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RESEARCH ARTICLE

Both synergism and interaction diversity explain the mixtures of defensive monoterpenes in spruce oleoresin

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Abstract

1. Chemical defences, such as the monoterpenes of conifer oleoresin, frequently occur as complex blends of many components, but the selective pressures that maintain these mixtures are not yet known. Several theories attempt to explain the existence of chemical defence mixtures in plants. However, due to limited empirical evidence, it is unclear which theories might best apply.
2. Here, we tested the vapour phase activity of 12 individual Norway spruce monoterpenes and their naturally occurring mixtures to two types of natural spruce enemies, the adult Eurasian spruce bark beetles, *Ips typographus*, and their three major symbiotic fungi, using survival and growth bioassays. Next, we evaluated whether spruce trees could alter their monoterpene profile in response to fungal infection.
3. Individual monoterpenes had generally opposite effects on bark beetles compared to symbiotic fungi. The compounds that were most toxic to beetles were the least inhibitory to fungal growth and vice versa. The least abundant monoterpenes had the strongest activity against beetles or fungi, while the most abundant monoterpenes showed intermediate activity against both groups of enemies. Additionally, the activity of monoterpene mixtures was significantly stronger against beetles and some symbiotic fungi than the additive effects of individual compounds. Among the symbiotic fungi tested, one (*Grosmannia penicillata*) exhibited high tolerance to monoterpenes, and its growth was even stimulated by the monoterpenes most toxic to the beetle. Interestingly, spruce bark responded to *G. penicillata* inoculation by accumulating higher concentrations of specifically fungistatic monoterpenes.
4. Our results support the predictions of the interaction diversity hypothesis, which posits that defence mixtures are maintained in plants because the individual components target different attackers, as well as the synergy hypothesis, which predicts that mixtures will exhibit stronger activity than single compounds. Thus,

[Correction added on 1 July 2025 after first online publication: Tables 1 and 2 have been relabelled and citations have been updated.]

these two theories may deserve increased emphasis in explaining the widespread occurrence of mixtures in plant chemical defence.

KEYWORDS
chemical mixtures, interaction diversity, inverse toxicity, monoterpene toxicity, Norway spruce, ophiostomatoid fungi, spruce bark beetle

1 | INTRODUCTION

Plant chemical defences are characterized by the vast number of compounds produced, their extraordinary structural diversity and their variable distribution among taxa, organs and developmental stages. Moreover, individual defence compounds frequently occur in complex mixtures. The pyrrolizidine alkaloids of comfrey flowers (Stegemann et al., 2019), the cardenolides of milkweed leaves (Züst et al., 2019) and the monoterpenes of conifer oleoresin (Cates, 1996) are all present as multi-component blends. Yet researchers have only rarely investigated the ecological factors that maintain mixtures (Gershenzon et al., 2012; Richards et al., 2016; Whitehead et al., 2021).

Several theories aim to explain the existence of defence mixtures in plants. Among the most commonly cited, the synergy hypothesis predicts that the biological activity of blends can be more potent than that of individual compounds (Richards et al., 2016). The interaction diversity hypothesis posits that mixtures are maintained since plants interact with multiple organisms, and different defences are required to target different attackers (Berenbaum & Zangerl, 1996). On the other hand, the screening hypothesis proposes that biosynthetic processes to generate mixtures will be favoured by natural selection since this makes it more likely that active compounds will be formed (Jones et al., 1997). These theories may not be mutually exclusive, but researchers have seldom conducted appropriate bioassays of mixtures and their components to determine which best apply.

Conifers are among the oldest lineages of woody plants and often dominate boreal and temperate forest ecosystems. The remarkable success of conifers may be partly due to their complex defences against natural enemies, particularly terpenoid oleoresins, formed both constitutively and after herbivore or pathogen attack (Franceschi et al., 2005; Trapp & Croteau, 2001). Oleoresins were identified to be present in early gymnosperms ca. 320 Mya, representing the oldest known chemical defence of land plants (Bray & Anderson, 2009; Lange, 2015). Oleoresins are mixtures dominated by terpenes of varying carbon length, comprising volatile monoterpenes (C_{10}), non-volatile diterpenes (C_{20}) and a smaller portion of volatile sesquiterpenes (C_{15}) (Celedon & Bohlmann, 2019). Norway spruce contains over 20 different monoterpenes that vary in composition and concentration among constitutive and inducible oleoresins (Krokene, 2015; Schiebe et al., 2012).

Monoterpenes may have multiple modes of biological activity against conifer enemies. They serve as toxins by killing insects and inhibiting microbial growth either in the vapour phase or upon contact with the fluid resin (Chiu et al., 2017; Groot, 1972; Klepzig et al., 1996; Reid et al., 2017). Oleoresin also physically deters attackers by forming a viscous solution, which is forcefully released upon wounding or damage by herbivores (Cabrita, 2018; Phillips & Croteau, 1999). Upon exposure to air, the monoterpenes evaporate and the diterpenes solidify, leading to the entrapment of insects and sealing of wounds. Thus, plant oleoresins may act in more than one way. Yet, we have little information on why they contain complex multi-component mixtures of monoterpenes.

One of the major current threats to conifers worldwide is the increasing outbreaks of bark beetles, which, amplified by climate change, have had large ecological and economic consequences (Huang et al., 2020; Seidl et al., 2014). In Eurasia, the spruce bark beetle *Ips typographus* attacks living Norway spruce (*Picea abies*) trees. These beetles feed and breed in the phloem of spruce bark and introduce symbiotic fungi into the phloem, ultimately causing the tree's death (Netherer et al., 2021). Pioneer adult males of *I. typographus* select suitable hosts and emit aggregation pheromones to attract conspecifics. Once the tree defence is overcome, females join males, lay eggs in tunnels beneath the bark and inoculate ophiostomatoid fungi (Ascomycota: Ophiostomales and Microascales). These fungi may facilitate the development of larvae and immature adults by providing nutrition, detoxifying plant defence and inhibiting the growth of beetle pathogens (Kandasamy et al., 2019).

Throughout their life cycle, all stages of bark beetles encounter monoterpenes, but the role of these compounds is not always clear as they function both as attractants and defences depending on concentration and identity (Raffa, 2014; Seybold et al., 2006). Bark beetles may use host monoterpenes as kairomones to locate suitable hosts and to synthesize aggregation pheromones. For instance, *I. typographus* oxidizes (−)-α-pinene from Norway spruce into (4*S*)-*cis*-verbenol, which, together with de novo-produced 2-methyl-3-buten-2-ol, attracts conspecific beetles (Ramakrishnan et al., 2022; Rudinsky et al., 1971). The fungal symbionts of *I. typographus* also metabolize host monoterpenes into distinct oxygenated derivatives, which may aid in successful beetle colonization (Kandasamy et al., 2023). In other bark beetle-fungus systems, monoterpenes have been previously reported to be toxic to bark beetles and inhibit symbiotic fungi (Cates, 1996; Raffa & Berryman, 1986). However, the individual roles of monoterpenes in the mixture are still uncertain for most conifer bark beetles and especially for their fungal symbionts.

In this study, we examined the activity of the major and some minor Norway spruce monoterpenes against a major native enemy complex of the tree–the bark beetle *Ips typographus*, and its symbiotic fungi using bioassays to measure survival and growth. A few prior studies in other bark beetle systems have tested the insecticidal and fungistatic activity of monoterpenes (Chiu et al., 2017; Reid et al., 2017), but these have focused on the dominant compounds. We determined the insecticidal and fungistatic activity of 12 individual monoterpenes and two mixtures formulated to represent the natural blend of constitutive and inducible monoterpenes in the oleoresin. We used the results to evaluate the following hypotheses: (1) that individual spruce monoterpenes have different effects on beetles compared to symbiotic fungi (interaction diversity), (2) that monoterpene mixtures act synergistically to negatively affect beetle survival and fungal growth (synergy), and 3) that most compounds have no effect on the performance of beetles and fungi (screening). In addition, we asked whether monoterpenes that are most toxic to fungi would be specifically induced when we infected trees with fungi alone without bark beetles. Our findings demonstrate that both the interaction diversity and synergy hypotheses explain the existence of monoterpene mixtures in Norway spruce.

2 | MATERIALS AND METHODS

2.1 | Study organisms

For all the bioassays, bark beetles (*I. typographus*) were reared in logs, collected and sex determined as previously described (Kandasamy et al., 2019, 2023). The initial population originated from naturally attacked trees near Jena, Germany. We chose three different symbiotic fungi of *I. typographus* that vary in virulence to the host tree (Table S1). *Endoconidiophora polonica* and *G. penicillata* have been reported to be highly virulent as they cause large lesions in the bark, and *O. bicolor* was reported to be moderately virulent (Zhao et al., 2018). However, the virulence of these fungi may be strain-specific, since the *E. polonica* strain used in this study (Table S2) was less virulent than the strains of the other two species. Ethical approval was not required for this study, as it involved invertebrates.

2.2 | Effect of vapour phase spruce monoterpenes on *Ips typographus* survival

To assess the toxicity of monoterpenes to adult beetles, we exposed them to individual spruce monoterpenes and mixtures in the vapour phase (Table S2). The composition of monoterpene mixtures mimicking constitutive and induced resin was chosen according to an earlier report (Schiebe et al., 2012). The bioassay was performed according to the method described by Chiu et al. (2017) with slight modifications. Briefly, 23 mm Whatman circular filter papers (No. 3) were affixed to the inner side of the screw cap of 20 mL scintillation glass vials. A single beetle was placed at the bottom of each vial before pure monoterpenes were applied to the filter paper. The weight of experimental beetles ranged from 10 to 13 mg. Glass vials were then secured tightly with caps and were kept in darkness at room temperature for 24 h and checked for mortality. Beetles were considered dead if they did not respond after rubbing gently with soft forceps. The concentrations used for the assay ranged from 1 to 3000 μL of monoterpene /L of vial headspace (Supporting Information: Method 1a). Blank filter papers were used as a control. In total, 2360 beetles were used (Table 1). The beetle mortality was expressed as LC_{50}, the lethal concentration of the monoterpene that killed 50% of the beetles.

Given the varying vapour pressures of monoterpenes tested (Figure 1), some trials at high concentrations were conducted at monoterpene pressures over saturation. In these assays, the test compound remained partially in the liquid state after application on the filter paper and may have also condensed on the walls of the vial. Thus, we may have tested both vapour phase and contact toxicity with beetles and fungi. However, since we used monoterpene doses covering the natural range of concentrations found in Norway spruce bark, this should mirror the situation in bark beetle galleries.

2.3 | Effect of vapour phase spruce monoterpenes on growth of symbiotic fungi

The growth of three major fungal symbionts of *I. typographus*-*Grosmannia penicillata*, *Endoconidiophora polonica* and *Ophiostoma bicolor* (Table S1) was assessed in the presence of individual monoterpenes and their mixtures. Briefly, 35 mL of 4% PDA was poured into a 90 mm Petri dish (total volume 75 mL) and a fungal plug was taken from the outer edge of an actively growing 4-day-old mycelium using a no. 3 cork borer (1 cm diameter) and placed at the centre of agar with mycelium facing the agar. Different volumes of monoterpenes were applied to a filter paper (23 mm no. 3 Whatman) that was secured underneath the Petri dish lid using double-sided sticky tape. After the addition of monoterpenes, the Petri dishes were immediately sealed first with a layer of polyvinyl chloride (PVC) electrical tape and then with a layer of Parafilm. The controls used filter papers without monoterpenes. Each compound was tested with each fungus at variable concentrations ranging from 31.25 to 10,000 μL of monoterpenes/L of Petri dish headspace in five replicates. After 4 days post-inoculation, Petri dishes were photographed, and images were analysed using ImageJ software to determine the surface area of mycelial growth in mm^2. Experiments were conducted multiple times, with controls included in each round. In total, 1370 fungal cultures were used (Supporting Information: Methods, part 1b) (Table 1). Fungal growth inhibition was expressed as IC_{50}, the concentration of the monoterpene that inhibits fungal growth by 50% in the range between maximum and minimum growth.

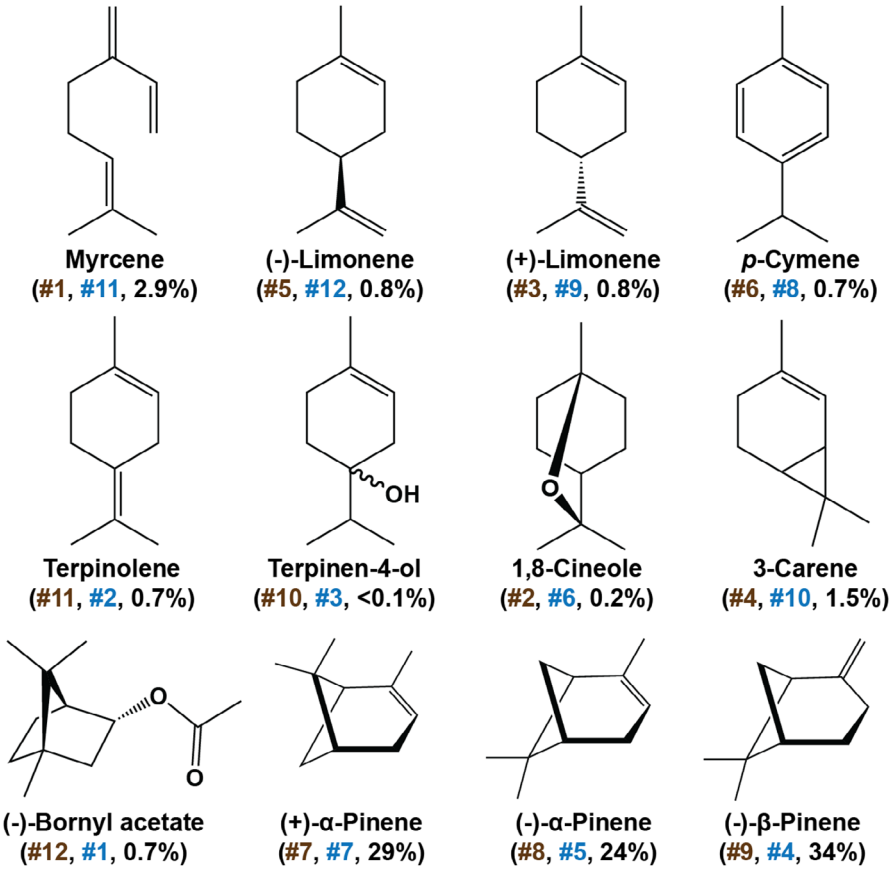

FIGURE 1 Chemical structures of monoterpenes of Norway spruce oleoresin tested in this study. Number below structure in brown is the rank of toxicity to *Ips typographus* (#1 = most toxic, #12 = least toxic). Number below structure in blue is the rank in growth inhibition towards the symbiotic fungus *G. penicillata* (#1 = most inhibitory, #12 = least inhibitory). The last number is the proportion of the total monoterpene composition represented by each compound of the constitutive oleoresin according to (Schiebe et al., 2012). The chirality of terpinen-4-ol in Norway spruce oleoresin has not yet been determined.

2.4 | Fungal inoculation of spruce logs

Five logs approximately 50 cm long and 15 cm in diameter were cut from a 40-year-old spruce tree and inoculated with different *I. typographus* symbiotic fungi on the next day. Fungi were first grown on PDA at 25°C for 5 days. Two rings were drawn along the circumference of each log 15 cm away from the cut ends. Four equidistant inoculations were made along the circumference of each ring, and the bark was replaced with agar plugs containing fungal mycelium or agar plugs only using a cork borer. The holes were made alternating between the two rings to prevent the overlapping of the fungal lesions. The fungi *G. penicillata*, *E. polonica* and *O. bicolor* were used for inoculating logs, and a PDA plug alone without fungus was used as a control Table 1. A sample was collected (pre-control) from each log before fungal infection, flash-frozen in liquid nitrogen and stored at −80°C. Five logs were then incubated for 14 days under the conditions used for rearing beetles. After 10 days, bark from each log was peeled around the fungal inoculations, and necrotic lesions caused by fungal infections and wounding on bark were marked on transparent sheets. Immediately, necrotic phloem samples from each treatment were collected. Additional samples were collected from the uninfected part (post-control) of the logs after a 14-day incubation period of the logs to estimate any change in the bark chemistry due to experimental conditions. All bark samples were ground to powder using a cryo-vibratory micro mill and then stored at −80°C until analysis. In cut logs, resin monoterpenes were less induced after fungal inoculation compared to the response in living trees, which showed a strong induction in response to fungi (Zhao et al., 2011).

2.5 | Extraction of monoterpenes from spruce bark and their quantification using GC-FID/MS

Approximately 100 mg of frozen bark powder was placed in a preweighed 4 mL glass vial on dry ice, and 1 mL of methyl tert-butyl ether spiked with 50 μg/mL of nonyl acetate (internal standard) was added to the vial. For extractions, the vials were shaken gently using a rotary shaker for 24 h. Then, 0.4 mL of 0.1 M $(NH_4)_2CO_3$ (pH 8.0) was added to each vial and vortexed for 10 s. The samples were then centrifuged at 3320 *g* for 10 min. After freezing at −80°C for 2–3 h to precipitate the debris, the supernatant was transferred to clean 2 mL amber glass vials with the help of 200 μL glass capillaries attached to a micropipette. Samples were stored at −20°C until analysed. The eluted

TABLE 1 Replication statement of the study design.

	Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
1	Population (*Ips typographus*)	Individuals*	2360 trials
2	Species (Ophiostomatoid fungi)	Individuals*	3 species (1370 trials)
3	Tree (*Picea abies*)	Tree logs#	5 spruce logs

Note: Factor of interest: *monoterpene vapours, #Ophiostomatoid fungi.

volatile samples were subjected to GC–MS analysis for identification and GC-FID analysis for quantification using the protocol described in (Kandasamy et al., 2023).

2.6 | Data analysis

The dose–response effect of monoterpenes on the mortality of beetles and the growth response of symbiotic fungi was tested using a constrained four-parameter logistic model to estimate parameters such as $\log LC_{50}$, $\log IC_{50}$, hillslope, LC_{50}, and IC_{50}. Since beetles and fungi vary in their responses to monoterpenes, different concentration ranges were tested to obtain their respective dose–response curves to accurately estimate LC_{50} and IC_{50} values. Monoterpene concentrations were log-transformed, and beetle mortality and fungal growth were normalized to percentages (Supporting Information: Methods, part 2). For both beetles and fungi, normalized responses were constrained between 0% and 100% when fitting the model using the least square method without weighing. The adjusted R^2 goodness-of-fit measure was used to evaluate how well the model fit the data. Differences in LC_{50} and IC_{50} values among chemicals and fungal species were analysed using Welch's ANOVA due to heterogeneity of variances (Bartlett's test, $p < 0.0001$). A single value $\log LC_{50}$ or $\log IC_{50}$ of each chemical was used as a fixed effect. Weights were based on a standard deviation of each $\log LC_{50}$ or $\log IC_{50}$, calculated from the standard errors and total sample size (N), followed by Dunett's T3 post hoc test for multiple comparisons. All the above analyses were performed using GraphPad Prism version 8.4.

To test for synergy in mixtures, an interaction index was calculated as $\alpha = Z/\text{sum}(fi^*Ai)$ (described in Richards et al., 2012, 2016), where Z is the LC_{50} or IC_{50} of mixtures, fi represents fractions of individual chemicals in the mixture (Table S3) and Ai represents the LC_{50} or IC_{50} of chemicals when tested individually. Interaction indices were calculated separately for constitutive and inducible mixtures tested on beetles and fungi. Values of α significantly <1 indicate synergy, values of $\alpha = 1$ indicate additive effects, and values of $\alpha > 1$ indicate antagonism. The significance of interaction indices was assessed using the standard error of α calculated using the bootstrap method (Supporting Information: Method 3).

Lesion area and the concentrations of individual, total and oxygenated monoterpenes in spruce logs inoculated with different fungi were subjected to ANOVA followed by Tukey's test for normally distributed data. The normality was assessed using both the Kolmogorov–Smirnov and Shapiro–Wilk tests. For data that did not follow a Gaussian distribution (terpinen-4-ol and γ-terpinene), a Kruskal–Wallis test followed by a Bonferroni pairwise comparison was used (Table S9). Non-metric multidimensional scaling (NMDS) analysis was performed on Bray–Curtis dissimilarity matrices using 23 compounds, including monoterpene hydrocarbons and oxygenated monoterpenes quantified from fungus-infected and uninfected spruce bark in R version 4.4.1 using the vegan package. NMDS was performed with a maximum of 100 iterations and the two-dimensional ordination ($k = 2$) was selected based on stress value. PERMANOVA (permutational multivariate analysis of variance) was applied with 999 permutations to test significant differences among treatments. To identify chemical features most strongly associated (both in strength and direction) with NMDS ordinates, vector fitting was performed using the envfit() function in vegan with a significance level of 0.05 (Table S10). A Spearman correlation was performed to test the relationship between the fungal lesion area and the monoterpenes that were identified within the lesion using auto-scaled data and with a significance level of 0.05 in Metaboanalyst 5.0 software (Chong et al., 2019).

3 | RESULTS

3.1 | Norway spruce monoterpenes are not equally toxic to adult *I. typographus*

To evaluate the toxicity of Norway spruce monoterpenes (Figure 1) to adult bark beetles, insects were exposed to individual compounds or mixtures in the vapour phase for 24 h, and concentrations resulting in 50% mortality (LC_{50}) were calculated. For the tested compounds, these values ranged from ~71 μL of monoterpene/L of headspace for myrcene to 2475 μL of monoterpene/L of headspace for (−)-bornyl acetate, a 35-fold quantitative difference in toxicity (Welch's ANOVA: $W(13, 824.6) = 58{,}308$, $p < 0.001$; Figure 2a, Table S4). Myrcene, 1,8-cineole, (+)-limonene and 3-carene were the most toxic among tested compounds. The enantiomers of α-pinene differed in their toxicity to beetles by 2-fold with (+)-α-pinene being more toxic than (−)-α-pinene (Welch's ANOVA, Dunnett's T3 test, $p < 0.05$). Similarly, (+)-limonene was more toxic than (−)-limonene. The three most abundant monoterpenes in Norway spruce oleoresin, (+)-α-, (−)-α- and (−)-β-pinene, were of intermediate toxicity to *I. typographus*, while some of the least abundant monoterpenes (−)-terpinen-4-ol, (−)-bornyl acetate, and terpinolene were the least toxic (Figure 2a, Table S4). The monoterpene blend

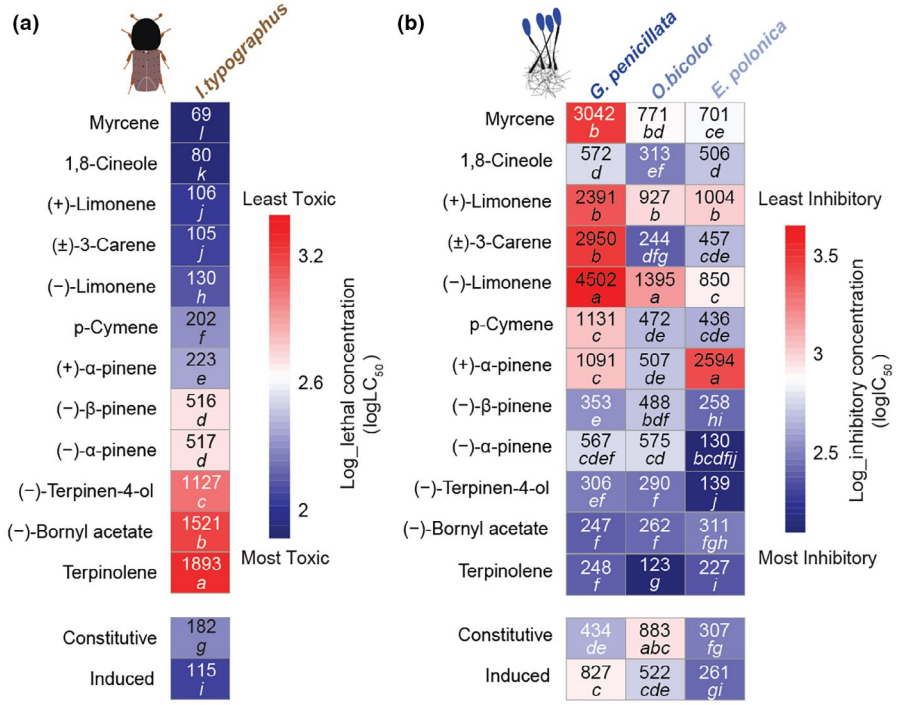

FIGURE 2 Norway spruce monoterpenes are inversely toxic to adult *Ips typographus* beetles and their symbiotic fungi in the vapour phase. Heat map representation of toxicity of different Norway spruce monoterpenes to (a) bark beetles based on 50% log lethal concentration ($logLC_{50}$) and to (b) their dominant symbiotic fungi based on 50% log inhibitory concentration ($logIC_{50}$). Each box in the heat map represents beetle $logLC_{50}$ or fungi $logIC_{50}$ to either an individual monoterpene or a monoterpene blend. The heatmap colours indicate log-transformed LC_{50} or IC_{50} values, while the numbers in the cells show the original LC_{50} or IC_{50} values. The most active monoterpenes are in the darkest blue and the least active in red. Beetles, $n = 10$ for each sex per trial; fungi, $n = 5$ for each fungus per trial). Different small letters denote significantly different LC_{50} or IC_{50} values within species at $p < 0.05$, Welch's ANOVA followed by Dunnett's T3 post hoc test.

induced by jasmonate treatment was slightly more toxic compared to the constitutive blend, but neither blend was as toxic as the individual compounds, myrcene, 1,8-cineole and (+)-limonene. Yet the LC_{50} values of the blends were lower than those of (−)-α-pinene, (+)-α-pinene, and (−)-β-pinene, which constitute the major proportion (ca. 85%) of the blends (Table S2). Among sexes, there was strong evidence that female beetles were more susceptible to some monoterpenes, such as 1,8-cineole, (±)-3-carene, *p*-cymene, (+)-α-pinene, (−)-α-pinene, (−)-β-pinene, terpinen-4-ol, and (−)-bornyl acetate compared to males (Unpaired Welch's *t*-test, $p < 0.05$, Table S5). Effect sizes were the largest for 1,8-cineole, *p*-cymene and (−)-α-pinene, indicating these monoterpenes have the strongest effect on females compared to males. All LC_{50} values were within natural concentration ranges of monoterpenes in Norway spruce bark, assuming that all of the monoterpenes in a given sample of tissue are in the volatile phase.

3.2 | Monoterpenes with the highest toxicity to *I. typographus* are the least inhibitory to symbiotic fungi and vice versa

Like *I. typographus*, the symbiotic fungi carried by these beetles are also exposed to monoterpenes when inoculated into the bark during a bark beetle attack. Therefore, we calculated the inhibitory concentration (IC_{50}) of monoterpenes to three fungal associates of this beetle. Overall, there was a 35-fold difference in activity between the least and the most inhibitory compounds (Tables S6–S8), and the fungi themselves varied in their sensitivity. The order of inhibitory effect of the individual monoterpenes on the fungi was generally opposite to their order of toxicity towards bark beetles, though *E. polonica* deviated slightly from this pattern (Figure 2b). For example, the three most inhibitory monoterpenes to fungi were among the least toxic to bark beetles (Figure 2a). These included (−)-bornyl acetate, terpinolene and (−)-terpinen-4-ol for *G. penicillata*; terpinolene, (−)-bornyl acetate and (−)-terpinen-4-ol for *O. bicolor*; and (−)-α-pinene, (−)-terpinen-4-ol and terpinolene for *E. polonica* (Welch's ANOVA, Dunnett's T3 test, $p < 0.05$). Conversely, the three least inhibitory monoterpenes to the fungi were among the most toxic to bark beetles. These included (−)-limonene, myrcene, and 3-carene for *G. penicillata*; (−)-limonene, myrcene, and (+)-limonene for *O. bicolor*; and (+)-α-pinene, (+)-limonene and (−)-limonene for *E. polonica*. Intriguingly, enantiomers of α-pinene revealed a 20-fold difference in the growth inhibition of *E. polonica* (Table S7). (+)-α-Pinene was the least fungistatic compound to *E. polonica* compared to (−)-α-pinene, which was one of the most fungistatic of the tested compounds (Figure 2b, Table S7). Concerning mixtures, the induced monoterpene blend tested inhibited *E. polonica* and

O. bicolor similarly to the constitutive monoterpene blend. In contrast, the constitutive blend was more inhibitory to *G. penicillata* growth than the induced blend (Welch's ANOVA, Dunnett's T3 test, $p < 0.05$).

3.3 | Monoterpene mixtures showed synergistic effects against *I. typographus* and some fungi

For *I. typographus*, both mixtures showed a synergistic effect on beetle mortality in which their toxicity was greater than the additive toxicity of the individual monoterpene components. The inducible blend showed the strongest synergy ($\alpha = 0.27$, bootstrap $\alpha \pm SE = 0.23 \pm 0.13$, Table 2), while the constitutive blend had a slightly weaker synergy ($\alpha = 0.46$, bootstrap $\alpha \pm SE = 0.44 \pm 0.24$, Table 2). For the fungal symbionts of *I. typographus*, both mixtures had similar synergistic inhibitory effects on *E. polonica* growth (constitutive mixture: $\alpha = 0.34$, bootstrap $\alpha \pm SE = 0.25 \pm 0.09$; inducible mixture: $\alpha = 0.34$, bootstrap $\alpha \pm SE = 0.26 \pm 0.12$, Table 2). In contrast, only the constitutive mixture showed synergistic inhibition of *G. penicillata* ($\alpha = 0.58$, bootstrap $\alpha \pm SE = 0.47 \pm 0.29$), while the inducible mixture had an additive effect ($\alpha = 1.12$, bootstrap $\alpha \pm SE = 1.24 \pm 0.46$). We found no evidence for synergistic effects of either mixture against *O. bicolor*, with both mixtures showing additive interactions (constitutive mixture: $\alpha = 1.72$, bootstrap $\alpha \pm SE = 1.2 \pm 0.69$; inducible mixture: $\alpha = 1.05$, bootstrap $\alpha \pm SE = 0.76 \pm 0.57$, Table 2).

3.4 | Monoterpenes with toxicity to *I. typographus* promote the growth of the symbiotic fungus *G. penicillata*

Since it was observed that the lowest concentrations of monoterpenes may even have stimulated the growth of some of the symbiotic fungi, we quantified the effects of individual monoterpenes on the growth rate of fungi. Surprisingly, the five monoterpenes most toxic to *I. typographus* promoted the growth of the fungal symbiont, *G. penicillata* (Figure 3). Both enantiomers of limonene strongly promoted the growth of this fungus at all tested concentrations, compared to its growth on terpene-free agar. Similarly, the other monoterpenes most toxic to *I. typographus*, 3-carene, myrcene, and 1,8-cineole, promoted the growth of *G. penicillata* at least at the lower concentrations tested. Turning to the other fungal symbionts, the *G. penicillata* growth-promoting monoterpenes, (+) and (−)-limonene, myrcene, and 3-carene, inhibited the growth of *E. polonica* and *O. bicolor* at all tested concentrations (Figure 3).

3.5 | Lesions from symbiotic fungi in the bark contain high concentrations of fungistatic monoterpenes

To determine which monoterpenes are produced by spruce trees in response to infection by the symbiotic fungi, we quantified the lesion area and monoterpene profile of bark after fungal infection (Figure 4; Table S9). Among the symbiotic fungi, *G. penicillata* induced a larger lesion area in bark compared to the lesion areas of the other fungi and the lesion caused by the mock wounded control (ANOVA, $F_{(3,35)} = 134.6$, $p < 0.001$, Tukey's test; Figure 4a). A non-metric multidimensional scaling (NMDS) ordination plot based on a Bray–Curtis dissimilarity matrix showed that the monoterpenes induced by *G. penicillata*, which caused the largest lesions, clustered separately from the monoterpenes induced by the other two fungal species and the controls. Vector fitting analysis identified several fungistatic oxygenated monoterpenes, including terpinen-4-ol, bornyl acetate, and terpinolene, to be significantly correlated with the NMDS ordination axes ($r^2 > 0.6$, $p < 0.001$, Table S10). Interestingly, the fungistatic monoterpene terpinen-4-ol (Kruskal–Wallis, $Z = 33.8$, $p < 0.001$, Bonferroni's test) and (−)-bornyl acetate (ANOVA, $F_{(4,29)} = 14.2$, $p < 0.001$, Tukey's test; Figure 4c,d) also increased in *G. penicillata*-infected tissues compared to controls. However, there was little or no increase in the concentrations of monoterpenes toxic to *I. typographus* in fungal lesions compared to controls (Table S9). The increase in fungistatic monoterpenes upon fungal infection was also correlated with fungal lesion area (Figure 4e).

TABLE 2 Evaluation of the potential synergistic effects of constitutive and induced monoterpene mixtures against *Ips typographus* and their symbiotic fungi.

Organism	Monoterpene mixture	Expected LC_{50}/ IC_{50}	Observed LC_{50}/ IC_{50}	Interaction index (α)	Bootstrap mean (α) ± SE	Interaction outcome
I. typographus	Constitutive	401.6	184.5	0.46	0.44 ± 0.24	Synergy
	Induced	420.7	114.7	0.27	0.23 ± 0.13	Synergy
G. penicillata	Constitutive	745.6	434.2	0.58	0.47 ± 0.29	Synergy
	Induced	740.9	834.3	1.12	1.24 ± 0.46	Additive
O. bicolor	Constitutive	514.6	882.6	1.72	1.2 ± 0.69	Additive
	Induced	508.8	522.2	1.05	0.76 ± 0.57	Additive
E. polonica	Constitutive	914.7	307.2	0.34	0.25 ± 0.09	Synergy
	Induced	766.4	261.3	0.34	0.26 ± 0.12	Synergy

Note: Expected LC_{50} values for *I. typographus* and IC_{50} values for the fungi, plus the interaction index (α) were calculated as described in the methods. Values of $\alpha < 1$ indicate synergy. The statistical significance of α was tested via the bootstrap method.

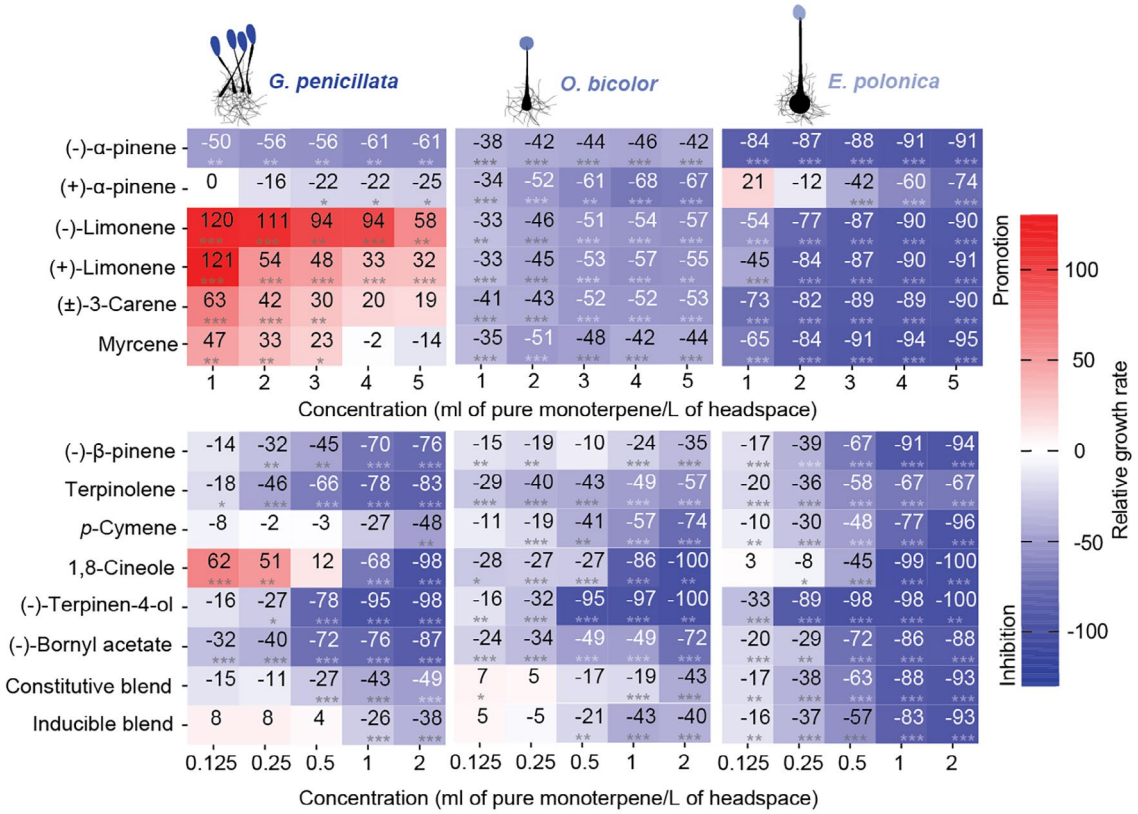

FIGURE 3 Norway spruce monoterpenes promote or inhibit the growth of bark beetle symbiotic fungi depending on the fungal species and monoterpene tested. Heatmap representation of relative growth rate of three fungal symbionts when fumigated with different concentrations of individual monoterpenes and their mixtures. Each number in the heatmap represents the relative growth rate of a fungus in the presence of a monoterpene in the vapour phase compared to growth in a monoterpene-free environment. The darkest red and highest positive number indicate the strongest promotion of fungal growth by monoterpenes; the darkest blue and lowest negative number indicate the strongest inhibition of fungal growth by monoterpenes. Monoterpenes were tested in two different concentration ranges depending on the initial toxicity demonstrated in preliminary experiments. (see Table S11 for different concentrations tested) Asterisks denote the significant difference between each treatment and control-*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (unpaired t-test) ($n = 5$).

4 | DISCUSSION

4.1 | Both the interaction diversity and synergy hypothesis work together to explain the existence of mixtures of monoterpene chemical defences

Despite the presence of complex, multi-component mixtures of chemical defence compounds in many plant species, there is still much discussion about the underlying causes of this phenomenon. Several plausible explanations for mixtures have been put forth over the years, including the interaction diversity hypothesis, the synergy hypothesis and the screening hypothesis (Berenbaum & Zangerl, 1996; Jones et al., 1997; Richards et al., 2016). Our investigation of the mixture of monoterpenes found in Norway spruce trunk resin showed good support for the first two. The interaction diversity hypothesis predicts that the different components of a mixture are needed for their activity against different enemies. The top three most effective monoterpene defences against the spruce bark beetle based on vapour phase toxicity (myrcene, 1,8-cineole, (+)-limonene) proved to be completely different from the three monoterpenes most effective at inhibiting the growth of the spruce bark beetle symbiotic fungi in the vapour phase ((−)-bornyl acetate, terpinolene, (−)-terpinen-4-ol) (Figures 1 and 2). As bark beetles and their associated fungi invade the tree at the same time, it is essential that defences are present that are effective against both types of attackers. Norway spruce seems to have solved this problem by deploying a mixture containing some compounds with strong activity against one of the two attackers but not necessarily against the other. Meanwhile, the three major monoterpene constituents of Norway spruce oleoresin (+)-α-, (−)-α- and (−)-β-pinene (each making up 20%–35% of the total monoterpene fraction) exhibit intermediate toxicity to *I. typographus* and intermediate inhibitory activity to some but not all of the symbiotic fungi. Thus, the strategy for constructing the mixture appears to involve smaller amounts of compounds that are potent defences towards one group of enemies or the other, with the bulk of the oleoresin composed of compounds with intermediate activities against both groups.

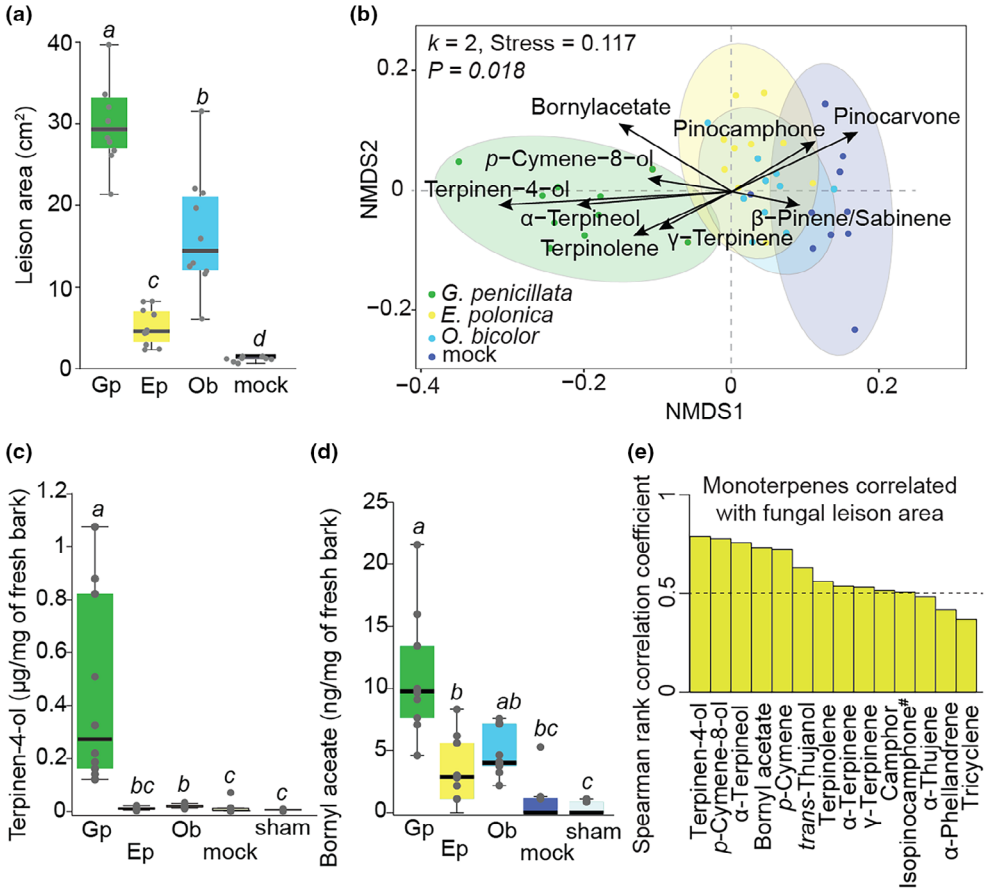

FIGURE 4 Fungistatic monoterpenes are accumulated in spruce bark in response to infection by symbiotic fungi of the spruce bark beetle. (a) Symbiotic fungi associated with spruce bark beetles differed in their virulence to spruce. *Grosmannia penicillata* produced significantly larger lesion areas when inoculated in spruce logs, followed by *O. bicolor*, *E. polonica*, and the mock-infected control (wounding only, no fungus). (b) Non-metric multidimensional scaling (NMDS) ordination plot based on the monoterpenes measured in fungal-infected and control bark showed that the monoterpene profile of *G. penicillata* clustered separately from the monoterpene profiles of the other fungi and the mock-infected control, which clustered closely together. (c) and (d) The most fungistatic monoterpenes from Figure 2, terpinen-4-ol and bornyl acetate, are more abundant in fungus-infected bark than in mock-infected (wounding only, no fungus) and sham (no wounding, no fungus) controls. (e) Significantly strong positive Spearman rank correlation coefficients between fungistatic compounds and lesion area indicate that the larger lesion areas produced in response to fungal infection are associated with high production and accumulation of fungistatic monoterpenes in spruce bark. Only monoterpenes with Spearman coefficients above 0.35 and $p < 0.05$ are shown here. Gp = *G. penicillata*, Ep = *E. polonica*, Ob = *O. bicolor*. Different letters in a, c, and d indicate significant differences among treatments ($p < 0.05$; (d) ANOVA followed Tukey's test; (c) Kruskal-Wallis followed by Bonferroni's test) ($n = 10$).

The same strategy may be used by other conifers as well. The monoterpene chemotypes of the lodgepole pine that most inhibited the growth of the mountain pine beetle were found to be least inhibitory to the major symbiotic fungus of this insect, and vice versa (Ullah et al., 2021). Among other classes of chemical defences, a well-designed study on the phenolics of apple fruits demonstrated that the individual compounds tested also seemed to be most effective against different insect herbivores and fungal pathogens (Whitehead et al., 2021). Additionally, the individual components of glucosinolate hydrolysis product mixtures found in the Brassicaceae appear to be most effective against different plant enemies (Gershenzon et al., 2012; Lankau, 2007).

Our results also support the synergy hypothesis, that mixtures are inherently more active than individual compounds against a single enemy. We compared the biological activity of the naturally occurring, constitutive mixture of spruce oleoresin monoterpenes, as well as a mixture induced by jasmonate treatment, to the activity of individual compounds. Both mixtures showed synergistic effects on bark beetle mortality (Table 2), although neither was as toxic as the top four individual compounds: myrcene, 1,8-cineole, (+)-limonene and 3-carene. The constitutive mixture also showed synergistic effects on the growth of two out of the three fungal symbionts, while the induced mixture was only synergistic on the growth of *E. polonica*. Other studies on mixtures have found little support for the synergistic effects of monoterpenes against the mountain pine beetle (Reid et al., 2017) and phenolics against diverse insect herbivores and fungi (Whitehead et al., 2021), but there are convincing counter examples from studies of furanocoumarins, *Piper* amides

and pyrrolizidine alkaloids (Berenbaum & Zangerl, 1996; Richards et al., 2016).

Synergy may also exist between Norway spruce monoterpenes and the other major constituents of the trunk oleoresin, sesquiterpenes and diterpene resin acids, in influencing the toxicity, growth inhibition or even the physical properties of the resin, such as viscosity, stickiness to insects, and evaporation rate, which could also have significance in defence against insect or fungal invaders. Synergism among defence compounds has been attributed to a number of mechanisms, including the ability of some mixture components to facilitate the absorption of others or inhibit detoxification enzymes active on others (Berenbaum & Zangerl, 1993' López-Goldar et al., 2024; Richards et al., 2016).

Our results do not favour the screening hypothesis, whose main assumption is that biological activity is rare among the arsenal of potential chemical defences produced by plants. We found that all of the monoterpene components of Norway spruce trunk oleoresin tested were active against either the spruce bark beetle or their symbiotic fungi (Figure 2). Another explanation for the presence of mixtures is that these are consequences of a biosynthetic machinery that makes multiple products because of the enzyme mechanism involved. The last step in the formation of monoterpene hydrocarbons is catalysed by enzymes called monoterpene synthases, which often make multiple products from a single substrate via a carbocation-based mechanism (Degenhardt et al., 2009). While some spruce monoterpene synthases are indeed multiple product-forming enzymes, it appears that Norway spruce employs at least eight separate enzymes to make its twelve major products, based on those characterized to date (Keeling et al., 2011; Martin et al., 2004) of which at least five are single-product enzymes. Thus, multi-product enzymes make only a small contribution to the mixture of monoterpenes in Norway spruce oleoresin.

4.2 | Monoterpenes are toxic to *I. typographus* bark beetles

The individual monoterpenes of the mixture from Norway spruce trunk resin are variably toxic to *I. typographus*, with myrcene, 1,8-cineole and (+)-limonene being the most poisonous in the vapour phase (Figure 1). Myrcene was described as a contact toxin to *I. typographus* in an earlier study (Everaerts et al., 1988), while 1,8-cineole inhibited the mass attack of *I. typographus* by reducing its sensitivity to aggregation pheromones, possibly a consequence of its toxicity (Andersson et al., 2010). For other bark beetle species, limonene (chirality unspecified) has been cited in the older literature as one of the most toxic of all conifer oleoresin monoterpenes (Cates, 1996; Seybold et al., 2006). (−)-Limonene was the most toxic monoterpene in the vapour phase to *Dendroctonus ponderosae* (Chiu et al., 2017), and this insect was twice as sensitive to (−)-limonene as *I. typographus*, based on the LC_{50} value. However, both species exhibit similar sensitivity to myrcene. While the results of this and previous studies have shown clear hierarchies of monoterpene toxicity, it is difficult to know if our bioassays in air-tight containers accurately reflect the conditions in the native gallery system of *I. typographus*. In galleries, monoterpenes from oleoresins may evaporate quickly due to ventilation. However, pioneer bark beetles might come into prolonged contact with monoterpene vapours from fresh oleoresins in their initial tunnelling and nuptial chambers. Higher levels of toxic monoterpenes may aid in tree resistance to bark beetles at early stages of attack. In fact, Norway spruce that survived *I. typographus* attack contained higher amounts of 1,8-cineole and limonene than trees that succumbed to beetle attack (Schiebe et al., 2012; Zhao et al., 2011). During attack, monoterpenes may act as toxins or as deterrent cues indicating reduced host susceptibility.

4.3 | Spruce monoterpenes that most inhibited the growth of bark beetle symbiotic fungi are least toxic to beetles, but accumulate in response to fungal invasion

The monoterpenes most consistently fungistatic to all three symbiotic fungi tested, (−)-bornyl acetate, terpinolene and terpinen-4-ol, were among the least toxic to *I. typographus*. Two of these compounds are oxygenated, in keeping with previous reports that oxygenated monoterpenes tend to be more fungistatic than monoterpene hydrocarbons (Achotegui-Castells et al., 2016; Kusumoto et al., 2014; Marei et al., 2012). Interestingly, infection of Norway spruce bark with the symbiotic fungus *G. penicillata* alone (without beetles) led to an enrichment of resin with oxygenated monoterpenes. For example, the concentration of terpinen-4-ol in *G. penicillata* lesions was 34-fold higher than in wounded (no fungus) controls, and the strong fungistatic compounds, (−)-bornyl acetate and terpinolene, showed a 10-fold higher concentration in *G. penicillata* lesions. Terpinen-4-ol may be produced by the fungus as a catabolite of α- and β-pinene, as well as being a component of induced host tree oleoresin (Kandasamy et al., 2023). These findings suggest that spruce bark recognizes the infection of bark beetle fungal symbionts and responds by elevating fungistatic compounds to restrict further advancement of fungi.

4.4 | Some spruce monoterpenes even promote the growth of the symbiotic fungi

Several Norway spruce oleoresin monoterpenes that were toxic to *I. typographus* not only failed to inhibit the growth of the symbiotic fungi, but even promoted fungal growth. This was especially true for *G. penicillata*, which exhibited pronounced tolerance to induced monoterpene mixtures and other spruce defence compounds in a previous study (Zhao et al., 2018). The stimulation of *G. penicillata* growth

by monoterpenes may allow this species to out-compete other fungi in terpene-rich bark, and so better deliver its benefits to its bark beetle partners, such as nutritional supplementation (Six, 2012; Zaman et al., 2023). Fungal growth, despite the presence of high concentrations of monoterpenes, may result from direct detoxification reactions (Lah et al., 2013; Wang et al., 2014) or excretion via an efflux transporter (Wang et al., 2013), as described for the congeneric *G. clavigera*. The stimulation of fungal growth by monoterpenes may be due not only to the ability of *G. penicillata* to metabolize monoterpenes to less toxic derivatives or excrete them, but also to their use of the metabolites as carbon sources for central metabolism (Cale et al., 2016; DiGuistini et al., 2011; Wang et al., 2014).

5 | CONCLUSIONS

The complexity of chemical defence mixtures in plants has long puzzled researchers, but few studies have sought the underlying causes. Here, we investigated the naturally occurring monoterpene mixtures in Norway spruce oleoresin and tested them against native enemies. Our results support two hypotheses explaining the existence of mixtures of defence compounds in plants. The interaction diversity hypothesis predicts that the individual components of mixtures act against different enemies, while the synergy hypothesis postulates that mixtures are more active than would be expected from the simple addition of the individual components. While mixtures of many more plants need to be studied before large-scale generalizations are possible, future progress in understanding the function of mixtures also depends on knowing more about the range of enemies that infest plants, the causes of synergy and the molecular mode of action of defences.

AUTHOR CONTRIBUTIONS

Dineshkumar Kandasamy, Rashaduz Zaman, Jonathan Gershenzon and Almuth Hammerbacher conceived the ideas. Dineshkumar Kandasamy and Rashaduz Zaman designed methodology for experiments and models; Rashaduz Zaman and Akanksha Jain collected the data; Dineshkumar Kandasamy and Rashaduz Zaman analysed the data; Dineshkumar Kandasamy, Jonathan Gershenzon and Rashaduz Zaman led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at https://doi.org/10.6084/m9.figshare.26495722.v1 (Kandasamy et al., 2024).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. List of fungi used in this study.

Table S2. List of monoterpenes used in bioassays along with their purities and suppliers.

Table S3. Composition of constitutive and induced blends used in bioassays.

Table S4. Toxicity of Norway spruce monoterpenes to *Ips typographus*.

Table S5. Toxicity of Norway spruce monoterpenes to *Ips typographus* males and females.

Table S6. IC_{50} values of Norway spruce monoterpenes for *Grosmannia penicillata*.

Table S7. IC_{50} values of Norway spruce monoterpenes for *Endoconidiophora polonica*.

Table S8. IC50 values of Norway spruce monoterpenes for *Ophiostoma bicolor*.

Table S9. Concentrations (ng mg^{-1} of fresh bark) of monoterpenes identified within bark lesions caused by different fungal and control treatments (wounded and unwounded bark without fungus) ($n = 5$ or 10).

Table S10. Vector fitting analysis of monoterpenes that strongly correlate with NMDS axes.

Table S11. List of monoterpene concentration ranges tested on fungi.

Appendix S1. Supplementary methods.

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