels, respectively. The seedlings inoculated with PAA started to die 8 and 9 p.i. at elevated and ambient CO_2 levels, respectively. There were no differences in seedling survival probability between PAA and PAU isolates at both ambient and elevated CO_2 level. Compared to the control groups, PAU showed significant differences at ambient ($\chi^2 = 8.67$, $p = 0.003$) and elevated CO_2 level ($\chi^2 = 6.07$, $p = 0.014$). Also, PAA showed a significant difference from the control groups at ambient ($\chi^2 = 4.02$, $p = 0.045$) and elevated ($\chi^2 = 6.07$, $p = 0.014$) CO_2 level (Figure 5).

At the beginning of the experiment, plant height and collar diameter were variable with a mean height of 47.6 ± 15.6 cm and a mean collar diameter of 0.8 ± 0.2 cm (Table 5). Wilcoxon test revealed no significant differences in plant height or collar diameter between *Phytophthora* species at any CO_2 level ($p > 0.05$). The majority of plants reached their final height by the time of inoculation, with minimal height increases observed until the end of the experiment. In contrast, surviving plants exhibited a noteworthy increase in collar diameter (16–70% after 13 weeks). Conversely, plants that died first showed a decrease in collar diameter as the tissue shrunk due to water loss.

A statistically significant negative correlation between mortality and stem girth was found for both PAA and PAU (Welsh *t*-test, $p < 0.05$), i.e., higher mortality was associated with plants of smaller stem girth (Figure 6).

The percentage of lesion length from total plant height ranged in dead plants approximately from 30% to 80%. In surviving plants, the lesions extended approximately over 1% to 31% of the total plant height. This percentage correlated significantly with the mortality in both PAA and PAU ($r = 0.804$ and $r = 0.851$, respectively) (data not shown). Moreover, plant height showed a significant negative correlation with mortality for PAA ($r = -0.389$), PAU ($r = -0.357$) and PAA-PAU combined ($r = -0.27$) (Figure 7), i.e., higher plants died later or survived until the end of the experiment. The first plants to die were those with the shortest height and lesion lengths exceeding 60 % of the total plant height.

Plant biomass

The above-ground biomass at the end of the experiment was affected by various plant reactions to the pathogen infection. In many plants, successive leaf loss occurred over the whole experiment. Symptoms, such as wilting, leaf drying, chlorosis, sudden leaf dropping, and subsequent regeneration leaf flush, caused variations in plant biomass across all treatments (Figure 1). No significant differences were observed in dry biomass between above-ground plant tissues and roots. The average values for above and below-ground biomass for all plants were 8.89 ± 6.50 g and 6.96 ± 5.06 g, respectively (Table 5).

The effect of *Phytophthora* species on dry biomass did not show statistical significances. However, when comparing the ratio between above- and below-ground biomass, a statistically significant negative effect of elevated CO_2 was observed in control ($p = 0.0013$) and PLU–inoculated plants ($p = 0.0013$) (Supplementary Figure S3). In both cases, higher ratios were found at the ambient level compared to the elevated level (control: 1.60 ± 0.24 vs. 1.14 ± 0.24, PLU: 1.64 ± 0.28 vs. 1.17 ± 0.24). In plants inoculated with PAA or PAU the ratio between above- and below-ground biomass showed neither a significant effect of the CO_2 level ($p > 0.05$; Supplementary Figure S3) nor a statistically significant correlation with mortality ($p > 0.05$; data not shown).

Physiological measurements and chemical analysis

Physiological responses of alder seedlings to the three *Phytophthora* species showed significant variations in time vs. CO_2 levels, and their interplay across treatments for net photosynthesis (Pn), stomatal conductance (Gs), and intracellular CO_2 concentration (Ci). The Ci/Ca ratio, denoting the intra- and extracellular CO_2 concentration ratio, displayed

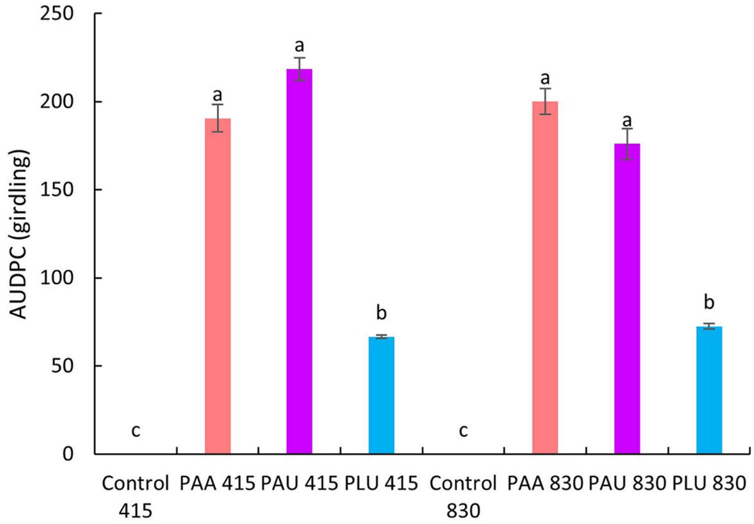

FIGURE 3
Comparison of area under the disease progress curve (AUDPC) in stems of *Alnus glutinosa* seedlings inoculated with *Phytophthora ×alni* (PAA), *P. uniformis* (PAU) and *P. plurivora* (PLU) at two CO_2 levels (415 ppm and 830 ppm). Error bars show the standard deviation. Different letters indicate significant differences (Fisher's LSD Test, $p < 0.05$).

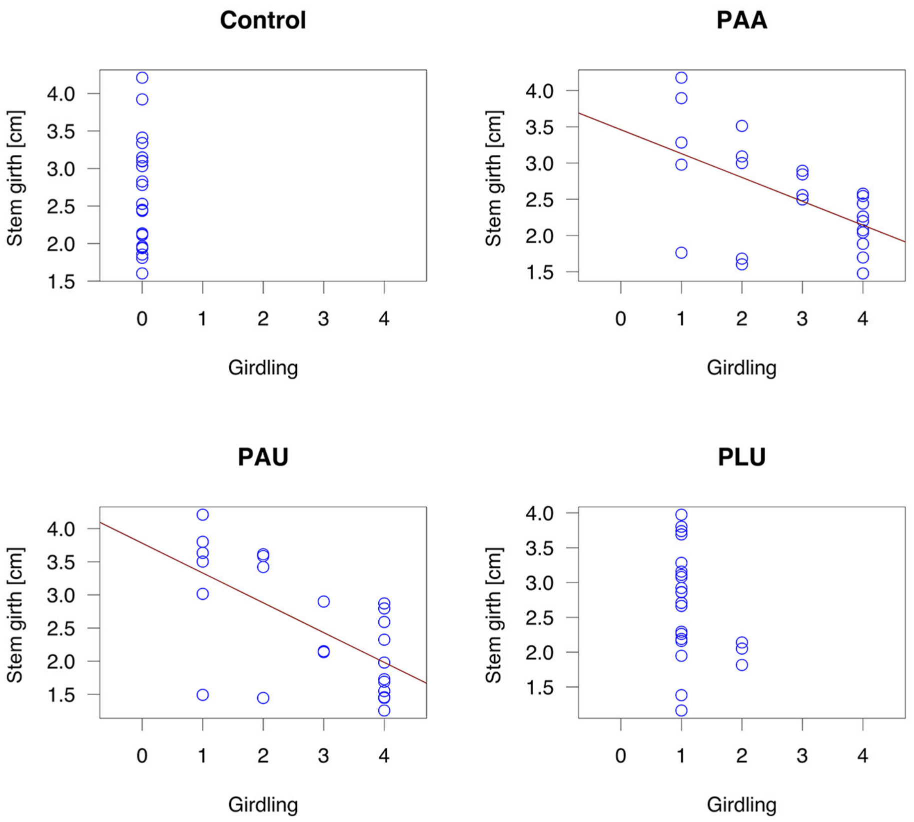

FIGURE 4
Relationship between the stem girth of *Alnus glutinosa* seedlings and girdling (PAA, *P. ×alni*; PAU, *P. uniformis*; PLU, *P. plurivora*): Linear regression model (PAA: $R^2 = 0.272$, $p = 0.0052$; PAU: R2 = 0.368, $p = 0.001$).

differences between measurements and CO_2 concentrations. Net photosynthesis (Pn) exhibited a declining trend throughout the measurement period, with no significant differences between *Phytophthora* species and the control, excluding CO_2 concentration and week interaction. However, significant differences were found between the first and second measurements after the inoculation

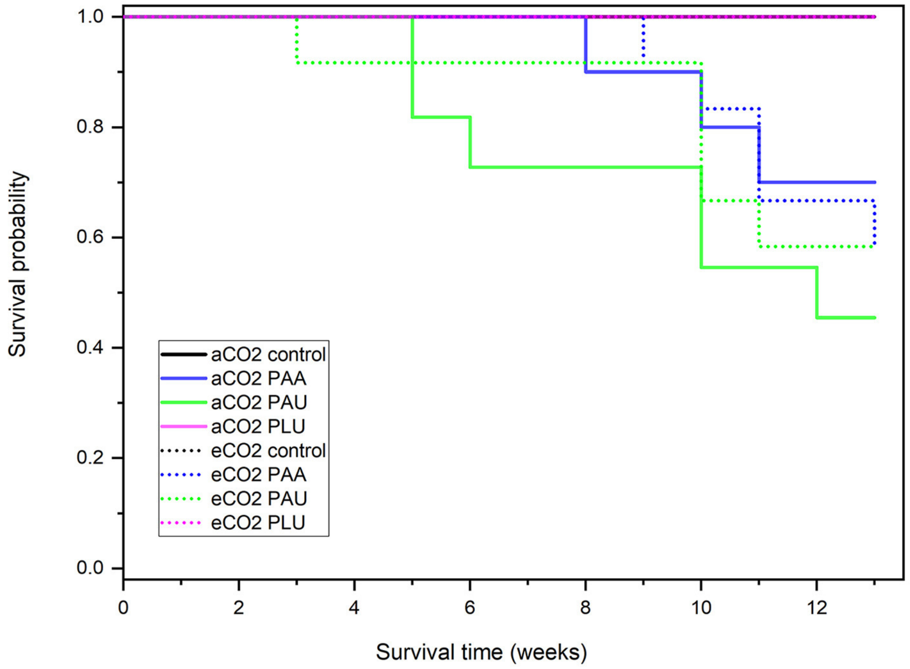

FIGURE 5
Survival probabilities of *Alnus glutinosa* seedlings 13 weeks after the inoculation: Kaplan–Meier test at ambient CO_2 level ($aCO_2 = 415$ ppm) and elevated CO_2 level ($eCO_2 = 830$ ppm). PAA, *Phytophthora ×alni*; PAU, *P. uniformis*; PLU, *P. plurivora*.

TABLE 5 Results from the underbark inoculation test on *Alnus glutinosa* seedlings with three *Phytophthora* species after 13 weeks.

CO_2 conc. [ppm]	*Phytophthora* species	No. of inoculated plants	Collar diameter [cm][a]	Plant height [cm][a]	Dry weight of above-ground biomass [g][a]	Dry weight of below-ground biomass [g][a]	Above-/below-ground biomass ratio[b]
415	Control	12	0.83 ± 0.22	49.79 ± 14.16	9.86 ± 7.22	6.05 ± 3.96	1.60 ± 0.24*
	P. ×alni	12	0.81 ± 0.22	46.30 ± 19.71	7.63 ± 6.33	5.75 ± 3.95	1.36 ± 0.86
	P. uniformis	12	0.82 ± 0.29	51.95 ± 21.03	9.80 ± 8.83	8.29 ± 6.62	1.40 ± 0.65
	P. plurivora	12	0.80 ± 0.30	49.33 ± 16.41	9.59 ± 7.10	6.10 ± 4.67	1.64 ± 0.28**
830	Control	12	0.83 ± 0.22	44.13 ± 12.17	8.25 ± 5.45	7.68 ± 5.30	1.14 ± 0.24*
	P. ×alni	12	0.81 ± 0.25	42.50 ± 12.26	8.97 ± 7.01	7.80 ± 6.16	1.20 ± 0.45
	P. uniformis	12	0.78 ± 0.31	49.39 ± 15.73	8.99 ± 7.14	7.25 ± 5.63	1.23 ± 0.25
	P. plurivora	12	0.86 ± 0.21	47.33 ± 13.02	8.75 ± 5.02	7.88 ± 5.01	1.17 ± 0.24**

[a]No significant differences were found between treatments. [b]*Control ($p = 0.0013$) and **PLU ($p = 0.0013$) values differ significantly between CO_2 levels.

(two and 4 weeks p.i., respectively) in all inoculated plants at each CO_2 level ($p < 0.05$) (Figure 8). A non-significant decrease in Pn, observed between the first and second measurements in control plants at ambient CO_2 level, was induced by competition for light and space. Elevated CO_2 notably increased Pn, particularly in the pre-inoculation stage, but the values in inoculated plants were gradually decreasing over the experimental period.

Stomatal conductance (Gs) remained stable throughout the experiment, with the control having the lowest average values (0.27 ± 0.14 mol H_2O m^{-2} s^{-1}) and PLU exhibiting the highest values (0.32 ± 0.18 mol H_2O m^{-2} s^{-1}). Elevated CO_2 led to a 7% Gs reduction throughout the experimental period. Intriguingly, Ci responded to higher CO_2 levels, gradually reaching its peak during the third measurement (4 weeks p.i.), while Ci/Ca ratio increased in elevated CO_2. Transpiration (Tr) showed decreasing trend in all inoculated plants till 4 weeks p.i. with no significant differences among *Phytophthora* species within each CO_2 level ($p > 0.05$) (Figure 8). Water use efficiency (WUE) ranged from 5.7 to 9.4 µmol CO_2 per mmol H_2O, with higher values at the elevated CO_2 level. Fast kinetics of fluorescence indexes, quantum efficiency of PSII (Fv/Fm) and performance index (PI) exhibited common values for healthy plants. Similarly, the course of the OJIP curve did not reveal any significant differences in any step of the graph (Supplementary Figure S4).

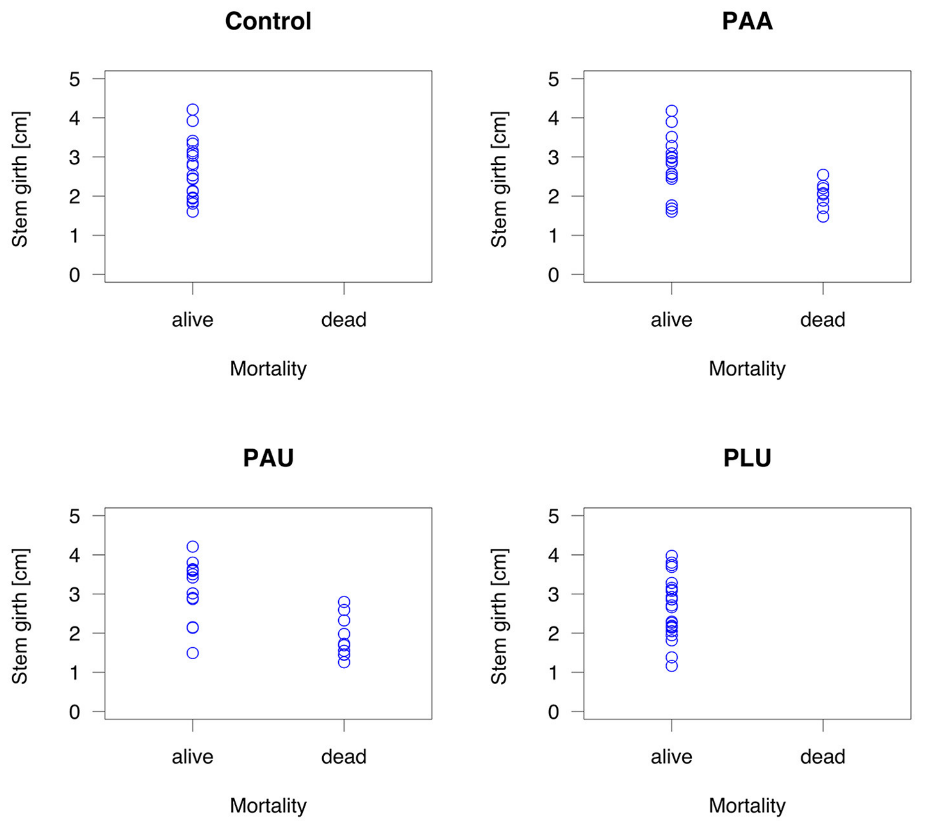

FIGURE 6
Relationship between the stem girth of *Alnus glutinosa* seedlings and mortality (PAA, *Phytophthora ×alni*; PAU, *P. uniformis*; PLU, *P. plurivora*): Welsh *t*-test (PAA: $p = 0.0018$; PAU: $p < 0.001$).

In root samples, five sugars and eight phenolic compounds were identified, with no significant differences between treatments (*Phytophthora* species/CO_2 level) (Supplementary Table 2). Total sugar content was lower in infected alders, but no statistical significance was found between treatments. A higher content of mannitol was recorded in both PAA- and PAU-inoculated plants which were considered dead or dying compared to living plants. In PLU-inoculated plants approximately ten times lower values of mannitol were measured at elevated as compared to ambient CO_2 level. In contrast, the content of total sugars (mannitol excluded) was higher at ambient CO_2 level, and glucose and fructose showed approximately double concentrations compared to the elevated CO_2 level. Phenole concentrations in roots of infected and non-infected plants displayed extensive variation without significant differences (Supplementary Table 2). However, for several phenolic compounds an effect of CO_2 level was recorded in both control and PLU-inoculated plants. Higher mean values of catechin, epicatechin, gallic acid, and taxifolin were found at elevated CO_2 level compared to the ambient level.

Discussion

The devastating alder dieback epidemic, caused by the three species from the "*Phytophthora alni* complex," was first noticed in the mid 1990s in the UK (Brasier et al., 1999; Gibbs et al., 1999) and is currently widespread along rivers and in alder plantings of most European countries (Jung et al., 2016, 2018). *Phytophthora ×alni* (PAA) has been identified as the main causal agent of the disease in most affected regions with temperate climatic conditions but *P. ×multiformis* (PAM) and *P. uniformis* (PAU) also cause bark lesions and mortality in the field (Brasier et al., 1999; Nagy et al., 2003; Jung and Blaschke, 2004; Thoirain et al., 2007; Solla et al., 2010; Aguayo et al., 2013; Jung et al., 2013, 2018; Štěpánková et al., 2013; Redondo et al., 2015; Corcobado et al., 2023). Only two of the three species, i.e., PAA and PAU, have been recorded in the Czech Republic yet, with PAA strongly prevailing in declining alder stands (88% of 59 declining alder stands studied; Štěpánková et al., 2013). In one riparian forest in the Czech Republic, PAA and PAU were isolated from the same declining *A. glutinosa* tree (Macháčová, unpublished). A range of other *Phytophthora* species can contribute to the decline of riparian alder stands, most important *P. plurivora* (PLU) a widespread forest pathogen in Europe with a broad host range that includes *A. glutinosa*, *A. incana* and *A. viridis* (Jung and Blaschke, 2004; Jung and Burgess, 2009; Jung et al., 2013, 2016, 2018; Mrázková et al., 2013; Haque et al., 2014; Corcobado et al., 2023; Tkaczyk et al., 2023).

The pathogenicity of species from the "*Phytophthora alni* complex" to all European alder species was demonstrated in several studies with aggressiveness of the different *Phytophthora* species varying between different studies (Brasier and Kirk, 2001; Santini et al., 2003; Jung and Blaschke, 2006; Černý and Strnadová, 2010;

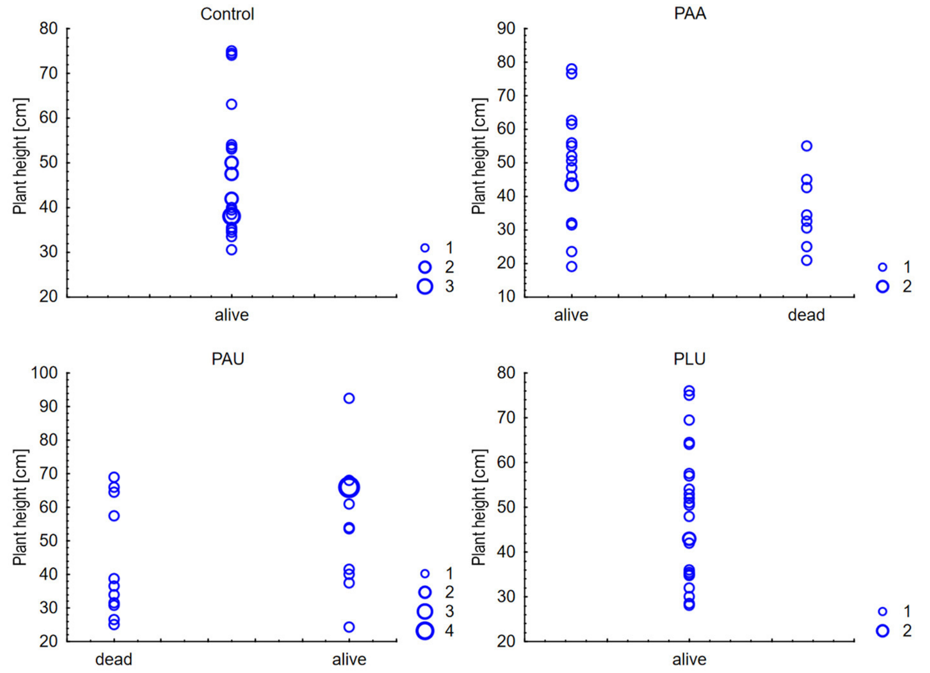

FIGURE 7
Correlations between plant height of *Alnus glutinosa* seedlings and mortality (PAA, *Phytophthora ×alni*; PAU, *P. uniformis*; PLU, *P. plurivora*): Spearman Rank Order Correlations (PAA: r = −0.389, PAU: r = −0.357, PAA and PAU: r = −0.27).

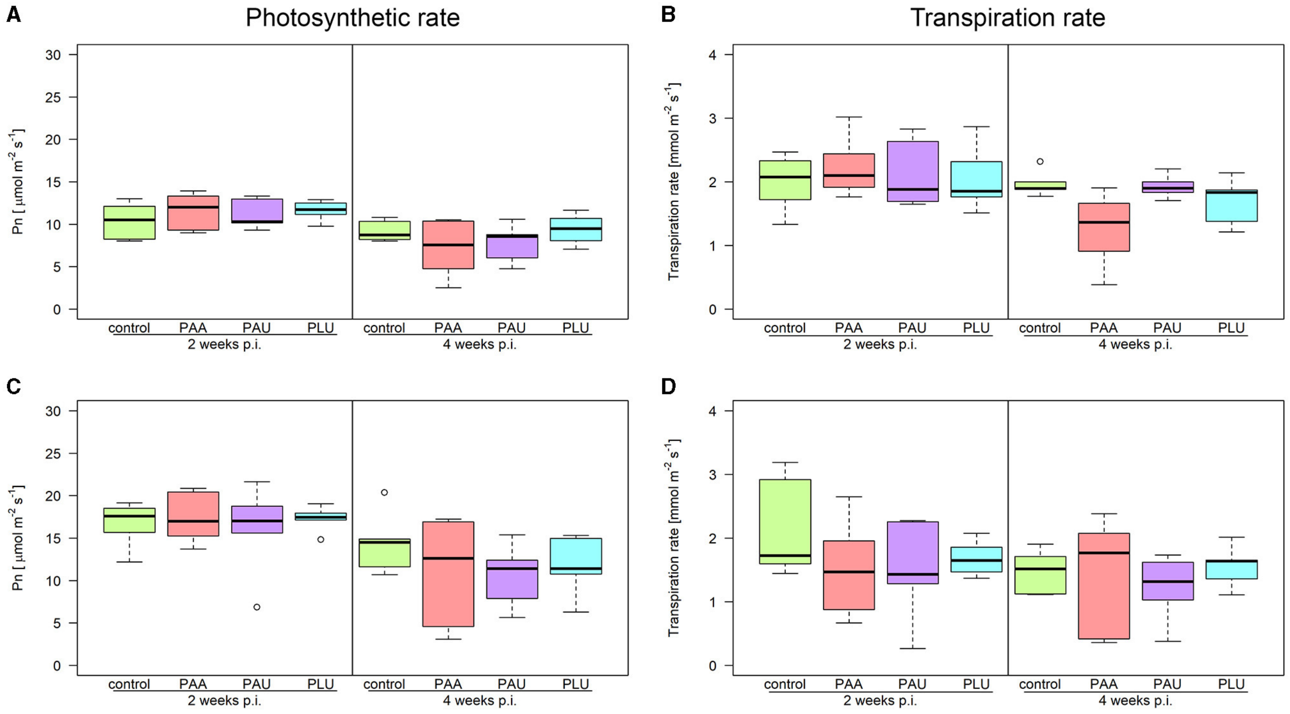

FIGURE 8
Physiological parameters of *Alnus glutinosa* seedlings inoculated with *Phytophthora ×alni* (PAA), *P. uniformis* (PAU), and *P. plurivora* (PLU) measured 2 and 4 weeks after underbark inoculation (p.i.). Rates of net photosynthesis (Pn) measured at **(A)** ambient (415 ppm) and **(B)** elevated CO_2 level (830 ppm). Transpiration rate measured at **(C)** ambient and **(D)** elevated CO_2 level.

Haque and Diez, 2012; Haque et al., 2015; Bjelke et al., 2016; Chandelier et al., 2016; Romportl et al., 2016; Zamora-Ballesteros et al., 2017). Extensive bark inoculation trials with mature logs of *A. glutinosa* revealed high aggressiveness of multiple PAA strains and of Dutch PAM strains whereas German PAM strains and Swedish PAU strains were only moderately pathogenic (Brasier and Kirk, 2001). Pathogenicity tests from other studies indicate, however, that PAU can be quite aggressive to *A. glutinosa* (Corcobado et al., 2017; Zamora-Ballesteros et al., 2017) and also to the North American red alder (*A. rubra*; Navarro et al., 2015). In this study, both PAA and PAU caused large bark lesions and high mortality in *A. glutinosa* seedlings, whereas PLU produced only small lesions not leading to mortality. The lesions caused by PAA and PAU did not differ significantly from each other. However, PAU lesions led to a higher mortality rate (45.8%) compared to PAA (33.3%), although this difference was not statistically significant. In a similar underbark inoculation study by Zamora-Ballesteros et al. (2017), PAA and PAU showed similar aggressiveness to *A. glutinosa* with lesion lengths of ca. 35–40 mm. In their study, the survival probability at 37 weeks p.i. was 50–70% for PAA (depending on the isolate used) and 50% for PAU. Similar mortality rates were found in our study at 13 weeks p.i., but in contrast, the lesions were considerably longer reaching on average 110 mm in PAA and 117.8 mm in PAU. Lesions extended rapidly in some plants during the first 2 weeks p.i. and sometimes reached their maximum length within this time. A similar scenario was observed in other underbark inoculation studies (Zamora-Ballesteros et al., 2017; Marques Gomes et al., 2019). Gibbs (2003) reported on 15-months old *A. glutinosa* seedlings inoculated with species from the "*Phytophthora alni* complex" 3 weeks p.i. mean lesion lengths ranging from 28 to 68 mm. Comparison to results from other studies suggests a higher aggressiveness of the PAA and PAU isolates or higher susceptibility of the *A. glutinosa* genotypes used in our study. Isolate genotype seems to be an important factor affecting lesion development and mortality, as demonstrated by various experiments with PAA (Haque and Diez, 2012; Chandelier et al., 2016; Štochlová et al., 2016; Zamora-Ballesteros et al., 2017). The host plant susceptibility or resistance depends on many variables. Significant differences in susceptibility/tolerance of individual *A. glutinosa* trees to PAA were demonstrated in Belgium, Germany and the Czech Republic (Jung and Blaschke, 2006; Chandelier et al., 2016; Štochlová et al., 2016). In an extensive study including 90 genotypes of *A. glutinosa* sampled across the Czech Republic Štochlová et al. (2016) found that differences in susceptibility to PAA were also depending on the geographic origin and altitude of the alder genotypes. Geographic patterns of resistance could be related to past exposures to invasive *Phytophthora* pathogens (Frampton et al., 2013), as trees in river corridors, including alders, are particularly exposed to many alien pathogenic oomycetes (Černý et al., 2011; Milenković et al., 2018; Jung et al., 2019; Corcobado et al., 2023).

The age of a host plant is another important factor for the susceptibility to a pathogen. The thinner bark of young *A. glutinosa* shoots with smaller diameter may be more vulnerable to tissue colonization than mature bark tissues of older thicker shoots (Haque et al., 2015). This is consistent with the results of our study where we observed the first dying plants being those with the smallest collar diameter (ca. 4–6 mm). On the contrary, plants with thicker collars (>9 mm) survived until the end of the experiment.

Phytophthora plurivora produced lesion lengths of ca. 10–30 mm but induced no mortality. All inoculated seedlings healed the wounds with the callus tissue and did not show wilting or dieback symptoms. However, as a widely distributed pathogen, PLU was found quite aggressive to different host plants in numerous pathogenicity tests (Jung et al., 2003; Rytkönen et al., 2012; Henricot et al., 2014; Milenković et al., 2018; Oszako et al., 2018; Milanović et al., 2020; Ďurkovič et al., 2021). Several studies proved PLU to be pathogenic to alder seedlings, which is inconsistent with our results. Zamora-Ballesteros et al. (2017) reported in an underbark inoculation test on 1-year old *A. glutinosa* seedlings lesion lengths of 35–40 mm and a seedling survival probability of approximately 50% at 37 weeks p.i. (90% after 13 weeks). Similarly, Mrázková et al. (2013) observed 35–40 mm lesion length on 2-years old alder seedlings after 6 weeks p.i. In another underbark trial, Haque et al. (2014) found all inoculated plants wilted and died 3 months p.i.. In a zoospore inoculation test with excised *A. glutinosa* shoots, Jung and Nechwatal (2008) found PLU (under its former name *P. citricola*) causing within 3 weeks significantly longer lesions than PAA (123 vs. 72 mm). These contrasting results indicate that different genotypes of PLU show considerable differences in aggressiveness to *A. glutinosa*. In our study, despite having been constrained early by the formation of callus tissue the pathogen was successfully reisolated from all inoculated plants. Apparently, this PLU genotype has low virulence on *A. glutinosa* but has the ability to survive in dead tissue for quite a long time (13 weeks) which is probably related to the formation of oospores (Jung and Burgess, 2009). However, the pathogen would probably lose its viability and die after a certain time. This was demonstrated by Ďurkovič et al. (2021), who inoculated stems of 10-years old poplar trees under field conditions with *P. cactorum* (Lebert and Cohn) Schröt. and *P. plurivora* and found the wounds after 4 years completely healed with no presence of the pathogens.

Studies on *Phytophthora*-host interaction at elevated CO_2 levels are limited and showed contrasting results. Oszako et al. (2016) did not find any effect of elevated CO_2 concentration (800 ppm) on the root and shoot development of oak seedlings growing in non-infested soil or in soil infested with the oak-specific fine root pathogen *P. quercina*. In this study, a statistically significant negative correlation between the elevated CO_2 level and the above-/below-ground biomass ratio of *A. glutinosa* seedlings was found for the control and for plants inoculated with the moderately aggressive PLU strain but not for plants inoculated with the aggressive PAA and PAU strains. Although the mortality rate did not differ significantly between ambient and elevated (830 ppm) CO_2 levels, there was an effect of the CO_2 levels on the extent of the lesions for PAU-inoculated plants. Thus, at the elevated CO_2 level PAU caused a significantly smaller lesion area and width as well as a tendentially lower lesion length. Similarly, in a tomato trial, a tendency of increased tolerance to *P. nicotianae* Breda de Haan (referred to as *P. parasitica*) was found under elevated CO_2 (Jwa and Walling, 2001). However, in other studies, *Phytophthora* pathogens generally displayed higher aggressiveness to host plants at elevated CO_2. Tkaczyk et al. (2014) reported *P. cactorum* and *P. plurivora* causing more extensive fine root destructions of beech seedlings at elevated CO_2 level compared to the ambient condition. Similar results with increased susceptibility of beech seedlings to *P. plurivora* (referred to as *P. citricola*) at elevated CO_2 level were recorded by Fleischmann et al. (2010).

As the pathogen gradually colonizes the conductive tissues, the girdling extends, and the plant's response can be observed in both lesion development and physiological parameters. Many studies have shown that the common reactions after *Phytophthora*-induced bark infection are a gradual reduction in water uptake, the fast closure of stomata and in consequence a decrease in photosynthesis (Osswald et al., 2014). In our study, the lesions length reached their maximum in most of the inoculated plants 4 weeks p.i. Therefore, the most profound reactions were measured within this period −2 and 4 weeks post inoculation (p.i.). The biggest decrease of transpiration (Tr) was recorded in PAU 4 weeks p.i. when the amount of transpired water dropped to the half value (49%) of the control values (1.2 mmol m^{-2} s^{-1}) at elevated CO_2 level, and to 68% (1.0 mmol m^{-2} s^{-1}) at ambient level. Similarly, net photosynthesis (Pn) reached significantly lower values in PAA- and PAU-inoculated plants 4 weeks p.i. compared to 2 weeks p.i. at regardless the CO_2 levels. Generally, it would have been expected that Pn increases under elevated CO_2 conditions as it occurred for control plants and also in many studies (e.g., Pritchard et al., 1999; Badiani et al., 2023). It seems that the influence of the CO_2 conditions on Pn disappears under acute infections caused by aggressive pathogens. Surprisingly, no significant differences in Pn were recorded between control and inoculated plants at both CO_2 levels. At ambient CO_2 level, these results are consistent with Clemenz et al. (2008), who found no significant differences between control and inoculated plants in a trial with 3-years-old alders stem-inoculated with *Phytophthora ×alni* during the 1st month after the inoculation. Only after 3 months were differences recorded, indicating the late effect of the pathogen on plant physiology.

The decrease of stomatal conductance (Gs) as a common physiological trait was observed across various *Phytophthora* and tree species (Gallego et al., 1999; Robin et al., 2001). In contrast, Gs remained relatively stable in our experiment, but PAA- and PAU-inoculated plants decreased their Gs by approx. 30% 4 weeks p.i. in comparison to 2 weeks p.i.. This trend was not observed in PLU-inoculated plants, as these plants showed very small lesion sizes and no mortality. Moreover, no effect of elevated CO_2 on Gs was observed in any treatment. Our comparison of Pn and Gs trend 2 and 4 weeks p.i. revealed that Pn decreased with Gs simultaneously at ambient CO_2 level. A similar observation was made by Fleischmann et al. (2005) who performed soil inoculation of beech seedlings with *P. plurivora* (referred to as *P. citricola*). As reported in many studies, plants close their stomata at elevated CO_2 level due to surplus of CO_2 in the atmosphere (e.g., Kupper et al., 2006). Nevertheless, in our study, elevated CO_2 caused disbalance between Pn and Gs in PLU and PAA with lower Pn value and the same value of Gs.

Elevated CO_2 also resulted in higher WUEinst values in all treatments 2 weeks p.i. as expected according to general trends (Running and Nemani, 1991). However, the positive influence of CO_2 diminished over time and the later values were comparable between treatments. Similarly, Clemenz et al. (2008) reported that WUE of leaf gas exchange hardly differed between inoculation treatments and controls. On the contrary, another study with 1-year-old beech plants infected with *P. plurivora* showed that WUE data indicated that infected plants suffered from severe drought, even though a significant decrease in net assimilation and transpiration was not observed (Fleischmann et al., 2002).

Chlorophyll a fluorescence is a useful tool for evaluating the plant health status (Oxborough, 2004). Pfanz et al. (2015) measured bark chlorophyll fluorescence in alders 10 weeks after basal stem inoculation with *Phytophthora ×alni* and revealed that Fv/Fm and DF/Fm$'$ of the cortex chlorenchyma decreased to almost zero, indicating tissue necrosis. Similarly, in other studies at ambient CO_2 level, when *Phytophthora* soil inoculation was performed, differences were recorded in leaf chlorophyll fluorescence between control and inoculated plants (Angay et al., 2014; Dalio et al., 2017; Corcobado et al., 2022). Furthermore, Corcobado et al. (2022) observed differences in chlorophyl fluorescence of beech seedlings only 2 weeks p.i. but 3 weeks p.i. these differences disappeared. Similarly, at elevated CO_2 level, Fleischmann et al. (2010) found electron quantum yield of PSII decreased only shortly before the first wilting symptoms occurred in inoculated dying plants. In contrast, our results did not reveal any difference between treatments regardless of the CO_2 level. The discrepancy between our findings and those of Fleischmann et al. (2010) and Corcobado et al. (2022) might be influenced by the different type of applied inoculation methods. In soil infestation experiments with *Phytophthora* pathogens, root damage can be rapidly reflected in decreased foliage vitality, including decreasing performance of chlorophyll a. On the contrary, underbark inoculation leads primarily to necrosis of cortex and phloem tissue, resulting in limited assimilate transport (Osswald et al., 2014) that is minimally shown in chlorophyll fluorescence. Therefore, only when the pathogen starts to move deep into phloem and xylem tissue and the stem becomes completely girdled, chlorophyll a fluorescence would be possibly affected.

Plant responses to pathogen infection can be observed in changing levels of secondary metabolites. Levels of sugars may decrease notably, as reported by Corcobado et al. (2022) who found sugar pool decrease in beech roots in a soil infestation trial with different *Phytophthora* species, suggesting reduced phloem transport. Levels of mannitol may rise dramatically during plant infection by biotrophic or necrotrophic fungi, as mannitol has a multitude of functions in fungal metabolism (Calmes et al., 2013). In our study, at ambient CO_2 level, total sugar content was lower in infected alders, but statistical significance was only found for mannitol, which showed increased levels in PAA and PAU-inoculated plants compared to the control. Similarly, Camisón et al. (2019) reported a temporary increase of sugar alcohols in chestnut clones resistant to *P. cinnamomi* during disease progression. In our study, plants with an advanced stage of dieback exhibited particularly high values of mannitol, which can be explained by the role of mannitol as an antioxidant agent in fungi and oomycetes that can suppress host defense responses by quenching the reactive oxygen species (Meena et al., 2015; Puig et al., 2018). Interestingly, in our study, an effect of elevated CO_2 level on the concentrations of several sugars and phenolic compounds in the roots was found, although the differences to ambient CO_2 were not statistically significant. The elevated CO_2 level showed an inhibiting effect on the total sugars content, with glucose and fructose reaching only half of the concentrations measured at the ambient CO_2 level. High variability in phenolic levels was recorded in all root samples tested in this study, regardless of the treatment. Generally, phenolic compounds level tends to rise in leaves in response to tissue damage, as demonstrated by other studies (e.g., Brown et al., 2018). In our study, PAA and PAU-inoculated plants showed a high variability of phenolic compounds in roots. Regarding elevated CO_2 levels, a positive effect was recorded for several phenolic compounds in control and PLU-inoculated plants. Similar results have been shown at elevated CO_2 by Roth et al. (1998). In general, the lack of differences in secondary metabolites between treatments was probably due to the low number of sampled plants which exhibited significantly different health status ranging from severe dieback to healthy-looking plants.

In conclusion, this study demonstrated that PAU can show similar aggressiveness to *A. glutinosa* as PAA. The results indicate that inoculation experiments should be performed over longer periods to observe for different *Phytophthora* species statistically significant differences in disease incidences and defense responses of inoculated plants. Statistically significant effects of elevated CO_2 concentration on the disease symptoms of underbark-inoculated *A. glutinosa* plants, such as mortality, lesion development, plant biomass or chemical and physiological parameters were not recorded. On the contrary, PAU caused significantly larger lesions at ambient CO_2 and, although not significant, a trend of smaller lesion area at elevated CO_2 was also observed for PAA. Further experiments using soil infestation methods which simulate the natural infection process (Jung et al., 1996, 1999, 2003, 2018) and longer exposition to various CO_2 levels to enable a significant effect on growth and above-ground biomass production of plants exposed to elevated CO_2 level (Ainsworth and Long, 2005) and a potentially more pronounced effect of *Phytophthora* infections on above-ground plant growth are needed to clarify the effect of elevated CO_2 on the *Phytophthora* root and collar rot disease of *A. glutinosa*.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

MM: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. IT: Conceptualization, Formal analysis, Investigation, Writing – review & editing. TC: Conceptualization, Investigation, Writing – review & editing. ZN: Formal analysis, Investigation, Writing – review & editing. SM: Formal analysis, Writing – review & editing. JJ: Investigation, Writing – review & editing. VP: Investigation, Supervision, Writing – review & editing. JC: Data curation, Formal analysis, Writing – review & editing. SG: Formal analysis, Writing – review & editing. ON: Investigation, Writing – review & editing. VZ: Investigation, Writing – review & editing. AK: Formal analysis, Writing – review & editing. IM: Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing. TJ: Conceptualization, Investigation, Writing – review & editing.

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Conflict of interest

SG was employed by VSN International.

The remaining 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.2024.1379791/full#supplementary-material

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Tubakia spp., *Didymella macrostoma* and *Apiognomonia errabunda* causing leaf spot and anthracnose of Quercus robur in the Mura-Drava-Danube Biosphere Reserve

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The Mura-Drava-Danube transboundary UNESCO Biosphere Reserve represents one of the best-preserved wetlands in Europe. The Reserve's riparian forests play a significant role in ecosystem functioning and pedunculate oak (Quercus robur) is one of the keystone species of these forests. In recent years, pedunculate oak trees in the Reserve displayed symptoms of necrotic lesions on their leaves. The lesions varied in size, from small, circular to irregular reddish brown to grayish spots to larger necrotic areas that resembled leaf anthracnose and extended along the leaf nerves. In 2021, symptomatic leaves were collected in three countries of the Reserve, i.e. Austria, Slovenia, and Serbia to identify the causative agents of these diseases. Fungal cultures were obtained from symptoms and identified using morphology and multilocus phylogenetic analyses of the ITS rDNA, partial LSU rDNA, tef 1-α, BT2, CAL, ACT, and RPB2 genes. The fungi were identified as *Tubakia dryina*, *Tubakia* sp. (*Tubakia dryinoides sensu lato*), *Didymella macrostoma*, and *Apiognomonia errabunda*. Pathogenicity tests done by inoculating the leaves of one-year old pedunculate oak plants revealed that the isolated fungi caused symptoms as those seen in the forest. To our knowledge, this study represents the first report of *D. macrostoma* as the cause of pedunculate oak leaf spot disease in Serbia and worldwide. It is also the first finding of *Tubakia* leaf spot disease of pedunculate oak caused by *T. dryina* in Austria and Serbia. Moreover, *Tubakia* sp. was proven to be another causative agent of *Tubakia* leaf spot disease. Additionally, oak anthracnose caused by *A. errabunda* was found for the first time on pedunculate oak leaves in Austria and Slovenia. During the past decade, pedunculate oak trees have been facing increasing threats from multiple abiotic and biotic factors which has resulted in decline and absence of natural regeneration of these trees. The results of this study add to the understanding of the contributing factors to the decline of pedunculate oak in riparian forests and are important for the development of management strategies to counteract this decline.

KEYWORDS

Mura-Drava-Danube Biosphere Reserve, riparian forests, leaf spot diseases, *Tubakia* leaf spot, *Didymella macrostoma*, oak anthracnose, pedunculate oak

1 Introduction

The transboundary UNESCO Mura-Drava-Danube Biosphere Reserve represents one of the best-preserved European wetlands in the border area between Austria, Slovenia, Hungary, Croatia, and Serbia. An almost 700 km long "green belt" formed by the Danube, Mura and Drava is the world's first biosphere reserve spanning five countries, and the largest riverine protected area in Europe; hence it was named the "Amazon of Europe" (UNESCO, 2023). The Reserve consists of four wetland habitats protected by the Ramsar (2023) convention and fulfills many ecosystem services that contribute to environmental and human well-being (Oettel et al., 2022; Ramsar, 2023). It is a major carbon storage and drinking water reservoir and acts as a natural buffer by providing bank protection and climate regulation (Dybala et al., 2019; Riis et al., 2020). Moreover, its unique river landscape offers a range of recreational and nature-based tourism opportunities, and it represents a biodiversity hotspot that provides food and water resources for many rare and endangered plant, fish, insect, and animal species possibly acting as a future refugia against impacts of climate warming (Köck et al., 2022; Trišić et al., 2022; Zhang et al., 2023).

Despite their breathtaking natural features, in the past decades, floodplain ecosystems have faced numerous challenges that have resulted in their degradation (Xu et al., 2019; Tadić et al., 2022). The riverine area of the Reserve has suffered joint impacts from climate change and human intervention such as river regulation, water abstraction and dam constructions for the extension of agricultural land, protection of settlements from floods and industry needs. These impacts have resulted in changing of the hydrological regime and overall ecological conditions which have had a negative impact on the unique floodplain biotopes including the riparian forests (Skiadaresis et al., 2019; Tadić et al., 2022). Riparian forests of the Reserve are dominated by forest tree species, such as Quercus robur L., *Alnus glutinosa* L. (Gaertn.), *Fraxinus angustifolia* Vahl., *Fraxinus excelsior* L., *Populus nigra* L., *Ulmus laevis* Pall., and *Ulmus minor* Mill. Which depend on appropriate soil moisture content and water table level, as well as periodical floodings (Galić et al., 2019; Kostić et al., 2021). Therefore, the latest tree species distribution models predict a possible loss of these habitats and because of the above-mentioned perturbations, loss of biodiversity and ecosystem function (Sallmannshofer et al., 2021).

Pedunculate oak (*Q.robur*) is a long-lived deciduous tree species native to most of Europe and western Asia with major cultural, ecological, and economic importance (Eaton et al., 2016; Mölder et al., 2019). It is widespread in lowlands and often found close to rivers as it prefers moist soils with higher belowground water (gleysols: hydromorphic soils) and intermittent floodings (Eaton et al., 2016; Kostić et al., 2021, 2022; Kesić et al., 2023). It is a keystone species in many countries, and a major feature of internationally important habitats such as floodplain riparian forests of the Mura-Drava-Danube Biosphere Reserve (Sallmannshofer et al., 2021). Across Europe, pedunculate oak is experiencing decline of health, growth, and regeneration due to the synergistic stressors which include frequent and long-lasting droughts, lowering of the water table coupled with the absence of occasional flooding, or water table rise caused by river regulations like damming, as well as pests and diseases (Csóka et al., 2020; Stojanović et al., 2021; Kesić et al., 2023). Among the pests, the oak lace bug *Corythucha arcuata* (Say, 1832), spongy moth *Lymantria dispar* (Linnaeus, 1758), gall wasps (Cynipidae), leaf miners like *Tischeria ekebladella* (Bjerkander, 1795) and early spring defoliators (e.g. winter, tortrix and owlet moths, Lepidoptera: Geometridae, Tortricidae, Noctuidae) are the most notable (Csóka et al., 2020; Rađević et al., 2020; Hoch et al., 2023). Moreover, stem and root diseases caused by fungi, such as *Diplodia seriata* De Not., *Fusarium sporotrichioides* Sherb.; bacteria, such as *Brenneria* spp., *Gibbsiella quercinecans* Brady et al., 2010; *Rahnella victoriana* Brady et al., 2017, and pseudo fungi such as *Phytophthora* spp. are becoming increasingly problematic in European pedunculate oak forests (Zlatković et al., 2018; Milanović et al., 2020; Ruffner et al., 2020; Jankowiak et al., 2022).

Leaf diseases of pedunculate oak are numerous and some, for example powdery mildew can be symptom-specific (Demeter et al., 2021). Oak powdery mildew is caused by several cryptic species among which *Erysiphe alphitoides* (Griffon & Maubl.) U. Braun & S. Takam most commonly occurs on pedunculate oak (Bradshaw et al., 2022; Kebert et al., 2022). The disease symptoms are white powdery patches on the leaf surface with round, closed fruit bodies named chasmothecia (Demeter et al., 2021; Bradshaw et al., 2022). Other diseases, like *Tubakia* leaf spot and *Apiognomonia* anthracnose can be distinguished if fungal reproductive structures (i.e., fruiting bodies) are present on the leaf lesions (Kowalski, 2006; Boroń and Grad, 2017). Leaf spot disease caused by *Tubakia dryina* (Sacc.) Sutton (1973) is characterized by necrotic spots on the leaves and sometimes on the petioles. The spots can enlarge and merge to form leaf blotch, whereas petiole necrosis can cause premature defoliation (Kowalski, 2006). *Tubakia* species produce unique umbrella-like pycnothyrial conidiomata that consist of convex scutella made from pigmented setae-like cells fixed to the leaf surface by a central columnella. Underneath scutella mostly globose to elliptical, hyaline, subhyaline to pigmented conidia and sometimes microconidia are born on conidiogenous cells (Braun et al., 2018). Oak leaf anthracnose caused by *Apiognomonia errabunda* (Roberge ex Desm.) causes necrosis that develops along leaf veins with apothecia formed on the necrotic lesions

(Kowalski, 2006). On the other hand, *Taphrina caerulescens* (Desm. & Mont.) Tul. produces small yellowish round spots on the leaves that swell upwards resembling blisters (Kowalski, 2006). However, there are leaf diseases with nonspecific symptoms such as small leaf spots of varying shape and color caused by e.g., *Septoria* spp., *Mycosphaerella* spp. or members of the Didymelaceae including *Phoma*-like species like *Ascochyta* spp., *Phoma* spp. and *Didymella* spp. (Butin, 1996; Kowalski, 2006) which challenges identification. The taxonomy of the species rich Didymellaceae has been demanding as the species identification relied on morphology and host association, but with the application of molecular phylogenetics substantial progress in species delimitation has been made (Chen et al., 2015, 2017; Hou et al., 2020a).

The synergistic effect of abiotic and biotic threats of pedunculate oak relates to "acute oak decline", a phenomenon that has been known to occur throughout Europe, including the Mura-Drava-Danube Biosphere Reserve (Denman et al., 2014; Zlatković et al., 2018). In the Reserve, the pedunculate oak is threatened by the abiotic challenges, insect pests as well as fungal and pseudo fungal stem and root pathogens, namely *D. seriata* and *Phytopthora* spp., respectively (Zlatković et al., 2018; Milanović et al., 2020; Kostić et al., 2021). However, until 2019 nothing was known about the leaf diseases of this tree species in the Reserve. Thus, in the summer of 2019, disease symptoms such as small necrotic spots and larger necrotic areas along the leaf veins resembling leaf anthracnose were observed on the leaves of pedunculate oak in the Mura-Drava-Danube Biosphere Reserve. The presence of necrotic lesions was roughly assessed using binoculars or a branch was cut off and leaves were examined for the presence of fungal fruit bodies using hand lenses in the forest (de Groot et al., 2022). Symptoms and signs (lesions that extended along the leaf nerve and brownish cushion-like fruit bodies) resembled those of an *Apiognomonia* leaf anthracnose. On the other hand, certain lesions had blackish, round fungal fruit bodies resembling pycnidia/perithecia and were non-specific. Thus, these lesions were of unknown etiology and suspected to be caused by either *Tubakia* spp., *Phoma* species complex, *Septoria* spp., *Mycosphaerella* spp. and/or other leaf pathogens. Nonetheless, the true identity of the pathogens causing disease symptoms remained unknown. Therefore, in 2021 we collected leaves of pedunculate oak with symptoms such as leaf spots and anthracnose in three countries of the Reserve, i.e. Austria, Slovenia, and Serbia intending to identify the causal agents of these symptoms. We approached the diagnosis by conducting isolations from symptomatic leaves, followed by multigene phylogenetic and morphological analyses of the isolated fungi. Finally, we performed the pathogenicity tests to fulfil Koch's postulates and determine whether the isolated fungi were the causative agents of the leaf spot and anthracnose observed in the forest.

2 Materials and methods

2.1 Sample collection and fungal isolations

In total, 33 randomly selected mature trees (average diameter at breast height 100 cm, average tree heigh 35 m) were sampled in 11 previously designed transects in Austria, Slovenia, and Serbia in the Mura-Drava-Danube Biosphere Reserve (**Supplementary Table 1**, three trees per transect, de Groot et al., 2022). Three leaves per tree displaying necrotic lesions were collected in paper envelopes and transferred to the laboratory for examination. Within the same day leaf spots were examined for the presence of fungal fruit bodies using Olympus SZX10 stereo microscope (Olympus Co., Tokyo, Japan). When found, fruit bodies were sectioned by hand, mounted in distilled water, and examined using Olympus BX53F light microscope with differential interference contrast (DIC) illumination equipped with Olympus SC50 digital camera and accompanying software. Leaves with symptoms were then separated into groups according to lesion dimensions, lesion position in relation to the leaf nerve, type of fungal fruit bodies found within lesions (i.e., pycnidia, pycnothyria or apothecia), color and dimensions of spores (**Supplementary Table 2**). Thereafter, two leaves per group per country were chosen for fungal isolation. Isolations were done immediately after leaf examinations.

To isolate fungi from the leaf spots small pieces (approximately 1 × 1mm) were cut at the margin of diseased and apparently healthy tissue of symptomatic leaves. The leaf pieces were shortly washed in tap water, then surface sterilized using 70% ethanol (1 min.), followed by 10% bleach (1 min.), washed in sterile distilled water, and blotted dry with sterile paper towels before they were put on AMEA (MEA Neogen, UK supplemented with lactic acid) to suppress bacterial growth. Fungi were also isolated from fruit bodies. Lesions were surface sterilized by spraying with 70% ethanol followed by spraying with sterile water and a fruit body was removed from the leaf using a sterile hypodermic needle and plated on AMEA. Petri dishes were kept in the dark in a cooled microbiological incubator (VWR international, Darmstadt, Germany) at 21°C for 1 week and checked daily. The mycelium was then hyphal tipped to obtain pure cultures. Isolates were separated into morphologically similar groups and depending on the number of available isolates, at least one representative isolate per country from each morphological group was chosen for further molecular phylogenetic identification and morphological characterization (**Supplementary Table 2**). Isolates used in the phylogenetic analyses (**Supplementary Table 2**) were stored in water or under mineral oil on +4°C and in 40% (v/v) glycerol on −80°C in Culture collection of the Laboratory of Forest Protection at the Slovenian Forestry Institute (ZLVG) as well as Collection of microorganisms of the Institute of Lowland Forestry and Environment (ILFE). The exception was four isolates with the morphology of *Penicillium* spp., *Biscogniauxia* sp., and *Aureobasidium* sp., respectively, which are well recognized as endophytes and biocontrol agents in the leaves of forest tree species (Terhonen et al., 2018). These isolates were therefore not used for further analyses.

2.2 DNA extractions, PCR, and sequencing

Since the oak leaves were collected in different countries, fungal isolations, DNA extractions, and PCR reactions of different fungal isolates were done in the country where the leaf was collected to avoid the transfer of potentially pathogenic isolates across international borders. The only exception was *Phoma*-like isolates (symptom group 2) which were solely obtained from leaves collected in Serbia and thus all the molecular work for these isolates was done at the Institute of Lowland Forestry and Environment (ILFE). At ILFE, to extract the DNA, fungal cultures were grown for 2 weeks on 2% MEA overlaid with sterile polypropylene discs with micro perforation (Macropack, Ledinci) to allow the fungus to reach MEA. The mycelium was then scraped from the disk surface using sterile scalpel or inoculation loop. The genomic DNA was extracted using Prepman Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, USA) following manufacturer's instructions with the following modifications: instead of vortexing, the mycelium was grinded using sterile micro pestles (Carl Roth, Germany) and spinning for two minutes was replaced with up to three ten minutes long centrifugation steps. Alternatively, at Austrian Research Center for Forests (BFW) and Slovenian Forestry Institute (SFI) the DNA was extracted using a NucleoSpin Plant II (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions, after homogenizing the fungal material with a Lysing Matrix A tube (MP Biomedicals, Solon, USA) using a Precellys Evolution device (Bertin Technologies, Montigny-le-Bretonneux, France). At ILFE, the DNA quality and concentrations were assessed using BioSpec-nano spectrophotometer (Shimadzu-Biotech, Japan), whereas at BFW and SFI the DNA was checked using biophotometer plus (Eppendorf, Germany). The isolates were initially screened and identified up to the genus level using universal barcode marker for fungi, i.e., ITS rDNA and primers ITS1F/ITS4 (White et al., 1990; Gardes and Bruns, 1993). Thereafter, the combination of other genes and primer sets used for amplification depended on the fungal genus. Thus, tef 1-α and BT2 genes were additionally amplified for *Tubakia* spp. isolates; LSU, RPB2 and BT2 were amplified for *Didymella* sp., whereas ACT and CAL genes were amplified for *Apiognomonia* sp. isolates (e.g., Sogonov et al., 2007; Braun et al., 2018; Boroń et al., 2019). The tef 1-α, BT2, LSU rDNA, RPB2, ACT, and CAL gene were amplified using the EF1/EF2 (O'Donnell et al., 1998), Bt-2a/Bt-2b (Glass and Donaldson, 1995), LR0R/LR6 (Vilgalys and Hester, 1990), RPB2-5F2/7CR (Liu et al., 1999; Sung et al., 2007), ACT-512-F/783-R (Carbone and Kohn, 1999; Udayanga et al., 2014), and CAL-228F/737R (Carbone and Kohn, 1999) primer sets, respectively.

PCR reactions for *Phoma*-like isolates (symptom group 2) were done at ILFE in a total volume of 25 μl, with the following components: 2 μl of previously diluted DNA template (20 ng/μl), 2.5 μl of 10 × Taq buffer with KCl (Thermo Scientific, Vilnius Lithuania), 3.5–5.5 μl of 25 mM MgCl2 (3.5 μl: BT2; 4.5 μl: ITS, LSU; 5.5 μl: RPB2), Thermo Scientific, Vilnius, Lithuania), 1μl of 100 mM of each dNTPs (Thermo Scientific, Vilnius, Lithuania), 0.5 μl of 100 mM of each primer (Invitrogen, Thermo Fisher Scientific, Paisley, UK), 0.3 μl of 5 U/μl Taq DNA polymerase (recombinant, Thermo Scientific, Vilnius, Lithuania) and sterile deionized filtered ultra-pure water. The PCR conditions were as described in Kovač et al., 2021 with annealing temperatures 55°C (ITS, BT2) and 60°C (LSU). The exception was the RPB2 gene which was amplified using a touchdown protocol at annealing temperatures of 65–55°C (Kovač et al., 2021). The PCR amplifications were done in MiniAmp Plus thermal cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA). The size of the amplified PCR products was estimated visually using O'RangeRuler 100bp DNA ladder (Thermo Scientific, Vilnius, Lithuania) after electrophoresis on 1.75% agarose gels stained with GelRed (Biotium, Hayward, USA). PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sent to Macrogen Europe laboratories in the Netherlands for sequencing. While PCR reactions for *Tubakia*-like isolates (symptom group 1) were conducted at BFW, SFI and ILFE, PCR reactions for *Apiognomonia*-like isolates (symptom group 3) were done at BFW and SFI. Since chemicals and equipment differed in the three laboratories, the PCR mixtures and amplification conditions for these fungi are presented in **Supplementary Table 3**. The strands of all isolated fungi were sequenced in both directions with the primers as in PCR reactions.

2.3 Phylogenetic analyses

Consensus sequences were made using CLC Main Workbench 23 (Quiagen, Hilden, Germany), aligned using MAFFT v.7 online service with G-INS-1 strategy (Katoh et al., 2019), and manually improved where necessary in MEGA 11 (Tamura et al., 2021). Sequences were compared to those present in NCBI GenBank using BLASTn search, and sequences from this study were aligned with closely related reference sequences obtained from GenBank. Phylogenetic analyses including Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian Inference (BI) were done first for the single gene sequence datasets and then also for the combined datasets (ITS-tef 1-α-BT2 for *Tubakia*; ITS-LSU-RPB2-BT2 for *Didymella*; ITS-ACT-CAL for *Apiognomonia*). ML analyses were done using PhyML online v.3.0 (Guindon et al., 2010) by employing an automatically selected substitution model and AKAIKE information criterion. The aligned sequences were analyzed for MP with PAUP v.40b10 and a partition homogeneity test (PHT) was performed to determine if the different gene datasets could be combined (Swafford, 2002). Posterior probability (BI) estimates were determined using Mrbayes v.3.2.7a and using the substitution model previously selected in ML analyses (Ronquist et al., 2012). MP and BI analyses were done as explained in Zlatković et al., 2016. Bootstrap analyses were conducted with 1000 bootstrap replications. Phylogenetic trees were viewed with Mega v.11 (Tamura et al., 2021) and further processed by Corel Photo Paint 2018, Microsoft Paint and Microsoft Paint3D. Nucleotide sequences from this study were deposited in GenBank, and the GenBank accession numbers of all sequences used in the analyses are provided in **Supplementary Tables 4–6**.

2.4 Morphological characterization

For the descriptions of colony morphology fungi were grown on 2% MEA for 2 weeks at 21°C in the dark. Colony colors were determined using the color charts of Rayner (1970). To promote fruit body production *Tubakia*-like isolates (symptom group 1) and *Apiognomonia*-like isolates (symptom group 3) were inoculated onto 2% MEA overlaid with triple autoclaved *Pinus nigra* J.F. Arnold needles and kept under room temperature (20 ± 2 °C) for up to two months under near UVA light (12h dark/12h light regime). Morphological characteristics of the fruit bodies and spores were checked by means of light microscopy using stereo and light microscopes described above and measurements of up to 20 conidia, microconidia and fruit bodies were made. Sections of pycnidia were made using rotary microtome cryostat MEV (SLEE medical GmbH, Mainz, Germany). Morphological characteristics of the isolated fungi were compared to those described in recent literature, e.g., Braun et al., 2018; Zhang et al., 2021; Zhu et al., 2022 (*Tubakia*); de Gruyter et al., 2002; Chen et al., 2015; (*Didymella*), Sogonov et al., 2007; Bensaci et al., 2021 (*Apognomonia*).

2.5 Pathogenicity test

To confirm that the isolated fungi were the cause of the disease symptoms observed in the forest, a pathogenicity test was conducted using one-year old pedunculate oak plants grown from acorns. Acorns were collected in pure pedunculate oak stand on alluvial soil (fluvisol, hydromorphic soil) in Apatin district (Serbian part of the Mura-Drava-Danube Biosphere Reserve; 45.62162°N 18.94562°E) in October 2021. To ensure that just one genotype of pedunculate oak was selected for the experiment, acorns were collected within a meter of the stem beneath the crown of a single, mature, dominant tree. The other mature dominant trees that produced acorns were spaced 23, 26, and 19 meters away in the east, south-west, and north, respectively, so their crowns did not overlap or touch the selected tree. Also, there were no acorns on the nearby codominant, smaller trees. Soil was taken from the same location, mixed with peat moss (3:1, Pešterski treset Production, Tutin), sterilized (by autoclaving at 121°C for 35 min.) and used to saw acorns in 3l pots. To remove damaged acorns before sowing, the acorns were soaked in tap water for 24 h and all floating acorns were discarded. Pots were kept in the greenhouse (20 ± 2°C day temperature, 17 ± 2°C night temperature, 70 ± 3% relative humidity, photoperiod of 16 h light/day) and watered as needed until the pathogenicity test was performed in April 2022.

One representative isolate of each of the previously identified *T. dryina*, *Tubakia* sp. (*Tubakia dryinoides* C. Nakash. *sensu lato*), *Didymella macrostoma* (Mont.) Qian Chen & L. Cai and *A. errabunda* was used to complete Koch's postulates and confirm pathogenicity of these fungi on pedunculate oak leaves (**Supplementary Table 2**). This is because, aside from isolates of *A. errabunda*, the isolates of *T. dryina*, *Tubakia* sp. and *D. macrostoma* represented single haplotypes. For inoculation isolates were grown for 2 weeks at 21°C on MEA in a microbiological incubator (INCU-Line, VWR International, USA) in the dark. Leaves were first sterilized using 70% ethanol and then ruptured between major veins using a sterile needle and mycelial discs of 6 mm diameter, taken from the edges of fungal cultures were placed on the upper leaf surface, with the mycelium faced downwards. Mycelial plugs were used for inoculation instead of spore suspension since it was a more convenient method to test pathogenicity of fungi from three different genera with varying sporulation ease and times (Bhunjun et al., 2021). A total of ten plants per isolate was used for inoculations and on each plant two fully expanded leaves were inoculated giving a total of 20 leaves per isolate. Twenty leaves on ten plants served as mock-inoculated controls and these plants were inoculated using sterile MEA plugs. For the next 48h, to ensure sufficient humidity, plants were covered with transparent polyethylene bags that had tiny needle-punched ventilation holes. The plants were grown in the greenhouse with temperature ranging from 19 ± 2 °C (night) to 21 ± 2 °C (day), air humidity 80 ± 2%, and photoperiod of 16 h light/day. The plants were arranged in a completely randomized design and watered twice a week to field capacity. Leaves were monitored for disease symptoms every day until lesions emerged, then once a week. The experiment lasted for 4 weeks as by that time all the inoculated fungi developed lesions and produced fruit bodies on the leaves.

3 Results

3.1 Disease symptoms and isolations

Based on disease symptoms and signs, collected leaves were separated into three groups. The first group represented leaves with leaf spots ranging from 1 to 2.5 cm in diameter. Lesions were purplish brown, reddish brown to brown with indefinite margin or darker border. The older lesions were often with cracks, and superficial fungal fruit bodies resembling pycnothyria with scutella of radiating, pigmented cells and ellipsoid, hyaline to brown *Tubakia*-like spores (**Figures 1**, **2**; Braun et al., 2018). Based on the presence of pycnothyria on necrotic areas, leaf lesions of this group were classified as *Tubakia*-like leaf spots. This type of lesions was found on leaves from each sampled country. Apart from three isolates of *Biscogniauxia* sp., *Aureobasidium* sp. and *Penicillium* sp., twelve isolates resembling *Tubakia*-like morphology (cultures initially white, fluffy and with rosette-like appearance turning dark with age) were obtained from these symptoms (eight from Austria, one from Slovenia, and two from Serbia). The *Tubakia*-like isolates were further separated into two subgroups with cultures that turned gray with age belonging to the first subgroup (seven from Austria and one from Serbia) and isolates turning blackish with age as the second subgroup (one from Austria, one from Slovenia, one from Serbia). Six representative isolates (three isolates from each subgroup with representatives from each country) were chosen for further molecular phylogenetic identification and morphological characterization (**Supplementary Table 2**).

The lesions of the second group were reddish brown to grayish brown, ranging from 0.2 to 0.7 cm in diameter containing pycnidial fruit bodies with conidia like those of the *Phoma*-like species (**Figure 3**), thus lesions of this group were classified as *Phoma*-like lesions. This type of lesions was present only on leaves collected in Serbia. One isolate with *Penicillium*-like and two isolates with *Phoma*-like morphology were obtained from these lesions, and *Phoma*-like isolates were used in subsequent phylogenetic and morphological analyses (**Supplementary Table 2**).

The lesions of the third group were reddish brown to grayish extending along leaf veins, measuring 1.5–3 cm in diameter. These lesions contained brownish, cushion-shaped acervuli with conidia like those of the *Apiognomonia* species and they were characterized as *Apiognomonia*-like lesions. Four *Apiognomonia*-like isolates were isolated from these lesions (two from Austria and two from Slovenia) and these were further used in the analyses (**Supplementary Table 2**).

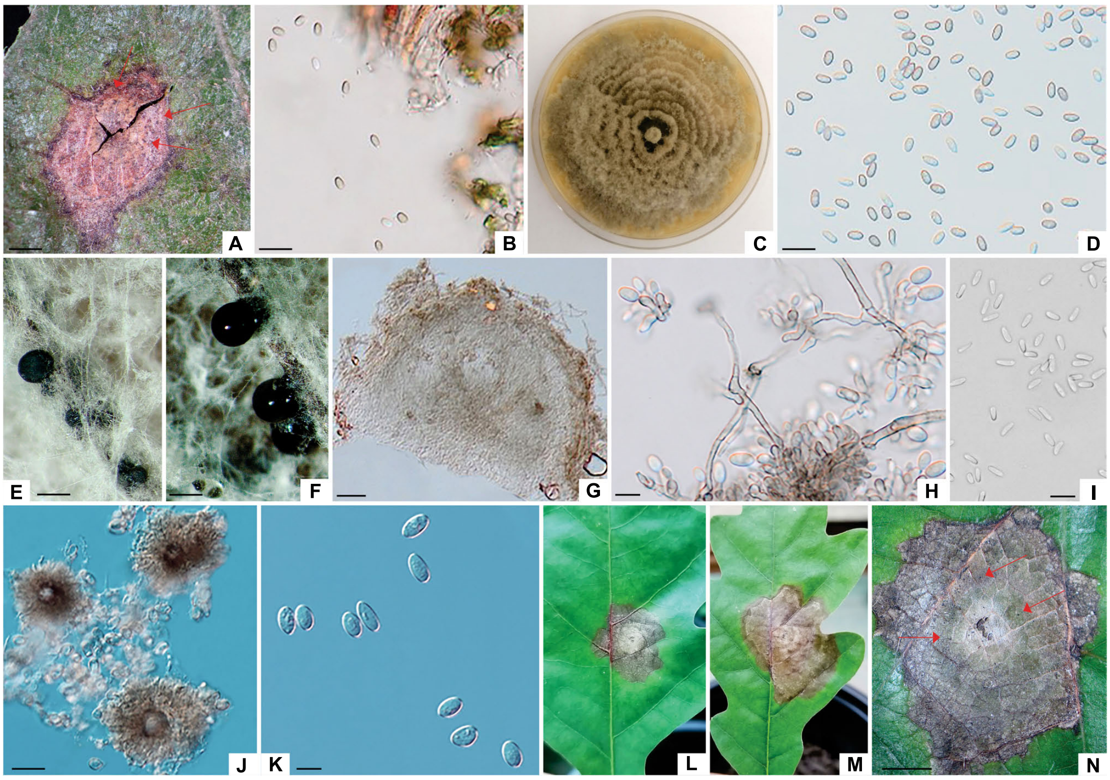

FIGURE 1
Tubakia dryina (ILFE 6). **(A)**: Leaf lesion with conidiomata (pycnothyria, arrows) on leaves of *Q. robur* in Serbian part of the Mura-Drava-Danube Biosphere Reserve. **(B, D)**: Conidia from pycnothyria on leaf lesions in the field. **(C)**: Colony morphology of ILFE 6 on MEA after 2 weeks at 21°C in the dark. **(E)**: Pycnidia formed on needles. **(F)**: Sporodochial conidiomata formed on needles with blackish conidial masses. **(G)**: Cross section of pycnidium formed on pine needles. **(H)**: Conidiogenous cells giving rise to conidia. **(I)**: Microconidia. **(J)**: Pycnothyria with scutella. **(K)**: Conidia. **(L–N)**: Lesions formed during pathogenicity test with superficial pycnothyria (arrows). Scale bars: **(A)**, **(N)** = 1 mm; **(B)**, (D) = 30 μm; **(E,F)** = 0.5 mm; **(G–I)**, **(K)** = 10μm; **(J)** = 20μm.

3.2 Molecular phylogenetic identification

A combined dataset (ITS-tef 1-α-BT2) of *Tubakia* species contained 42 sequences including two outgroup sequences of *Paratubakia subglobosa* (T. Yokoyama & Tubaki) U. Braun & C. Nakash. (CBS 193.71, CBS 124733). The matrix consisted of 1736 characters (ITS: 609, tef 1-α: 603, BT2: 524) of which 451 were parsimony-informative. The TN93 substitution model was automatically selected in the ML analyses and used in the BI analysis. There were 16 most parsimonious trees with TL = 726, CI = 0.78, RI = 0.94. The PHT value indicated that the three datasets could be combined (PHT = 0.01). The topologies of MP, BI and ML trees were substantially congruent, and the best ML tree is presented (Figure 4).

The multigene phylogenetic tree grouped isolates from the first subgroup of *Tubakia*-like leaf spots (symptom group 1, subgroup 1) in a highly supported single clade with *T. dryina* (99/100/1, ML, MP, BI bootstrap/posterior probability support) (Figure 4). In individual gene trees, these isolates also resided within *T. dryina* (Supplementary Figure 1).

The multigene phylogenetic analyses grouped isolates from the second subgroup of *Tubakia*-like leaf spots in a highly supported clade with the ex-type strain of *T. dryinoides* (*T. dryinoides sensu stricto*) and strains of *T. dryinoides sensu lato* (100/98/1, ML, MP, BI bootstrap/posterior probability support, Figure 4). Within this clade, our isolates clustered with isolates of *T. dryinoides sensu lato* (*s.l.*) in a highly supported sub clade (99/99/1 bootstrap/posterior probability support, Figure 4). In the ITS phylogeny isolates from this study clustered with *T. dryinoides s.l.*, *T. dryinoides sensu stricto* (*s.s.*), *Tubakia koreana* H.Y.Yun and *Tubakia paradryinoides* C. Nakash. In the tef 1-α and BT2 phylogenies these isolates resided in a clade with *T. dryinoides s.s.* and *T. dryinoides s.l.* and within this clade in a subclade with strains of *T. dryinoides s.l.* (Supplementary Figure 1). Shared polymorphisms between *Tubakia* sp. isolates from this study, *T. dryinoides s.s* and *T. dryinoides s.l.* are shown in Supplementary Table 7. Molecular phylogenetic analyses identified *Tubakia*-like isolates as *T. dryina* and *Tubakia* sp. (*T. dryinoides s.l.*) (Figure 4).

A combined dataset (ITS-LSU-RPB2-BT2) of *Didymella* species contained 27 sequences including an outgroup sequence of *Epicoccum nigrum* Link (CBS 173.73). The matrix consisted of 2386 characters (ITS: 493; LSU: 964; RPB2: 596; BT2: 333) of which 295 were parsimony informative. The TN93+G+I substitution model was automatically selected in the ML analyses and used in the BI analysis. There were two most parsimonious trees with TL = 730, CI = 0.56, RI = 0.79. The PHT value was low but still acceptable and indicated that the datasets could be combined (PHT = 0.01). The topology of MP and BI trees was like that of the ML tree, and the best ML tree is presented (Figure 5).

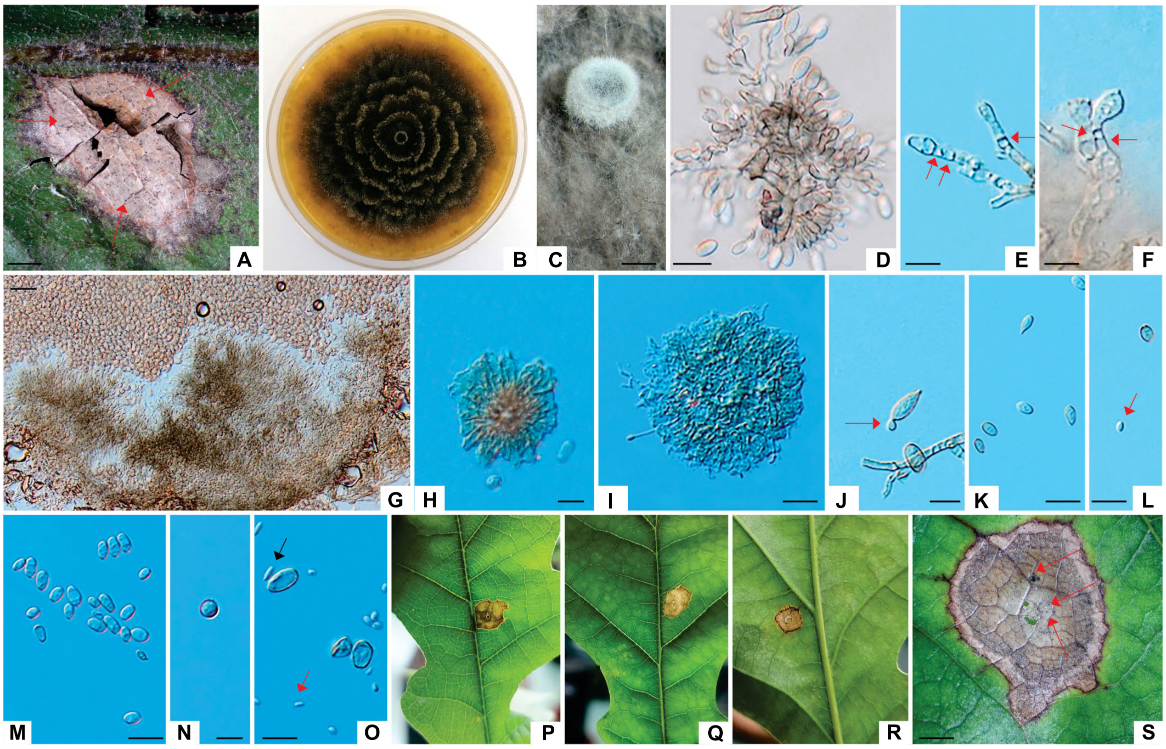

FIGURE 2
Tubakia dryinoides (ZLVG 972). **(A)**: Leaf lesion with conidiomata (pycnothyria, arrows) on leaves of *Q. robur* in Slovenian part of the Mura-Drava-Danube Biosphere Reserve. **(B)**: Colony morphology of ZLVG 972 on MEA after 2 weeks at 21°C in the dark. **(C)**: A pycnidium formed on needles. **(D–F)**: Conidiogenous cells giving rise to conidia (arrows indicate annelations). **(G)**: Cross section of pycnidium formed on needles. **(H,I)**: Underdeveloped pycnothyrium. **(J)**: Microcycle conidiation (arrow). **(K,M,N)**: Conidia. **(L,O)**: Conidia and microconidia (arrows). **(P–S)**: Lesions formed on inoculated leaves (pycnidia are denoted by arrows). Scale bar: **(A,S)** = 1 mm; **(C)** = 0.5 mm; **(G)** = 50 μm; **(E,F,H,J,O)** = 10 μm; **(K, M,D)** = 20μm.

The multigene phylogenetic analyses grouped isolates from the second group of *Phoma*-like leaf spots in a clade strongly supported in ML and BI analyses with *D. macrostoma* (100/1, ML, BI bootstrap/posterior probability support) (Figure 5). In the single gene analyses of RPB2 and BT2 isolates from the *Phoma*-like leaf spots grouped with isolates of *D. macrostoma*. In the ITS analyses isolates from this study are grouped with *D. macrostoma*, *Didymella subrosea* L.W. Hou, L. Cai & Crous, *Didymella finnmarkica* Crous & Rämä and *Didymella pteridis* L.W. Hou, Crous & L. Cai, whereas in the LSU analyses isolates from this study grouped with *D. macrostoma*, *D. subrosea*, *Didymella aquatica* Q. Chen, Crous & L. Cai, *Didymella rumicicola* (Boerema & Loerakker) Qian Chen & L. Cai, and D. *finnmarkica* (Supplementary Figure 2). Fixed polymorphisms unique for isolates of *D. macrostoma* from this study are shown in Supplementary Table 8. Molecular phylogenetic analyses identified *Phoma*-like isolates as *D. macrostoma* (Figure 5).

A combined dataset (ITS-ACT-CAL) of *Apiognomonia* species (third group of symptoms), had 14 taxa including an outgroup sequence of *Plagiostoma aesculi* (Fuckel) Sogonov (AR 3640). The matrix contained 1223 characters (ITS: 525, ACT: 260, CAL: 438) of which 64 were parsimony informative. The model GTR+I was selected in the ML analyses and used in the following BI analyses. The MP analyses gave 14 most parsimonious trees with TL = 72, CI = 1, RI = 1 and the PHT test showed that the three datasets could be combined (PHT = 1). The topologies of MP, BI and ML trees were concordant, and the best ML tree is presented (Figure 6).

In the multigene phylogenetic analyses, isolates from this study grouped with isolates of *A. errabunda* forming a fully supported clade (100/100/1, ML, MP, BI bootstrap/posterior probability support) (Figure 6). In the single gene phylogenies isolates from this study grouped with *A. errabunda* in the ACT and CAL analyses, whereas in the ITS analyses they clustered with *A. errabunda* and *Apiognomonia platani* (Lév.) L. Lombard (Supplementary Figure 3). Molecular phylogenetic analyses identified *Apiognomonia*-like isolates as *A. errabunda* (Figure 6).

3.3 Morphological characterization

Based on phylogenetic analyses and morphology, this study identified three known species, i.e. *T. dryina* (symptom group 1, subgroup 1), *D. macrostoma* (symptom group 2) and *A. errabunda* (symptom group 3), while *Tubakia* sp. (*T. dryinoides s.l.*, symptom group 1, subgroup 2) represented an undescribed cryptic species that is closely related to *T. dryinoides.* This species has been tentatively maintained in *T. dryinoides* by Braun et al., 2018. who preferred not to describe it as a new species due to the insufficient sampling. In this study, we also prefer not to name it for the same reason as Braun et al., 2018. However,

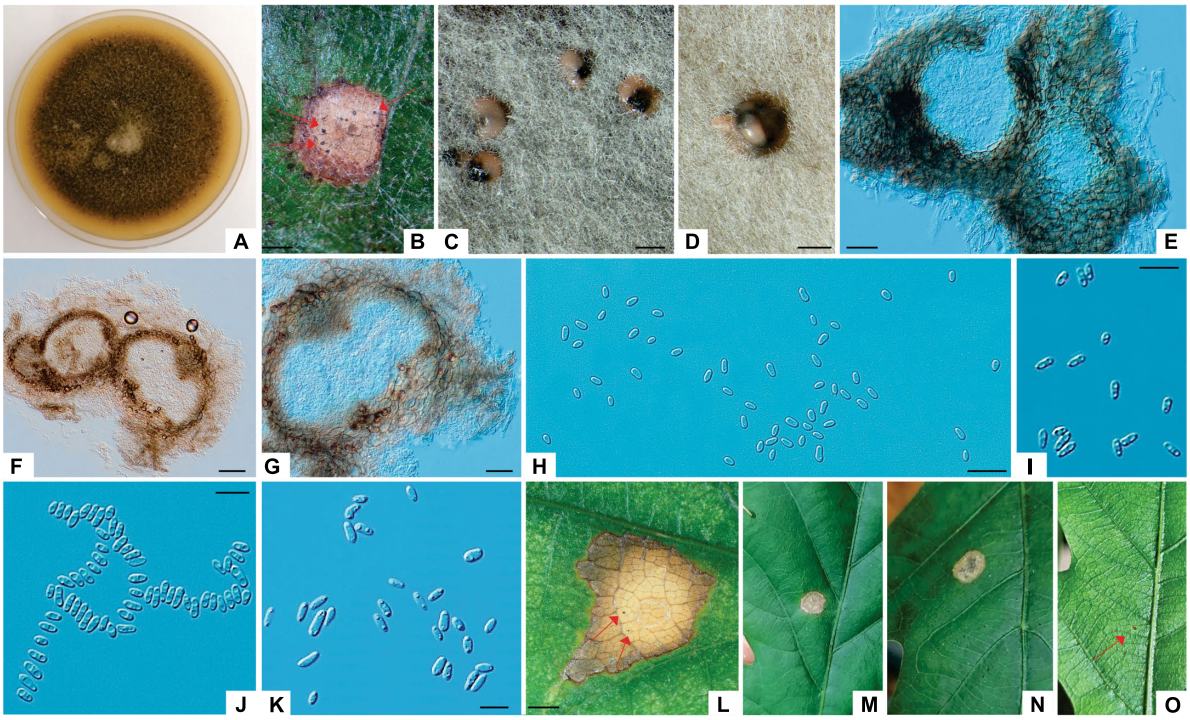

FIGURE 3
Didymella macrostoma (ILFE 8). **(A)**: Colony morphology of ILFE 8 on MEA after 2 weeks of growth at 21°C in the dark. **(B)**: Leaf lesion with conidiomata (pycnidia, arrows) on leaves of *Q. robur* in the Serbian part of the Mura-Drava-Danube Biosphere Reserve. **(C,D)**: Pycnidia formed on MEA extruding spores in creamy colored spore masses. **(E,G)**. Cross section of pycnidia. **(H–K)**. Spores. **(L–N)**. Lesions formed on inoculated leaves (pycnidia are denoted by arrows). **(O)**: Control plant without disease symptoms showing only a small discoloration as a result of wounding (arrow). Scale bar: **(B–D,L)** = 1 mm; **(F)** = 50 μm; **(E)** = 25 μm; **(H–J)** = 20 μm; **(K)** = 10 μm.

to facilitate future studies, we have provided and illustrated its unique morphological characteristics. Moreover, since there are not many recent collections of *T. dryina*, *D. macrotoma* and *A. errabunda* from Europe, this section also contains morphological characteristics of these fungi, especially those that differ from what has been described in other studies.

3.3.1 Taxonomy

Tubakia dryina (Sacc.) Sutton (1973), Trans. Brit. Mycol. Soc. 60 (1): 165 (1973), **Figures 1**, **4**, **Supplementary Figure 1** and **Supplementary Tables 1–4**.

Colonies of *T. dryina* (symptom group 1, subgroup 1) initially white, fluffy and with a rosette-like appearance turning grayish with age, reverse grayish. *Conidiomata* (pycnothyria) on leaves black, scutellate, up to 200 μm diameter; conidiomata on previously autoclaved pine needles black, pycnidial, up to 500 μm diameter, often sporodochial extruding whitish or blackish conidial masses. *Conidiophores* reduced to hyaline to pale brown conidiogenous cells that proliferate percurrently to form periclinal thickenings or rarely annelations. *Conidia* 1-celled, broadly ellipsoidal, ellipsoidal or obovoid, pyriform with round or rarely truncate base, rarely globose, initially hyaline, turning pale brown with age, with rough outer wall, rarely smooth, often thick-walled, 9.82–15.20 μm × 5.26–7.93 μm (av. 11.32 × 6.25 μm, length/width ratio: 1.8, conidia in pycnothyria on leaves, n = 20); 12.76–15.66 μm × 8.01–8.46 μm (av. 13.9 × 8.46 μm, length/width ratio: 1.6, conidia in pycnidia on autoclaved pine needles, n = 20). *Microconidia* in pycnothyria on leaves 1-celled, fusiform, hyaline, smooth, 6.05–8.13 μm × 1.32–2.21 μm (av. 7.1 × 1.7 μm, length/width ratio: 0.6, n = 20).

Isolates examined: Serbia, Mura-Drava-Danube Biosphere Reserve, on necrotic lesion of a leaf of *Q. robur*, June 2021, M. Zlatković/M. Sallmannshofer (living isolate ILFE 6).

Notes: The morphology of an isolate of *T. dryina* from this study was in accordance with that described by Braun et al., 2018, with an exception that conidia were mostly with rough outer wall, and conidiogenous cells were hyaline, but also pale brown similarly to what was observed by Lee et al., 2018. Moreover, in this study, the fungus formed pycnidia and sporodochia instead of scutelloid pycnothyria on autoclaved pine needles. Similarly, in the work of Holdenrieder and Kowalski, 1989 pycnidia instead of pycnothyria had formed on previously autoclaved pedunculate oak twigs. Thus, it appears that pycnothyria may serve as a useful diagnostic character for *T. dryina* only when the fungus is found in nature on a necrotic lesion of a living plant that serves as its host.

Tubakia* sp.** (Tubakia dryinoides*** Nakash (2018). *sensu lato*), Fungal Systematics and Evolution 1: 80 (2018), **Figures 2**, **4**, **Supplementary Figure 1** and **Supplementary Tables 1–7**.

Colonies of *Tubakia* sp. (symptom group 1, subgroup 2) initially white and with rosette-like appearance, becoming blackish with time, reverse blackish. *Conidiomata* (pycnothyria) on leaves black, scutelloid, up to 200 μm diameter; conidiomata on previously autoclaved pine needles pycnothyrial or pycnidial covered with whitish or grayish hairs, up to 700 μm diameter.

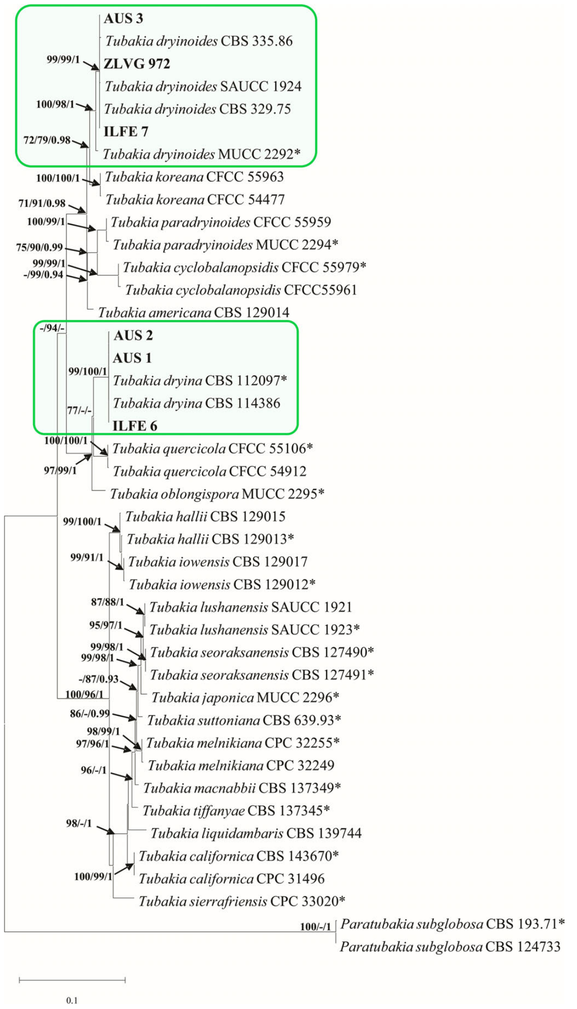

FIGURE 4
Phylogram of *Tubakia* species based on combined dataset of ITS, tef 1-α and BT2. Maximum likelihood (ML), maximum parsimony (MP) bootstrap support values (ML, MP > 70%), and Bayesian Posterior Probabilities (BPP ≥ 0.9) are shown at the tree nodes. The tree is rooted with *Paratubakia subglobosa* (CBS 193.71, CBS 124733). Ex-type strains are marked with an asterix, whereas strains from this study are indicated in bold type. The scale bar represents the number of changes per site. Species identified in this study are indicated with color blocks.

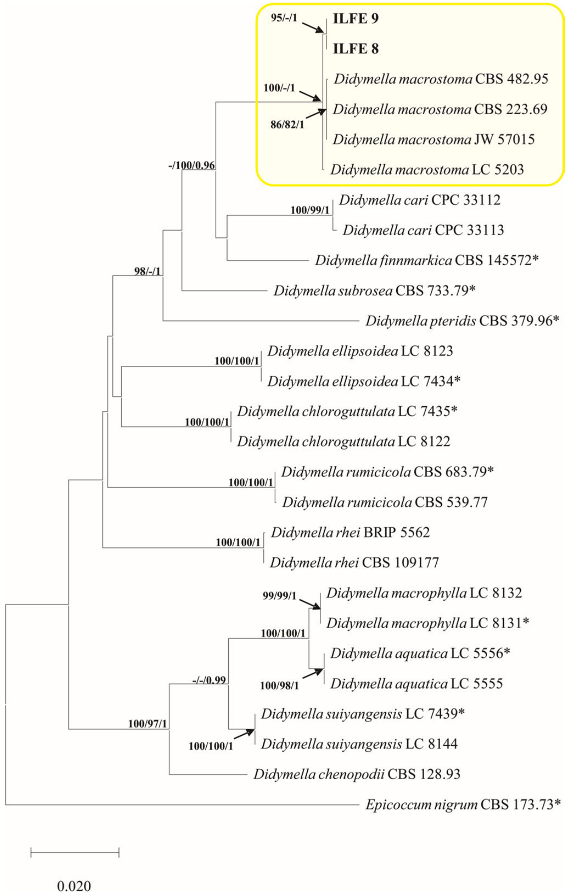

FIGURE 5
Phylogram of Didymellaceae based on combined ITS, BT2, RPB2 and LSU dataset. Maximum likelihood (ML), maximum parsimony (MP) bootstrap support values (ML, MP > 70%), and Bayesian Posterior Probabilities (BPP ≥ 0.9) are shown at the tree nodes. The tree is rooted with *Epicoccum nigrum* (CBS 173.73). Ex-type strains are marked with an asterix, whereas strains from this study are indicated in bold type. The scale bar represents the number of changes per site. Species identified in this study are indicated with color blocks.

Conidiophores reduced to hyaline to pale brown conidiogenous cells, forming indistinct periclinal thickenings or annelations. Rarely, conidia produced by macrocycle conidiation. *Conidia* 1-celled, broadly ellipsoidal, ellipsoidal or obovoid, sometimes spindle shaped with one round and one tapered end, initially hyaline, turning pale brown with age, with rough outer wall, rarely smooth, sometimes thick-walled, 8–13.10 μm × 5.56–7.93 μm (av. 9.22 × 6.62 μm, length/width ratio: 1.4, conidia in pycnothyria on leaves, n = 20); 7.16–12.39 μm × 5.63–7.71 μm (av. 9.39 × 6.52 μm, length/width ratio: 1.4, conidia in pycnidia on autoclaved needles, n = 20); 8.10–13 μm × 5.6–8 μm (av. 9.10 × 6, length/width ratio: 1.5, conidia in pycnothyria on autoclaved needles, n = 20). *Microconidia* in pycnidia on autoclaved needles, 1-celled, hyaline, smooth, fusiform, 6–7.9 μm × 1.46–2 μm (av. 7 × 1.8 μm, n = 10) or pyriform to ellipsoid, 2.5–3.3 × 1.7–2.3 (av. 2.9 × 2.6 μ m, n = 20).

Isolates examined: Serbia, Mura-Drava-Danube Biosphere Reserve, necrotic lesion on the leaf of *Q. robur*, June 2021, M. Zlatković/M. Sallmannshofer (living isolate ILFE 7); Slovenia, necrotic lesion on the leaf of *Q. robur*, June 2021, N. Ogris/B. Piškur (living isolate ZLVG 972).

Notes: Braun et al., 2018 noticed that the ex-type strain of *T. dryinoides* MUCC 2292, isolated from *Quercus phillyreoides* A. Grey in Japan, differs from the European collections of *T. dryinoides*. However, the authors decided to maintain European isolates in *T. dryinoides* due to the lack of morphological and ecological data and the insufficient number of isolates. Phylogenetic analyses from this study showed that isolates from this study AUS3, ZLVG 972 and ILFE 7, other European isolates of *T. dryinoides* (*T. dryinoides s.l.*) CBS 329.75 and CBS 335.86 and an isolate SAUCC 1924 from China represent cryptic species closely related to *T. dryinoides*. Nonetheless, since there are only two living isolates of *Tubakia* sp. available from this study, we prefer not

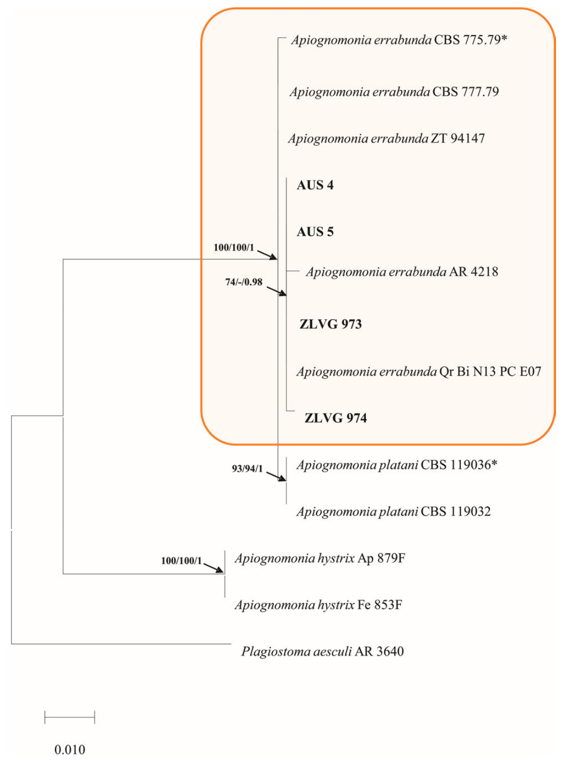

FIGURE 6
Phylogram of Apiognomoniaceae based on concatenated ITS, CAL and ACT sequence alignment. The maximum likelihood (ML) and maximum parsimony (MP) bootstrap support values (> 70%) as well as Bayesian Posterior Probabilities (BPP ≥ 0.9) are shown at the tree nodes. The tree is rooted to *Plagiostoma aesculi* AR 3640. Strains from the current study are in bold, whereas ex-type strains are marked with an asterix. The scale bar represents the number of changes per site. Species identified in this study are indicated with color blocks.

to name the species. Future studies should morphologically and phylogenetically analyze all available living isolates of this cryptic taxon.

Isolates of *Tubakia* sp. from this study were morphologically similar to the type strain of *T. dryinoides* described by Braun et al., 2018 (*T. dryinoides s.s.*), but conidia of *Tubakia* sp. from this study were hyaline and dark like those of *T. dryina* and apart from a study of Zhu et al., 2022 differed from Asian collections of *T. dryinoides*. Moreover, our isolates were characterized by microcycle conidiation and conidiogenous cells were often pale brown, similar to isolates of *T. dryina* from this work. Furthermore, *T. dryinoides* has been reported to produce pycnothyrhia and sporodochial conidiomata (Braun et al., 2018; Zhang et al., 2021; Zhu et al., 2022), but *Tubakia* sp. from this study also produced pycnidia covered with whitish or grayish hairs.

Didymella macrostoma (Mont.) Chen and Cai (2015), Stud. Mycol. 82: 177 (2015). **Figures 3, 5, Supplementary Figure 2** and **Supplementary Tables 1, 2, 5, 8.**

Colonies of *D. macrostoma* (symptom group 2) initially white, becoming dark reddish gray with age, reverse dark gray. *Conidiomata* pycnidial, up to 180 μm diameter, ostiolate, single or often aggregated. *Conidia* aseptate, variable in shape, ellipsoidal to oblong or allantoid, eguttulate or with one or two polar guttules, hyaline, smooth walled, extruding from pycnidia in buff colored spore masses, 3–9.6 μm × 2.5–4.3 μm (av. 6.6 × 3.4 μm, length/width ratio: 1.9, conidia in pycnidia on leaves, n = 20), 3.13–10.50 μm × 2.68–4.54 μm (av. 7.05 × 3.41 μm, length/width ratio: 2.1, conidia in pycnidia on MEA, n = 20).

Isolates examined: Serbia, Mura-Drava-Danube Biosphere Reserve, necrotic lesion on the leaf of *Q. robur*, June 2021, M. Zlatković/M. Sallmannshofer (living isolates ILFE 8, ILFE 9).

Notes: The *D. macrostoma* isolates obtained in this study exhibited similar morphological features as those reported by de Gruyter et al., 2002 and Jayasiri et al., 2017, with the difference that conidia were aseptate and pycnidia were frequently aggregated. Moreover, conidia were often eguttulate and some pycnidia contained only this type of conidia. Thus, it is important to look at multiple pycnidia because guttulate conidia may not always be the best diagnostic trait. Moreover, it appears that *D. macrostoma* is uncommon in Europe, or at the very least, the current sampling is poor. This is likely the cause of the name's lack of stabilization through epitypification, which is crucial for the taxonomic and phylogenetic analysis of the taxon.

Apiognomonia errabunda (Roberge (1918) ex Desm.) Höhn., Ann. Mycol. 16 (1–2): 51 (1918). **Figures 6, 7, Supplementary Figure 3** and **Supplementary Tables 2, 3, 6.**

Colonies of *A. errabunda* (symptom group 3) white becoming whitish gray at the center with age, with dense aerial hyphae, reverse grayish. *Conidiomata* on leaves brownish, cushion-like, mostly ellipsoid acervuli, up to 0.8 mm in length. Conidiomata on MEA with pine needles pycnidial, multilocular, aggregated in the black stroma, mostly ellipsoidal, up to 1mm in length, extruding spores in whitish or grayish spore masses or amber colored, sporodochial, gregarious, up to 2 mm in length. Conidiomata on autoclaved pine needles black with white to gray hairs, extruding spores in white, buff, pink or purple spore masses, up to 0.8 mm in diameter. *Conidiophores* hyaline, septate. *Conidiogenous cells* proliferating percurrently to form annelations or periclinal thickenings. *Conidia* variable in shape, broadly fusiform, oval to obovoid, ellipsoidal, occasionally obpyriform or oblong, usually tapered at one end, often slightly curved, with rough outer wall, often with 1–5 guttules, aseptate, older conidia prior to germination rarely 1-septate, 7.29–14.10 μm (av. 10.58 × 4.8 μm, length/width ratio: 2.2, n = 20). *Microconidia* oval, oblong to cylindrical, sometimes obpyriform, hyaline, occasionally with 1–2 guttules, in pycnidia on autoclaved pine needles and sporodochia on MEA with pine needles 3.85–7.08 μm (av. 5.39 × 2.74 μm, length/width ratio: 1.97, n = 20).

Isolates examined: Slovenia, Mura-Drava-Danube Biosphere Reserve, necrotic lesion on the leaf of *Q. robur*, June 2021, N. Ogris/B. Piškur (living isolates ZLVG 973, ZLVG 974).

Notes: The morphology of *A. errabunda* isolates from this study was similar to that reported by Sogonov et al., 2007 and Li et al., 2020. However, pycnidia from this study were also amber colored, sporodochial and conidia were variable in shape, with rough outer wall, sometimes 1-septate.

3.4 Pathogenicity test

One week after inoculation, small lesions started to appear on plants inoculated with each fungal isolate used in the pathogenicity

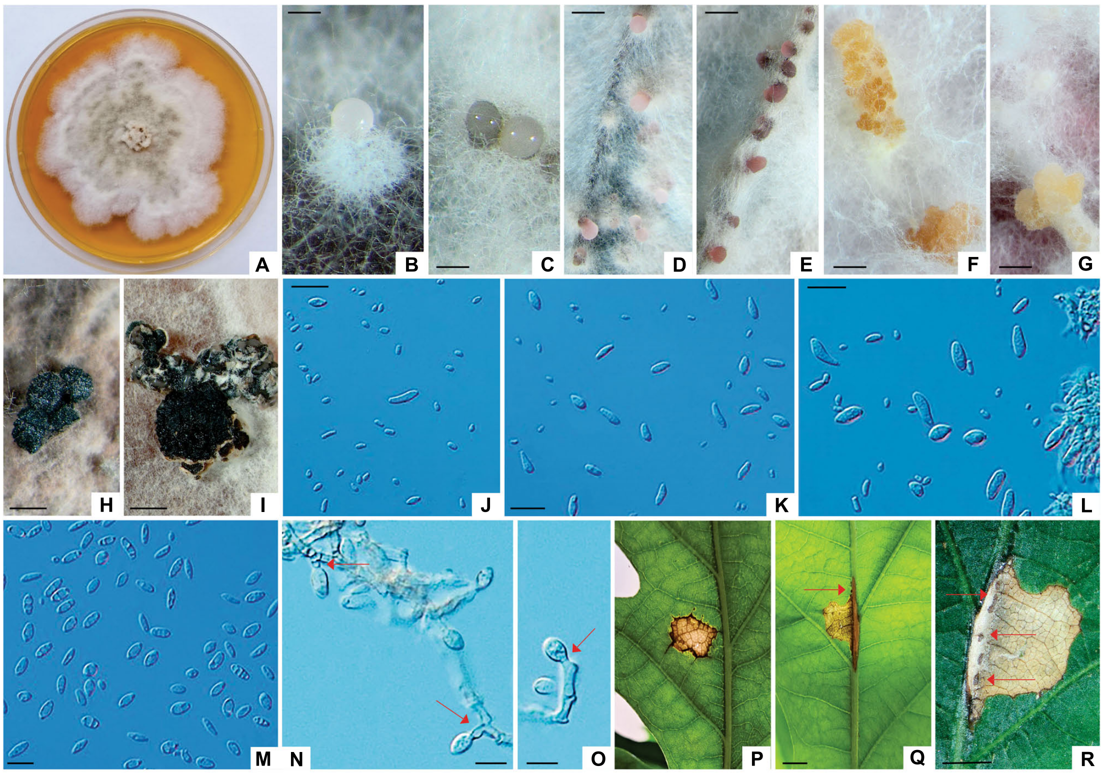

FIGURE 7
Apiognomonia errabunda (ZLVG 973). **(A)**: Colony morphology of ZLVG 973 on MEA after 2 weeks at 21°C in the dark. **(B–D)**: Pycnidial conidiomata formed on pine needles extruding spores in creamy white, greyish, pink or purpule spore masses. **(F,G)**: Amber colored sporodochia formed on MEA with pine needles. **(H,I)**: Pycnidium formed on MEA. **(J,K,M)**: Conidia. **(L)**; Conidia and conidiogenous cells. **(N,O)**: Conidia formed on conidiogenous cells (annelations and periclinal thickenings are denoted by arrows). **(P–R)**: Lesions formed on inoculated leaves (cushion-shaped brown acervulae are denoted by arrows). Scale bar: **(B,C)** = 200 μm; **(D–F,H,I)** = 0.5 mm; **(G)** = 1 mm, **(J–O)** = 20 μm; **(Q,R)** = 2mm.

test and 4 weeks later fungal fruit bodieswere found on leaves of the inoculated plants. Small reddish-brown lesions were observed on each leaf inoculated with *T. dryina* and on leaves inoculated with *T. dryinoides*. The lesions gradually enlarged in size; 4 weeks after inoculation the lesions were irregular in shape and all inoculated leaves were with pycnothyrial conidiomata containing *Tubakia*-like spores. The lesions of plants inoculated with *T. dryina* resembled leaf blight, covered large portions of the leaf and measured 1.5–2 × 1.7–2.2 cm. Lesions caused by *Tubakia* sp. were with a distinctive margin and measured 0.5–0.7 × 0.6–0.9 cm. Pycnothyria formed on ten leaves inoculated with *T. dryinoides*. Small grayish lesions were observed on plants inoculated with *D. macrostoma*. These remained grayish and small and measured 0.4–0.6 × 0.3–0.5 cm. Pycnidial conidiomata with *Phoma*-like spores formed on one leaf. Reddish-brown irregular lesions that measured 1.5–2 × 1.4–2.6 cm were present on the leaves of plants infected with *A. errabunda*. Brownish apothecia with *Apiognomonia*-like spores formed along and across leaf veins of two leaves.

On the control plants that were mock-inoculated no disease symptoms were observed. Inoculated leaves with necrotic lesions were collected and the fungi were re-isolated as described previously. Re-isolations resulted in cultures with morphology and ITS sequences of fungi used for inoculations, thus fulfilling Koch's postulates.

4 Discussion

The current study represents the first attempt to identify the causative agents of the leaf spot and anthracnose diseases of pedunculate oak in the Mura-Drava-Danube Biosphere Reserve. Using the molecular phylogeny of multiple genes (ITS rDNA, partial LSU rDNA, tef 1-α, BT2, CAL, ACT and RPB2) the isolated fungi were identified as *T. dryina*, *Tubakia* sp. (*T. dryinoides s.l.*), *D. macrostoma* and *A. errabunda*. The pathogenicity of the fungi was confirmed by inoculating pedunculate oak leaves, which resulted in symptoms similar to those found in the forest. The study has identified *T. dryina* for the first time in Austria and Serbia; *A. errabunda* in Austria and Slovenia, *D. macrostoma* in Serbia, and an undescribed species of *Tubakia* in all three countries. *Didymella macrostoma* and *Tubakia* sp. (*T. dryinoides s.l.*) have been described for the first time as causes of a leaf spot disease in pedunculate oak.

The well-known pathogen of oaks, *T. dryina* (Holdenrieder and Kowalski, 1989; Kowalski, 2006) was isolated from lesions on pedunculate oak leaves collected in this study. *Tubakia dryina* is a causative agent of *Tubakia* leaf spot disease (Holdenrieder and Kowalski, 1989; Munkvold and Neely, 1990; Kowalski, 2006; Lee et al., 2018). The species is widespread in Europe (Germany, Italy, Poland, Romania, Russia, UK, Netherlands), and it has been speculated that *T. dryina* has been introduced in other parts of the world like New Zealand and USA with pedunculate oak which is its main host (Kowalski, 2006; Braun et al., 2018; Jankowiak et al., 2022). In this work, *T. dryina* was isolated for the first time from pedunculate oak in Austria and Serbia and this work adds to the knowledge on its global distribution. The pathogenicity test conducted in this study confirmed that *T. dryina* is a causative agent of *Tubakia* leaf spot disease of pedunculate oak in the Mura-Drava-Danube Biosphere Reserve.

A cryptic *Tubakia* species, *Tubakia* sp. (*T. dryinoides s.l.*) was isolated for the first time from lesions found on pedunculate oak leaves collected in Austria, Slovenia, and Serbia. The *Tubakia* leaf spot was initially described from pedunculate oak in Poland in 2006, but molecular phylogenetic analyses later revealed that *T. dryina* represents species complex comprising several cryptic species, including *T. dryinoides s.s.* (Kowalski, 2006; Braun et al., 2018; Zhang et al., 2021; Zhu et al., 2022). However, *T. dryinoides s.s.* was described based on Japanese isolate from *Q. phillyraeoides* and phylogenetic analyses of Braun et al., 2018 showed that European isolates of *T. dryinoides* (*T. dryinoides s.l.*) might represent another cryptic species closely related to *T. dryinoides*. This study confirmed this suggestion as our isolates were morphologically and phylogenetically different from what has been described as *T. dryinoides* in Braun et al., 2018; Zhang et al., 2021; Zhu et al., 2022. Host range and distribution of *T. dryinoides s.l.* are still largely unraveled and most reports of the *Tubakia* leaf spot disease were based on collections of leaf spots with pycnothyria and without living cultures and molecular phylogenetic identification which is necessary to identify cryptic species of *T. dryina* and *T. dryinoides* species complexes (Braun et al., 2018). Therefore, further sampling and re-examination of all isolates of *T. dryinoides s.l.* are urgently needed to unravel the identity, distribution, and ecology of members of the *T. dryinoides* species complex.

By conducting pathogenicity tests in this study and fulfilling Koch's postulates, *Tubakia* sp. (*T. dryinoides s.l.*) was shown to represent a pathogen of pedunculate oak. Since its discovery, *T. dryinoides s.s.* has been known to live as an endophyte in its host leaves (Braun et al., 2018). However, Zhang et al., 2021 isolated *T. dryinoides* from lesions on *Q. palustris* leaves in China, but a test of pathogenicity was not done. Moreover, the fungus was isolated from the margins between apparently healthy and diseased tissues and thus could also represent an endophyte or saprophyte feeding on leaves already killed by another pathogen as has been shown by Taylor and Clark, 1996 for *T. dryina s.l.* Furthermore, it may be possible that *T. dryina s.l.* isolates used for pathogenicity tests done before molecular taxonomy in the past on pedunculate oak leaves (e.g., Holdenrieder and Kowalski, 1989; Munkvold and Neely, 1990; Kowalski, 2006) represented *T. dryinoides s.s.* or *s.l.* but this can only remain a matter of speculation. The current study confirmed that *Tubakia* sp. (*T. dryinoides s.l.*) represents an additional causative agent of *Tubakia* leaf spot disease of pedunculate oak.

Interestingly, *D. macrostoma* was found to cause a leaf spot disease of pedunculate oak in this study. The fungus was isolated from spots found on leaves in the Serbian part of the Mura-Drava-Danube Biosphere Reserve. To our knowledge, this is the first report of *D. macrostoma* causing leaf spot disease of pedunculate oak in Serbia, and anywhere worldwide. *Didymella macrostoma* is known as an endophyte, saprophyte and weak parasite commonly found on woody members of the Rosaceae, *Ailanthus altissima* (Mill.) Swingle, *Larix decidua* Mill., *Acer pseudoplatanus* L., *P. nigra* J.F. Arnold and *F. excelsior* (de Gruyter et al., 2002; Chen et al., 2015; Jayasiri et al., 2017; Barta et al., 2022). It is often found in lesions caused by other pathogens, in soil, and it has also been described as human pathogen causing onychomycosis (de Gruyter et al., 2002; Chen et al., 2015; Hou et al., 2020b; Kukhar et al., 2020). However, in this work we showed that *D. macrostoma* is a pathogen of pedunculate oak, capable of causing leaf spot disease. Inoculated leaves from this study were previously wounded, which is in accordance with the description of the fungus provided by de Gruyter et al., 2002 who considered it to be a wound pathogen.

Apiognomonia errabunda was isolated from lesions formed on pedunculate oak leaves in Austria and Slovenia in this work. *Apiognomonia errabunda* has been known as a leaf parasite, endophyte, and saprophyte of various broadleaved trees including those from *Fagaceae*, *Salicaceae* and *Tiliaceae* as well as herbaceous plants (Sogonov et al., 2007; Vainio et al., 2017; Li et al., 2020; Bensaci et al., 2021). For example, in Austria, *A. errabunda* has been isolated as an endophyte from sessile oak leaves as well as from necrotic lesions of European beech leaves (Halmschlager et al., 1993; Cech, 2010). Moreover, *A. errabunda* was described as the cause of the *Tilia cordata* Mill. leaf petiole necrosis that has led to defoliation of *T. cordata* trees in urban areas of Finland in 2016 (Vainio et al., 2017). In addition, it is a well-known cause of oak anthracnose disease in temperate regions of Europe, Russia, USA, and Canada (Hepting, 1971; Sinclair et al., 1987; Bensaci et al., 2021). *Apiognomonia errabunda* has been isolated from pedunculate oak in Switzerland, Germany, Poland, and Russia (Sogonov et al., 2007; Boroń et al., 2019). However, numerous isolates identified using morphology only and before molecular phylogenetics were found to represent closely related *A. platani* which causes anthracnose of plane trees or *Apiognomonia hystrix* (Tode) Sogonov which causes necrosis of leaf petioles of sycamore maple, whereas some isolates were characterized as intraspecific hybrids between *A. errabunda* and *A. hystrix* (Sogonov et al., 2007; Boroń et al., 2019; Kowalski et al., 2021). In this study *A. errabunda* was described for the first time as a pathogen of pedunculate oak in Slovenia, Austria, and the Mura-Drava-Danube Biosphere Reserve.

This research identified new leaf diseases (*Tubakia* and *Didymella* leaf spot, *Apiognomonia* anthracnose) of pedunculate oak in riparian forests of the Mura-Drava-Danube Biosphere Reserve. As recently demonstrated for the *T. koreana* infection of *Quercus acutissima* Carruth., leaf diseases can reduce photosynthetic capacity even if they usually do not kill the trees (Park et al., 2021). The *Tubakia* leaf spot disease is especially important as the fungus can cause stem necrosis of pedunculate oak seedlings (Jankowiak et al., 2022). Moreover, lesions of *Tubakia* leaf spot disease can coalesce in large necrotic areas leading to leaf blotch and if petioles are necrotized the tree may experience premature defoliation (Taylor and Clark, 1996; Kowalski, 2006). Similarly, *T. dryina* produced extensive lesions that covered a significant section of the leaves in our inoculation experiments and resembled leaf blight. Reduced photosynthetic activity caused by leaf pathogens can be particularly harmful to young seedlings and can change the host's biochemical defenses against subsequent biotic attack from different pathogens and pests (Oliva et al., 2014; Pap et al., 2014; Hossain et al., 2019; Amaral et al., 2022). Moreover, *T. dryina* caused larger lesions in this study than did *Tubakia* sp. (*T. dryinoides s.l.*), *D. macrostoma* and *A. errabunda*.

Given that this is the first study to look at the pathogenic potential of *D. macrostoma* and *Tubakia* sp. (*T. dryinoides s.l.*) it seems likely that these pathogens have less of an impact on the oak decline phenomenon than *T. dryina*. Furthermore, during our 4-week experiment, lesions caused by *A. errabunda* developed apothecia that followed a leaf nerve, like what has previously been reported for this pathogen (Kowalski, 2006). However, these lesions did not proceed towards leaf petioles. Therefore, the pedunculate oak genotype from this study may have been less susceptible to the disease since acorns originated from the Serbian portion of the Reserve, where *A. errabunda* has not yet been detected. Nevertheless, the pedunculate oak seedlings were not genotyped using molecular markers and other factors, such as temperature, might not have been optimal for *A. errabunda* infection and growth. However, since the isolates from this study were not examined for their temperature requirements, this may only be speculated. Forest management options in the Mura-Drava-Danube Biosphere Reserve are rather limited (Vastag et al., 2020; Zlatković et al., 2021). Therefore, ecological requirements of the fungi isolated in this study as well as susceptibility of different genotypes of pedunculate oak towards *Tubakia*, *Didymella* and *Apiognomonia* diseases should be a matter of further research.

Pedunculate oak trees sampled in this study might have been under stress. In the past decade, health, and growth of pedunculate oak in the Reserve has been affected by various abiotic and biotic stress factors (Rađević et al., 2020; Kostić et al., 2021). The most significant stress is related to changes in the hydrological regime due to climate change and human-generated river alterations (Kostić et al., 2021; Stojanović et al., 2021; Tadić et al., 2022). This has been negatively affecting pedunculate oak because due to its ecological traits, like deep root system and preference to moist soils with occasional flooding, pedunculate oak is much more sensitive to soil moisture fluctuations compared to other oak species (Eaton et al., 2016; Kostić et al., 2022). Moreover, pedunculate oak trees suffer from continuous infestations by oak lace bug, gall wasps, spongy moth, early oak defoliators, leaf miners and leaf aphids (Nikolić et al., 2019; Csóka et al., 2020; Rađević et al., 2020; Hoch et al., 2023). However, we can only speculate that various abiotic and biotic stresses to which pedunculate oak trees have been exposed in the Reserve might have increased susceptibility of oak trees to pathogens, including those that feed and grow in the leaves. In our pathogenicity test all the fungi isolated in this study induced disease symptoms. During the experiment plants were watered regularly and kept in controlled conditions because the aim of the study was not to subject the plants to water stress. However, since it is well known that stress conditions can enhance fungal diseases of plants (Desprez-Loustau et al., 2006; Hossain et al., 2019; Zolfaghari et al., 2022) the possible influence of predisposing stress to the diseases found in this study should be further investigated in the future.

Because of competing ground vegetation (mostly invasive plants), we were unable to find any seedlings beneath the crowns of pedunculate oak trees during sample collection for this study. As a result, we were unable to determine the occurrence and severity of leaf spot and anthracnose diseases on seedlings. However, we successfully completed Koch's postulates and demonstrated the susceptibility of seedlings to the disease by conducting pathogenicity tests on the leaves of pedunculate oak seedlings. This is important since, in the Mura-Drava-Danube Biosphere Reserve, pedunculate oak regeneration is seriously endangered (Rađević et al., 2020). As was previously mentioned, several stresses that mature trees experience result in physiological weakening of the trees which in turn reduces the number of acorns produced (Nikolić et al., 2019; Pilipović et al., 2020). Moreover, the development of acorns is inhibited by the galls produced on female catkins by the gall wasp *Andricus querquscalicis* (Burgsdorf, 1783; Rađević et al., 2020). Also, the feeding habits of acorn weevils *Curculio* spp. cause acorns to drop off early, whereas the fungus *Ciboria pseudotuberosa* (Cooke) Rehm infects ripe acorns after they fall to the ground and cause serious problems during acorn storage (Drekić, 2006; Rađević et al., 2020). Furthermore, there isn't enough periodic flooding which damages *Curculio* spp. and provides pedunculate oak trees root system with water (Rađević et al., 2020; Kostić et al., 2022; Kesić et al., 2023). In addition, extreme high temperatures, competition from ground vegetation (i.e., *Rubus* spp.), invasive alien species (i.e., *Amorpha fruticosa* L. and *Ambrosia artemisiifolia* L.) and woody species (i.e., *F. excelsior*, *Carpinus betulus* L.), game browsing, *E. alphitoides* and *D. seriata* infections and *C. arcuata* infestation are some of the additional stressors that these seedlings face (Pap et al., 2014; Zlatković et al., 2018; Rađević et al., 2020; Drekić et al., 2023). Therefore, leaf spot and anthracnose diseases from this work may pose a further risk to the regeneration of pedunculate oak. This is especially important in the core zones of the Mura-Drava-Danube Biosphere Reserve, where it is prohibited to use common human assistance techniques including sawing acorns from local seed sources and removing competitive vegetation, to assist in regeneration of pedunculate oaks. Pedunculate oak is one of the keystone species of the Reserve (SUPERB, 2023). Therefore, follow up studies are urgently needed to investigate the occurrence, severity and distribution of leaf spot and anthracnose diseases from this study on pedunculate oak seedlings in riparian forests of the Mura-Drava-Danube Biosphere Reserve.

Located along rivers, riparian forests are of major importance to overall biodiversity and floodplain ecosystem functioning (Dybala et al., 2019; Riis et al., 2020; Zhang et al., 2023). However, riparian forests of the Mura-Drava-Danube Biosphere Reserve are threatened by multiple abiotic and biotic stressors (Lapin et al., 2021; Zlatković et al., 2021; de Groot et al., 2022), including *Tubakia*, *Didymella* and *Apiognomonia* leaf diseases of pedunculate oak identified in this study. Future directions for studying leaf diseases in the Reserve should include sampling of other valuable riparian tree species like poplars and willows, and more thorough surveys with greater number of examined leaves and fungal isolates. Moreover, *Penicillium* spp., *Biscogniauxia* sp., and *Aureobasidium* sp. isolated in this research should be tested for biological control potential against *Tubakia*, *Didymella* and *Apiognomonia* leaf diseases of pedunculate oak.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Author contributions

MZ: Writing—review and editing, Writing—original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. MS: Writing—review and editing, Visualization, Project administration, Investigation. SSc: Writing—review and editing. TC: Writing—review and editing, Resources, Investigation. MD: Writing—review and editing, Visualization, Software, Formal analysis, Data curation. GH: Writing—review and editing. KL: Writing—review and editing. NO: Writing—review and editing, Validation, Investigation, Data curation. BP: Writing—review and editing, Software, Resources, Formal analysis, Data curation. KS: Writing—review and editing, Formal analysis, Data curation. SSt: Writing—review and editing, Methodology. MW: Writing—review and editing, Funding acquisition. SO: Writing—review and editing, Supervision, Resources, Methodology, Funding acquisition.

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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.2024.1363141/full#supplementary-material

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Early detection of bark beetle infestation using UAV-borne multispectral imagery: a case study on the spruce forest in the Czech Republic

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Over the last decade, biotic disturbances caused by bark beetles have represented a serious environmental and economic issue in Central Europe. Great efforts are expended on the early detection and management of bark beetle infestation. Our study analyses a time series of UAV-borne multispectral imagery of a 250-ha forest in the Vysočina region in the Czech Republic. The study site represents a typical European spruce forest with routine silvicultural management. UAV-borne data was acquired three times during the vegetation period, specifically (a) before swarming, (b) at the early stage of infestation, and (c) in the post-abandon phase, i.e., after most bark beetle offspring left the trees. The spectral reflectance values and vegetation indices calculated from orthorectified and radiometrically calibrated imageries were statistically analyzed by quadratic discriminant analysis (QDA). The study shows that healthy and infested trees could be distinguished at the early stage of infestation, especially using NIR-related vegetation indices (NDVI and BNDVI in our case). Detecting infested trees is more significant by vegetation indices than spectral bands and increases with the increasing time after infestation. The study verified the usability of UAV-borne multispectral imageries for early detection of bark beetle infestation at the level of individual trees. Thus, these methods can contribute to precise and effective forest management on a local level.

KEYWORDS

bark beetle detection, green-attack stage, unmanned aerial vehicles (UAVs), multispectral sensors, spectral change, vegetation indices, time-series analysis

1 Introduction

In the last decades, we are witnessing the destruction of the central European Norway spruce *Picea abies* (L.) *H. Karst*. forests by unprecedented bark beetle outbreaks. These are augmented by climatic drivers (hot weather and periods of lower precipitation causing drought) and the increasing frequency and severity of wind disturbances (Schelhaas et al., 2003; Modlinger and Novotný, 2015; Hlásny et al., 2021c; Patacca et al., 2023) associated with climate change globally affecting forest ecosystems (Seidl et al., 2017; Hlásny et al., 2021a). Since 2015 to the present, spruce forests have been demonstrably threatened in the Czech Republic, mainly by the European spruce bark beetle *Ips typographus* (L.) (Hais et al., 2016; Minařík and Langhammer, 2016; Fernandez-Carrillo et al., 2020; Minařík et al., 2020; Bárta et al., 2022); this situation is similar in other Central European countries (Gdulová et al., 2021) such as Germany (Zimmermann and Hoffmann, 2020), Austria (Immitzer and Atzberger, 2014), Slovakia (Havašová et al., 2015), and Poland (Stereńczak et al., 2020). The Czech Republic has become Europe's epicenter of the bark beetle outbreak, and a short time prognosis (Hlásny et al., 2021b) does not promise early improvement of the situation.

A detailed field survey with an emphasis on the early detection of newly infested spruce trees in the so-called "green-attack" stage (Wulder et al., 2006) just after bark beetle swarming (Hlásny et al., 2019) is one of the most effective tools of forest management; it is, however, often difficult to achieve at the time of outbreaks (Vošvrdová et al., 2023). Moreover, it is often costly and time-consuming (Abdullah et al., 2018, 2019b). Therefore, solutions are searched for to replace or supplement and simplify field surveys. The information about infested trees is crucial not only for the minimization of economic losses (allowing the stoppage or at least slowing down of bark beetle outbreaks) but also from the environmental point of view (Zimmermann and Hoffmann, 2020) as it can help understand the spatial spreading of bark beetle infestation and help predict its future development (Hlásny et al., 2021b,c).

Remote sensing (RS) is the approach with the highest potential to overcome the limitations of field surveys, and its importance in bark beetle detection is gradually growing. The RS applicability relies on significant differences in spectral reflectance of healthy and infested trees, which can be recorded by sensors placed on various platforms. This theoretical assumption was confirmed by Foster et al. (2017) and Abdullah et al. (2018) using *in situ* spectroscopy measurements. The use of RS methods for the detection of trees infested by bark beetle has been the subject of many studies [see, e.g., reviews published by Wulder et al. (2006), Senf et al. (2017), Abd El-Ghany et al. (2020), Zabihi et al. (2021), and Kautz et al. (2024)]. However, most of these studies are on coarse (national) scales, typically using data from high-resolution multispectral satellites (Latifi et al., 2014; Havašová et al., 2015; Senf et al., 2015; Hais et al., 2016; Stych et al., 2019). At present, the applicability of the data from the Copernicus program (Sentinel1 and Sentinel-2) has been extensively studied (Abdullah et al., 2019b; Zimmermann and Hoffmann, 2020; Bárta et al., 2021; Huo et al., 2021). For fine-scale (local) analysis, methods using Unmanned Aerial Vehicle (UAV) based technology are potentially valuable, especially in forests with high conservation or economic value. In such areas, very detailed and repeated field surveys are needed, which well fits the advantages of UAVs (Näsi et al., 2015; Minařík and Langhammer, 2016; Dash et al., 2017; Brovkina et al., 2018; Stoyanova et al., 2018; Klouček et al., 2019; Minařík et al., 2020, 2021; Slavík et al., 2020; Huo et al., 2023). The regional scale analysis, a "compromise" between the above-mentioned RS approaches, uses airborne RS with, in particular, hyperspectral sensors for bark beetle detection (Lausch et al., 2013; Fassnacht et al., 2014; Näsi et al., 2018; Bárta et al., 2022). As discussed by Bárta et al. (2021) and Abdullah et al. (2019b) existing studies are mainly focusing on the mapping of later stages, tree mortality, or forest disturbances in general. However, these approaches are not suitable for effective stoppage or slowing down of the bark beetle outbreaks (Immitzer and Atzberger, 2014). UAVs bring many benefits to forest management from the perspective of bark beetle detection issues. For instance, their spatial resolution allows the detection at the level of the individual tree (Dash et al., 2017). The current fixed-wing UAVs can cover areas of square kilometers (Li et al., 2020). Thanks to available cloud processing platforms (Moeyersons et al., 2021) and the necessary knowledge of UAV-borne image processing, even the analysis is getting more user-friendly.

The presented study aims to answer the question of whether multispectral imagery acquired by fixed-wing UAVs is able to detect the early stage of bark beetle infestation at the level of individual trees. The investigation is evaluated using three partial hypotheses, namely: (a) the reflectances in the individual bands differ between infested and healthy spruces at a stage when the bark beetles have not yet left the trees, (b) the same can be said for the selected vegetation indices, (c) these differences are more pronounced for the vegetation indices than for the reflectances in the individual bands, and their use sufficiently distinguishes between healthy and early infested spruces.

2 Materials and methods

2.1 Study site

The study area is situated in the Vysočina region, in the center of the Czech Republic, near the town of Nové Město na Moravě (Figure 1). The study area occupies 250 ha and is covered mainly by a Norway spruce forest (*Picea abies*), growing at elevations between 610 and 780 m a.s.l. The site is a representation of a typical central European coniferous production forest. 10% of trees in the study area were under 18 m in height, 47% of trees were within the height range of 19–24 m, 25% in the range of 25–30 m, and 18% were over 31 m. 21% of trees were younger than 40 years, 41% were 41–80 years old, 32% 81–120 years old, and 6% were over 120 years old, respectively. The canopy was 100% closed in 39% of the area, 90% closed in 32% of the study area, and 80% closed in 28% of the area (FMI, 2024).

Besides silviculture, however, these forests also have an important recreation function (the center of many sports activities). Thanks to this, the forest management in this part of Vysočina area is particularly meticulous (field surveys, early felling, and removal of infested trees, etc.) and although the entire region counts among the areas most affected by the bark beetle infestation in the Czech Republic (Hlásny et al., 2021c), but locally in the area of study the damage to this area remains minimal.

2.2 Bark beetle season and development

Compared to several previous vegetation seasons, the year 2020 was colder and higher in precipitation (see Table A1). The bark beetle activity depends on air temperatures; the swarming can start when the minimum air temperature reaches 16.5°C (Wermelinger, 2004). In the study area, swarming typically occurs in late May (Matějka and Modlinger, 2023) but in 2020, the cold and wet spring/early summer period delayed the peak of the first swarming and mass attack of standing trees to the 25th week of the year (15–21 June 2020). We applied the phenological model PHENIPS (Baier et al., 2007) to

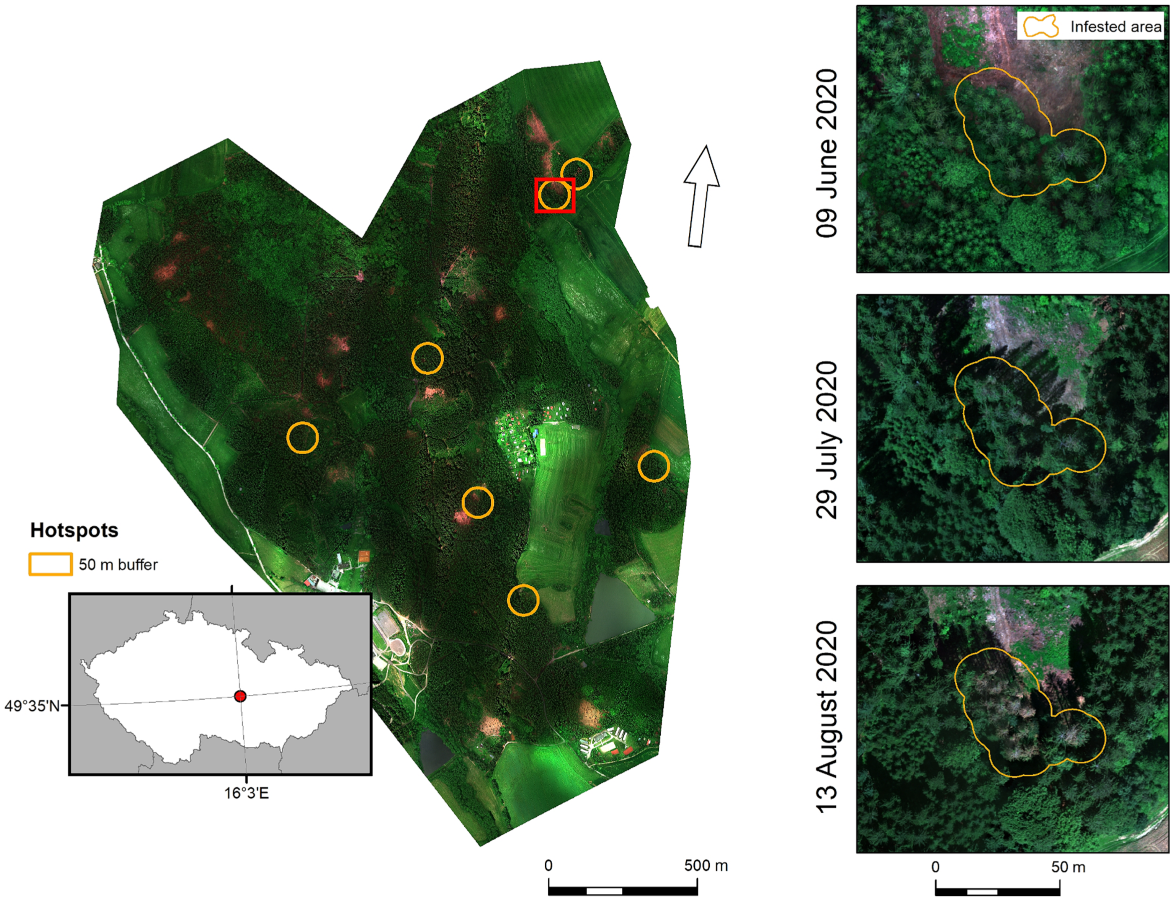

FIGURE 1
The location of the study area (red dot on the Czech Republic map) and the study site's extent. The background image represents the true color composite for the first sensing period (09 June). Identified infested hotspots (orange circles) and the detail of one particular bark beetle hotspot (red square) in the study area shows UAV-borne imagery acquired: before bark beetle activity (09 June), after the swarming on 29 July, and in the phase of tree abandonment by most of the new generation bark beetles on 13 August.

project the development of the bark beetle offspring under the bark of infested trees. For the calculation of degree days (dd), data from the nearest meteorological stations of the Czech Hydrometeorological Institute (CHMI) Vatín (558 m a.s.l.) and Bystřice pod Pernštejnem (553 m a.s.l.) were used. Specifically, the mean daily temperature [°C], the maximum daily temperature [°C], and the daily sum of solar radiation in Wh.m^{-2} were included in the calculation. The mean values from both stations were always used and interpolated to the target altitude of 650 m a.s.l. At the time of the second acquisition, a value of 474 dd was achieved, which corresponds to the pupal stage. The adult phase development of the bark beetle was completed on 06 August. Field observations confirmed the predicted values based on calculation of degree days (dd).

2.3 Acquisition and processing of UAV imagery

The UAV-based imagery acquisition periods represent phases of bark beetle development with visible symptoms in the tree crown (for conditions in the Czech Republic) (a) before swarming on 09 June, (b) the early stage of infestation (green-stage) on 29 July, and (c) the phase of abandonment of trees by most of the bark beetle offspring (yellow/red-stage) on 13 August (Huo et al., 2021). The period of flights was planned with respect to the current conditions (temperature, precipitations) and the probability of bark beetle swarming based on the knowledge of local foresters. The acquisition of UAV images was performed using the fixed-wing eBee X (senseFly, Cheseaux-sur-Lausanne, Switzerland) equipped with the RedEdge-MX (MicaSense, Seattle, United States) multispectral camera (see Table 1 for sensor details). Flight missions predefined using the eMotion ground control software, version 3.20 (senseFly, Cheseaux-sur-Lausanne, Switzerland), were performed at approximately 150 m above ground level with regular 80% side and frontal overlaps; each mission consists of two flights taking approx. 60 min each. All flights were performed approximately at solar noon (±2 h). The weather was sunny, with occasional clouds, for all flights.

UAV-borne imagery was processed using the image-matching software Metashape version 1.7.3 (Agisoft LLC, Saint Petersburg, Russia). The workflow included standard Structure from Motion (SfM) and Multi-View Stereo processing steps [see Klouček et al. (2019) for details], yielding orthomosaics with a 0.16 m pixel size calibrated using high-reflectance targets and irradiance values. Subsequently, digital surface (DSMs) and digital terrain models

(DTMs) with 0.32 m pixel size were created. The calculation of accurate spectral reflectance values using radiometric calibration is a crucial step in UAV-borne data processing. This procedure is necessary when time-series images are analyzed or vegetation indices are calculated (Song et al., 2001). We used the processing workflow recommended by the RedEdge-MX sensor manufacturer using the Metashape software and calibration target with laboratory-measured calibration parameters to guarantee the acquisition of reliable reflectance data throughout the bark beetle season.

2.4 Field survey and creation of tree database

An individual tree detection method was applied using the local maxima filtering approach (Panagiotidis et al., 2017; Surový et al., 2018; Klouček et al., 2022; Komárek et al., 2022) to facilitate database creation. In this way, we created a tree database including all Norway spruce trees (almost 55,000 individuals, 441 were newly infested). As reference data, we used field data provided by the foresters based on their regular field inspections and the database provided by the national Forest Management Institute (FMI, 2022).

Based on the observation of local forest managers, the peak of swarming in the study area was on 15 June. The field survey of newly infested trees was performed by local forest managers on 06 August. The studied period corresponds to the dates of acquisition of UAV-borne images (a) before bark beetle activity (09 June), (b) during the time of offspring development (29 July), and (c) on 13 August, after the offspring left the trees.

Our study aimed to evaluate healthy and infested trees across sensing periods. For this reason, only infested trees determined by the forest manager that remained standing until the last flight served as reference ground truth data for subsequent analyses (i.e., we excluded from analysis trees that were felled to prevent bark beetle spread before the last flight). Finally, seven active bark beetle hotspots were identified (see Figure 1), with 84 Norway spruce trees newly infested during the first bark beetle generation in 2020.

2.5 Image analysis

Pixels representing shadows were masked by thresholding of near-infrared bands. The unique thresholds for orthomosaics were set manually (by trial-and-error approach to visually achieve masking of all shadows; one threshold for the entire orthomosaic acquired during one flight), and the results were visually inspected as in our previous work (Klouček et al., 2019). For every tree, we extracted the (a) mean spectral reflectance value in a 0.5 m buffer around the tree top for spectral bands of masking orthomosaics and (b) height information from the normalized digital surface model (nDSM) calculated by subtracting the DTM from DSM (acquired 09 June). ArcGIS Pro version 2.9.3 (ESRI, Redlands, CA, United States) was used for this process.

TABLE 1 Brief description of spectral properties of the RedEdge-MX (MicaSense, Seattle, United States) multispectral camera.

Spectral bands	Wavelength centre (nm)	Bandwidth (nm)
Blue	475	32
Green	560	27
Red	668	14
Red edge	717	12
Near-infrared	842	57

2.6 Vegetation indices calculation

From the list of available spectral indices (Bannari et al., 1995; Henrich et al., 2009), we selected typical representatives of broadband normalized vegetation indices (VIs) for further bark beetle detection analysis. Specifically, we calculated Green Leaf Index (GLI), Normalized Difference Green/Red Index (NDGRI), Normalized Difference Red Edge Index (NDRE), Normalized Difference Vegetation Index (NDVI), Green Normalized Difference Vegetation Index (GNDVI), and Blue Normalized Difference Vegetation Index (BNDVI), see formulas in Table 2. We considered only indices that, according to our previous study (Klouček et al., 2018), are very unlikely to mutually correlate. ArcGIS Pro was used for the calculation of vegetation indices.

2.7 Statistical analysis

The statistical evaluation aimed to detect appropriate spectral bands and spectral indices for distinguishing healthy and infested trees. *I. typographus* prefers mature, weakened trees (Wermelinger, 2004; Hais et al., 2016) and newly infested trees that are most likely to occur near the previous year's bark beetle infestation locations (Kautz et al., 2011). We used seven located bark beetle hotspots with a total of 84 infested trees. Consequently, we selected all healthy trees taller than 20 m in 50 m buffers around the hotspots, not covered by shadows on orthomosaics. In this way, we selected a total of 960 healthy spruce trees.

An individual regression model was created for each spectral band and vegetation index. Due to the experimental design, a mixed-effect model approach was used (Zuur et al., 2009). The random part of the model was, in all cases, two-way nested by the hotspot and by the individual tree. The fixed part was made up of two factors: the health condition of the tree (Infested, Healthy) and the time of UAV images acquisition; their interaction was also included. During model building and validation, an appropriate distribution function was selected (for most spectral bands and vegetation indices, the Gamma distribution function with log link was the best fit). The Gaussian distribution function was used for several indices with minimal values below zero. Model formulation and prediction were performed in the R package *glmmTMB* following the procedures described by Brooks et al. (2017).

The quadratic discriminant analysis (QDA) (Rencher, 2002) was used to distinguish the groups of healthy and infested trees based on the spectral bands and vegetation indices. Due to a large difference between the size of both groups of trees, the bootstrap resampling technique with 60 iterations was used for sampling the group of healthy trees used in the QDA analysis (McRoberts et al., 2023). To avoid overfitting caused by the fact that the vast majority of trees were

TABLE 2 **Calculated broadband normalized vegetation indices with formulas and examples of use (Henrich et al., 2009).**

Vegetation index	Formula	References
Green leaf index	$GLI = \frac{(2 * GREEN - RED - BLUE)}{(2 * GREEN + RED + BLUE)}$	Hunt et al. (2012)
Normalized difference green/red index	$NDGRI = \frac{(GREEN - RED)}{(GREEN + RED)}$	Hunt et al. (2011) and Abdullah et al. (2019c)
Normalized difference red edge index	$NDRE = \frac{(NIR - Red\,Edge)}{(NIR + Red\,Edge)}$	Abdullah et al. (2019c)
Normalized difference vegetation index	$NDVI = \frac{(NIR - RED)}{(NIR + RED)}$	Rouse et al. (1974), Gitelson and Merzlyak (1997), Dash et al. (2017), and Brovkina et al. (2018)
Green normalized difference vegetation index	$GNDVI = \frac{(NIR - GREEN)}{(NIR + GREEN)}$	Gitelson and Merzlyak (1997) and Dash et al. (2017, 2018)
Blue normalized difference vegetation index	$BNDVI = \frac{(NIR - BLUE)}{(NIR + BLUE)}$	Yang et al. (2004)

healthy (i.e., the prevalence of infested trees was minimal – approx. 1,000 healthy vs. 80 infested trees, which would influence the QDA discriminant function and resulting accuracy characteristics), an infested to healthy ratio of 1:3 was used for bootstrapping. The set of trees was divided into the training and testing group. The training set contained around 60% of the trees. The goodness of the discrimination was measured by the confusion matrix detailing the sensitivity (i.e., the percentage of infested trees accurately detected as infested) and specificity (i.e., the percentage of healthy trees detected as healthy), and overall accuracy (i.e., the total proportion of accurately classified trees). For the QDA method was used the function qda() in the R package MASS (Venables and Ripley, 2002). Analyses were performed in the R 4.0.2 environment (R Core Team, 2020).

3 Results

3.1 The potential of spectral bands and vegetation indices

The trends of spectral reflectance of individual bands (blue, green, red, red edge, and NIR) in healthy and infested spruce trees analyzed across the entire area changed depending on the time from bark beetle infestation in 2020, with the highest difference observed in the red band (Figure 2E). Over time, the green (Figure 2C), red edge (Figure 2G), and NIR (Figure 2I) bands' spectral reflectances decreased in both healthy and infested trees. The red band reflectance grew during the last acquisition (13 August). However, the differences between the healthy and infested trees in the first (09 June) and second (29 July) acquisition periods are negligible and does not allow reliable detection of infested trees. Therefore, it is evident that using individual spectral bands makes early detection of infested trees difficult.

For this reason, we went on to evaluate individual vegetation indices. A significant increase in the differences between healthy and infested trees with the later acquisition time was observed, similar to the visual evaluation based on the spectral bands (Figure 2). All analyzed vegetation indices with the exception of NDRE and GNDVI (which grew between the first two periods in healthy trees) constantly decreased over time for both healthy and infested trees. Before swarming (09 June), almost no difference between healthy and (future) infested trees was observed. However, at the following stages of the bark beetle development (29 July and 13 August), significant differences in fitted (Figures 3A,C,E,G,I,K) and image-based (Figures 3B,D,F,H,J,L) values between infested and healthy trees could be found in all tested vegetation indices (see Figure 3 for details). The use of vegetation indices, therefore, allowed healthy and infested trees to be distinguished at the time of early infestation.

3.2 Statistical analysis of early-stage detection

In addition to the visual assessment presented in Figures 2, 3, QDA was also performed. This analysis, took into account the calculated indices and the background differences between hotspots, showed a possibility of distinguishing between healthy and infested trees even while the bark beetle is still in the tree, in particular when using vegetation indices. Therefore, we specifically focused on evaluating vegetation indices calculated only from the UAV imageries acquired at the stage of early infestation (29 July), which is essential for effective forestry intervention (e.g., cutting down infested trees). The best overall accuracy was acquired using NDRE (81%), NDVI (80%), and GNDVI (81%; Table 3). However, the accuracy differed among individual indices as well as between healthy and infested trees. The best success rate for infested trees was achieved using NDVI (90%) and BNDVI (89%). The success of classification of healthy trees was generally lower, and the most promising results were achieved using NDRE (83%) and GNDVI (81%). The most balanced results for both tree groups (approx. 80%) were acquired using GNDVI (combination of green and NIR bands). In addition, relatively low

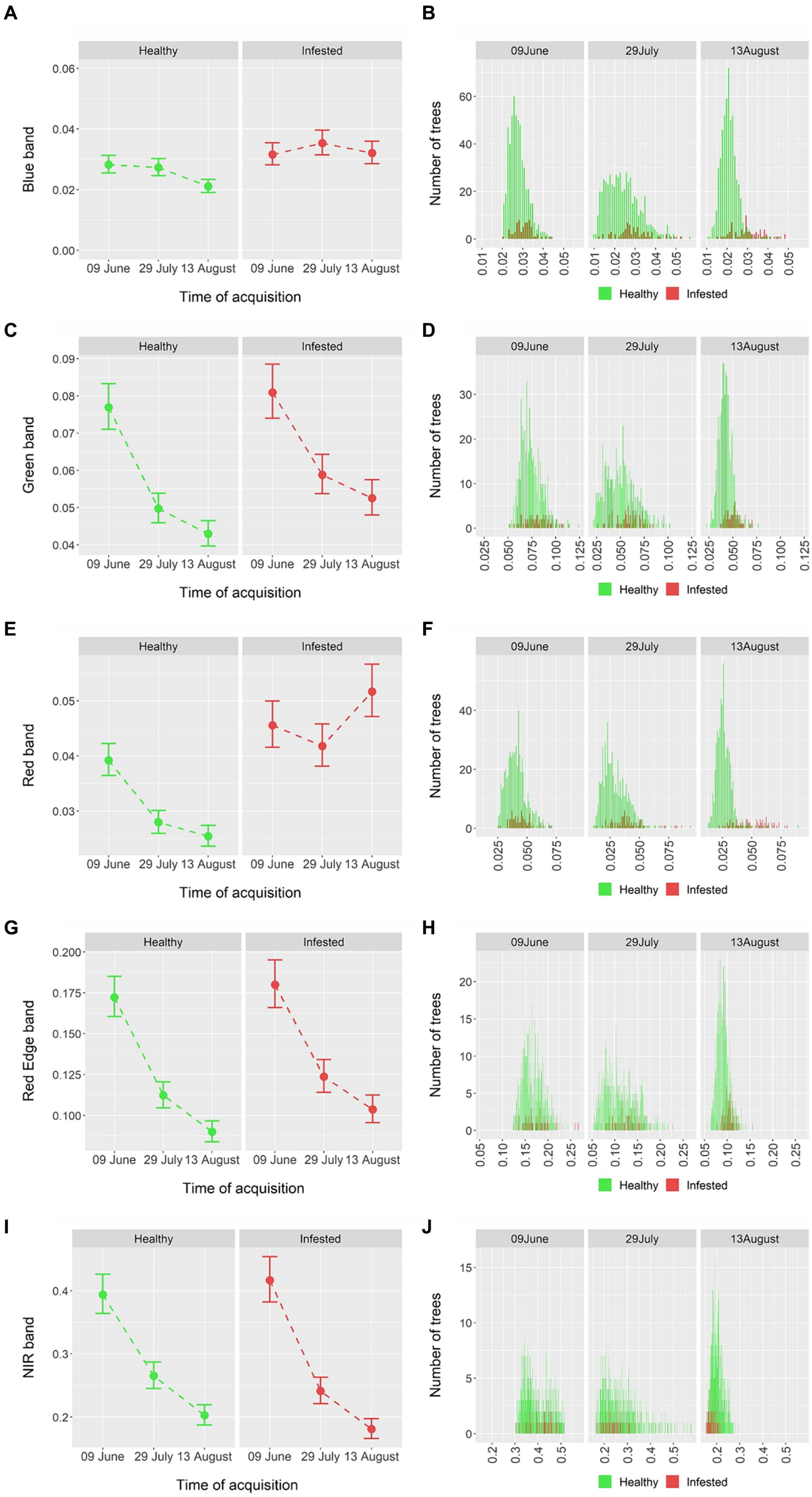

FIGURE 2

(A,C,E,G,I) Surface reflectance (*Y*-axis) of individual spectral bands (*X*-axis) at different time points showing healthy (green) and newly infested (red) trees. The color dots are fitted values resulting from the mixed-effect model, whiskers represent 95% confidence interval. **(B,D,F,H,J)** Histograms presenting the frequencies of surface reflectances of healthy (green) and infested (red) trees **(B,D,F,H,J)**.

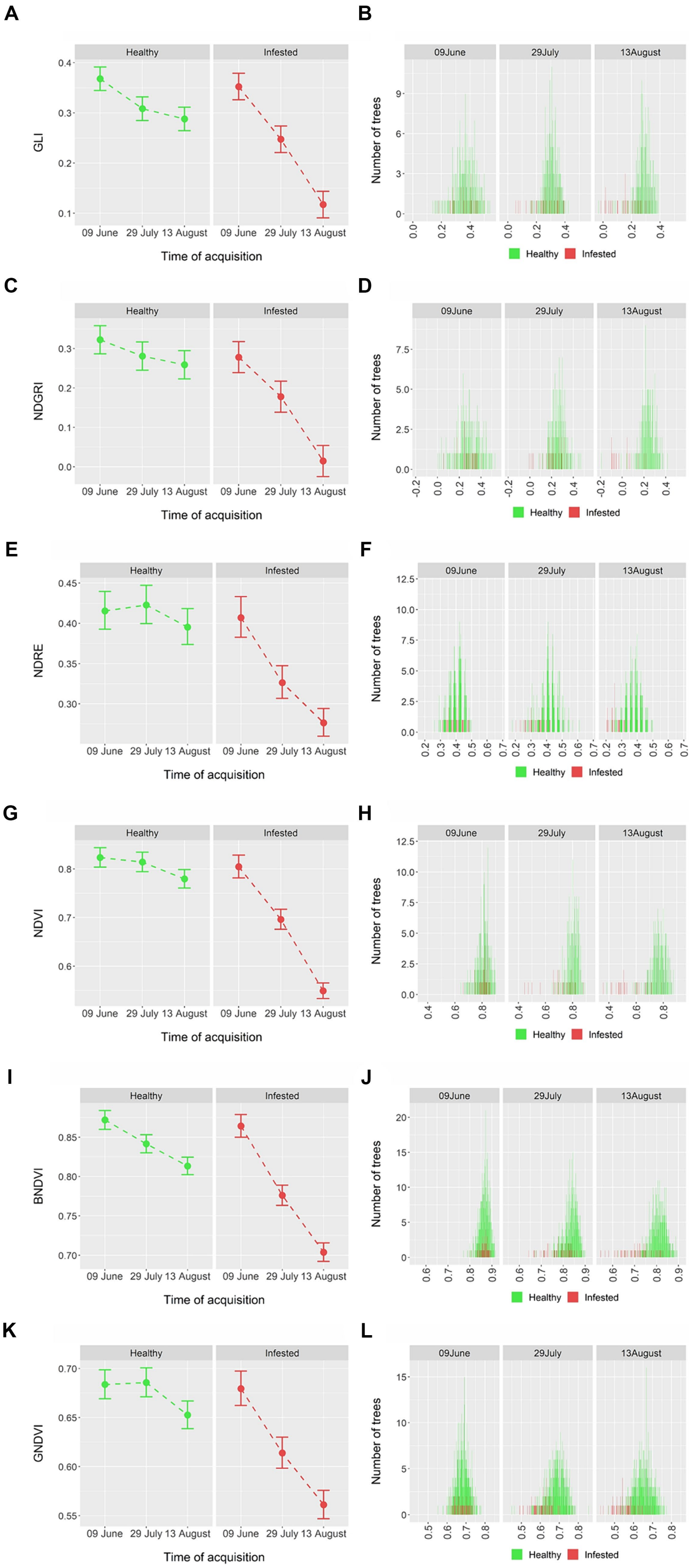

FIGURE 3
(A,C,E,G,I,K) Vegetation indices values (*Y*-axis) at different stages of the bark beetle infestation (*X*-axis), indicating healthy (green) and newly infested (red) trees. The color dots are fitted values resulting from the mixed-effect model, whiskers represent 95% confidence intervals; **(B,D,F,H,J,L)**: histograms showing frequencies of individual values.

TABLE 3 The results of the quadratic discriminant analysis (QDA) using individual vegetation indices for the early-stage bark beetle detection period (29 July).

	GLI	NDGRI	NDRE	NDVI	GNDVI	BNDVI
Overall accuracy	0.78 ± 0.03	0.78 ± 0.03	0.81 ± 0.03	0.80 ± 0.03	0.81 ± 0.03	0.78 ± 0.03
Sensitivity	0.86 ± 0.09	0.86 ± 0.09	0.75 ± 0.07	0.90 ± 0.08	0.80 ± 0.07	0.89 ± 0.08
Specificity	0.77 ± 0.04	0.77 ± 0.04	0.83 ± 0.04	0.79 ± 0.03	0.81 ± 0.04	0.77 ± 0.04

The accuracies of QDA are supplemented with standard deviations.

TABLE 4 The results of the quadratic discriminant analysis (QDA) using individual spectral bands and their combination for the early-stage bark beetle detection period (29 July).

	Blue	Green	Red	Red Edge	NIR	All
Overall accuracy	0.77 ± 0.03	0.64 ± 0.03	0.72 ± 0.03	0.59 ± 0.04	0.62 ± 0.03	0.87 ± 0.05
Sensitivity	0.00 ± 0.00	0.30 ± 0.05	0.38 ± 0.07	0.28 ± 0.05	0.34 ± 0.05	0.70 ± 0.10
Specificity	0.77 ± 0.03	0.81 ± 0.04	0.82 ± 0.03	0.81 ± 0.05	0.87 ± 0.03	0.93 ± 0.06

The accuracies of GDA are supplemented with standard deviations.

TABLE 5 The results of the QDA using a combination of all vegetation indices throughout the bark beetle season.

	09 June	29 July	09 June and 29 July	13 August
Overall accuracy	0.63 ± 0.04	0.84 ± 0.03	0.84 ± 0.03	0.94 ± 0.05
Sensitivity	0.37 ± 0.09	0.80 ± 0.08	0.77 ± 0.08	0.90 ± 0.10
Specificity	0.72 ± 0.05	0.86 ± 0.03	0.86 ± 0.03	0.94 ± 0.06

The parameters are supplemented with a standard deviation. The column "09 June and 29 July" represents a combination of vegetation indices calculated for both dates.

standard deviation values indicate the stability of calculated success rates based on the bootstrap resampling technique (60 iterations) in the QDA analysis (see Table 3).

The QDA results based on spectral bands are summarized in Table 4. The early-stage classification based on spectral bands might seem more accurate at first (87% overall accuracy using all available spectral bands) than using vegetation indices. However, the detection success of infested trees was lower (70%). Of the individual bands, the best results were obtained using the red band (which is consistent with the visual interpretation, see Figure 2E), although the sensitivity was still substantially inferior to that yielded by the indices (38%). The worst results, failing to identify any of the infested trees, was observed for the blue band. Therefore, we can consider vegetation indices as more appropriate inputs for the early detection of bark beetle infestation than individual spectral bands.

3.3 Detection of the infestation using vegetation indices at different time points

Since the use of vegetation indices leads to significantly better conclusions than detection using spectral bands (see Sections 3.1 and 3.2), we have focused in this section on the detection of infected trees using only vegetation indices. The overall accuracy, sensitivity and specificity using vegetation indices increase with time after infestation (Table 5). The lowest overall accuracy (63%) was obtained before bark beetle swarming (09 June), when only 37% of newly infested and 72% of healthy trees were correctly classified. In the early-stage of infestation (29 July), the overall classification accuracy was 84%, with sensitivity of 80% and specificity of 86%. The combination of before-swarming (09 June) and early-stage (29 July) vegetation indices did not improve the classification results compared to those from 29 July. The highest detection accuracy was achieved in the phase of abandonment of trees, when bark beetles probably left the trees (13 August). In this phase, overall detection accuracy was 94% (90% for infested, and 94% for healthy trees). The results of QDA are in detail summarized in Table 5.

4 Discussion

Our findings confirm that the use of vegetation indices allows detection of bark beetle-infested trees while the bark beetle offspring are still inside the infested tree. The accuracies of QDA increase with time after infestation (see Table 5). The high success rate of detecting infested trees during the second flight, when most of the bark beetle population was in the pupal stage, represents an important prerequisite for successful bark beetle management in forest stands (Wermelinger, 2004; Hlásny et al., 2019). Detection of bark beetle-infested trees was also evaluated by Bárta et al. (2022) at the canopy level or Abdullah et al. (2018) at the individual leaf/needle level using hyperspectral measurements. In the latter study, Abdullah et al. (2018) confirmed the potential usability of visible bands for early bark beetle detection.

The sensitivity of detecting infested trees using individual spectral bands was poor (Table 4). Even for the red band, which showed the best sensitivity during the second flight (6 weeks after infestation), the sensitivity was a mere 38%. The fact that the red band performed best in our study was not expected as other studies attributed greater importance to other bands. For example, Huo et al. (2023) reported the red edge band to perform best. They achieved 15% sensitivity 5 eeks after infestation and 90% sensitivity 10 weeks after infestation. In our study, we achieved better results with the bands alone (38% sensitivity with the red band and 70% in multifactorial analysis using all the bands, Table 4). When using indices, the results were much better – using NDVI, we detected as much as 90% of infested trees 6 weeks after infestation. We must, however, consider the fact that the study by Huo et al. (2023) took place in Scandinavia where the development of the bark beetle is slower than in the Central Europe (Baier et al., 2002). In effect, the acquisition 6 weeks after infestation in the Czech Republic may correspond to 10 weeks after infestation in Scandinavia. However, accurate information about the stage of *I. typographus* development for individual acquisitions is missing in their paper. In addition, their research was designed differently from ours – while our research is based on natural infestation of the bark beetle, Huo et al. (2023) used pheromone baited trees. Minařík and Langhammer (2016) found red edge and NIR UAV bands to be promising in detecting infested trees. Abdullah et al. (2019b,c) and Hellwig et al. (2021) reported the potential of red edge bands for early detection of *Ips typographus* infestations. Bárta et al. (2021) highlighted the importance of Sentinel-2 SWIR bands to perform best in early detection of infested trees by bark beetle. Trubin et al. (2023) indicated that the NIR band could be more reliable than other visible bands in detecting susceptible trees in the early detection stage of infestation.

On the other hand, the WorldView-2 study (Immitzer and Atzberger, 2014) using satellite imagery for detecting infestation in mountain regions of Central Europe, yielded results similar to our research, associating the red band with the highest detection accuracy. They called for more research focusing on early-stage detection in association with the change in the spectral signatures of infested trees and their difference from the surrounding environment. Also Huo et al. (2021) found the red band to be promising in detecting infested trees. The importance of the red band was also found in the case of UAVs by Klouček et al. (2019) and Minařík et al. (2021).

Vegetation indices performed better than individual spectral bands, with the differences between infested and healthy trees were obvious in the graphs (Figure 3) as early as the second flight (i.e., during the green attack stage, while the bark beetle offspring were still in the trees). The statistical evaluation using QDA confirmed this visual observation. In particular, the BNDVI (sensitivity of 89%) and NDVI (90%) indices were able to distinguish between healthy and infested trees with sufficient accuracy (Table 3). NDVI returned the overall best results and, therefore, appears to be the most promising of all tested indices. However, vegetation indices are highly correlated (Klouček et al., 2018) and the results provided by many of them are similar. This can also explain the fact that a combination of all indices (Table 5) did not improve the overall detection success compared to individual indices. Combining vegetation indices from different periods did not improve the detection capability, either – likely because the natural seasonal changes alter the natural reflectance of the needles, which confounds the changes associated with early infestation stages. The detailed summary of all 26 recent remote sensing studies focused on early bark beetle detection using individual spectral bands or vegetation indices is available in Appendix Table A1 of the recent review published by Kautz et al. (2024).

Based on our findings, the NIR band (although not providing usable results when detecting solely based on the bands) plays a key role in detecting trees infested by bark beetle using vegetation indices. This corroborates the findings published by Abdullah et al. (2019c), who concluded that the red edge and NIR bands (and, in particular, the indices derived from these bands) are crucial for successful distinguishing between healthy and infested trees. On the other hand, the results disagree with our previous study (Klouček et al., 2019). In that study, visible bands brought more valuable information for differentiation between healthy and infested trees than the NIR band. However, it may be due to the use of a home-made low-cost UAV sensors, instead of the NIR sensor that was used in the present study.

From the practical point of view, the maximum period from infestation that still allows implementation of forestry measures to prevent bark beetles from spreading is approximately 6 weeks in the conditions of the Czech Republic (Bárta et al., 2022). This corresponds to the second image acquisition (29 July), when all vegetation indices discriminated well between the healthy and infested trees (see Table 5). It should be, however, noted, that in countries with a colder climate (i.e., Scandinavia), this period might be longer, allowing more effective measures than is possible in the Czech Republic.

The possibility of finding vulnerable trees before the infestation could be a considerable advantage of RS approaches in bark beetle detection. Other methods react to chemical cues (Vošvrdová et al., 2023) or visual signs of infestation (Kautz et al., 2022) that develop only after the tree is successfully colonized by the bark beetle. Using the vegetation indices, we were able to classify 37% of trees that were later infested as early as the first flight carried out before swarming. A significant difference between healthy and infested trees before the attack was found also by Abdullah et al. (2019c), who, however, did not analyze the classification accuracy. The predisposition of Norway spruce to bark beetle infestation was also identified by Kozhoridze et al. (2023) using thermal satellite imagery. Identifying trees predisposed toward being infested depends, among other things, also on the resistance of the forest stand and on the bark beetle population density: as long as the bark beetle population density is low, *I. typographus* is able to establish offspring only in weakened trees, while once the infestation enters the epidemic phase, it is able to colonize healthy trees as well (Kausrud et al., 2012). The amounts of weakened (although apparently healthy) trees in forest stands increase with the length of the period between the bark beetle outbreaks (Økland and Bjørnstad, 2006). The study area has been well-managed against bark beetles for a long time, and only relatively small numbers of infested trees have been felled. Even so, the site is in a region with massive outbreaks (Hlásny et al., 2021a), and the supply of immigrant beetles is, therefore, considerable, which facilitates the infestation of healthy trees.

Studies analyzing such an extent of forest (250 ha) using time series of UAV-borne spectral reflectance data focusing primarily on early-stage bark beetle detection, such as that by Huo et al. (2023) are extremely rare (Kautz et al., 2024). This is, among other things, caused by the solar noon conditions allowing only a limited time for acquisition within a day. Considering this and fixed-wing UAV capabilities, the system used in this study can cover approximately 400 ha in 4 h. Of course, it could be possible to use simultaneously multiple UAVs, which would multiply the covered area. For this reason, it might be useful to use detailed airborne (Bárta et al., 2022) or satellite (Abdullah et al., 2019a,b,c; Abdollahnejad et al., 2021; Bárta et al., 2021) data to cover larger extents. The use of UAVs, on the other hand, has advantages over satellite and airborne data, such as high spatial resolution and possibility of optimal timing of the flights, thus allowing more accurate early detection at the individual tree level. Fixed-wings enabling vertical take-off and landing (so-called VTOL), capable of application in inaccessible terrain (Cromwell et al., 2021), are also available on the market. In addition, advanced sensors that combine RGB with multispectral or/and thermal cameras are already available, which reduces the number of necessary flights. In addition, the demands for the expertise required for UAV-borne image processing and analysis are decreasing thanks to the available cloud processing solutions [for example, the Forest Guard application in the Czech Republic, that has, unfortunately, been recently discontinued (Unicorn, 2024)].

As a partial limitation of our study, we can mention that even though all flights were performed approximately at the same time of the day, the sun position differed due to the seasonal changes. However, these differences were accounted for by the use of calibration targets and the imagery was radiometrically calibrated, so this is unlikely to cause any issues with the validity of results.

Early detection of bark beetle infestation is a complex issue influenced by many spatial variables (bark beetle life cycles, temperature, precipitation, the actual health status of trees, weather conditions during imagery acquisition etc.). The use of all RS possibilities and their combination with traditional forestry approaches, such as field surveys (Kautz et al., 2022; Kautz et al., 2024), trap installation, etc., could be a way to more effective prevention of bark beetle calamities.

5 Conclusion

Our study evaluated the effectiveness of UAV-borne multispectral imagery for detecting the early stages of bark beetle infestation. Input data were acquired during the bark beetle season (a) before swarming, (b) at the early stage of infestation, and (c) in the post-abandon phase. Results indicate that multispectral imagery allows good discrimination of healthy and early-infested Norway spruce trees at the so-called green attack stage using quadratic discriminant analysis (QDA). In particular, vegetation indices NDVI and BNDVI performed very well in identifying infested trees, with sensitivities up to 90%, which was much better than when using individual bands. The overall detection sensitivity increased with the increasing time from infestation. The discrimination success rate by QDA was similar for the combination of vegetation indices as for individual well-performing indices. The study shows that the use of professional multispectral UAV-borne sensors allows early bark beetle detection and can contribute to precise and effective forest management on a local level.

Data availability statement

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

Author contributions

All authors contributed in a substantial way to the manuscript. TK and JK conceived, designed, and performed the experiments and wrote the manuscript. RM and MZ performed the statistical evaluation. RM and MK supervised and discussed the research. All authors contributed to the manuscript revision, read, 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

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Appendix

TABLE A1 Precipitation (mm) and mean temperature (°C) values in the studied region in 1999–2022.

	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Precipitation	620	674	773	834	527	682	727	723	718	577	791	843
Temperature	7.8	8.7	7.3	8.3	7.9	7.3	7.3	7.8	8.6	8.5	8.0	6.8

	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
Precipitation	578	646	692	660	552	551	652	517	645	**834**	653	620
Temperature	8.2	8.0	7.7	9.0	9.1	8.3	8.3	9.3	9.1	**7.4**	7.7	8.9

Long-term air temperature normal (1981–2010) is 7.4°C; long-term precipitation normal (1981–2010) is 673 mm. Year **2020** is in **bold** (CHMI, 2024).

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Firewood transport and invasive insect spread in Michigan

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Transportation of firewood can be a vector for invasive insect spread resulting in damage to surrounding areas. In 2016 and 2021, surveys were conducted at campgrounds around Michigan to understand where campers were sourcing their firewood, awareness of the 'Do not move firewood' campaign, knowledge of invasive insects and pests, reactions to a potential ban on bringing firewood to campgrounds, and perspective on kiln-dried firewood – all potential policy levers to reduce the spread of invasive insects. Results indicated that campaign awareness slightly decreased between the survey years, personal firewood transport has decreased, and knowledge of invasives remains low. There is an opportunity for intensifying invasive species and firewood outreach efforts, however, regulation (and enforcement) may be more effective among those who would not comply or support a ban.

KEYWORDS

invasive (exotic non-native) species, recreation, behavior, outreach, campground

1 Introduction

Firewood has been documented as a vector for invasive insect spread in the United States (Solano et al., 2021), and camping has been identified as the main recreational driver for firewood transport (Solano et al., 2021). Although most campers visit federal campgrounds within 100 km miles of their home, at least 10% travel over 500 km to camp (Koch et al., 2012). Invasive pests often originate in metropolitan or suburban areas from infested nursery stock, pallets, or other shipping materials (Lovett et al., 2016); residents in these more densely populated areas may bring infested materials into more rural and forested areas, thereby spreading pests to vulnerable tree species. Emerald ash borer, for example, originated in lower Michigan, but was found in the Upper Peninsula of Michigan in 2005. Haack and Petrice (2021) reported that 581 vehicles were stopped from 2005 to 2011 on their way from the Lower Peninsula of Michigan to the Upper Peninsula, yielding 479 interviews (11 commercial drivers and 468 private vehicles) pertaining to transportation of firewood. Over 80% of interviewees reported that they were transporting firewood for camping and cottages and of the 59 vehicles transporting ash firewood, 15 had signs of emerald ash borer.

When invasive insects are introduced into forests it can lead to many different complications. Direct changes to tree species composition can be seen, with the most extreme cases resulting in whole tree mortality that removes entire species from forest stands. These changes to forest structure have cascading effects on the wildlife that rely on the forests for food and habitat, as well as the economic loss from the inability to harvest certain tree species for timber (Lovett et al., 2016). There is also a perceived decrease in aesthetic quality in forests that have been infested by these invasive species due to tree mortality, which reduces property values and reported enjoyment of public lands (Lovett et al., 2016). These aesthetic concerns are often also tree hazard concerns, when mortality occurs in areas used for recreation. Standing dead trees are much more susceptible to falling during wind, snow, or ice events and are fuel for wildfires.

Recreational users, in addition to being affected by tree mortality, may contribute to the spread of invasive insects if they bring woody material with them for camping and vacationing instead of purchasing at or near the campground. Campground users typically have three choices for sourcing firewood. They can bring it from home, purchase it at the campground, or purchase it locally outside the campground area (Borchert et al., 2010). In 2018, a camper survey was completed in three northeastern U.S. states found that 25% of campers brought their own wood (Daigle et al., 2019). The risk of personal wood transport can be mitigated by only transporting heat-treated or kiln-dried firewood.

2 Policy options and implications

Heat-treated firewood is firewood that has been sterilized by being heated for various amounts of time to at least 133°F (Wang et al., 2009). Kiln-dried firewood is heat-treated firewood that has been treated in a United States Department of Agriculture (USDA) certified kiln, with the wood reaching an internal temperature of 160°F for 75 min, to properly ensure that the produced wood is USDA Animal and Plant Health Inspection Service (APHIS) approved and pest free (Wang et al., 2009, 2014). While heat-treated and kiln-dried firewood use the same basic thermal treatment strategies, only APHIS approved kiln-dried firewood is approved for transport across state lines. However, since emerald ash borer (the target threat in Michigan) is no longer federally-regulated, this may limit the implementation of federal regulation unless a state decides to implement their own regulation. There are also several cost concerns with both treatment strategies for consumers and producers alike. Both strategies come with high time, energy, and equipment cost that leads to more expensive products for the consumers (Wang et al., 2009). Mandating heat-treatment and kiln-drying, or subsidizing the cost of heat-treated and kiln-dried firewood may be the only way to overcome the cost barrier that producers and consumers perceive.

Additional policy levers to address the issues caused by spreading invasive insects include outreach campaigns, incentives (e.g., free firewood at destinations), and regulations (e.g., firewood quarantines and park gate bans). While some studies have documented use and preferences for transporting firewood in recreational settings, little research has documented the habits of campground users in states severely impacted by invasive species or sought to understand if the "Do not Move Firewood" campaign, led by The Nature Conservancy and other partners, has led to an increased knowledge of the risks of moving firewood long distances, and what the effect of additional regulation might be on behavior. Solano et al. (2020, 2022) are two exceptions. Solano et al. (2020) analyzed 4,840 survey responses to firewood awareness campaigns over 15 years (2005–2016). As awareness increases they found a greater willingness to take action. Moreover, small increases in education resulted in greater public concern about firewood movement and invasive species. Solano et al. (2022) also reported that participants would be most likely to pay attention to an on-site flyer in a campground, that state forestry agencies were the most believable sources of information, and that older and more educated people were more aware of campaigns.

This Policy Brief reports data from multiple campground surveys in Michigan, U.S.A, to understand firewood/invasive insect knowledge, firewood purchase and transport behavior, and perception of firewood/invasive insect risk 5 years after the Solano dataset. Given the increased number of state, regional, and national awareness campaigns like "Do not Move Firewood," it is important to continue measuring camper awareness and behavior to determine if more outreach is needed, or if different policy approaches are warranted to change behavior.

3 Methods

We used data from a 2005 to 2010 inspection report, a 2016 survey and a 2021 survey. The inspection report includes data from 11 to 67 campgrounds (varied by year) in Michigan in emerald ash borer quarantine areas. DNR park staff checked all incoming campers for firewood from 2005 to 2010, tallying whether the visitor had (1) brought firewood, (2) where the camper originated, and (3) where the firewood originated. Those that arrived from EAB-infested areas were given information about the pest and were not allowed to bring their firewood into the park. In 2016, DNR interns were dedicated to surveying park users at campsites for three state parks: Sleepy Hollow, P. J. Hoffmaster, and Holland. At the gate when checking in visitors during the summer months, people were surveyed if there was firewood visible at check-in. They were informed it was voluntary, but that ash firewood was not allowed in the park. The questions asked about the origin of the firewood they had with them, knowledge of invasive species, knowledge of the do not move firewood campaign, and perceptions of a firewood ban. Most park users for all three sites came from the metro Detroit area [Department of Natural Resources (DNR), 2023]. Sleepy Hollow is a park most often used by in-state residents and offers roadside stands for firewood purchase. Holland is a more urban park with no rustic camping, often used as a stopping point on the way to Sleeping Bear National Dunes. While there is an RV park, there is not much access to roadside firewood. P. J. Hoffmaster has more out-of-state clientele, but also some local interest some rustic tent camping, along with roadside stands for firewood purchase (Department of Natural Resources (DNR), 2023).

In the summer of 2021, we randomly sampled 4 campgrounds in lower Michigan, and 1 in the Upper Peninsula. Campgrounds were located in Luzerne (private), Porcupine Mountains (state), Algonac (state), Metamora-Hadley (state), and Muskegon (state). At each site, surveys were administered at entrances (stationary), by walking through the campground (roving) and at other points of interest (e.g., hiking trails, common spaces). Refusals were noted, in addition to responses. At least 3 h were spent administering surveys at each location over a 2–3 day period, during varying times of day and early evening. Each of these sites offer firewood for purchase either at the campground store or from roadside stands. Surveys could be completed verbally or on paper. All survey questions asked in 2016 were repeated in 2021, but a few additional detailed questions were asked in 2021, such as intended source of firewood, nuanced level of concern/knowledge of invasive species, perspectives on cost for heat-treated or kiln-dried wood, and specific responses to a hypothetical firewood ban. Across both surveys, questions covered knowledge of invasive species, firewood consumption behaviors, and distance traveled to campground. After survey completion, participants were offered handouts and stickers (Do not Move Firewood campaign) on invasive insects. Data was double entered and R software (CRAN, R Core Team, 2022) was used for data analysis. For open-response questions (e.g., "what would you do if outside firewood was banned") two researchers categorized responses into thematic groups and compared coding. Adjustments were made until at least 90% agreement was reached with different data subsets. Human Subjects approval was granted by the Michigan State University Institutional Review Board.

4 Results

Data from the 2005–2010 inspection report revealed that 20,988 out of 84,753 campers inspected (25%) brought firewood with them, of which 17,979 (21%) came from EAB quarantine areas.

In 2016, there were 116 surveys collected at P. J. Hoffmaster, 86 at Holland and 31 at Sleepy Hollow for a total of 233 responses. In 2021, 44 responses were collected across all state parks (Table 1), out of 115 people asked (38% response rate).

Awareness of the Do not Move Firewood (DMF) Campaign was slightly higher in 2016, but the percentage of individuals bringing their own firewood was about half as much in 2021 (Figure 1).

When asked about their perceptions of a potential firewood ban (meaning campers could not bring outside firewood into the park), survey respondents in 2021 were generally more supportive (Table 2), despite less awareness of the DMF campaign.

To better understand how campers would respond behaviorally to a ban, the 2016 survey asked respondents to explain what they would do if a ban was to be put in place; responses were thematically grouped into 6 themes (Figure 2). Most responded they would buy locally for their firewood needs, while others would either not return to the park or find other items to burn.

The 2016 survey found that for those who brought their own firewood, the wood traveled an average of 98.5 km. In 2021, average travel distance was 142 km, but sample size was smaller, so a statistical comparison would not be meaningful. This firewood was brought instead of purchased because it was free and readily available. When asked if they were concerned about invasive insects, 76% of those surveyed in 2016 stated they were concerned, compared with 58% in

TABLE 1 Sample size from two surveys done in 2016 and 2021 at 8 campgrounds across Michigan.

Campground	2016	2021
P.J. Hoffmaster	116	–
Holland	86	–
Sleepy Hollow	31	–
Luzerne	–	12
Metamora	–	4
Muskegon	–	11
Porcupine Mountains	–	11
Algonac	–	6
Total	233	44

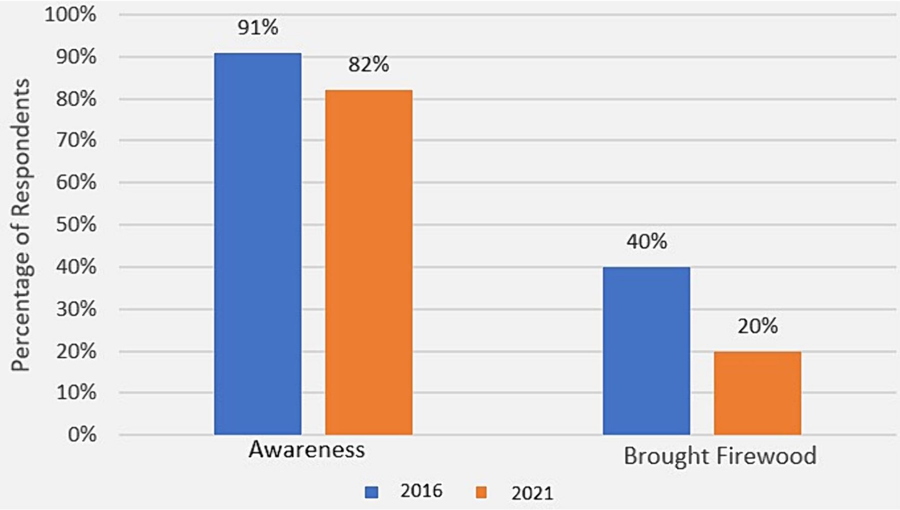

FIGURE 1
Awareness of do not move firewood campaign and percentage of campers bringing their own firewood compared across two surveys (2016 and 2021).

TABLE 2 Campers' perceptions of a firewood ban across two surveys (2016 and 2021).

Year	Do not support (%)	Neutral (%)	Support (%)
2016	30	30	40
2021	25	13.6	59.1

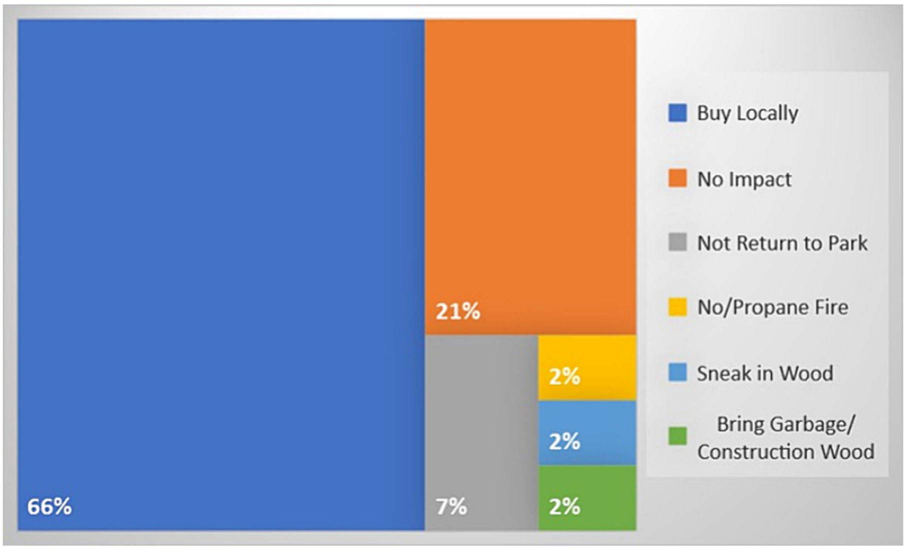

FIGURE 2
Campers' behavioral response to a ban on bringing firewood into parks.

2021. In 2021, 41% of campers purchased their firewood from the camp store, 2.3% from local vendors, and 20% from other sources (comparable data not collected in 2016). Most campers, 68%, felt they knew a little about the threat of invasive insects, 7% felt they knew nothing, 9% a moderate amount, 12% a lot, and 2% said they knew everything (comparable data not collected in 2016).

In 2021, campers were asked if they knew of kiln-dried firewood and if they would be willing to spend more for this certified pest free firewood that is permitted to travel across state lines and has no risk of spreading invasive insects. Just over half, 57%, of respondents have heard of kiln-dried firewood, but 75% of respondents are unwilling to pay extra for this pest free wood.

5 Actionable recommendations

There are two major policy instruments that would address firewood movement as a vector for invasive insect spread: outreach/education and regulation/enforcement. Outreach could include both the general awareness of the problem and suggest that campers buy locally or bring/use kiln-dried or heat-treated firewood. We found that interest in using kiln-dried firewood as an alternative to reduce the risk of spreading insects and pests when moving firewood is low, likely due to cost, and consistent with Daigle et al., 2019. The overwhelming majority of respondents are not willing to pay a higher price for this product, as Wang et al. (2009) identified as a possible limitation. If outreach campaigns do not clearly articulate the avoided cost of invasive insect damage for a consumer to compare with the increased price of kiln-dried firewood, there may be limited support. If kiln-dried/heat-treatment is a preferred option, subsidies or mandates may be the only viable way to change consumer behavior. If firewood prices were reduced (through subsidies to producers for technology), outreach campaigns have been shown to be effective (Solano et al., 2020, 2022).

There may also be an opportunity to scale up outreach efforts about both the threat of invasive insects and pests and how the use of kiln-dried firewood could reduce these threats, building on the success of the Do not Move Firewood Campaign (Solano et al., 2020). However, we found that a segment of those surveyed in 2021 (25–50%) are still opposed to these restrictions and some are willing to violate them; thus a regulatory approach paired with real enforcement may be the only option to influence visitor behavior. Conversely, nearly half of respondents supported a firewood ban measure. Although regulatory approaches can be costly, it is likely that the cost of doing nothing will be higher, due to expensive hazard tree removal (following insect-caused mortality) and decreased visitor satisfaction if widespread tree mortality changes the aesthetics, shade, and other tree-derived benefits from parks and campgrounds.

6 Conclusion and limitations

Awareness of the 'Do not Move Firewood' campaign from 2016 to 2021 decreased. We also found that fewer people are bringing their own firewood from home in 2021 when going camping. Campers are opting instead to either buy firewood from the camp store located within the campground or from local vendors in the surrounding areas. Similarly, most respondents stated they have concerns with invasive insects and pests in general, yet few feel they have any actual knowledge about them, in keeping with other findings (Solano et al., 2022). While fewer people are bringing their own firewood from home while camping, this may be due to convenience or coincidence, rather than any actual worry about the damage that could be done by pests and insects in firewood. The 2016 surveys were conducted at parks closer to the quarantine area, where visitors may have had higher awareness than those from the 2021 surveys. Between 2016 and 2021, the emerald ash borer invasion shifted from intense outbreaks to post-invasion urban and suburban forest (Ward et al., 2021), thus outreach efforts likely decreased during this time as well. These could be potentially confounding factors that limit the comparability of survey results, thus the focus should be on general perspectives and concern for invasive insects and support for policy mechanisms overall.

While support for firewood-restricted behaviors may be increasing as outreach campaigns percolate to the public, at least 25–50% of respondents are still opposed to these restrictions and some are willing to violate them, in keeping with other findings (Robertson and Andow, 2010). Our results include data from two surveys conducted at different campsites across Michigan. They may not be directly comparable in terms of camper demographics, thus impacting generalizability of the results. To move beyond descriptive statistics and enable statistically comparable trends over time, future research could investigate experimental work that compares a site with regulation and enforcement vs. outreach and voluntary compliance to determine which is most effective in reducing firewood transport. Future research should also provide a full economic cost–benefit analysis to compare the cost of a regulatory approach versus the cost of ‘doing nothing’ and instead experiencing insect-induced tree mortality for critical and high-value parks and campgrounds.

Author contributions

EB: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. EH: Conceptualization, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing. HF: Conceptualization, Data curation, Methodology, Writing – review & editing.

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Conflict of interest

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Mitigating Norway spruce mortality through the combined use of an anti-attractant for *Ips typographus* and an attractant for *Thanasimus formicarius*

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This study investigates the efficacy of combined treatment strategy, incorporating pheromones for bark beetle *I. typographus* (IT) and attractant of its natural enemy *T. formicarius* (TF), along with anti-attractants for IT (containing 1-hexanol, 1-octen-3-ol, 3-octanol, eucalyptol, trans-thujanol, and trans-conophthorin), to enhance protection methods for *Picea abies* against biotic disturbances. Two field experiments—trapping experiment and tree protection experiment—were conducted in June 2023 in managed spruce-dominated beetle-affected stands in Czechia. We anticipated higher catches of IT in traps baited with IT pheromone (containing s-ipsdienol, s-*cis*-verbenol, and 2-methyl-3-buten-2-ol) and TF attractant compared to traps using IT pheromone alone, since compounds intrinsic to IT pheromone, namely 2-methyl-3-buten-2-ol, ipsenol, and ipsdienol, are integral components of the attractant designed for TF. We hypothesized that application of TF attractant and IT anti-attractant would enhance the treatment's protective properties, assuming that attracted TF would function as a predator, reducing bark beetle population and increasing tree survival rates. Semiochemical composition declared by the producers was verified using gas chromatography-mass spectrometry analysis. In the trapping experiment, EcoTrap-type traps were baited with six combinations of lures and anti-attractant. In the tree protection experiment, 28 mature Norway spruce trees situated at newly created forest edges underwent four treatment types: TF attractant, IT anti-attractant, their combination, and no treatment ("control"). Traps baited solely with TF attractant did not capture either beetle, whereas traps lured with IT pheromone, TF attractant and anti-attractant showed no captures of IT but recorded the highest numbers of TF, suggesting significant potential for combined treatment efficacy. Surprisingly, tree mortality was observed exclusively among trees treated only with TF attractant and in their vicinity, suggesting unique bark beetles' response to the mixture of predator's attractant and host tree kairomones, a phenomenon that was not previously reported. Application of anti-attractant and TF treatment effectively prevented tree mortality, demonstrating the repellent potential of IT anti-attractant against bark beetles. However, mortality rates showed no significant differences among control trees, those treated with anti-attractants, or those treated with the combination of anti-attractants and TF attractant, underscoring necessity for further research to optimize treatment efficacy.

KEYWORDS

bark beetle, *Picea abies*, natural enemy, ipsdienol, ipsenol, drought, climate change, semiochemicals

1 Introduction

The escalating frequency and severity of drought waves and windstorms have induced widespread bark beetle disturbances, significantly impacting vast forested regions (Millar and Stephenson, 2015). In Europe, the preeminent economic pest, the bark beetle *Ips typographus* (L., 1758), has inflicted damage on tens of millions of Norway spruce trees in recent decades, resulting in pronounced ecological, economic, and social consequences (Senf and Seidl, 2018). Forest owners employ a spectrum of measures to protect their stands and mitigate the bark beetle outbreaks. These strategies encompass the timely removal of infested trees, the implementation of pheromone traps, and the application of anti-attractants (Fettig and Hilszczański, 2015). Nevertheless, the limited cost-effectiveness of such measures over extensive areas during severe outbreaks necessitates a continued search for efficient, economically viable, and environmentally friendly methods to control *I. typographus* population densities.

In the population control strategy of bark beetles, their natural enemies play a significant role (Wermelinger, 2004; Wegensteiner et al., 2015). One of the key predators of the critical forest pest *I. typographus* is the clerid beetle *Thanasimus formicarius* (L., 1758). *T. formicarius* has been observed in association with numerous species of bark beetles inhabiting both coniferous and deciduous trees (Wehnert and Müller, 2012). The species demonstrates an affinity for bark beetle pheromone components and host tree volatiles (Rudinský et al., 1971; Bakke and Kvamme, 1981; Schroeder and Lindelöw, 1989; Hulcr et al., 2006). Adult predators target adult bark beetles before they bore into host trees and lay their eggs in bark crevices of recently infested trees (Schroeder, 1999). The flight season of *T. formicarius* usually begins in March or April and lasts for several months (Schroeder, 2003). *T. formicarius* exhibits flight patterns similar to those *of I. typographus*, except in early spring. During this period, *T. formicarius* preys on other bark beetle species whose flight periods begin earlier than the *I. typographus* one (Schroeder, 1996; Wehnert and Müller, 2012). Adult individuals predominantly feed on adult bark beetles and their larvae, while the larvae of *T. formicarius* move within the galleries of bark beetles, hunting for bark beetle larvae (Koçoglu and Özcan, 2018). The density of the *T. formicarius* population positively correlates with the population density of bark beetles. Concurrently, elevated levels of bark beetle population density result in elevated larval mortality due to predation by *T. formicarius* (Weslien, 1994). Meshkova et al. (2021) demonstrated that the experimental realize of *T. formicarius* into pine stands resulted in a faster decline of *Ips sexdentatus* outbreaks compared to the control stands.

The field of chemical ecology pertaining to the interactions between *T. formicarius* and *I. typographus* witnessed significant progress since the mid-1980s (Bakke and Kvamme, 1981; Hansen, 1983), with the predominant focus of further research directed toward the examination of pheromone compounds produced by *I. typographus*, that elicit attraction in *T. formicarius* (Hulcr et al., 2006; Etxebeste et al., 2012). The chemical composition of *I. typographus* pheromone encompasses several compounds. The primary examples among them are 2-methyl-3-buten-2-ol (MB) and *cis*-verbenol (cV), which collectively serve as the principal aggregation pheromones for *I. typographus* (Vité et al., 1972; Bakke, 1977; Bakke and Kvamme, 1981; Birgersson et al., 1984, 1988). MB specifically influences the orientation of *I. typographus* at short distances, and has been observed to enhance the likelihood of their landing (Schlyter et al., 1987). Ipsdienol (Id), present in modest quantities in males prior to mating, augments the overall attractiveness of the *I. typographus* pheromone (Vité et al., 1972; Bakke, 1977; Bakke and Kvamme, 1981; Schlyter et al., 1992). Scientists supposed that 2-phenylethanol could be a minor component of the aggregation pheromone of *I. typographus* (Birgersson et al., 1984; Sun et al., 2006; Xie and Lv, 2013). Following copulation, males produce ipsenol (Ie), an anti-aggregation pheromone which, in conjunction with verbenone, assumes a pivotal role in regulating the density of bark beetle galleries under the bark. Additionally, at elevated concentrations, these two substances can redirect bark beetle attacks toward neighboring trees (Bakke and Kvamme, 1981; Hansen, 1983; Birgersson et al., 1984, 1988; Sun et al., 2006).

Previous studies have shown that *T. formicarius*, attracted by the aggregation pheromone of *I. typographus*, is specifically drawn to cV. MB, the second component of the aggregation pheromone, neither attracts *T. formicarius* nor enhances the attractiveness of cV when added to the mixture. In contrast, Id is highly attractive for *T. formicarius*, even more so than the aggregation pheromone of *I. typographus* (Hulcr et al., 2006). Etxebeste et al. (2012) found that Ie is also a highly attractive semiochemical for *T. formicarius*. The combination of Id and Ie was reported to be the most attractive mixture for *T. formicarius* (Bakke and Kvamme, 1981; Hulcr et al., 2006). Hansen (1983) concluded that *T. formicarius* has olfactory receptors for all four compounds (cV, MB, Ie, Id). Considering that MB has no attractive effect, the kairomonal response of *T. formicarius* to the mixture of *I. typographus* pheromonal compounds, cV, Id, and Ie is evident (Hansen, 1983). Tømmerås (1985) found that *T. formicarius* has highly specialized olfactory receptors on its antennae, specifically tuned to bark beetle pheromones. That author describes receptors that are capable of detecting (+)-ipsdienol, (–)-ipsdienol, (S)-*cis*-verbenol, (–)-ipsenol, (+)-lineatin, and (–)-verbenon, suggesting that the predator *T. formicarius* can distinguish among various species of bark beetles. However, it remains unclear how the commercial attractant developed for *T. formicarius* influences the trap catches of *I. typographus* and, more specifically, how it modifies the host colonization behavior of *I. typographus* in natural forest settings.

Numerous compounds that have been proven to deter *I. typographus* have been previously identified. Verbenone, the first compound, is synthesized either from the host compound α-pinene or by converting cis-verbenol, the primary pheromone component for *I. typographus* (Birgersson and Leufvén, 1988). Another category encompasses non-host volatiles, such as trans-conophthorin and green leaf alcohols, e.g., 1-hexanol and (Z)-3-hexen-1-ol, commonly found in species like birch and aspen (Zhang et al., 1999). Additionally, C8 alcohols emitted from the barks of these trees act as deterrents. Eucalyptol, a relatively new compound, has demonstrated field effectiveness, exhibiting better precision than verbenone by inhibiting cis-verbenol at the single-sensillum level (Andersson et al., 2010; Binyameen et al., 2014).

Recently, oxygenated monoterpenes derived from host trees, such as trans-thujan-4-ol, have been reported to possess anti-attractant properties (Kalinová et al., 2014; Blažytė-Čereškienė et al., 2016; Schiebe et al., 2019; Jirošová et al., 2022). These compounds have been incorporated into dispensers for tree protection, yielding various degrees of success (Jakuš et al., 2003, 2022, 2024; Schiebe et al., 2011; Deganutti et al., 2023). A novel dispenser, developed by Jakuš et al. (2024) and devoid of beetle-derived compounds, has exhibited promising efficiency. However, anti-attractants are still not widely used in practical forest protection measures due to their limited effectiveness and relatively high costs in tree protection.

Zuhlke and Mueller (2008) proposed a method for controlling bark beetle population density by attracting their predators, such as *T. formicarius*, to their habitats. The authors explored the concept of selective attraction using attractants that contain only some components of the bark beetle's attractant bouquet. They found that even with only one, two or three components, these attractants effectively lured predators of the target bark beetle species. However, if certain specific components are missing, these attractants fail to attract or only minimally attract the target beetles, even in areas where they are abundant (Zuhlke and Mueller, 2008). The potential of using the combined treatment comprising the attractant for *T. formicarius* and anti-attractant for *I. typographus* in Norway spruce protection against biotic disturbances has not been experimentally studied in field conditions. Investigating these questions could contribute to the improvement of tree protection measures and strategies involved in forest management pest control.

The aim of this study is to assess the efficacy of the attractant that has been developed for *T. formicarius* in capturing *T. formicarius and I. typographus* in pheromone traps and mitigating spruce tree mortality. Additionally, the paper examines the feasibility of using a combined treatment, that consists of the attractant for *T. formicarius* and anti-attractant for *I. typographus*, to protect trees against *I. typographus* attacks. We also aim to develop a basis for further reinforcing the effectiveness of our tree protection method based on the use of anti-attractants (Jakuš et al., 2024). We anticipated observing the highest *I. typographus* catches in traps baited with the pheromone of *I. typographus* and attractant developed for *T. formicarius*. As compounds that are intrinsic to the *I. typographus* pheromone constitute components of the attractant that was designed for *T. formicarius* (MB, Ie, Id), we hypothesized that the traps baited with *I. typographus* pheromone and attractant of *T. formicarius* would exhibit the highest catches of *I. typographus*. We also hypothesized that application of both an attractant for *T. formicarius* and an anti-attractant for *I. typographus* on spruce trees would enhance the treatment's protective properties, assuming that attracted *T. formicarius* would prey on potential pioneer bark beetles. We anticipated that the attractant of *T. formicarius* would not attract *I. typographus*. If any *I. typographus* lands on a tree, it would be killed by elevated numbers of *T. formicarius*, which function as predators, consequently mitigating tree mortality by reducing the bark beetle population density.

2 Materials and methods

2.1 Study areas

2.1.1 Kostelec nad Černými lesy

Field trapping experiments were established in a forest near the town of Kostelec nad Černými Lesy in Central Bohemia (coordinates 49.9146136° N, 14.8780744° E, altitude 460 m above sea level). The 90-year old forest stand predominantly consisted of *P. abies* (70%) with a mixture of *L. decidua* (20%) and *P. sylvestris* (10%). A recent bark beetle calamity led to the clearing of a gap in the middle of the stand, where a trapping experiment was conducted. The study plot is situated within the area managed by the School Forest Enterprise (SLP) near the town of Kostelec nad Černými Lesy in the Central Bohemian Region of the Czech Republic. The SLP spans approximately 5,700 ha of forest land and is administrated by the Czech University of Life Sciences Prague (CZU). The region experiences mild winters, with average annual temperatures ranging from 7.0 to 7.5°C. Annual precipitation averages 650 mm, and the vegetation season typically lasts from 150 to 160 days (Tolasz et al., 2007). Currently, the area is affected by an *I. typographus* outbreak, which began after the drought in 2018 (Pirtskhalava-Karpova et al., 2024).

2.1.2 VU Libavá

The tree protection experiment was conducted in 40-year old Norway spruce-dominated stands, with spruce comprising 90% of stand composition, situated in the Potštát Forest district, near Vojenský Újezd Libavá[1] (VU Libavá) in the Olomouc District, in the north-eastern sector of the Czech Republic (coordinates 49.670319° N, 17.545289° E). The area encompasses the Libavá administrative district, which is designated for military forestry and agricultural activities, and has functioned as the training grounds for the Czech army since 1946. The topography of the region is undulating, with elevations ranging between 500 and 650 m. The average annual air temperature is in the range of 5–6°C, with the average daily temperature during the growing season (April–September) not surpassing 12°C in VU Libavá. Annual precipitation averages between 700 and 800 mm/year (Tolasz et al., 2007). The prevalent monoculture of spruce trees, characterized by a low static stability, renders them susceptible to frequent wind-induced damage. The windstorm of 1991 provoked a sequence of enduring bark beetle infestations. Exacerbated by the impacts of climate change, this infestation induced a significant decline in the forested area. The region's military training activities impose constraints on the implementation of conventional forest management and pest control practices in the Potštát Forest district. In 2018, bark beetles proliferated over extensive spruce stand areas. Simultaneously, a severe drought, affecting the entire Central European region (Buras et al., 2020), induced large-scale forest dieback that persisted into 2019. Preceding the commencement of the experiment, a sanitary felling initiative was undertaken, involving the removal of deceased and infested trees within the stands that were designated for this study.

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[1] Available online at: https://www.vojujezd-libava.cz/vismo/dokumenty2.asp?u=9342&id_org=9342&id=3381 (accessed January 20, 2024).

2.2 Semiochemicals

A commercial pheromone lure, Pheroprax A (BASF GmbH, Germany), was used as attractant for *I. typographus*. According to the material safety data sheet (MSDS), the dispenser contains s-ipsdienol, s-*cis*-verbenol, and 2-methyl-3-buten-2-ol. The second attractant dispenser used in our study was ThanasiWit® (Witasek PflanzenSchutz GmbH, Feldkirchen in Kärnten, Austria), that was designed for *T. formicarius*, which contains 2-methyl-3-buten-2-ol, ipsenol, ipsdienol, and phenylethanol. As an anti-attractant for *I. typographus*, a pouch dispenser containing green leaf and non-host volatiles 1-hexanol, 1-octen-3-ol, 3-octanol, eucalyptol, trans-thujanol, and trans-conophthorin was produced by Synergy Semiochemicals Corp. (British Columbia, Canada), in accordance with the formula published in Jakuš et al. (2024).

2.2.1 Gas chromatography-mass spectrometry

To check the composition of the dispensers, solid phase microextraction (SPME) from the headspace over the dispenser was used. Freshly opened specimen from the dispenser was placed into a 5 L glass jar, which was then sealed using aluminum foil and a lid. Volatile sampling was conducted at room temperature, 5 min after a 5-min incubation. Compound separation was performed using a two-dimensional gas chromatograph coupled with a time-of-flight mass spectrometer (GC × GC-TOF–MS) (Leco Pegasus 4D, LECO Corp., Michigan, USA). The hot split/splitless injector (275°C) was operated in a split mode (100:1 split ratio). Separation of the compounds was performed on two chromatographic columns connected in a consumable-free modulator. The HP-5 MS UI column (0.25 mm i.d., 0.25 μm film thickness) and the VF-17 MS column (1.5 m, 0.1 mm i.d., 0.1 μm film thickness) were employed for the first- and second-dimension separations, respectively. Both columns were manufactured by Agilent Technologies (USA). The temperature programme for separation started at 40°C with a hold time of 2 min, followed by a gradient of 10°C min^{-1} to 120°C, and then at 20°C min^{-1} to 300°C with a hold time of 2 min. The secondary oven and the modulator had temperature offsets of 5 and 15°C, respectively. A 5-s modulation period was used. The separated compounds underwent ionization in the ion source of MSD at 70 eV, and full spectral (35–500 Da) information was acquired at 100 Hz. The compounds were identified using mass spectral similarity, and confirmed via retention index comparison. For comparative analyses, mass spectra and retention indexes were referenced from the NIST Mass Spectral Libraries (Mass Spectrometry Data Center, NIST, USA), except in the case of trans-conophthorin, for which the mass spectrum was sourced from Zhao et al. (2019).

2.3 Experimental design

2.3.1 Trapping experiment

Six EcoTrap-type traps were installed in a clearing of a stand that had previously been affected by bark beetles. The traps were situated along the edges of the spruce stand, which featured larch and pine admixture, spaced 15 m apart and positioned 20 m from the forest edge. The traps were baited with the pheromone lure for *I. typographus* Pheroprax (PhI), attraction lure for *T. formicarius* Thanasiwit (TA), a lure with a customized mixture of anti-attractants for *I. typographus* (AI), and their combinations (PhI; TA; AI; PhI + TA; AI + TA; PhI + TA + AI, Table 1). The experiment took place in June 2023, with traps being inspected at intervals of 2–3 days. Bait rotation was implemented using the Latin Square method. After each beetle collection, the count of *T. formicarius* was recorded, and the estimation of *I. typographus* numbers was derived from the volume of catches.

TABLE 1 Experimental variants (treatment types) used in the trapping experiment.

Dispenser variant	Pheroprax A	ThanasiWit®	Anti-attractant
PhI	+	–	–
TA	–	+	–
AI	–	–	+
PhI + TA	+	+	–
AI + TA	–	+	+
PhI + TA + AI	+	+	+

PhI, *I. typographus* pheromone; TA, *T. formicarius* attractant; AI, anti-attractant for *I. typographus*.

2.3.2 Tree protection experiment

On 2 June 2023, in the Libavá military forest study area, we conducted a tree protection experiment on 28 mature Norway spruce trees using *T. formicarius* attractant and *I. typographus* attractant. We selected visually healthy spruces that were upper-canopy or mid-canopy individuals. To achieve homogeneous experimental conditions, our seven plots and selected trees were situated alongside the extended recently created southern-oriented forest edge, which resulted from salvage cutting immediately preceding the experiment. The average diameter at breast height of the experimental trees was 17 cm, with an average height of 16 m, as indicated in the forest management plan. The goal of the experiment was to investigate the efficiency of using *T. formicarius* attracatant in protecting Norway spruce trees against *I. typographus* colonization. We also aimed to test the viability of employing an attractant for *T. formicarius*, coupled with an anti-attractant for *I. typographus*, as a preventive measure against mass attacks of *I. typographus*. In each plot, four trees were treated with four different treatment variants: (A) anti-attractant for *I. typographus* (AI); (B) attractant for *T. formicarius* (TA); (C) anti-attractant for *I. typographus* and attractant for *T. formicarius* (AI+TA; (D) control (no treatment). The minimal inter-tree distance was 12 m, in order to prevent the potential transfusion of olfactory signals among the treated trees that could potentially obfuscate the results (Schlyter et al., 1987). The plots were spaced 50 m apart. We monitored the statuses of the treated trees (beetle-killed vs. non-attacked) for 4 months.

2.4 Statistical methods used in the analysis of the experiment results of trapping and tree protection

To compare *I. typographus* and *T. formicarius* catches among six different trap treatments (variants) in the Kostelec nad Černými lesy part of the study area, and bark beetle-caused tree mortality among four treatment types in the Libavá military forest part of the study area, we used a one-way ANOVA. The normality of the distribution of residuals in the ANOVA model was checked using the Shapiro-Wilk test from the stats package in R (R development Core Team 2023). The Levene test of the equality of variances from the car package in R was also used. If assumptions regarding the ANOVA model were not met, we used a non-parametric Kruskal-Wallis rank sum test from the stats package in R to compare *I. typographus* and *T. formicarius* catches and tree mortality among different treatment types. Dunn's test of multiple comparisons (i.e., a *post-hoc* test) (FSA package in R) was used to identify the pairs of treatments, for which the catches of *I. typographus* and *T. formicarius,* and tree mortality were significantly different. We used Holm's method for the adjustment of *p*-values regarding multiple comparisons (Holm, 1979). To visualize the results of pairwise comparisons, we quantified a compact letter display at 0.05 significance level using a cldList() function from the rcompanion package in R. To compare the total trap catches between two bark beetle species, we used a non-parametric Mann-Whitney *U*-test for two independent non-normally distributed groups. All analyses were performed in R.

3 Results

3.1 Compounds identified in the tested dispensers

The results of GCMS analysis, performed for the pheromone designed for *I. typograpus*, the attractant designed for *T. formicarius*, and *I. typographus* anti-attractant revealed 11 compounds detected in the tested dispensers. The identified compounds corresponded to the components declared by the producers of semiochemicals. Particularly, the attractant designed for *T. formicarius* (ThanasiWit®) incorporated 2-methyl-3-buten-2-ol, ipsenol, ipsdienol, and phenylethanol. Apart from 2-methyl-3-buten-2-ol and ipsdienol, a commercial pheromone lure developed for *I. typographus* (Pheroprax A) contained verbenol. Anti-attractant components comprised green leaf and non-host volatiles 1-hexanol, 1-octen-3-ol, 3-octanol, eucalyptol, trans-thujanol, and trans-conophthorin (Supplementary Table S1). Two-dimensional chromatographs derived for *I. typographus* pheromone, *T. formicarius* attractant, and *I. typographus* anti-attractant are depicted in the form of contour plots (Figure 1).

3.2 Trapping experiment

The total trap catches of *I. typographus* measured for all treatments (19,340 beetles) were incomparably larger than the total catches of *T. formicarius* (25 beetles) ($p = 0.013$). The number of both *I. typographus* and *T. formicarius* catches significantly differed among six treatment types (Supplementary Table S2). Traps baited with *I. typographus* pheromone caught significantly more *I. typographus* (PhI) than traps baited with *T. formicarius* attractant (TA) (Figure 2A; Supplementary Table S3). Correspondingly, larger numbers of *I. typographus* were caught when PhI was added to TA than when traps were baited with only TA. Conversely, traps baited with TA and PhI did not catch increased numbers of *I. typographus* compared to traps baited with PhI alone. Similarly, we did not observe significant differences in *I. typographus* catches between traps baited with *I. typographus* anti-attractant (AI) and AI + TA. However, the number of catches were significantly higher in traps baited with PhI, TA and AI, compared to traps baited with AI and TA + AI alone (Supplementary Table S3). Traps baited with both species' attractants did not catch significantly larger numbers of *I. typographus* when AI was added to the traps. We identified significantly smaller number of *I. typographus* catches in traps baited with anti-attractant and anti-attractant coupled with *T. formicarius* attractant than in traps lured with the pheromone of *I. typographus* and attractant of *T. formicarius.* However, there was no significant difference in *I. typographus* catches observed in traps lured with the pheromone of *I. typographus* and attractant for *T. formicarius*, and traps in which both dispensers were coupled with anti-attractant (Figure 2A; Supplementary Table S3).

T. formicarius catches did not vary significantly among the treatments, except for the catches recorded in traps treated with *I. typographus* anti-attractant vs. traps treated with *I. typographus* pheromone, attractant for *T. formicarius*, and anti-attractant (Figure 2B; Supplementary Table S3), for which the highest number of *T. formicarius* catches was observed. The count of beetles in traps treated with both *I. typographus* pheromone, attractant for *T. formicarius*, and anti-attractant exceeded the corresponding value in traps without anti-attractant, yet the difference was statistically insignificant (Figure 2B; Supplementary Table S3). Traps baited with the attractant for *T. formicarius* (TA) failed to capture either *T. formicarius* or *I. typographus.*

3.3 Tree protection experiment

The results of the tree protection experiment that was conducted in the Libavá military forest part of the study area indicate that the largest number of bark beetle-killed trees was observed for individuals treated with TA (Figure 3). Actually, all trees treated with the attractant developed for *T. formicarius*

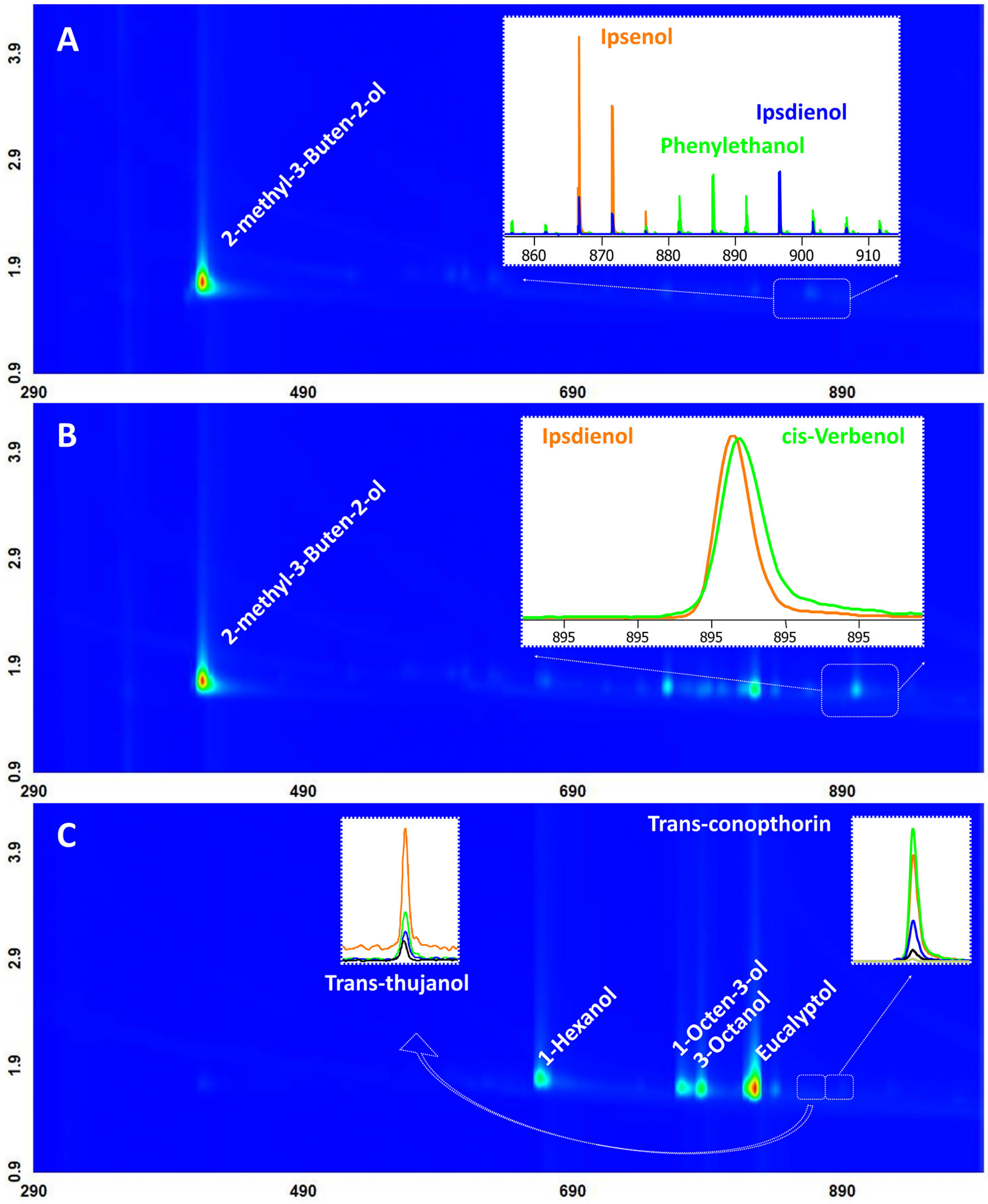

FIGURE 1
Two-dimensional chromatographs depicting the emissions from the tested dispensers. Panel **(A)** represents ThanasiWit®, Panel **(B)** showcases Pheroprax A, and Panel **(C)** delineates the customized anti-attractant. A total ion chromatogram is utilized for the contour plot. When necessary, different colors are employed to highlight characteristic masses of co-eluting compounds. Specifically, trace amounts of trans-conophthorin are marked with the mass spectra of m/z 84 in orange, 87 in green, 97 in blue, 112 in black, and 156 (molecular ion) in gold color. Additionally, trans-thujanol is indicated with m/z 93 in orange, 121 in green, 136 in blue, and 154 in black. Major signals in the chromatograms were identified as components of the dispenser, aligning with the formulae published in the respective MSDS, or in Jakuš et al. (2024).

were attacked and killed by *I. typographus*. The number of these trees significantly differed from the mortality rates recorded for the rest of the treatment types (Supplementary Table S4). Bark beetles did not attack individuals treated with AI and AI+TA, and also they did not colonize the untreated trees (i.e., control samples).

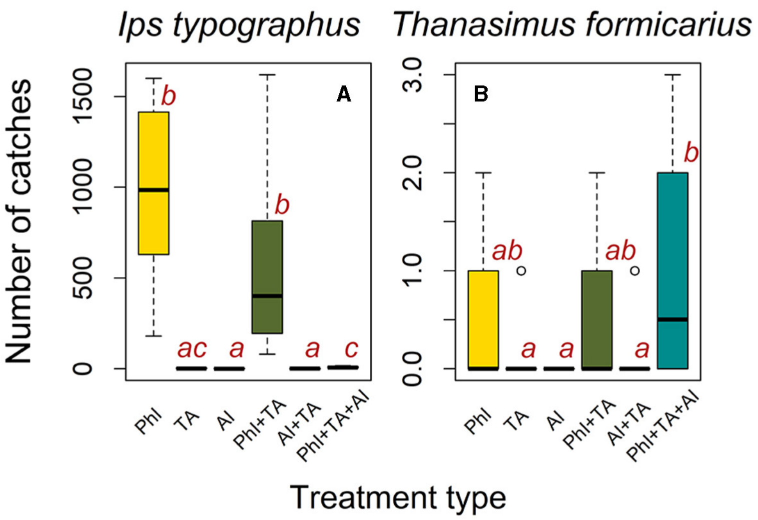

FIGURE 2
Boxplots showing the numbers of *I. typographus* **(A)** and *T. formicarius* **(B)** catches in Kostelec nad Černými lesy study area among six treatment types indicated by color. Definitions are Phl, *I. typographus* pheromone; TA, *T. formicarius* attractant; AI, *I. typographus* anti-attractant. The differences in catches among treatment types were checked using the Kruskal-Wallis rank sum test. Pairwise comparisons were performed using Dunn's multiple comparison (*post-hoc*) test with Holm's adjustment of *p*-values for multiple comparisons. Compact letter display (lowercase letters "a"–"c" and their combinations) indicates (in)significance in catches between treatment pairs at 0.05 significance level. If any two given treatment types within a panel share at least one common letter, the number of catches does not significantly differ between them. Conversely, if any two given treatment types within a panel do not share any common letters, the number of catches significantly varies between them. The horizontal lines inside the boxplots correspond to the median catch values. The boxes display the interquartile range, which represents the middle 50% of the data. The error bars are the 95% confidence intervals.

4 Discussion

4.1 *T. formicarius* attractant and *I. typographus* trap catches

In total, we caught a significantly larger number of *I. typographus* beetles than *T. formicarius* beetles, which approximately corresponds to the balance between predators and their prey existing in natural bark beetle communities (Reeve, 1997; Turchin et al., 1999). The *T. formicarius*/*I. typographus* ratio observed by us (1/774) and based on the number of catches in the baited traps is consistent with previously published empirical evidence on the same species (Warzée et al., 2006). Previous research (Warzée et al., 2006) has indicated that the proportion of pines within a 500-m radius significantly influences these ratios in traps. In this study, our objective was not to quantify the effects of stand composition on the abundance of predator/prey ratios. Given that our experimental plots were situated in a spruce-monodominated forest, with *P. abies* comprising nearly 100% of the trees, we aimed to ensure homogeneous experimental conditions, including consistent proportions of host trees, across all plots. Consequently, drawing conclusions about the impact of stand composition on predator/prey ratios and the number of beetle catches would be hindered by the lack of available data resulting from our experimental settings.

Contrasting our expectations, the results of the trapping experiment conducted in the Norway spruce stands in the Kostelec nad Černými lesy part of the study area indicate that traps treated with the *I. typographus* pheromone and attractant for *T. formicarius* caught smaller, though insignificantly, numbers of *I. typographus* compared to the traps treated with *I. typographus* pheromone alone (Figure 2A). Interestingly, the number of *T. formicarius* catches also did not vary significantly between the traps treated with the *I. typographus* pheromone and attractant for *T. formicarius*, and traps baited with *I. typographus* pheromone alone (Figure 2B). A larger sample size may be needed in order to detect a more pronounced number of the clerid beetle caught in traps baited with *I. typographus* pheromone and attractant for *T. formicarius*. The number of *I. typographus* catches varied more often among the treatments than the number of *T. formicarius* catches, presumably due to an inherently smaller overall population density that is commonly observed for *T. formicarius*, being a natural enemy of *I. typographus* (Warzée et al., 2006).

Surprisingly, traps treated solely with the attractant designed for *T. formicarius* failed to capture either *T. formicarius* or *I. typographus*. However, in traps treated with both attractants (*I. typographus* pheromone and *T. formicarius* attractant) and anti-attractant, no *I. typographus* were caught, while the highest numbers of *T. formicarius* were observed (Figure 2). The absence of *I. typographus* catches in the combined treatment traps may be attributed to the strong repellent effects of tree-based anti-attractants comprising the anti-attractant dispenser (Jakuš et al., 2024). The deterrent effect might have outweighed the luring effect of the *I. typographus* attractant, despite the latter presumably being enhanced by the presence of Ie, Id, and MB—compounds constituting the *T. formicarius* attractant dispenser. While, to our knowledge, there is no evidence in the literature in support of *T. formicarius* attraction to the compounds comprising the anti-attractant, previous studies have reported catches of *Thanasimus dubius* in traps baited with eucalyptol (Munro et al., 2020),

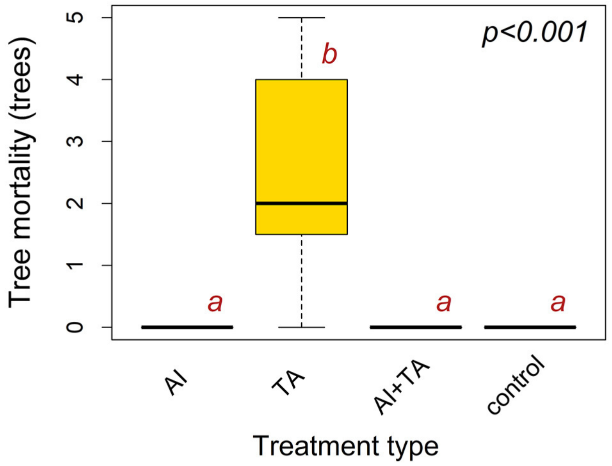

FIGURE 3
Boxplots showing tree mortality during the tree protection experiment, conducted in the Libavá military forest part of the study area, by treatment type. The y-axis represents the number of bark beetle-killed Norway spruce trees. Definitions: AI is *I. typographus* anti-attractant, TA is *T. formicarius* attractant, control designates the absence of treatment. The differences in tree mortality among treatment types were checked using the Kruskal-Wallis rank sum test. Pairwise comparisons were performed using Dunn's multiple comparison (*post-hoc*) test with Holm's adjustment of *p*-values for multiple comparisons. Compact letter display (lowercase letters "a", "b") indicates (in)significance in tree mortality between the treatment pairs at 0.05 significance level. If any given two treatment types do not share a common letter, the number of bark beetle killed trees significantly differs between them. The horizontal lines inside the boxplot correspond to the median tree mortality value. The box displays the interquartile range, which represents the middle 50% of the data. The error bars are the 95% confidence intervals.

a compound known to act as a repellent for *I. typographus* (Andersson et al., 2010; Binyameen et al., 2014), and a key component of our anti-attractant mixture. The results of our trapping experiment demonstrate the considerable potential of the combined use of an attractant for *T. formicarius* and anti-attractants in tree protection.

4.2 *T. formicarius* attractant and *I. typographus* caused spruce mortality

We recorded tree mortality only in trees baited with attractant for *T. formicarius* and trees in their proximity, which contradicts our expectations (Figure 3). The influence of *T. formicarius* on the population dynamics of *I. typographus* was reported to be substantial (Mills, 1985, 1986; Weslien, 1992; Weslien and Regnander, 1992), primarily owing to its considerable reproductive capacity (106–162 eggs per female) and its significant voracity both in the adult stage (consuming 0.86 to 2–3 adult *I. typographus* day^{-1}) (Weslien and Regnander, 1992; Faccoli and Stergulc, 2004) and during the larval stage (preying upon 44–57 larvae throughout its entire larval life) (Mills, 1985; Hérard and Mercadier, 1996; Dippel et al., 1997). Thus, we anticipated that the attractant of *T. formicarius* will not attract *I. typographus* and if any *I. typographus* lands on tree, it will be killed by the elevated numbers of *T. formicarius*, functioning as a predator. The mortality of individuals treated with attractant designed for *T. formicarius* was possibly caused by a synergistic effect of spruce primary attractants and the components of *T. formicarius* attractant (2-methyl-3-buten-2-ol, ipsenol, ipsdienol) on *I. typographus* colonization behavior. To the best of our knowledge, the previously published literature does not provide evidence for a spruce mortality increase in response to such a mixture of compounds. However, traps baited with *T. formicarius* attractant did not catch either of the beetles, supposedly due to the low population densities of the clerid beetle and potentially higher comparative attractiveness of the traps baited with both *I. typographus* pheromone and *T. formicarius* attractant. The absence of tree mortality observed among the control trees in our experiment may be attributed to the relatively low bark beetle population in the study area, notwithstanding the fact that it is sufficient to cause mortality among individuals treated with *T. formicarius* attractant.

We observed that trees treated with a combination of anti-attractant and *T. formicarius* attractant were not affected by the bark beetles (Figure 3). This outcome suggests that the repelling effect of the anti-attractant, composed of green leaf and non-host volatiles (1-hexanol, 1-octen-3-ol, 3-octanol, eucalyptol, trans-thujanol, and trans-conophthorin), could be strong enough to overwhelm the attractiveness of the compounds that are present in the *T. formicarius* attractant. The absence of variation in tree mortality rates among the control trees, trees treated with anti-attractants, or trees treated with a combination of anti-attractant and *T. formicarius* attractant, hinders a comprehensive understanding of the effects of the combined treatment. Results from trapping experiments suggest that the combined treatment could held the greatest potential for improving tree protection methods. To unravel the mechanisms underlying the efficacy of the combined treatment, further experiments employing pheromone traps would be required that are aimed at identification of the optimal composition of dispensers with compounds repelling *I. typographus* and attracting *T. formicarius*.

4.3 Limitations of tree protection experiment

The number of replications (7) in the tree protection experiment was largely constrained by the availability of suitable forest edges in the study area. We acknowledge that the number of replications used in this study may be perceived as relatively small. However, it is comparable to the number of replications used in similar tree protection experiments conducted in spruce stands in the field of chemical ecology of bark beetles, typically ranging from 10 to 25 (Christiansen and Krokene, 1999; Graves et al., 2008; Mageroy et al., 2020). Our results showed statistically significant differences in the number of bark beetle-killed trees among the treatment types, suggesting that the number of replications implemented can be deemed adequate. Considering the observed pattern of bark beetle attacks experienced exclusively by the individuals treated with *T. formicarius* attractant, we assume that increasing the number of replications would be unlikely to alter the statistical significance of our findings.

The second limitation of our tree protection experiment is the absence of a variant involving the attachment of *I. typographus* pheromone dispensers to the spruce trees. Application of this treatment would likely result in augmented numbers of bark beetle attacks on all treated trees, a pattern reported in previous studies, especially in forest edge conditions exposed to relatively high bark beetle pressure (Mulock and Christiansen, 1986; Weslien et al., 1989; Hübertz et al., 1991). The potential outcomes of using *I. typographus* pheromone dispensers are predictable and suggest significant infestations, including the possibility of widespread *I. typographus* proliferation throughout the entire stand, which could disrupt the homogeneous experimental conditions established for the rest of the treatments. Additionally, obtaining permission from the local authorities to apply such treatment would be challenging, if not impossible. Overall, implementing this variant correctly would require significant alterations to the experimental design, including a substantial increase in the spacing between experimental trees, which was unfeasible due to constrained availability of suitable forest edges in the study area.

We have made every effort to maintain homogeneous experimental conditions, employing the maximum number of replications feasible in our study area, and applying all permitted treatment types that would not potentially bias the outcome. We argue that the absence of tree mortality in all other groups, except for the individuals treated with *T. formicarius* attractant, could be explained by the luring effect of the *T. formicarius* attractant, coupled with host tree volatiles, on bark beetle behavior. Contrary to our expectations, this effect could overwhelm the anticipated predatory activity of the increased numbers of *T. formicarius* presumed to be lured by the attractant designed for this species. Individuals treated with attractant for *T. formicarius* seem to be more attractive for *I. typographus* than stressed untreated forest edge trees that escaped infestation.

4.4 Forest management applications

Our experiments demonstrate the potential to enhance the efficacy of anti-attractant treatments for tree protection by combining *I. typographus* anti-attractant dispensers with attractants designed for *T. formicarius*. Another implication of our findings for enhancing forest management practices suggests that attractant dispensers designed for *T. formicarius* should not be employed to boost predator numbers in the absence of simultaneously applied *I. typographus* anti-attractant, which repels bark beetles. The green leaf and non-host volatiles emitted by the anti-attractant act as deterrents for bark beetles (Zhang and Schlyter, 2003, 2010; Unelius et al., 2014). The deterrent effect could outweigh the attractive influence of the *T. formicarius* attractant combined with host tree volatiles on *I. typographus* aggregation behavior, as suggested this study. However, we contend that additional development and field testing of semiochemical mixtures are necessary to clarify the potential effects of the attractants designed for both beetle species, *I. typographus* anti-attractant, and host and non-host volatiles on the beetles' behavior. Specifically, for the enhancement of forest protection measures, it would be advantageous to investigate how the behavior of *I. typographus* and *T. formicarius* under field conditions modifies, with different proportions of constitutive compounds in admixtures, varying bark beetle population densities, and predator–prey ratios.

The application of combined dispensers may prove particularly effective in the later stages of bark beetle gradation, where higher population densities of bark beetle predators are anticipated (Weslien, 1994). This approach may be beneficial in localities near unmanaged areas, where we expect a higher population of bark beetle predators compared to managed stands (Weslien and Schroeder, 1999). Additionally, ThanasiWit®, a *T. formicarius* dispenser employed in our experiment, can be used for attracting the clerid beetles to localized areas of infestation and to wood stacks in order to diminish bark beetle populations, as well as to prevent the predator from being caught in pheromone traps. One possible direction for further development of an improved dispenser could involve using compounds that have not demonstrated any potential attraction to *I. typographus* but are attractive to *T. formicarius*. According to Zuhlke and Mueller (2008), chemical compounds comprising the pheromones of various bark beetle species, including those attacking broad-leaved trees, could potentially be used to attract *T. formicarius* to its prey, *I. typographus*, without simultaneously elevating the risk of bark beetle infestations in host trees.

5 Conclusions

Our trapping experiment has shown that traps baited with a combination of anti-attractant for *I. typographus* and attractant for *T. formicarius* did not catch any *I. typographus*. Concurrently, these traps caught the highest numbers of *T. formicarius* specimens. This synergistic combination indicates promising potential for enhancing tree protection measures. However, the observed mortality of Norway spruce, exclusively in trees treated with attractant dispensers designed for *T. formicarius*, highlights potential risks associated with such applications. This suggests that further investigation is necessary to optimize the composition and compound proportions of the combined dispenser.

Data availability statement

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

Author contributions

NK: Data curation, Formal analysis, Investigation, Software, Visualization, Writing—original draft, Writing—review & editing. JB: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Writing—original draft, Writing—review & editing. JH: Investigation, Visualization, Writing—original draft, Writing—review & editing. JK: Conceptualization, Data curation, Investigation, Methodology, Writing—review & editing. BD: Investigation, Visualization, Writing—review & editing. RJ: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Writing—original draft, Writing—review & editing.

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Conflict of interest

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Bark beetle detection method using electronic nose sensors. A possible improvement of early forest disturbance detection?

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Forest ecosystems are long-term exposed to dry periods in Europe, which leads to a significant loss of vitality and higher mortality, especially in coniferous forests. Identifying stress in the early stages when measures can be taken to protect the forest and living trees is crucial. Current detection methods are based on field surveys by forest workers or remote sensing methods to cover larger areas, which use changes in spectral reflectance of the forest canopy. In some cases, the attacked trees do not change their appearance, and based on calculations of vegetation indices from remote sensing data, the attack cannot be mapped. We present an innovative methodology based on non-optical analysis, namely identifying a group of volatile compounds and microclimate signs in forest stands that indicate stress factors in forest stands. An attacked tree by a bark beetle produces increased amounts of biogenic volatile organic compounds associated with defense, and the microclimate changes due to interrupted transpiration. In addition, the bark beetle uses the aggregation pheromone to attract more individuals and to attack the tree massively. In this study, we tested three electronic noses (Miniature Bosch sensor device with 25,419 samples, Sensory device for environmental applications with 193 samples, Handheld VOC Detector Tiger with 170 samples) in a freshly infested spruce stand. The measurement was conducted at ground level with the help of a human operator and was repeated six times to verify the detection capability of the electronic noses. To verify the capability of electronic noses to predict tree infestation, we used machine learning Random Forest. The results demonstrated that electronic noses can detect bark beetle infestation start (within 1 week of the first attack). The Miniature Bosch sensor device achieved the highest accuracy with a value of 95%, in distinguishing forest sections that are healthy and infested; the second most accurate electronic nose is the Sensory device for environmental applications, with an accuracy of 89%. Our proposed methodology could be used to detect bark beetle presence.

KEYWORDS

electronic noses, forest disturbances, odor mapping, stress detection, bark beetle, early detection

1 Introduction

Coniferous forest stands have been grappling with intense stress recently, including fires, drought, windstorms, and insect pests. The leading causes of damage are biotic disturbances, especially insect infestation. The resistance of the forest stand to stress is directly related to its vitality and the level of insect attack. Among the most significant pests are insects that feed on the bast fibers of living trees, such as the Eurasian spruce bark beetle (*Ips typographus* (L.)) (Hais et al., 2016; Fernandez-Carrillo et al., 2020; Bárta et al., 2021). The emergence of bark beetles is often associated with increased temperature and drought, and the intensity of infestation depends on the complex interaction between the bark beetle, climatic conditions, forest conditions, and extreme natural events such as storms and fires (Schelhaas et al., 2003; Jönsson et al., 2012; Marini et al., 2017; Hlásny et al., 2019; Patacca et al., 2023; Netherer et al., 2024).

Stressed trees under bark beetle attack secrete several times more biogenic volatile organic substances (mostly α-pinene, camphene, myrcene) than under resting conditions (Ghimire et al., 2016; Jaakkola et al., 2022; Hakola et al., 2023; Lehmanski et al., 2023; Netherer et al., 2024). In the event of an attack by the Eurasian spruce bark beetle, as part of the chemical communication between these individuals, an aggregation pheromone is secreted, composed of 2-methyl-3-buten-2-ol and cis-verbenol (Birgersson et al., 1984; Netherer et al., 2021; Moliterno et al., 2023). In addition to chemical changes, tree damage by bark beetles brings other non-optical symptoms, such as a change in the microclimate. Forest understories locally influence the microclimate, and compared to open areas, provide lower average temperatures and higher air humidity (Geiger et al., 1995; Morecroft et al., 1998; Aussenac, 2000; Kašpar et al., 2021). A healthy tree has transpiration 200–300 liters of water per day; in case of damage, the ability to transpire is impaired or completely stopped. In the case of reduced transpiration capacity of damaged trees, the temperature buffering decreases, and thus, leads to a higher temperature and lower air humidity (Kopáček et al., 2020).

Monitoring natural disturbances in a forest ecosystem is a key element for forest management and in forest-protected areas like national parks for taking precautionary measures (tourism safety, fire, etc.). Early identification of stress conditions of forest stands can prevent significant economic and ecological damage. A very accurate but time-consuming method is a field inspection by forest experts; during a field visit, each tree can be carefully mapped, and it is also possible to record the initial state of infestation based on observing the first beetle entrance holes, boring dust on the trunk, and resin flows (Birgersson et al., 1984; Abdullah et al., 2019a; Bárta et al., 2022; Bozzini et al., 2024). This technique cannot be used to check extensive forest stands or hard-to-reach locations and requires a very close visual examination of each tree to identify symptoms of infestation. For larger areas, remote sensing methods are used, and a suitable carrier (satellite, aircraft, drone) is chosen according to the required resolution and characteristics of the study area. Health status can be observed based on the different spectral reflectance of vegetation, for example, Piecewise index PI B(710 + 738–522), Greenness GI, NDVI GREEN/NIR, Normalized difference photochemical reflectance index PRI, and ANCB index (Gitelson and Merzlyak, 1997; Zarco-Tejada et al., 2001; Le Maire et al., 2004; Zhang et al., 2018; Bárta et al., 2022). Canopy change methods monitoring deviations in spectral reflectance cannot identify early attacks; approximately only 40% of attacked trees change their spectral expression in the crown, and if a change occurs after 6–10 weeks, the red or gray attack is not timely enough (Kautz et al., 2023; Bozzini et al., 2024). True early methods deal with bark beetle pheromone detection based on a specially trained dog, which can upwind orientation to the pheromone plume from single trees under attack up to 150 m (Johansson et al., 2019) and, compared to visual human detection, achieves significantly better results in identifying an early attack (Vošvrdová et al., 2023).

Non-optical mapping, namely the monitoring of a group of volatile compounds and microclimate cues, appears to be a promising method. New sensors utilizing microchip architecture and electronic nose technology have recently emerged. These sensors can convert the concentration of chemical substances into electrical signals, which are then transformed into digital numbers. Over the past decade, there has been a significant increase of interest for using electronic sensors across various applications (food product quality control, air quality monitoring, disease diagnosis, and environmental monitoring). This trend reflects the growing awareness of the possibilities electronic sensors offer in ensuring accurate, reliable, and efficient data collection, which significantly benefits research, industry, and public health (Pobkrut et al., 2016; Cellini et al., 2017; Xing et al., 2019; Tiele et al., 2020; Fuentes et al., 2021). Due to continuous technological advances, weight reduction, and better integration with other devices, electronic sensors are becoming capable of greater detection accuracy. Electronic sensors are evaluated based on sensitivity given in units of ppb (parts per billion). These criteria are key in assessing the ability of sensors to provide reliable and accurate measurements in various environments and applications (Deshmukh et al., 2015; Ye et al., 2021).

In this research, we followed up on our previous study (Hüttnerová et al., 2023), where we demonstrated the detectability of a substance indicating the presence or proximity of infested and dead trees with an electronic nose Sniffer4D. Data collection occurred on three different height levels (ground, 60 m, 80 m); the best results were achieved by a wide-range Hydrogen Chloride (HCL) Sensing Module at ground level. No correlation between stress compounds and distance from infested trees was recorded above the forest canopy, which can be caused by higher airflow divergence in the area above the forest canopy. The study by Vošvrdová et al. (2023), which focuses on identifying synthetic semiochemicals in the forest by a specially trained dog, confirms the possibility of ground detectability. In this study, we focus on ground-taken data for benchmarking several sensor responses to the presence of attacked trees.

We hypothesized that specific chemical compounds present in the forest stand during a stress event (bark beetle attack) would be detectable by electronic nose. We assumed that trees that are infested by bark beetles would produce more volatile organic compounds, and the stress of trees would lead to a change in their temperature and humidity profile. At the same time, aggregation pheromones, which bark beetles use to communicate, will be present in the forest. In the case of the ability of electronic noses to detect an increased amount of volatile organic substances or the presence of an aggregation pheromone or changes in the temperature or humidity profile, we will be able to identify an attack in the early stage of infestation and thus prevent enormous economic and ecological losses. The main goals of this research were (1) to evaluate the ability of electronic sensors to detect bark beetle infestation (2) to determine which factors influenced the most often measured increased values near attacked trees. We assessed the measured values with electronic noses in a spruce stand attacked by the Eurasian spruce bark beetle and in a healthy stand using a machine learning algorithm.

Following our previous study and the ever-expanding insect pests destroying valuable forest ecosystems, we asked the following specific research questions:

1. Can the tested electronic sensors capture specific substances that identify stress in the forest ecosystem?
2. Which electronic nose achieved the most accurate results based on the machine learning evaluation?
3. What model variables were the most significant predictors for stress detection in the forest stand?

2 Materials and methods

2.1 Study area

The study area of 1.42 ha was located 35 km south-east of Prague; the forest stands to fall into category 32d – Forests with aiming forestry research and forestry education; the area was represented by monoculture Norway spruce [*Picea abies* (L.) Karst.] in age class 5 (81–100 years). Data collection took place on September 4th, 2023 (11:10 a.m. – 1:10 p.m.); the outside temperature was around 19°C, and humidity 46%. The mean annual temperature of the study area in 2023 was 10.4°C; the long-term average temperatures in the Central Bohemian Region and Prague is 9.3°C. The maximum temperature, 19.0°C, was recorded in July, and the minimum temperature, −0.6°C, was recorded in January. The mean annual precipitation in 2023 was 607 mm, whereas the long-term average in the region is 583 mm (Czech Hydrometeorological Institute, 2023).

Data collection was performed six times, and the measurement trajectory was kept identical for a possible evaluation of the sensitivity of the sensors (Figure 1). The trajectory was chosen in the shape of figure eight to evaluate the sensors' response to the occurrence of stress factors in the forest ecosystem. The research area was one with a size of 1.42 ha; the invaded area was approximately 80 × 50 m. Each collection took approximately 15–20 min (6 repetitions) when the operator slowly walked through the study area and simultaneously collected data by all electronic sensors at ground level. Our approach is not based on detecting individual trees but on detecting an area (area-based method).

2.2 Materials

Three electronic noses were used as part of data collection to verify their suitability for stress mapping in forest ecosystems. Two sensors are commercially available on the regular market, and one sensor was specifically designed for environmental measurements. Therefore, it is described in detail below.

2.2.1 Miniature Bosch sensor device © Bosch Sensortec GmbH 2023 (Bosch)

This sensor was the BME688 AI miniature environmental sensing device, which can detect volatile organic compounds, volatile sulfur compounds and other gases like carbon monoxide and hydrogen in the ppb resolution. The sensor includes a unit for measuring temperature in the range of −40-85°C with sensitivity ±1°C, a humidity sensor in the range of 0–100% with sensitivity ±3%, a unit for measuring pressure in the range of 300–1,100 hPa with sensitivity ±1 hPa, and a Metal-Oxide Semiconductor (MOX) gas sensor. The manufacturer does not directly state the range and sensitivity of the MOX sensor unit but based on the general characteristics of MOX sensors and the focus on the Air Quality Index (AQI), which Bosch company describes in the datasheet, it can be estimated that the sensor can detect VOCs in the tens of ppb.

2.2.2 Sensory device for environmental applications (SDEA)

The next device for monitoring the bark beetle is designed as a mobile unit powered by an accumulator and containing two basic parts—a source part that is fixed in the lower part of the carrier and a sensor part located on a support rod at a height of about 60 cm above the head of the experimenter (Figure 2).

The source unit and the sensor part are connected to each other by a cable, enabling both power supply to the sensor part and communication between the two units. The source unit contains the accumulator, the circuits for charging the accumulator, and the circuits for monitoring the status of the source unit. The sensor unit is in a removable cover and contains sensors in a tunnel formed by an aluminum profile. Air enters the tunnel through a dust filter and is extracted by a fan on the opposite side of the aluminum profile. Holes are made in the profile, in which sensors for individual types of gas are stored, and their outputs are processed by the control unit located on the handle on the side of the profile.

Individual sensors include:

- NO_2 electrochemical sensor, range 20 ppm, sensitivity in the order of ppb
- H_2S electrochemical sensor, range 400 ppm, sensitivity in the order of ppb
- VOC photoionization sensor, range 40 ppm, sensitivity in the order of ppb (isobutylene)
- NO electrochemical sensor, range 20 ppm, sensitivity in the order of ppb
- SO_2 electrochemical sensor, range 50 ppm, sensitivity in the order of ppb
- CO electrochemical sensor, range 500 ppm, sensitivity in the order of ppb
- CO_2 IR/thermopile sensor, range 5,000 ppm, sensitivity 1 ppm
- O_3 electrochemical sensor, range 20 ppm, sensitivity in the order of ppb

The sensors, together with the electronics, are placed on the sensor board and their measuring part extends into the space of the measuring tunnel. The exception is the temperature and humidity sensor, which is located on the side (against the control unit holder, not visible in the picture). The entire sensor unit is controlled by a Raspberry microcomputer, which takes analog values from individual sensors, converts them into numerical data, and converts these data according to set conversion coefficients and data in measurable units, namely ppb or ppm. At the same time, it controls the work of the sensors, because some sensors have time delays between individual measurements, or it is necessary to turn them on and off to save batteries.

The last part of the system is a regular notepad, which communicates with the Raspberry control unit using the Bluetooth interface and is used to control the sensor unit, display, and save the measured values. The statuses of the individual processes are displayed in the informative section at the top left. As long as the source is

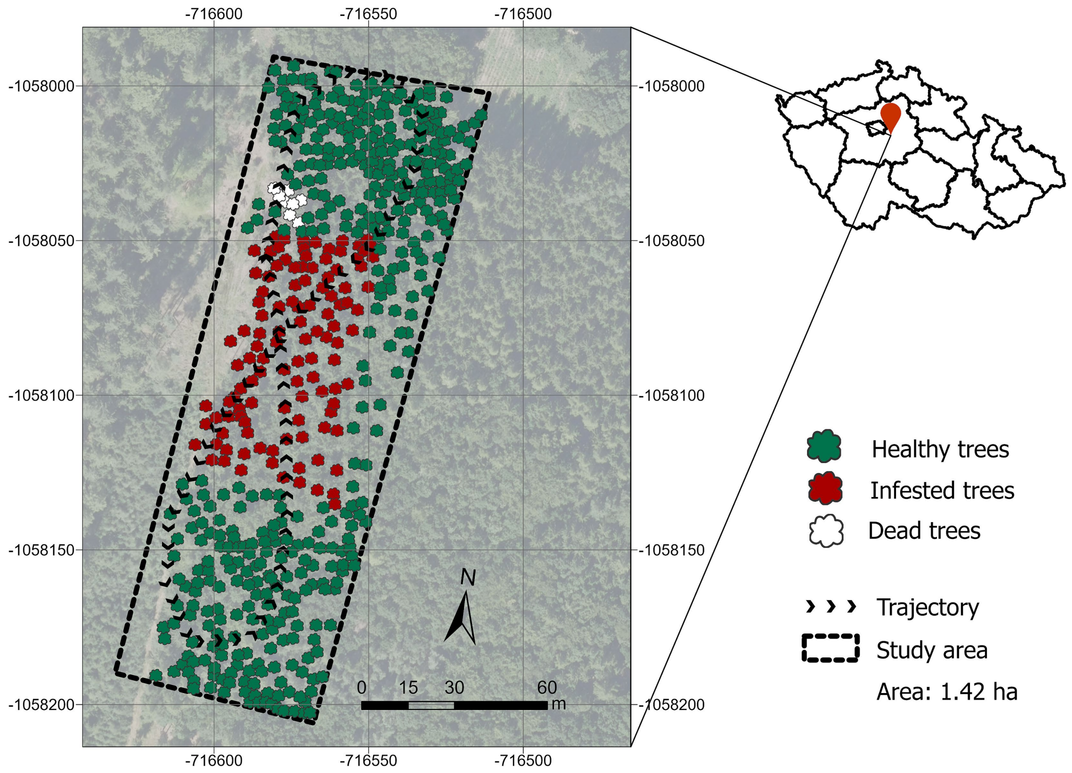


FIGURE 1
The study area 35 km away from Prague (Prague West district, Czechia) displays a forest stand, which was heavily infested by bark beetles (infested trees are marked red, healthy ones in green, and dead trees in white). Black arrows mark the trajectory of data collection.

turned off or the measuring application is not running, which means that Bluetooth communication is not turned on, the status of the source is red and cannot be measured. After establishing a connection with the measuring device, the status wheel turns green, which means measuring is possible. The correct GPS function is indicated separately, as the location data is part of the recording.

Furthermore, the upper part contains environmental data on the state of the air in the measured location (temperature, humidity, and pressure) and the state of the measuring device, especially the temperature of the individual measuring modules. These are important for correcting the measured values of electrochemical sensors, as they are highly temperature dependent. Battery status and airflow rate in the measuring unit are on the far right of the display.

The last icon on the display is a green button that sets one of three values:

- Measurement initialization (from the measurement status off),
- Turn on the measurement (from the measurement initialization state),
- Turn off the measurement (from the measurement status on).

Simultaneously with online measurement, a set of measured data is written at regular intervals, which has the following structure (in .csv format separated by semi-colons):

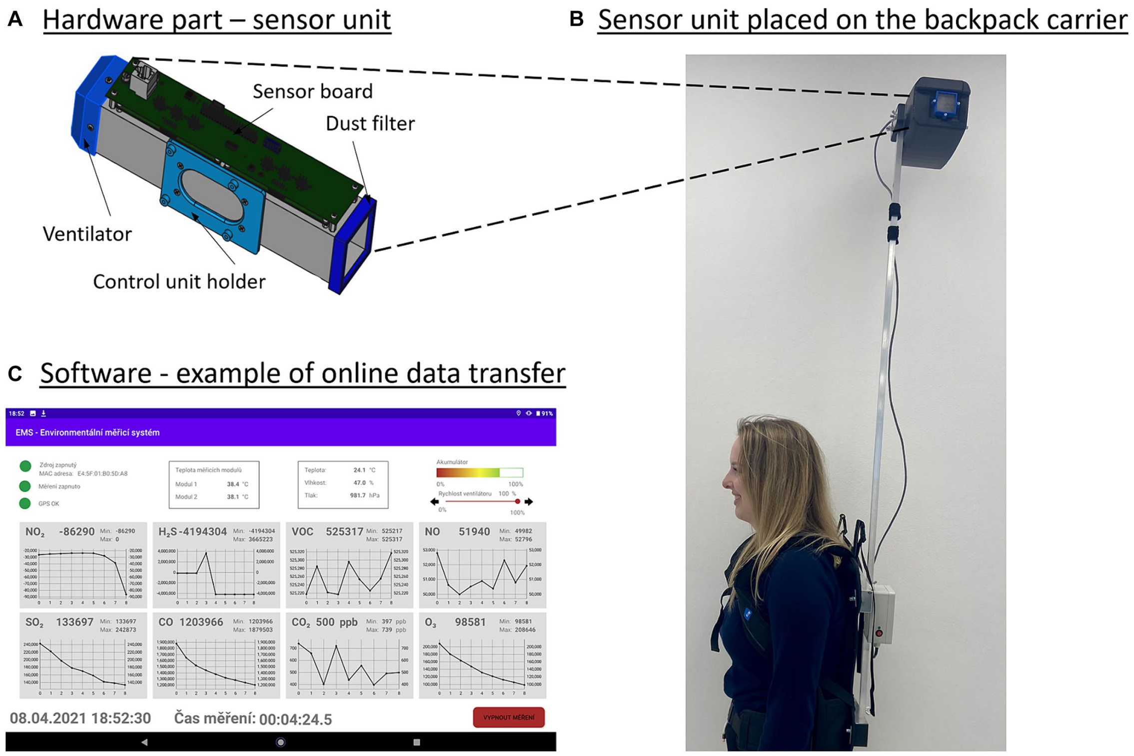
> <Date and Time>; <GPS: number of satellites>; <GPS: accuracy [m]>; <Latitude [°]>; <Longitude [°]>; <Fan speed [%]>; <NO_2 [ppb]>; <H_2S [ppb]>; <VOC [ppb]>; <NO [ppb]>; <SO_2 [ppb]>; <CO [ppb]>; <CO_2 [ppb]>; <O_3 [ppb]>; <Temperature of module 1 [°]>; <Temperature of module 2 [°]>; <Battery status [%]>; <Outside temperature [°]>; <Pressure [hPa]>; <Relative Humidity [%]>.

2.2.3 Handheld VOC Detector Tiger © 2024 Ion Science UK. Ion Science is registered trademark on Ion Science Ltd. (Tiger)

Detector Tiger has photoionization technology (PID) for detecting volatile organic compounds. The detector can display measured values very quickly and accurately. It is suitable for screening and locating, for example, leaking dangerous gases. The sensor system has a response time of 2 s and can detect up to 750 volatile organic substances. The range of the sensor is 0–20,000 ppm with sensitivity 1 ppb. Figure 3 shows the Miniature Bosch sensor device and Handheld VOC Detector Tiger.

2.3 Data processing

First, we evaluated the health status of the trees in the study area; the trees were classified into three categories (dead, infested, and healthy). The assessment was carried out in two ways; first, based on a field investigation, the bark of the trees was inspected with a focus on locating the bark beetle entrance holes, boring dust on the trunk, and resin flows. Natural infestation (without chemical baiting) of coniferous trees was within a week of the first attack by the bark beetle,



Zdroj zapnutý
MAC adresa: E4:5F:01:B0:5D:A8
Měření zapnuto
GPS OK

Teplota měřicích modulů
Modul 1 38.4 °C
Modul 2 38.1 °C

Teplota: 24.1 °C
Vlhkost: 47.0 %
Tlak: 981.7 hPa

Akumulátor 0% 100%
Rychlost ventilátoru 100 % 0% 100%

NO_2 -86290 Min: -86290 Max: 0
H_2S -4194304 Min: -4194304 Max: 3665223
VOC 525317 Min: 525217 Max: 525317
NO 51940 Min: 49982 Max: 52796
SO_2 133697 Min: 133697 Max: 242873
CO 1203966 Min: 1203966 Max: 1879503
CO_2 500 ppb Min: 397 ppb Max: 739 ppb
O_3 98581 Min: 98581 Max: 208646

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FIGURE 2
Comprehensive overview of the Sensory device for environmental applications: **(A)** sensor unit assembly consisting of the ventilator, control unit holder, dust filter, and sensor board; **(B)** the main sensory part is located 60 cm above the operator's head; **(C)** software part: layout of data on the notepad screen. The notepad screen is divided into four parts: an informative part at the top left (three items marked with status—source, measurement, and GPS), measured values at the top of the screen + fan speed, a part containing eight graphs for estimated quantities, and a status line at the bottom of the screen.

and the study area was selected based on consultation with Field technician, who performs regular checks of the status of trees. The second method for determining the state of health was based on optical data obtained from a UAV; the area was captured with a DJI Phantom4 Pro multirotor with an RGB camera at the height of 90 m, and the images were processed using the Structure from Motion method. We visually evaluated the spectral characteristics of the tree canopy from the orthophoto mosaic (Figure 4). Spruce trees in the study area were categorized [healthy, attacked, dead] and stored as a point layer.

The measurement results from the sensors were exported in .csv format, we used the R Studio and ArcGIS Pro software's for all data analysis. Each electronic nose measurement dataset was annotated by two labels (infested forest, healthy forest). These labels were assigned based on the position of the measurement within the forest. Using the *Select by Location* functionality, measurement positions in the attacked stand were selected and assigned the "Infested" attribute. The procedure was repeated for measurement positions in the healthy part of the forest, and the "Healthy" attribute was assigned. Due to the low number of dead trees, they were not considered in the machine learning models.

We train machine learning algorithm Random Forest (Breiman, 2001), which is widely used for classification tasks in environmental studies. The input data for the model does not have to be standardized, which ensures a more straightforward interpretation of the results (Müller et al., 2022). We used the R package *randomForest* (Liaw and Wiener, 2002), *caret* (Kuhn, 2008), *kernlab* (Karatzoglou et al., 2004), and *boot* (Davison and Hinkley, 1997) to create three binary classifications for each dataset measured by the electronic nose (Sensory device for environmental applications, Handheld VOC Detector Tiger, Miniature Bosch sensor device). The dataset was split 80:20 into training and validation sets. We used the resampling method *repeatedcv.*, which is used to set K-fold cross-validation, and for the *repeats* argument, we chose the value three, and this argument controls the number of repetitions; the K parameter is regulated by the *number* argument, which we chose 20 (Kuhn, 2008). The goal was to find out which electronic nose has the most sensitive predictive ability and what variables affect the values of electronic nose sensors. We selected the explanatory variables based on the technical equipment of the sensor. The machine learning model was based on 25,419 samples for the Miniature Bosch sensor device, 193 samples for the Sensory device for environmental applications, and 170 samples for the Handheld VOC Detector Tiger. The number of samples varied depending on the time resolution of the electronic nose for recording values; data collection was carried out simultaneously by all three electronic sensors along the same trajectory.

2.3.1 Miniature Bosch sensor device

We used input explanatory variables of outside temperature (*Temperature*), pressure (*Pressure*), relative humidity (*Humidity*), and data collection number (*Data_col*). The Bosch device provides one information about chemical measurement, and that is Resistance. Gassenzor (*Gas*), which we used for the dependent variable in the model. We used the following model formula:

$$rf\_classifier < -train\begin{pmatrix} Location \sim Temperature + Pressure \\ + Humidity + Gas + Data\_col, data \\ = trainData, method = "rf," trControl \\ = train.control \end{pmatrix}$$

FIGURE 3
On the left side is Handheld VOC Detector Tiger, in the upper right in the red rectangle is Miniature Bosch sensor device.

2.3.2 Sensory device for environmental applications

For Sensory devices for environmental applications, we used input explanatory variables such as outside temperature (*Outside_tem*), temperature of module 1 (*TEM_mod1*), temperature of module 2 (*TEM_mod2*), pressure (*Pressure*), relative humidity (*Humidity*), and data collection number (*Data_col*). This electronic nose is equipped with several cross-sensitive sensors which can record values for all sensors simultaneously, so the explanatory variables of the gases were NO_2, H_2S, VOC, NO, SO_2, CO, CO_2, O_3. We used the following model formula:

$$rf\_classifier < -train\begin{pmatrix} Location \sim NO_2 + H_2S + VOC + NO \\ + SO_2 + CO + CO_2 + O_3 + TEM\_mod1 \\ + TEM\_mod2 + Outside\_tem + Pressure \\ + Humidity + Data\_col, data = trainData, \\ method = "rf," trControl = train.control \end{pmatrix}$$

2.3.3 Handheld VOC Detector Tiger

This sensor device is not equipped with a temperature, humidity, and pressure monitoring unit. The Tiger detector contains a gas library primarily focused on safety monitoring; within the software settings, measuring and recording information on one compound at a time is possible. We collected data for pinene in the first and second

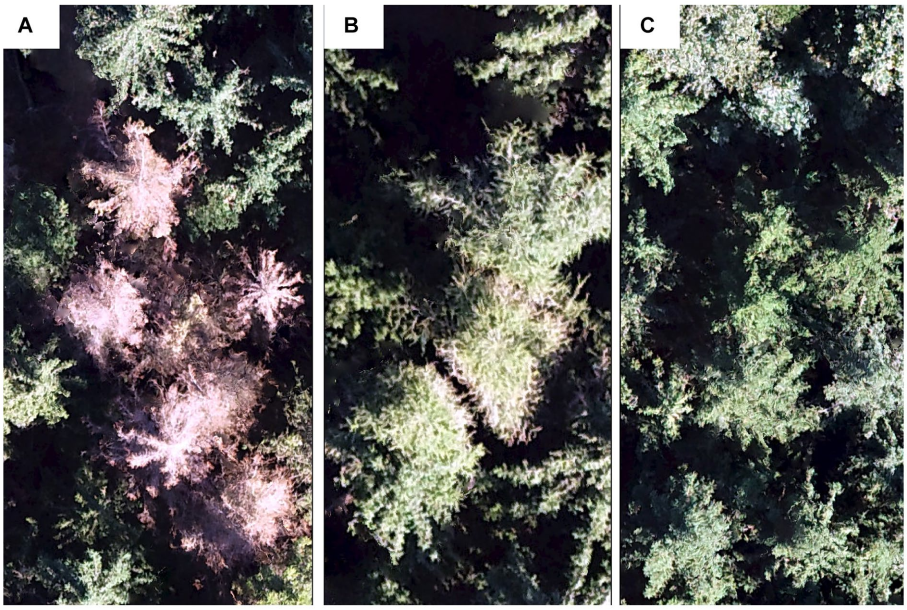

FIGURE 4
An example of the health status of the trees on the orthomosaic: **(A)** dead trees, **(B)** infested trees, **(C)** healthy trees.

measurements, methyl-butanol in the third, camphene in the fourth measurement, carene in the fifth, and pinene again in the sixth measurement. For evaluation we used explanatory variables of gas response (*Gas*), and data collection number (*Data_col*). We used the following model formula:

$$rf\_classifier <- train\left(\begin{matrix} Location \sim Gas, data = trainData, \\ method = "rf", trControl = train.control \end{matrix}\right).$$

3 Results

First, the results achieved for individual electronic noses will be described from the highest predictive ability of the models to the lowest. Then, the importance of the variables will be presented.

3.1 Miniature Bosch sensor device

The best model is selected automatically by the random forest algorithm, based on the highest accuracy, the outputs also provide set of best solutions based on the amount of variables included in the model (mtry), and kappa accuracy which is another way of measuring the performance especially in imbalanced datasets (Cohen, 1960). The results show the same accuracy for all the combinations of variables, mean the simpler model (the one with two variables) might be easier for future practical applicability (Table 1).

The Confusion Matrix is used to evaluate the performance of the classification model; it shows how often the actual classes were confused with the predicted ones. In the prediction part of the testing, accuracy 0.95 was achieved with a Kappa value 0.89. The sensitivity (true positive rate) was 0.97, and the specificity (true negative rate) was 0.91. The Confusion Matrix is used to evaluate the performance of the classification model; it shows how often the actual classes were confused with the predicted ones (Table 2).

3.2 Sensory device for environmental applications

The best model is selected automatically by the random forest algorithm, based on the highest accuracy. The results in Table 3 show the highest accuracy (0.89) for the combination with eight variables (mtry).

In the prediction part of the testing, accuracy 0.93 was achieved with a Kappa value 0.84. The sensitivity (true positive rate) was 1.00, and the specificity (true negative rate) was 0.81. Detailed confusion matrix values for the Sensory device for environmental applications are shown in Table 4.

TABLE 1 Results for different amount of random variables for the Miniature Bosch sensor device.

mtry	Accuracy	Kappa
2	0.95	0.88
3	0.95	0.89
5	0.95	0.88

3.3 Handheld VOC Detector Tiger

The tuning parameter "mtry" was held constant at a value of 2 (Table 5). The third of the sensors Handheld VOC Detector Tiger, received Kappa 0.16, and its predictive ability for forest stress detection was the lowest.

In the prediction part of the testing, accuracy 0.65 was achieved with a Kappa value 0.21. The sensitivity (true positive rate) was 0.85, and the specificity (true negative rate) was 0.35. Detailed confusion matrix values for the Handheld VOC Detector Tiger are shown in Table 6.

The importance of the variables for all tested electronic noses is shown in Figure 5. It was determined by the varImp function from the R software caret package, which provides information about the importance of variables for machine learning algorithms. The importance score of 100 indicates that the variable affects the model predictions most, while the variable with an importance score of 0 is irrelevant to the model's predictive ability, i.e., the least.

For the Miniature Bosch sensor device, the relative humidity sensor (100.00) had the most important influence on the predictive ability to detect infestation or healthy forest, followed by the pressure sensor (98.12) and temperature sensor (57.97). In the case of infested trees, it reduces the transpiration flow in the tree; with less water capacity, the trees cannot regulate the temperature profile and thus can lead to overheats. The reduction of transpiration close to the tree affects the air humidity and the pressure in the environment (slight increase); dry air is denser than humid air. A sensor unit measuring gases had a moderate effect on the model, and by 22.86. The variable "Data collection" did not affect the model (0.00), which means that the model performed very well for all data collections. The SDEA, which also achieved a very high predictive ability, had the most significant variable, the pressure-sensitive sensor (100.00), followed by the chemical sensor cross-sensitive to NO_2 gas (11.17). The cross-sensitive sensor to CO_2 gas had an effect of 9.92 on the model, and the sensor sensitive to H_2S (5.34). The outdoor temperature slightly affected the predictive ability, namely 3.73 and the humidity sensor (1.75). Measurement time (Data Collection variable) also did not affect the model, and predictive ability was achieved for the entire measurement. The Handheld VOC Detector

TABLE 2 Confusion matrix values for the Miniature Bosch sensor device.

Prediction	Healthy	Infested
Healthy	4,017	194
Infested	121	2034

TABLE 3 Results for different amount of random variables for the Sensory device for environmental applications.

mtry	Accuracy	Kappa
2	0.84	0.67
8	0.89	0.77
14	0.88	0.75

TABLE 4 Confusion matrix values for the Sensory device for environmental applications.

Prediction	Healthy	Infested
Healthy	27	3
Infested	0	13

TABLE 5 Results for the Handheld VOC Detector Tiger.

mtry	Accuracy	Kappa
2	0.60	0.16

TABLE 6 Confusion matrix values for the Handheld VOC Detector Tiger.

Prediction	Healthy	Infested
Healthy	22	11
Infested	4	6

Tiger did not achieve a good prediction ability for identifying stress in the forest ecosystem; based on the comparison of the importance of the variables, the gas sensor achieved 100.00, and the variable "Data collection" was also 0.00. The complete variable importance results for all three electronic noses are shown in Appendix.

4 Discussion

Based on our research findings, we can answer the scientific questions posed. We conclude that electronic noses can detect changes in volatile compounds and microclimate cues after bark beetle infestation. The Miniature Bosch sensor device achieved the highest accuracy in distinguishing forest sections that are healthy and infested (more addressed in Section 4.2 Electronic noses), and a more significant indicator of infestation was relative humidity, temperature, and pressure sensors (more discussed in Section 4.3 Influence of variables from statistical evaluation).

The presented methodology is a potential solution for the early identification of stress in forest stands, focusing on fresh attacks till 1 week old caused by bark beetle infestation. Early detection is essential for minimizing the spread of bark beetles to surrounding trees and thus reducing ecological and economic loss. In the case of late detection of the infestation, the bark beetle will spread to other trees, and from the point of view of safety and preventing further spread, the best solution is to cut down the trees and take them safely away from the forest stands. The financial costs must be allocated to the cutting and restoring forest stands. On small-scale clearings created because of natural disturbances, it is possible to reforest by natural regeneration if there are mature forest stands in the surrounding stands; in the case of large-scale sites, the natural distribution of sowing may be insufficient, and it is necessary to use artificial afforestation.

4.1 Detection methods

Conventional methods of infestation detection are field visits by forest experts and marking infested trees based on visual inspection; this method is very time and physically demanding (Stadelmann et al., 2013; Leverkus et al., 2021; Bárta et al., 2022). Remote Sensing methods can detect the deteriorated condition of the stand based on the change in the spectral reflectance of tree needles. Still, it is not possible to detect early infestation with this method. In the case of an attacked tree, the spectral reflectance of the canopy will be changed after 6–10 weeks, and only approximately 40% of the attacked trees show crown degradation; these findings do not confirm the potential of satellite or aerial detection systems (Kautz et al., 2023). Stress detection based on the analysis of crowns from a remote sensing image is challenging; the most accurate results are achieved at the end of the growing season (Latifi et al., 2018; Bárta et al., 2021; Huo et al., 2021). When using single spectral channels, the quality is insufficient; better results are achieved when using several spectral channels and calculating the vegetation index (Kautz et al., 2024). The authors of several studies argue about the importance of using the red-edge and NIR band (Abdullah et al., 2019a,b; Minařík et al., 2020; Hellwig et al., 2021; Trubin et al., 2023).

The scientific community has been mainly concerned with mapping and analyzing the healthy status of forests in the optical and near-infrared bands in the last decade. Analysis of chemical substances can bring new information about natural disturbances and help with early detection. In the case of monitoring bark beetle infestation of spruce stands, it is possible to focus on odor mapping; on the one hand, the bark beetle uses an aggregation pheromone to communicate with its individuals; still, the presence of pheromones in the forest is below the mark of the sensitivity and selectivity of electronic sensors; on the other hand it is possible to map biogenic volatile organic substances, which are secreted from the bark of trees; in the case of stress events, these substances are secreted in several times larger quantities. Therefore, chemical mapping could provide a very effective source of information about the attack, even at an early stage. We proved the detection ability of early infestation by electronic noses up to 1 week from the first attack. Early attack can be detected using specially trained snifferdogs, which can detect synthetic semiochemicals (identical to the species-specific major pheromone components of Eurasian spruce bark beetle) (Johansson et al., 2019; Vošvrdová et al., 2023). Chemical mapping is already conventionally used in security and industry, e.g., for detecting the leakage of dangerous gases or mapping air quality in cities, and several studies have already appeared in agriculture that intend to detect crop pests (Zhou and Wang, 2011; Abdullah et al., 2018; Arroyo et al., 2020; Rahman et al., 2020; Fuentes et al., 2021; Sudama et al., 2022).

4.2 Electronic noses

Of the tested electronic noses, the Miniature Bosch sensor device achieved the best capabilities, followed by a Sensory device for environmental applications. The Miniature Bosch sensor device, with a very favorable price, could be used to create a more comprehensive network of stationary measurements in spruce stands for early identification of stress factors. So, it would perform a function like the detection sensors for triggering the smoke detection alarm. The sensors could send the values to the cloud in real-time and display them on the map portal. We recommend and will further use a Sensory device for environmental applications to map local areas with a risk of a bark beetle outbreak due to its software equipment, which can show measurement results in real-time and has GPS. A possible interaction here for both mentioned electronic noses is with a drone platform, where a larger area of the forest ecosystem could be explored; the detection capability above the forest canopy has not yet been proven, and a potential solution appears to be collecting data under the forest canopy with manual flight mode.

Paczkowski et al. (2021) research focused on testing the applicability of sensors GGS1330, GGS2330, and GGS5330 to detect aggregation pheromone and biogenic volatile organic compounds; the results show the potential use of this device to verify the detectability of alpha-pinene, which is the main component of biogenic volatile organic compounds from spruce stands when their sensor was able to capture different concentrations. The detection capability of elevated concentrations has not been demonstrated by UAV data collection above canopies. Similar results were also obtained by Hüttnerová et al. (2023), where the electronic nose Sniffer4D with DJI Matrice 600 Pro was verified for early detection of bark beetles; the ability of the electronic nose to detect increased concentrations of chemical substances was also not confirmed, but the

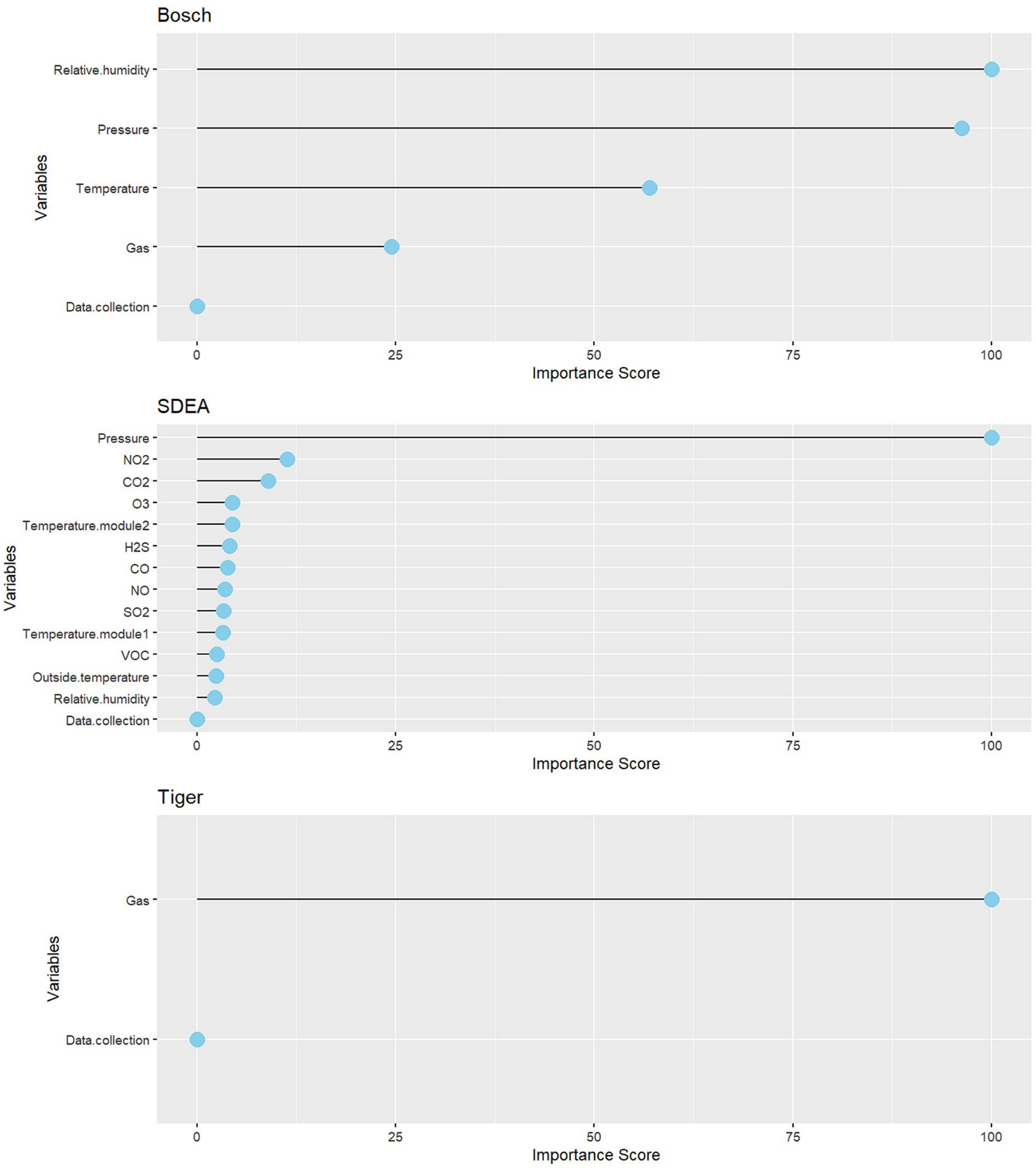

FIGURE 5
Overview of the importance of variables for sensor the Miniature Bosch sensor device, the Sensory device for environmental applications, the Handheld VOC Detector Tiger. The importance score of 100 indicates that the variable affects the model predictions most, while the variable with an importance score of 0 is irrelevant to the model's predictive ability, i.e., the least.

detection ability of the electronic nose was demonstrated under the crowns when they were data collected on the ground and the research find out that wide-range Hydrogen Chloride (HCL) sensor performed most reliably. The results of this study and the conclusions published by Paczkowski et al. (2021) and Hüttnerová et al. (2023) demonstrate the ability of electronic noses to detect specific substances present in forest stands when attacked by bark beetles.

4.3 Influence of variables from statistical evaluation

Our research proved the influence of variables that represented non-optical measurement. The relative humidity, temperature, and pressure sensors were a more significant indicator of infestation. Bark beetle is associated with symbiotic ophiostomatoid fungi that degrade spruce toxins, help to exhaust tree defenses, and thus lead to impaired conductive tissues and reduce transpiration (Netherer et al., 2021, 2024). Transpiration has a cooling effect on trees and an effect on air humidity; reduced transpiration leads to changes in local humidity, and this can increase air pressure. Differences in humidity are logical, but identifying differences between infected and healthy forests on rainy or cold cloudy days will not work because healthy trees do not transpire under these conditions.

In the case of infested trees, the transpiration flow is reduced in the tree; with less water capacity, the trees cannot regulate the temperature profile and thus can lead to overheats. The reduction of transpiration close to the tree affects the air humidity and the pressure in the environment (slight increase); dry air is denser than humid air. Another factor that can cause an increase in air temperature is canopy loss due to bark degradation (Anderegg et al., 2013; Wehner and Stednick, 2017).

Long-term microclimate changes were demonstrated in study Kopáček et al. (2020), and the changes were attributed to reducing or stopping tree transpiration of trees; there was an increase in daily mean air temperature (2 m above ground) of 1.6 and 0.5°C per year, and relative humidity was on the disturbed plots of land on average 4% lower. In our research, we noted much faster changes in microclimate clues, which suggests that the changes will manifest themselves significantly faster.

4.4 Limitations and future challenges

The presented methodology offers the possibility of early detection of bark beetle infestation based on identifying volatile compounds and microclimate cues found in the forest environment during bark beetle attack. With this methodology, it is not necessary to rely only on the spectral changes of the tree canopy to identify infestations. The limitation of this methodology is currently the size range of the area we can analyze. A time-efficient innovative method is the use of snifferdogs, which are twice as fast in detecting bark beetle infestation as human experts; the searching abilities of a dog are four times higher than human experts in the case of randomized plots because the dog can smell the pheromone at a greater distance (Vošvrdová et al., 2023).

For the possibilities of a large-scale detection system, it would be advisable to use the UAV platform and first try to verify the detection above the tree canopy, which has not been confirmed for the time being, or to test the possibilities of flying under the tree canopy. The battery capacity limits the data collection by the drone; the average flight time is about 30 min, depending on its load. A potential solution would also be creating a warning mapping system, where each electronic nose would be connected to a cloud system and send the currently measured values from the forest area in real-time. The system could serve as a suitable screening method of a preventive nature; for this purpose, the use of the Miniature Bosch sensor device would be appropriate in terms of its dimensions and financial availability.

In the case of global use, it would be necessary to recalibrate our model to local conditions; for example, a different climate can be predicted in Northern Europe than in the tested conditions in Czechia. However, we know from research which variables are significant for tested electronic noses and identify early bark beetle infestation.

5 Conclusion

In this study, we focused on evaluating three electronic noses for stress detection in forest stands, which were heavily attacked by bark beetles. This research aims at early detection of bark beetle infestation by novel technology of odor mapping as a more viable alternative to optical detection, which may be unreliable if the trees do not demonstrate signs of beetle attack. The possibility of detecting beetles by odor signals was clearly proven to be possible using dogs (Johansson et al., 2019; Vošvrdová et al., 2023). The predictive ability of electronic noses was evaluated based on machine learning model with two classes ("Healthy", "Infested"), we found that electronic noses can obtain sensitive information about stressors in forest ecosystems and thus help detect problematic areas for further analysis or action. The Miniature Bosch sensor device achieved the highest values for the ability to predict the infested trees, followed by Sensory devices for environmental applications. The presented methodology provides a very effective and fast solution for stress monitoring in forest stands, mainly for detecting bark beetle outbreaks. This monitoring could contribute to more effective mapping and prediction of the spread of infestations in forest ecosystems, thus radically minimizing the ecological and economic damages caused by insect pests.

Data availability statement

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

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any identifiable images or data included in this article.

Author contributions

TH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project

administration, Resources, Software, Visualization, Writing – original draft. PS: Conceptualization, Formal analysis, Investigation, Methodology, Software, Supervision, Writing – review & editing, Funding acquisition.

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Conflict of interest

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

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Appendix

TABLE A1 Output data from the Random Forest machine learning model used for evaluation of variables importance.

Variable importance	Bosch	SDEA	Tiger
Relative humidity	100.00	1.75	X
Pressure	98.12	100	X
Temperature	57.97	3.73	X
Gas	22.86	X	100
Data collection	0.00	0.00	0.00
NO_2	X	11.64	X
CO_2	X	9.92	X
H_2S	X	5.34	X
O_3	X	4.06	X
CO	X	4.89	X
NO	X	3.42	X
SO2	X	3.82	X
VOC	X	2.62	X
Temperature module 1	X	5.11	X
Temperature module 2	X	5.05	X

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Transgenic poplar for resistance against pest and pathogen attack in forests: an overview

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Forests are potential habitats for immense terrestrial ecosystems and aquatic biodiversity, performing an essential role in ecological preservation and regulation of climate. The anthropogenic pressures on the forests lead to forest loss, fragmentation and degradation. Requirements for sustainable methodologies for forest protection are of utmost priority under the climate change regime. Among forest trees, poplar trees (*Populus* L.) have attracted attention in global forestry as a promising material for improving the quality and quantity of urban landscapes. These plants provide wood, which can be utilized as raw resources for the paper industry and as a potential source of biofuel. However, several biotic stresses, such as attacks by pests and pathogens, severely affect poplar production and productivity. The improvement of *Populus* trees through conventional tree breeding methods is restricted due to their long-life cycles and the lack of suitable donors with resistance genes. *Populus* has been utilized as a model plant for studying gene functions due to its highly efficient genetic transformation capabilities. The present review will provide a comprehensive overview of pest and pathogen attacks on poplar, focusing on their infection mechanisms, transmission routes, and control strategies. Additionally, it will examine the most widely used genetic transformation methods (gene gun-mediated, *Agrobacterium tumefaciens*-mediated, protoplast transformation, micro-RNA mediated and micro-RNA clustered regularly interspaced short palindromic repeats (CRISPR)-associated (CRISPR-Cas) systems methods and RNA interference) for improving tolerance in poplar trees against pest and pathogens attack. Furthermore, it will delve into prospects, challenges, and recent advances in molecular biology tools and their safe application for genetic transformation to improve insect and pest resistance in poplar trees. Finally, the regeneration of transgenic poplar trees with enhanced resistance, developed through various genetic engineering techniques, is discussed.

KEYWORDS

forest protection, genetic transformation, protoplast transformation, *Agrobacterium*-mediated transformation, salicylic acid (SA), methyl jasmonate (MeJA)

1 Introduction

Forest trees have several pivotal roles, such as maintaining ecology, climate regulation, providing raw materials for the construction of buildings, greening roads, and being an energy source (Trumbore et al., 2015). Among these forest trees, poplar (*Populus* spp.) (known as 'the people's tree') is one of the most widespread trees in the world (Xi et al., 2021; Yevtushenko and Misra, 2019). The poplars are essential for maintaining the world's ecological balance and socio-economic wellbeing (Häggman et al., 2013). The first use of the poplar cultivar started in 1700–1720 when *Populus nigra* 'Italica' (*P. nigra, Lombardy nigra*) was used in Italy, Europe and North America. There was a rapidly increased demand for poplar plants after World War II when Europe was devastated by the lack of readily available wood for construction and fuel. Consequently, the domestication and cultivation of the genus poplar started in Europe by introducing eastern cottonwood (*Populous deltoides*) and followed by hybrids black poplar (*P. nigra*) (a hybrid of P. ×*canadensis*) for fulfilling the increased demands for woods (Stanton et al., 2009).

Poplar trees (family *Salicaceae*) are tall, deciduous, dioecious, paleopolyploids, or ancient polyploids, naturally diverse, fast-growing and widely distributed globally (Lubrano, 1992), especially in temperate, sub-temperate and sub-tropical regions of Northern hemisphere and in tropical Africa also (Tuskan et al., 2006; Guleria et al., 2022). These poplars, except aspens and Asian mountain balsam poplars, grow widely in several regions like hot-arid and desert-like regions of central Asia and Africa, alpine forests in Europe and North America, as well as riparian zones like river banks and flood plains (Guleria et al., 2022). Poplars are the dominant species in these habitats for tolerating and sustaining in the complete flood. China, Turkey, France, India and the Po River plain of Italy are the largest poplar farming areas for worldwide wood supply, whereas Italy, Spain, France and Hungary provide a landscape of 0.5 out of 0.61 million hectares for poplar farming only. Poplars shape global forests and woodlands in their natural habitats. Besides being domesticated as an agroforestry tree, they provide timber, fuel wood, plywood, industrial roundwood, sports materials, pallets, paper pulp for the paper industry and fodder (Kollert et al., 2014). In addition, *Populus* species are cultivated as energy crops/biofuel (ethanol) in Europe (England and Italy) for carbon sequestration and sustainable bioenergy production in USA because of its high biomass production in a relatively short time (Dou et al., 2017). They are also used in the phytoremediation of toxins, e.g., heavy metals (Cd, Pb, As, and Hg) from contaminated soils, indicating ozone pollution as a bio-indicator, rehabilitation of fragile ecosystems and restoration of forest landscapes (Alahabadi et al., 2017).

Poplar (Genus: *Populus*) comprises 32–40 species based on taxonomic and morphological traits (Cronk, 2005; Douglas, 2017). There is a record of a total 582 *Populus* species, with more than 100 species names recognized worldwide. The higher number of species is due to the presence of naturally occurring hybrids (The Plant List, 2013). According to Eckenwalder (1996), the genus *Populus* is classified into six groups including the cottonwoods (*Aigeiros*), aspens (*Populus*), balsam poplars (*Tacamahaca*), large-leaf or swamp poplars (*Leucoides*) and (*Abaso*) and Afro-Asian poplars (Turanga), and but the Flora of China recognized 71 species from five sections (except *Abaso*) (Park et al., 2004; Table 1). Species boundaries among poplars are sometimes variable as intrasectional and intersectional hybridization occurs among them. However, this has not been supported by molecular evidence. Hence relationships between these sections are reported to be controversial (Wang et al., 2014; Liu et al., 2017; Zhang et al., 2018). During 1950s, poplar was introduced in India from the United States of America. Since then, *P. deltioides,* have been cultivated in India and cover an area of 270,000 ha in India, according to the report of the Indian Council of Forestry Research and Education (ICFRE, 2016; Eqbal and Ansari, 2024). Several researches have proved that the genus *populus* is a rich source of active metabolites, like phenolic compounds, terpenoids, and flavonoids in different parts like stems, buds, leaves and bark (Guleria et al., 2022). These poplar trees have been used to cure various ailments and have several pharmacological properties such as antioxidants, antimicrobials, anticancer, and anti-inflammatory (Guleria et al., 2022).

Poplar was the first woody perennial tree used as a model/experimental tree species among forest trees worldwide for understanding several aspects such as taxonomy, genetics, evolution and the genomics of wood formation for decades because of their small genome size, clonal propagation, fast growth, easy transformation, and long-life cycle (Taylor, 2002). In addition, poplar genome has been completely sequenced after *Arabidopsis* and rice. Moreover, it is the most advanced genomics resource of any forest tree, having reference genomes for several tree species. The black cottonwood (*P. trichocarpa*) was the first forest tree whose genome was sequenced entirely, and currently, there are massive genomic resources available for other poplar species. Interestingly, *P. tomentosa* and *P. euphratica* genomes have been extensively studied, while others have been ignored. All *Populus* species contain 19 haploid genomes (Shi et al., 2024). However, massive rearrangement and diploidization of the whole genome of poplar have been reported. The arabidopsis-poplar genome comparative model approach has been used efficiently in many cases (Rottmann et al., 2000; Jansson and Douglas, 2007). The genus *Populus* is an excellent model for studying the molecular genetic mechanisms involved in pathogen defense responses in several forest trees. The availability of *Populus* genome sequences enhances the efficiency of the substantial molecular tool kit that already exists for *Populus* species, including expressed sequence tags (ESTs) collection and microarrays for transcriptome. In addition, these tools can be applied with valuable pedigrees and genetic maps developed for *Populus* breeding for decades (Sterky et al., 1998; Frewen et al., 2000; Hertzberg et al., 2001; Cervera et al., 2001; Bhalerao, 2003; Andersson Gunnerås et al., 2006). Such pedigrees have been proven useful and efficient in revealing *Populus* loci responsible for conferring resistance to fungal pathogens attacks (Goué-Mourier et al., 1996). Poplar cultivation is severely affected by several ranges of pest insects and pathogens such as fungi, bacteria, and viruses, resulting in reduced growth and quantity and quality of wood. These biotic stresses comprise the complex interactions between hosts, pests, pathogens, and environmental factors, negatively affecting the poplar population (Seserman, 2018). The efficiency of traditional methods for protecting poplar farming from attack of pests and pathogens is hindered by factors like climatic instability, global warming, flood, drought, high temperatures and humidity. Consequently, new biotechnological approaches like genetic transformation and genome editing are required to overcome these limitations of traditional breeding. These tools are utilized to increase the quality and yield of wood and improve pest and pathogen

TABLE 1 Taxonomic categorization of different species of the genus *Populus*.

Divisions	Names	Occurrence	References
Turanga Bunge	Subtropical Asian poplars (*P. lasiocarpa* Oliv, Chinese necklace poplar), *P. ilicifolia* (Engl.) Rouleau (Kenyan poplar), *P. euphratica* Oliv (Euphrates poplar)	China, Northeast Africa, Southwest Asia, East Africa (subtropical and tropical)	Gai et al. (2021) and Du et al. (2024)
Populus or *Leuce Duby*	True white poplars and aspens *P. adenopoda* Maxim (Chinese aspen), *P. monticola Brandegee* (White poplar), *P. alba* L. (White poplar) *P. tremuloides* Michx (Quaking aspen) *P. tremula* (Japanese aspen)	China, Europe, North Africa, India, Mexico, North America, Northeast Asia	Chanda et al. (2010) and Du et al. (2024)
Aigeiros Duby	Black poplars, *P. trichocarpa* Torr. (Black cottonwood poplar), *P. fremontii S. Watson* (Fremont's cottonwood), *P. deltoides* Marshall (Eastern cottonwood)	Europe, Western Asia, Temperate region of North America, Central Asia	Gai et al. (2021) and Porth et al. (2024)
Abaso Eckenwalder	Endemic Mexican poplars (*P. pruinosa* Schrenk, Desert poplar), *P. Mexicana*	Mexico	Liu et al. (2017), Wang W. et al. (2022a), and Wang Y. et al. (2022b)
Leucoides Spach	Big leaf poplars (*P. heterophylla* L., *Swamp cottonwood poplar*), *P. jacquemontiana* Dode (Sichuan poplar)	Eastern North America (USA), Eastern Asia China, India (warm and temperate)	Wang W. et al. (2022a), Wang Y. et al. (2022b), and Zhang et al. (2019)
Tacamahaca *Spach*	Balsam poplars *P. ciliata* Wall. ex-Royle (Himalayan poplar), *P. angustifolia* E. James (Narrow leaf cottonwood poplar), *P. suaveolens Fisch.* ex-Loudon (Asian poplar), *P. trichocarpa* Torr. (Black cottonwood poplar)	North America, Asia [India, Pakistan, Bhutan, Nepal, Myanmar (Cool temperate), Northeast China, Japan]	Chanda et al. (2010) and Gai et al. (2021)

resistance in forest trees, including poplar (Li P. et al., 2024; Li Y. et al., 2024; Li Z. et al., 2024). This paper primarily summarized the details of pest and pathogen attacks that cause diseases in poplar trees. We also examined the mechanism of infection, transmission route, the role of lignin in protecting poplar against pests and pathogens and the mode of control of diseases in poplar. Here, we also emphasized conventional breeding and its limitation for poplar plantations on a large scale. The application of different tools of genetic transformation and genome editing for developing transgenic poplar resistant to pests and pathogens was discussed in detail. Finally, we summarize the regeneration of transgenic poplar trees that have been modified by incorporating defense genes, such as those conferring pest and pathogen resistance.

2 Poplar susceptibility to biotic stresses

Poplars are frequently impacted by infestations of insect pests such as mites, aphids, and caterpillars, as well as bacterial, viral, and fungal infections. These pests and pathogens target all parts of the tree, damaging buds and leaves, inducing gall formation, sucking sap, altering bark structure, and boring into shoots and roots, which facilitates the transmission of plant diseases. Over time, these attacks can lead to complete defoliation, reduced tree growth, and even tree death. As a result, affected poplars become unsuitable for various uses, including furniture production, biofuel generation, and veneering (Charles et al., 2018).

2.1 Pest attacks on poplar

Insect pests are a limiting factor affecting *Populus* productivity worldwide (Table 2). Globally, outbreaks of pests are boosted due to climate change (Fenning, 2013). Around 525 and 300 species of insects and mites feeding on *Populus* have been identified as serious threats for causing economic and ecological losses in poplar plantations in Europe and North America, respectively (Charles et al., 2018). Ahmad and Faisal (2012) documented that around 133 insect species feed on poplar plantations in India. These pests hinder plant growth and increase tree mortality (Dickmann, 2001; Coyle et al., 2005). In North America, the cottonwood leaf beetle (CLB) (*Chrysomela scripta*) is reported as the most widespread and severe defoliator of young *Populus* cultivation (Coyle et al., 2005). In the Mediterranean, *Saperda carcharias* (large poplar borer) is reported to be one of the most damaging insects for young poplar plantations (Biselli et al., 2022). In China, major pest species destroying poplar plantations are trunk borers and defoliators such insects of the *Lepidoptera* (*Hyphantria cunea* Drury), *Apocheima cinerarius* Ershoff, *Lymantria dispar* Linnaeus, *Malacosoma neustria* Motschulsky, and other moth species belonging to the *Notodontidae* and *Limacodidae* and *Coleoptera* (*Apriona germari* Hope, *Anoplophora glabripennis* Motschulsky, and *Plagiodera versicolora* Laicharting). In addition, up to 40% loss of hybrid *Populus* plantations is reported due to poplar looper (*Apochemia cinerarua*) and the spongy moth (*Lymantria dispar*) (Hu et al., 2001; Wang et al., 2018) in China. *Anaplophora glabripennis* also causes massive destruction of hectares of Chinese poplar (*P. simonii*) plantations. Biselli et al. (2022) observed that *Phloemyzus passerinii* [Wooly Poplar Aphid (WPA)] causes 10% of production losses of poplar, mainly in European and American countries. Other insects, for example, *Cossus cossus*, *Agrilus suvorovi*, *Megaplatypus mutatus*, *Paranthrene tabaniformis*, *Melanophila picta*, and *Gypsonoma aceriana,* also threaten poplar farming. Recently, transcriptomic and metabolomic analyses were conducted to investigate the species-specific defense responses of *Populus tremula* against herbivores such as spongy moths (*Lymantria dispar*) and aphids (*Chaitophorus populialbae*). The insights gained from these studies could

TABLE 2 List of pests/pathogens/bacteria/viruses affecting poplar plantations.

Plants	Pest/pathogens	Diseases	Affected Countries	References
Insects/pests				
Populus fremontii, P. tremula, P. nigra and P. angustifolia, P. alba	*Aceria parapopuli*	Soap sucker, galls, irregular, warty, cauliflower-like growth	North America, Iran	McIntyre and Whitham (2003) and Mehri et al. (2020)
P. tremula, P. deltoides, P. alba	*Agrilus suvorov*	Borer	Germany, Greece, Guernsey, Hungary, Ireland, Israel, Italy	Cavalcaselle (1972)
P. tacamahaca, P. tremuloides	*Altica populi*	Defoliation	North America	De Tillesse et al. (2007) and Ostry et al. (2014)
P. deltoides, P. alba P. nigra, P. x euramericana hybrids	*Leucoma wiltshieri*	Defoliation	Europe, Middle East, Japan, Iran, America, China	Sadeghi et al. (2009) and Charles et al. (2014)
P. × euroamericana, P. euphratica, P. alba, P. nigra L., P. deltoides Marsh.	*Melanophila picta*	Wood borer	Bulgaria, Southern France, Italy, Spain, Portugal, Turkey Pakistan	Mazhar and Sadeghi (2024)
P. tremuloides, P. purpurea, P. nigra	*Phratora laticollis*	Defoliation	North America, Belzium, Germany	Nagaraju et al. (2023)
P. deltoides × P. nigra [P. × euramericana (Dode) Guinier], P. alba	*Sesia apiformis*	Borer, root sucker	Europe, Canada, Asia Minor, Middle East, China, North America	Martín García et al. (2011) and Meert (2022)
P. deltoides, P. tremula	*Byctiscus populi*	Defoliation	Europe	Urban (2013) and Schroeder and Fladung (2018)
P. tremuloides, P. deltoides, P. gradidentata	*Choristoneura conflictana*	Defoliation	Canada, Northeastern and Central USA, Alaska,	De Tillesse et al. (2007) and Charles et al. (2014)
P. deltoides	*Dasineura salicis*	Galls	Europe, North America	De Tillesse et al. (2007) and Charles et al. (2014)
Fungus				
P. davidiana × P. bollena, P. euphratica, P. deltoides.	*Alternaria alternata*	Leaf blight	India, China, Iran	Osdaghi et al. (2014), Uniyal et al. (2018), and Huang et al. (2022)
Populus× canescens 'Tower'	*Apioplagiostoma populi*	Bronze lea	North America	Wijekoon et al. (2021)
P. deltoides × P. nigra	*Botrydiplodia populea*	Canker	China, Poland	Kwaśna et al. (2021b)
P. deltoides, P. tremuloides, P. maximowiczii × P. x. berolinensis, P. serotina	*Ceratocystis fimbriata*	Black and target canker	USA, North America, Alaska, Poland, Quebec, India, Poland	Johnson et al. (2017)
P. tremuloides	*Ciborinia whetzelii*	Ink-spot disease	Northern USA, Canada	Zegler et al. (2012) and Kowalski (2013)
P. ×euramericana, P. yunnanensis, P. deltoides	*Corticium salmonicolor*	Pink disease	India	Saxena et al. (2017)
P. tremuloides, P. balsamifera, P. tremula	*Diplodia tumefaciens*	Bark alterations, woody gall	Canada, Europe, Northern USA	Kwaśna et al. (2021b)
P. trichocarpa ×P. deltoides, P. tremula, P. alba, P. grandidentata, P. tremuloides	*Linospora tetraspora*	Leaf blight	USA, Canada	Zobrist et al. (2023)
P. deltoides	*Melampsora medusae*	Leaf rust	Europe, New Zealand, Australia, South Africa, Argentina, North America, India, Canada, Japan	Zeng et al. (2023)
P. deltoides	*Septoria musiva*	Canker and leaf spot	Europe, North America	Feau et al. (2010) and Dunnell and LeBoldus (2017)
P. alba	*Venturia tremulae*	Spring leaf, shoot blight	North America, China, Africa	Martínez-Arias et al. (2019)

(Continued)

TABLE 2 (Continued)

Plants	Pest/pathogens	Diseases	Affected Countries	References
Bacteria				
P. alba, P. trichocarpa, P. deltoides	*Erwinia herbicola, Erwinia carotovora*	Bacterial twig canker with gall like formations	Europe, North America	Fabi et al. (2008)
P. tremula L. 70 × (Populus × canescens)	*Phytophtora. cactorum and P. plurivora.*	Root rot	Asia, Europe, Africa, USA, Australia, New Zealand, Serbia	Cerny et al. (2022)
P. ×euramericana	*Lonsdalea populi*	Bark canker	China, Europe	Li and He (2019)
P. trichocarpa	*Pseudomonas syringae*	Bacterial blight	Worldwide	Saint-Vincent, et al. (2020)
P. tomentosa, P × euramericana	*Sphingomonas sanguinis*	Bark canker	Worldwide	Deng et al. (2023), Li P. et al. (2024), Li Y. et al., (2024), and Li Z. et al. (2024)
P. trichocarpa	*Xanthomonas populi*	Canker	Europe and America	Kwaśna et al. (2021b)
Virus				
P. nigra, P. trichocarpa, P. deltoides, P. candicans, P. ×euramericana	*Poplar mosaic virus*	Leaf mosaic	Worldwide	Smith and Campbell (2004)
P. tremuloides	*Tobacco necrosis virus*	Necrosis of leaf	Worldwide	Shen et al. (2015)
P. ×euramericana	*Arabis mosaic virus*	Leaf mosaic	Japan, New Zealand, America, Europe	von Bargen et al. (2020), Li P. et al. (2024), Li Y. et al., (2024), and Li Z. et al. (2024)
P. tremuloides	*Potato virus Y*	Mottling/yellowing of leaf, leaf drop leaf crinkling	Worldwide	Lawrence and Novak (2006)
P. euphratica and P. × canescens	*Tobacco rattle virus*	Mottling, chlorotic or necrotic local lesion, ringspots or line patterns, necrosis	Worldwide	Shen et al. (2015)
P. balsamifera	*Tomato black ring virus*	Mottling, deformation, leaf necrosis	Worldwide	Li P. et al. (2024), Li Y. et al., (2024), and Li Z. et al. (2024)

be valuable for developing transgenic poplar varieties with enhanced resistance to pest attacks (Pastierovič et al., 2024).

2.2 Pathogen attacks on poplar

Poplar trees are also constantly challenged by various pathogens like fungi, bacteria and viruses (Table 2). These pathogens inhibit the growth of poplar, impacting the quality and quantity of wood biomass. The different poplar culture practices and the introduction of exotic pathogens promote the widespread distribution of some specific pathogens (Newcombe et al., 1996).

2.2.1 Fungal attack on poplar

Fungi are usually considered as "primary parasites." They infect healthy plants, which can eventually affect poplar growth and hence decrease the quality and production of wood. The diseased poplars can exhibit reduced leaf photosynthetic areas. Leaf scars created allows entry of secondary pathogens. Repeated infections and premature poplar defoliation may weaken plants, making them susceptible to insect attack, high temperatures and drought (Kebert et al., 2022). Generally, plant pathogens are categorized into three groups: (a) biotrophs (feed on living plant tissue), (b) necrotrophs (feed on dead plant tissue), and (c) hemibiotrophs (first infect living plant tissue and make them dead and then feed on dead tissues) (McCombe et al., 2023). Examples of biotrophs infecting poplar are powdery mildews by *Phyllactinia* spp. or *Uncinula* spp., leaf rust by fungus *Melampsora* spp., while necrotrophs including leaf blight by *Septoria* spp. and leaf spot by *Coryneum* spp. and *Marssonin*a spp., canker (*Septoria* spp.) (Feau et al., 2010; Zeng et al., 2023). Among fungi, the genus *Melamspora* (biotrophic rust fungi), especially (*Melamspora. larici-populina*) is reported as the most severe and widespread fungi in poplar plantations (Polle et al., 2013). Infection with this genus is characterized by premature defoliation and reduced photosynthetic ability, resulting in loss of wood production (Polle et al., 2013). Moreover, *M. larici-populina* is also responsible for severe economic poplar losses in Europe and America (Duplessis et al., 2009), while *Melamspora medusae* caused leaf rust in *P. deltoides* in East-North America and the North-West USA (Newcombe et al., 1996). The other primary poplar diseases like stem canker and leaf spot in North America and Europe are caused by fungus *Septoria musiva* (also known as *Sphaerulina musiva*) (Zhao et al., 2023). *Venturia* spp. are found to cause shoot and leaf blight in poplar plantations in Asia, Europe and North America (Gennaro and Giorcelli, 2019). Other major fungal pathogens of *Populus* affecting leaf are *Apioplagiostoma populi* (causing bronze leaf disease) and *Taphrina* spp. (causing yellow blister of leaves), *Entoleuca mammata* (causing Hypoxylon canker), *Cytospora chrysosperma*, (causing canker) and *Phellinus tremulae* (causing aspen bracket) (Duplessis et al., 2009). The poplar blister canker disease develops upon infection with the Botryosphaeria pathogen during drought stress, commonly observed in southern China (Xing et al., 2022). Recently, black spot disease in poplar has been reported to be one of the major diseases in China affected by fungi such as *Marssonina castagnei*, *Marssonina populi*, and *Marssonina brunnea* (Xiong et al., 2021).

2.2.2 Bacterial diseases in poplar

A few bacteria also negatively affect the growth of poplar plantations. The attack of bacteria (*Xanthomonas populi*, *Erwinia genus* and *Lonsdalea populi*) causes canker, resulting in reduced wood biomass yield of poplar (Li et al., 2019). *Xanthomonas populi* (Ridé) Ridé and Ridé and *Pseudomonas syringae* Van Hall are responsible for necrosis, wilting, injury, cankers, rots and tumors in poplar vegetations (Kalinichenko et al., 2017). The fluctuating temperatures cause *P. syringae* growth in poplar bark (Ramstedt et al., 1994). *Lonsdalea quercina* caused bark canker in *Populus×euramericana* (Tóth et al., 2013). Recently, *Pseudomonas aeruginosa* (Schröter) Migula was reported to cause disease in poplar plants. It causes rot, resulting in fast wilting, with trees dying within 48 h. *Agrobacterium radiobacter* Beijerinck and van Delden and *Agrobacterium tumefaciens* cause crown gall disease upon transfer and integration of the bacterial transfer DNA (T-DNA) into the plant genome (Kwaśna et al., 2021a). Currently, the large population of the hybrid poplar *Populus × euramericana* in Hungary and China is severely affected by *Lonsdalea populi* (Zlatković et al., 2020). Bacterial wetwood of poplar (*Populus alba* L.) by *Lelliottia nimipressuralis* has been common in the territory of Ukraine since 1974. The poplar wetwood disease was also reported in Bulgaria, USA, and other countries. The primary bacterial pathogens of poplar are *Xanthomonas populi, Pseudomonas syringae*, *Enterobacter cancerogenus* in the coastal zone of Western Europe, Eastern Europe and Central Europe, respectively (Goychuk et al., 2023).

2.2.3 Viral attack in poplar

Viral pathogens such as the poplar mosaic virus, poplar decline virus, tobacco necrosis virus, tobacco mosaic virus, rhabdoviruses, cucumber mosaic virus, tobacco rattle virus, arabis mosaic virus and tomato black ring virus are also severe threats to the poplar population other than fungus and bacteria (Table 2; Wang P. et al., 2023; Wang S. et al., 2023). Poplar mosaic virus (PopMV), with a single-stranded RNA, is the most common dangerous filamentous plant virus and is widespread worldwide (UK, "former Czechoslovakia and former Yugoslavia" Holland, France, Germany, Switzerland, Denmark, Italy, Bulgaria, USA and Canada) where poplar is grown at large scale. It attacks almost all the poplar plants in the *Aigeiros* section, including several clones of *P. x euramericana*. Members of the *Tacamahaca* section and crosses between these species and the *Aigeiros* section are also affected by viruses. The symptoms of a viral attack on poplar include stunted growth, leaf discoloration, necrosis, wilting and deformities in poplar. It causes severe losses in the quantity and quality of wood (Smith et al., 2004; Naylor et al., 2005; Smith et al., 2009). The virus is generally spread by cutting diseased parts (Berg, 1964).

3 Transmission route, infection, and defense mechanism in poplar attacked by pest and pathogens and their control

The vast diversity of insect pests and pathogens poses significant challenges to forest trees, severely impacting their health and productivity. These threats are particularly serious for poplar plantations worldwide. Climate change also plays a crucial role in altering the occurrence and spread of native and invasive insect outbreaks. Insects typically target susceptible trees for feeding or establishing habitats, further exacerbating the problem. These insects attack and affect all tree parts like shoot, xylem, phloem leaves, flowers, barks, and roots (Balla et al., 2021). In addition, most insects are generally introduced into a non-native area other than their native range and spread rapidly across the country. Imported alive plants and wood materials can act as carriers for introducing many pests (Dara et al., 2019). Fungi, the most common disease agent of poplar trees, have several invasion mechanisms and an array of virulent factors. In root rot disease, rhizomorphs (clusters of intertwining fungal hyphae) and secondary metabolites play a crucial role in infection. The rhizomorphs aggregate around the tree roots, feeding on the host tissues, and can persist in the dead tissues of infected plants for extended periods. This disease is marked by root decay, premature defoliation, wilting, and the production of dwarf fruits and leaves (Balla et al., 2021). Warmer winters, due to climate change, have increased the frequency of sporulation and the rate of fungal infections. Notably, poplar's defense mechanisms vary depending on the type of fungus involved. Rust diseases caused by the *Melampsora* spp. are the most common diseases in forest trees, such as poplar. Cankers are mainly caused by attacks of fungal pathogens which affect tree branches, shoots, and twigs. It has been noticed that canker-related diseases occur because of functional failure of the cambium and phloem, carbon starvation, and hydraulic failure. For instance, the fungus inoculations *Botryosphaeria* disease in poplar (*P. alba* var. *pyramidalis* = *Populus bolleana*) arrested the regeneration of callus and phloem and decreased the rate of photosynthesis and transpiration, as well as arrested the opening of the stomatal aperture and disrupted electron transport (Xing et al., 2022). Bacteria affect plants by forming colonies on their surface or within their tissues. Unlike fungi, they cannot penetrate host cells directly. Instead, they typically enter through natural openings like stomata or through wounded areas. Once inside, these bacteria secrete extracellular enzymes that break down host cells, allowing them to colonize plant tissues. Additionally, they produce polysaccharides that clog the plant's vascular system, reducing water transport through the xylem. Beetles and leafhoppers can also act as vectors, carrying pathogens and transmitting diseases to plants. Bacterial infections often manifest through symptoms such as spots, cankers, burns, tissue rot, and hormonal imbalances, which can lead to excessive root branching and leaf epinasty (Chatterjee et al., 2008). Certain bacteria, like *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, inject their plasmids into plant host cells through wounded areas, integrating them into the host genome. This results in tumor gall diseases and the production of hairy roots, respectively (Sharan et al., 2019). Viral pathogens are widespread in plant ecosystems, serving two roles: as agents of plant diseases and as natural enemies of pests and tree pathogens, offering indirect protection to trees. Viral infections often cause significant tissue damage and can lead to symptoms like yellowing, chlorotic lesions, necrotic spots, and ring spots on plant parts. Some stable viruses, such as tobacco mosaic virus, do not require vectors to spread, while other viruses rely on vectors, such as aphids, mites, leafhoppers, fungi, beetles and nematodes, soil, water, other plants and debris for transmission (Balla et al., 2021). Smith and Campbell (2004) reported that the poplar mosaic virus (PopMV) infection and spread depend on the poplar genotypes.

Insects are typically controlled by the release of toxic phytochemicals from plants, which either inhibit pest growth or kill the insects (Fernandez-Conradi et al., 2021). To defend against pathogen attacks, poplars utilize two types of defense mechanisms: induced and constitutive defenses. Induced defenses are activated in response to external stimuli and involve complex processes, while constitutive defenses, the first line of defense, involve non-host resistance through physical barriers and the accumulation of phytochemicals in the plant (Alkan and Fortes, 2015; Zeng et al., 2023). Induced resistance can be further classified into locally induced resistance and systemic induced resistance (SIR). SIR provides broad-spectrum, long-lasting protection against secondary infections. The exogenous application of signal molecules that trigger these defenses can enhance plant immunity and help manage pest populations (Balla et al., 2021). Some observations demonstrate that signal molecules such as salicylic acid (SA) and methyl jasmonate (MeJA) are involved in local and systemic defense responses. Recent research proved that both SA and MeJA pathways are induced in leaves of poplar upon infection with the fungus *M. larici-populina* proving that both hormone pathways are essential for defense response (Ullah et al., 2019; Chen et al., 2021). Upon fungus attack, the poplar trees activates constitutive defenses involving several processes such as recognition of the fungus by receptor proteins and resistance (R) proteins (PR) resulting into pattern-triggered immunity (PTI) (receptors of plant membranes recognize molecular patterns (PAMPs) of pathogens) and effector-triggered immunity (ETI) (intracellular receptors, (a nucleotide-binding leucine-rich repeat (NLR) class recognize effectors released by pests and pathogens) as well as noncoding RNA (ncRNA)-mediated defense (non-coding RNA having more than 200 nucleotides in length with having role in plant growth and development, and stress responses), initiation of hormone signaling network pathways (mitogen-activated protein kinase (MAPK) cascades and calcium-dependent protein kinase (CDPK) involved in plant growth and development and stress response), activation of defense-related genes and transcription factors (TFs) involved in controlling gene expression by binding DNA elements at 5′ non-coding regions (promoters) of desired genes and modulating transcription rate) and accumulation of phytoconstituents (De Kesel et al., 2021; Zeng et al., 2023). In addition, the pathogen-associated protein 1 (PR1) gets activated as a plant response to abiotic and biotic stresses. Total 17 PtPR1 genes were found in *Populus trichocarpa* (Wang P. et al., 2023; Wang S. et al., 2023). A total of 1888 lncRNAs and 52,810 mRNAs were recognized in poplar coma (Song et al., 2024). The 30 CDPK genes and 20 closely related kinase genes were identified in *Populous* spp. (Zuo et al., 2013), The 11 MAPKKs (PtMKKs) and 21 MAPKs (PtMPKs) were identified in the *Populus trichocarpa* (Hamel, 2006). A total of 104 WRKYs (TFs) have been identified in poplar (He et al., 2012). Recently, the integrated transcriptomic and transgenic analyses were applied to understand mechanisms of poplar resistance against *Alternaria alternata* attack (Wang W. et al., 2022; Wang Y. et al., 2022).

The most effective method for preventing leaf diseases caused by pests, fungi, bacteria, and viruses is selecting and planting pathogen-resistant poplar clones. Another approach involves using fungicides, such as copper- and carbamide-based treatments, to prevent infections. Fungal diseases can also be managed by maintaining proper spacing between poplars, reducing weed competition, and optimizing plant density, as high relative humidity contributes to disease development. Infected leaves, roots, stems, and branches should be pruned, particularly during the dormant season, to minimize pest and pathogen attacks. Additionally, poplars should be planted in appropriate soil conditions within nurseries to promote healthy growth. Additionally, the soil from infected areas must not be used and moved with equipment (Kebert et al., 2022). Proteomic and genomic technologies offer valuable tools for precisely identifying and characterizing bacterial infections by analyzing their genetic and protein markers (Zubair et al., 2022). Recent studies have shown that lactic acid bacteria (LAB) can effectively combat plant pathogens due to their high biosecurity and ability to promote plant growth (Jaffar et al., 2023). Quorum sensing (QS) molecules, such as 3-OH PAME, regulate the virulence genes in bacteria and fungi, making the identification and development of QS-quenching genes and enzymes promising for disease control (Wang P. et al., 2023; Wang S. et al., 2023). Additionally, eucalyptus oil, known for its antibacterial properties and ability to stimulate plant defense mechanisms, has been shown to reduce plant diseases and could be used to protect poplar in the future (Montesinos et al., 2023). A few genes have been reported whose expression can impart disease resistance in poplar trees. For example, the overexpression of PdbLOX2 was able to induce the resistance in *P. davidiana* × *P. bollena* against *A. alternata* attack, while silencing this gene increased the susceptibility of the poplar tree to *A. alternata* infection (Huang et al., 2022). Furthermore, the study reported that PtoMYB142 can regulate transcription of wax biosynthesis genes [fatty acid hydroxylase (CER4) and 3-ketoacyl CoA synthase (KCS6)] mediating adaption of poplars against drought conditions were highly expressed upon infection with fungal pathogens (Song et al., 2022). In addition, lignin has a vital role in protecting poplar from pest and pathogen attacks. It is a primary three-dimensional phenolic biopolymer of the secondary cell wall in vascular plants (Ma et al., 2024). It imparts strength and imperviousness to cell walls, mediating long-distance water transport in vascular tissues. In addition, it acts as a barrier to the spread of invading pathogens as it is non-degradable to pathogens, thereby preventing their penetration into the plant cell wall and the supply of water and nutrients from plant cells to pathogens. It is noticed that the gene expression of lignin increased with higher lignin content upon pathogen infection. The genes (phenylalanine ammonia lyase (PAL), HCT4-Coumarate: coenzyme A ligase (4CL), cinnamate 4-hydroxylase (C4H), cinnamoyl-CoA reductase (CCR), cinnamyl alcohol dehydrogenase (CAD) and hydroxycinnamoyl transferase) are involved in lignin biosynthesis and highly expressed during fungal infection leading to increase in lignin content (Lee et al., 2019; Zeng et al., 2023; Ma et al., 2024; Riseh et al., 2024). Hence, regulating the lignin biosynthesis pathway may be critical for improving poplar resistance against pathogen attacks (Polle et al., 2013). It is reported that the higher expression of Pto4CL1 of *P. tomentosa* increased the lignin content from 33.11 to 46.65%, leading to the decreased formation of cellulose, hemicellulose, and pectin (Hu et al., 2019). RNAi technology was used to down-regulate the expression of 4CL gene to modify lignin biosynthesis in *P. tremula* (Kovalitskaya et al., 2016). A significant 30% reduction in lignin content has been observed in poplars due to the downregulation of cinnamate 4-hydroxylase (C4H) genes (Bjurhager et al., 2010). Similarly, the downregulation of CAD genes in *Populus tremula* × *Populus alba* led to reduced lignin levels (Özparpucu et al., 2017). Dirigent (DIR) proteins have also been identified as crucial players in lignin biosynthesis. Li et al. (2022) reported that the overexpression of PtDIR11 in poplars enhanced lignin biosynthesis, thereby increasing the trees' resistance to *Septotis populiperda*. Hence, gene editing can be utilized to regulate the expression of these genes to enhance lignin biosynthesis, which confers pest and pathogen resistance in poplar trees.

4 Conventional breeding in poplar

The recent global temperature, drought, humidity and climate instability render poplar plants vulnerable to pests and pathogens, severely affecting wood quality and quantity (Gullino et al., 2022). Plants have physical and physiological barriers against microbial pathogens, preventing their access to plants (Kumudini et al., 2018). Plants produce antimicrobial peptides and other molecules that cause detoxification and the inhibition of virulence factors (Silva et al., 2016). Moreover, plants also apply RNA interference (RNAi), which detects invading viruses and cleaves the RNAs of viruses (Bocos-Asenjo et al., 2022). However, these pathogens have evolved to cope with the defense systems of their plant cells by secreting cell-wall degrading enzymes, which gain access for molecules into plant cytoplasm, inhibiting host defenses and promoting susceptibility of plants toward pathogens (Kaur et al., 2022). Some viral pathogens are also reported to attack and silence the host RNAi system, promoting viral pathogenicity (Leonetti et al., 2021). The increased demand for wood and its sustainability requires approaches to improve efficient production even under environmental constraints and minimize the threats due to pests affecting wood properties for industrial purposes (Polle et al., 2013). However, controlling pests and pathogens with chemicals is increasingly considered unsafe due to their high toxicity, environmental accumulation, and harmful impacts on beneficial insects, non-target organisms, and humans (Ahmad et al., 2024). The commonly adopted traditional methods for the protection of plants from attack of poplar pests and pathogens are the use of resistant clones that have good adaptability to different soil (salinity, calcium level and pH), drought and climatic conditions coupled with the adoption of proper cultivation practices (i.e., fertilization, low plant density and irrigation) (Biselli et al., 2022). Enormous progress has been made in enhancing traits such as plant growth rate, pest and pathogen resistance, and environmental adaptations in poplar plantations by applying conventional breeding practices (Ye et al., 2011). However, these traditional methods are time-consuming because of their long-life span, costly land demand, and high labor costs. In addition, because of the heterozygosity of most *Populus* genotypes and inbreeding depression, it is difficult to estimate the genetic control of particular traits (Ye et al., 2011). Genetic engineering and molecular breeding methods for developing transgenic poplar plants can address the limitations of traditional breeding, such as the challenges of distant hybridisation, the complexity of cultivation, and issues with interspecific hybridisation (Begna, 2021).

These tools have enormous potential to improve two or more traits simultaneously by introducing desired exogenous genes of donor plant or non-plant origin into a particular plant genome, enabling the improvement of poplar against pests and pathogens, herbicide resistance, abiotic stress, wood properties, flowering regulation and phytoremediation (Thomas, 2022).

5 Genetic engineering and different transformation methods in poplar

Genetic transformation has been widely employed in research on various forest trees. This process involves introducing exogenous genes into tree cells, thereby altering their genetic traits (Li et al., 2023). Poplar trees were among the first forest trees used successfully for genetic engineering for gene research (Ye et al., 2011). For more than 20 years, several progress has been made in *Populus* transformation. The most widely used transgenic tools involve vector-mediated transformation, such as *Agrobacterium tumefaciens*-mediated and *A. rhizogenes* mediated and non-vector mediated transformation (Gene gun-mediated, pollen tube pathway, and protoplast transformation methods). The Genome editing method is an advanced approach for adding, deleting, or modifying genes within the specific genome (Li et al., 2023). Mobile genome editing techniques, such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated (CRISPR-Cas) systems, RNA interference (RNAi), and nanoparticle-meditated gene transformation have been recently applied to improve poplar tree (Yin et al., 2021; Figure 1). Among these methods, *Agrobacterium*-mediated and gene gun-mediated transformations are the most widely used techniques for forest trees (Lv et al., 2020).

5.1 *Agrobacterium tumefaciens*-mediated transformations: basic mechanisms

Agrobacterium tumefaciens-mediated transformation is the most preferred method for the genetic transformation of forest trees. *A. tumefaciens* (a gram-negative soil bacteria) infects the wounded sites in many dicotyledons, gymnosperms and a few angiosperms. It delivers its transfer DNA (T-DNA) molecules into plant cells and then integrates them into the plant genome (Chilton et al., 1980; Sekine and Shinmyo, 2020). The *Agrobacterium*-mediated transformation method involves removing oncogenes causing tumorigenesis, inserting exogenous genes in disarmed T-DNA, and delivering and integrating foreign genes into the plant genome (Pratiwi and Surya, 2020). The success of *Agrobacterium*-mediated transformation depends on different parameters such as the virulence of *Agrobacterium* cells, explant types and plant genotypes and regeneration of transgenic populations. *Agrobacterium rhizogenes* is also a relative of *A. tumefaciens*, which develops hairy root at the wounded site of plant cells (also known as "hairy root disease") and can be used to transfer the T-DNA into a binary vector into developing root cells (Limpens et al., 2004; Sharan et al., 2019). Various wild-type strains of *A. tumefaciens* and *A. rhizogenes* have transformed various trees. *A. tumefaciens*-mediated genetic transformation system has been widely applied in various poplars, such as *Populus alba* × *Populus glandulosa*, *Populus simonii* × *Populus nigra*, and *Populus tomentosa*. Several attempts were made to improve *A. tumefaciens*-mediated transformation in poplar by optimizing several parameters such as types of explants, different strains of *Agrobacterium* and culture densities, incubation time and concentration of acetosyringone and sucrose (Movahedi et al., 2014, Sharan et al., 2019). Pest infestation and bacterial, fungal and viral diseases are limiting factors which affect the healthy growth of poplar trees (Li P. et al., 2024; Li Y. et al., 2024; Li Z. et al., 2024). By introducing insect and disease-resistance genes into poplar trees using *A. tumefaciens*, these trees can protect themselves from invading pests and diseases, enhancing their survival rate and disease-resistance capabilities. However, *Agrobacterium*-mediated transformation has been done in several poplars, but many other poplars remain recalcitrant to *Agrobacterium*-mediated transformation (Song et al., 2019).

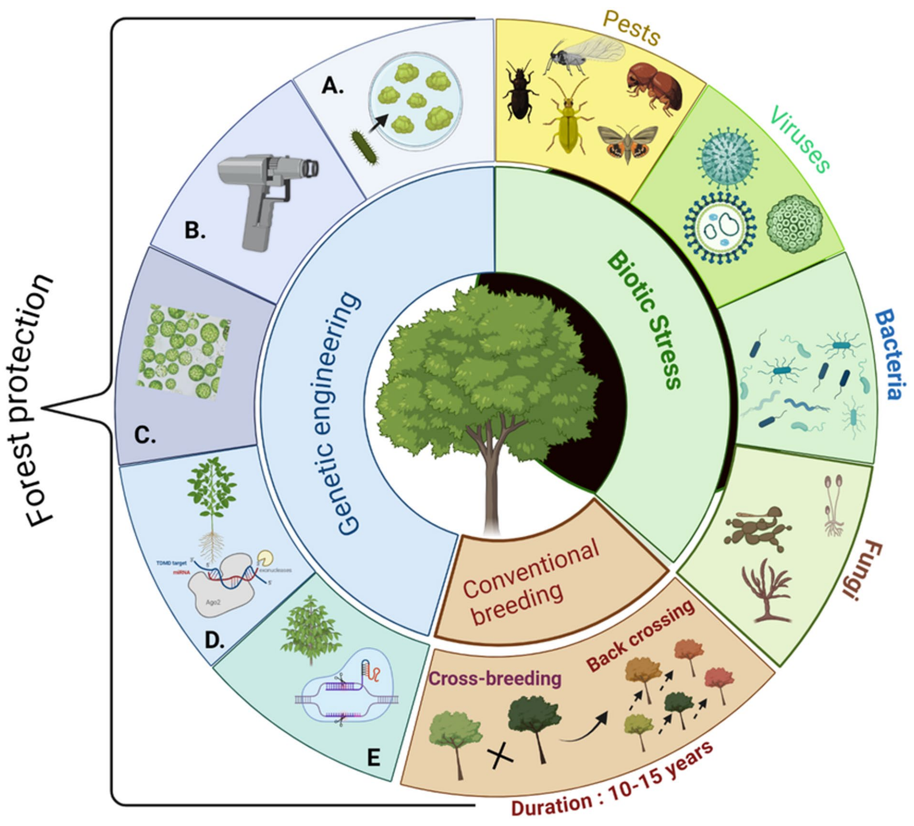

FIGURE 1
Summarizing forest protection using genetic engineering. Increasing biotic stressors (i.e., pests and pathogens) cause dramatic tree mortality in the forests worldwide. The conventional breeding method is time-consuming and fails to cope with the demands. Transgenic pest and disease resistance trees (i.e., Poplar) can potentially mitigate the challenges. Transgenic trees can be generated using various transformation techniques such as (A) agrobacterium-mediated transformation, (B) gene-gun mediated transformation, (C) protoplast-mediated transformation, (D) mi-RNA or RNAi-mediated transformation, and (E) CRISPR-mediated transformation techniques. (The figure is prepared using biorender.com).

5.2 *Agrobacterium*-mediated transformation for pest resistance in poplar

Several transgenic poplars have been developed that overexpress genes encoding different serine proteinase inhibitor proteins (Heuchelin et al., 1997; Confalonieri et al., 1998) and *Bacillus thuringiensis*-derived genes (Cry/Bt genes) (McCown et al., 1991; Wang et al., 1996), *Androctonus australis* hector insect toxin, Kunitz trypsin inhibitor (KTI) and chitinase gene for conferring pests resistance (Clemente et al., 2019; Ren et al., 2021; Table 3). However, Bt gene is the most widely used for generating pest-resistant poplar trees. The first stable transfer of Bt was reported in *Populus nigra* (McCown et al., 1991). Recently, the simultaneous introduction of two Bt genes into the trees' genomes expanded the scope of insect resistance in transgenic forest trees (Dong et al., 2015; Wang et al., 2018). China has been the first nation to generate and commercialize two transgenic lepidopteran-resistant poplar lines since 2002 (Thakur et al., 2021). The plant *P. alba*×*P. glandulosa* was transformed with a Bt Gene (CRY3A) using *Agrobacterium*-mediated transformation method, which resulted in the development of transgenic line BGA-5 and toxic to the larvae of *Anoplophora glabripennis* with a growth inhibition rate of 78.6% (Zhang et al., 2006). *P.* × *euramericana* was transformed with Cry1AC and Cry3A genes to confer resistance to the poplar plants against *H. cunea* exhibiting mortality rate of 42.2–66.1 and 100% of *Plagiodera versicolora* larvae of L1 and L2 stages, respectively (Yang et al., 2016). Transgenic poplar lines 'Shanxin' (*Populus davidiana*×*Populus bolleana*) were developed through *Agrobacterium*-mediated transformation method carrying Cry1Ac + SCK, Cry1Ah3, and Cry9Aa3, respectively against fall webworm (*Hyphantria cunea*) and gypsy moth (*Lymantria dispar*) as these genes Cry1Ac + SCK, Cry1Ah3, and Cry9Aa3 were toxic to the larvae of both insects (Ding et al., 2017). Two Bt toxin genes, Cry1Ac and Cry3A, were simultaneously integrated into the genome of *Populus* × *euramericana* 'Neva' with the help of *Agrobacterium tumefaciens,* to develop transgenic poplar, which was highly resistance to *Lepidopteran and Coleopteran* pests (Satish et al., 2021). Other than Bt, many other genes, such as cowpea trypsin inhibitor (CPTI), cysteine proteinase inhibitor (Atcys) gene, *glycine max* trypsin proteinase inhibitor (KTi3 and PtdPP01 genes, etc.) were inserted into *Populus* species, which conferred some degree of resistance against insect pests (Table 3). La Mantia et al. (2018) observed that the overexpression of *Arabidopsis* AlgolS3 (AtGolS3) and *Cucumber sativus* Raffinose synthase (CsRFS) in *Populus alba* × *P. grandidentata* antagonizes leaf rust defense mechanism by inhibiting reactive oxygen species (ROS) and attenuating phosphatidic acid and calcium signaling pathways leading to salicylic acid (SA) defense. Lin et al. (2006) generated transgenic *P. simonii*×*P. nigra* plants by inserting the spider neurotoxin gene along with C-terminal of CryIA(B) gene resistance against *Lymantria dispar*. Moreover, the scorpion neurotoxin AaIT expression in hybrid *Populus* was responsible for developing resistance against the spongy moth (Lin et al., 2006).

TABLE 3 *Agrobacterium*-mediated transformation for imparting pest resistance in poplar species.

Poplar species	Gene	Targets	Percentage of transformation	Percentage of tolerance	References
P. alba ×P. grandidentat	Maize Ac transposable element and *Bt*	–	67–100% with Ac gene 67–75% with Bt gene	–	Howe et al. (1994)
P. alba×P. grandidentata	Cry1A	Spongy moth	–	91.9%	Kleiner et al. (1995)
P. deltoides × *P. simonii*	Bt	*Lymantria dispar* and *Clostera anchoreta*	17.8%	45%	Rao et al. (2001)
P. tremula × *P. tremuloides*	Cry3Aa	*Chrysomela tremulae*	–	100%	Génissel et al. (2003)
P. simonii × *P. nigra*	Spider insecticidal peptide and Bt	*Lymantria dispar*	–	92%	Cao et al. (2010)
P. tomentosa Carr	Cry1Ac; API	*L. dispar* and *C. anachoreta* larvae	39.3%	80%	Yang et al. (2006)
P. euramericana	cry1AC-cry3A-NTHK1	*Hyphantria cunea and Plagiodera versicolora*	–	60% (*Hyphantria cunea*) 100% (*Plagiodera versicolora*)	Liu et al. (2016)
P. euramericana	cry1Ac, cry3A,	*Hyphantria cunea* and *Plagiodera versicolora*	–	42.2–66.7% (for *Hyphantria cunea 100%*) (for *Plagiodera Versicolora*)	Yang et al. (2016)
P. davidiana × *P. bolleana*	cry1Ac + SCK, cry1Ah3, cry9Aa3	*Lymantria dispar* and *Hyphantria cunea*	–	97% (for *Lymantria dispar*) 91% (for *Hyphantria cunea*)	Ding et al. (2017)
P. deltoides×P. euramericana	Cry1Ah1	*Hyphantria cunea*	–	90%	Xu et al. (2019)
Populus × *euramericana 'Neva'*	Cry1Ac Cry3A	*Lepidopteran* and *Coleopteran pests*	–	100%	Ren et al. (2021)
P. nigra L. *mtlD*	Cry3A, Cry1Ac	*Hyphantria cunea* larvae and *Plagiodera versicolora* larvae	–	More than 80% (for *Hyphantria cunea*) larvae 100% for *Plagiodera versicolora*	Zhou et al. (2020)
P. simonii × *P. nigra*	spider neurotoxin gene fused with C-terminal of cryIA(B) gene	*Lymantria dispar*	–	37 and 92%	Lin et al. (2006)
P. alba	ATCYS	*Chrysomela populi*	11%	77–100%	Delledonne et al. (2001)
P. alba × *P. grandidentata*	Arabidopsis AlgolS3 (AtGolS3) and Cucumber sativus Raffinose synthase (CsRFS)	Leaf rust	–	100%	La Mantia et al. (2018)
P. tremula × *P. alba*	PtdPPO1	*Malacosoma disstria*	–	50-fold greater PPO activity relative to untransformed controls	Wang and Constabel (2004)
P. tomentosa × *P. bolleana* × *P. tomentosa*	CPTI	*Malacosoma disstria* and *Stilpnotia candida*	–	40–55%	Zhang et al. (2005)

5.3 *Agrobacterium*-mediated transformation for disease resistance in poplar

A diverse array of bacterial and fungal attacks and viral infestation causes significant losses in the poplar yield. Transgenic poplars have various antibacterial and antifungal genes encoding proteins capable of breaking down mycotoxins and inhibiting cell-wall-degrading enzymes such as rabbit defensin (NP-1), osmotin, glucanases, chitinases (CH5B), lysozyme and thaumatin were able to combat pathogens mentioned in Table 4 (Zhao et al., 1999; Juge, 2006; Karlovsky, 2011; Thakur et al., 2021). Noël et al. (2005) generated

TABLE 4 *Agrobacterium*-mediated transformation for imparting disease resistance in poplar species.

Plant	Genes	Target	Percentage transformation	Percentage resistance	References
P. trichocarpa×P. deltoides and *P. trichocarpa×P. nigra*	Bacterio-opsin resistance	*Melampsora occidentalis and Septoria populicola*	–	Ineffective	Mohamed et al. (2001)
P. euramericana × P. canadensis and *P. nigra× P. maximowiczii*	AcAMP1,2 and ESF12	*Septoria musiva*	–	40%	Liang et al. (2002)
P. tremula×P. alba against	D4E1 resistance	*Xanthomonas populi*	–	57%	Mentag et al. (2003)
P. alba	BS	*Melampsora pulcherrima*	2.5%	40–63%	Giorcelli et al. (2004)
P. nigra × P. maximowiczii	ECH42	*Melampsora medusae*	–		Noël et al. (2005)
P. tomentosa × P. alba	Antisense and sense PtWRKY23	*Melampsora* species	–	Expression level was of 10-fold after infection	Boyle et al. (2010)
P. tomentosa	LJAMP2	*Alternaria alternata* and *Colletotrichum gloeosporioides (Penz.)*	–	–	Jia et al. (2010)
P. trichocarpa Torr. and *P. tomentosa Carr*	PtrWRKY18 and PtrWRKY35 transcription factors	*Melampsora* rust	–	Enhanced expression level of these genes	Jiang et al. (2017)
P. nigra L. × *P. maximowiczii* A. Henry (NM6)	MsrA2, N-terminally modified amphibian host defense peptide (HDPs)	*S. musiva*	–	95%	Yevtushenko and Misra (2019)

transgenic hybrid poplar plants harboring the ECH42 (*Trichoderma harzianum* endochitinase) gene responsible for imparting an enhanced level of resistance against *Melampsora medusa*, a leaf rust pathogen of poplar. Levée et al. (2009) functionally identified and characterized the transcription factor PtWRKY23 gene in *P. tomentosa* × *P. alba* whose silencing is responsible for enhanced susceptibility of transgenic poplars toward *Melampsora* infection. In addition, the overexpression of a transcription factor PtoWRKY60 in *P. tomentosa* clone 741 was noticed for conferring resistance to the fungal pathogen *Dothiorella gregaria* (Ye et al., 2014). Jiang et al. (2017) observed that over-expression of PtrWRKY18 and PtrWRKY35 transcription factors increased resistance in poplar transgenics against *Melampsora* rust. Hybrid *Populus* having over-expressed a wheat (*Triticum aestivum*) germin-like oxalate oxidase gene encoding enzyme responsible for metabolizing the oxalic acid molecules secreted by fungal pathogen *Septoria musiva*, showed delayed infection by the fungal pathogen (Liang et al., 2001). Interestingly, developing genetically engineered transgenic poplar resistant to bacterial pathogens is less common as bacterial damage is rare in poplar plantations. However, severe infections by *Xanthomonas* spp. on poplar plantations are reported (Ye et al., 2011). Mentag et al. (2003) generated transgenic *P. tremula* × *P. alba* having a gene encoding a synthetic antimicrobial peptide D4E1 imparting resistance to several fungal and bacterial pathogens. The nucleotide MsrA2 [N-terminally modified amphibian host defense peptide (HDPs) from *the skin secretion of arboreal frogs*] was inserted into the hybrid poplar *Populus nigra L.* × *P. maximowiczii A* through *Agrobacterium-mediated* transformation method. The peptide was reported to inhibit *S. musiva* conidia germination but is non-toxic to poplar (Yevtushenko and Misra, 2019). Certain viruses, such as the poplar decline virus, poplar mosaic virus, and arabis mosaic virus, pose significant threats to the poplar population by stunting plant growth and severely impacting wood biomass and quality (Pinon and Frey, 2005). To date, there have been no reports of developing transgenic poplar plants with improved viral resistance using the *Agrobacterium tumefaciens*-mediated transformation method. Therefore, this method holds potential for future use in enhancing viral resistance in poplar.

5.4 Gene gun-mediated transformations

The gene gum method (biolistic particle delivery system) has excellent potential in forest tree research. This physical method is commonly applied for genetic transformations of several plants. This method was first developed by Sanford and colleagues in 1982. The process involves the transfer of gold or tungsten microparticles (or microcarriers) coated with exogenous donor genes into receptor cells or tissues or organs with the help of accelerators like pressurized helium (He) gas and integration of genes into receptor genome and expression of the genes (Zhang et al., 2014; Cunningham et al., 2018). The efficiency of gene gun-mediated transformation depends on the factors, for example, types of receptors, culture and transformation conditions (Wang et al., 2018). In addition, this method is independent of plant genotypes compared to *A. tumefaciens*-mediated transformation. This method is commonly applied to generate transgenic poplars, as mentioned in Table 5 (Ozyigit and Yucebilgili Kurtoglu, 2020). The insect resistance Bt gene was co-transformed into *P. nigra* through gene gun mediated transformation protocol (Li et al., 2000). The Bt gene (cry3Bb) gene was successfully incorporated into the genome of poplar plastid through biolistic bombardment, generating transformed poplar with a mortality rate of 100% to *Plagiodera versicolora* (Xu et al., 2020). Wang et al. (2007) inserted three foreign *Bacillus subtilis* genes vitreoscilla hemoglobin (vgb), fructan sucrase (SacB), and bivalent stem borer resistance (BtCry3A + OC-I), and the regulatory gene (JERF3) into *Populus* × *euramericacana* 'Guariento' through particle bombardment method. No incorporation of pathogen and disease-resistant genes in poplar trees with the help of gene gun mediated transformation tools has been reported. The disadvantages of this gene gun-mediated transformation are low efficiency, silencing the transformed genes, inserting multiple gene copies and unstable expression of exogenous genes (Yin et al., 2021).

5.5 Protoplast transformation

The use of protoplasts for genetic transformation in plants has grown significantly in recent years. This technique involves introducing and incorporating exogenous genes into plant protoplasts, leading to the generation of transgenic plants with stable gene expression. The protoplast method has proven to be easy, fast, and efficient, with minimal or no interference from surrounding cells or the microenvironment (Yin et al., 2021; Adjei et al., 2023). Because of their versatility and efficiency, protoplast transformation systems have been optimized, established and applied to many recalcitrant non-model plants, along with the efficient delivery of several genes (Rehman et al., 2016; Naing et al., 2021; Ojuederie et al., 2022). This method is affected by several parameters such as explant types, tissue types, the composition of the digestion solutions, the pH of the digestion solution, the digestion time, the concentration of polyethene glycol (PEG) and the transformation time (Rezazadeh et al., 2011, Biswas et al., 2022). The protoplast transformation method is easy and efficient in annual herbaceous plants such as *Oryza sativa, Arabidopsis thaliana* and *Nicotiana tabaccum* (Jiang et al., 2013; Sun et al., 2018). The separation and transformation of protoplasts and regeneration from transformed protoplasts are difficult in forest trees. Advances have been made in PEG-mediated transformation method by applying liposome-mediated shock perforation and *A. tumefaciens* co-culture transformation method (Wu et al., 2014). The PEG-mediated method is the widely used protoplast transformation system in plants (Lenaghan and Neal Stewart, 2019). In addition, protoplasts can be transformed directly by imbibing DNA followed by PEG pre-treatment, microinjection, and electroporation. However, protoplast isolation and its transformation are complex and challenging for woody trees like poplar and have not been fully optimized and developed. Xu et al. (2020) used the leaf protoplast of poplar (*P. davidiana *P. bollaena*) to introduce cry3Bb genes for developing insect-resistant transgenic poplar. This method has not yet been utilized to generate transgenic poplar with pathogen-resistant genes.

5.6 Micro RNA mediated transformation

MicroRNAs (miRNAs) are endogenous, short, single-stranded, non-coding RNAs of 20–24 nucleotides, processed from hairpin RNA precursors by Dicer-like (DCL) enzymes. These are found in all eukaryotic cells and negatively regulate gene expression. After their discovery in plants, several miRNAs have been recognized with the help of high-throughput sequencing technology and bioinformatics and for there essential roles in regulating critical genes involved in plant-pathogen interactions at the transcriptional or post-transcriptional levels (Islam et al., 2022; Nizamani et al., 2023). According to the host and the specific pathogen, miRNAs can be up- or down-regulated, thereby promoting plant disease resistance by participating in hormone signaling and regulating and moderating resistance (R) genes (Yang et al., 2021). The first report of plant microRNAs was reported in *Arabidopsis* by Llave et al. (2002). Several studies established the pivotal roles of microRNAs in regulating biotic and abiotic stresses in several plants (Kar and Raichaudhuri, 2021). Transgenic poplar overexpressing miR159a (OX-159) showed enhanced resistance to necrotrophic fungi *C. chrysosperma* while enhanced susceptibility to infection by *L. populi (bacterial canker)* and hemi-biotrophic fungi *C. gloeosporioides* (Yang et al., 2023). Furthermore, in transgenic poplar (*P. trichocarpa*), miR472a positively regulated resistance to *Colletotrichum gloeosporioides* by targeting nucleotide-binding site and leucine-rich repeat domains (largest R proteins, NBS-LRR) and regulated negatively resistance to *Cytospora*

TABLE 5 Gene gun mediated transformation in poplar species.

Plant	Gene	Target	Percentage of transformation	Percentage of tolerance	References
P. alba× P. grandidentata and P. nigra× P. trichocarpa	Bt	*Malacosoma disstria* and *Lymantria dispar*	–	60% (for *Malacosoma disstria*) 24% (for *Lymantria dispar*)	McCown et al. (1991)
P. nigra	Chimaeric TA29-barnase gene	Insect	16.1	–	Li et al. (2000)
P. euramericana 'Guariento	SacB/vgb/BtCry3A, OC-I/JERF36/NPT II	*Coleopterus* insect	–	–	Wang et al. (2007)
P. davidiana ×P. bollaena	(Bt) cry3Bb	*Plagiodera versicolora*	–	100%	Xu et al. (2020)

chrysosperma (Su et al., 2018). miR156a was found to be the most stable miRNA examined as a reference gene in *P. tomentosa* under canker pathogen stress (Zhang et al., 2021). Several evidences proved that miRNAs can regulate and mediate biological processes during plant-insect and plant-viral interactions, ultimately conferring pest/viral resistance in plants (Zhang et al., 2022; Satish et al., 2021). To date, miRNA molecules have not yet been used to generate transgenic poplar with pest and viral resistance genes. We believe that with the growing recognition of miRNA molecules, as highlighted by the 2024 Nobel Prize, artificial mi-RNA holds the potential to be used for manipulating tree traits in the future.

5.7 RNA interference-mediated transformation

RNA interference (RNAi) is a naturally occurring cellular defense system in most eucaryotic cells. It is mediated by double-stranded RNA (dsRNA) as either a source of virus infection or because of transposon activity, both seeking need to be suppressed (Obbard et al., 2009). RNAi pathway involves the formation of several interfering molecules, i.e., small interfering RNAs (siRNAs) and microRNAs (miRNAs), generated through the activity of a dicer enzyme. These interfering molecules are then loaded on an RNA-induced silencing complex (RISC) containing argonaute protein (AGO). RISC directs the interfering molecules to their target gene, and homology-based cleavage of target mRNA occurs in the cells (Mamta and Rajam, 2017). RNAi has an important role in functional genomics research and is also a promising species-specific pest/pathogen management strategy in agroforestry (Mamta and Rajam, 2017; Joga et al., 2021; Mogilicherla et al., 2023; Sandal et al., 2023; Sellamuthu et al., 2024). RNAi tool is a sequence-based method that suppresses target gene expression for pest growth, development, and reproduction without affecting non-targeting other pest species (Whyard et al., 2009; Christiaens et al., 2020). Transgenic poplar plants harboring dsRNA targeting CYP6B53 from *Lymantria dispar* via *A. tumefaciens*-mediated transformation exhibited inhibited larval feeding and delayed growth (Sun et al., 2022). Such studies endorse the potential for using trees expressing dsRNA against target forest pests. RNAi-mediated lignin modification has also been successfully applied in poplar. The overexpression of microRNA, such as miR6443 reduces S lignin biosynthesis during shoot development in *Populus tomentosa,* making the plant susceptible to pathogens (Fan et al., 2020). Thus, RNAi can be utilized to modulate gene expression miR6443 to produce more lignin in poplar to confer resistance against pathogens.

5.8 Microparticle-mediated CRISPR DNA delivery for genome editing in poplar

The clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system is the most promising technique used for precise genetic engineering in plants, including poplar (Bewg et al., 2018; Anders et al., 2023; Sulis et al., 2023). This method is harnessed to improve sustainable production and introduce precise alterations at target sites, thereby altering plant architecture and floral development and developing biotic/abiotic resistance in trees (Borthakur et al., 2022). This method does not introduce foreign genes into the forest trees, making it safer than other genetic engineering methods. In this process, CRISPR gene-editing reagents, i.e., Cas9 protein and the guide RNA (gRNA), are generally delivered through *A. tumefaciens,* resulting in the stable genome integration and expression of the transfer DNA (T-DNA) in the plant genome (Hoengenaert et al., 2023). Alternative strategies other than *Agrobacterium*-mediated method for the delivery of gene-editing reagents into plant genomes are either through the expression of a gRNA- and Cas9-coding DNA/RNA or ribonucleoproteins (RNPs) into callus or protoplasts (Lin et al., 2018). However, this approach has drawbacks, such as inducing somaclonal variation and large genome rearrangements resulting in altered phenotype of plants (Serres et al., 1991; Fossi et al., 2019). Another commonly used method applies mechanical force like a gene gun to deliver gene-editing reagents coated with microparticles into plant tissue. Several researchers applied CRISPR-mediated gene editing for wood quality improvement and drought/pest/disease resistance in forest trees (Dort et al., 2020). In addition, microparticle-mediated DNA delivery technology has previously been used to deliver the CRISPR gene in poplar trees (Devantier et al., 1993; Nowak et al., 2004; Canto, 2016). Jang et al. (2021) and Huang et al. (2022) utilized this method for knocking out caffeoyl shikimate esterase (CSE) to improve lignocellulose biomass and root growth transcription factor PDNF-YB21 for repression of root and inducing drought resistance in transgenic poplar, respectively. However, it has not yet been applied to develop pest and pathogen resistance in poplar trees.

6 Regeneration methods used in *Populus* species

An efficient regeneration system is crucial for successful genetic transformation, as it enables the development of transgenic plants from a single cell carrying the desired genes. However, genetic transformation and regeneration remain significant challenges in many forest trees, including poplar. Various plant regeneration methods have been developed for poplars (Thakur et al., 2005; Li et al., 2017), which can be employed to produce transgenic trees with resistance to pests and diseases. In recent decades, significant research efforts have focused on creating transgenic poplars with enhanced resistance to abiotic stress and improved wood quantity and quality. There have been a few reports on regenerating transgenic poplar trees with biotic stress resistance. The established suspension cultures of *P. alba x P. grandidentata* cv. 'Crandon' were transformed with vectors *A. tumefaciens* carrying the maize Ac transposable element and an insect toxin gene isolated from *Bacillus thuringiensis* (Bt). These transgenic plants were regenerated by subculturing the transformed callus on the medium, supplemented with a growth regulator Thidiazuron (TDZ) of 0.11–27.0 μM (Howe et al., 1994). *A. tumefaciens* mediated genetic transformation and regeneration of hybrid poplar (*P. alba x P. grandidentata*) and transgenic quaking aspen from cuttings from young leaves were also readily achieved (Tsai et al., 1994). *A. tumefaciens*-mediated transformation of leaf explants of *P. nigra* L. was done with a Kunitz trypsin proteinase inhibitor (KTi3) gene for pest resistance, and regeneration of this transgenic leaf explants was successfully achieved (Confalonieri et al., 1998). The stems and petioles of transformed hybrid aspen (*Populus tremula* × *P. alba*) clones containing PtdPPO1 genes (conferring pest and pathogens resistant in plants) of *in vitro* plantlets were used for regeneration (Wang and Constabel, 2004). Further research is needed to establish a protocol for regenerating transgenic poplars with enhanced resistance to pests and pathogens from modified cells.

7 Conclusion and future perspectives

Poplars play a crucial role in supporting global ecological and socioeconomic wellbeing. The growing demand for poplar products has driven genetic engineering efforts to enhance various traits, particularly pest and disease resistance, as these trees are highly susceptible to numerous pests, fungi, and viruses. Considering the long growth cycle with low transformation tendency in forest trees, including poplar trees, it is necessary to establish a stable and efficient transformation system. Adopting pest and disease-resistant transgenic poplar plants to minimize yield loss and pesticide consumption has been successful. Many researchers have employed genetic transformation methods, including *Agrobacterium tumefaciens*, protoplast, gene gun, RNA interference, and miRNA-mediated transformations, to improve poplar resistance to pests and pathogens. These techniques, along with genome editing to introduce pest resistance genes and modulate lignin biosynthesis, offer promising avenues for developing transgenic poplar trees capable of withstanding pest and disease attacks, thus improving their survival rates. However, no research has been conducted on the pollen tube method, *A. rhizogenes* mediated and nanoparticle-mediated transformation to enhance pest/pathogen or virus resistance in *Populus* species. Further efforts are required to establish transgenic poplars with single/multiple genes for increasing biotic stress tolerance limits (pest/pathogens infestations) using nanoparticles, *A. rhizogenes*, and the pollen tube method. It will be optimal if the methods developed in poplar can be used in other forest trees to make them resistant to biotic and abiotic stresses. The use of omics technologies (i.e., genomics, transcriptomics, and proteomics), along with high-throughput screening and selection methods, accelerates the identification of successful transgenic poplar lines. Integrating big data, machine learning, and artificial intelligence (AI) into poplar breeding programs (i.e., data-driven breeding) can enhance the accuracy of predicting genetic alteration outcomes (Farooq et al., 2024), enabling more targeted and efficient transgenic strategies for trees. A key factor for success is the competence to regenerate transgenic plants from modified cells. Addressing the genotype dependency in poplar transformation is crucial for expanding the applicability of transgenic approaches. Developing "transgene-free" or non-GMO techniques, such as transient CRISPR expression, could alleviate regulatory and public concerns, facilitating the adoption of genetically improved poplar.

Author contributions

AS: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. SS: Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. AC: Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. AR: Formal analysis, Funding acquisition, Investigation, Resources, Software, Visualization, Writing – review & editing. IS: Data curation, Formal analysis, Investigation, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

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

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Ectomycorrhizal response to bark beetle attack: a comparison of dead and surviving trees

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Bark beetle outbreaks have become increasingly prevalent and intense, causing widespread tree mortality and altering forest ecosystems globally. In this study, we investigate the dynamics between tree root systems of the Norway spruce and ectomycorrhizal fungi in the aftermath of bark beetle-induced tree mortality, focusing on the changes in density of vital and non-vital mycorrhizal tips. The survey was carried out in the Bohemian Switzerland National Park, Czechia. The sampling sites were chosen based on polygon layers delineating individual years of mortality status, obtained by PlanetLab imagery for 2018–2022, classified by Support Vector Machine, a machine learning tool (SVM). Fieldwork involved the collection of soil and root samples. Mycorrhizal tips were examined and counted using a stereomicroscope. Soil pH and root dry weight were determined. Wood decay fungi were identified using a comprehensive approach, combining macroscopic examination with genetic analysis. Due to the favorable conditions, the density of vital mycorrhizal tips (VM) on living trees gradually increased, peaking in the 2nd and 3rd years after the surrounding forest decay. VM on bark beetle snags was significantly lower compared to living trees, with minimal variation over time. The dry biomass of fine roots was significantly greater in living trees. Fine root abundance showed a slight decrease over time in living and dead trees; however, there was a lack of statistical significance. Most of the fine root biomass decomposes within the first half year after tree death. This might be influenced by wood decay fungi identified on dead trees, including genera *Armillaria*, *Fomitopsis*, and *Pleurotus ostreatus*. Overall, the study provides insights into the complex dynamics of mycorrhizal associations, root system biomass, and wood decay fungi in the aftermath of bark beetle-induced tree mortality. Our study demonstrates that mycorrhizal activity increases in trees surviving a bark beetle disturbance, and they probably become another ECM refugia. This research contributes valuable insights into the ecological consequences of bark beetle infestations on below-ground interactions, offering a deeper understanding of forest ecosystem responses to widespread tree mortality. Such knowledge is essential for developing effective strategies to manage and mitigate the ecological impacts of bark beetle outbreaks in forested environments.

KEYWORDS

Norway spruce, *Ips typographus*, root, deadwood, disturbance, forest health, stability, conservation forests

Introduction

Dead trees play a key role in natural forest ecosystems (Rondeux and Sanchez, 2010). Since they are associated with a wide range of microorganisms, animals, and fungi, dead trees contribute significantly to forest biodiversity (Stokland et al., 2012). Deadwood also binds carbon and other biogenic elements, allowing them to return gradually to forest soils (Russell et al., 2015). In general, the emergence and presence of dead trees strongly influence the dynamics of forest ecosystems in the temperate zone (Král et al., 2018). Production-oriented silviculture, which has an over 300-year-long tradition in Central Europe, changed the composition of the forest to spruce-dominated stands and has practically ruled out the presence of dead trees (Dieler et al., 2017). The occurrence of dead trees was limited only to small and isolated forest reserves. This situation changed in the 1990s when non-intervention management began to be implemented in large Central European protected areas (Parviainen et al., 2000). This led to an increase in snag deadwood in relatively large areas, such as the Bavarian Forest (Müller et al., 2008), the Bohemian Forest (Svoboda, 2005) or the Tatra Mountains (Potterf et al., 2019), due to the European spruce bark beetle *Ips typographus* (L.). The spruce bark beetle is a key species that maintains spatial, structural, and age heterogeneity of the spruce forests and provides higher resistance and resilience against most of disturbance agents (Hlásny et al., 2021a). Over the past 20 years, however, there has been a doubling in bark beetle-induced forest disturbances on a Europe-wide level (Patacca et al., 2022), Central Europe's epicenter. As a result of the ongoing bark beetle outbreak in the Czech Republic, there has been a dramatic increase in dead trees extending beyond protected areas into a large proportion of production forests (Hlásny et al., 2021b; Washaya et al., 2024). Despite the apparent positive impact on biodiversity and carbon storage (Vítková et al., 2018), the increased presence of dead trees raises several questions concerning their stability and, thus also, the safety of the public, forestry personnel, machinery, and infrastructure (Mortimer and Kane, 2004; Schmidlin, 2009).

Degradation processes in the spruce deadwood lead to a gradual change in the physical properties of snags (Löwe et al., 2022; Jelonek et al., 2020), eventually resulting in their collapse. Depending on the tree's species and size, climatic conditions, and the management of its surroundings, the tree collapses usually within 3 to 20 years after its death (Oettel et al., 2023; Gärtner et al., 2023). Following death by fire, the average time for the spruce to fall is 32 years to collapse (Aakala, 2010). However, trees killed by bark beetles collapse on average after only 13 years (Gärtner et al., 2023). With the Norway spruce, *Picea abies* (L.) Karst., in the boreal areas of Europe, an average time to fall of 12 to 27 years was determined (Aakala, 2010); however, the cause of death was not considered. In the case of the lodgepole pine, *Pinus contorta* Douglas ex Loudon, killed by *Dendroctonus ponderosae* Hopkins, half of the snags fell within 15 to 20 years (Rhoades et al., 2020). According to Rhoades et al. (2020), 53.2% of the dead lodgepole pines fell due to butt rot (i.e., breakage at or below ground level with visible signs of decay), 14.3% due to bole snap (i.e., breakage at 1 m above ground or higher) and 32.5% of trees were tipped up (i.e., trees remained attached to exposed roots and soil mound). Decaying root systems likely cause the decrease in stability of bark beetle snags and their resulting tip-up. However, the long persistence of dead trees, in the case of the spruce, even several decades (Přívětivý et al., 2018), suggests that root degradation takes longer than might be expected.

The spruce's root system dynamics are mainly determined by soil conditions, such as pH or moisture (Puhe, 2003). Suboptimal soil conditions stimulate the formation of mycorrhizal associations, which facilitate tree nutrition (Cheng et al., 2016). Mycorrhizal fungi form symbiotic connections with most terrestrial plants (Read, 1998). The relationship between a mycorrhizal fungus and its plant symbiont is based on a mutually beneficial exchange of nutrients. The fungus provides minerals (especially nitrogen and phosphorus); in return, the plant provides photosynthetically produced carbon compounds (saccharides). Simultaneously, the hyphal threads of the mycorrhizal fungus provide the plant with a significantly increased surface area where nutrient ions can be absorbed (Maheshwari, 2008). Within a functional mycorrhizal symbiosis, the above-ground biomass and the root network of the host plant increase considerably compared to plants without mycorrhizal associations. This results from an adequate supply of nutrients and water through hyphal threads that are three to five times thinner than root hairs and thus have access to areas of the soil that remain inaccessible to root hairs (Graf et al., 2018). For example, measurements of mycorrhizal hyphae associated with roots of *Pinus sylvestris* L. have yielded values of 10–80 m of hyphae per 1 cm of root length (Read and Boyd, 1986). This results in a vastly increased surface area and, consequently, a much greater volume of soil that the host plant can exploit (Jansen, 1992). Thus, apart from their significant role in plant nutrition and their influence on species composition of plant communities, mycorrhizal fungi also affect soil aggregation and soil stability by weaving together free soil particles and binding them with sticky substances, such as saccharides and other extracellular metabolites (Rillig and Mummey, 2006; Enkhtuya and Vosátka, 2005). Based on these findings, it can be supposed that the presence of mycorrhizae in a tree's root system influences its mechanical stability within the soil. The presence of mycorrhizae is, therefore, not only an indicator of a tree's vitality but also of its mechanical stability.

Ectomycorrhizal fungi (ECM) encompass a great diversity of species. Tens of species can be found even in small forest monocultures (Bruns, 1995). In coniferous forests, they represent roughly one-third of the microbial biomass (Högberg and Högberg, 2002). It is supposed that the activity of an ECM species is directly proportional to the number of mycorrhizal tips (Landeweert et al., 2003). The vitality of mycorrhizae on a given root system can be determined by comparing the numbers of vital and non-vital ECM tips (Pešková, 2007; Corcobado et al., 2014). The number of ECM tips per milligram of fine root biomass is constant. Trees increase nutrient uptake by increasing fine root production, hence the number of ECM tips (Helmisaari et al., 2009). Studies of fine roots and mycorrhizae of the Norway spruce were conducted by Blanck et al. (1995), Feil et al. (1988), Majdi (2001), Palátová (2004), Gaul et al. (2008), Eldhuset et al. (2012), and Konôpka et al., (2013). Morphological properties of ECM roots provide a good indication of a tree's adaptation to local conditions. These include both absolute markers (e.g., root length, root surface area, root diameter, or number of ECM tips) and relative markers, usually related to dry root biomass (e.g., specific root length, surface area and diameter, and specific number of ECM tips) (Peterson et al., 2004; Ostonen et al., 2013). Specific root length (i.e., length divided by mass) is probably used most commonly. The most significant changes of specific length in reaction to changes in the soil environment can be observed with fine roots. An increase in specific root length gives access to a greater volume of soil and is thus one possible adaptation of plants ensuring sufficient nutrient supply (Ostonen et al., 2007).

The changes that occur over time in a root system following a tree death by bark beetles have not been directly quantified. Bark beetle disturbances are known to lead to wide-ranging changes of forest ecosystems, both on the macroscopic and microscopic levels. *I. typographus* is the major disturbance agent in Norway spruce dominated forests, causing ecosystem changes in case of eruptive outbreaks. *Ips typographus* occurs almost the entire Palearctic region: from mountaineous regions in the south to the lowlands in the north. According to the climatic region and current condition, *I. typographus* can have one to three generations during vegetation season. The tree is colonized by a pheromone-mediated mass attack of beetles. If successful, the infested tree dies after a short period (Lieutier et al., 2004; Wermelinger, 2004; Kausrud et al., 2012; Vega and Hofstetter, 2015, Hlásny et al., 2021a). A gradual discoloration and shedding of needles occur when a tree is infested (Kautz et al., 2022). This results in a gradual decrease in the supply of photosynthetic products to the soil (Štursová et al., 2014). On a macroscopic scale, a fungal community reacts to bark beetle-induced tree dieback by a significant decrease in overall fungal biomass and by changes in its species composition. While mycorrhizal species dominate at first, the relative abundance of saprotrophic species gradually increases. The overall species composition, however, remains constant. This suggests that mycorrhizal species can persist for a long time, even without symbiotic trees. Some species, such as Russulales, can switch from a mycorrhizal to a saprotrophic mode, thus persisting in the community and contributing to wood decomposition (Veselá et al., 2019).

Following the death of the above-ground part of a tree, the root system begins to disintegrate. At first, mycorrhizal community changes, followed by the decomposition of root hairs and fine roots. Later, more massive roots are also decomposed. This work compares the root systems of bark beetle snags at different stages after dying to the root systems of nearby living trees. The work aimed to determine the extent of root system degradation over 5 years from tree death using ECM tip counts as a degradation marker. Concomitantly, the rate at which mycorrhizal parameters change following tree death was investigated. We addressed these main research questions: (i) How long do VM and NVM persist on dead trees? We suppose the immediate decline of the VM after tree death and the successive disappearance of the NVM. (ii) The second research question is connected to the stability of the dead trees through the root system. We assume that roots smaller than 2 mm will degrade quickly following the tree's dead. (iii) The last question is the species representation of wood fungi in the decomposition process in different years after a tree dead.

Materials and methods

The study was carried out in the Bohemian Switzerland National Park located in northwest Czechia on the eastern bank of the river Elbe, on the borders of Saxony (DE). This is a warm to temperate area with average yearly temperatures between 6 and 8°C and annual precipitation of 800 mm (Tolasz et al., 2007). The 79 km^2 area has a high relief and is mostly forested. Relict pinewoods occupy sandstone platforms, while lower areas are populated by various cold-adapted plant communities.

However, most of the national park is covered with non-native pure and even-aged stands of Norway spruce – *Picea abies* (L.) H. Karst. In 2018, an outbreak of the European spruce bark beetle, *Ips typographus* (L.), occurred in the area. The infestation began in the warmer western areas and gradually spread across the national park. During the first 2 years of the outbreak, partial sanitation harvests were undertaken. From 2019 onwards, however, all interventions aimed at containing the spread of the bark beetle were abandoned, due to possible massive clear-cuts resulting from a bark beetle intervention, which are unsuitable in a national park. By the end of 2022, 19,953 ha of spruce forest had been destroyed by the bark beetle in the national park and surroundings (Washaya et al., 2024). In 2022, a large wildfire broke out in the western regions of the park (Kudláčková et al., 2023). The area affected by fire was excluded when selecting sites for sample collection.

Due to the extensive large-scale changes in the national park forests, remote sensing techniques were used to monitor the development of bark beetle disturbance. The chronosequence of dead tree areas was provided from an inventory of the mortality dynamics. The data from PlanetScope satellites were used; they were obtained for years 2018–2022 (Figure 1) at the end of the thermal growing season (mainly September and the first half of October, depending on the cloud coverage), 1 pixel was referred to 3 × 3 meters. The rasters were processed by the Support Vector Machine (SVM) machine learning algorithm in ArcGIS Pro for every period dataset, and the spread and enlargement of attacked trees were delineated based on the color change in the given area. The training and validation samples were supported by visual verification corresponding to available aerial imagery in 2020 and 2021. A complete description of the methods, including the classification details, can be found in Matějčíková et al. (2024).

These layers were then matched with data from the forest management plan and, using an attribute query, areas with the following parameters were selected: more than 90% spruce, 80–100 years of age, in *Abies* – *Fagus* vegetation zone, acidic, somewhat stony and meagre (5 K resp. 5 N by Viewegh et al., 2003). These parameters correspond to the most common spruce stands in Bohemian Switzerland. The classified stands were randomly visited, and if the surviving spruce was found, a dead tree with a similar dimension was chosen in the nearby surroundings (See Figure 2i). The selection continued until a balanced number of strata for each year of tree death was achieved.

In total, samples were collected on 17 sites corresponding to different years of tree dieback (Figure 1; for details of layers, see Supplementary material 1). Sample collection was performed using a soil sampling probe with a 6 cm inner diameter and 15 cm sample tube length (Pešková, 2007). On each site, two trees were selected - one snag and a nearby living tree of similar dimensions. Five soil samples were collected randomly up to 1 m from the bole of the living tree and snag (2 × 5 samples, see Figure 2). The bark beetle snag was subsequently felled, and samples were taken in places with visible wood decay. These were used for the macroscopic determination of wood-decaying fungi. Samples were also collected for DNA analysis, they were held in stored in 35% ethanol at −32°C.

In the laboratory, roots were manually extracted from the soil samples and separated according to diameter into <1 mm, 1–2 mm,

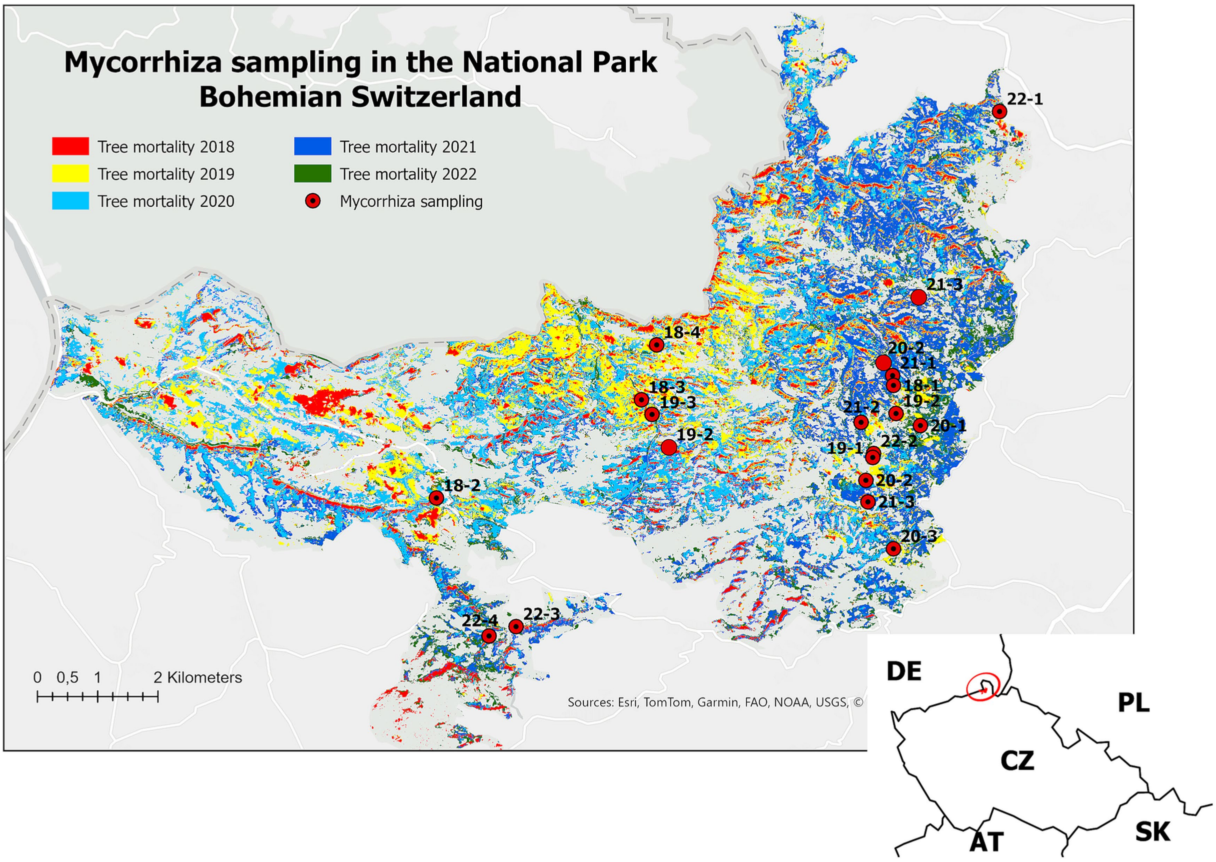

FIGURE 1
Map of the national park Bohemian Switzerland with indicated mycorrhiza sampling locations and classified layer of the tree mortality in 2018–2022. Mycorrhiza sampling sites are labeled by the year of stand death (18–2018, etc.) and the serial number of the location.

and 2–5 mm fractions. Roots smaller than 1 mm in diameter were stored in 2.5% glutaraldehyde for further analysis. Roots 1–2 mm and 2–5 mm diameter were dried in a drying cabinet at 105°C for 24 h and weighed with 0.01 g precision. Numbers of mycorrhizal tips were determined on root segments with a diameter of <1 mm using a stereomicroscope with 40× magnification. Smooth, light-colored ECM tips with a well-developed hyphal sheath and high turgor were considered "vital mycorrhizae" (VM). In contrast, flaccid, dark-colored ECM tips without a visible hyphal sheath were categorized as "non-vital mycorrhizae" (NVM) (Corcobado et al., 2014). From each soil sample, 20 root segments (5 cm in length) were evaluated this way. The density of mycorrhizal tips was calculated as the mean number of VM or NVM per 1 cm of root length (Pešková et al., 2015). Soil pH (H_2O) was determined in each soil sample by suspending 10 g of soil in 100 mL of 0.1 M aqueous calcium chloride and measuring the pH using a pH 50 VioLab meter (Germany) with a glass electrode until a constant value was displayed. The measurements were carried out in triplicates.

Identification of fungal species from the trees where mycorrhizae were evaluated involved a multi-faceted approach: (i) observation of fruiting bodies on the bole and stump, (ii) search for rhizomorphs and/or mycelial fans beneath the bark, and (iii) assessment of wood rot. Fruiting bodies found on the boles or stumps of the trees were collected for further determination using the identification key for European polypores (Ryvarden and Melo, 2014). If wood rot was present, trees were inspected in more detail. Additionally, samples of rhizomorphs were collected from beneath the bark of stumps or root swellings. Samples of decaying wood were also collected from the felled trees. In the laboratory, wood samples were cut into smaller pieces (3–4 mm) with a sterilized scalpel, superficially sterilized in 70% ethanol, and washed with distilled water. The sterilized pieces were placed onto a malt extract agar (MEA; 33.6 g/L of Malt Extract Agar, Carl Roth), with 4–5 pieces per Petri dish. The samples were incubated at 20°C in the dark, and after the appearance of the first hyphae, they were promptly subcultured onto a fresh MEA medium. Collected rhizomorphs were cut into smaller parts using a sterilized scalpel, superficially sterilized using 70% ethanol and sodium hypochlorite, and subsequently washed with distilled water. These samples were plated onto a MEA amended with 100 mg/L streptomycin (Carl Roth). Additionally, samples of rhizomorphs and mycelium growing beneath the bark were collected and stored in 35% ethanol at −32°C for further molecular analysis, employing the same procedures as the fungal cultures. All obtained isolates were morphologically studied and identified using fungal DNA barcoding. Mycelium taken from developed cultures was lyophilised and used for genetic identification. DNA from these samples was isolated using a modified CTAB-PVP method (Porebski et al., 1997; Schenk et al., 2023). The primer combination ITS1/ITS4 for amplifying the ITS region of the ribosomal RNA gene and LR3/LR0R primers (nuclear large subunit rDNA) were used for PCR. The PCR products were

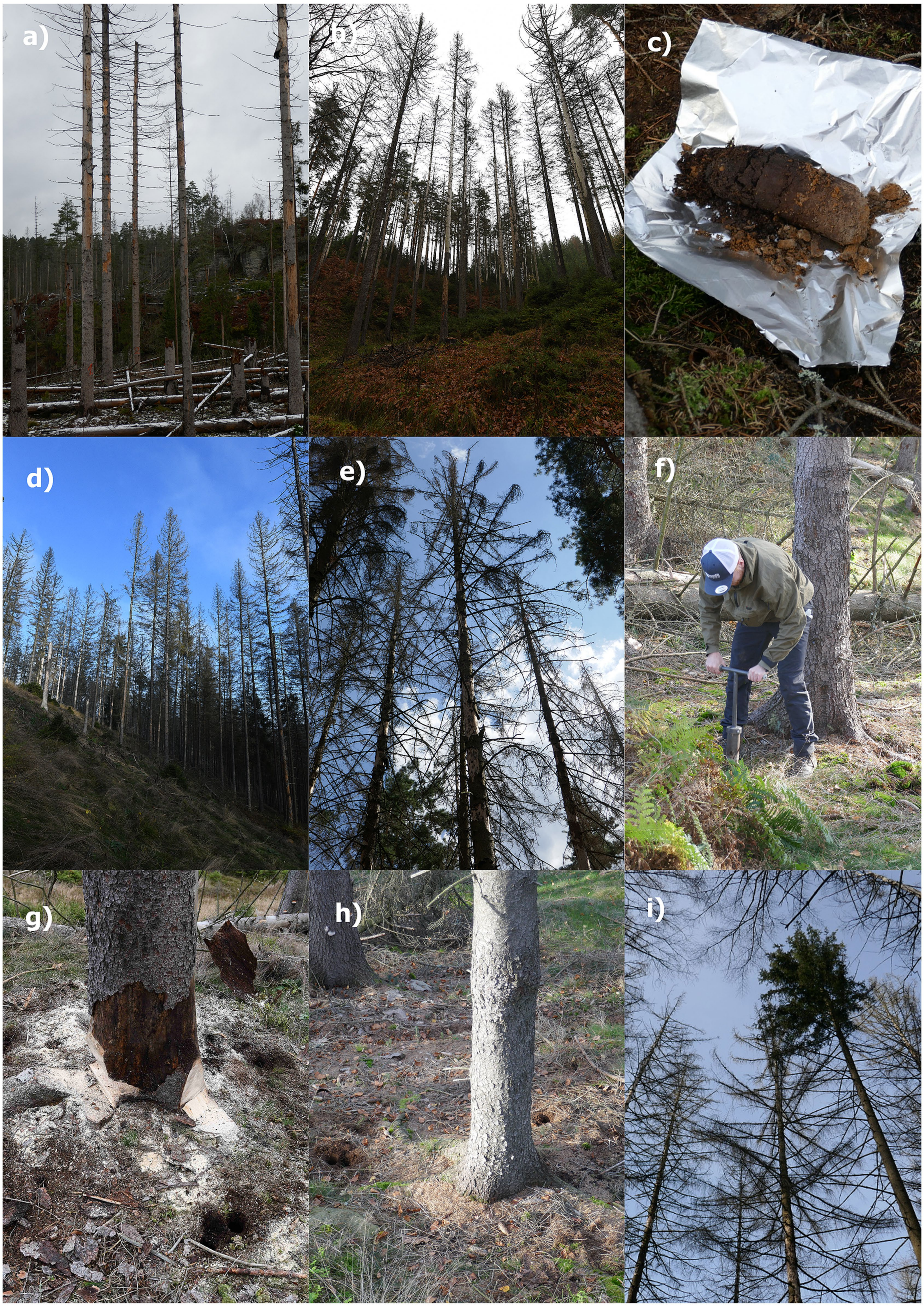

FIGURE 2
Field sampling: **(a)** dead standing trees 4 years after disturbance; **(b)** death standing trees 3 years after disturbance; **(c)** soil and root sample; **(d)** death standing trees 2 years after disturbance; **(e)** death standing trees 1 year after disturbance; **(f)** collection of soil and root samples using a sampling probe; **(g)** dead tree sampling; **(h)** living tree sampling; **(i)** a surviving tree surrounded by bark-beetle snags.

sequenced using the Sanger sequencing method by SEQme s.r.o. (Dobříš, Czech Republic). The nucleotide Basic Local Alignment Search Tool (BLAST) was used to compare the obtained sequences with those in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov, accessed on 15 October 2023).

The differences between snags and live trees, regarding densities of VM and NVM and dry root biomass, were assessed using independent regression models created according to Zuur et al. (2010). The independent variables were (i) *Tree*, a categorical variable with two levels – bark beetle snag and live tree; (ii) *Year*, an ordinal variable representing the time elapsed between tree death and sample collection with five levels – 0.5 years; 1 year; 2 years; 3 years; 4 years. Since the live and dead trees originated from the same site and multiple samples were taken from the same tree, a generalized mixed effect model (GLMM) with the two-time nested factor was applied. A suitable family of distributions was chosen according to the Akaike information criterion (AIC). The fit quality was evaluated visually by plotting the model's residuals against the quantiles of a normal distribution. In all cases, the negative binomial family was most suitable. The significance of factors was subsequently determined by χ^2 tests using the command *drop1*. Differences between the levels of *Tree* and *Year* were tested in the selected model using a contrast matrix (Pekár and Brabec, 2016). The model formulation was performed in R version 4.3.1 (R Core Team, 2023) in the package *glmmTMB* following the procedures described by Brooks et al., (2017). The connections between Tree mycorrhizal parameters (*Roots12* – mean dry weight mass of roots with 1–2 mm; *Roots25* – mean dry weight mass of roots with 2–5 mm; *VM* – mean density of vital mycorrhizae; *NVM* – mean density of non-vital mycorrhizae) and environmental, respectively, specific factors (*pH* – mean pH of soil samples, *decay* – sum of trees with wood decaying, *tree* – categorical variable: Dead standing tree / Living tree) were determined using a principal component analysis (PCA) according to Meloun and Militký (2011) and visualized using the *factoextra* package (Kassambara and Mundt, 2020).

Results

Mycorrhizal density and dynamics

The density of vital mycorrhizae on bark beetle snags was significantly lower compared to living trees (χ^2: df = 1; $p < 0.001$). The number of VM tips changed with different dynamics between living and dead trees (χ^2: df = 4; $p < 0.01$). On living trees, a gradual increase of VM, peaking in the 2nd and 3rd year after the bark beetle disturbance of the surrounding forest stand, was observed (Figure 3). The difference between VM density on live trees in the 1st, 2nd, and 3rd year after the bark beetle disturbance and VM density 0.5 years after the disturbance was statistically significant (Table 1). In the 4th year, however, a pronounced decrease in VM was observed (Figure 3). The density of VM on the root systems of dead trees was very low from the beginning and randomly fluctuated in later years (Figure 3, Table 1, and Supplementary Table S1). A statistically significant

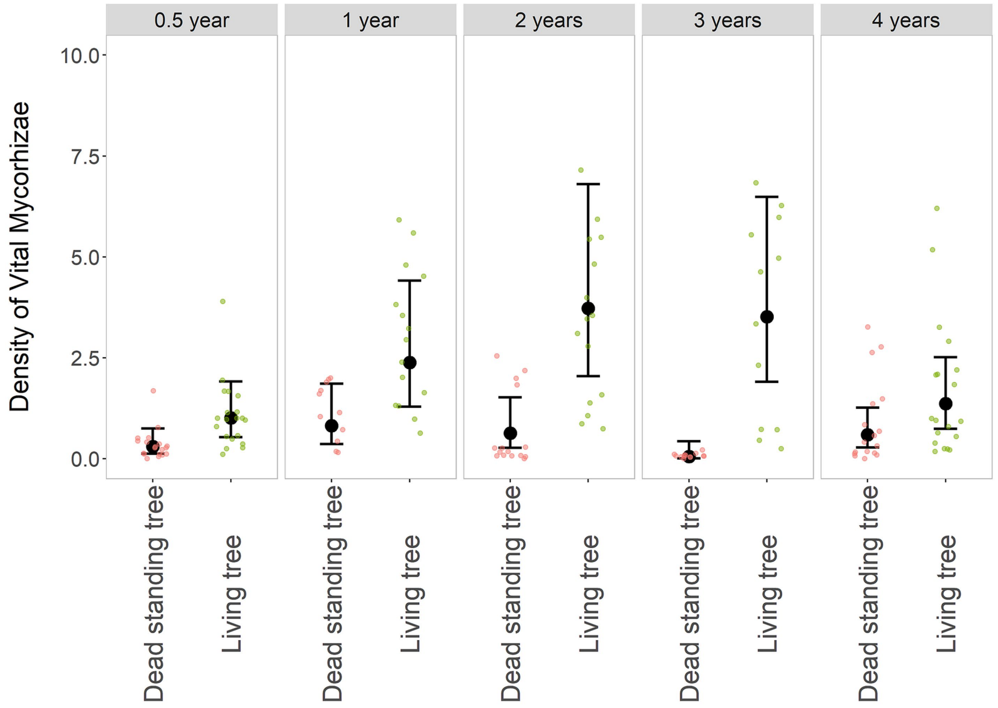

FIGURE 3
Density of vital mycorrhizae (VM) in dead and living trees over time. The black dot represents the fitted value and whiskers 95% confidence intervals of the GLMM model *VM ~ Tree * Year + (1 | Locality | Individual)*, family = negative binomial. The smaller dots in the background are original values, red-dead standing trees, and green-living trees. A comparison of the dead and living trees is grouped along the years from the disturbance of the surrounding forest stand, resp—the tree death.

TABLE 1 Result of the contrast comparison (*p*-values) for GLMM model *VM ~ Tree * Year + (1 | Locality/TreeIndividual)*, family = negative binomial.

Years	Relative density of VM		Live vs. Dead Tree
After disturbance	Dead trees	Living trees	
0.5	Reference level	Reference level	0.017 *
1	0.075	0.020 *	0.014 *
2	0.233	0.002 **	9.94E-05 ***
3	0.126	0.001683 ***	7.17E-05 ***
4	0.208	0.411	0.0416 *

The density of vital mycorrhizae (VM) was compared to the density determined by 0.5 years after stand disturbance. The last column represents a comparison of the two types of trees at a given point in time. Stars mark a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

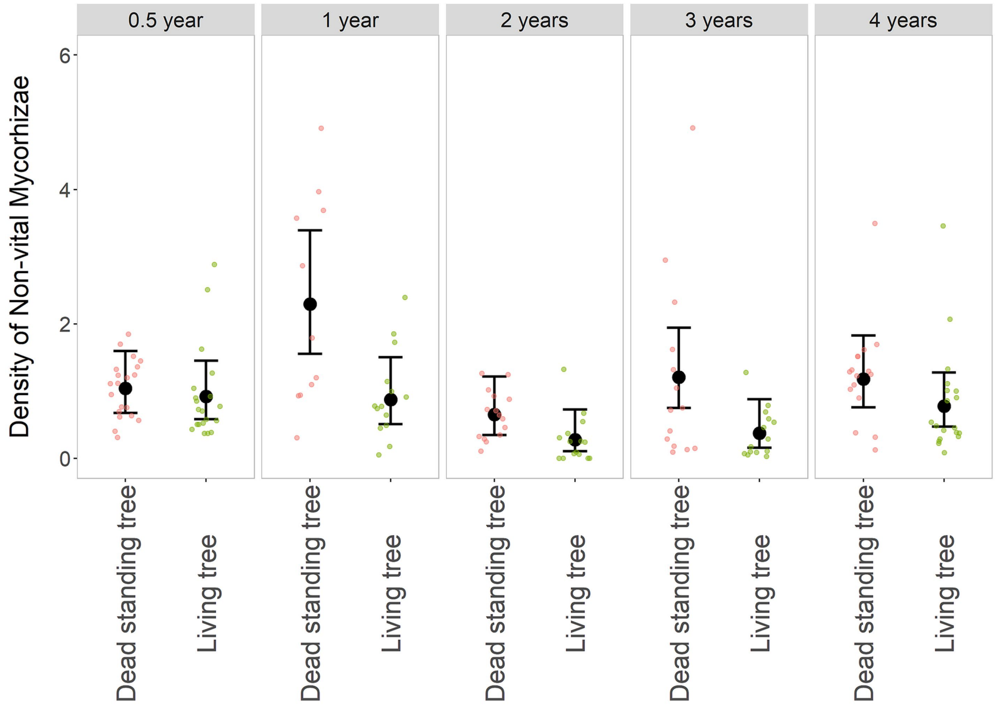

FIGURE 4
Relative density of non-vital mycorrhizae (NVM) in dead and living trees over time. The black dot represents the fitted value and whiskers 95% confidence intervals of the GLMM model *NVM ~ Tree * Year + (1 | Locality | Individual)*, family = negative binomial. The smaller dots in the background are original values, red-dead standing trees, and green-living trees. A comparison of the dead and living trees is grouped along the years from the disturbance of the surrounding forest stand, resp—the tree death.

difference was observed in all periods when comparing VM density between live and dead trees (Table 1).

The density of non-vital mycorrhizal tips was significantly higher on snags compared to living trees (χ^2: df = 1; $p < 0.001$) and did not change as markedly over time as the density of VM. Nevertheless, it was unstable (χ^2: df = 4; $p < 0.01$). The dynamics of change in the density of NVM was also significantly different between live and dead trees (χ^2: df = 4; $p < 0.01$). With dead trees, a significant increase in NVM was observed within the 1st year of tree death (Figure 4, Table 2, and Supplementary Table S2). This is likely due to a change of vital mycorrhizal tips to non-vital ones. After the 2nd and 3rd years, living trees had a low density of NVM but did not differ owing to the situation 0.5 years after the disturbance (Table 2). A significant difference between dead and surviving trees was observed in the 1st and 3rd year (contrast *t*-test; $p < 0.01$ and $p < 0.05$, respectively), where bark beetle snags had a higher density of NVM tips.

Root system biomass

The volume of the root system, as represented by the dry biomass of roots smaller than 2 mm in diameter, was significantly greater in living trees (χ^2: df = 1; $p < 0.01$). The abundance of fine roots gradually decreased as the surrounding tree cover died (Supplementary Table S3). However, the change between individual periods was statistically insignificant (χ^2: df = 4; $p = 0.12$). A higher ratio of non-vital mycorrhizae was associated with dead trees. The gradual decrease in root biomass in living and dead trees also occurred at a similar rate.

TABLE 2 **Result of the contrast comparison (*p*-value) for GLMM model *NVM ~ Tree * Year + (1 | Locality/TreeIndividual)*, family = negative binomial.**

Years	Relative density of NVM		Live vs dead tree
After disturbance	Dead trees	Living trees	
0.5	Reference level	Reference level	0.705
1	0.007 **	0.889	0.005 **
2	0.228	0.028 *	0.147
3	0.649	0.069 ·	0.019 *
4	0.686	0.624	0.218

The relative density of non-vital mycorrhizae (NVM) of dead trees and living trees was compared to the density determined 0.5 years after stand disturbance. The last column represents a comparison of the two types of trees at a given point in time. Stars mark a significant difference (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

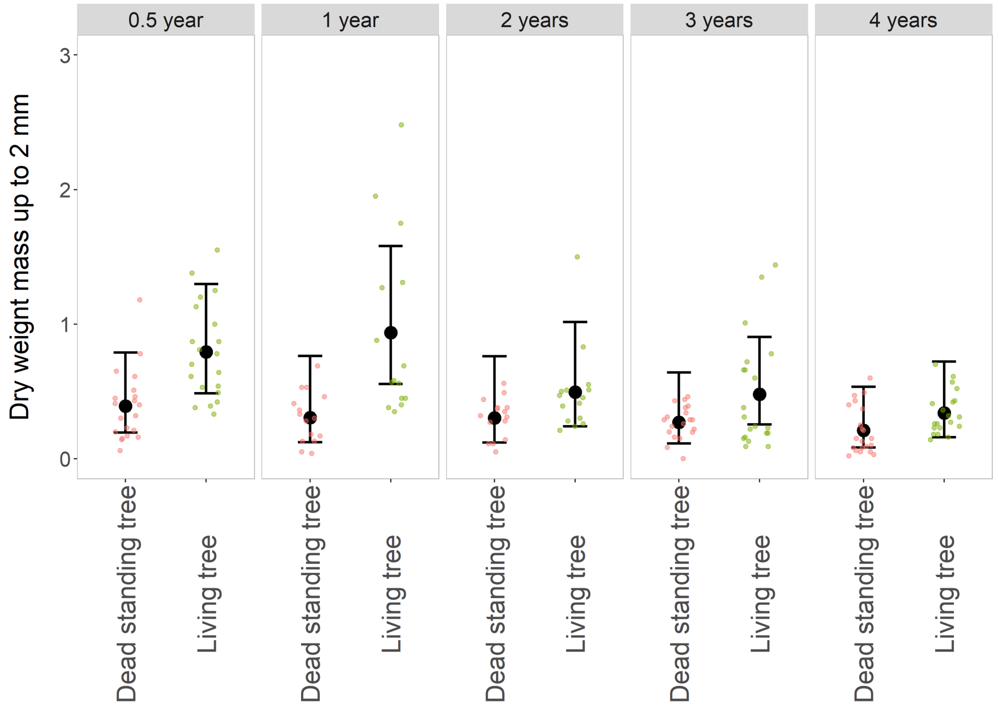

FIGURE 5
Dry biomass of roots up to 2 mm in diameter. The black dot represents the fitted value and whiskers 95% confidence intervals of the GLMM model *Roots < 2 mm ~ Tree * Year + (1 | Locality | Individual)*, family = negative binomial. The smaller dots in the background are original values, red-dead standing trees, and green-living trees. A comparison of the dead and living trees is grouped along the years from the disturbance of the surrounding forest stand, resp—the tree death.

The interaction between factors was statistically insignificant (χ^2: df = 4; $p = 0.92$; Figure 5). The dry weight of roots <2 mm in size was negatively associated with an increasing pH value (Figure 6). The higher the soil pH, the fewer fine roots are present. The connection between the mass of larger roots and soil pH was rather indifferent (only a weak negative association was found).

Identification of fungal species

The most frequently identified fungal species belonged to the genus *Armillaria* (*Armillaria ostoyae* (Romagn.) Herink, *Armillaria cepistipes* Velen.) and *Fomitopsis pinicola* (Sw.) P. Karst. (Table 3 and Figure 7). Fungi from the genus *Armillaria* were detected and isolated on four snags between 2018 and 2021. The genus *Fomitopsis* was detected between 2018 and 2021 on seven snags. The white-rot fungus *Pleurotus ostreatus* (Jacq.) P. Kumm. was observed on one snag from 2021. Results of PCA analysis (Figure 6) show the more complex view of the relationships between mycorrhizal parameters, root biomass, decaying processes and selected habitat characteristics. The first component (Dim1) is associated with habitat richness (characterized by pH), which is positively connected to the density of VM and negatively connected to NVM. The second component (Dim2) directly separated living and dead trees according to the presence and number of wood-decaying fungi. The third component (Dim3) is probably led by the wetness of the habitat, where sites more enriched with water had more VM and NVM, and the opposite, relatively dryer sites contained more roots with a 2–5 mm diameter. Projections of the three principal components explained 72% of the variability. The general trend across the dimensions was the following–density of VM

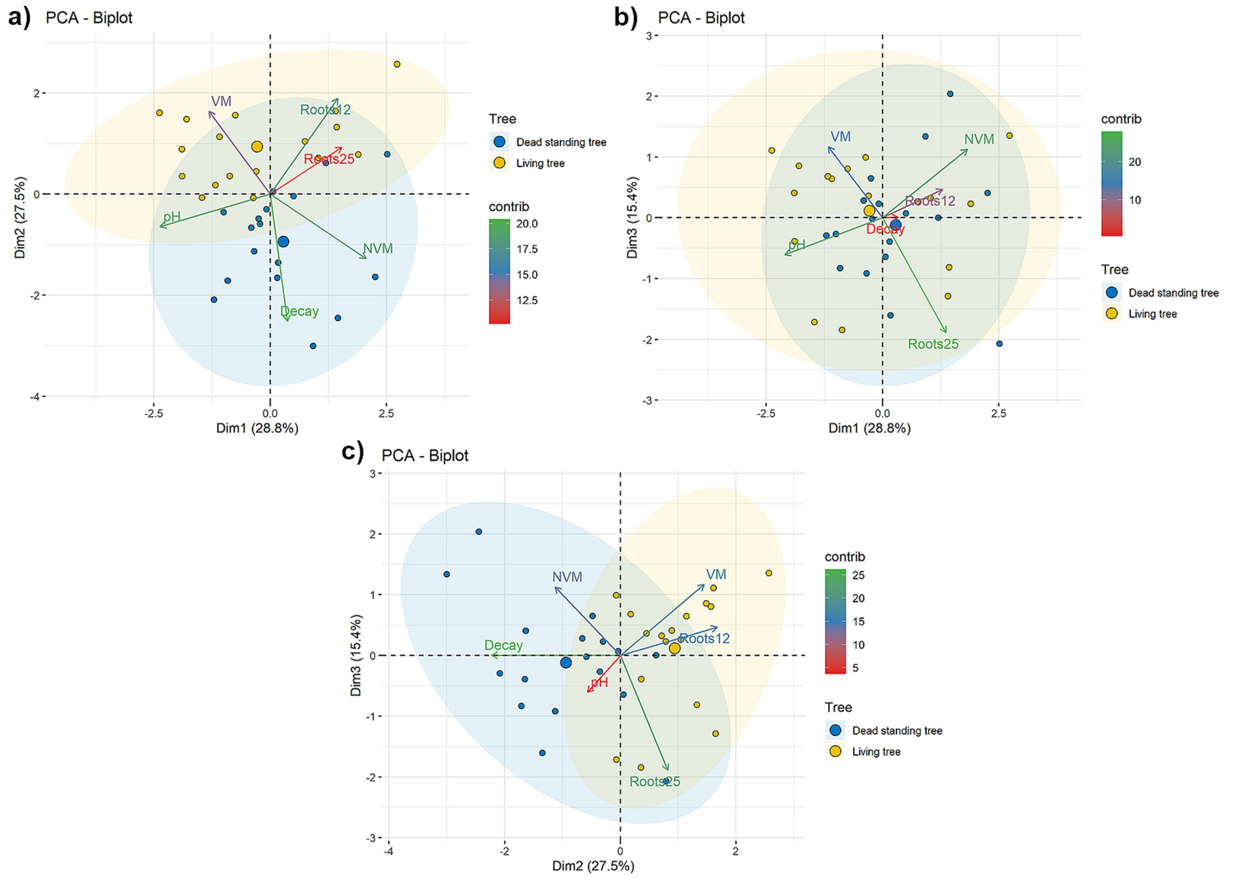

FIGURE 6
BiPlots for PCA analysis, displayed are the first three principal components (first – Dim 1, second – Dim2, and third – Dim3 components); **(a)** Dim1 and Dim2; **(b)** Dim1 and Dim3; **(c)** Dim2 and Dim3. Dead and living trees are represented by blue and yellow dots, respectively. The size of the dots represents the importance of the observation. Loadings are color-coded according to their contribution to explaining the variability of the plotted principal components.

was positively associated with living trees. Conversely, decaying processes and the presence of saprotrophic fungi were associated with dead trees.

Discussion

The bark beetle outbreak of the *I. typographus* in the Bohemian Switzerland National Park has been intense since 2018, with vast areas being infested (Hlásny et al., 2021c; Washaya et al., 2024). It can be assumed that all standing snags in the area result from the bark beetle attack. The few surviving spruces were mostly of lower canopy, an example of classification by Pommerening et al. (2021), and thus somewhat smaller than the surrounding trees. Contrary to bark beetle-surviving trees in the Šumava National Park (cf. Korolyova et al., 2022), the surviving trees in Bohemian Switzerland did not show any regular spatial distribution pattern. The trees in Šumava, however, belonged to the natural spruce zone, often in waterlogged locations with a high abundance of natural forests. In contrast, the studied locations in Bohemian Switzerland consisted mostly of azonal, artificially planted growths.

During the first years after the disturbance, the surviving trees gradually increased VM tips. The mean values of VM density on live trees in the 2nd and 3rd years after the disturbance were more than double the values reported in previous studies (Pešková, 2007; Pešková et al., 2011; Lorenc et al., 2018). An increased access to sunlight, water, and nutrients will likely cause an increase in VM density in the surviving trees. Most of the current Central European spruce forests are overly dense. Thinning can improve tree vitality manifested, for example, by increases in transpiration rate (Özçelik et al., 2022) and photosynthetic activity. A higher allocation of photosynthetic products to the soil can stimulate ECM species' development (Štursová et al., 2014; Corcobado et al., 2014). In addition to improved tree conditions, fungal growth conditions are directly affected after stand decay. For example, an increase in soil temperature leads to the development of ECM fungi (Mohan et al., 2014), which has also been observed in areas where trees were removed following a windstorm (Veselá et al., 2019). Temperature can directly affect mycorrhizae both positively and negatively. The higher temperatures above 35–40°C damage hyphae of ECM fungi (Smith and Read, 2008), but an increase in soil temperature usually leads to a rise in ECM fungi density (Mohan et al., 2014). However, the temperature optimum varies among species (Domisch et al., 2002). Low temperatures can have a lethal effect, especially on fungi colonizing the upper soil horizons (Gryndler et al., 2004). Many species of ECM fungi can survive exposure to low temperatures, but

TABLE 3 Wood decay fungal species identified in bark beetle snags of different ages (number of identifications).

Fungal species	Years after disturbance				
	0.5	1	2	3	4
Pleurotus ostreatus	–	1	–	–	–
Fomitopsis subpinicola/pinicola	–	1	1	2	3
Armillaria cepistipes	–	1	–	–	–
Armillaria ostoyae	–	–	–	1	1
Armillaria sp.	–	1	–	–	–

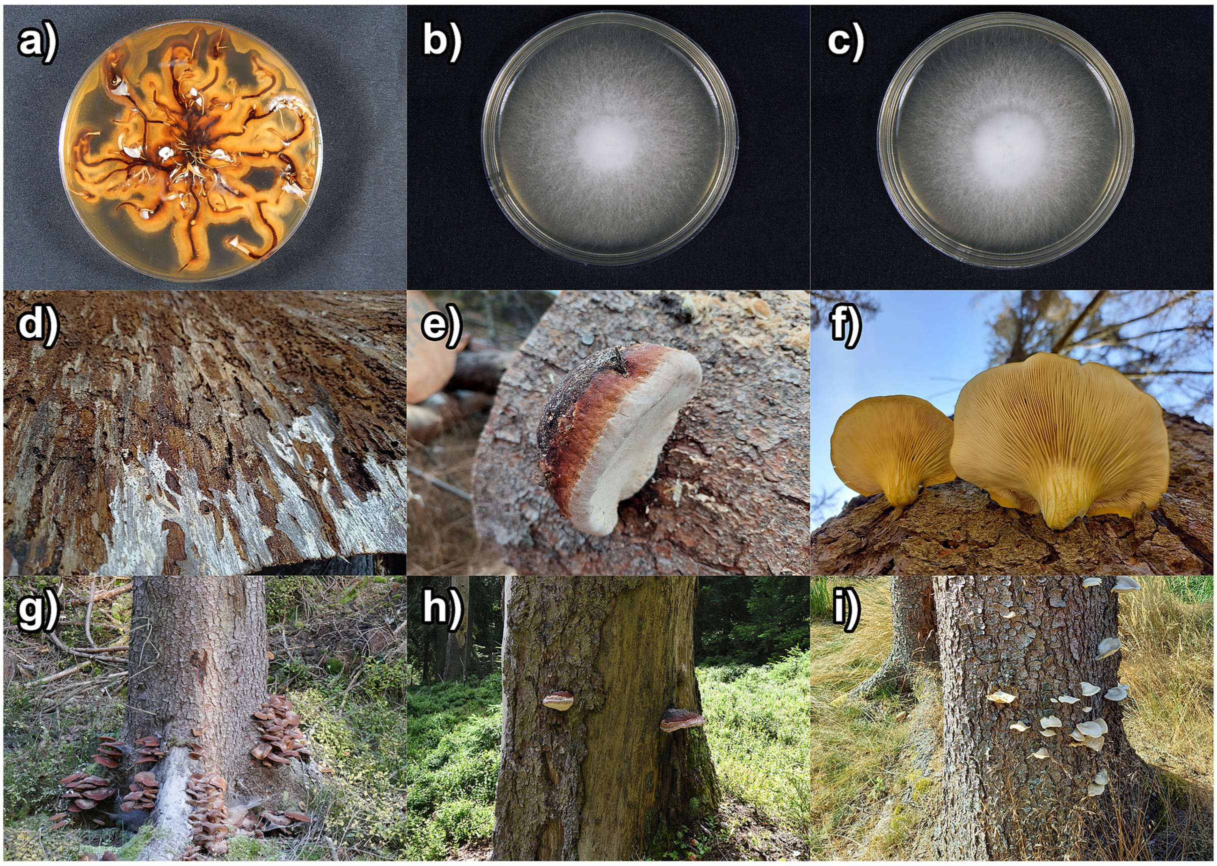

FIGURE 7
Selected wood decay fungi found on bark beetle snags: **(a)** culture of *Armillaria cepistipes* after 60 days on PDA agar medium; **(b)** *Fomitopsis pinicola* after 8 days on PDA agar medium; **(c)** *Pleurotus ostreatus* after 8 days on PDA agar medium; **(d)** syrrocium of *Armillaria* sp.; **(e)** fruiting body *Fomitopsis pinicola*; **(f)** fruiting bodies *Pleurotus ostreatus*; **(g)** massive fructification of *Armillaria* sp. in the autumn; **(h)** bole with fruiting bodies *Fomitopsis pinicola*; **(i)** bole with amounts of fruiting bodies *P. ostreatus.*

the time required to resume active growth and their subsequent growth rates vary inter- and intraspecifically (France et al., 1979). It can be assumed that mycorrhizal tips formed gradually, in an immature form at first, and subsequent years transitioned to a mature vital form, as was the case during drought stress in spruce stands (Pešková et al., 2015).

The decline in vital mycorrhizae in the fourth year in living trees may be related to the overall decline of ECM fungal species in sites disturbed by bark beetle outbreaks (Štursová et al., 2014; Mayer et al., 2022; Choma et al., 2023) or to increased drought stress due to stronger exposition to sunlight. Similar results were reached by Sterkenburg et al. (2019), who investigated the effect of logging in pine stands on ECM's abundance and species diversity. After 3 years, they observed a 95% decrease in ECM fungi in logged or partially logged forests compared to the unlogged area. This decline was due to the increased stress and stronger soil exposure to sunlight. Preserved were only a few common species of ECM fungi, while rarer species gradually disappeared. Thus, it can be agreed that trees surviving bark beetle disturbance are crucial for maintaining the community of ECM fungi (Mayer et al., 2022; Choma et al., 2023).

The number of VM tips on the roots of dead trees was very low in our study compared to live or drought-stressed trees (Pešková et al., 2015; Lorenc et al., 2018). Trees infested by the spruce bark beetle gradually change their physiological functions, i.e., they must invest available resources into various defense responses, thus stopping assimilate deposition and slowing down photosynthesis. Subsequently, the transpiration stream is interrupted, and the tree dies within about a month (Krokene, 2015). The lower rate of photosynthesis (or its gradual cessation) leads to a decrease in C deposition in the soil. Carbon balance and its allocation are closely linked to the vitality of the tree and its ability to respond to stress factors such as drought, nutrient deficiency or pest incidence. In healthy trees, carbon obtained through photosynthesis is primarily used for growth, with some of this carbon being transported to the roots, which also supports symbiosis with mycorrhizal fungi (Finzi et al., 2015; Brunner et al., 2015). However, environmental stress or pest infestation can significantly disrupt this process. After tree death, the ectomycorrhizal symbiosis can be temporarily maintained due to previously stored carbon stocks, as studies from boreal forests suggest (Lindahl et al., 2007). These changes could lead to the subsequent alteration in the composition of the soil fungal community with the dominance of saprotrophic species and the gradual disappearance of ECM fungi (Yarwood et al., 2009, Štursová et al., 2014, Mayer et al., 2022, Choma et al., 2023). Despite that, vital mycorrhizal tips were found even 4 years after tree death. It has been suggested that ECM fungi could temporarily switch to a saprotrophic mode of nutrition (Štursová et al., 2014). The presence of ECM fungi on dead roots was demonstrated 1 year after the tree was felled (Veselá et al., 2019). The number of NVM tips was statistically significantly higher in bark beetle snags compared to live trees in all surveyed years. Declines in mycorrhizal diversity due to insect damage to trees, pathogens, nutritional deficiency in the soil, drought, and other disturbances have been described in several studies (Jones et al., 2008; Blom et al., 2009; Corcobado et al., 2014). In the case of dead trees, there was a significant increase in the number of NVM tips in the first year after tree death. This significant increase in the proportion of NVM tips probably occurred due to a change from the vital to the non-vital form and their gradual death in the following years (Scatollin et al., 2008).

Root volume, represented by the dry weight of roots <2 mm in diameter, was significantly larger in live trees than in bark beetle snags. The fact that the fine root biomass of bark beetle snags was so low compared to live trees and did not decline significantly over time suggests that most of the fine roots had already decomposed before sampling was initiated. This is in accordance with our initial supposition, based on Rhoades et al. (2020), that fine root decay will occur soon after tree mortality. In live trees, root dry weight was relatively high during the first year after stand dieback and gradually decreased in fine roots. The difference between periods, however, was not statistically significant. The development of fine roots in the first year after disturbance followed an improved supply of solar energy and water, as in the case of vital mycorrhizae. However, the increase in root volume can also be associated with the surviving tree's need to improve its stability against the action of wind. While the surrounding dead trees may temporarily slow wind speeds, their eventual fall exposes the surviving trees to conditions they may not be adapted to Oberle et al. (2018). This situation is also well known in commercial forests, where individual trees left in clearings after clear-cutting are often subject to the forces of winds, as is also the case with newly exposed stand edges (Modlinger and Novotný, 2015). A higher risk of storm damage is also well-known after the thinning. However, the number of fine roots was relatively lower from the second year after the bark beetle disturbance. This may be partly due to the same unfavorable climatic conditions of 2018 and 2019, which caused the large outbreak of bark beetles in the first place (Netherer et al., 2019; Hlásny et al., 2021c).

The extreme dry season of 2018 may have influenced biological processes for multiple years ahead (Bose et al., 2022). Drought-stressed trees typically experience a decline in mycorrhizae (Gehring et al., 1997; Kuikka et al., 2003; Ostonen et al., 2013) and an increase in the relative abundance of roots compared to above-ground parts. However, the total amount of root biomass, especially in fine roots, decreases during drought stress (Brunner et al., 2015). A gradual decrease in root mass also occurred in dead trees. This corresponds with the gradual decrease in the dry weight of fine roots we observed. When tree mortality is high, there is an increase in wood-decaying fungi, at least in the short term. This may also have implications for ECM fungi diversity. Extremes in environmental conditions (e.g., drought, high temperatures, CO_2, insect infestation) predispose stressed trees to subsequent infestation by wood decay fungi (Kim et al., 2021). These fungi represent a specific physiological-ecological group of heterotrophic organisms that initiate successional processes, leading to humification and, in some cases, mineralization of deadwood (Murray and Leslie, 2021).

Genetic analyses have identified several pathogens in our research, the most prominent of which belonged to the genus *Armillaria*. *Armillaria* causes root rot and a decline in dry root biomass in infected trees (Kubiak et al., 2017; Murray and Leslie, 2021). *Armillaria* species are primarily saprotrophic, with occasional opportunistic pathogenicity, causing primary infections of the root system (Coetzee et al., 2018). Another identified fungal species was *Fomitopsis*, a pathogen typically infecting fresh bark-beetle snags (Vogel et al., 2017). As saprotrophs, *Fomitopsis* species can rapidly decompose their hosts, leading to a collapse within several years. Fungi of both genera, *Armillaria* and *Fomitopsis*, can significantly influence local conditions through the decomposition of newly emergent deadwood and influence nutrient cycles in the soil. Therefore, wood decay fungi can contribute to changes in vegetation composition that generally impact mycorrhizae, likely due to their obligately symbiotic relationships (Anderson et al., 2010).

Multivariate analysis of correlations between mycorrhizal abundance, root dry weight, and environmental factors, as well as detected fungal species, confirmed an association between VM density, live trees, and root abundance. In contrast, NVM tips' density and wood-decaying fungi's presence were associated with dead trees. Soil pH was not directly positively associated with any of the observed factors, but the analysis showed a relatively strong negative association with the number of fine roots. The range of measured pH values was not very large at the individual sites, ranging from 2.78 to 3.65, i.e., these were relatively acidic soils, which corresponds to the soil classification assigned by foresters (Viewegh et al., 2003). Mycorrhizal root formation is significantly affected by soil acidity in mature stands of Norway spruce (Nowotny et al., 1998), and even small changes in pH are sufficient to induce long-term changes in the composition of ECM communities, as demonstrated by Kjøller and Clemmensen, (2009). However, these differences in our study were only small because of the relatedness of soil and habitat originating from variability within the same classification.

Our study demonstrates that mycorrhizal activity increases in trees surviving a bark beetle disturbance, and they probably become another ECM refugia. In active management of the spruce bark beetle, in addition to felling infested trees, living trees are usually also removed for easier mechanized restoration. With the current extensive deforestation that has occurred in Czechia as a result of the spruce bark beetle (see Washaya et al., 2024), the chances of survival of symbiotic root organisms are considerably reduced (Sterkenburg et al., 2019). The success of subsequent restoration of clearings is thus made even more complicated. The goal of forest management should, therefore, be to preserve trees that have survived bark beetle disturbance and, previously, increase their chances of survival when the collapse of the surrounding forest stand comes. However, this can only be achieved through targeted stand tending at a young age (up to approximately 40 years), supporting the tree's stability and resistance to bark beetle attacks. In the context of the increasing frequency and intensity of disturbances in forests, enhancing tree resistance also brings other benefits that will strengthen forest resilience due to higher ECM persistence.

Conclusion

The findings of this study underscore the complex relationships between tree mortality, mycorrhizal dynamics, and forest ecosystem processes in the aftermath of bark beetle-induced dieback. We confirmed that the density and dynamics of vital mycorrhizae (VM) differ markedly between living trees and bark beetle snags, with living trees exhibiting a temporal increase in VM density. In contrast, snags showed a higher prevalence of non-vital mycorrhizal (NVM) tips, reflecting a pronounced shift from vital to non-vital mycorrhizae within the first year following tree death. This transition highlights the immediate below-ground consequences of tree mortality.

The study further reveals that the biomass of fine roots, represented by the dry weight of roots smaller than 2 mm, is substantially greater in living trees compared to dead ones. The rapid decomposition of fine root biomass—primarily within 0.5 years of tree death—emphasizes the transient nature of below-ground structures. However, the differences in fine root biomass between living and dead trees 2, 3, and 4 years after mortality aren't surprisingly so markable.

From an ecological perspective, the role of wood decay fungi is significant. Identifying fungi such as *Armillaria*, *Fomitopsis*, and *Pleurotus ostreatus* through macroscopic analysis and DNA barcoding underscores their contribution to the decomposition process. Additionally, environmental factors, particularly soil pH, influenced these below-ground dynamics, as evidenced by the negative correlation between fine root abundance and pH levels.

In conclusion, our findings highlight the intricate interplay between tree mortality, mycorrhizal dynamics, and forest ecosystems in the wake of bark beetle infestations. This research contributes valuable insights into the broader ecological consequences of such disturbances, offering another insight into below-ground processes and providing useful information for future strategies in managing and mitigating the impacts of bark beetle outbreaks on forest ecosystems.

Data availability statement

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

Author contributions

RM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. VP: Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. JZ: Investigation, Writing – original draft. MM: Investigation, Methodology, Writing – original draft. ML: Investigation, Writing – original draft. DT: Investigation, Visualization, Writing – original draft, Writing – review & editing. ON: Writing – original draft. VZ: Writing – original draft. PS: Data curation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2025.1492622/full#supplementary-material

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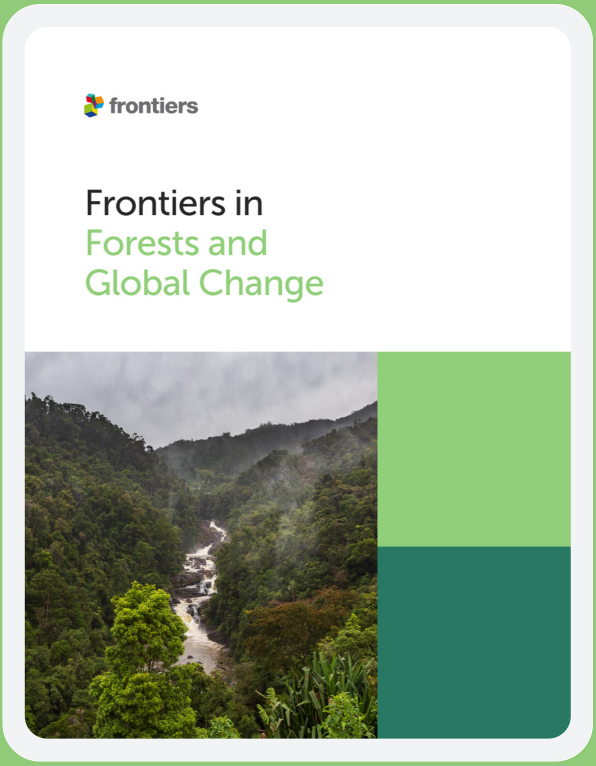

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