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PLANT-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Inducibility of chemical defenses in Norway spruce bark is correlated with unsuccessful mass attacks by the spruce bark beetle

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Abstract Secondary attraction to aggregation pheromones plays a central role in the host colonization behavior of the European spruce bark beetle *Ips typographus*. However, it is largely unknown how the beetles pioneering an attack locate suitable host trees, and eventually accept or reject them. To find possible biomarkers for host choice by *I. typographus*, we analyzed the chemistry of 58 Norway spruce (*Picea abies*) trees that were subsequently either (1) successfully attacked and killed, (2) unsuccessfully attacked, or (3) left unattacked. The trees were sampled before the main beetle flight in a natural Norway spruce-dominated forest. No pheromones were used to attract beetles to the experimental trees. To test the trees' defense potential, each tree was treated in a local area with the defense hormone methyl jasmonate (MeJ), and treated and untreated bark were analyzed for 66 different compounds, including terpenes, phenolics and alkaloids. The chemistry of MeJ-treated bark correlated strongly with the success of *I. typographus* attack, revealing major chemical differences between killed trees and unsuccessfully attacked trees. Surviving trees produced significantly higher amounts of most of the 39 analyzed mono-, sesqui-, and diterpenes and of 4 of 20 phenolics. Alkaloids showed no clear pattern. Differences in untreated bark were less pronounced, where only 1,8-cineole and (−)-limonene were significantly higher in unsuccessfully attacked trees. Our results show that the potential of individual *P. abies* trees for inducing defense compounds upon *I. typographus* attack may partly determine tree resistance to this bark beetle by inhibiting its mass attack.

Keywords Resistance · Mass attack · Terpenes · Phenolics · Alkaloids

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Introduction

The spruce bark beetle *Ips typographus* L. (Coleoptera: Curculionidae: Scolytinae) is a serious pest of Norway spruce, *Picea abies* (L.) H. Karst., in Eurasia. Conifer-killing bark beetles have been described as minute

pack-hunting predators (Berryman et al. 1985), killing large numbers of well-defended mature trees, and causing great economic losses, and changing landscapes and CO_2-budgets of whole biomes (Kurz et al. 2008). Small endemic populations of *I. typographus* colonizing downed or severely weakened host trees can increase dramatically to epidemic levels, usually following windstorms or extended drought periods, and then destroy large areas of Norway spruce forest (Långström et al. 2009). The tree-killing behavior is mediated by a rapidly produced aggregation pheromone (Birgersson et al. 1984) that can attract thousands of attacking beetles to a single tree within hours or days. When the breeding substrate within a tree is occupied, beetles arriving later switch their attack to neighboring trees (Birgersson et al. 1984; Schlyter and Anderbrant 1989). This process continues until the pool of dispersing beetles within the attraction range of a pheromone plume is depleted or until unsuitable flight conditions prevent more beetles from joining.

The first *I. typographus* to approach a tree, however, cannot rely on guidance from pheromone plumes. These pioneer beetles are thought to use semiochemicals from both *P. abies* and non-hosts in their search for suitable habitats (Zhang and Schlyter 2004), since they have similar numbers of olfactory receptor neurons for host- and non-host odors as for pheromones (Andersson et al. 2009). But how pioneers assess host tree suitability is largely unknown. Tree suitability includes traits such as nutrient content and defensive capacity. Phloem from vigorous trees is thicker and more nutritious than phloem from dying trees, and beetles consequently reproduce better in vigorous trees (Reid and Robb 1999; Hedgren and Schroeder 2004). On the other hand, the ability to avoid trees with a strong defensive capacity may be a matter of life or death for the pioneering beetles (Raffa 2001). The spatial distribution of trees attacked by *I. typographus* within stands clearly suggests that beetles are able to discriminate between suitable and unsuitable host trees. Attacked trees are often found in groups at sun-exposed forest edges and other sites exposed to drought or other stress factors (Jakuš 1998; Wermelinger 2004). However, even during severe outbreaks, there may be surviving trees within such pockets of destruction (Wallin and Raffa 2004), suggesting that some trees are inherently resistant to attack.

Despite these field observations of host discrimination by the beetles, it has not been possible to demonstrate a clear behavioral response to host stimuli alone in *I. typographus*. Two alternative hypotheses have been provided to explain the host selection of tree-killing bark beetles (Schlyter and Birgersson 1999): primary attraction, guided by host monoterpenes, or other stress signals to individual trees (Reddemann and Schopf 1996; Baier et al. 1999), or random landing followed by post-landing choices (Moeck et al. 1981; Byers 1996). Regardless of the initial mechanism of host finding, final host acceptance or rejection is thought to be dependent on inherent chemical properties of the host.

However, Raffa and Berryman (1982) found no correlations between beetle host choice and the terpene chemistry of the constitutive bark of *Pinus contorta* Douglas var. *latifolia* taken before natural attacks by high populations of *Dendroctonus ponderosae* Hopkins. In contrast, resistance was correlated with the induced responses of the tree, including increased monoterpene content in bark that previously had been inoculated with fungi vectored by this beetle. For *I. typographus*, Zhao et al. (2011b) recently demonstrated a significant correlation between terpene induction following fungal inoculation and beetle colonization of trees baited with artificial pheromones.

Previous studies of conifer defense traits have almost exclusively addressed the monoterpene composition of the host. However, phenolic compounds, a major chemical class in *P. abies* bark, have rarely been investigated even though they have been suggested as resistance markers (Lieutier 2004; Zhao et al. 2011a, b), and also have been demonstrated to serve as feeding inhibitors in laboratory bioassays with *I. typographus* (Faccoli and Schlyter 2007). In addition, tissues of *Picea* spp. have been shown to contain small amounts of alkaloids (Stermitz et al. 2000), that are known as antifeedants to insects in low concentrations (Kamm et al. 1998 and references therein). To our knowledge, no studies to date concerning resistance of conifers to bark beetles have included alkaloids. Moreover, the simultaneous responses of these three groups of compounds have never been assessed in conifer–bark beetle systems.

Untangling host selection and colonization success of bark beetles under field conditions is a challenging task. To be able to characterize trees that are susceptible to pioneer attacks, it is necessary to distinguish between trees that are the original focal trees of attraction and trees that are attacked as a result of the "spill over" of attack from a neighboring focal tree due to pheromone attraction (Raffa and Berryman 1982). Even relatively resistant trees may succumb to attack if they are growing next to a successfully attacked tree. Such studies are further complicated by the resources needed for sampling sufficient numbers of experimental trees a priori, since it is difficult to predict where attacks will occur. However, attack probability increases with beetle population size, proximity to previous attacks, and tree exposure to solar radiation, drought, and other stress factors (Mulock and Christiansen 1986; Wichmann and Ravn 2001).

A recent beetle epidemic triggered by heavy storms in 2005 and 2007 (Enander and Svensson 2007) provided an opportunity to test host choice by *I. typographus* under

natural conditions without using synthetic pheromones. We hypothesized that: (1) focal trees of successful bark beetle attack would differ in the constitutive chemical properties of their bark compared to neighboring trees not killed by the beetles, or that (2) focal trees would have weaker induced defenses than surviving trees. By screening host bark sampled before flight of *I. typographus* for a wide range of defensive compounds, including terpenes, phenolics, and alkaloids, we hoped to identify chemical markers for host acceptance or rejection that later may be tested for possible behavioral activity in *I. typographus*. We also assessed the formation of traumatic resin ducts (TRD) in the xylem as an indicator for defensive responses to beetle challenge (Franceschi et al. 2005). In order to test the induced defensive capacity of every tree, we analyzed bark sampled 4 weeks after a very localized treatment with the defense hormone methyl jasmonate (MeJ) for the same variables as untreated samples.

Materials and methods

Study area, sampling of trees

The experiment was performed in a forest dominated by Norway spruce [*Picea abies* (L.) Karst.], in Parismåla, SE Sweden (56°35′N, 15°29′E, 120–135 m a.s.l.). Trees were 90–100 years old and were naturally regrown except for a minor area that was planted 50 years ago. The experimental area covered ~15 ha (Online Resource 1). In 2005, the largest tree-felling storm in recorded history, with ~75 million m^3 of forest wind-felled, followed by a second big storm in 2007, triggered a serious bark beetle epidemic in southeastern Sweden. Warm and partly dry summers impaired the vigor of the forests in 2006 and 2007, and 2.3 million m^3 were killed by *I. typographus* attacks in 2006–2008 (Enander and Svensson 2007; Långström et al. 2009). In March 2008, we selected 290 experimental trees for sampling along forest edges created by removal of trees that had been killed by bark beetles the previous years, or on elevated terrain where trees are more exposed to drought and thus more likely to be attacked.

Five observation traps (Lindgren funnel traps; Pherotech, Canada) baited with commercial pheromone dispensers (IT ECOLURE; Fytofarm, Slovakia) were placed on clear-cuts with at least 30–50 m distance to experiment trees, and with >100 m inbetween, and checked weekly. The attraction range of pheromone traps for bark beetles are estimated not to exceed 50 m (Schlyter 1992) and recommendations from the Swedish forest agency state a minimum distance of 15–20 m to standing trees to avoid infection.

One bark sample (1 × 4 cm), including outer bark and the outermost sapwood, designated as "untreated", was removed from the shady side of each tree at 2 m above ground, and the wound was sealed with grafting wax. Samples were immediately frozen in liquid nitrogen and transferred to a −80 °C freezer the same day. The day of sampling each tree was treated with MeJ to induce chemical defenses by removing a bark plug (9 mm diameter) at 0.5 m above ground using a cork borer, placing a filter paper (9 × 9 × 0.5 mm) soaked with 50 mM MeJ on the exposed cambium surface, and replacing the bark plug to seal the wound. Sampling for analysis of untreated bark chemistry and local MeJ treatment was performed between 3 April and 15 May from all 290 selected trees before any bark beetle attack. One month later, two 1 × 2 cm samples of bark were taken immediately above and below the MeJ treatment point and designated as "treated". In two cases, treated samples were collected after attack, but none of these trees showed beetle damage in the vicinity of the MeJ application on the lower 1 m of the stem.

Analysis of terpenes

Sample preparation and extraction

For chemical and anatomical analyses, we selected the samples from 19 out of the 290 sampled trees that were attacked after sampling and from 39 unattacked sampled ("control") trees growing adjacent to attacked trees. For extraction of terpenes, 150–300 mg of frozen bark sample (without sapwood, but including cork bark) was ground in liquid nitrogen and immediately submerged in 1.5 ml 90:10 pentane/ether (v/v %) containing 50 μg ml^{-1} heptyl acetate and 200 μg ml^{-1} heptadecanoic acid as internal quantification standards. The powder was extracted for 30 min in an ultrasonic water bath at 20 °C. To remove polar contaminants, the solvent was extracted with 0.3 ml saturated $(NH_4)HCO_3-$ solution and the pentane/ether phase was then filtered through 0.3 g silica gel (0.2–0.5 mm/Merck) presaturated with solvent and 0.2 g anhydrous Na_2SO_4. The silica gel was rinsed with 1 more ml of the pentane/ether mixture and the rinse was added to the eluate.

Diterpene acids were derivatized by methylating 0.4 ml of the pentane-ether extract with 50 μl of 0.2 M *N*-trimethylsulfonium hydroxide (TMSH) in methanol (Macherey–Nagel, Germany) at room temperature for 2 h. The solvent was evaporated to ~150 μl for untreated samples and ~250 μl for MeJ-treated samples. The remaining non-methylated part of the extract was used for the analysis of mono- and sesquiterpenes. For treated samples, the extract was analyzed without further evaporation, whereas it was evaporated to ~250 μl for the untreated samples. The dry weight of each sample was obtained by drying the remaining bark powder overnight in a fume hood at room temperature followed by 30 min at 80 °C in an oven prior to weighing.

Terpene analysis

Terpenes were analyzed by combined gas chromatography and mass spectrometry (GC–MS) using an Agilent 6890–5973 instrument (Agilent Technologies, Palo Alto, CA, USA), fitted with a fused silica capillary column (30 m × 0.25 mm) coated with HP-5MS stationary phase (film thickness 0.25 μm; Agilent Technologies 19091S-433). All samples were injected with an Agilent 7683 autosampler, at an inlet temperature of 220 °C with helium as carrier gas (flow rate 35 cm/s). The mass spectrometer was operated using electron impact ionisation (70 eV) in scan mode with a mass range of *m/z* 40–550, and with temperatures for interface 280 °C, quadropole 150 °C, and source 230 °C.

Mono- and sesquiterpenes

Split injections (1 μl extract) at a ratio of 1:5 were applied at an initial oven temperature of 40 °C for 3 min, increased by 3 °C min^{-1} to 80 °C, followed by 5 °C min^{-1} to 180 °C, 5 min at 180 °C, and a final increase of 15 °C min^{-1} to 240 °C.

Methylated diterpenoid acids

Split injections (1 μl extract) at a ratio of 1:10 were applied. The initial oven temperature was 120 °C for 5 min, increased at a rate of 1 °C min^{-1} to 150 °C, followed by 5 °C min^{-1} to 250 °C and held at 250 °C for 10 min.

Analysis of monoterpene enantiomeric composition

All mono- and sesquiterpene samples were reanalyzed on an Agilent 6890–5975 GC–MS, fitted with a fused silica capillary column (30 m × 0.25 mm) coated with HP-Chiral 20B stationary phase (β-cyclodextrin, film thickness 0.25 μm; Agilent Technologies 19091G-B233), and helium as carrier gas (flow rate 35 cm/s). Using the 7683 autosampler, splitless injections were applied (1 μl extract) with 0.1 min purge time and an inlet temperature of 220 °C. The temperature program started at 30 °C for 3 min, increased by 8 °C min^{-1} to 150 °C, followed by 12 °C min^{-1} to 225 °C, and held at 225 °C for 5 min. The mass spectrometer was operated in scan mode with a mass range of *m/z* 29–400 and an initial transfer line temperature of 150 °C, ramped to 225 °C. All other parameters were as described above.

Compound identification

Terpenes were identified by comparison of retention times and mass spectra with authentic standards, or to mass spectra in the Wiley library or Pherobase (El-Sayed 2010). Compounds that could not be reliably identified were assigned as unknowns to a terpenoid class, based on their mass spectra. One minor phenolic compound (estragole, also called 4-allylanisole) was extracted and identified together with the terpenes.

Quantification

All peaks were quantified using the software Agilent MSD Chemstation D.02.00.275. The amount of each terpenoid was based on the peak area of each compound in the total ion chromatograms (TIC), normalized with the peak area of the internal standard heptyl acetate for mono- and sesquiterpenes and the methylated heptadecanoic acid for methylated diterpenic acids. Standard curves for representative mono-, and sesquiterpene hydrocarbons and methylated diterpenic acids using concentrations spanning five orders of magnitude, together with equal amounts of their internal standards, were evaluated. All measured amounts of these compounds in our samples fell within the concentration range of the standard curve that was linear ($R^2 \geq 0.99$). The enantiomeric ratios determined on the chiral column were also used to recalculate and confirm the amounts of mono- and sesquiterpenes quantified using the HP-5MS column. No separation of limonene and β-phellandrene was possible on the HP-5MS. However, their mass spectra are different, and their quantifications were based on the extracted ion chromatograms of *m/z* 68 for limonene and *m/z* 93 for β-phellandrene, adjusted by the ratio of *m/z* 68 and 93 in limonene. 1,8-cineole did not separate from limonene and β-phellandrene on the HP-5MS column, so the quantification of 1,8-cineole was based only on the chiral analysis. The peak for β-pinene overlapped with that for sabinene on the HP-5MS. Therefore, the amount of sabinene was calculated from the chiral analysis, and the amount of β-pinene on the HP-5MS was adjusted accordingly. To be able to compare quantifications from HP-5MS and the chiral column, response factors for known monoterpenes were calculated using commercial standards, and relative amounts of 1,8-cineole and sabinene from the chiral column were adjusted with these factors.

Analysis of phenolics and alkaloids

Sample preparation

Spruce bark was ground to a fine powder in liquid nitrogen and freeze-dried using an Alpha 1–4 LD plus freeze dryer (Martin Christ, Germany). Approximately 100 mg powder was extracted for 12 h at 4 °C in 4 ml methanol containing 10 μg ml^{-1} chlorogenic acid as internal standard. The extract was filtered, dried under nitrogen, and re-dissolved in 1 ml methanol. Undiluted samples were used for the analysis of alkaloids. For analysis of phenolics, samples were diluted 1:40 in methanol.

Liquid chromatography-mass spectrometry with electrospray ionization (LC–ESI–MS)

Phenolics and alkaloids were identified and quantified by LC–ESI–MS using a Bruker Esquire 6000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). For analysis of phenolics, the mass spectrometer was operated in negative ionization mode, scanning in the range *m/z* 50–1,200 with an optimal target mass of *m/z* 405. Other parameters were: Skimmer voltage −60 V; capillary exit potential −121.4 V; capillary voltage 4,000 V; nebulizer pressure 35 psi; drying gas 11 L min^{-1}; and gas temperature 330 °C. Alkaloids were analyzed using positive ionization mode scanning in the range of *m/z* 50–1,000 with optimal target mass at *m/z* 140, and skimmer voltage: −60 V; capillary exit potential −101.5 V; capillary voltage: −4,200 V; nebulizer pressure: 35 psi; drying gas: 11 L/min; and gas temperature: 330 °C.

The MS was coupled to an Agilent 1100 series HPLC. Compound separation was accomplished using a Nucleodur Sphinx RP column (250 × 9 × 4.6 mm, 5 l m; Macherey–Nagel) at a flow rate of 1 ml min^{-1}. Mobile phases were 0.2 % formic acid (v:v) (A) and acetonitrile (B), starting with 100 % A for 5 min, followed by a gradient to 75 % B in 25 min, 100 % B for 3 min and a re-equilibration step with 100 % A for 5 min. Esquire software (Brucker Daltronics) was used for data acquisition and Quant Analysis software (Brucker Daltronics) was used for quantification of extracted mass traces.

Linearity in ionization efficiencies was verified by analyzing dilution series of spruce bark extracts. Peak areas of compounds were normalized relative to the internal standard to account for process variability during analysis. External calibration curves for catechin, astringin, pinidine, and dehydropinidine were created by linear regression. Flavan-3-ol and flavonoid concentrations were determined relative to the catechin calibration curve (mg catechin equivalents), stilbenes, and neolignan concentrations were determined relative to the astringin calibration curve (mg astringin equivalents), and alkaloids relative to the pinidine or dehydropinidine calibration curves (mg pinidine equivalents).

Anatomical analysis

A subset of MeJ-treated tissue samples was examined microscopically for formation of traumatic resin ducts (TRD) in the young sapwood. We examined sapwood from 10 killed, 6 resistant and 8 control trees. Frozen tissue pieces of ~0.4 cm^2 were placed in fixative (1.25 % glutaraldehyde and 2 % para formaldehyde in 0.05 M Pipes buffer, pH 7.2) for 24 h. Semi-thin cross-sections (16 μm thick) were cut using a cryotome (Cryo-Star HM 560; Microm International, Walldorf, Germany) at −18 °C, mounted on glass slides, stained with Stevenel's blue, and photographed at ×20 magnification under a Leica S8APO Stereo Zoom Microscope fitted with a Leica DC 300F CCD camera. The percent coverage of TRD in the sapwood was measured across the tangential width (4 mm) of each cross section using the software ArcView GIS 3.2a (ESRI, Redlands, CA, USA).

Statistical analysis

Correlation structures between the three groups of trees assigned after beetle flight (attacked and killed trees, attacked but surviving trees, unattacked control trees) for 66 chemical variables were analyzed using principal component analyses (PCA) (SIMCA-P+ software v.12.0.1.0; Umetrix, Umeå, Sweden). The scaling method for PCA was the unit-variance method with values normalized in units of standard deviation, and residuals were standardized and autolog-transformed according to the software's standard settings. The α-level for Hotellings T2 was at 0.05. Comparison of means by ANOVA was performed in SPSS 11.0.0. (SPSS, Chicago, IL, USA) after $\log(x + 1)$ transformation of the data. Normality was tested with the Kolmogorov–Smirnov test and the homogeneity of variances with Levene. Tukey's HSD test (α at 0.05) was used as post hoc, and Dunnett's T3 was used for compounds that showed unequal variances. To compare the effect of MeJ treatment on the abundance of different chemical compounds, and on the formation of anatomical defense structures (TRD), we calculated the effect size, which scales the differences of a pair of means by their pooled standard deviation (bias corrected Hedges' *g*) (Nakagawa and Cuthill 2007). Canonical discriminant function analysis was run on SPSS 11.0.0.

Results

Extent of bark beetle attack

Flight activity of *Ips typographus* started in late April and continued until late August. The first attacks were recorded 5–7 May, and new attacks occurred from the end of May until the first week of June, in early July, and in early August. Of the 290 sampled *Picea abies*, 19 trees were attacked by *Ips typographus* in 2008, which accounted for approximately a third of the total number of trees attacked in the 15-ha study area during that year. In 2007, there were about 180 killed trees in the same area, and in 2006 the number was around 300. Even though beetle populations on the landscape scale remained high in 2008, it is likely that improved tree vigor (due to high precipitation in late summer 2007) resulted in fewer successful attacks, higher pheromone trap catches, and smaller groups of attacked

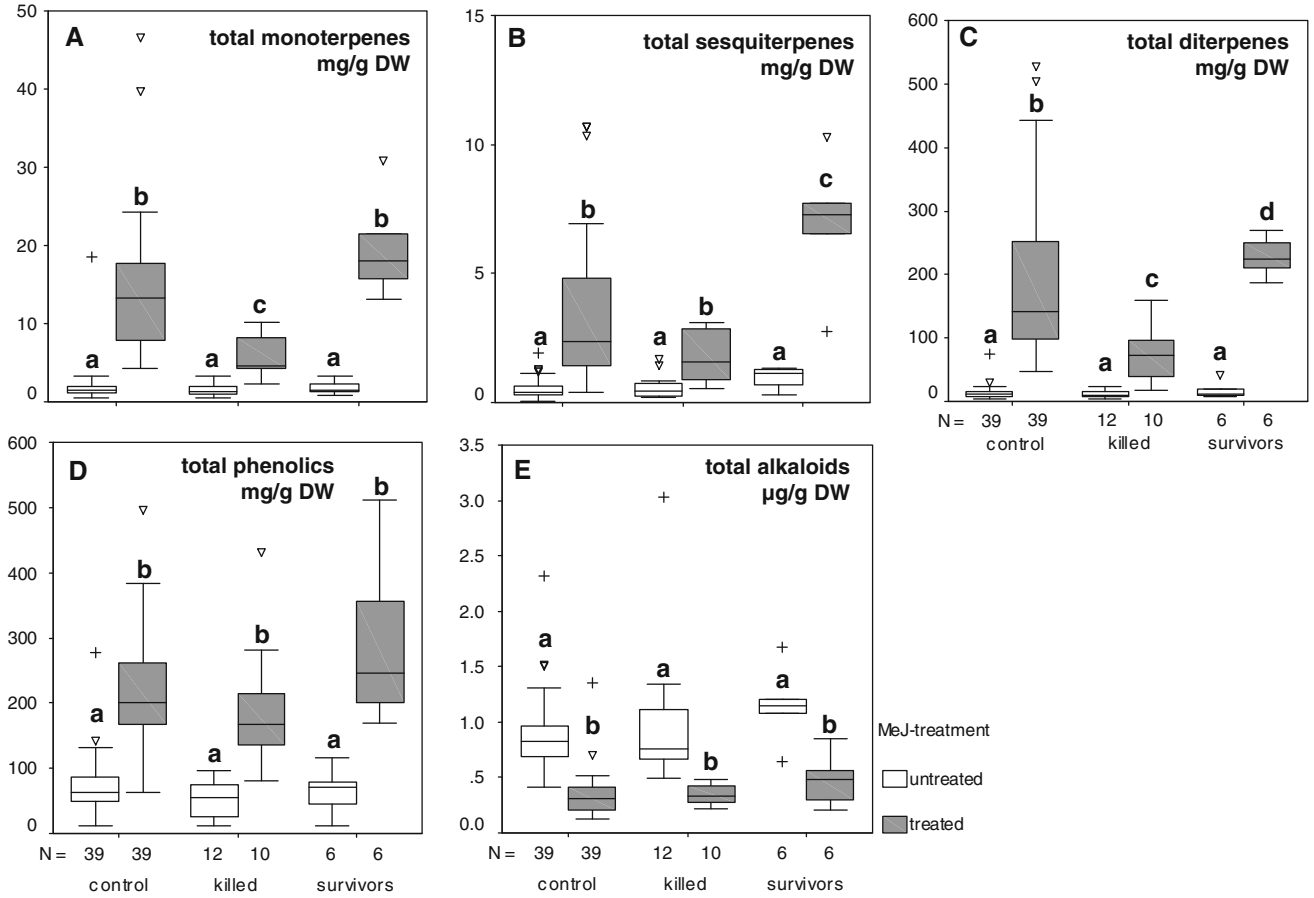

Fig. 1 Amounts of chemical classes in the bark of sampled trees. Samples were chosen from 290 trees sampled before beetle flight and conferred to the groups "killed", "surviving" or unattacked "control" *P. abies* trees after beetle attack. Each tree is represented by two samples either treated with MeJ or untreated: **a** monoterpenes (23 compounds), **b** sesquiterpenes (9 compounds), **c** diterpenes (6 compounds), **d** phenolics (20 compounds), and **e** alkaloids (8 compounds). In treated samples, two killed trees (G19 and E43) and one surviving tree (C40) are excluded (see Fig. 3a and text). *Light boxes* show distributions for untreated and *dark boxes* for treated bark samples. *Boxes* show lower and upper quartile divided by the median, *whiskers* assign minimum and maximum values, *inverted triangle* outliers, *plus* extreme values. *Boxes* with the same *letters* are not significantly different [ANOVA of $\log(x + 1)$ transformed values with Tukey HSD and Dunnett's T3 for diterpenoids]. Note the different scale for alkaloids (µg g^{-1})

trees than in the two previous years (Långström et al. 2009). A total of 98,000 beetles were caught in the five observation pheromone traps maintained in the vicinity of the experimental area in 2008.

The 19 attacked trees were distributed among five small, independent spots of attacked trees with 1–9 trees in each. Twelve of the attacked trees were killed, but seven survived. On the seven surviving trees, we noted only 1–10 attack holes at the lower 4 m of the stem. From frequent visits, we observed that the pioneering beetles on these trees had either been drowned in resin or had left their entrance holes after 3 days, and no more beetles joined their attack. Beetle activity was high during this period, with optimal flight conditions and high beetle counts in the observation traps. The seven trees apparently resisted attack even though one sampled and some non-sampled trees were successfully attacked within 20 m. These seven resisting trees are designated as "survivors" in the analysis. We selected "control" trees from 271 unattacked sampled trees that stood near the attacked trees. The positions of killed, surviving, and control trees in the study area are shown in a map (Online Resource 1).

Induction of potential defense metabolites by MeJ

The local MeJ treatment at the stem base resulted in a strong overall increase in terpenes and phenolics in all samples taken adjacent to treatment points, independent of subsequent attack success. The monoterpenes increased 4.6-fold (corresponding to an effect size of $g = 1.5$), sesquiterpenes 7.1-fold, ($g = 1.0$); diterpenes 7.6-fold ($g = 1.8$), and phenolics 1.5-fold ($g = 2.0$), whereas alkaloid levels decreased (a 0.4-fold change, $g = -0.5$) (Fig. 1). However, overall chemical changes, measured by effect

size $g \pm$ SE (all 66 compounds included), differed significantly in strength between trees that survived the attack ($g = 1.3 \pm 0.12$), and trees that were killed ($g = 0.9 \pm 0.09$) (two-tailed t tests, $t = 4.7$, df 65, $p \ll 0.05$), and between trees that survived and unattacked controls ($g = 1.0 \pm 0.09$) ($t = 5$, df 65, $p \ll 0.05$). Absolute amounts and effect sizes for all 66 quantified compounds (23 monoterpenes, 9 sesquiterpenes, 6 diterpenoids, 20 phenolics, and 8 alkaloids) are shown in Table 1.

Multivariate analysis of 66 compounds in relation to bark beetle attack

To compare the chemical composition of killed, surviving, and unattacked control trees, we applied separate models for MeJ-treated and untreated bark in principal component analyses (PCA). In untreated bark, the score scatter plot with all 66 quantified compounds showed no clear grouping with regard to success of attack (Fig. 2a). However, for MeJ-treated bark, 10 of the 12 killed trees separated well from all 7 surviving trees in the PCA score scatter plot. Unattacked control trees were evenly distributed between the two groups (Fig. 3a). Two killed trees (E43 and G19 in Fig. 3a) that were chemically more similar to surviving trees had grown next to other successfully attacked trees (Map, Online Resource 1). Tree E43 resisted beetle attack for 1 week after five neighboring trees in the attack spot had been successfully attacked. Tree G19 stood directly adjacent to a tree damaged by lightning strike, which was attacked in August. These two trees were excluded from further comparisons, since it appeared that they were victims of attack not because of their intrinsic properties but simply by being located next to other successfully attacked trees. A surviving tree with extremely high levels of defensive compounds in both untreated and MeJ-treated bark (C40) was classified as an outlier and was also excluded from further comparisons. The two components in the score scatter plot (Fig. 3a) explained 47.6 % of the variation in the dataset. A third component explained an additional 5.8 % of the variation. To test the correct assignment of samples to predetermined groups, a discriminant analysis was performed. In this analysis, the 10 killed and 6 surviving trees (without excluded trees) and 35 out of the 39 control trees were correctly classified. The four misclassified controls were classified as killed trees.

Monoterpenes and diterpene acids contributed more to the separation between killed and surviving trees than sesquiterpenes, phenolics, and alkaloids, as shown in the PCA loading plots (Figs. 2b, 3b). The weighted differences of single compounds between two groups in a score scatter plot can be derived from a contribution plot (not shown) that explains why one group of points deviates from an other. The weighted differences for the group of surviving trees versus the group of killed trees had values >2.5 for the monoterpenes (−)-α-pinene, tricyclene and (+)- and (−)-camphene, values >2 for the diterpenes neoabietic acid, phenanthrene carboxylic acid and sandaracopimaric acid, and values >1.5 for the sesquiterpenes α-cubebene/α-longipinene (not chromatographically separated) and δ-cadinene . All phenolics scored below 1, except for myricetin (2.3), estragole (1.8), and laricitrin (1.7), which grouped together with the monoterpenenes on the right-hand side of the first principal component (Fig. 3b).

Differences in individual compounds and compound classes in relation to attack

Untreated bark

Comparisons of means of individual compounds by ANOVA showed only a few differences between killed, surviving, and unattacked control trees in their constitutive chemistry prior to MeJ treatment. Killed trees had significantly lower amounts of the monoterpenes 1,8-cineole ($p = 0.002$) and (−)-limonene ($p = 0.006$) (Fig. 4) than surviving trees, and lower amounts of bornyl acetate ($p = 0.027$) than control trees (Table 1). The sesquiterpenes as a group had nearly significantly higher amounts in surviving trees than in controls ($p = 0.064$) (Fig. 1).

MeJ-treated bark

In treated bark, all individual monoterpenes (except (−)-β-pinene, α-terpinene, (−)- and (+)-β-phellandrene and 1, 8-cineole) had significantly lower levels in killed than in surviving trees (Table 1). The same was true for 3 of the 9 sesquiterpenes (unknown SqT 2, δ-cadinene, and α-cubebene/α-longipinene), all 6 diterpenes, and 4 of 20 phenolics (myricetin, estragole, laricitrin, and an unknown coniferyl alcohol derivative). The phenolic *Z*-astringin had significantly higher levels in killed trees than in surviving trees. Alkaloid levels were very low in all samples; only pipecoline and *trans*-pinidinol showed means above 0.5 μg g^{-1} DW in untreated samples, and alkaloid levels decreased even further after MeJ treatment. Some surviving trees and some unattacked control trees showed a huge increase of euphococcinine, *trans/cis*-methylseridine, and pinidine after MeJ treatment (Table 1; Fig. 4). Sums of monoterpenoids, sesquiterpenes, and diterpenes were significantly lower in the bark of killed trees than in surviving trees after MeJ treatment, whereas phenolics and alkaloids did not differ significantly between these groups (Fig. 1a–e).

Formation of traumatic resin ducts after MeJ treatment

The formation of traumatic resin ducts (TRD) in the secondary xylem following MeJ treatment was quantified in

Table 1 Amounts of terpenes, phenolics and alkaloids in MeJ-treated and untreated *P. abies* bark-samples

Compound	Mean in untreated samples μg g^{-1} DW ± SD			Mean in treated samples μg g^{-1} DW ± SD			Effect size Hedges' *g* of MeJ-treatment		
	Control	Killed	Survivors	Control	Killed	Survivors	Control	Killed	Survivors
Monoterpenes									
Tricyclene[b]	6 ± 6	5 ± 2	13 ± 22	42 ± 31	**26 ± 30**	**62 ± 21**	1.6	1.0	2.1
α-Thujene[b]	5 ± 6	5 ± 6	14 ± 25	28 ± 29	**20 ± 24**	**39 ± 22**	1.1	0.8	1.0
(−)-α-Pinene[a]	448 ± 554	341 ± 165	1,080 ± 1,700	3,400 ± 2,480	**1,860 ± 1,360**	**5,980 ± 2,250**	1.6	1.5	2.3
(+)-α-Pinene[a]	531 ± 439	469 ± 236	797 ± 892	3,040 ± 1,820	**1,900 ± 1,730**	**3,230 ± 1,240**	1.9	1.2	2.1
(−)-Camphene[a]	18 ± 17	13 ± 9	59 ± 107	120 ± 80	**73 ± 95**	**277 ± 213**	1.8	0.9	1.2
(+)-Camphene[a]	6 ± 7	5 ± 2	15 ± 25	47 ± 34	**25 ± 26**	**67 ± 28**	1.6	1.1	1.9
Verbenene[b]	0.3 ± 0.9	0.1 ± 0.1	0.81 ± 2	2 ± 2	**2 ± 2**	**6 ± 8**	1.4	1.4	0.8
(−)-Sabinene[a]	7 ± 11	4 ± 2	6 ± 4	103 ± 212	**35 ± 38**	**106 ± 103**	0.6	1.2	1.3
(+)-Sabinene[a]	34 ± 35	24 ± 24	54 ± 37	226 ± 212	**139 ± 171**	**288 ± 151**	1.3	0.9	2.0
(−)-β-Pinene[a]	629 ± 1170	458 ± 285	1,580 ± 2,880	4,970 ± 3,590	3,160 ± 3,270	5,540 ± 3,000	1.6	1.2	1.2
Myrcene[a]	54 ± 101	42 ± 40	247 ± 523	444 ± 524	**208 ± 273**	**713 ± 367**	1.0	0.9	0.9
α-Phellandrene[b]	3 ± 4	2 ± 2	19 ± 41	38 ± 40	**17 ± 23**	**61 ± 65**	1.2	0.9	0.7
3-Carene[a]	27 ± 78	9 ± 20	108 ± 270	479 ± 1117	**134 ± 211**	**675 ± 728**	0.6	0.8	0.9
α-Terpinene[a]	3 ± 4	3 ± 3	20 ± 44	32 ± 34	17 ± 22	43 ± 37	1.2	0.9	0.6
Para-cymene[a]	6 ± 15	2 ± 2	37 ± 93	19 ± 18	**12 ± 12**	**65 ± 107**	0.8	1.2	0.3
(−)-β-Phellandrene[b]	171 ± 354	103 ± 85	553 ± 1140	1,330 ± 1,560	724 ± 897	1,960 ± 1,400	1.0	1.0	1.0
(+)-β-Phellandrene[b]	14 ± 18	10 ± 7	28 ± 46	68 ± 56	42 ± 46	75 ± 36	1.3	1.0	1.0
(−)-Limonene[a]	15 ± 15	**15 ± 24**	**70 ± 80**	113 ± 170	**36 ± 27**	**504 ± 529**	0.8	0.8	1.0
(+)-Limonene[a]	15 ± 11	13 ± 10	25 ± 24	111 ± 71	**67 ± 76**	**124 ± 41**	1.9	1.0	2.8
1,8-Cineole[a]	3 ± 5	**2 ± 1**	**13 ± 24**	46 ± 50	31 ± 23	56 ± 28	1.2	1.7	1.5
γ-Terpinene[a]	6 ± 8	6 ± 6	43 ± 94	60 ± 75	**31 ± 39**	**86 ± 70**	1.0	0.9	0.5
Terpinolene[a]	12 ± 14	9 ± 6	65 ± 147	172 ± 281	**65 ± 79**	**255 ± 221**	0.8	1.0	0.9
Bornyl acetate[a]	**12 ± 18**	**4 ± 8**	11 ± 20	112 ± 102	**48 ± 76**	**152 ± 73**	0.8	0.6	2.2
Sesquiterpenes									
α-Cubebene + α-longipinene[b]	38 ± 39	46 ± 66	67 ± 53	293 ± 358	**152 ± 163**	**428 ± 191**	1.4	0.8	2.4
Unknown SqT 1[c]	70 ± 48	70 ± 78	177 ± 270	637 ± 613	313 ± 323	820 ± 466	1.0	0.9	2.4
Unknown SqT 2[c]	64 ± 104	88 ± 163	120 ± 104	245 ± 211	**259 ± 278**	**577 ± 223**	1.3	1.0	1.6
β-Caryophyllene[b]	14 ± 20	14 ± 10	62 ± 104	152 ± 286	90 ± 144	265 ± 264	1.1	0.8	2.4
Unknown SqT 3[c]	18 ± 27	20 ± 17	60 ± 39	106 ± 166	64 ± 49	189 ± 134	0.7	0.7	0.9
Germacrene-D[b]	168 ± 231	179 ± 153	523 ± 324	1,380 ± 2,910	640 ± 457	3,190 ± 1,850	0.7	1.2	1.2
α-Cedrene[b]	17 ± 22	38 ± 32	109 ± 252	218 ± 335	264 ± 382	338 ± 323	0.6	1.4	1.9
Unknown SqT 4[c]	35 ± 36	38 ± 32	152 ± 220	224 ± 386	122 ± 134	348 ± 268	0.8	0.8	0.7
δ-Cadinene[b]	111 ± 87	117 ± 131	261 ± 251	768 ± 811	**337 ± 186**	**939 ± 256**	0.7	0.9	0.7

Table 1 continued

Compound	Mean in untreated samples μg g^{-1} DW ± SD			Mean in treated samples μg g^{-1} DW ± SD			Effect size Hedges' *g* of MeJ-treatment		
	Control	Killed	Survivors	Control	Killed	Survivors	Control	Killed	Survivors
Diterpenes									
Methyl sandaracopimarate[b]	592 ± 629	493 ± 370	1,090 ± 1,250	9,380 ± 6,590	**6,000 ± 6,960**	**14,700 ± 5,440**	1.1	1.4	2.5
Phenanthrenecarboxylic acid methyl ester[b]	1,936 ± 1,442	1,848 ± 1,338	4,383 ± 5,590	26,160 ± 18,500	**16,200 ± 15,700**	**39,100 ± 18,000**	1.9	1.1	3.3
Unknown diterpenoid[c]	5,700 ± 4,950	4,700 ± 2,740	11,900 ± 13,800	79,400 ± 53,700	**48,600 ± 40,600**	**1,03,200 ± 26,800**	1.8	1.3	2.4
Methyl dehydroabietate[b]	2,920 ± 3,270	2,230 ± 829	6,210 ± 8,200	30,600 ± 22,000	**23,600 ± 19,000**	**50,900 ± 30,000**	1.9	1.4	4.0
Methyl abietate[b]	1,250 ± 1,180	994 ± 676	3,770 ± 5,400	18,100 ± 12,800	**11,000 ± 12,200**	**27,300 ± 15,800**	1.8	1.5	1.9
Methyl neoabietate[b]	1,510 ± 1,170	1,500 ± 947	3,980 ± 5,220	19,722 ± 13,700	**12,000 ± 15,000**	**29,900 ± 12,300**	1.9	1.2	1.9
Phenolics									
Estragole[a]	3 ± 3	2 ± 2	4 ± 5	28 ± 42	**13 ± 28**	**55 ± 31**	1.9	1.0	2.5
E-Astringin[a]	35,300 ± 17,500	26,800 ± 16,200	23,800 ± 15,100	61,500 ± 24,100	60,700 ± 15,000	52,000 ± 21,400	1.2	2.1	1.4
Z-Astringin[a]	3,510 ± 3,460	1,330 ± 686	1,100 ± 678	12,800 ± 10,000	**9,280 ± 7,330**	**3,770 ± 3,280**	1.2	1.4	1.0
E-Isorhapontin[a]	5,620 ± 3,910	5,820 ± 4,160	8,660 ± 6,890	10,400 ± 5,720	14,400 ± 9,490	14,700 ± 6,900	1.0	1.2	0.8
Z-Isorhapontin[a]	1,000 ± 1,900	360 ± 401	620 ± 581	4,590 ± 5,990	3,480 ± 3,440	2,770 ± 4,280	0.8	1.3	0.7
Piceid[a]	1,060 ± 602	579 ± 273	704 ± 711	2,080 ± 1,270	1,930 ± 1,370	1,410 ± 845	1.0	1.4	0.8
Catechin[a]	900 ± 723	582 ± 472	794 ± 508	1,1500 ± 7,350	13,300 ± 7,160	10,800 ± 5,870	2.0	2.4	2.2
Gallocatechin[a]	3 ± 9	1.7 ± 1.7	2.1 ± 1.2	66 ± 87	79 ± 107	99 ± 125	1.0	1.0	1.0
Unknown coniferyl alcohol derivative[c]	77 ± 97	23 ± 24	91 ± 112	754 ± 1090	**566 ± 892**	**1,790 ± 2,040**	0.9	0.9	1.1
Unknown neolignan 1[c]	48 ± 152	6 ± 20	56 ± 75	343 ± 680	360 ± 1250	482 ± 834	0.6	0.4	0.7
Unknown neolignan 2[c]	586 ± 1167	169 ± 231	77 ± 121	1,180 ± 2,700	528 ± 419	416 ± 505	0.3	1.1	0.8
Unknown coniferyl alcohol derivative[c]	1,000 ± 1,040	380 ± 258	1,000 ± 848	604 ± 1410	30 ± 104	311 ± 678	−0.3	−1.7	−0.8
Unknown phenolic glycoside[c]	3,480 ± 3,510	2,640 ± 2,570	3,280 ± 4,650	57,500 ± 64,900	41,300 ± 60,000	72,000 ± 47,500	1.2	0.9	1.9
Kaempferol glucoside[a]	36 ± 64	1 ± 3	36 ± 63	103 ± 134	55 ± 57	138 ± 295	0.6	1.3	0.5
Unknown flavonol glucoside[c]	84 ± 142	41 ± 48	21 ± 41	415 ± 539	270 ± 298	396 ± 645	0.8	1.1	0.7
Laricitrin glycoside[a]	1,440 ± 2,110	652 ± 792	1,180 ± 830	2,910 ± 3,200	2,250 ± 1,360	3,690 ± 4,420	0.5	1.4	0.7
Quercetin glucoside[a]	361 ± 548	231 ± 616	201 ± 159	746 ± 855	456 ± 485	690 ± 549	0.5	0.4	1.1
Myricetin[a]	285 ± 612	107 ± 68	983 ± 2180	7,940 ± 4,750	**4,900 ± 3,520**	**12,700 ± 3,590**	2.3	1.8	3.7
Laricitrin[a]	1,190 ± 2,230	374 ± 322	1,640 ± 3,560	12,700 ± 7,440	**10,900 ± 8,910**	**25,800 ± 9,130**	2.1	1.6	3.3
Taxifolin glucoside[a]	16,800 ± 31,500	13,100 ± 11,700	19,300 ± 13,300	30,300 ± 36,800	36,400 ± 31,300	63,200 ± 78,800	0.4	1.0	0.7
Akaloids									
Trans-Pinidinol[d]	0.5 ± 2	0.3 ± 0.6	0.1 ± 0.1	4E−02 ± 0.1	0.1 ± 0.3	0.1 ± 0.2	−0.3	−0.4	0.1
Cis-N-Methylseridine[d]	0	0	0	2E−04 ± 7E−04	9E−04 ± 3E−03	4E−04 ± 6E−04	0.4	0.4	0.9

Table 1 continued

Compound	Mean in untreated samples µg g^{-1} DW ± SD			Mean in treated samples µg g^{-1} DW ± SD			Effect size Hedges' g of MeJ-treatment		
	Control	Killed	Survivors	Control	Killed	Survivors	Control	Killed	Survivors
Trans-N-Methylseridine[d]	0	0	0	3E−05 ± 1E−04	8E−05 ± 3E−04	4.E−05 ± 5E−05	0.3	0.4	0.9
Pipecoline[d]	0.7 ± 0.2	0.7 ± 0.1	0.9 ± 0.4	0.3 ± 0.1	0.3 ± 9E−02	0.4 ± 0.2	−2.6	−3.0	−1.6
N-Methylpellietrine[d]	7E−03 ± 1E−02	1E−02 ± 2E−02	1E−02 ± 3E−02	3E−04 ± 1E−03	4E−04 ± 7E−04	4E−02 ± 9E−02	−0.7	−0.9	0.3
Pinidine[a]	3E−05 ± 8E−05	0	2E−04 ± 4E−04	1E−03 ± 5E−03	3E−03 ± 1E−02	8E−02 ± 0.2	0.3	0.4	0.5
Euphococcinine[d]	6E−05 ± 3E−04	0	0	1E−03 ± 6E−03	2E−02 ± 7E−02	4E−03 ± 7E−03	0.3	0.4	0.7
Dihydropinidine[a]	8E−03 ± 1E−02	3E−02 ± 5E−02	7E−03 ± 6E−03	1E−03 ± 4E−03	1E−02 ± 4E−02	0.3 ± 0.8	−0.7	−0.3	0.5

Columns show values for trees that were later killed by *Ips typographus* attack, survived the attack, or were unattacked controls

Differences between columns with boldface values are significant for α set at 0.05

[a] Identified by comparison of retention time and mass spectrum to those of authentic standard

[b] Identified by comparison of retention time and mass spectrum to those found in the literature

[c] Identical mass spectrum at same retention time

[d] Tentatively identified by comparison of retention time and mass spectrum to those found in the literature relative to standard samples of pinidine and dihydropinidine

10 killed trees, 6 surviving trees, and 8 unattacked control trees. Killed trees had fewer and smaller TRD than either control trees or surviving trees. The effect of MeJ treatment on TRD formation was $g = 1.11$ for killed trees, $g = 2.77$ for surviving trees, and $g = 3.07$ for control trees (Hedges bias-corrected ES) (Fig. 5a, b, d). Some surviving trees had rows of TRD also in 2- to 4-year-old sapwood, indicating that they had been previously challenged by, e.g., drought or bark beetle attack (Fig. 5c).

Discussion

Our results suggest that the ability of trees to mobilize induced defenses, rather than qualitative and quantitative aspects of the constitutive chemistry, influence the success of host colonization by *I. typographus*. Trees that were subsequently successfully colonized and killed had an overall lower inducibility of defensive compounds and much lower formation of TRD than unsuccessfully attacked surviving trees. To test the defensive capacity of individual trees, we used a localized treatment with the defense hormone methyl jasmonate (MeJ) (Franceschi et al. 2005), carried out before bark beetle attack. MeJ sprayed upon the bark has been shown to elicit responses comparable to pathogen infection in Norway spruce bark 4 weeks after treatment (Krokene et al. 2008), and also to induce responses only locally (up to 7 cm from the application zone) within a similar time frame (Franceschi et al. 2005). Thus, we expected that our very localized treatment would not markedly influence the trees' systemic defenses and, by that, the beetles' host choice. In spite of the large between-tree variation in the naturally regrown forest, and the huge quantitative differences between different compound groups in the bark (ng/g DW in alkaloids and tens of mg/g DW in phenolics and diterpenes), we achieved a good grouping of killed versus surviving trees in the PCA of MeJ-treated bark. These sources for variation, however, probably explain why we achieved a relatively low percentage of explanation in our PCA including two components. Instead, we have to rely on a thorough exploration of the compounds with the highest impact on the grouping accounted for in the loading plot.

The seven surviving trees, with strong induced defenses, were also attacked by pioneer attackers, but these were not joined by enough conspecifics in a mass attack that could overcome the trees' defenses. A few of the attackers were drowned in resin, while others had abandoned their entrance holes after a couple of days, indicating that they may have sensed or realized the trees' defenses before they physically interfered with them. The same could be observed again in 2010, when three of our resistant trees from 2008 were found with new unsuccessful attacks

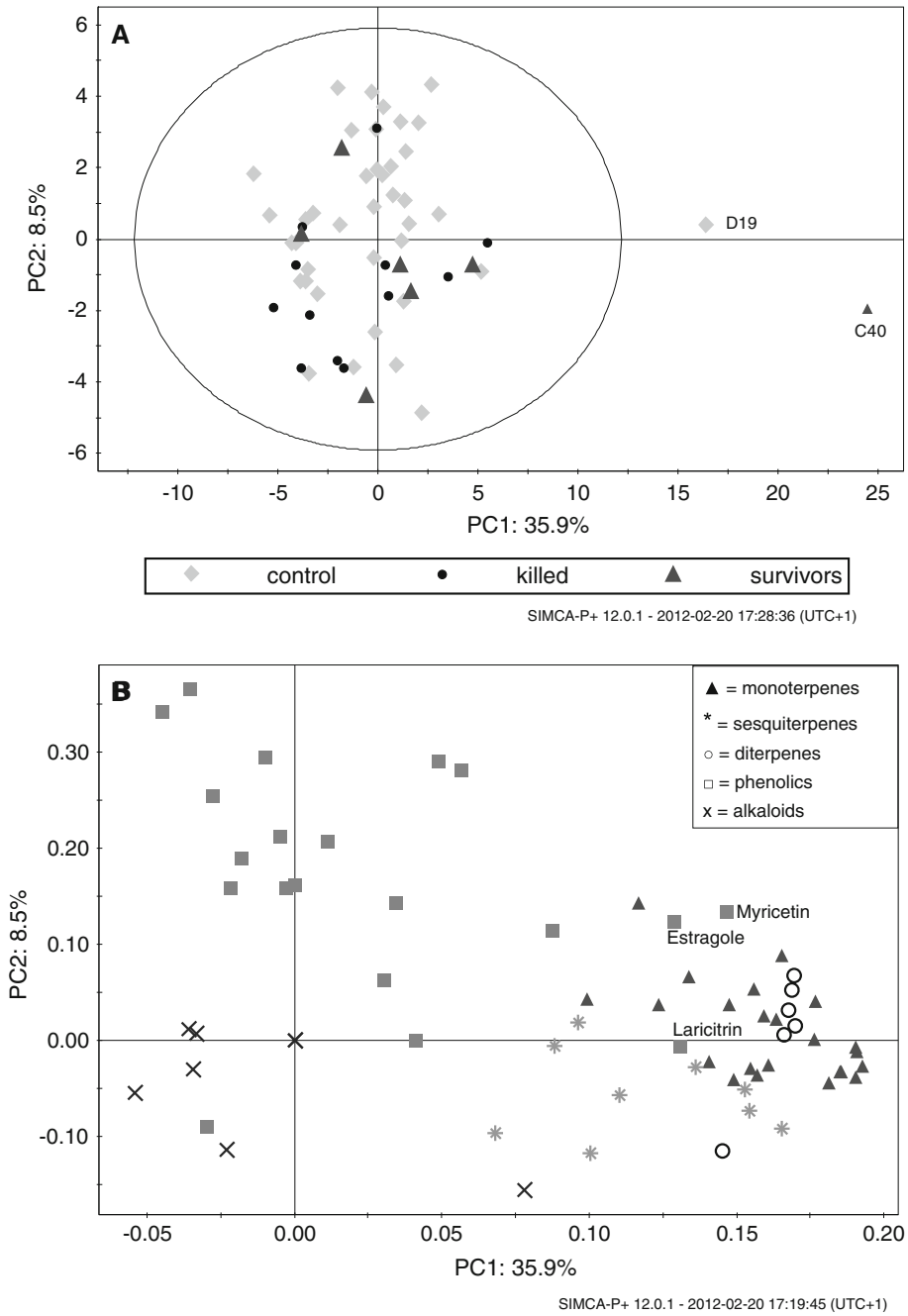

Fig. 2 Principal component analysis of untreated *Picea abies* bark samples. **a** Score scatter plot for the first and second components of all 58 untreated bark samples and 66 chemical variables. Scores are autolog-transformed. The first two components explain 44.5 % of the variance in the data. Trees C40 and D19, lying outside the 95 % range of the Hotelling T^2 ellipse, are considered to be outliers. **b** *Loading scatter plot* showing the influence of the 66 chemical variables in explaining the distances along the component axes between the samples shown in (**a**). The longer the distance to the origin, the more important is a compound in explaining the position of a sample along either the *x* or *y* axis in (**a**)

following colonization of a wind-felled tree nearby. Since these trees were chosen repeatedly by pioneering beetles, but survived, while others were attacked and killed nearby at the same time, we interpret the resistance shown by the surviving trees as an inability of the pioneering beetles to start a mass attack on trees with strong defenses.

Our finding that the ability to induce strong defenses, rather than the constitutive defense level, determines beetle colonization success, is in agreement with older and more recent studies. These previous studies, however, either used synthetic pheromones to attract beetles and addressed only physiological responses or terpene chemistry (Mulock and Christiansen 1986; Zhao et al. 2011b), or they were performed on different beetle–tree systems (Raffa and Berryman 1982). In a recent study in the *Dendroctonus ponderosae–Pinus contorta* system, Boone et al. (2011) found that induced defenses were crucial for repelling mass attacks during endemic low populations, but became less important during incipient conditions and were positively correlated to colonization success in sites with epidemic populations. *I. typographus*, however, might be more sensitive to increasing defenses abilities of healthy trees even during epidemics, since trap catches in 2008 were generally higher in the whole region than the previous years, and the

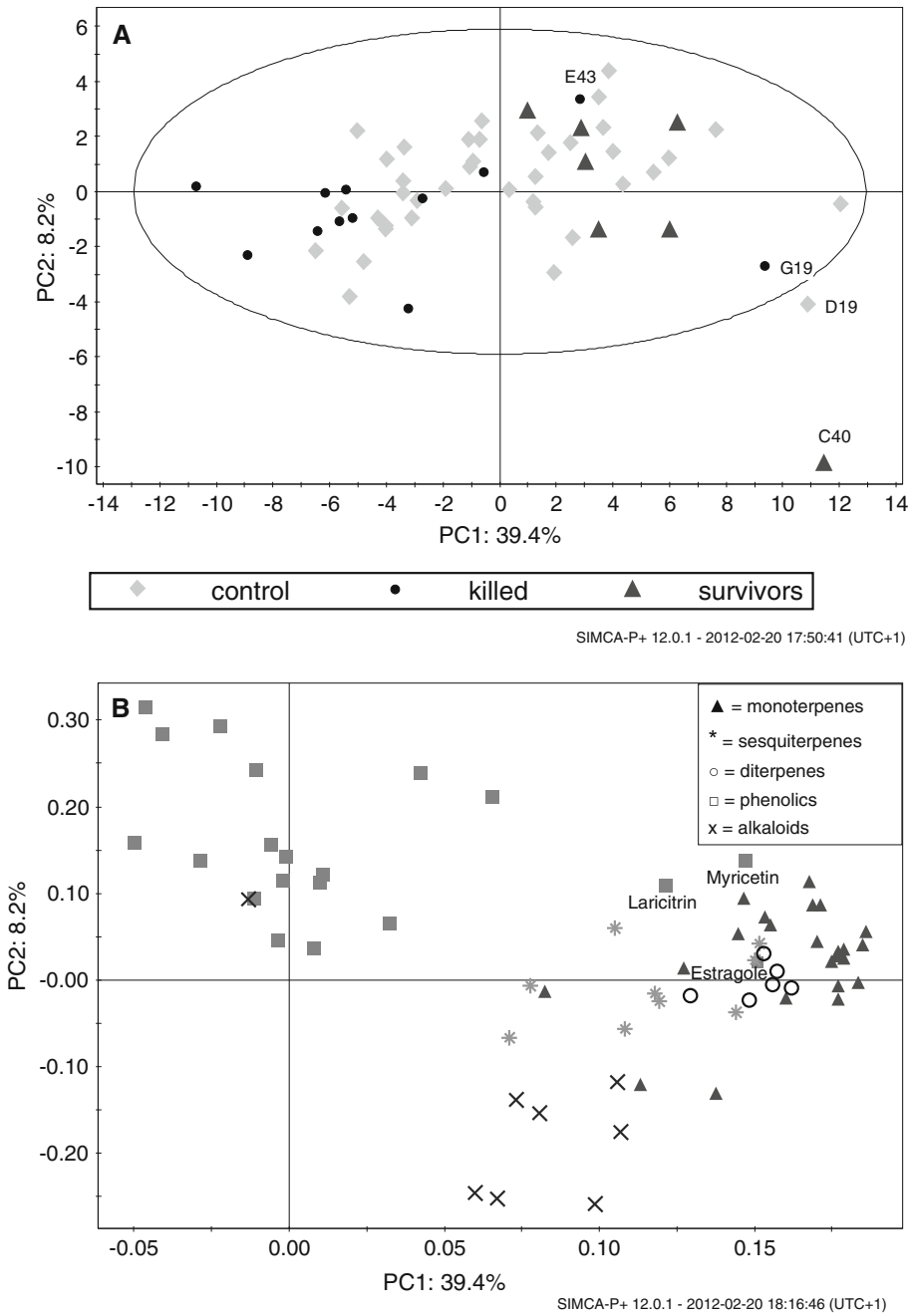

Fig. 3 Principal component analysis of MeJ-treated *Picea abies* bark samples. **a** Score scatter plot for the first and second components of all 58 MeJ-treated bark samples and 66 chemical variables. Scores are autolog-transformed. The first two components explain 47.6 % of the variance in the data. Samples lying outside the 95 % range of the Hotelling T^2 ellipse, are considered to be outliers. **b** *Loading scatter plot* showing the influence of the 66 chemical variables in explaining the distances along the component axes between the samples shown in (**a**). The longer the distance to the origin, the more important is a compound in explaining the position of a sample along either the *x* or *y* axis in (**a**)

amount of killed standing trees decreased (Långström et al. 2009). Also, our catches in the observation traps indicate a continued high population that was not attracted, however, to the few pioneers attacking the resistant trees, but to trees with low defenses.

Several underlying mechanisms could explain the resistance shown by our surviving trees. If the induction of defensive compounds is rapid enough, their concentrations will become deleterious at some level and deter pioneer beetles from the tree before others are attracted (Raffa and Smalley 1995; Wallin and Raffa 2000; Faccoli et al. 2005). However, the higher chemical levels in the treated bark of our surviving trees show the state of induction after 4 weeks, but the beetles had already abandoned the trees after a few days. Zhao et al. (2011a), have shown that bark of *P. abies* sprayed with MeJ 1 month earlier induced up to 20-fold increases in terpene levels within only 24 h after mechanical wounding. Correspondingly, we could hypothesize that our surviving trees may have shown a similar rapid induction enabled by previous challenges in the life history of these almost 100-year-old trees. As an indication of previous challenges, we found rows of TRD in the wood from previous years in some of these trees. Zhao et al. (2011a) also suggests an alternative resistance mechanism

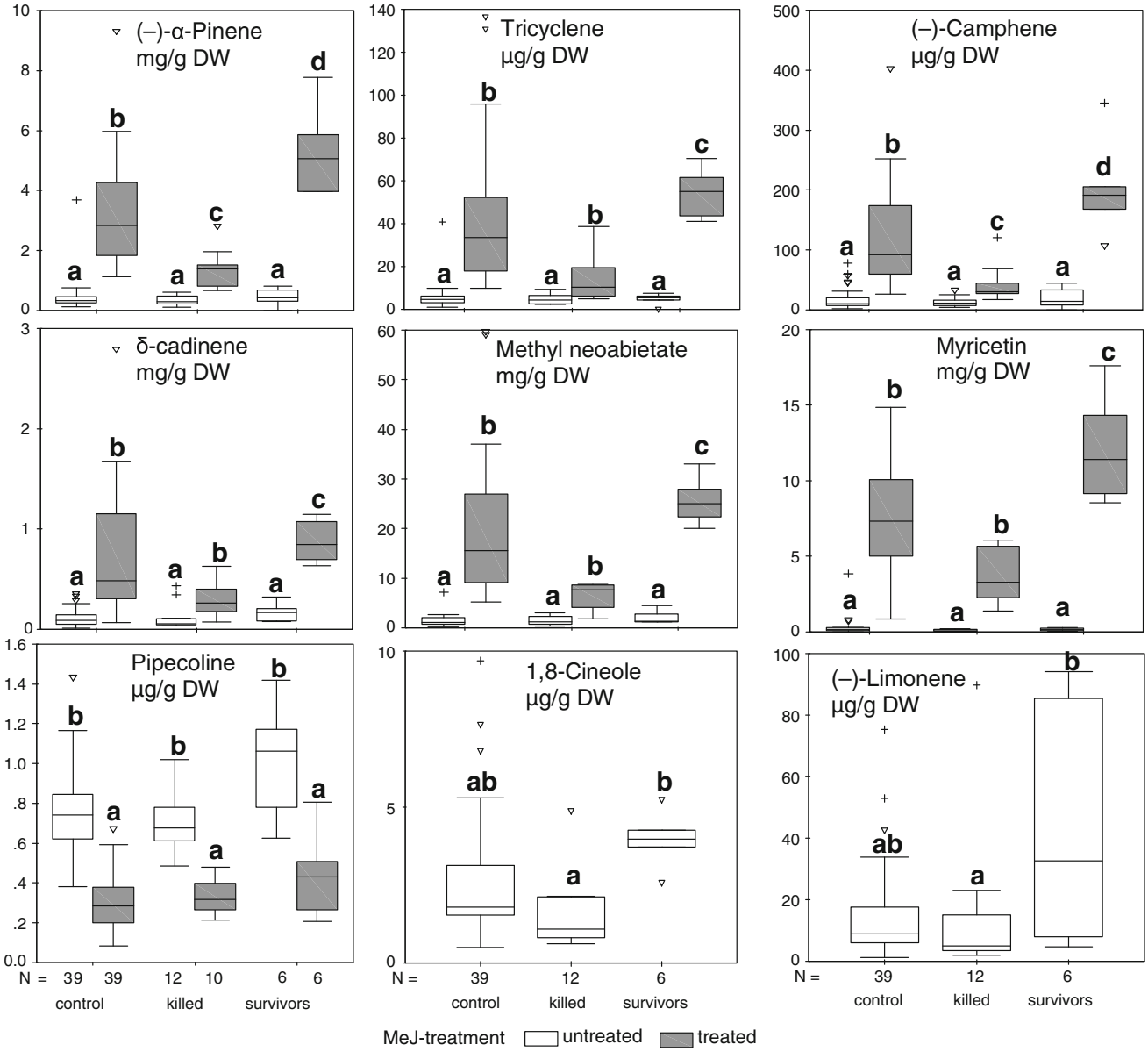

Fig. 4 Content of selected terpenes, phenolics, and alkaloids that show significant differences among the bark of killed, surviving, or unattacked control trees. In treated samples, two killed trees (G19 and E43) and one surviving tree (C40) are excluded (see Fig. 3a and text). Pipecoline is the alkaloid with the highest mean amounts found in bark samples. *Light boxes* show content for untreated and *dark boxes* for MeJ-treated bark samples. For 1,8-Cineole and (−)-Limonene, only untreated samples are shown to clarify the differences in the constitutive bark. *Boxes* show lower and upper quartile divided by the median, *whiskers* show minimum and maximum values and *circles* indicate outliers. Extreme values are not shown. *Boxes* with different *letters* are significantly different [ANOVA of log(x + 1) transformed values with Tukey HSD and Dunnett's T3 for diterpenoids]

that could have been active in the surviving trees: a rapid induction of defensive compounds that inhibit the beetles' pheromone production and prevent or delay the initiation of a mass attack (see also Erbilgin et al. 2006).

In spite of the strong indications for the importance of inducible host defenses for bark beetle colonization success, the question remains if and by which means pioneering *I. typographus* are able to distinguish between suitable and resistant hosts either before host entrance or during the first attack attempts. The resistance mechanisms discussed above do not exclude the possibility that a perception of host suitability by the beetles is part of the attack process. Volatiles released from unattacked bark would probably be the most important cues for evaluation of host quality prior to host entry. The monoterpene composition commonly varies considerably between individual trees within conifer species (Latta et al. 2000), and this was also shown in our study. Despite this variability, 1,8-cineole and

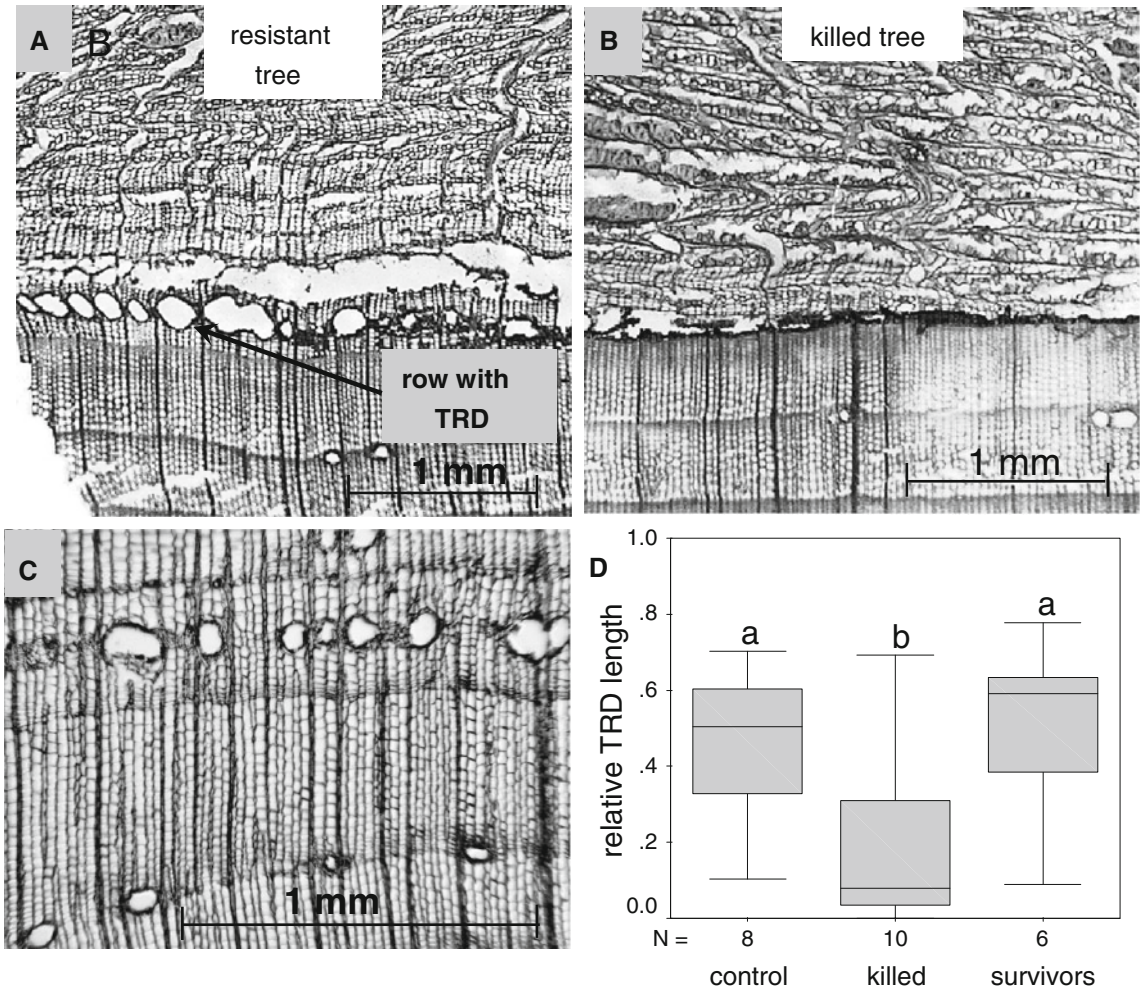

Fig. 5 Micrographs of *P. abies* wood cross-sections showing (**a**) a well-established row of TRD in the MeJ-treated bark of a surviving tree, **b** poorly-developed TRD in the MeJ-treated sample of a killed tree, and **c** TRD in the second- and third-year wood in a surviving tree. Pictures taken under ×20 magnification from 16-µm cryotome sections stained with Stevenel's blue. **d** *Box* plot of the relative length of TRD formation per sample, 4 weeks after induction with MeJ. TRD formation in 10 killed trees was significantly shorter than in 8 control or 6 surviving trees (ANOVA with Tukey's HSD: $p = 0.037$ for differences between control and killed trees; $p = 0.022$ for differences between surviving and killed trees)

(−)-limonene were significantly more abundant in untreated bark of surviving trees compared to killed trees. Our study could not prove any influence of these differences on beetle host choice, but the compounds should be taken into consideration as possible semiochemicals in connection with the abandoned colonization attempts. Both compounds were also strongly induced after MeJ treatment. The compound 1,8-cineole is particularly interesting in this context. In single-cell recordings, it elicits strong selective responses on neurons situated in the same sensilla as neurons responding to the *I. typographus* pheromone compound *cis*-verbenol (Andersson et al. 2009). Simultaneous responses to high doses of cineole and low doses of cV significantly reduced the response to cV, and cineole also inhibited the attraction to pheromones in the field (Andersson et al. 2010). We hypothesize that a strong and rapid induction of 1,8-cineole in induced trees could interfere with pheromone perception and be involved in inhibiting a mass attack. Limonene has been suggested to be the most toxic monoterpene among those that have been tested against different bark beetles (Raffa and Berryman 1987). The higher amounts of (−)-limonene could therefore be related to repellence in the surviving trees, at least after levels have exceeded a critical threshold after attack.

The phenolics were less induced than terpenes in this study, but the fact that some phenolics (estragole, the flavanoids myricetin and laricitrin, and an unknown coniferyl alcohol derivative) diverged from the phenolic pattern and showed a significantly stronger induction in surviving than in killed trees is in line with earlier reports proposing phenolics as resistance markers. Their inducibility has been shown to correlate with resistance against the bark beetle-associated

fungus *Ceratocystis polonica* (Brignolas et al. 1995), and antifeeding properties of some phenolics against *I. typographus* have been shown in bioassays (Faccoli and Schlyter 2007). Phenolics with strong inducibility following challenges may therefore be particularly expected to possess antifeeding properties against bark beetle attack.

The diterpenes generally showed the strongest inducibility following MeJ treatment, and their content differed significantly between killed trees and survivors. To our knowledge, no studies to date have specifically addressed the biological activity of diterpenes on *I. typographus* colonization success, survival, or host recognition. In *Ips pini* Say, however, no direct inhibition of adults or larvae by specific diterpine resin acids has been shown, but the inhibition of germination and growth of the associated fungus *Ophiostoma ips* was strong (Kopper et al. 2005). Hence, the biological effects of diterpenes may be more related to indirect reproduction success, due to inhibition of fungal associates, than to host choice by the beetles. Studies related to recognition and perception of diterpenes by bark beetles are strongly required.

The alkaloids showed a strong induction in a few resistant trees, but their extreme variability does not allow us to draw any firm conclusions about their importance in tree resistance. Little is known about the role of alkaloids in the defense of *Picea* sp. (Stermitz et al. 2000), but alkaloids are generally known to possess antifeedant activity against herbivore insects (Schneider et al. 1991).

In summary, our study found evidence for the critical importance of induced defenses in *P. abies* on the colonization success of *I. typographus*. Bioassays on behavioral effects of individual compounds or blends of compounds should be performed to evaluate their importance for beetle perception of host suitability. Assessing how host defenses affect the pheromone system of *I. typographus* could be crucial for the understanding of host resistance.

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